Arch. Mikrobiol. 76, 252—264 (1971)
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Autotrophic Growth and Synthesis of Reserve Polymers in *Nitrobacter winogradskyi*

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Received December 3, 1970

Summary. 1. A rapid decrease in the rate of CO_2 fixation and nitrite oxidation was observed in autotrophically growing cultures of *Nitrobacter winogradskyi*.

2. This decline was accompanied by the accumulation of poly-β-hydroxybutyrate (PHB) and was also reflected in the decrease of the free energy efficiency of nitrite oxidation. PHB was depolymerized as soon as nitrite was depleted in the culture medium.

3. Besides that of PHB the synthesis of glycogen and polyphosphate by *Nitrobacter winogradskyi* was also demonstrated.

Since the report of Smith *et al.* (1967) on the biochemical basis of obligate autotrophy in blue-green algae and thiobacilli the earlier data on the incorporation and metabolism of organic compounds by autotrophic bacteria (Butler and Umbreit, 1966; Clark and Schmidt, 1967a; Clark and Schmidt, 1967b; Delwiche and Finstein, 1965; Ida and Alexander, 1965; Kelly, 1967; London and Rittenberg, 1966; Schön, 1965; Van Gool and Laudelout, 1967) became of particular interest and significance. The same report also stimulated several workers (Butler and Umbreit, 1966; Butler and Umbreit, 1969; Hooper, 1969; Trudinger and Kelly, 1963; Williams and Watson, 1968) to check the obligate autotrophic nature of several bacteria using the criteria given by Smith *et al.* (1967).

Nitrobacter agilis was extensively studied by Smith and Hoare (1968) particularly in relation to the assimilation of acetate and the distribution of the acetate carbon in growing and resting cell suspensions. They concluded that this particular *Nitrobacter* species should be considered as a "facultative" rather than an "obligate" autotroph since it could be grown heterotrophically and was capable to oxidize acetate and to assimilate this compound in the absence of nitrite. They also reported that this species contained all the enzymes of the tricarboxylic acid cycle.

In this report the development of important parameters of growth and biosynthesis was examined during autotrophic growth of *Nitro-*

bacter winogradskyi. At the same time acetate assimilation and synthesis of poly-β-hydroxybutyrate (PHB) and polyphosphate reserve polymers were carefully examined.

Materials and Methods

Culture Methods. Nitrobacter winogradskyi (strain Dr. H. Engel, Hamburg) was grown in 10 l carboys using the medium and cultural conditions previously described (Boon and Laudelout, 1962). A 1% inoculum was used and derived from stock cultures that had oxidized approximately 40 millimoles of nitrite. Cells were harvested throughout different growth phases and the cultures were not allowed to oxidize more than 100 millimoles of nitrite i.e. a maximum of two aseptic additions of nitrite resulting each time in a final nitrite concentration in the culture medium of 40 mM. In most instances growth was measured by taking the OD of samples of the growing culture at 600 nm. The cells were harvested in the cold by centrifugation at 10,000 g for 15 min. The pellet was washed twice in Tris-HCl buffer (25 mM; pH 7.65) and resuspended in the same buffer unless otherwise stated. Nitrite was measured by the method described by Shinn (1941).

Measurement of the Rate of Nitrite Oxidation and CO_2 Fixation. The rate of nitrite oxidation was measured at 30° C using the conventional Warburg technique. Flasks contained in the main compartment 2.7 ml cell suspension (approximately 0.8 mg dry weight) and 0.3 ml of a 160 mM sodium nitrite solution in Tris buffer (25 mM; pH 7.65), the center cup contained 0.1 ml KOH 20% when required. The rate of CO_2 fixation was measured as previously described (Laudelout *et al.*, 1968). The particular composition of the scintillation liquid was selected to obtain minimal quenching. The samples were counted using a MBLE Model PNP 101 liquid scintillation counter.

When *Nitrobacter* cells were incubated with ^{14}C-labelled organic molecules or with ^{32}P-inorganic phosphate the cells were collected on a millipore filter (pore size: 0.22 μm). They were subsequently washed on the filter with Tris buffer (25 mM; pH 7.65) containing the cold compound until the filtrate did not give any measurable activity. The filter was either put directly in a scintillation vial or glued on an aluminium planchet and counted in a Nuclear Chicago gas flow counter.

