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Title: Hydrogen-Stimulated CO_2 Fixation and Coordinate Derepression of Hydrogenase and Ribulosebisphosphate Carboxylase in a H_2-uptake Positive Strain of Rhizobium japonicum

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Abstract approved: _____
Professor Harold J. Evans

H_2-uptake positive strains (122 DES and SR) and H_2-uptake negative strains SR2 and SR3 of Rhizobium japonicum were examined for ribulose-bisphosphate (RuBP) carboxylase and H_2-uptake activities during growth conditions which induced formation of the hydrogenase system. The rate of $^{14}CO_2$ uptake by hydrogenase-induced cells was about six times greater in the presence than in the absence of H_2. RuBP carboxylase activity was observed in free-living R. japonicum strains 122 DES or SR only when the cells were derepressed for their hydrogenase system. Hydrogenase and RuBP carboxylase activities were coordinately induced by H_2 and both were repressed by added succinate. Hydrogenase-negative mutant strains SR2 and SR3 derived from R. japonicum SR showed no detectable RuBP carboxylase activities under hydrogenase derepression conditions. No detectable RuBP carboxylase was observed in bacteroids formed by H_2-uptake positive strains R. japonicum 122 DES or SR. Propionyl CoA carboxylase activity was consistently observed in extracts of cells

from free-living cultures of R. japonicum but activity was not appreciably influenced by the addition of H_2. Neither phosphoenolpyruvate carboxylase nor phosphoenolpyruvate carboxykinase activity was detected in extracts of R. japonicum.

Hydrogen-Stimulated CO_2 Fixation and Coordinate Derepression of Hydrogenase and Ribulosebisphosphate Carboxylase in a H_2-Uptake Positive Strain of *Rhizobium japonicum*

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Typed by Karen Krause for Frank Berry Simpson

DEDICATION

This thesis is dedicated to Parker Creek on the western slope of Mary's Peak, Oregon.

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I would also like to thank Professor Harold J. Evans for having high and demanding standards which forced me to work hard and produce a thesis for which I can be proud.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Illustrations, continued

ABBREVIATIONS

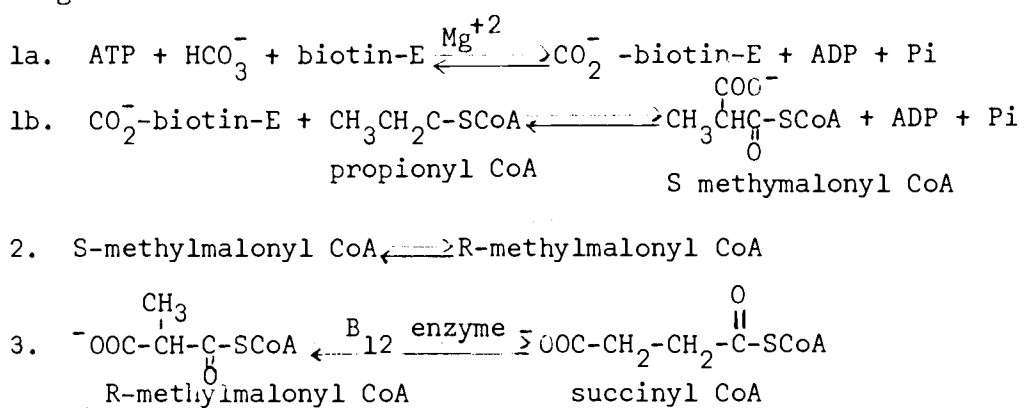
Ribulose 1,5-bisphosphate	(RuBP)
Ethylenedinitrilo-tetraacetic acid disodium salt	(Na_2EDTA)
Phenazine methosulfate	(PMS)
Propionyl coenzyme A	(propionyl CoA)
Phosphoenolpyruvate	(PEP)
Reduced glutathione	(GSH)
N-tris (hydroxymethyl)-methylglycine	(Tricene)

Hydrogen-Stimulated CO_2 Fixation and Coordinate Derepression of Hydrogenase and Ribulosebisphosphate Carboxylase in a H_2-Uptake Positive Strain of Rhizobium japonicum

INTRODUCTION

Propionyl CoA and ribulose 1,5-bisphosphate carboxylases. It has been known for many years that bacteria require CO_2. Valley and Rettger (1927) examined 109 different strains representing widely different families and genera and reported that all of them required CO_2 for growth. The essentiality of CO_2 for growth of five Rhizobium species and the presence of propionyl CoA carboxylase and phosphoenolpyruvate (PEP) carboxylase in soybean nodule bacteroids was reported by Lowe and Evans (1962). More recent evidence has indicated that most, if not all, of the phosphoenolpyruvate carboxylase is confined to the non-bacteroid soluble protein fraction of soybean nodules (Peterson and Evans, 1979) and lupine nodules (Christeller et al., 1977).

Propionyl CoA carboxylase catalyses the first step in the conversion of propionyl CoA to succinyl CoA as outlined in the following diagram.

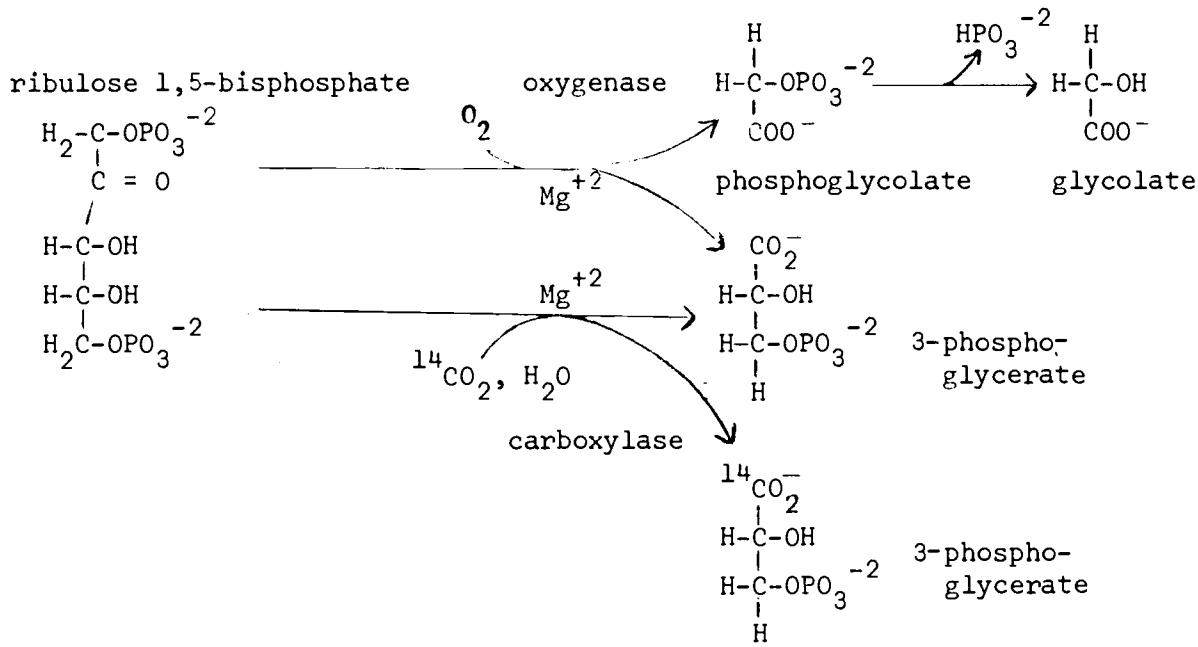


Equations 1a. and 1b. (Alberts and Vagelos, 1972) represent the two step reaction catalyzed by propionyl CoA carboxylase, and equations 2. and 3.

No page 2

represent reactions catalyzed by methylmalonyl CoA racemase (Barker, 1972) and methylmalonyl CoA mutase (Lehninger, 1970), respectively. Purified propionyl CoA carboxylase isolated from pig heart has a molecular weight of 770,000 and has one bound molecule of biotin per 175,000 daltons (Alberts and Vagelos, 1972). Presumably the enzyme is composed of four subunits. The biotin molecule is covalently bound to the carboxylase through the ϵ-amino group of lysine (Alberts and Vagelos, 1972). Methylmalonyl mutase is a B_{12}-containing enzyme (Barker, 1972). The presence of the cobalt-containing corrin ring of vitamin B_{12} coenzyme in methylmalonyl CoA mutase suggests an explanation for the significantly reduced capacity to oxidize propionate in cobalt-deficient cells of R. meliloti (De Hertogh et al., 1964) and also explains the cobalt requirements observed in symbiotically grown soybean plants (Ahmed and Evans, 1960) and in free-living Rhizobia (Lowe et al., 1960).

Another carboxylase which will be frequently mentioned in this thesis is ribulose 1,5-bisphosphate (RuBP) carboxylase. RuBP carboxylase is the most abundant enzyme in nature comprising more than 50% of the soluble leaf protein in plants (Jensen and Bahr, 1977). The purified enzyme from spinach (Jensen and Bahr, 1977) and from the hydrogen bacterium Alcaligenese eutrophus (Bowein et al., 1976) catalyses both the carboxylation of ribulose 1,5-bisphosphate to form two molecules of 3-phosphoglycerate and the oxygenation of RuBP to form one molecule of 3-phosphoglycerate and one molecule of phosphoglycolate as shown in the equations below (Jensen and Bahr, 1977).


ribulose 1,5-bisphosphate
oxygenase
O_2
Mg^{+2}
HPO_3^{-2}
phosphoglycolate
glycolate
Mg^{+2}
$^{14}CO_2$, H_2O
carboxylase
3-phospho-glycerate
3-phospho-glycerate

In plants phosphoglycolate is then dephosphorylated and converted to 3-phosphogylcerate via the enzymes of the glyoxylate cycle (Hatch, 1976). O_2 and CO_2 are competitive inhibitors of the carboxylation and oxygenation reactions, respectively (Jensen and Bahr, 1977). Recently Brändén (1978; Brändén and Brändén, 1978) claimed to have separated RuBP carboxylase and oxygenase activities from parsley using a DEAE A 25 Sephadex column at pH 8.3. However, this result has not been repeatable (Tolbert, 1978), and there is a large amount of accumulated evidence in favor of a single enzyme species catalyzing both activities (Jensen and Bahr, 1977).

RuBP carboxylase as isolated from higher plants is a large complex of molecular weight 557,000 composed of eight heavy subunits of 55,800 daltons each, and eight light subunits of 12,000 daltons each (Lane and Miziorko, 1978). The RuBP binding site, i.e. the catalytic center, appears to reside on the large subunit (Lane and Miziorko, 1978), while some data suggest that the small subunit may have a regulatory role (Schloss et al., 1979). The large subunit is catalytically active in the absence of the small subunit (Jensen and Bahr, 1977). Although RuBP carboxylase isolated from the hydrogen bacterium Alcaligenes eutrophus is approximately the same size and structure as the plant enzyme (Bowein et al., 1976), the RuBP carboxylases isolated from some other prokaryotes lack the smaller subunit and are composed of six to eight large subunits per molecule (Schloss et al., 1979; Jensen and Bahr, 1977). Rhodopseudomonas sphaeroides has been shown to produce two forms of RuBP carboxylase, one containing both large and small subunits, the other containing only large subunits (Schloss et al., 1979). The

smallest RuBP carboxylase yet found has been isolated from Rhodospirillum rubrum. A simple dimer of two 56,000 dalton subunits, the R. rubrum enzyme contains no small subunit (Schloss et al., 1979).

Experiments in which rates of ^{14}C incorporation into 3-phosphoglycerate by RuBP carboxylase were measured in the presence of $^{14}CO_2$ or $H^{14}CO_3^-$ showed that CO_2 was the preferred substrate of RuBP carboxylase (Lane and Miziorko, 1978). Until recently the activities reported for purified RuBP carboxylase were inadequate to explain the CO_2 fixation rates observed in nature, and the reported Km values for CO_2 were between 70 and 600 uM, or seven to 60 times the concentration of dissolved CO_2 in water in equilibrium with one atm of air containing 0.03% CO_2 at 25°C (Jensen and Bahr, 1977). It was found, however, that exposure of the enzyme to CO_2 and Mg^{+2} caused a conformational change which increased the affinity of substrate CO_2 for the enzyme and also increased the Vmax (Jensen and Bahr, 1977; Lane and Miziorko, 1978). Km values now reported for CO_2 for the activated enzyme are ten to 20 uM (Jensen and Bahr, 1977). Preincubation of RuBP carboxylase for five to ten min in saturating Mg^{+2} and CO_2 is thus necessary to insure that the fully active enzyme is observed (Jensen and Bahr, 1977).

Evidence suggests that the Calvin cycle is the major assimilatory pathway during growth of photosynthetic prokaryotic organisms and chemolithotrophic bacteria using CO_2 as the main or sole carbon source (McFadden, 1973). Two enzymes believed to be unique to the Calvin cycle are RuBP carboxylase and phosphoribulokinase (Schlegel and Eberhardt, 1972). In Pseudomonas facilis phosphoribulokinase is inhibited by AMP (McFadden and Tu, 1967) and phosphoenolpyruvate, while NADH has been

shown to be a positive allosteric effector (McFadden, 1973). Fructose-6-phosphate has been shown to activate RuBP carboxylase in plants (Hatch, 1976), and gluconate-6-phosphate has been shown to inhibit the RuBP carboxylases from several organisms (McFadden, 1973). Although plants use NADPH as reductant in the Calvin cycle, lithotrophic bacteria utilize NADH (McFadden, 1973). Evidence that RuBP carboxylase and phosphoribulokinase may not be coordinately regulated in P. facilis has been presented (McFadden, 1973).

