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Factors Affecting the Synthesis and Degradation of Ribulose-1,5-Diphosphate Carboxylase in *Hydrogenomonas facilis* and *Hydrogenomonas eutropha*

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Hydrogenomonas facilis and *H. eutropha* cultured in fructose medium retained high levels of ribulose-1,5-diphosphate carboxylase only when the following conditions were fulfilled: low aeration, $FeCl_3$ addition to fructose medium, and cell harvest at or prior to mid-exponential phase of growth. Repression of carboxylase synthesis was demonstrated under conditions of high oxygen tension during growth of *H. eutropha* on fructose. Upon depletion of fructose in the growth medium, carboxylase activity fell abruptly in both organisms. The decline could not be attributed to a repressive mechanism. Rapid inactivation of carboxylase was promoted by transfer of mid-exponential-phase *H. eutropha* to a basal salts medium lacking fructose. During severe fructose starvation, N_2, H_2, 80% H_2 to 20% air, 2,4-dinitrophenol, actinomycin D, streptomycin, bicarbonate, and magnesium ion deficiency spared carboxylase. Nitrogen starvation or chloramphenicol afforded no protection during severe starvation. In vitro inactivation was also demonstrated in crude cell-free extracts from nonstarved, fructose-grown *H. eutropha*. Substrate bicarbonate protected against this loss. Inactivation of the carboxylase could not be demonstrated either by starvation of autotrophically grown cells or in autotrophic extracts. Autotrophic extracts mixed with heterotrophic extracts lost their carboxylase activity, but mixing with heterotrophic extracts that had been heated to 50 C resulted in no loss of activity. Mechanisms are proposed to accommodate these observations.

Evidence from many laboratories has established that ribulose diphosphate carboxylase (RDPC), EC 4.1.1.39, catalyzes the primary fixation of carbon dioxide in most autotrophs. Thus, the fate of the enzyme in facultative organisms growing heterotrophically has posed some interesting questions in regulatory biology. It is now clear that, in general, levels of this catalyst are decreased by growth on organic substrates (1, 2, 10, 13, 15, 24, 33). Although repression has been implicated (25) to account for these gross variations in levels of RDPC, little is known about other factors which contribute to the variation in its level during the autotrophic to heterotrophic conversion.

Among the facultative chemosynthetic bacteria, evidence for the occurrence and variation of levels of RDPC was first presented for *Hydrogenomonas facilis* (24). Although a high specific activity was noted for organisms grown under strictly autotrophic conditions, growth on sugars resulted in comparable levels of this enzyme. Because large-scale autotrophic cultivation of the hydrogen bacteria is difficult, this retention of RDPC was of interest. The observation suggested that *H. facilis* grown on sugars would be an excellent source of RDPC for purification trials. However, subsequent work in our laboratory on both *H. facilis* and *H. eutropha* established that the specific activity of RDPC varied as much as 30-fold in separate cultures harvested during exponential growth on fructose. This suggested that the control of RDPC was not related solely to the nature of the carbon source, but that additional factors were involved. A study of these factors is the subject of the present communication.

MATERIALS AND METHODS

Special chemicals. The following special reagents were commercial preparations: streptomycin sulfate

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from Mann Research Laboratories, New York, N. Y.; $Na_2{}^{14}CO_3$ from Nuclear-Chicago Corp., Des Plaines, Ill.; dibarium ribulose-1,5-diphosphate from Sigma Chemical Co., St. Louis, Mo.; chloramphenicol from Parke, Davis and Co., Detroit, Mich.; actinomycin D from Merck Sharp and Dohme Research Laboratories, Rahway, N.J.; 2,4-dinitrophenol from Matheson Co., East Rutherford, N.J.

Organisms and media. Autotrophic culture medium for *H. facilis* and heterotrophic culture medium for *H. facilis* and *H. eutropha* were prepared as described by McFadden and Homann (23), except that fructose was autoclaved separately in deionized water and filter-sterilized $FeCl_3 \cdot 6H_2O$ was added to a final concentration of 0.0008%. *H. eutropha* was cultured autotrophically on medium described by Repaske (32). Provision of H_2, O_2, and CO_2 during starvation studies and during autotrophic growth of both species was as described by McFadden and Atkinson (21). The culture of *H. facilis* was a subculture of the original isolate of Schatz and Bovell (35), and has been maintained autotrophically since 1953. *H. eutropha* was kindly provided by R. Repaske.