Poly-β-Hydroxybutyrate (PHB) Isolation and Measurement. Extraction, purification and spectrophotometric analysis of PHB were carried out as previously described (Tobback and Laudelout, 1965). When PHB was labelled with ^{14}C-acetate, ^{14}C-sodium bicarbonate or ^{14}C-β-hydroxybutyrate 1 ml of the *Nitrobacter* chloroform extract was removed and the activity measured as described above.

Fractionation and Analysis of Phosphorus Compounds. A heavy cell suspension (approximately 10 mg protein N) was thoroughly washed until no inorganic phosphate could be revealed in the washings. Aliquots of the final suspension were taken for analysis of total P and total N content. The suspension was centrifuged and extracted at 0° C for 1 hour with 5% icecold TCA. This extract and washings gave the *acid soluble fraction*. The residue was extracted with 10 ml 94% ethanol at 30° C for 24 hours followed by a second extraction in 94% ethanol-ether (1:3 v/v) during 5 hours at 50—60° C. This extract and combined washings gave the *ethanol-ether soluble fraction*. The remaining residue was treated with 10 ml N NaOH for 15 hours at 20° C and analyzed to measure the P content in the *acid-insoluble fraction*. Acidification of this alkaline solution with 5 M acetate buffer resulted in a precipitate which was centrifuged and treated with 3 ml N NaOH containing the *DNA phosphorus* (soluble) and *phosphoprotein* (insoluble) fraction. The supernatant after acidification contained *polyphosphate-* and *RNA-phosphorus*. *Orthophosphate*

was determined by the method of Fiske and Subbarow (1925). *Acid-labile phosphate* was measured as the excess of inorganic phosphate after a 7 min treatment with 1 N HCl at 100° C. *Total phosphorus* was measured by the method of Umbreit *et al.* (1957). Polyphosphate was determined by precipitation with Ba^{++} at pH 4.0 and 0° C. The precipitate was dissolved in 1 N HCl and determined as acid-labile P.

Results

When a resting *Nitrobacter* cell suspension was incubated for a short time with nitrite and ^{14}C-bicarbonate, the label was incorporated by the cells at a constant rate (Fig. 1). This allowed an accurate measurement of the rate of CO_2 fixation and enabled us to study the influence of temperature on carbon dioxide fixation by intact cells. Fig. 2a shows a fairly sharp temperature optimum around 40° C when the initial rate of CO_2 fixation was plotted over a temperature range from 14 to 45° C. The calculation of the apparent Arrhenius activation energy from the ascending branch of the curve (Fig. 2b) gave a value of 23.9 Kcal.

Although the rate of carbon dioxide fixation remained constant in shortterm incubation experiments on resting cells, significant fluctuations were observed for the rate measured on growing cells (Fig. 3). A maximum rate (which could not be revealed with certainty) was pre-

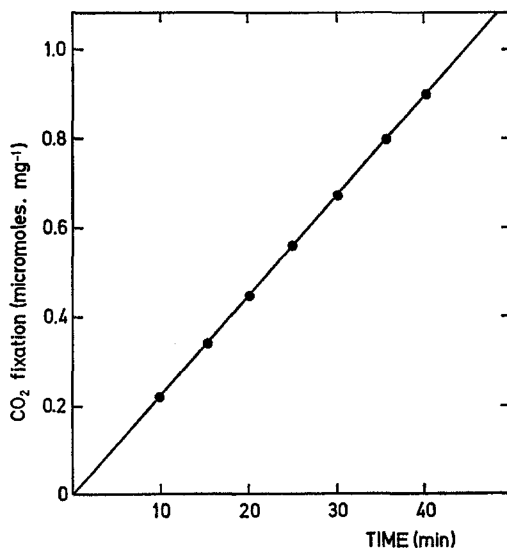

Fig. 1. Rate of CO_2 fixation by a resting *Nitrobacter* cell suspension. Cells (1.8 mg dry weight) were suspended in 70 ml Tris-HCl buffer (25 mM; pH 7.65) and incubated under continuous shaking with 10 micromoles $NaH^{14}CO_3$ and 16 mM sodium nitrite (final concentration). Incubation temperature 30° C. The buffer solution was saturated with oxygen during 1 hour prior to the addition of cells