The hydrogenase enzyme sytem of Rhizobium bacteroids. H_2 evolution via nitrogenase may consume up to 40% of the energy supplied to the N_2-fixing system in legumes (Schubert and Evans, 1976). Some legume-rhizobium symbioses, however, contain a H_2-recycling system which may reutilize some of the energy lost as H_2 from nitrogenase (Emerich et al., 1979). An uptake hydrogenase was first reported in Rhizobium leguminosarum bacteroids by Phelps and Wilson (1941). These initial findings were not confirmed by Shug et al. (1956) but were confirmed in a rediscovery by Dixon (1967). The extent of H_2 loss from nodules during N_2 fixation was shown to vary greatly among a series of legumes surveyed by Schubert and Evans (1976), and the level of hydrogenase in bacteroids was shown to be a major factor that prevented H_2 loss from nodules (Ruiz-Argüeso et al., 1978; Evans et al., 1977). Dixon (1972) postulated three roles for the hydrogenase system in N_2-fixing bacteroids. 1. Oxidation of hydrogen may scavenge excess oxygen and thus assist in maintaining an anaerobic environment for nitrogenase. 2. The hydrogenase system may prevent the accumulation of H_2 evolved by nitrogenase and thus prevent H_2 inhibition of nitrogenase. 3. The energy lost as H_2 by

nitrogenase may be recovered by oxidation of H_2 via the hydrogenase enzyme system. Protection of nitrogenase from O_2 damage by H_2 oxidation has been observed in blue-green algae and Azotobacter, and H_2-dependent ATP formation has been observed in blue-green algae, A. chroococcum and in R. leguminosarum bacteroids (Dixon, 1968). Recently Emerich et al. (1979) have reported that the oxidation of H_2 by R. japonicum bacteroids increased intracellular ATP levels and led to increased nitrogenase activity at increased optimal O_2 partial pressures. These results imply that the oxidation of H_2 can support nitrogenase activity and provide respiratory protection for the nitrogenase system in R. japonicum.

Oxidation of H_2 by bacteroids has been shown to inhibit respiratory CO_2 evolution thereby conserving carbon substrates (McCrae et al., 1978). Dixon (1968) observed inhibition of H_2 uptake when R. leguminosarum bacteroids were provided with pyruvate or succinate and suggested that electron flow from metabolism of substrates could compete with H_2 oxidation for carriers in the electron transport pathway. Ruiz-Argüeso et al. (1979) reported that succinate, and especially formate and acetate, caused inhibition of H_2 uptake in R. japonicum bacteroids. In contrast, Maier et al. (1979) found no inhibition of H_2 uptake on addition of succinate or any other carbon substrates tested in hydrogenase-derepressed free-living R. japonicum cells.

Electron acceptors for the hydrogenase system in bacteroids include O_2, methylene blue, potassium ferricyanide, phenazine methosulfate (PMS), 2,6-dichloroindophenol and triphenyl tetrazolium (Ruiz-Argüeso et al., 1979). Benzyl and methyl viologen did not serve as acceptors for H_2 uptake. In contrast to observations in Azotobacter

(Smith et al., 1976), neither C_2H_2 nor CO appeared to inhibit hydrogenase activity in R. japonicum bacteroids (Ruiz-Argüeso et al., 1979), however inhibitors of respiration such as NaCN, NH_2OH, and NaN_3 inhibited H_2 uptake when O_2 was the electron acceptor but did not appreciably inhibit H_2 uptake in the presence of non-physiological acceptors (Evans et al., 1978). Iodoacetate strongly suppressed ATP formation due to endogenous respiration in R. japonicum bacteroids but only partially inhibited ATP production from the oxidation of H_2 (Emerich et al., 1979). The uncoupler carbonyl cyanide m-chlorphenyl hydrazone (CCCP) was found to inhibit ATP production associated with both endogenous respiration and H_2 oxidation (Emerich et al., 1979).

Ruiz-Argüeso et al. (1979) have found that the ratio of H_2 consumed to O_2 consumed by H_2 oxidation in bacteroids is very nearly 2:1. The Km of the hydrogenase system for O_2 is difficult to determine accurately, however values between 2.5 and 1.3 uM have been observed (Ruiz-Argüeso et al., 1979; Evans et al., 1978). The uptake hydrogenase from R. japonicum is membrane-bound and catalyzes an irreversible reaction (Ruiz-Argüeso et al., 1979).

The hydrogen bacteria. The hydrogen bacteria are capable of living autotrophically with H_2 and CO_2 or heterotrophically with organic substrates (Schlegel and Eberhardt, 1972). These microorganisms are a very heterogeneous group and contain either a soluble or a membrane-bound hydrogenase, or both (Schlegel, 1976), and utilize CO_2 via ribulose-bisphosphate carboxylase and the pentose phosphate (Calvin) cycle. The most studied hydrogen bacterium, Alcaligenes eutrophus, contains both a soluble and a particulate hydrogenase (Schlegel and Eberhardt, 1972).

The soluble hydrogenase reversibly catalyzes the direct reduction of NAD^+ by H_2, while the particulate hydrogenase oxidizes H_2 via the respiratory chain with concommitant ATP production (Schlegel and Eberhardt, 1972). The primary acceptor for the particulate hydrogenase *in vivo* is not known, though the enzyme will reduce methylene blue (Schlegel and Eberhardt, 1972) and phenazine methosulfate (PMS) (Aragno and Schlegel, 1978). Mutants of *A. eutrophus* containing either the soluble or the particulate hydrogenase but not both were still able to grow autotrophically (Schink and Schlegel, 1978). Lack of the membrane-bound hydrogenase did not affect growth rates, however lack of the soluble hydrogenase resulted in decreased autotrophic growth rates. Evidence for reverse electron transport in membranes of *A. eutrophus* has been observed by Ishaque and Aleem (1970).

Bongers (1967), working with cell-free preparations of *Hydrogenomonas* H20, found that succinate and H_2 could serve as electron donors for oxidative phosphorylation. Evidence was provided for a unique phosphorylation site between H_2 and cytochrome b and also for the possible occurrence of a cytochrome b oxidase. From growth studies using *A. eutrophus* the oxidation of one mole of H_2 was calculated to yield two moles of ATP (Bongers, 1970). Ishaque and Aleem (1970) determined, however, that three phosphorylation sites are involved in H_2 oxidation in *A. eutrophus*. Recently Beatrice and Chappell (1979) using spectroscopic techniques have shown that *Hydrogenomonas* H20 has a branched respiratory pathway and two terminal oxidases, one CN^- sensitive and the other CN^- insensitive. Similar respiratory pathways exist in both autotrophically grown and fructose grown cells except that the latter

cells cannot oxidize H_2. The locations of phosphorylation sites were not investigated by these workers.

The stimulation of H_2 uptake by addition of CO_2 is a response known as the "Bartha effect" (Schlegel and Eberhardt, 1972). The molecular mechanism of this effect is unknown, though it does not appear to be due to the increased ATP demand of CO_2 fixation (Schlegel et al., 1970). Schink and Schlegel (1978) have shown that the "Bartha effect" is observed only in mutants of A. eutrophus containing the soluble hydrogenase. Interestingly, Aquaspirillum autotrophicum, which contains only the particulate hydrogenase does not exhibit the "Bartha effect" (Aragno and Schlegel, 1978).

The H_2 bacteria differ profoundly in their patterns of enzyme regulation (Schlegel and Eberhardt, 1972; Aragno and Schlegel, 1978). In Alcaligenes eutrophus no definite conclusions concerning the regulation of the hydrogenase enzymes have been found (Schlegel and Eberhardt, 1972), while RuBP carboxylase levels depend upon growth and substrates used. In Pseudomonas facilis low oxygen tensions are required for RuBP carboxylase expression (Schlegel and Eberhardt, 1972). Addition of H_2 to cultures of A. eutrophus supressed utilization of carbon substrates by repressing the enzymes of the Entner Doudoroff pathway (Schlegel and Trüper, 1966), and also inhibited fructose utilization by cells fully induced for this enzyme system (Schlegel and Eberhardt, 1972). The inhibitory effect of H_2 is believed to be attributable to a product of H_2 oxidation such as ATP or NADH (Schlegel and Eberhardt, 1972). This suppressive effect of H_2 on carbon substrate utilization is also seen in mutant strains of Alcaligenes eutrophus containing either the soluble

or the membrane-bound hydrogenase but not both (Schink and Schlegel, 1978). Thus it is not possible to attribute the suppressive effect of H_2 specifically to one of the hydrogenase enzymes.

The regulatory patterns observed in Aquaspirillum autotrophicum are entirely different from those observed in Acaligenes eutrophus. In A. autotrophicum RuBP carboxylase and hydrogenase are both entirely inducible by autotrophic growth conditions and are both repressed by heterotrophic growth (Aragno and Schlegel, 1978). Autotrophically grown cells contain only a particulate, membrane-bound hydrogenase capable of donating electrons to methylene blue or PMS but not to NAD^+ (Aragno and Schlegel, 1978). No stimulation of H_2 uptake by CO_2 is observed in autotrophically grown cells (Aragno and Schlegel,1978). In atmospheres containing 50% H_2, 10% CO_2, variable O_2 and the remainder N_2, hydrogenase induction is completely repressed by atmospheric O_2 concentrations greater than 30%, while hydrogenase induction rates continue to increase down to 2.5% O_2, the lowest O_2 concentration tested (Aragno and Schlegel, 1978). However in whole cells,hydrogenase activity, once induced, is not inhibited by O_2. The control exerted by O_2 on hydrogenase is believed to occur at the transcriptional level (Aragno and Schlegel, 1978). The effect of addition of carbon substrates during hydrogenase induction depends upon the substrate added. Gluconate had a repressive effect similar to that observed under high O_2 tensions; addition of succinate had no apparent effect on the rate of hydrogenase induction, while addition of citrate increased the rate of hydrogenase synthesis (Aragno and Schlegel, 1978). In A. autotrophicum the enzymes

of the Entner Doudoroff pathway are constitutively formed (Aragno and Schlegel, 1978).

Expression of hydrogenase activity in free-living Rhizobium japonicum.

Definition of the conditions necessary for expression of hydrogenase activity in free-living R. japonicum has been accomplished only recently (Maier et al., 1978a). Expression of enzyme activity occurred only when cells were cultured in a medium containing a low concentration of carbon substrates and provided with a source of H_2 and low O_2 partial pressures (Maier et al., 1978a). Nitrogenase activity was not necessary for expression of hydrogenase activity provided H_2 was added exogenously, though cells with nitrogenase activity did contain measurable amounts of hydrogenase (Maier et al. 1978a). Repression of hydrogenase in R. japonicum results from high partial pressures of O_2 and excessive levels of carbon substrates (Maier et al. 1978a; Maier et al., 1979).

Since some similarities were observed in the physiological characteristics of H_2 uptake positive Rhizobium strains and previously characterized hydrogen bacteria, we decided to investigate the activities of several carboxylases in R. japonicum SR during different growth conditions. This thesis describes the coordinate derepression[1] of hydrogenase and RuBP carboxylase in an H_2-uptake positive strain of R. japonicum that was grown under free-living conditions.

[1]In order to obtain expression of hydrogenase in free-living R. japonicum it is necessary to lower the partial pressure of O_2 and supply a decreased level of carbon substrates. In addition it is necessary to

1 (cont.)

supply H_2. Expression of activity, therefore, is a consequence of a combination of derepression and induction. In this thesis I am using the term "derepression" unless the effect is known to be specifically related to the induction of enzyme activity by H_2.

MATERIALS AND METHODS

Rhizobium strains. Rhizobium japonicum strain USDA 122 DES (hereafter referred to as 122 DES) is a small, single colony isolate obtained by detergent treatment of R. japonicum strain USDA 122. The original culture of USDA 122 was obtained from Dr. Deane Weber of the U. S. Department of Agriculture and the DES isolate from this culture was prepared by Dr. David W. Emerich of this laboratory (see Emerich et al., 1979). Strain SR is a kanamycin and streptomycin resistant mutant derived from the 122 DES parent strain by Dr. John R. Postgate. Strains SR2 and SR3 are H_2-uptake negative and kanamycin and streptomycin resistant strains of R. japonicum derived from the SR parent by procedures described by Maier et al. (1978b). The organisms used in the various experiments retained their antibiotic resistance characteristics during the different growth conditions and produced a single colony type on yeast extract-mannitol agar plates.

Chemicals. $NaH^{14}CO_3$ of specific activity of 0.083 mCi per mg was obtained from New England Nuclear Corporation, Boston, Mass. Biochemicals were obtained from Sigma Chemical Corporation, St. Louis, and other chemicals from Matheson, Coleman and Bell, Norwood, Ohio; Mallinckrodt Chemical Corporation, St. Louis, Missouri; J. T. Baker Chemical Company, Phillipsburg, New Jersey, and Packard Instruments Company, Inc., Downers Grove, Illinois.