Cell-free preparations. Unless otherwise designated, cell-free extracts were prepared from 300-ml cultures. The relationship between cell mass and turbidity was established by use of 0.5-inch Klett tubes and a Klett-Summerson colorimeter equipped with red filter. Subsequent growth data were obtained turbidimetrically by using growth flasks onto which side arms (0.5 inch in diameter) had been sealed. Cells were collected by centrifugation at 2 C, washed once with TM buffer [made up of 0.01 M tris(hydroxymethyl)aminomethane (Tris) containing 2 mM β-mercaptoethanol]. The *p*H of this buffer (7.4), and of all other buffers used, was measured at 25 C after adjustment with sulfuric acid. Cells were suspended in TM containing 0.01 M $MgCl_2 \cdot 6H_2O$, to give a 10% suspension (w/v). These suspensions were disrupted by passage through a French pressure cell at 5 C and a pressure of 15,000 psi, unless otherwise indicated. The cell debris was separated from the supernatant fraction by centrifugation at 105,000 $\times$ *g* at 2 C for 1 hr. The supernatant fraction was then used in the experiments described. Protein concentration was estimated by the method of Lowry et al. (17) with appropriate corrections for Tris or β-mercaptoethanol interference where necessary.

Ribulose diphosphate carboxylase was assayed as reported by McFadden and Tu, with the exception that assays were carried out at *p*H 8.0 (25). One unit of enzyme is defined as that amount which catalyzes the carboxylation of 1 mμmole of ribulosediphosphate per min. Specific activity is given as units per milligram of protein.

Chemical assays. Fructose and ammonia were determined according to the methods of Seliwanoff (37) and of Milton and Waters (28), respectively.

Starvation experiments. To demonstrate the effect of starvation on RDPC expression, cells were routinely grown in fructose medium and harvested at room temperature near mid-exponential stage of growth. Unless otherwise noted, cells were not washed but were immediately suspended in 200 ml of basal inorganic medium (*p*H 7.0) containing 0.2% $(NH_4)_2SO_4$, 0.02% $MgCl_2 \cdot 6H_2O$, 0.1% K_2HPO_4, and 0.0008% $FeCl_3$. Additional components were either added or deleted from this basal medium as indicated. Cell densities during starvation corresponded to 1.20 to 1.60 mg (dry weight) per ml. All starvation experiments were performed at 30 C in 500-ml flasks which were shaken at 115 cycles per min under air, unless otherwise specified. In time-course studies, samples of suspensions were periodically removed, chilled, harvested by centrifugation at 6,000 $\times$ *g* for 5 min (2 C), and washed once in TM at 2 C. Cells were then collected by centrifugation, ruptured, and assayed as described.

RESULTS

Optimal conditions for RDPC synthesis in fructose-grown cultures. Early in the present work, it was recognized that the age of the autotrophic inoculum of both *H. eutropha* and *H. facilis* affected the ultimate expression of RDPC synthesis during fructose growth. Autotrophic cells stored at 2 C for periods longer than approximately 3 weeks lost their capacity to synthesize RDPC when grown in fructose medium. Consequently, fresh, autotrophic inoculum was routinely used for all studies.

In incipient studies of the regulation of RDPC in *H. facilis* and *H. eutropha* during fructose growth, the specific activity of RDPC varied as much as 30-fold in different exponential-phase cultures. Cultures which invariably yielded low RDPC activity had growth rates approximately twice those of cultures which produced high RDPC activity. In addition, rapidly grown cells were markedly bleached as compared to pink cells harvested from slowly grown cultures. Pink cells yielded amber extracts and bleached cells yielded pale yellow extracts.

In one experiment, a cell-free extract from *H. eutropha* was prepared from pink cells and was fractionated with a Sephadex G-150 column equilibrated with TM buffer. The only amber-colored band, which was a major protein component in the extract, was eluted from the column. After a portion was reduced with sodium borohydride, samples were spectrally characterized. The oxidized state showed absorption maxima at 265 and 406 mμ and the reduced material at 265, 416, 520, and 549 mμ. Thus, the spectra were identical with those for a cytochrome of class C (18). This suggested that bleached cells which had not produced this amber protein were presumably experiencing a Fe^{+3} ion deficiency. This was indeed found to be the case. Fructose-grown cultures supplemented with 0.0008% filter-sterilized $FeCl_3$, shaken at low speed, and harvested before two-thirds maximal growth was attained (see subsequent supporting data) thereafter consistently contained high levels of RDPC. Figure 1 illustrates a correlation between RDPC

