# RELATIONSHIP BETWEEN POLY-β-HYDROXYBUTYRATE CONTENT AND NITROGENASE AND HYDROGENASE ACTIVITY IN SOME STRAINS OF *Rhizobium*

G. A. Bonartseva, V. L. Myshkina, and E. D. Zagreba

UDC 579.841.31.013

The poly-β-hydroxybutyrate content of cells of nodule bacteria was determined when these were reared under conditions inducing nitrogenase activity and promoting hydrogen recycling. There was shown to be an inverse relationship between the poly-β-hydroxybutyrate content and nitrogenase activity and a direct relationship between the same and hydrogenase activity.

Many nitrogen-fixing microorganisms synthesize poly-β-hydroxybutyrate. Its synthesis and expenditure are closely connected with the energy requirements of the cell.

It is well known that the process of fixation of molecular nitrogen requires the expenditure of a large amount of energy. In some free-living nitrogen-fixing microorganisms

---

Institute of Microbiology, Academy of Sciences of the USSR, Moscow. Translated from Mikrobiologiya, Vol. 58, No. 6, pp. 920-922, November-December, 1989. Original article submitted November 4, 1988.

 0026-2617/89/5806-0742$12.50 

there has been found to be a close inverse correlation between the intensity of nitrogen fixation and the poly-β- hydroxybutyrate content [9] In a number of publications of Romanov et al [4, 5] data are cited for bacteroids of nodule bacteria of lupine and pea relating to a connection between poly-β-hydroxybutyrate metabolism and processes of nitrogen fixation and photosynthesis. These authors showed that initially a portion of the photoassimilation is included in poly-β-hydroxybutyrate and subsequently used for purposes of nitrogen fixation.

Molecular hydrogen released during nitrogen fixation in the presence of hydrogen-assimilating hydrogenase may be secondarily involved in the energy metabolism of Rhizobium [6], thus reducing the expenditure of energy-producing material on nitrogen fixation by 1/3 [7]. Moreover, recycled hydrogen may be used by microorganisms not only as an electron donor for nitrogenase but also to bring about $CO_2$ assimilation in other reduction-type reactions including those connected with the synthesis of reserve substances [8, 9].

The aim of the present work was to determine the content of poly-β-hydroxybutyrate in some strains of Rhizobium differing in their ability to recycle hydrogen and reduce acetylene under optimum conditions for these processes.

## OBJECTS AND METHODS OF INVESTIGATION

Use was made, in this work, of the active strains Rhizobium japonicum 646, R. vigna 164, R. phaseoli 673, R. meliloti 35, and the low-activity strains R. phaseoli 680 and R. meliloti $A_3$. Strains 35 and $A_3$ were obtained from the Latvian Agricultural Academy (Elgav); all the other strains investigated were obtained from the collection of the All-Union Research Institute of Agricultural Microbiology, Lenin All-Union Academy of Agricultural Sciences. Collection strains of nodule bacteria from soybean and cowpea were kept on a glucose-peptone agarized medium composed as follows (in g/liter): peptone 2, agar 15, glucose 20, yeast extract 2, with pH 6.8-7.0; collection strains of nodule bacteria from Phaseolus and lucerne were kept on an agarized pea medium composed as follows (in g/liter): pea 50, $K_2HPO_4$ 0.5, and saccharose 5, with pH 7.

Experiments to determine nitrogen fixation in a pure culture of nodule bacteria were carried out under anaerobic conditions in 50-ml bottles on a CS-7 medium promoting induction of nitrogenase in a pure culture of Rhizobium and composed as follows (in mM): $KH_2PO_4$ 2.2; $CaCl_2 \cdot 2H_2O$ 0.7; KCl 0.9; $MgSO_4 \cdot 7H_2O$ 0.14; glutamine 2; inositol 5.6; Na-succinate 25; L-arabinose or glucose 25; and (in μm) $MnSO_4 \cdot 4H_2O$ 58; $H_3BO_3$ 82; $ZnSO_4 \cdot 7H_2O$ 3.5; KI 6; $CuSO_4 \cdot 5H_2O$ 0.8; $Na_2MoO_4 \cdot 2H_2O$ 0.4; $CoCl_2 \cdot 6H_2O$ 0.4; $FeSO_4 \cdot 7H_2O$ 54; $Na_2$-EDTA 54; nicotinic acid 41; vitamin $B_6$ 2.4; and vitamin $B_1$ 15; with pH 6.8. Each bottle received 25 ml medium. $KNO_3$ was introduced at a rate of 5 g/liter (50 mM). Inoculatory material for the experiments was provided in the form of a 5-day culture grown on an agarized glucose-peptone medium. Each bottle received 1 ml bacterial suspension with a density as determined by photoelectric colorimetry of 0.5 (optical path length of cuvette, 1 mm). Following inoculation, the cotton-wool bungs were exchanged for rubber ones and the air was evacuated from the bottles, which were washed twice with Ar and then filled with a gaseous mixture of the following composition: $CO_2$ 0.03%; $C_2H_2$ 5%; and the remainder Ar. To give steady agitation, the bottles were placed on a rotary shaker. The incubation temperature was 30°C. Formation of ethylene was registered with a Chrom-3 gas chromatograph with flame-ionization detector, ASK silica gel column (length 120 cm, internal diameter 5 mm), vaporizer temperature 80°, thermostat temperature 50°, and flow-rate of the carrier gas (nitrogen) 40 ml/min.