Cultural and depression conditions. All cells were grown on slants or plates containing the H_2 uptake medium described by Maier et al. (1978a) except that 1.5% Noble agar from Difco (Detroit, Mich.) was

used. The cells were grown and derepressed for hydrogenase and ribulosebisphosphate (RuBP) carboxylase enzymes by one of two procedures: In procedure A, cultures on agar slants or plates were grown in air at 28°C for three days then were placed in 3.8 liter jars containing an atmosphere of 10% H_2, 5% CO_2, 1% O_2 and 84% N_2 for two or three additional days prior to harvest. In procedure B, cells from cultures grown in air for four or five days were removed from plates by washing and diluted to an optical density of 0.5 (540 nm) with 0.05 M potassium phosphate buffer, pH 7.0, containing 2.5 mM $MgCl_2$ (hereafter referred to as K-phosphate-$MgCl_2$ buffer). A sample of 25 ml of this suspension was then placed in a 250 ml bottle which was sealed with a serum stopper and flushed for 10-15 minutes with N_2. Sufficient H_2, CO_2 and O_2 were added to obtain an atmosphere composed of 9.3% H_2, 3.3% CO_2, 0.9% O_2 and 86.5% N_2 (after equilibration). These bottles were then placed on a rotary shaker at 28°C for the desired incubation period (see tables and figures). Cells not induced for hydrogenase were subjected to conditions identical with those in procedure A or B above, except that N_2 replaced H_2 in the gas phase. All cultures were five or six days-old when hydrogenase and carboxylase assays were performed.

Preparation of cell-free extracts. Cell-free extracts were prepared by methods outlined by McFadden and Tu (1965). Cells grown on agar plates or slants were removed by washing with 0.85% NaCl, thoroughly mixed, centrifuged at 8,000 x g for 20 minutes, and then resuspended with 2 to 3 ml of freshly prepared 0.1 M N-tris (hydroxymethyl)-methylglycine (Tricine) buffer, pH 7.5, containing 2 mM reduced glutathione (GSH). This suspension was kept in an ice bath, passed twice through a French

pressure cell at 1125 kg per cm^2 at 4^o and then centrifuged at 13,000 x g for 20 minutes at 0 to 2^o. The supernatant liquid was stored in an ice bath until it was used for carboxylase or protein assays.

Cells derepressed in bottles by procedure B were centrifuged at 8,000 x g, resuspended in 0.1 M Tricine buffer, pH 7.5, centrifuged again at 8,000 x g and resuspended in 3 ml of freshly prepared 0.1 M Tricine buffer, pH 7.5, containing 2 mM GSH. The cells in this suspension were disrupted by passing twice through a French pressure cell and extracts were prepared as described above.

Bacteroids were isolated from soybean nodules by the methods of Klucas et al. (1967) except that the pH of the buffered-ascorbate medium was adjusted to pH 6.9. Bacteroids were then washed in 0.1 M Tricine buffer, pH 7.5, prior to resuspension in 0.1 M Tricine buffer, pH 7.5, containing 2 mM GSH. The cells were disrupted by passing through a French pressure cell as described for free-living cells above and centrifuged at 13,000 x g for 20 minutes. The supernatant liquid was collected and stored as described above.

Carboxylase assays. Assays for four different carboxylase activities were performed by published methods as follows: RuBP carboxylase, Wishnick and Lane, 1971; propionyl CoA carboxylase, Lane and Halenz, 1962; phosphoenolpyruvate (PEP) carboxylase and PEP carboxykinase, Vennesland, 1962. Reaction volumes of 1 ml contained the following additions in umoles unless otherwise indicated. Tricine buffer, pH 8.0, 100; $MgCl_2 \cdot 6H_2O$, 5.0; (ethylenedinitrilo)-tetraacetic acid disodium salt (Na_2EDTA) 0.03; reduced glutathione, 3; extract, 0.2 ml containing approximately 0.3 mg protein; $NaHCO_3$, 35.7 containing 0.07 uCi of ^{14}C per

umole. The following substrates in umoles were added to the designated assays with starred (*) reagents added to initiate reactions: assays for RuBP carboxylase, RuBP* 0.25; assays for propionyl CoA carboxylase, propionyl CoA* 0.25 and tris (hydroxymethyl)-aminomethane salt of ATP, 5; assays for PEP carboxylase, phosphoenolpyruvate* 0.25; assays for PEP carboxykinase, phosphoenolpyruvate* 0.25 and Na_2ADP 5. Control reactions were conducted in which appropriate substrates or cell-free extracts were omitted. In order to conserve reagents, some reactions were conducted in a final volume of 0.27 ml rather than 1 ml. The concentration of components in these reaction mixtures were the same as in the 1 ml reactions with the exception that 13.3 umoles of $NaHCO_3$ containing 0.047 uCi of ^{14}C per umole were added. The two assays gave comparable results. Before adding substrates, reaction mixtures were preincubated in a water bath at 30° for 10 minutes. Substrates were then added and reactions were incubated at 30° for 30 minutes, then terminated by the addition of 0.4 ml 6 N HCl (1 ml assay volumes) or 0.1 ml of 6 N HCl (0.27 ml assay volumes). An aliquot of each reaction mixture was transferred to a Whatmann 3 MM filter paper disc, dried and transferred to a scintillation vial (Davidson, 1962). Radioactivity was determined as described below. Although rates of RuBP-dependent $^{14}CO_2$ fixation at varying RuBP concentrations were not determined, the quantities of $^{14}CO_2$ fixed in RuBP carboxylase assays were approximately linear with the period of incubation (Figure 1), and reaction rates were proportional to the volume of enzyme extract added (Figure 2).

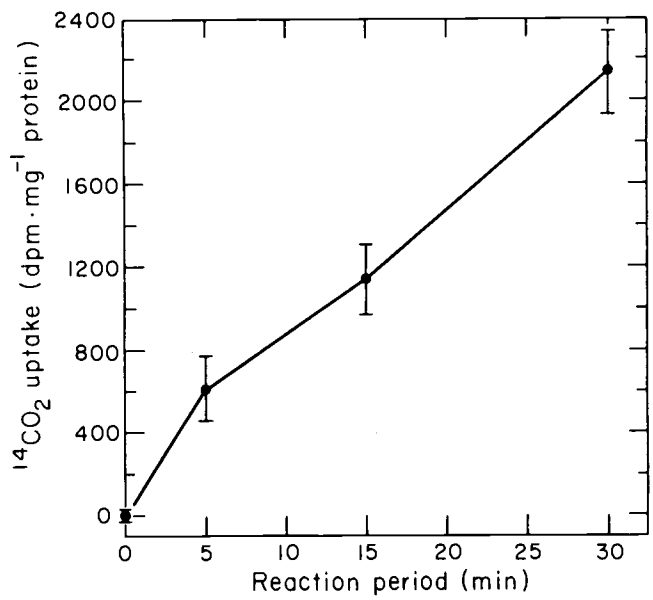


Figure 1. The relationship between the amount of $^{14}CO_2$ fixed by RuBP carboxylase and the period of incubation. RuBP carboxylase assays were performed as described in Materials and Methods. Reactions were terminated at 5, 15 and 30 min. Values reported are means (± SEM) of 3 replicate determinations and were corrected for $^{14}CO_2$ incorporation in assays without added RuBP.

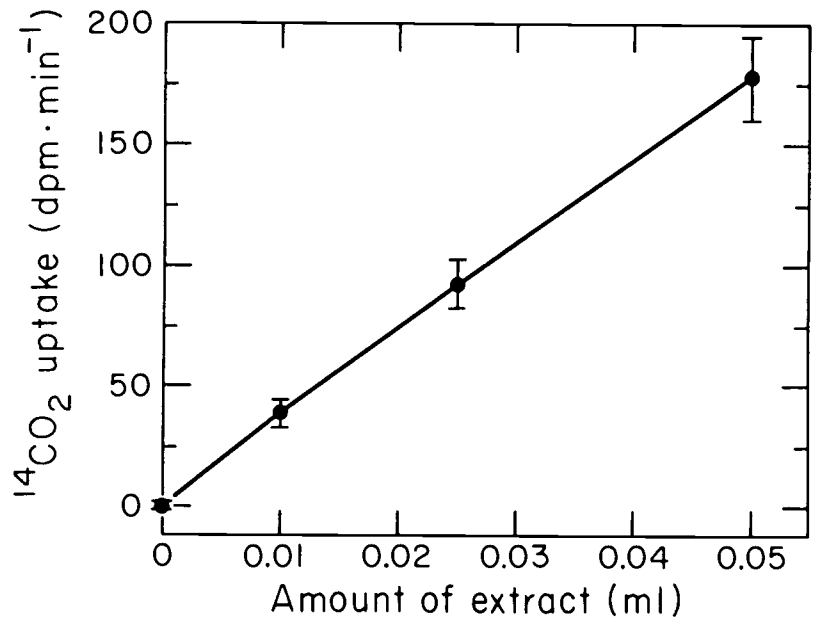


Figure 2. The relationship between the amount of extract added to assays and the rate of $^{14}CO_2$ fixation by RuBP carboxylase. RuBP carboxylase assays were performed as described in Materials and Methods. Reactions were terminated after 30 min. Values reported are means (± SEM) of 3 replicate determinations and are corrected for $^{14}CO_2$ incorporation in assays without added RuBP.

Hydrogen uptake assays. H_2-uptake assays were performed with whole cell suspensions using an amperometric method (Wang et al., 1971; Schubert and Evans, 1976). Cells from cultures were suspended in K-phosphate-$MgCl_2$ buffer, pH 7.0, and the suspension was added to the 5.8 ml chamber of the amperometric device. H_2 and O_2 uptake rates were monitored after injection of 75.4 nmoles of H_2 and 122 nmoles of O_2 each as saturated solutions in K-phosphate-$MgCl_2$ buffer at pH 7.0.

Protein determinations. Whole cells were hydrolyzed with NaOH using the procedure described by Stickland (1951). Proteins in cell hydrolysates or proteins in cell-free extracts were determined by the microbiuret method of Goa (1953) or by the method of Lowry et al. (1951). Bovine serum albumin was used as the standard in both cases.

^{14}C uptake determinations. For the determination of ^{14}C in whole cells on slants, stoppers were removed and slants were placed in an ice bath to terminate reactions. The cells were then rapidly removed by washing with two 2.5 ml aliquots of 0.85% NaCl and thoroughly mixed. Samples were then prepared for counting using one of the following methods. In the experiment described in Table 1, cell suspensions were filtered through a 0.45 micron Millipore filter and the filter discs washed with two 1.0 ml portions of 0.85% NaCl. Filter discs containing cells were then dried and added to scintillation vials for counting. In the experiments described in Table 2 and Figure 3 known aliquots of cell suspensions were taken to dryness in the scintillation vials before adding fluor. Identical samples counted using both of these methods showed the latter method to give fewer counts by a factor of 0.576, thus

values in Table 2 and Figure 3 have been corrected for this factor. Samples of cell-free extracts were prepared for counting as described in the "Carboxylase assay" section of Materials and Methods.

Each scintillation vial received 5 ml of scintillation-grade toluene containing 4.0 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene per liter. The radioactivity was determined by use of a Packard 2425 scintillation spectrometer. Values reported as disintegrations per minute (dpm) were determined from counting efficiencies which ranged from 0.68 to 0.76. Samples were counted for a 10 minute period. The background radioactivity usually ranged from 25 to 30 counts per minute. Control carboxylase assays lacking added substrates had radioactivities ranging no more than 5% above background.

RESULTS

CO_2 fixation in whole cells. In an initial experiment it was shown that R. japonicum cells that had been induced for hydrogenase and supplied with H_2 during the assay period showed markedly higher rates of $^{14}CO_2$ fixation than cells that had not been induced for hydrogenase (Table 1). Hydrogenase non-induced cells and induced cells assayed without H_2 fixed CO_2 at approximately the same rates. In an experiment where a saturating partial pressure of 0.05 atm CO_2 was added over cultures, the rate of CO_2 fixation by hydrogenase-induced cells assayed in the presence of H_2 was 6.0 times higher than the rate observed with comparable induced cells that were not provided with H_2 during the CO_2 fixation assay period. These results (Table 1) suggested the possibility that the increased CO_2 fixation rates in cells induced for hydrogenase and assayed with H_2 were dependent upon H_2 as an exogenous energy source. However, in another experiment (Table 2) it was found that the addition of 15 mM succinate in place of H_2 to either hydrogenase induced or non-induced cells markedly stimulated the rates of CO_2 fixation. Since CO_2 fixation requires energy, the stimulatory effects of H_2 or succinate on CO_2 fixation may be related to increased ATP level (Emerich et al., 1979).