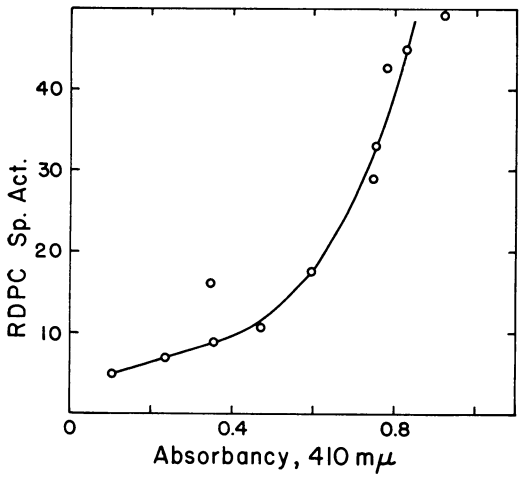

FIG. 1. *Correlation of ribulose diphosphate carboxylase (RDPC) specific activity with cytochrome concentration. Extracts obtained from separate cultures of Hydrogenomonas eutropha were adjusted to 2.5 mg of protein/ml for equivalent comparison at 410 mμ. This biphasic curve was obtained by plotting data from several types of experiments involving: (i) starvation of cells in an inorganic basal medium under air, (ii) variations in the shaking rate of fructose-grown cultures, and (iii) cell growth in fructose medium deficient in* Fe^{+3}.

levels and the presence of cytochrome pigment in cell-free extracts of *H. eutropha*. Similar data were obtained with *H. facilis*.

Marked reduction in cytochrome content, accompanied by low levels of RDPC, could be achieved in Fe^{+3}-ion supplemented cultures by increasing the shaker rate and thereby the growth rate. For example, consecutive cultures grown at slower shaker speeds (105 to 110 rev/min) had specific activities of RDPC of 51.9 and 48.9. In contrast, enzyme in consecutive cultures shaken more rapidly (160 to 170 rev/min) had specific activities of 11.3 and 7.1.

The effect of shaker speed on *H. facilis* was markedly different. Rapidly shaken cultures (170 cycles per min) underwent growth inhibition, and the terminal cell yield was only one-half that achieved at slower shaker speeds (110 cycles per min). Moreover, highly aerated cells were bleached and almost deficient in RDPC.

Repressive effect of high aeration on RDPC. When autotrophically grown *H. eutropha* was transferred to fructose medium and subsequently aerated at a rapid shaker speed, RDPC activity declined in accord with the expectation for cessation of synthesis and dilution by growth (Fig. 2). These results suggest that high aeration represses RDPC synthesis. Similar data have also been obtained with *Hydrogenomonas* H_{16} (7) and *Pseudomonas oxalaticus* (31).

Levels of RDPC obtained during growth in the presence of nitrate. When *H. eutropha* was grown on fructose medium in which NH_4NO_3 and $MgSO_4$ had been substituted for $(NH_4)_2SO_4$ and $MgCl_2$, a twofold increase in growth rate (Fig. 3) for a given shaker speed was observed, as compared to cultures grown on the usual medium. Again, cells which had experienced rapid growth were bleached and nearly devoid of RDPC. *H. eutropha* could also be grown in standing cultures in the presence of nitrate, although the growth rate was slow. Cells harvested from standing cultures were distinctly pink and contained high autotrophic levels of RDPC (Fig. 3).

Repeated subculture of H. eutropha on fructose. When the type of inoculum, shaker speed, and Fe^{+3} concentration were optimally controlled, *H. eutropha* could be subcultured continuously in fructose medium without loss of RDPC expression, provided that inoculum was derived from cultures in early exponential phase of growth (Table 1). The downward trend in the specific activity of RDPC suggested a slow loss of derepression in the presence of fructose. Data obtained with *H. facilis* revealed a more rapid loss in the specific activity of RDPC during subculturing on fructose. For example, by the third subculture a 10-fold decrease had been observed when all inocula had been obtained from the preceding culture in the late log phase of growth.

Correlation of fructose or nitrogen utilization, pH change, and culture growth with levels of RDPC. RDPC rapidly disappeared in the latter stages of exponential growth of *H. eutropha*. This suggested that carboxylase disappearance paralleled the depletion of an essential growth substrate. Fructose was found to be the limiting substrate (Fig. 4) and, although the data are not shown, its depletion was accompanied by a decline in *p*H to 4.5. RDPC activity decreased

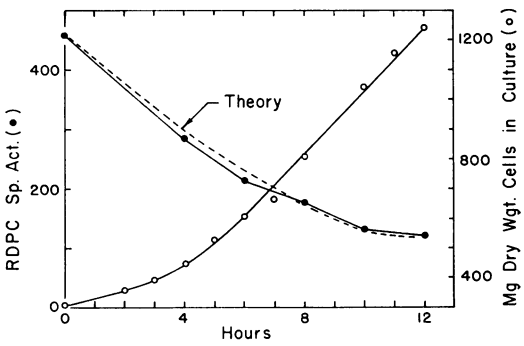

FIG. 2. *Repression of ribulose diphosphate carboxylase (RDPC) synthesis in fructose-grown Hydrogenomonas eutropha under conditions of rapid shaking (175 cycles/min).*