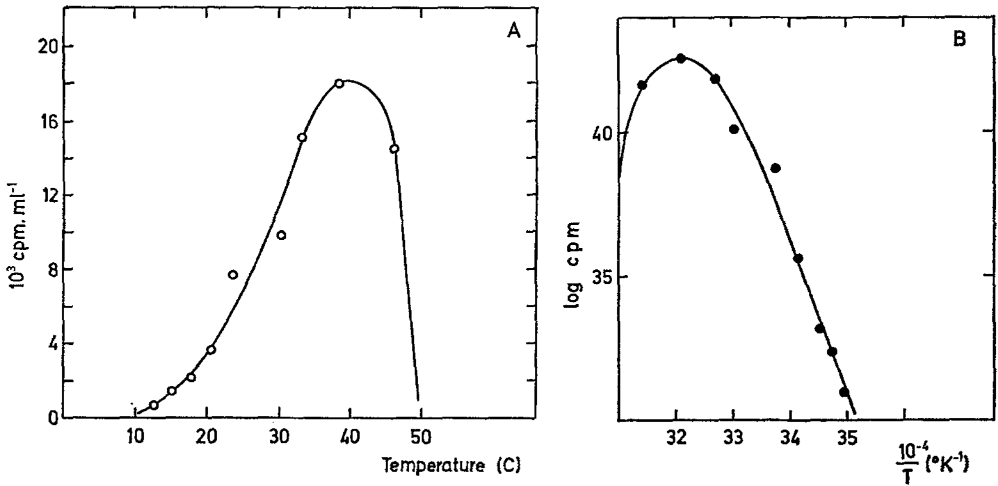

Fig. 2 A and B. Influence of temperature on the initial rate of CO_2 fixation. A Cells were incubated in Tris-buffer (25 mM; pH 7.65) with 10 micromoles $NaH^{14}CO_3$ and 16 mM nitrite (final concentration). After 20 min incubation the suspension was sampled and the net activity of the cells measured. The reagents were all thermally equilibrated, at the temperatures indicated, during 30 min prior to the reaction. B Arrhenius plot of the data of Fig. 2 A

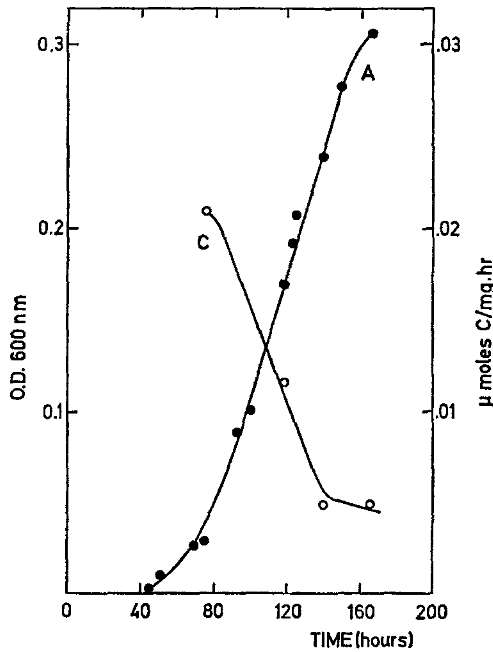

Fig. 3. Growth (*A*) and rate of carbon dioxide fixation (*C*) of *Nitrobacter*

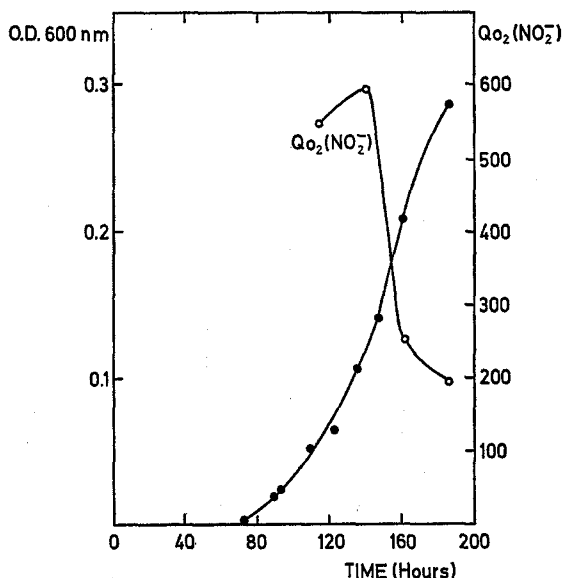

Fig. 4. Growth (•) and rate of oxygen uptake (○) during nitrite oxidation Q_{O_2} (NO_2^-) and PHB content in a growing *Nitrobacter* culture