The effect of the partial pressure of CO_2 on CO_2 uptake in hydrogenase-induced and non-induced whole cells of R. japonicum is shown in Figure 3. The rates of fixation are higher in hydrogenase induced cells than in non-induced cells at all CO_2 concentrations tested. CO_2 fixation rates appear to reach a maximum value at 5% atmospheric CO_2 (after equilibration with a pH 7.5 medium) in hydrogenase-induced or non-induced

Table 1. The effect of H_2 addition on $^{14}CO_2$ uptake by hydrogenase induced and non-induced R. japonicum SR[a]

Type of Cells	H_2 uptake (nmoles·min^{-1}·mg^{-1} protein)	$^{14}CO_2$ uptake (dpm·min^{-1}·mg^{-1} protein)	
		With 10% H_2	Without H_2
Induced for hydrogenase	66	50.7 ± 2.5	8.2 ± 1.0
Not induced for hydrogenase	<0.5	8.8 ± 1.6	8.2 ± 1.1

[a] Cells were cultured on slants for three days and induced for hydrogenase activity for two additional days as described in procedure A of Materials and Methods. Cells not induced for hydrogenase activity were placed under comparable conditions except that N_2 was added in place of H_2. After equilibration of culture tubes in air for 15 minutes, each was sealed with a serum stopper and gases added to obtain final equilibration partial pressures of 10% H_2, 5% CO_2, 20% O_2, 65% N_2 (cultures with H_2) or 5% CO_2, 20% O_2, 75% N_2 (cultures without H_2). At the start of the incubation period sufficent $^{14}CO_2$ was added to each culture to obtain a specific activity of 13 uCi per mmole CO_2. The assays were allowed to run 4.5 h at 28°C and then stoppers were removed and culture tubes were placed into an ice bath. Cells were then removed and $^{14}CO_2$ uptake was determined as described for whole cells in Materials and Methods. Immediately before initiation of the $^{14}CO_2$ uptake experiments,

Footnotes for Table 1. (cont.)

H_2 uptake rates were measured as described in Materials and Methods. $^{14}CO_2$ uptake rates are means (± SEM) of results from 4 cultures and have been corrected for background counts. Total nmoles CO_2 fixed·min^{-1} ·mg^{-1} protein may be calculated by multiplying dpm·min^{-1}·mg^{-1} protein values by 0.035 nmoles CO_2·dpm^{-1}. A comparable experiment produced similar results.

Table 2. Effect of succinate and H_2 on $^{14}CO_2$ uptake by hydrogenase induced and non-induced cells of R. japonicum SR [b]

Type of cells	H_2 uptake (nmoles·min^{-1}·mg^{-1} protein)	$^{14}CO_2$ uptake (dpm·h^{-1}·mg^{-1} protein) in assays with:		
		No H_2 or succinate	H_2	Succinate
Induced for hydrogenase	19.9	45 ± 1	359 ± 24	919 ± 94
Not induced for hydrogenase	<0.5	85 ± 18	85 ± 4	571 ± 37

[b] Hydrogenase induced cells were grown on plates and induced for hydrogenase for 15 h by procedure B in Materials and Methods. Non-induced cells were subjected to comparable conditions, except that N_2 replaced H_2. In assays for $^{14}CO_2$ fixation, 2 ml of cell suspension were added to each 6 ml vial before sealing with a serum stopper. Additions were made as follows: vials with H_2, 10% H_2, 1% O_2 and 84% N_2; vials with succinate; sufficient sodium succinate to obtain a final concentration of 15 mM succinate, 10% H_2, 1% O_2 and 84% N_2; vials lacking H_2 and succinate, 10% Ar, 1% O_2 and 84% N_2. Sufficient $NaHCO_3$ was added to the vials to obtain 5% CO_2 in the gas phase (after equilibration) and reactions were initiated by adding 0.36 umoles of $NaH^{14}CO_3$ of specific activity 7 uCi ·umole^{-1} in a final volume of 2.3 ml (pH 7.0). After incubation for 3 h reactions were terminated by the addition of 6 N HCl and $^{14}CO_2$ uptake was determined as described in Materials and Methods. Total nmoles of CO_2 fixed·h^{-1}·mg^{-1} protein may be determined

Footnotes for Table 2. (cont.)

by multiplying $dpm \cdot h^{-1} \cdot mg^{-1}$ protein by 4.5×10^{-2} nmoles $CO_2 \cdot dpm^{-1}$. Values for $^{14}CO_2$ fixation are means ($\pm$ SEM) of 3 replicate assays. Rates of H_2 uptake of the cell suspensions were determined as described in Materials and Methods.

Figure 3. CO_2 saturation of CO_2 uptake in hydrogenase induced and non-induced whole cells of R. japonicum SR. Activities plotted are the following: ●——● cells induced for hydrogenase; o——o cells not induced for hydrogenase. Cells were cultured and induced or not induced for hydrogenase on slants as described in Table 1. Gases were added to tubes as described in Table 1 with the exception that CO_2 levels were varied, being 0%, 0.03%, 0.5%, 1%, 2%, 3%, and 5% in the atmosphere after equilibration with pH 7.5 medium and prior to addition of $^{14}CO_2$. Tubes with 0% CO_2 were flushed for 20 min with a stream of N_2 to remove air. Assays were initiated by adding 0.357 umole $^{14}CO_2$ of specific activity 7.0 uCi umol^{-1} CO_2. Final specific activities in dpm·nmole^{-1} CO_2 were 1.555×10^4, 3.666×10^3, 2.618×10^2, 1.384×10^2, 70.53, 47.41, and 28.57 in the 0%, 0.03%, 0.5%, 1%, 2%, 3%, and 5% tubes, respectively. Assays were run for 4 h and then terminated as described in Materials and Methods. 6N HCl was added to cell suspensions in scintillation vials prior to evaporating to dryness and counting as described in Materials and Methods. Points represent means of three replicate determinations and have been corrected for background counts. H_2 uptake rates measured immediately before starting assays were 46.3 nmol $H_2 \cdot min^{-1} \cdot mg^{-1}$ protein for hydrogenase induced cells and <0.5 nmole $H_2 \cdot min^{-1} \cdot mg^{-1}$ protein for non-induced cells.

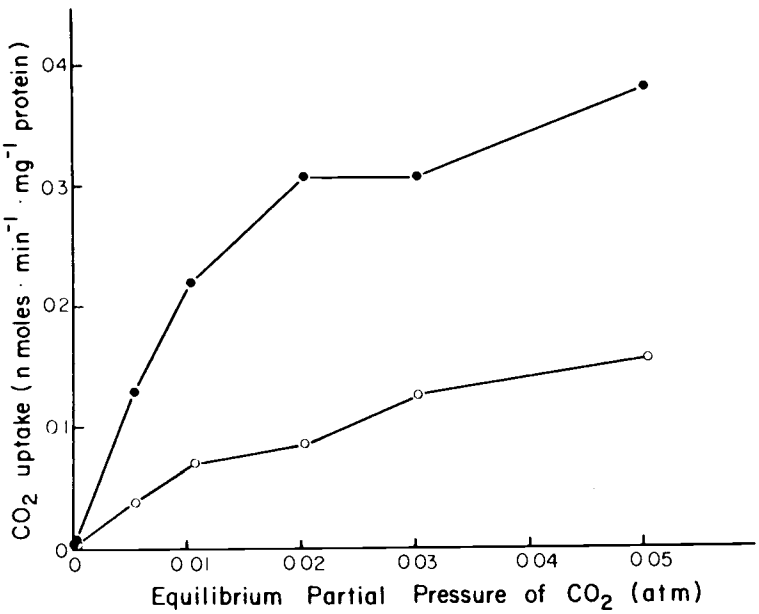


Figure 3. CO_2 saturation of CO_2 uptake in hydrogenase induced and non-induced whole cells of R. japonicum SR.

cells. For this reason, all whole cell assays were conducted under atmospheres containing 5% CO_2.

Effect of CO_2 on H_2 uptake. It has been reported that the addition of CO_2 to suspensions of certain hydrogen bacteria increased the rate of H_2 uptake, and this response has been referred to as the "Bartha effect" (Schlegel and Eberhardt, 1972). In an experiment designed to determine whether the "Bartha effect" could be observed with hydrogenase-induced R. japonicum SR, the addition of increasing amounts of $NaHCO_3$ to an amperometric assay chamber which contained cells induced for hydrogenase did not appreciably affect the rate of H_2 uptake during the assay period (Figure 4). Thus, no evidence of the "Bartha effect" was observed in R. japonicum SR. The data in Figure 4, however, demonstrates an O_2 requirement for H_2 oxidation by R. japonicum SR.

Carboxylase activities. It seemed essential to examine R. japonicum SR for enzymes considered most likely to participate in CO_2 fixation reactions. Results of assays for four different carboxylases in cell-free extracts from hydrogenase induced and non-induced R. japonicum cells are presented in Table 3. RuBP carboxylase activity was observed only in hydrogenase-induced R. japonicum cells. This observation provides a logical basis for interpretation of the differences in rates of CO_2 fixation between hydrogenase induced and non-induced cells reported in Table 1. In the experiment described in Table 3 no indication of PEP carboxylase or PEP carboxykinase activities was observed in reactions containing cell-free extracts of free-living R. japonicum SR. A control experiment similar to that described in Table 3 where an extract of the

Figure 4. The influence of the concentration of added bicarbonate on the rate of H_2 uptake by suspensions of R. japonicum strain SR. Slants of R. japonicum SR were grown for three days and derepressed for hydrogenase activity for an additional three days in an atmosphere of 10% H_2, 5% CO_2, 1% O_2 and 84% N_2 (see Materials and Methods, derepression procedure A). After removing cells and suspending in K-phosphate-$MgCl_2$ buffer (0.05 M potassium phosphate, pH 7.0, containing 2.5 mM $MgCl_2$), 15 ml of this suspension was added to a sealed 21 ml vial and flushed for 20 min with N_2. The H_2 uptake assay was performed in a 5.8 ml chamber by the method described in Materials and Methods. The chamber was flushed with N_2 until no O_2 was detected by the O_2 electrode. Syringes used for all transfers were flushed several times with N_2 prior to additions. The suspension of cells (.045 mg protein per ml) was then added to the closed chamber. H_2 saturated K-phosphate-$MgCl_2$ buffer (100 ul) containing 75.4 nmoles H_2 was then added to the chamber and the instrument was allowed to equilibrate. The H_2 uptake reaction was initiated by adding 122 nmoles of O_2 (100 ul of O_2-saturated K-phosphate-$MgCl_2$ buffer). The following aliquots (ul) of 0.70 M $NaHCO_3$ were added sequentially at the indicated arrows: A, 10; B, 10; C, 20; D, 20; F, 40; G, 40. The final concentrations of $NaHCO_3$ in solution in the chamber after all additions was 18 umoles per ml. The rate of H_2 uptake was 37 nmoles$\cdot min^{-1}\cdot mg^{-1}$ protein throughout the experiment.

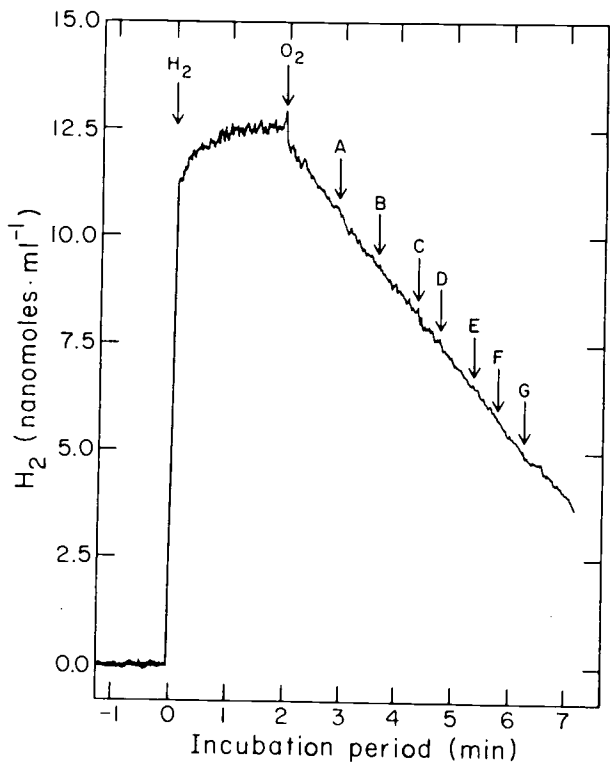


Figure 4. The influence of the concentration of added bicarbonate on the rate of H_2 uptake by suspensions of R. japonicum strain SR.

Table 3. Carboxylase activities of R. japonicum SR cells induced and non-induced for hydrogenase[c]

Enzyme	Rates of $^{14}CO_2$ fixation (dpm·min^{-1}·mg^{-1} protein)	
	Cells induced for hydrogenase	Cells not induced for hydrogenase
PEP carboxylase	0 ± 5	-5 ± 1
RuBP carboxylase	296 ± 14	-1 ± 4
Propionyl CoA carboxylase	243 ± 13	151 ± 16
PEP carboxykinase	-7 ± 1	3 ± 3

[c] Cells were induced for hydrogenase by derepression procedure A of Materials and Methods. Cells not induced for hydrogenase were grown under similar conditions except that N_2 replaced H_2. Hydrogenase induced and non-induced cells showed rates of H_2 uptake of 49.5 and <0.5 nmoles·min^{-1}·mg^{-1} protein, respectively, in assays performed by an amperometric method (Materials and Methods). Cell-free extracts were prepared and carboxylase assays determined as described in Materials and Methods. Control assays were conducted in which substrates for PEP carboxylase, RuBP carboxylase, propionyl CoA carboxylase and PEP carboxykinase were omitted. The rates of $^{14}CO_2$ fixation presented are means (± SEM) of three replicate determinations and have been corrected for fixation obtained in assays lacking substrates. $^{14}CO_2$ fixation as dpm·min^{-1}·mg^{-1} protein may be converted to nmoles CO_2 fixed·min^{-1}·mg^{-1} protein by multiplying values by 6.43 x 10^{-3} nmoles CO_2·dpm^{-1}.

non-bacteroid (cytosol) fraction of soybean nodules was assayed showed high PEP carboxylase activity (Peterson et al., 1979). Propionyl CoA carboxylase was easily detected in extracts of both hydrogenase-induced and non-induced cells (Table 3).