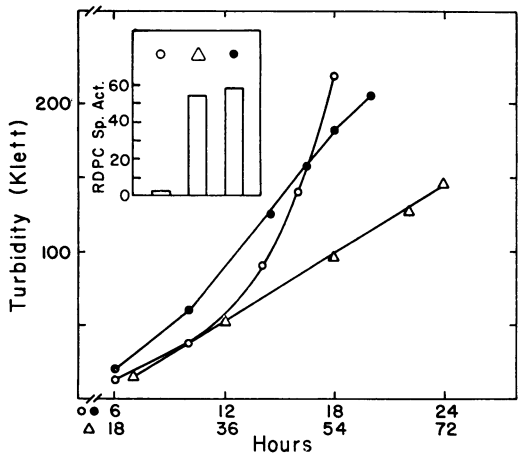

FIG. 3. *Correlation of ribulose diphosphate carboxylase (RDPC) levels with growth rate of Hydrogenomonas eutropha in fructose medium containing:* (○) *0.3% NH_4NO_3, 0.3% $MgSO_4$;* (●) *0.1% $(NH_4)_2SO_4$, 0.01% $MgCl_2 \cdot 6H_2O$. Shaker speed was 120 cycles/min.* (△) *Connotes the same supplements as* (○) *provided in a standing culture. Inset shows the corresponding levels of RDPC in extracts prepared from the latest recorded Klett value (see Table 1, footnote a).*

TABLE 1. *Levels of ribulose diphosphate carboxylase (RDPC) during continuous culture of Hydrogenomonas eutropha on fructose*

Culture	Klett readings		RDPC[b] (specific activity)
	Inoculum[a]	Harvest	
1	2 slopes	258	51.9
2	70	224	48.9
3	100	232	49.5
4	67	210	53.3
5	105	246	53.3
6	70	216	49.8
7	216	277	59.8
8	270	210	48.0
9	210	225	36.3
10	225	220	19.4

[a] For cultures 2 through 6, one-tenth volume of the preceding culture in the early log phase of growth was used as an inoculum. For cultures 7 through 10, one-tenth volume of the preceding culture in the mid- to late-log phase of growth was used as an inoculum. Klett readings of 100 and 250 correspond to cell suspensions of 0.50 and 1.10 mg (dry weight)/ml, respectively.

[b] Extracts were prepared after harvest by sonic treatment at 2 C for 30 sec at full power with a Bronwill 20-kc sonic oscillator. The activity was measured in the supernatant fluid remaining after centrifugation at 2 C at 105,000 × *g*.

rapidly and reached a basal level before actual stationary phase was attained. Attempts to induce this loss in intact cells by suspending exponential cultures in fructose media of *p*H values of 4, 5, and 6 were unsuccessful. As is evident, the loss in RDPC activity was far greater than could be accounted for by dilution, through growth, of preformed enzyme and thus was suggestive of rapid degradation or inactivation. A similar loss was observed in *H. facilis* cultures, but the observed decline, which was associated with fructose depletion, was more abrupt and profound (Fig. 5).

Starvation for fructose. When conditions during fructose depletion in normal culture growth were artificially approximated by harvesting mid-exponential-stage cells and resuspending them in inorganic basal medium lacking fructose, disappearance of RDPC activity was even more pronounced. Figure 6 represents such an experiment and shows protection afforded by the presence of fructose during harvesting and incubation of *H. eutropha*. Similar degradation was obtained when cells were suspended in 0.01 M Tris buffer containing 0.01 M $MgCl_2$, *p*H 7.4. Closely parallel results were obtained by starvation of *H. facilis;* essentially all RDPC disappeared after 30 min of severe starvation. Addition of fructose to *H. eutropha* at any time after the onset of starvation

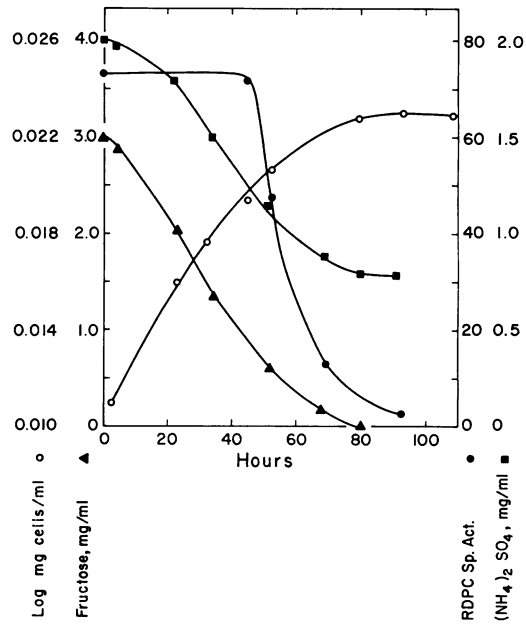

FIG. 4. *Correlation of (RDPC) specific activity with culture growth, and fructose and NH_4^+ utilization for a 5-liter culture of Hydrogenomonas eutropha.*