sumably obtained very early during growth. Subsequently the rate of CO_2 fixation decreased rapidly and attained a minimal value near the stationary growth phase. A similar decrease was also observed in the rate of nitrite oxidation (Fig. 4).

These observations on the decline of important parameters of autotrophic growth led to a parallel study on the synthesis and degradation of reserve polymers in growing *Nitrobacter winogradskyi* cells. Since considerable amounts of PHB were shown to occur in these cells (Tobback and Laudelout, 1965) the concentration of this reserve polymer was measured in growing cultures. As shown in Fig. 5 the increase in PHB content of the cells ran parallel to the decrease observed in the rate of CO_2 fixation and nitrite oxidation (Figs. 3 and 4). The polymer concentration reached a maximum and decreased rapidly as soon as nitrite was depleted (Fig. 5, arrow). When acetate or β-hydroxybutyrate was supplied in addition to nitrite (Table 1) in the presence of ^{14}C-bicarbonate the total PHB content increased; but the specific activity of the PHB-polymer ^{14}C derived from labelled bicarbonate was considerably reduced and the rate of CO_2 fixation slightly depressed. The possibility of dilution of the ^{14}C-polymer-carbon by carbon dioxide which could be derived from the oxidation of acetate or β-hydroxybutyrate was severely controlled and found to be either not detectable or negligible.

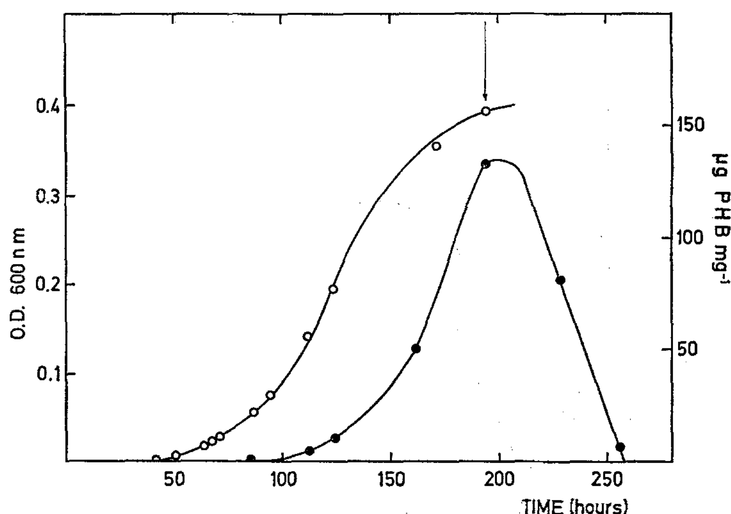

Fig. 5. Synthesis and breakdown of PHB (•) during the growth (o) of *Nitrobacter* cells. Arrow indicates nitrite depletion

Table 1. *Influence of the exogenous supply of acetate or β-hydroxybutyrate on the synthesis of PHB and the rate of CO_2 fixation*

Exp. No.	Substrates	CO_2 fixation rate cpm · min^{-1}	Total PHB content in µg	Specific activity cpm/µg PHB
I	Nitrite	1333	236.4	354
	Nitrite plus acetate	1200	337.5	270
II	Nitrite	1135	249.0	117.2
	Nitrite plus β-hydroxybutyrate	927	265.0	69.2

Cells (1 mg dry wt/ml) were incubated in 50 ml Tris buffer (25 mM; pH 7.65) over 100 min at 30° C with 25 micromoles $NaH^{14}CO_3$. Final concentration: $NaNO_2$: 16 mM; acetate: 20 mM; β-hydroxybutyrate: 20 mM.