Before a time course induction of hydrogenase and RuBP carboxylase experiment could be run, methods had to be developed for reproducibly obtaining high rates of H_2 uptake in large volumes of cells. Maier, et al., (1979) utilized a method in which 6 ml of cell culture were induced for H_2 uptake activity, however, a method for inducing 75 ml or more of cell suspension was required. Table 4 shows results of H_2 uptake derepression experiments in which cell optical density at 540 nm, volume of cell suspension per bottle, and shaking rates were varied. Results from stationary cultures after a 16 h derepression period showed that rates of H_2 uptake increased as cell optical densities and volumes of cell suspension decreased. Further, shaking cultures increased H_2-uptake rates 4 to 18 fold over rates observed in standing cultures. These results implied that diffusion of an essential component becomes rate limiting in derepression of H_2-uptake ability in standing cultures.

In a more refined experiment, the induction of uptake hydrogenase and RuBP carboxylase activities was followed at 4 h intervals during a 24 h period (Figure 5). After the first 4 h interval, hydrogenase and RuBP carboxylase activities rapidly increased in an approximately parallel pattern. The two enzymes, therefore, appeared to be coordinately induced. In contrast, the activity of propionyl CoA carboxylase remained relatively constant during the 24 h derepression period.

The specific activity of RuBP carboxylase in the experiment

Table 4. Effects of varying derepression conditions on rates of H_2 uptake in R. japonicum SR[d]

Culture conditions during derepression	Volume (ml)	Optical density of cultures at 540 nm	H_2 uptake (nmoles·h^{-1}·10^8 $cells^{-1}$)
Stationary	6	0.85	20.3 ± 0.2
Stationary	6	0.50	26.7 ± 0.3
Stationary	6	0.20	52.1 ± 4.5
Stationary	12	0.50	15.4 ± 0.4
Stationary	18	0.50	7.6 ± 0.4
Stationary	24	0.50	4.8 ± 0.2
Shaking	6	0.50	106.9 ± 0
Shaking	30	0.50	86.7 ± 5.9
Shaking	60	0.50	66.7 ± 0

[d] Cells grown for four days on slants in air were washed from slants, combined and mixed, and then diluted to the optical densities indicated with K-phosphate-$MgCl_2$ buffer. Volumes of these suspensions were placed into 250 ml bottles as indicated, sealed with a serum stopper, and flushed for 10-15 minutes with N_2. Sufficient H_2, CO_2, and O_2 were added to obtain an atmosphere of 4.7 H_2, 1.7% CO_2, 0.9% O_2, and 92.7% N_2 (after equilibration). These bottles were then allowed to stand or were placed on a rotory shaker at 28°C for 16 h. At the end of this time period, stoppers were removed and H_2-uptake assays performed as

Footnotes for Table 4. (cont.)

described in Materials and Methods. Values are means (± SEM) of two replicate bottles.

Figure 5. Time course of induction of hydrogenase and RuBP carboxylase in R. japonicum SR. Activities plotted are represented as follows: ●———● hydrogenase, x———x RuBP carboxylase; o———o propionyl CoA carboxylase. Cells were grown five days on plates and induced for hydrogenase and RuBP carboxylase activities as described in procedure B of Materials and Methods. H_2 uptake measurements were conducted with cell suspensions by use of the amperometric procedure described in Materials and Methods. At indicated intervals, cells were removed from derepression conditions, cell-free extracts prepared and RuBP and propionyl CoA carboxylase assays conducted as described in Materials and Methods. The dpm values for RuBP and propionyl CoA carboxylase activities were corrected for activities in reaction mixtures lacking RuBP or propionyl CoA respectively. Each point plotted is the mean ($\pm$ SEM) of three replicate determinations. Total CO_2 fixed in nmoles$\cdot$ $min^{-1}\cdot mg^{-1}$ protein may be determined by multiplying dpm values by 9.657 x 10^{-3} nmoles $CO_2\cdot dpm^{-1}$. Similar results were obtained in a duplicate experiment.

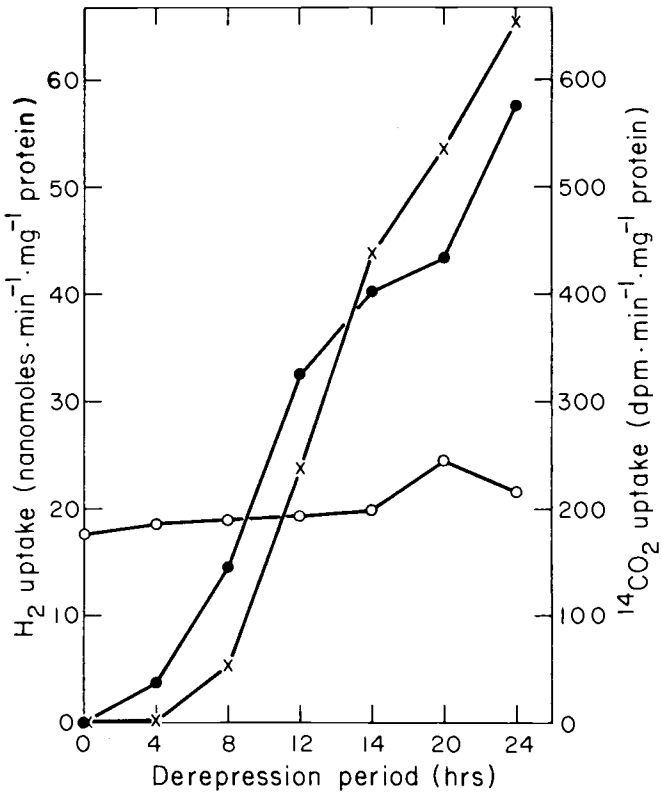


Figure 5. Time course of induction of hydrogenase and RuBP carboxylase in R. japonicum SR.

reported in Figure 5 is considerably higher than that observed in the experiment described in Table 3. The lower specific activity of RuBP carboxylase in Table 3 very likely was caused by the presence of carbon substrates in the medium used during derepression (derepression method A).

Factors affecting expression of RuBP carboxylase. Addition of H_2 or succinate to hydrogenase containing R. japonicum nodule bacteroids has been shown to increase intracellular levels of ATP (Emerich et al., 1979). A possible explanation for the coordinate induction of RuBP carboxylase and hydrogenase in R. japonicum SR was the assumption that H_2 oxidation via the hydrogenase system produced an essential metabolite (i.e. ATP) that promoted RuBP carboxylase synthesis. This hypothesis was examined by assaying for hydrogenase and RuBP carboxylase activity in R. japonicum SR after 24 h of derepression in the presence and absence of each of the following: H_2; succinate; or a combination of H_2 and succinate (Table 5). As expected cells supplied with H_2 during derepression showed easily measurable H_2 uptake and RuBP carboxylase activities, and cells not supplied with H_2 or succinate during the derepression period showed negligible H_2 uptake or RuBP carboxylase activities. No appreciable RuBP carboxylase or hydrogenase activities were observed, however, in cells supplied with succinate, and no detectable RuBP carboxylase and decreased hydrogenase activities were observed in cells supplied with a combination of H_2 and succinate. These latter results indicate that 10 mM succinate not only failed to induce the activities of these enzymes, but that it actually repressed the expression of both hydrogenase and RuBP carboxylase in R. japonicum SR

Table 5. Succinate repression of RuBP carboxylase and hydrogenase in *R. japonicum* SR[e]

Conditions of cultures during derepression period	H_2 uptake (nmoles·min^{-1}·mg^{-1} protein)	RuBP carboxylase (dpm·min^{-1}·mg^{-1} protein)	Propionyl CoA (dpm·min^{-1}·mg^{-1} protein)
Without H_2 or succinate	<0.5	2 ± 3	118 ± 9
With H_2	49	319 ± 7	121 ± 12
With succinate	<0.5	-2 ± 3	117 ± 3
With H_2 and succinate	12	5 ± 3	106 ± 4

[e] Cells were grown for four days on plates in air prior to derepression in bottles as described in procedure B of Materials and Methods. Each bottle contained 25 ml of a cell suspension of *R. japonicum* SR (OD 0.55 at 540 nm), 3.3% CO_2 (after equilibration), 0.9% O_2, plus the following: bottles without H_2 or succinate, 95.8% N_2; bottles with H_2, 9.3% H_2 and 86.5% N_2; bottles with succinate, 10 mM succinate and 95.8% N_2; bottles with H_2 and succinate, 9.3% H_2, 10 mM succinate and 86.5% N_2. The bottles were incubated with shaking for 24 h at 28°C. Hydrogen uptake rates on whole cells and carboxylase assays with cell-free extracts were performed as described in Materials and Methods. Rates of $^{14}CO_2$ fixation are means (± SEM) of 3 replicate determinations and were corrected for $^{14}CO_2$ fixation rates in assays lacking substrates. $^{14}CO_2$ fixation rates in dpm·min^{-1}·mg^{-1} protein may be converted to nmoles CO_2 fixed·min^{-1}·mg^{-1} protein by multiplying values by 9.66 x 10^{-3} nmoles CO_2·dpm^{-1}. A replicate experiment gave similar results.

supplied with H_2. In the experiment described in Table 5, the various derepression and repression conditions had no appreciable effect on the level or activity of propionyl CoA carboxylase.

An experiment was conducted to determine whether the addition of H_2 during the derepression period would induce the synthesis of RuBP carboxylase in mutant strains of R. japonicum which were derived from R. japonicum SR and known to be H_2-uptake negative (Hup^-) (Table 6). No appreciable RuBP carboxylase or hydrogenase activities were detected in the two Hup^- strains of R. japonicum tested, but activities of both these enzymes were observed in Hup^+ SR strains of R. japonicum. Although the specific lesions in the Hup^- mutant strains have not been completely defined (Maier et al., 1978) these data suggest a regulatory relationship in the synthesis of both hydrogenase and RuBP carboxylase.

The experiment described in Table 7 was designed to determine whether RuBP carboxylase could be detected in bacteroids isolated from nodules of soybeans inoculated with R. japonicum 122 DES or R. japonicum SR. Freshly prepared bacteroids formed in soybean nodules by either 122 DES or SR actively oxidized H_2, but no RuBP carboxylase activity was detected in three different preparations using either the procedure of Klucas et al. (1967) or a modification of this procedure in which bovine serum albumin was added to buffers used for the isolation of bacteroids and to buffers used for the preparation of cell-free extracts (Laane et al., 1978). RuBP carboxylase activity was present in extracts of hydrogenase-induced free-living 122 DES and propionyl CoA carboxylase was present in all extracts of either bacteroids or free-living 122 DES.

Table 6. The effect of H_2 on the expression of hydrogenase and RuBP carboxylase activities in H_2-uptake positive and H_2-uptake negative strains of R. japonicum[f]

R. japonicum strain	Conditions during derepression	H_2 uptake (nmoles·min^{-1}·mg^{-1} protein)	RuBP carboxylase activities ($^{14}CO_2$ uptake in dpm·min^{-1}·mg^{-1} protein)
SR (Hup^+)	$+H_2$	40	207 ± 0
	$-H_2$	0.5	3 ± 1
SR2 (Hup^-)	$+H_2$	0.5	3 ± 1
	$-H_2$	0.5	6 ± 2
SR3 (Hup^-)	$+H_2$	0.5	0 ± 1
	$-H_2$	0.5	1 ± 1

[f] Cells were grown on plates for three days and derepressed for hydrogenase activity for three additional days as described in procedure A of Materials and Methods. H_2 uptake rates were determined, cell-free extracts were prepared, and carboxylase assays were performed by procedures described in Materials and Methods. RuBP carboxylase activities are means (± SEM) of 2 replicate determinations and have been corrected for rates of $^{14}CO_2$ incorporation by reaction mixtures lacking RuBP. All extracts contained propionyl CoA carboxylase activity. Total CO_2 uptake as nmoles·min^{-1}·mg^{-1} protein may be determined by multiplying given values by 6.4×10^{-3} nmoles CO_2·dpm^{-1}. Two replicate experiments gave similar results.

Table 7. Carboxylase activities of free-living and bacteroid cells of R. japonicum[g]

Type of cells assayed	Enzyme activities ($^{14}CO_2$ incorporation as $dpm \cdot min^{-1} \cdot mg^{-1}$ protein)	
	RuBP carboxylase	Propionyl CoA carboxylase
Free-living 122 DES induced for hydrogenase	241 ± 11	90 ± 1
122 DES bacteroids	1 ± 1	132 ± 1
122 DES bacteroids (BSA)	3 ± 2	111 ± 2
SR bacteroids	1 ± 1	104 ± 3

[g] Free-living R. japonicum 122 DES was derepressed for RuBP carboxylase activity by procedure B of Materials and Methods. Bacteroids were isolated from nodules of 5-week-old soybean plants (cultivar Wilkin) and cell-free extracts were prepared as described in Materials and Methods. All whole cell preparations showed active H_2 uptake by the amperometric method described in Materials and Methods. 122 DES bacteroids (BSA) were isolated and extracts were prepared by the method described in Materials and Methods except that all buffers contained 3% BSA (bovine serum albumin) (Laane, et al., 1978). Protein contents of bacteroid preparations containing added BSA were determined on a comparably prepared extract in which BSA was omitted. Total CO_2 uptake as $nmoles \cdot min^{-1} \cdot mg^{-1}$ protein may be determined by multiplying values by 9.7×10^{-3} nmoles CO_2 dpm^{-1}. Values have been corrected for rates

Footnotes for Table 7. (cont.)

of $^{14}CO_2$ incorporation in reaction mixtures lacking substrates and are means (± SEM) of three replicate determinations.