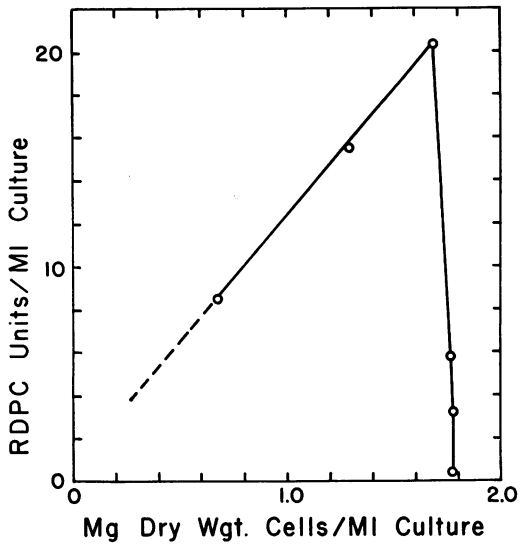

FIG. 5. *Differential rate of ribulose diphosphate carboxylase (RDPC) synthesis in Hydrogenomonas facilis throughout a complete growth cycle. Data were taken from an experiment that was similar to that described in Fig. 4.*

failed to arrest RDPC destruction. In all cases, after more than 30 min of starvation, there always remained a residual "constitutive" level of RDPC activity.

The observed decrease of RDPC activity in *H. eutropha* was accompanied by a sevenfold increase in 30 min in extracellular material absorbing at 260 mμ. This material had a 280:260 mμ absorbancy ratio of 0.55, implying some breakdown and release of intracellular material. However, RDPC could not be detected extracellularly. It is well-known that viability of pseudomonads is retained during extended starvation (6; R. E. Hulbert, Master's Thesis, Univ. of Southern Calif., 1959), and a recent report has confirmed this for *Hydrogenomonas H-16* (12).

Factors affecting RDPC loss during starvation of H. eutropha. In an effort to identify requirements for expression of the RDPC inactivating process, cells grown to mid-exponential phase on fructose were subjected to fructose starvation in the absence of other growth substrates. Magnesium ion deprivation, or addition of substrate bicarbonate, protected RDPC for up to 6 hr during fructose starvation (Table 2). In addition, nitrogen starvation in the presence of fructose resulted in no loss in RDPC.

Since bicarbonate was found to spare RDPC from inactivation in starved cells derived from fructose growth, the question arose concerning the existence of the inactivating system in autotrophic cells. Autotrophically grown *H. eutropha* was therefore washed and resuspended in inorganic basal medium and then shaken in air at 30 C for 18 hr. Complete retention of RDPC activity was observed (Table 2).

Experiments were also carried out with chloramphenicol to see whether protein synthesis was necessary for inactivation of RDPC. In one experiment, chloramphenicol (100 μg/ml) was added to log-phase, fructose-grown cells 20 min before they were harvested and resuspended in inorganic basal medium containing the antibiotic. In a second experiment, cells were exposed to chloramphenicol only after they had been harvested. The results of the latter experiment are shown in Table 2. In the former experiment, immediate cessation of exponential growth demonstrated that entry of the antibiotic into the cells had occurred. In both experiments, RDPC disappeared as rapidly as in controls lacking chloramphenicol. In marked contrast, the addition of actinomycin D completely spared RDPC, and streptomycin addition partially spared the enzyme. Streptomycin at the level employed did not inhibit growth. The effect of actinomycin D upon growth was not tested.

The effects of 2,4-dinitrophenol were next

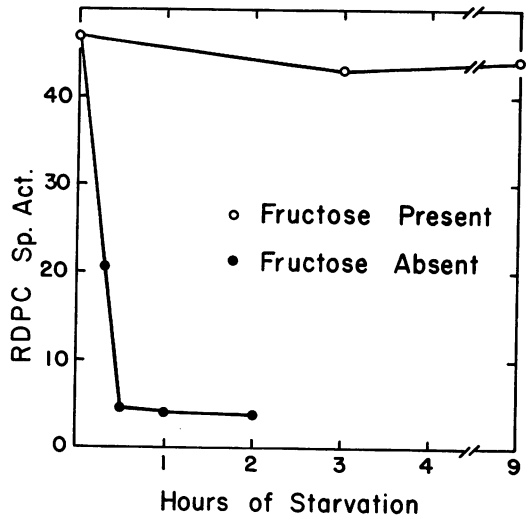

FIG. 6. *Effect of harvest and transfer of exponential-phase, fructose-grown Hydrogenomonas eutropha to: (●) inorganic basal medium, and (○) inorganic basal medium containing 0.3% fructose. Mid-exponential-phase cells were harvested by centrifugation at 6,000 × g. The collected cells were then resuspended in the respective media, and portions were removed for rupture and assay at the specified times.*