Since acetate was more readily incorporated in the polymer than β-hydroxybutyrate, the uptake of labelled acetate by a growing *Nitrobacter winogradskyi* culture was examined. Fig.6 shows the incorporation of ^{14}C acetate. The total activity of the cells (curve b) levelled off near the end of the growth period while the specific activity (curve a) reached a constant value before nitrite was completely oxidized (arrow). Compared to the total activity of the cells the acetate label was diverted into

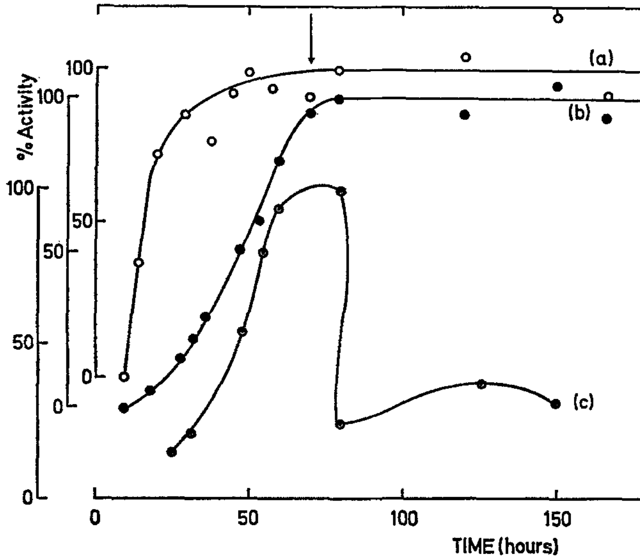

Fig. 6. Uptake of methyl-labelled ^{14}C acetate by growing cells. To a growing *Nitrobacter* culture 100 μC acetate-2-C^{14} was added and the specific activity (cpm/mg dry wt) of the cells (*a*), the total activity of the cells (*b*) and the activity of the PHB-fraction (*c*) was followed. As the uptake of acetate proceeded rapidly, a constant activity in the culture solution was maintained by additional quantities (50 μC) of labelled acetate after 36 and 55 hours of growth. The arrow indicates the depletion of nitrite. Activities were expressed in arbitrary units, the average activity during stationary phase of growth was considered as the 100% value

PHB with a certain delay and the activity of the PHB fraction decreased immediately as soon as the nitrite supply was depleted. This loss in activity, however, was not accompanied by a complete oxidation of depolymerized β-hydroxybutyrate to CO_2 since the total activity of the cells remained constant after depletion of nitrite (Fig. 6; curve b). Moreover attempts to study the respiration of the β-hydroxybutyrate monomer by crude cell free extracts of *Nitrobacter* failed and only after addition of NAD faint activities could be detected by measuring either oxygen uptake or cytochrome reduction.

Besides the mere observation on the decline of the rate of nitrite oxidation and CO_2 fixation during growth the variation of these parameters was related to the free energy efficiency of nitrite oxidation which gave an estimate of the overall yield of autotrophic biosynthesis. To obtain reliable data this efficiency was evaluated in two ways either indirectly measuring the rate of CO_2 fixation and nitrite oxidation or by direct microcalorimetric measurement of the molar heat of nitrite oxi-

Table 2. *Free energy efficiency of nitrite oxidation during growth and subsequent incubation*

Hours of growth[a]	Incubation time (hrs)[b]	Specific rate of NO_2^- oxidation $\mu M \cdot mg^{-1} \cdot h^{-1}$	Rate of CO_2 fixation $\mu M \cdot mg^{-1} \cdot h^{-1}$	Molar heat of nitrite oxidation Kcal/M NO_2^-	η_1[c]	η_2[d]
72	—	58.9	2.7	17.1	30	40
96	—	27.8	0.64	20.8	15	19
120	0	27.1	0.49	21.8	12	14
—	18	23.8	0.36	21.8	10	14
—	41	19.0	0.19	21.8	6	14

[a] Growth allowed to proceed until 0.120 M of nitrite had been oxidized per liter of culture medium.

[b] The cells were centrifuged and incubated in Tris-HCl buffer (25 mM; pH 7.65) at 30° C.

[c] Efficiency in per cent calculated from the ratio of the rate of CO_2 fixation and nitrite oxidation.

[d] Efficiency in per cent calculated from microcalorimetric data.