An experiment was conducted to test the possibility that the RuBP carboxylase activity observed in cell-free extracts of our R. japonicum SR strain may have been due to a bacterial contaminant (Table 8). Three known axenic strains of R. japonicum, all of which were derived from single colony isolates, were tested for RuBP carboxylase activity in hydrogenase-induced cells, and all three showed RuBP carboxylase activity higher than that observed on a control R. japonicum SR strain not induced for hydrogenase. Thus the RuBP carboxylase activities observed in R. japonicum SR do not result from a bacterial contaminant.

Table 8. Carboxylase activities of axenic cultures of R. japonicum 122 and SR[h]

R. japonicum strain	Conditions during derepression	RuBP carboxylase activities ($^{14}CO_2$ uptake in dpm·min^{-1}·mg^{-1} protein)	Propionyl CoA carboxylase ($^{14}CO_2$ uptake in dpm·min^{-1}·mg^{-1} protein)
SR A	$-H_2$	13.5 ± 7.0	192.4 ± 18.9
SR A	$+H_2$	104.5 ± 12.8	195.4 ± 22.9
122 DES	$+H_2$	109.9 ± 12.2	191.9 ± 5.0
SR B	$+H_2$	71.2 ± 4.4	200.4 ± 18.7

[h] Stock cultures used in assays were as follows: SR A, a R. japonicum SR stock culture from Dr. Robert Maier; 122 DES, a stock culture of R. japonicum 122 DES stored in glycerol and prepared by Mr. Sterling Russell; SR B, a single colony isolate of R. japonicum SR prepared by Dr. Robert Maier. Cells were grown and derepressed for hydrogenase as indicated in the table and described in procedure A of Materials and Methods. Carboxylase assays were performed as described in Materials and Methods. Values reported are means (± SEM) of 3 replicate determinations and were corrected for $^{14}CO_2$ incorporation in assays without added substrates. Total nmoles CO_2 fixed·min^{-1}·mg^{-1} protein may be calculated by multiplying dpm·min^{-1}·mg^{-1} protein values by 9.7×10^{-3} nmoles CO_2·dpm^{-1}. All strains placed under hydrogenase induction conditions showed active H_2 uptake, while strain SR A cultured without

Footnotes for Table 8. (cont.)

added H_2 showed no H_2 uptake (<0.5 nmoles$\cdot min^{-1}\cdot mg^{-1}$ protein) when assayed by the amperometric method described in Materials and Methods.

DISCUSSION

In the initial investigation of the CO_2 requirements of rhizobia (Lowe and Evans, 1962), activities of both propionyl CoA and phosphoenolpyruvate carboxylases in soybean nodule bacteroids were reported. More recent evidence by Peterson and Evans (1979) and Christeller et al. (1977) indicates that phosphoenolpyruvate carboxylase in nodules of soybeans and lupines, respectively, is limited to the non-bacteroid (or cytosol) fraction of the nodules. These latter results are consistent with those reported here showing that both free-living and bacteroid forms of R. japonicum contain propionyl CoA carboxylase activity but no measurable phosphoenolpyruvate carboxylase activity. Our discovery of RuBP-dependent fixation of CO_2 in cell-free extracts of H_2-uptake positive strains of R. japonicum (SR and 122 DES) grown under free-living conditions shows that these strains are capable of fixing CO_2 not only via propionyl CoA carboxylase, but also through RuBP carboxylase, which presumably would participate in the pentose phosphate pathway. These observations indicate similarities between the H_2-uptake positive strains of R. japonicum and certain hydrogen bacteria and suggest the possibility that hydrogenase-containing R. japonicum strains supplied with H_2 and CO_2 may be capable of growing autotrophically (Hanus et al., personal communication).

The observed rate of 1.7 nmoles CO_2 fixed$\cdot min^{-1}\cdot mg^{-1}$ protein for R. japonicum SR cells (Table 1) and the highest observed rate of 6.3 nmoles $CO_2\cdot min^{-1}\cdot mg^{-1}$ protein in cell-free extracts of R. japonicum SR (Figure 5) are low when compared with CO_2 fixation rates of whole cells and cell-free extracts of H_2 bacteria. For example, the Gram-positive

hydrogen bacterium 12/60/X showed CO_2 fixation rates of 150 nmoles $CO_2 \cdot min^{-1} \cdot mg^{-1}$ protein in whole cells (Eberhardt, 1969), while cell-free extracts of Alcaligenes eutrophus and Aquaspirillum autotrophicum catalyzed RuBP-dependent CO_2 fixation at rates of 107 nmoles $CO_2 \cdot min^{-1} \cdot mg^{-1}$ protein (Bowien et al., 1976) and 2120 nmoles $CO_2 \cdot min^{-1} \cdot mg^{-1}$ protein (Aragno and Schlegel, 1978), respectively. In comparison the rates of CO_2 fixation by RuBP carboxylase were 67.3 nmoles $CO_2 \cdot min^{-1} \cdot mg^{-1}$ protein in extracts of H. facilis (McFadden and Tu, 1967) and 15 nmoles $CO_2 \cdot min^{-1} \cdot mg^{-1}$ in extracts of the hydrogen bacterium strain 12/60/X (Eberhardt, 1969). These activities are greater than those observed in R. japonicum SR. The R. japonicum cells, however, were not grown under autotrophic conditions.

Although relatively low rates of CO_2 fixation in hydrogenase-derepressed R. japonicum SR were observed by us, some reasonable explanations may be offered. Firstly, R. japonicum SR cells used in these experiments were grown on a medium containing low concentrations of sucrose, gluconate, glutamate and arabinose. These carbon sources are known to repress the synthesis of the hydrogenase system in R. japonicum (Maier et al., 1979). In addition, carbon substrates are known to repress the expression of hydrogenase and RuBP carboxylase in heterotrophically-grown H_2 bacteria (Aragno and Schlegel, 1978). Secondly, since we have made no exhaustive effort to obtain conditions for maximal CO_2 fixation in R. japonicum, greater rates may be obtained when optimal conditions for RuBP carboxylase derepression have been established. Thirdly, R. japonicum grows relatively slowly under heterotrophic conditions with a doubling time of 6 to 8 h (Vincent, 1974), and one

would expect even longer doubling times under autotrophic or mixotrophic conditions if R. japonicum is capable of these modes of growth. In contrast, the hydrogen bacterium, A. autotrophicum, has a doubling time of 4 h under autotrophic conditions (Aragno and Schlegel, 1978). The comparatively low rate of CO_2 fixation in R. japonicum SR thus appears to be consistent with its growth rate.

RuBP carboxylase and hydrogenase activities appear to be coordinately induced by H_2 (Figure 5) and are both repressed by the addition of 10 mM succinate (Table 5) in free-living R. japonicum SR under conditions of hydrogenase derepression. The coupled expression of two enzymes required for the utilization of an inorganic energy source and for the fixation of CO_2 suggests a conversion from heterotrophic growth to a mixotrophic or autotrophic type of metabolism. Induction of hydrogenase in R. japonicum by H_2 and repression of hydrogenase by the addition of succinate have been reported by Maier et al. (1979). The repressive effects of succinate on RuBP carboxylase and hydrogenase activities and the expression of hydrogenase and RuBP carboxylase activities in R. japonicum SR under conditions where H_2 is supplied and O_2 and carbon substrates are limited are similar to the effects of these conditions on A. autotrophicum (Aragno and Schlegel, 1978).

A coordinate regulatory relationship between hydrogenase and RuBP carboxylase is further supported by the observation that the Hup^- mutant strains of R. japonicum SR did not express RuBP carboxylase activity under conditions which led to expression of hydrogenase and RuBP carboxylase activities in the Hup^+ parent strain SR of R. japonicum (Table 6). Although the specific mutation in the Hup^- strains of SR2

and SR3 have not been identified, these results suggest that an unknown regulatory relationship exists between hydrogenase and RuBP carboxylase in free-living R. japonicum.

That exogenous energy sources other than H_2 can support increased $^{14}CO_2$ fixation in hydrogenase-induced whole cells of R. japonicum is supported by the results of Table 2. Increases in $^{14}CO_2$ uptake in hydrogenase-induced cells compared with non-induced cells were approximately equal in the presence of H_2 or succinate and may be due to increased intracellular ATP levels (Emerich et al. 1979).

The findings that bacteroids formed by H_2-uptake positive R. japonicum 122 DES or SR contained no detectable activity of RuBP carboxylase were surprising in view of our findings with H_2-uptake positive free-living cells of the same strains (Table 7). However, the nodule is a very complex environment containing enzymes (Peterson and Evans, 1979), organic acids (Bergerson, 1974; Jackson and Evans, 1966), fatty acids (Johnson et al., 1966) and other components that could exert control over expression of enzymes such as hydrogenase and RuBP carboxylase. The results of Table 7 thus suggest that the interaction between and the regulation of the hydrogenase and RuBP carboxylase enzymes in soybean nodule bacteroids is complex, and that the regulation of RuBP carboxylase and hydrogenase in free-living and bacteroid cells of R. japonicum requires further study.

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APPENDICES

APPENDIX 1: Calculations

I. Carbon dioxide, bicarbonate, and carbon-14

A. Whole cell assays - Two types of whole cell assays were performed, one using cells on agar slants, the other using cells in liquid culture. I will present sample calculations for the slant assays first.

1. Slant assays - Slant assays were conducted in 22 ml test tubes sealed with a serum stopper and containing 7 ml of hydrogen uptake agar medium (pH 7.5) and 16 ml of gas space. The amount of added gaseous CO_2 necessary to reach the desired atmospheric CO_2 concentration was calculated using the following equations (Umbreit et al., 1972).

eq. 1. $CO_{2\ tot} = \text{moles } CO_{2\ (g)} + \text{moles } CO_{2\ diss} + \text{moles } HCO_3^-$

eq. 2. $pH = pK' + \log \frac{[HCO_3^-]}{[CO_{2\ diss}]}$ (pK' = 6.348 at 30°C)

eq. 3. $[CO_{2\ diss}] = .665\ [CO_{2\ (g)}]$ (at 30°C, 1 atm.)

eq. 4. $\frac{\%\ CO_2\ \text{in atmosphere}}{(100)\ 22.414\ l \cdot mole^{-1}} =$ concentration of CO_2 in $moles \cdot l^{-1}$ of atmosphere = $[CO_{2\ (g)}]$

where

$CO_{2\ tot}$ = total moles CO_2 present

$CO_{2\ diss}$ = dissolved CO_2

$CO_{2\ (g)}$ = gaseous CO_2

[ ] = concentration in $moles \cdot l^{-1}$

a. Calculations for an atmosphere of 5% CO_2

Calculations for an atmosphere of 5% CO_2 proceed as follows:

From equation 4

$$5\%\ CO_2 = \frac{.05}{22.144} = 2.231 \times 10^{-3}\ M = [CO_{2\ (g)}]\ .$$

From equation 3 at 30°C, 1 atm. $.665\ [CO_{2\ (g)}] = [CO_{2\ diss}] = (.665)\ (2.231 \times 10^{-3}M) = 1.484 \times 10^{-3}M$.

From equation 2

$$pH = pK'_a + \log \frac{[HCO_3^-]}{[CO_{2\ diss}]}\ .$$

At pH 7.5 and 30°C

$$7.5 = 6.348 + \log \frac{[HCO_3^-]}{[CO_{2\ diss}]}$$

$$1.15 = \log \frac{[HCO_3^-]}{[CO_{2\ diss}]}$$

Taking the antilog of both sides of the above equation

$$14.13 = \frac{[HCO_3^-]}{[CO_{2\ diss}]}$$

or $14.13\ [CO_{2\ diss}] = [HCO_3^-]$.

Substituting, we find

$$[HCO_3^-] = 14.13\ [1.484 \times 10^{-3}M] = 2.096 \times 10^{-2}M.$$

Knowing that $CO_{2\ (g)}$ is in the gas phase and $CO_{2\ diss}$ and HCO_3^- are dissolved in the agar medium, and that there are 16 ml of gas space and 7 ml of agar per tube, one may calculate the number of moles of each CO_2 species present per slant.

$$\text{moles } CO_{2\ (g)} = (16 \times 10^{-3}l)\ (2.231 \times 10^{-3}\text{moles}\cdot l^{-1}) = 3.5696 \times 10^{-5}\ \text{moles}$$

$$\text{moles } CO_{2\ diss} = (7.0 \times 10^{-3}l)\ (1.484 \times 10^{-3}\ \text{moles}\cdot l^{-1}) = 1.03852 \times 10^{-5}\ \text{moles}$$

moles HCO_3 = $(7.0 \times 10^{-3} l)$ $(2.105 \times 10^{-2}$ moles$\cdot l^{-1})$ =

1.4676×10^{-4} moles

$CO_{2\ tot}$ is the sum of those values (eq. 1)

$CO_{2\ tot}$ = moles $CO_{2\ (g)}$ + moles $CO_{2\ diss}$ + moles HCO_3^- =

$(3.5696 \times 10^{-5}$ moles$)$ + $(1.03852 \times 10^{-5}$ moles$)$ +

$(1.4674 \times 10^{-4}$ moles$)$

$CO_{2\ tot} = 1.928 \times 10^{-4}$ moles

CO_2 was added to slants as gaseous CO_2. Therefore, to achieve a 5% atmosphere in each slant after equilibration one had to add

$(1.928 \times 10^{-4}$ moles$)$ $(22.414 l \cdot mole^{-1})$ =

4.32×10^{-3} or 4.32 ml CO_2 per slant culture

b. Calculations for the CO_2 contribution from air

The number of moles of CO_2 contributed to each tube by atmospheric CO_2 at 1 atm. and 30°C could be calculated in two ways.