Hydrogenase activity was determined by the method of 2,3,5-triphenyltetrazolium sulfate reduction [1]. To do this, the cultures were reared in 50-ml flasks on a liquid synthetic medium promoting induction of hydrogenase activity in a liquid culture of Rhizobium composed as follows (in mg/liter): $NaH_2PO_4 \cdot H_2O$ 150; $CaCl_2 \cdot 2H_2O$ 150; $MgSO_4 \cdot 7H_2O$ 250; Fe-EDTA 28; $MnSO_4 \cdot H_2O$ 10; $H_3BO_3$ 3; $ZnSO_4 \cdot 7H_2O$ 2; $NaMoO_4 \cdot 2H_2O$ 0.25; $CuSO_4 \cdot 5H_2O$ 0.04; $CoCl_2 \cdot 6H_2O$ 0.025; KI 0.78; inositol 100; vitamin $B_1$ 10; vitamin $B_6$ 1; nicotinic acid 1; and (in g/liter) saccharose 0.5; L-arabinose 1; Na gluconate 0.5; Na glutamate 0.5; yeast extract 0.1; and distilled water; with pH 6.8. The flasks were placed in an exsiccator in an atmosphere of air containing 3% hydrogen, the incubation temperature being 30° and the incubation time 5 hours. After termination of incubation, a part of the biomass was used for determination of the poly-β-hydroxybutyrate in it, while in the other part the hydrogenase activity was determined. To do this, the cell suspension was concentrated by sterile centrifugation at 18,000 rpm for 20 min. The cells were washed and suspended in a small quantity of 0.1 M phosphate buffer, pH 7. Thick suspensions were obtained with a density of ~1.0 as determined

by photoelectric colorimetry (in a cuvette with optical-path length 1 mm). Next, 1 ml suspension of the washed bacterial cells and 1 ml 0.25% aqueous tetrazolium solution were placed in chemically pure, sterile test tubes; these were hermetically sealed with rubber bungs provided with foil, agitated, and incubated for 24 h in a hydrogen atmosphere at 30°. After this, the triphenylformazan formed was removed from the bacterial cells with glacial acetic acid (1 ml, 1 h) and the formazan was then transferred from the acetic-acid solution to 3 ml chloroform under agitation (24 h). The optical density of the colored layer was further measured with an SF-16 spectrophotometer at a wave length of 250 nm. At the same time, controls were set up to determine the level of reduction of 2,3,5-triphenyltetrazolium sulfate due to endogenous reserves in the absence of $H_2$ and check for possible 2,3,5-triphenyltetrozolium sulfate reduction by molecular hydrogen in the absence of cells [1].

The poly-β-hydroxybutyrate (PHB) content in the cells was determined by the method of infra-red spectroscopy [3]. For this purpose, the bacterial biomass was separated from the culture liquid by centrifugation and washed twice with sterile tap water. The washed biomass was lyophilized, carefully stirred, and milled with KBr, and disks were then pressed out for recording the IR spectra. The spectra were recorded with an IK-20 infrared spectrophotometer (slot program 4; speed of registration 160 cm/min). The number of replications in each of the experimental variants was 5. The mean data are cited in Tables 1 and 2.

The range of variation did not exceed ±5% in the determination of hydrogenase activity, ±10% in the determination of PHB content, and ±3% in the determination of nitrogenase activity.

## RESULTS AND DISCUSSION

Content of Poly-β-hydroxybutyrate in Cells of Slow-growing Strains of Nodule Bacteria in Relation to Nitrogenase Activity. It can be seen from Table 1 that during growth under anaerobic conditions on a CS-7 medium the investigated strains R. japonicum 646 and R. vigna 164 synthesize large quantities of poly-β-hydroxybutyrate: 21 and 18%, respectively, of the weight of the dry cells; nitrogenase activity under these conditions was low. With the addition of nitrates to the medium under anaerobic growth conditions, acetylene reduction was observed for slow-growing cultures [2]. It follows from the data presented in Table 1 that nitrogenase activity in the investigated strains R. japonicum 646 and R. vigna 164 was 10 times greater in the presence of $NO_3^-$ than when the strains were grown under the same conditions without nitrates. The poly-β-hydroxybutyrate content, on the other hand, was minimal in the presence of $NO_3^-$. The data cited indicate a close inverse correlation between nitrogenase activity and the poly-β- hydroxybutyrate content in the strains investigated. It seems probable that the relationship results from the expenditure of poly-β-hydroxybutyrate on the process of nitrogen fixation under anaerobic conditions in the presence of nitrates.