Table 3. ^{32}*P-phosphate uptake by* Nitrobacter *cells in the presence or absence of nitrite and/or 2,4-dinitrophenol*

Exp. No.	Substrates	Incubation time (hrs)		Net variation of activity (%)
		0 cpm · mg^{-1}	25 cpm · mg^{-1}	
1	cells minus nitrite minus 2,4 DNP	15,263	13,790	− 10
2	cells plus nitrite	15,474	16,974	+ 10
3	cells plus nitrite plus 2,4 DNP	14,316	18,448	+ 29

Cells were grown in 0.5×10^{-3} M phosphate buffer and labelled with $H_3{}^{32}PO_4$ (pH 6.93). When the initial amount of nitrite had been oxidized (31.5 mM) the culture was divided aseptically in three parts. Nitrite (16 mM) was added to No. 2 and nitrite (16 mM) plus 2,4 DNP (0.2×10^{-3} molar) to No. 3. No. 1 was kept without nitrite or 2,4 DNP. Growth was continued for the next 25 hours at 30° C.

dation (Laudelout *et al.*, 1968). Table 2 lists the free energy efficiency values obtained by both methods for a cell suspension during growth and a subsequent 41 hrs period of incubation. In both instances the free energy efficiency declined initially during growth and levelled off at a considerably lower value near the end of the growth. The discrepancy between the values of the free energy efficiency depending on the method

Table 4. *Distribution of the total phosphorus in intact* Nitrobacter *cells*

Fractions		Phosphorus (μg P/mg N)	Content (%)
Cell suspension	Total P	224.17	100
Acid-soluble	Ortho P	32.10	14.3
	Poly P	3.60	1.6
	Other acid-labile P	0.86	0.4
Ethanol, ethanol-ether soluble		48.59	21.7
Acid-insoluble	RNA P	74.53	33.2
	DNA P	14.84	6.6
	Poly P	39.08	17.4
	"Phosphoprotein" P	1.36	0.6

Intact *Nitrobacter* cells (100 mg dry weight or 8.5 mg protein N) were washed and fractionated according to the procedure outlined in the methods section.

Table 5. *Uptake of ^{32}P-orthophosphate and distribution of label among the various P-containing fractions*

	P content (μg P/mg N)	(%)	P uptake with nitrite (μg P/mg N)	(%)	P uptake without nitrite (μg P/mg N)	(%)
Original cell suspension Total P	207.11	100	10.48	100	6.51	100
Acid-soluble:						
Ortho P	43.54	21.0	2.78	26.5	1.28	19.7
Poly P	2.03	1.0	2.54	24.2	1.78	27.3
Other acid-labile P	3.68	1.8	—	—	—	—
Ethanol, ethanol-ether-soluble:	39.44	19.0	0.42	4.0	0.32	1.9
Acid-insoluble:						
RNA P	78.23	37.8	3.05	29.1	1.78	27.3
DNA P	14.72	7.0	0.44	4.2	0.29	4.5
Poly P	35.87	17.3	0.44	4.2	0.71	10.9
"Phosphoprotein" P	1.99	0.9	0.06	0.6	0.04	0.6

A concentrated cell suspension (2 mg N/ml) was equilibrated in phosphate buffer (20 mM; pH 7.65) at 30° C during 30 min. At zero time it was divided in 3 equal parts. One part was analyzed directly for the P content in the different fractions. The two other fractions were incubated under aeration for 24 hours in the presence of 100 nC ^{32}P-phosphate and to one of them 25 mM nitrite (final concentration) was added. The net uptake of the label in the different fractions was examined at the end of the incubation period.

of determination has been previously reported (Laudelout *et al.*, 1968) and hypothetically attributed to the storage of energy, derived from nitrite oxidation, in non-carbon containing compounds presumably in polyphosphate.

This hypothesis was verified carrying out experiments on growing cells incubated in the presence of ^{32}P-inorganic phosphate. Table 3 shows that the addition of nitrite resulted in a net uptake of the ^{32}P label and that the activity of the cell was increased considerably after inhibition of CO_2 fixation by adding 2,4-dinitrophenol (2×10^{-4} M final concentration).