tested. This reagent, at a concentration of 1 mM preserved RDPC during extended starvation (Table 2). This concentration also immediately arrested growth of *H. eutropha*. Prior experiments have suggested that similar concentrations of 2,4-dinitrophenol uncouple oxidative phosphorylation in *H. facilis* (22). Anaerobiosis also spared RDPC activity as evidenced by fructose starvation of *H. eutropha* in atmospheres of pure N_2 or H_2 (Table 2).

Inactivation of RDPC in cell-free extracts. Loss in RDPC activity could also be consistently demonstrated in cell-free extracts prepared from fructose-grown *H. eutropha*. Figure 7 shows the effect of dialysis of crude cell-free extracts prepared from the same culture against buffers of varying composition. Complete preservation of RDPC was afforded only by the presence of the substrate $NaHCO_3$. Magnesium ion was not required for RDPC inactivation during dialysis, in contrast to its requirement for in vivo loss in activity. Parallel experiments with *H. facilis* extracts have yielded variable results, suggesting that release of the inactivating system by cell rupture is more variable.

Figure 8 shows that dialysis of a cell-free preparation from autotrophically grown *H. eutropha* against Mg^{+2}-supplemented buffer produced no loss in enzyme activity. Under identical conditions, extracts from fructose-grown cells lost 70% of their RDPC activity in a 22-hr period. A mixture of extracts derived from fructose- and autotrophically-grown cells lost 66% of its activity in 22 hr. The factor in heterotrophically derived extracts that inactivates RDPC is heat-labile (Fig. 8).

In vitro loss of RDPC activity in cell-free extracts of *H. eutropha* (13 mg of protein/ml) during a 22-hr period was not diminished by chloramphenicol (100 μg/ml), actinomycin D (100 μg/ml), 2,4-dinitrophenol (1 mM), N_2, or H_2.

DISCUSSION

Three important factors favoring the retention of high levels of RDPC during growth of the hydrogen bacteria on fructose have been identified: (i) low aeration rate, (ii) supplementation of the medium with $FeCl_3$, and (iii) harvest of cells at or prior to mid-exponential phase of growth. These observations emphasize the necessity of careful experimental design in estimates of RDPC content of the hydrogen bacteria and, perhaps, of other facultative organisms. They may account for the twofold to threefold variations observed with a given growth substrate for *Hydrogenomonas H-16* (7).

An inverse relationship between cytochrome content and oxygen tension has been observed in a variety of pseudomonads and denitrifying bacteria (16). In the present work this relationship has also been observed. In addition, a parallel existence and disappearance of RDPC and cytochrome has been observed under a variety of conditions, suggesting some relationship between the two. It is tempting to speculate that the synthe-

TABLE 2. *Effect of starvation of Hydrogenomonas eutropha under various conditions upon ribulose diphosphate carboxylase (RDPC) activity*

Cells grown	Starvation medium[a]	RDPC activity[b] (%)
On fructose	Inorganic basal	9
On fructose	+ Fructose	(100)
On fructose	− Mg^{+2}	100
On fructose	− NH_4^+ + 0.3% fructose	100
On fructose	+ HCO_3^-	92
Autotrophically[c]	Inorganic basal	91
On fructose	+ Chloramphenicol, 100 μg/ml	4
On fructose	+ Streptomycin, 100 μg/ml	34
On fructose	+ Actinomycin D, 50 μg/ml	100
On fructose	+ 1 mM 2,4-Dinitrophenol	102
On fructose	Under 100% N_2	100
On fructose	Under 100% H_2	106
On fructose	Under 80% H_2, 20% O_2	95
On fructose	+ Chloramphenicol, 100 μg/ml, under 80% H_2, 20% O_2	97

[a] Minus (−) denotes deletions from the inorganic basal medium; plus (+) denotes additions to the inorganic basal medium.

[b] Experiments yielding >90% loss in RDPC activity were terminated after 1 hr or less after starvation.

[c] Autotrophic cells were starved 18 hr. Starvation was allowed for 6 hr in all other experiments.