Method 1

Air contains 0.03% CO_2 volume:volume.

From the result of part a. that a slant with a 5% CO_2 atmosphere contained 1.928×10^{-4} moles CO_2, one can calculate the following.

$\frac{.03\%}{5.0\%}$ $(1.928 \times 10^{-4}$ moles$)$ = 1.156×10^{-6} moles $CO_2 \cdot slant^{-1}$ from air.

Method 2

The second method of calculating the contribution of CO_2 per slant from CO_2 in the air is by means of

equations 1 through 4.

$$[CO_{2\ (g)}] = \frac{.03}{(100)(22.414)} = 1.338 \times 10^{-5}M$$

$$[CO_{2\ diss}] = 0.665\ [CO_{2\ (g)}] = 0.665\ (1.338 \times 10^{-5}M)$$

$$[CO_{2\ diss}] = 8.90 \times 10^{-6}M$$

$$[HCO_3^-] = (14.13)\ (CO_{2\ diss})$$

$$= (14.13)\ (8.90 \times 10^{-6}M)$$

$$[HCO_3^-] = 1.25 \times 10^{-4}M$$

There are 16 ml of air space and 7 ml of agar.

Therefore

$$\text{moles } CO_{2\ (g)} = (16 \times 10^{-3}\text{l})\ (1.338 \times 10^{-5}\text{M}) = 2.14 \times 10^{-7} \text{ moles}$$

$$\text{moles } CO_{2\ diss} = (7 \times 10^{-3}\text{l})\ (8.90 \times 10^{-6}\text{M}) = 6.23 \times 10^{-8} \text{ moles}$$

$$\text{moles } HCO_3^- = (7 \times 10^{-3}\text{l})\ (1.258 \times 10^{-4}\text{M}) = 8.806 \times 10^{-7} \text{ moles}$$

The sum of the above values equals $CO_{2\ tot}$ (equation 1).

$$CO_{2\ tot} = \text{moles } CO_{2\ (g)} + \text{moles } CO_{2\ diss} + \text{moles } HCO_3^-$$

$$CO_{2\ tot} = 2.14 \times 10^{-7} + 6.23 \times 10^{-8} + 8.806 \times 10^{-7}$$

$CO_{2\ tot} = 1.157 \times 10^{-6}$ moles CO_2 per slant contributed by air.

c. Calculations for $^{14}CO_2$

We received $NaH^{14}CO_3$ from New England Nuclear Corporation of specific activity 0.25 mCi·3 mg^{-1}. This entire amount (3 mg) was dissolved in 10.0 ml H_2O and kept in the refrigerator at 4°C. (This solution will hereafter be called the stock $NaH^{14}CO_3$ solution.) To prepare $NaHCO_3$

for addition to slants it had to be converted to gaseous CO_2. This was done by adding 2.0 ml of the above-mentioned solution to 6N HCl to produce 10.0 ml of $^{14}CO_2$ in air (see Figure A1). Reactions were initiated by adding 0.5 ml of $^{14}CO_2$.

The number of moles of CO_2 and the mCi and dpm of ^{14}C per 0.50 ml injection may be calculated as follows. Our stock $NaH^{14}CO_3$ solution contained 0.25 mCi or 3 mg $NaHCO_3$ in 10.0 ml H_2O. The molecular weight of $NaHCO_3$ is 84.0 g.mole^{-1}.

$$\frac{3 \times 10^{-3} \text{ g}}{(10.0 \text{ ml})(84 \text{ g.mole}^{-1})} = 3.57 \times 10^{-6} \text{ moles } NaH^{14}CO_3 \text{ per 1.0 ml}$$

$$\frac{.25 \text{ mCi}}{10 \text{ ml}} = 0.25 \text{ mCi} \cdot \text{ml}^{-1}$$

Two ml of this solution were added to acid and collected in 10.0 ml of air. Assuming 100% conversion of $NaH^{14}CO_3$ to $^{14}CO_2$, the following calculations may be made for 0.5 ml of $^{14}CO_2$ added per slant

$$\frac{(0.5)(3.57 \times 10^{-6} \text{ moles} \cdot \text{ml}^{-1})\ 2.0 \text{ ml}}{10 \text{ ml air}} = 3.57 \times 10^{-7} \text{ moles per 0.5 ml gas}$$

$$\frac{(0.5)(.025 \text{ mCi} \cdot \text{ml}^{-1})(2.0 \text{ ml})}{10 \text{ ml air}} = 2.5 \times 10^{-3} \text{ mCi per 0.5 ml gas}$$

One mCi equals 3.70×10^7 dps

$$\frac{(2.5 \times 10^{-3} \text{mCi})(3.70 \times 10^{7} \text{ dps} \cdot \text{mCi}^{-1})}{60 \text{ sec} \cdot \text{min}^{-1}} = 5.55 \times 10^{6} \text{ dpm per 0.5 ml of air}$$

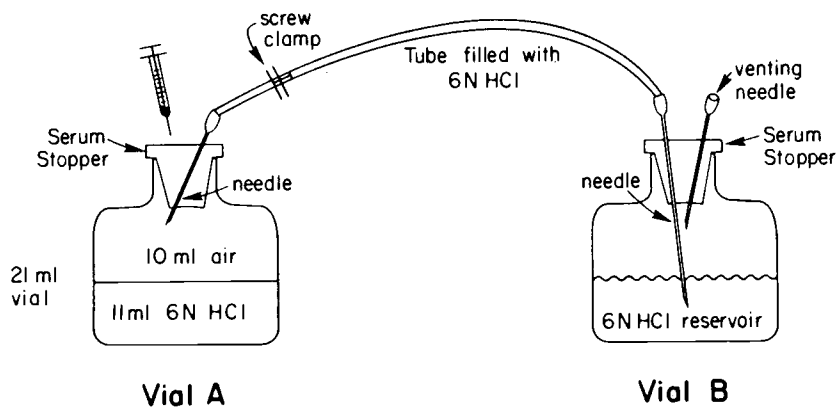

Figure A1. Apparatus for preparing $^{14}CO_2$ from dissolved $NaH^{14}CO_3$. Two ml of $NaH^{14}CO_3$ solution were added to vial A with the screw clamp closed. After one to two minutes, the screw clamp was opened and the pressurized gas pushed into the tube. Gas samples (0.5 ml) were then removed from vial A while gas, followed by acid solution, flowed into vial A from the tube and acid reservoir (vial B).

d. Calculations for moles CO_2 contributed by 0.5 ml of air

The number of moles of CO_2 contributed by 0.5 ml of air is

$$\frac{(0.03)(0.5 \times 10^{-3})}{(100)(22.414\,l \cdot mole^{-1})} = 6.69 \times 10^{-9} \text{ moles } CO_2$$

This small amount of CO_2 equals

$$\frac{(6.69 \times 10^{-9})(100)}{3.57 \times 10^{-7}} = 1.87\%$$

of that CO_2 added as $^{14}CO_2$ per tube in part c which is negligible, so the CO_2 contribution per tube from this 0.5 ml sample is not included in further calculations.

e. Additions of CO_2 to each 5% CO_2 slant

The following CO_2 additions were made to each 5% CO_2 slant:

i. CO_2 added to obtain 5% atmospheric CO_2 after equilibration = 1.928×10^{-4} moles.

ii. CO_2 from the air per slant = 1.156×10^{-6} moles.

iii. CO_2 from the addition of $^{14}CO_2$ = 3.57×10^{-7} moles containing 5.55×10^{6} dpm of ^{14}C.

f. CO_2 additions for experiment described in Figure 3

For the experiment described in Figure 3, Table A1 may be constructed. The factors listed in the tube containing 5% CO_2 were used in the calculations for Table 1. Specific activities in Table 1A were calculated from

$$\frac{\text{dpm } (10^{-9} \text{ moles} \cdot \text{nmole}^{-1})}{\text{Total moles } CO_2} = \text{dpm} \cdot \text{nmole}^{-1}$$

Table A1. Results of $^{14}CO_2$ calculations for Table 1 and Figure 3.

Tube No.	Amount of CO_2 added (%)	Moles CO_2 added ($\times 10^5$)	Moles CO_2 from air ($\times 10^6$)	Moles CO_2 added as $^{14}CO_2$ ($\times 10^5$)	Total moles CO_2 ($\times 10^5$)	dpm ^{14}C added ($\times 10^{-6}$)	Specific activity dpm·nmole^{-1} CO_2 ($\times 10^{-2}$)	(Specific activity)$^{-1}$ nmole CO_2·dpm^{-1} ($\times 10^3$)
1	0	0	0	3.57	.0357	5.550	155.5	.06432
2	0.03*	0	1.157	3.57	.1514	5.550	36.66	.2738
3	0.5%	1.927	1.157	3.57	2.1197	5.550	2.618	3.819
4	1%	3.855	1.157	3.57	4.01	5.550	1.384	7.225
5	2%	7.718	1.157	3.57	7.869	5.550	0.7053	14.18
6	3%	11.555	1.157	3.57	11.706	5.550	0.4741	21.09
7	5%	19.274	1.157	3.57	19.425	5.550	0.2857	35.00

*Air was added (0.03% CO_2)

2. Liquid culture assays - The specific activities of $^{14}CO_2$ in whole cell liquid culture assays were calculated similarly to those in slant assays. In the experiment described in Figure 2, in which the effect of succinate and H_2 on $^{14}CO_2$ uptake by hydrogenase-induced and non-induced cells was assayed, an atmosphere containing 5% CO_2 was used. Cells were suspended in K-phosphate-$MgCl_2$ buffer pH 7.0 and 2.0 ml of this cell suspension were placed in a 6.0 ml vial which was then sealed with a serum stopper. The sealed vial contained 4.0 ml of air space. Since cells were in liquid medium, bicarbonate was added rather than gaseous CO_2. Preparation of this bicarbonate solution is now described.

   a. Calculations for preparation of stock bicarbonate solution

      Equations 1 through 4 are employed once again. For an atmosphere containing 5% CO_2 at pH 7.0 and 30°C the following may be calculated.

      $$[CO_{2\ (g)}] = \frac{5.0}{(100)\ 22.414\ l \cdot mole^{-1}} = 2.23 \times 10^{-3} M$$

      $$[CO_{2\ diss}] = (.665)(2.23 \times 10^{-3} M) = 1.48 \times 10^{-3} M$$

      $$[HCO_3^-] = (4.49)(1.48 \times 10^{-3} M) = 6.66 \times 10^{-3} M$$

      Thus one 6 ml vial contains the following amounts of CO_2. (There are 4 ml of gas space and 2 ml of suspension.)

      $$\text{moles } CO_{2\ (g)} = (4 \times 10^{-3} l)(2.23 \times 10^{-3} M) = 8.92 \times 10^{-6} \text{ moles}$$

$$\text{moles } CO_{2\ diss} = (2 \times 10^{-3}\,l)(1.48 \times 10^{-3}\,M) = 2.96 \times 10^{-6} \text{ moles}$$

$$\text{moles } HCO_3^- = (2 \times 10^{-3}\,l)(6.66 \times 10^{-3}\,M) = 1.33 \times 10^{-5} \text{ moles}$$

$$CO_{2\ tot} = \text{moles } CO_{2\ (g)} + \text{moles } CO_{2\ diss} + \text{moles } HCO_3^-$$

$$= 8.92 \times 10^{-6} \text{ moles} + 2.96 \times 10^{-6} \text{ moles}$$

$$+1.33 \times 10^{-5} \text{ moles}$$

$$= 2.52 \times 10^{-5} \text{ moles } CO_2$$ neglecting the CO_2 contribution from the air

$$CO_{2\ tot} = (2.521 \times 10^{-5} \text{ moles})(84 \text{ g} \cdot \text{mole}^{-1})$$

$$= 2.12 \times 10^{-3} \text{ g } NaHCO_3 \text{ per vial}$$

A stock solution was then prepared containing

2.12×10^{-3} g $NaHCO_3$ per 0.1 ml

or 0.212 g $NaHCO_3$ per 10 ml H_2O

10 ml of this solution was always freshly prepared in a 21 ml vial which was immediately sealed with a serum stopper.

b. Calculation for $NaH^{14}CO_3$

To initiate reactions 0.1 ml of the stock $NaH^{14}CO_3$ solution containing 0.25 mCi or 3 mg $NaH^{14}CO_3$ per 10 ml was added to each vial. Thus

$$\frac{3 \times 10^{-3}}{(84 \text{ g} \cdot \text{mole}^{-1})(100)} = 3.57 \times 10^{-7} \text{ mole } NaHCO_3$$

containing $\frac{0.25}{100} = 2.5 \times 10^{-3}$ mCi ^{14}C

was added as $NaH^{14}CO_3$.