Results of direct measurements of the polyphosphate content of *Nitrobacter* cells are given in Table 4. It shows for an intact cell suspension the distribution of the total phosphorus content (approximately 1.9% of the dry weight) among the different fractions obtained after subsequent TCA; ethanol and ethanol-ether extraction. An acid-soluble and acid insoluble polyphosphate fraction was found representing together 19% of the total amount of phosphorus in the cells. When a resting cell suspension, previously equilibrated in phosphate buffer, was incubated with ^{32}P-orthophosphate the label was diverted differently into the various fractions depending on the presence or absence of nitrite (Table 5). In the presence of nitrite the total amount of ^{32}P incorporated was considerably higher. A higher activity was found in the orthophosphate fraction at the expense of ^{32}P incorporation in the polyphosphate fractions.

Discussion

When *Nitrobacter winogradskyi* was grown autotrophically in batch culture an important decline was observed in the rates of CO_2 fixation and nitrite oxidation. Parallel to this decline, which was already apparent a few generations after the initiation of growth, a rapid increase in the PHB content of the cells was found.

The synthesis of this polymer required the presence of nitrite since the PHB content of the cell decreased very rapidly after depletion of nitrite in the culture medium (Fig. 5). Nitrite oxidation presumably provides the precursors for PHB synthesis through CO_2 fixation explaining the decrease in rate of CO_2 fixation and in the specific activity of PHB, when cold acetate or β-hydroxybutyrate were added to cell suspensions incubated in the presence of ^{14}C-bicarbonate (Table 1).

Although it has been shown that *Nitrobacter winogradskyi* has an active $NADH_2$ oxidase (Van Gool and Laudelout, 1967) and even if it has the complete set of TCA cycle enzymes as reported for *Nitrobacter agilis* (Smith and Hoare, 1968), it was observed that neither acetate, β-hydroxybutyrate nor pyruvate were efficiently respired suggesting

that none or very little energy could possibly be derived from the metabolism of these organic nutrients. Previous reports (Van Gool and Laudelout, 1966, 1967) on the utilization of formate by *Nitrobacter winogradskyi* showed that the oxidation of this substrate although able to support a very weak CO_2 fixation was characterized by an extremely low free energy efficiency.

Besides PHB other reserve polymers were also synthesized by *Nitrobacter winogradskyi*. From previous studies on the phosphate requirements and -metabolism (Fischer and Laudelout, 1965; Van Droogenbroeck and Laudelout, 1967) the occurrence of polyphosphate was presumed. The data in Table 4 confirmed this hypothesis. The dependence of the phosphate uptake on the presence of nitrite is shown in Table 3 and 5. The rather unexpected increase of ^{32}P uptake in the presence of 2,4-dinitrophenol and nitrite reported in Table 3 may be related to the report of Laudelout *et al.* (1968) on the increase of the free energy efficiency of nitrite oxidation in the presence of this inhibitor. This could indicate that energy from nitrite oxidation is diverted efficiently into polyphosphate when the assimilation of carbon dioxide is strongly inhibited by 2,4-dinitrophenol.

Evidence for the storage of polyphosphate has moreover been given in reports on the ultrastructure of *Nitrobacter winogradskyi* (Tsien *et al.*, 1968; Van Gool *et al.*, 1969) while recently using a specific staining procedure for the detection of glycogen in thin sectioned cells this polymer was shown to occur quite commonly (S. W. Watson and C. C. Remsen, private communication).

Although sufficient evidence (Smith and Hoare, 1968; Tobback and Laudelout, 1965; Van Gool and Laudelout, 1966) has been given that *Nitrobacter* does not rely exclusively on autotrophic CO_2 fixation energetically supported by nitrite oxidation it certainly must be considered as the main pathway of biosynthesis stages of growth. Only when unbalanced or unfavorable growth conditions occur heterotrophic metabolic pathways may become important. This heterotrophic metabolism, reflected in the synthesis of storage polymers was already apparent during early growth when a parallel decline of the parameters of autotrophic growth was observed. These reserve polymers support the maintenance of the microorganism providing either by carbon (PHB)-, energy (polyphosphate)-, or carbon and energy sources (glycogen). In this respect we are considering further studies on the role and metabolism of glycogen and polyphosphate.

Acknowledgement. The authors are grateful to Dr. D. S. Hoare for reading and criticizing the manuscript.

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