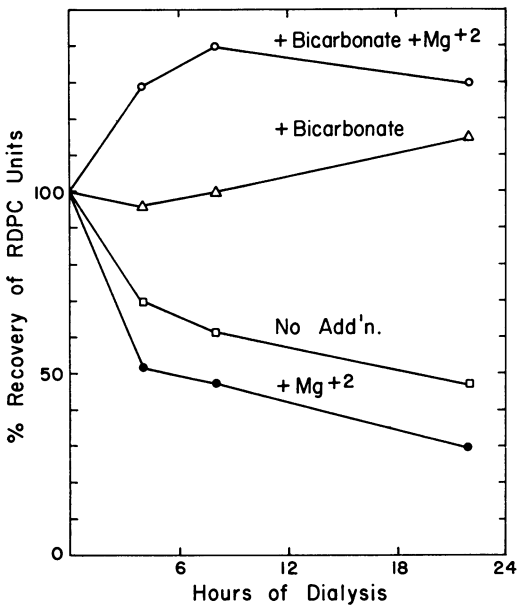

FIG. 7. *Dialysis of crude extracts from Hydrogenomonas eutropha at 2 C in TM buffer (made up of 0.01 M Tris containing 2 mM β-mercaptoethanol) containing 1 mM ethylenediaminetetraacetic acid, pH 8.0 (control: "no addition"), plus 0.01 M $MgCl_2 \cdot 6H_2O$ or 0.05 M $NaHCO_3$, or both. A single culture was divided into four equal portions and each portion was respectively pressure-ruptured in one of the buffers described above. After preparation, each crude extract was dialyzed against the buffer of identical composition to that in which the cells had been ruptured. During dialysis, portions were removed for assay of ribulose diphosphate carboxylase (RDPC).*

sis of these two proteins is regulated in a parallel manner.

In most cases, when the formation of an enzyme is suppressed as a result of either the withdrawal of an inducer or the addition of a repressing compound, the enzyme already existing is not destroyed. Rather, it is lost only through dilution by cell duplication (30). This was found to be the case for RDPC synthesis in *H. eutropha* grown under conditions of strong aeration. However, a different phenomenon was encountered in the rapid loss of activity when fructose-grown cells were cultured into the late exponential phase with low aeration. The rate of decline in the specific activity of RDPC was much more rapid than could be accounted for by dilution through new protein synthesis. A similar result has been obtained with *Chromatium* (13).

This rapid loss of RDPC activity occurred in cells during approach to fructose limitation of growth, in cells starved of fructose after growth on fructose, and in cell-free preparations from fructose-grown cells. Loss in RDPC activity could not, however, be demonstrated in autotrophically derived cells either by starvation or in cell-free extracts. This suggests that the RDPC-inactivating mechanism is synthesized or is functional only as a consequence of heterotrophic growth. The possibility that a resistant and susceptible carboxylase are synthesized during autotrophic and heterotrophic growth, respectively, was ruled out in the mixing experiments (Fig. 8).

In intact starving cells, disappearance of RDPC appeared to be an energy-dependent process. Addition of 1 mM 2,4-dinitrophenol afforded complete protection against destruction of RDPC during starvation for fructose. Similarly, effects of severe fructose starvation which destroyed enzyme activity under air could be prevented by an atmosphere of either pure N_2 or H_2. These three results indicated that loss in RDPC activity requires adenosine triphosphate (ATP).

That RDPC inactivation in vitro is the result of proteolysis was suggested by evidence for the involvement of a nondialyzable, heat-labile factor. Moreover, the observed protection by substrate parallels similar in vitro and in vivo

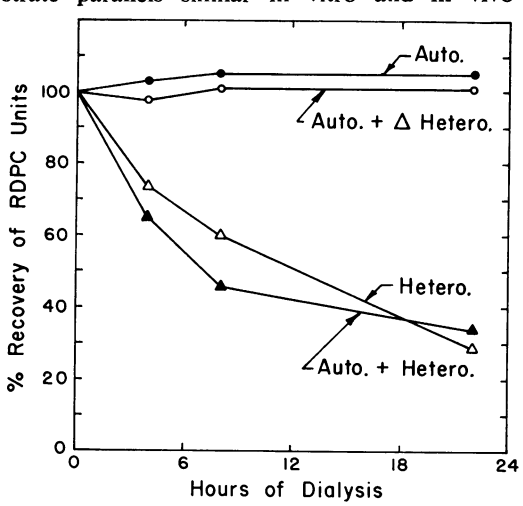

FIG. 8. *Demonstration of heat lability of ribulose diphosphate carboxylase (RDPC) inactivating factor and the effect of heterotrophic extracts on autotrophic RDPC activity. An extract prepared from autotrophic Hydrogenomonas eutropha was dialyzed in TM buffer supplemented with ethylenediaminetetraacetic acid (EDTA) and Mg^{+2} (see Fig. 7) separately (●); after addition of an equivalent amount of protein from an extract of exponential-phase, fructose-grown cells (▲); or after addition of the same heterotrophic extract previously heat-treated at 50 C for 10 min (○). The heterotrophic extract was also dialyzed separately in the TM buffer containing EDTA and Mg^{+2} (△).*