The in situ specific activity was thus

$$\frac{(2.5 \times 10^{-6}\ \text{Ci})(3.7 \times 10^{10}\ \text{dps} \cdot \text{Ci}^{-1})(60)}{(2.52 \times 10^{-5}\ \text{moles}) + (3.57 \times 10^{-7}\ \text{moles})} = 2.171 \times 10^{11}$$

$$\text{dpm} \cdot \text{mole}^{-1} = 217.1\ \text{dpm} \cdot \text{nmole}^{-1}\ CO_2$$

$$(\text{specific activity})^{-1} = 4.607 \times 10^{-3}\ \text{nmoles} \cdot \text{dpm}^{-1}$$

B. Carboxylase assays of cell-free extracts - Carboxylase assays of cell-free extracts were performed using assay volumes of 1.0 ml or 0.275 ml. Reactions in 1 ml vials contained 0.1 ml of a solution containing 0.2965 g $NaHCO_3$ in 10.0 ml H_2O. Assuming that all of the $NaHCO_3$ remained dissolved in this solution (21 ml vials were sealed with a serum stopper) one obtains

$$\frac{0.2965\ \text{g}}{(84\ \text{g} \cdot \text{mole}^{-1})(100)} = 3.53 \times 10^{-5}\ \text{moles}\ NaHCO_3^-\ \text{added per reaction vial}$$

To this amount of $NaHCO_3$ was added 0.1 ml of the stock $NaH^{14}CO_3$ solution. Thus 2.5×10^{-6} Ci ^{14}C in 3.57×10^{-7} moles of $NaHCO_3$ was added per vial. The specific activity in 1 ml reaction vials is thus

$$\frac{(2.5 \times 10^{-6}\ \text{Ci})(3.70 \times 10^{10}\ \text{dps} \cdot \text{Ci}^{-1})(60\ \text{sec} \cdot \text{min}^{-1})}{(3.53 \times 10^{-5}\ \text{moles}) + (3.57 \times 10^{-7}\ \text{moles})} =$$

$$1.556 \times 10^{11}\ \text{dpm} \cdot \text{mole}^{-1}$$

$$\frac{1}{(1.556 \times 10^{11}\ \text{dpm} \cdot \text{mole}^{-1})(10^9\ \text{nmole} \cdot \text{mole}^{-1})} = 6.425 \times 10^{-3}\ \text{nmoles} \cdot \text{dpm}^{-1}$$

Reactions in .275 ml volumes contained 0.025 ml of a solution containing 0.4471 g $NaHCO_3$ in 10 ml H_2O in a 21 ml vial sealed with a serum stopper. Thus each vial contained

$$\frac{(0.4471\ \text{g})(0.025\ \text{ml})}{(84\ \text{g} \cdot \text{mole}^{-1})(100)} = 1.33 \times 10^{-5}\ \text{moles}\ NaHCO_3$$

The CO_2 contributed by the air is not considered in this calculation. A volume of 0.025 ml of the stock $NaH^{14}CO_3$ solution on 6.25×10^{-7} Ci ^{14}C in 8.925×10^{-8} moles $NaHCO_3$ was then added to each reaction vial.

The specific activity of $NaH^{14}CO_3$ in .0275 ml reaction vials was thus

$$\frac{(6.25 \times 10^{-7}\ \text{Ci})(3.70 \times 10^{10}\ \text{dps} \cdot \text{Ci}^{-1})(60\ \text{sec} \cdot \text{min}^{-1})}{(1.33 \times 10^{-5}\ \text{moles}) + (8.925 \times 10^{-8}\ \text{moles})} =$$

$$1.035 \times 10^{11}\ \text{dpm} \cdot \text{mole}^{-1}$$

$$(\text{specific activity})^{-1} = 9.657 \times 10^{-3}\ \text{nmole} \cdot \text{dpm}$$

APPENDIX 2: Processing Data

Data were processed similarly in all cases so one example should suffice to explain the method used. For the experiment described in Table 3 the data reported in Table A2 were collected from the scintillation counter print out. Firstly the three cpm values for each enzyme assayed were averaged as were counting efficiencies, and the cpm values of all control vials. Standard errors of the mean were also determined. Average cpm $\pm$ SEM numbers for the data of Table A2 are listed in Table A3. The % error listed on the scintillation counter print out was not considered in any of these calculations. Average background counts were then subtracted from average cpm values but the SEM's of the average background counts were not considered. Average cpm $\pm$ SEM values were then divided by the average counting efficiency to obtain dpm $\pm$ SEM values.

Assays in the experiment described in Table 3 were 1.0 ml and contained 0.2 ml of cell-free extract. Reactions were terminated with 0.4 ml 6 N HCl (0.275 ml assays) and 0.1 ml of this terminated reaction mixture was transferred to a Whatman 3 MM filter paper disc and taken to dryness. This disc was then placed in a scintillation vial and counted as described in Materials and Methods. From this information and the mg protein $\cdot$ ml^{-1} values listed in Table A4, the mg protein per filter paper disc could be calculated.

Extract from cells induced for hydrogenase

$$(2.727 \text{ mg protein} \cdot \text{ml}^{-1})(0.2 \text{ ml})\left(\frac{0.1 \text{ ml}}{1.4 \text{ ml}}\right) = 0.0390 \text{ mg protein}$$

Extract from cells not induced for hydrogenase

$$(2.924 \text{ mg protein} \cdot \text{ml}^{-1})(0.2 \text{ ml})\left(\frac{0.1 \text{ ml}}{1.4 \text{ ml}}\right) = 0.0418 \text{ mg protein}$$

Dpm ± SEM values of Table A3 were then divided by the mg protein per filter paper disc numbers of Table A4 and the dpm · mg^{-1} protein ± SEM values listed in Table A5 were obtained. Carboxylase assays were run for 30 minutes in this experiment so dividing dpm · mg^{-1} protein ± SEM values by 30 min gave the dpm · min^{-1} · mg^{-1} protein ± SEM values also listed in Table A5. Rounding these latter numbers off gave the values reported in Table 3. Total nanomoles CO_2 fixed · min^{-1} · mg^{-1} protein may then be determined by multiplying dpm · min · mg^{-1} protein ± SEM values by the nmole · dpm^{-1} values given in legend of Table 3.

Data from whole cell assays as well as from assays of cell-free extracts were prepared in a similar manner to that described for Table 3 with corrections made for background counts, efficiencies of counting and the amount of sample counted in each case.

Table A2. Scintillation counter printout for results of Table 3.

Enzyme assayed	Efficiency	Vial #	Counting period (min)	Counts	% error[a]	Counts per minute
PEP	.7502	100	4.00	91	.0	22.7
carboxylase[b]	.7499	101	4.00	148	9.9	37.0
	.7296	102	4.00	111	9.9	27.7
RuBP	.7294	103	4.00	1138	4.0	284.5
carboxylase[b]	.7351	104	4.00	994	5.0	248.5
	.7186	105	4.00	1036	4.0	259.0
Propriony1-CoA	.7120	106	4.00	953	5.0	238.2
Carboxylase[b]	.7326	107	4.00	901	5.0	225.2
	.7333	108	4.00	812	5.0	203.0
PEP	.6515	109	4.00	90	.0	22.5
carboxykinase[b]	.6312	110	4.00	95	.0	23.7
	.6363	111	4.00	105	9.9	26.2
– Substrates[b]	.6318	112	4.00	105	9.9	26.2
	.6270	113	4.00	106	9.9	26.5
	.6407	114	4.00	107	9.9	26.7
+ATP[b]	.6241	115	4.00	103	9.9	25.7
+ADP[b]	.6233	116	4.00	83	.0	20.7
PEP	.6388	117	4.00	93	.0	23.2
carboxylase[c]	.6532	118	4.00	103	9.9	25.7
	.6542	119	4.00	108	9.9	27.0
RuBP	.6623	120	4.00	100	9.9	25.0
carboxylase[c]	.6747	121	4.00	143	9.9	35.7
	.6850	122	4.00	101	9.9	25.2

Table A2. Continued.

Enzyme assayed	Efficiency	Vial #	Counting period (min)	Counts	% error[a]	Counts per minute
Propionyl CoA	.7043	123	4.00	520	5.0	130.0
carboxylase[c]	.6764	124	4.00	683	5.0	170.7
	.6453	125	4.00	687	5.0	171.7
PEP	.6062	126	4.00	110	9.9	27.5
carboxykinase[c]	.6833	127	4.00	137	9.9	34.2
	.6918	128	4.00	134	9.9	33.5
Substrates[c]	.7034	129	4.00	136	9.9	34.0
	.7054	130	4.00	140	9.9	35.0
	.6880	131	4.00	138	9.9	34.5
+ATP[c]	.6919	132	4.00	137	9.9	34.2
+ADP[c]	.6784	133	4.00	119	9.9	29.7

[a] Percent error is a statistical value for the accuracy of counting based upon the total number of counts collected.

[b] Extracts of cells induced for hydrogenase.

[c] Extracts of cells not induced for hydrogenase.

Table A3. Results of calculations using data of Table A2.

Enzyme assayed	Ave. ± SEM	Ave-background	dpm ± SEM
PEP carboxylase[d]	29.1 ± 4.2	-0.2 ± 4.2	-0.3 ± 6.2
RuBP carboxylase[d]	264.0 ± 10.7	234.7 ± 10.7	345.8 ± 15.8
Propionyl CoA[d] carboxylase	221.1 ± 10.3	192.8 ± 10.3	284.1 ± 15.2
PEP carboxykinase[d]	24.1 ± 1.1	-5.2 ± 1.1	-7.7 ± 1.6
PEP carboxylase[e]	25.3 ± 1.1	-4.0 ± 1.1	-5.9 ± 1.6
RuBP carboxylase[e]	28.6 ± 3.5	-0.7 ± 3.5	-0.1 ± 5.2
Propionyl CoA[e] carboxylase	157.5 ± 13.7	128.2 ± 13.7	188.9 ± 20.2
PEP carboxykinase[e]	31.7 ± 2.1	2.4 ± 2.1	3.5 ± 3.1

Average of controls (of 10 vials) ± SEM = 29.3 ± 1.6

Average efficiency of counting ± SEM = .6787 ± .0071

[d]Extracts of cells induced for hydrogenase

[e]Extracts of cells not induced for hydrogenase

Table A4. Results of microbiuret protein determination for cell-free extracts of Table 3[f]

Cell extract	mg protein·ml^{-1}	mg protein per filter paper disc
induced for hydrogenase	2.73	.0390
not induced for hydrogenase	2.92	.0418

[f] Protein assays were performed as described in Materials and Methods.

Table A5. Results of calculations using results reported in Table A3.

Cell extract prepared from	Enzyme assayed	$dpm \cdot mg^{-1}$ protein ± SEM	$dpm \cdot min^{-1} \cdot mg^{-1}$ protein ± SEM
Cells derepressed for hydrogenase	PEP carboxylase	7.69 ± 159	0.26 ± 5.3
	RuBP carboxylase	8870 ± 405	296 ± 14
	Propionyl-CoA carboxylase	7285 ± 390	243 ± 13
	PEP carboxykinase	-197 ± 41.0	-6.6 ± 1.4
Cells not derepressed for hydrogenase	PEP carboxylase	-141 ± 38	-4.7 ± 1.3
	RuBP carboxylase	23.9 ± 124	.80 ± 4.2
	Propionyl-CoA carboxylase	4520 ± 483	151 ± 16
	PEP carboxykinase	84 ± 74	2.8 ± 2.5

APPENDIX 3: H_2-uptake calculations

The solubility (α) of H_2 at 30°C is 0.0175 ml $H_2 \cdot ml^{-1}$ H_2O (Umbreit et al., 1972). Thus there are 0.0175 $H_2 \cdot 1^{-1}$ H_2O in a saturated solution.

$$\frac{0.0175 \ H_2 \cdot {}^{-1} H_2O}{22.414 \cdot mole^{-1}} = 7.81 \times 10^{-4} \text{ moles } H_2 \cdot 1^{-1} H_2O$$

$$= 75.8 \text{ nmoles } H_2 \text{ per 100 ml } H_2O \text{ at } 30^{\circ}C$$

After calibrating the H_2 electrode against a known quantity of H_2 saturated buffer we were able to determine the rate of H_2 uptake from the slope of the line on the chart recorder as follows:

$$\left(\frac{\text{number of squares}}{\text{duration of assay (min)}}\right) \text{(ul } H_2 \text{ saturated buffer per square)}$$

$$\left(\frac{.758 \text{ nmoles } H_2 \cdot ml^{-1} H_2 \text{ saturated buffer}}{(\text{mg protein} \cdot ml)\ (5.8\ ml)}\right) = \text{nmoles } H_2 \cdot min^{-1} \cdot mg^{-1} \text{ protein}$$

The electrode chamber contained 5.8 ml. Values reported as nmoles $H_2 \cdot h^{-1}$. 10^8 cells^{-1} were obtained by dividing by the cell number per ml x 10^{-8} and multiplying by 60 min $\cdot$ h^{-1} in the last equation.