TABLE 1. Poly-β-hydroxybutyrate (PHB) Content and Nitrogenase Activity of R. vigna 164 and R. japonicum 646 under Anaerobic Conditions on a CS-7 Medium with $NO_3^-$ and without $NO_3^-$ (age of culture 8 days)

| Strain | CS-7 medium without $NO_3^-$ | | CS-7 medium with $NO_3^-$ | |
|---|---|---|---|---|
| | nitrogenase activity, nmole $C_2H_4$/mg dry biomass | PHB, % of weight of dry cells | nitrogenase activity, nmole $C_2H_4$/mg dry biomass | PHB, % of weight of dry cells |
| *R. vigna* | 0,52 | 18 | 5,55 | 0,5 |
| *R. japonicus* 646 | 0,15 | 21 | 2,00 | 0,9 |

TABLE 2. Poly-β-hydroxybutyrate Content in Cells of R. phaseoli and R. meliloti with Differing Hydrogenase Activity

| Indices | *R. phaseoli* | | *R. meliloti* | |
|---|---|---|---|---|
| | 673 | 680 | 35 | $A_3$ |
| Hydrogenase activity, μg formazan/h per 1 mg protein | 27,6 | 0.0 | 92,3 | 7,8 |
| PHB, % of weight of dry cells | 4.0 | 0.5 | 21,0 | 11,0 |

Content of Poly-β-hydroxybutyrate in Strains of Nodule Bacteria with Differing Hydrogenase Activity. It can be seen from the data presented in Table 2 that the strains R. meliloti 35 and R. phaseoli 673, which exhibit a high level of hydrogenase activity, contain considerable quantities of poly-β-hydroxybutyrate. The maximum level of hydrogenase activity of 92.3 μg formazan/h per 1 mg protein in strain R. meliloti 35 corresponds with the maximum poly-β-hydroxybutyrate content in the cell of 21% relative to the weight of the dry cells.

The strain R. phaseoli 680, which does not possess $H_2$-assimilating hydrogenase, contains virtually no poly-β-hydroxybutyrate.

The data cited show a direct correlation between hydrogenase activity and poly-β-hydroxybutyrate content in the strains of R. meliloti and R. phaseoli studied.

It may be assumed that the presence of hydrogen-assimilating hydrogenase gives the strains studied an advantage, in terms of energy, that is due to the secondary involvement of molecular hydrogen in metabolism and thereby enables surplus energy accumulating in the form of poly-β-hydroxybutyrate to be used more effectively.

The investigations carried out to determine the poly-β-hydroxybutyrate content in the cells of nodule bacteria in relation to processes of nitrogen fixation and the recycling of hydrogen may serve as a basis for further detailed study of the synthesis and expenditure of poly-β-hydroxybutyrate in relation to the energy requirements of Rhizobium.

## LITERATURE CITED


1. G. A. Bonartseva, V. L. Myshkina, and E. N. Mishustin, Izv. Akad. Nauk SSR. Ser. Biol., No. 4, 553 (1983).
2. G. A. Bonartseva and V. L. Myshkina, Mikrobiologiya, 58, No. 2, 181 (1989).
3. E. D. Zagreba, T. I. Seliverstova, M. K. Ginovska, et al., Izv. Akad. Nauk SSSR. Ser. Biol., No. 6, 906 (1982).
4. V. I. Romanov, Usp. Biol. Khimii, 18, 211 (1977).
5. V. I. Romanov, Nitrogen Fixation and Metabolism of Photoassimilates in the Nodules of Leguminous Plants, Author's abstract of dissertation for a doctorate in the biological sciences, Inst. Biokhimii im. A. N. Bakh AN SSSR, Moscow (1987).
6. R. O. D. Dixon, Arch. Microbiol., 85, 193 (1972).
7. H. J. Evans, D. U. Emerich, T. Ruiz-Argueso, et al., in: H. Schlegel, K. Schneider, and E. Golze (eds.) Hydrogenases, Gottingen (1978), p. 287.
8. F. B. Simpson, K. J. Maier, and H. J. Evans, Arch. Microbiol., 123, 1 (1979).
9. S. Tal and Y. Oron, Can. J. Microbiol., 31, No. 7, 608 (1985).