phenomena that have been attributed to substrate protection of protein against proteolysis (3, 36, 39). Results with intact starving cells using chloramphenicol suggested that a proteolytic system was not synthesized as a consequence of starvation. Instead, the inactivating system was latent and was "switched on" by conditions of fructose deficiency either during growth or starvation. We hypothesize that the inactivating system in vivo is proteolytic in nature. Indeed, the ability to demonstrate RDPC inactivation in vitro, with extracts derived from fructose-grown cells without prior starvation, is in accord with this hypothesis. Presumably, RDPC becomes accessible to intracellular proteases as a consequence of cell disruption.

It is difficult to rationalize the protection of RDPC during starvation exerted by 2,4-dinitrophenol, actinomycin D, streptomycin, and omission of Mg^{+2} in terms of the postulated proteolytic destruction. Perhaps these factors exert a secondary effect on a proteolytic system by affecting other processes essential to its activation or its action upon RDPC.

In this connection, ribosomal instability in glucose-grown, starving *E. coli* (5, 26) and *P. aeruginosa* (11) has been established. Latent ribosomal nucleases, which are activated by conditions that destroy the ribonucleoprotein particles containing them, have been reported in *Escherichia coli* (4, 9). Indeed, the observed release into starvation medium of material absorbing at 260 mμ by *H. eutropha* might partially reflect a similar process. Mandelstam has postulated that proteolytic enzymes might also be carried in a latent form by the ribosomes (20). From these reports and our compatible results, one might speculate on the requirements observed here for RDPC inactivation. RDPC could exist in vivo as a stable deoxyribonucleoprotein complex. During severe starvation, latent nucleases and proteases would be released as a result of Mg^{+2}-, energy-dependent intracellular alterations associated with ribosomal breakdown. Deoxyribonuclease would then destroy the normally stable RDPC-nucleic acid complex, thus sensitizing RDPC to protease or proteases, unless bicarbonate were present. In the presence of actinomycin D, the RDPC-deoxyribonucleic acid (DNA) complex would be refractory to partial degradation by deoxyribonuclease. Indeed, the inhibition of deoxyribonuclease by similar concentrations of actinomycin D to those used in the present study has recently been reported by Sarkar (34). Streptomycin, which partially protects RDPC, and omission of Mg^{+2}, which fully protects RDPC, are both known to favor the phenomenon of phage exclusion, which is thought to involve suppression of DNA degradation by deoxyribonuclease (38). Speculation concerning the nucleoprotein character of RDPC is not totally without support. Purification of this enzyme from both *H. eutropha* and *H. facilis* suggests that it may well be asociated with some nucleic acid (G. D. Kuehn, *unpublished observation*). A similar property has been observed for the rice (27), spinach, and tobacco leaf (8) enzymes.

Our observation that decreased ATP production may spare RDPC is consistent with those of Mandelstam (19) and Pine (29), who both reported that 2,4-dinitrophenol inhibited general protein degradation in *E. coli* during starvation. Moreover, anaerobiosis protects tryptophan pyrrolase from proteolytic inactivation in rat liver slices (36). An inhibition of further metabolism of inhibitory, accumulating, low molecular weight products of proteolysis was proposed by Mandelstam to account for the effect of 2,4-dinitrophenol on protein breakdown (21). While the concept of proteolysis is attractive, the apparent involvement of ATP for RDPC inactivation is reminiscent of the enzyme-catalyzed, ATP-requiring, nonproteolytic alteration of glutamine synthetase (14). Whether there is any parallel between these phenomena remains to be seen. It is impossible to assign a specific effect to magnesium ion in the present work. It may, however, be required for "release" or activation of the inactivating system, because RDPC degradation occurs in its absence in extracts. If RDPC indeed exists as a nucleoprotein complex in cells, cell disruption must sensitize the complex to direct proteolysis. Except for bicarbonate, none of the factors which preserved RDPC during starvation of intact cells diminished RDPC inactivation in extracts, thus suggesting that these factors interfered with "release" or operation of the inactivating system in vivo.

In conclusion, the experiments described here clearly establish two types of control that are exerted on RDPC in *H. eutropha* during growth on fructose: (i) repression, under conditions of high oxygen tension, and (ii) rapid inactivation, perhaps by proteolysis, under conditions of low oxygen tension and limiting fructose. The rapid RDPC inactivation process could be demonstrated in vivo and in vitro only after heterotrophic growth.

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