JOURNAL OF BACTERIOLOGY, Aug. 1967, p. 409-414

Vol. 94, No. 2
Printed in U.S.A.

Effect of Adenosine Monophosphate, Adenosine Diphosphate, and Reduced Nicotinamide Adenine Dinucleotide on Adenosine Triphosphate-dependent Carbon Dioxide Fixation in the Autotroph *Thiobacillus neapolitanus*[1]

JERRY V. MAYEUX[2] AND EMMETT J. JOHNSON[3]

Exobiology Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035

Received for publication 27 May 1967

The observation that adenosine triphosphate (ATP)-dependent CO_2 fixation in extracts of chemosynthetic and photosynthetic autotrophs may be regulated in part by adenosine monophosphate (AMP) was extended to the strict autotroph *Thiobacillus neapolitanus* (X). In addition, this report presents data which include adenosine diphosphate (ADP) in the regulatory role. When the primary CO_2 acceptor, ribose-5-phosphate, was replaced by ribulose-1,5-diphosphate, no inhibition of CO_2 fixation occurred unless the Mg^{++} concentration was limiting. A molar ratio of 5:1 AMP or ADP to ATP reduced the specific activity (micromoles of CO_2 fixed per milligram of protein per minute) of the extracts from 0.22 to 0.12 and 0.11, respectively. The reported stimulation of the carboxylative phase of ATP-dependent CO_2 fixation by reduced nicotinamide adenine dinucleotide ($NADH_2$) was investigated. Adding $NADH_2$ to the extracts did not stimulate CO_2 fixation, even at carbonate levels from 0.05 to 30 μmoles, except in the absence of ribose-5-phosphate. Slight increases in CO_2 fixation were noted when the assay system was incubated in air instead of the usual helium atmosphere.

It is now apparent that adenosine triphosphate (ATP)-dependent CO_2 fixation is inhibited by adenosine monophosphate (AMP) in several chemosynthetic and photosynthetic autotrophs (1, 6, 7). In addition, adenosine diphosphate (ADP) has been implicated in the inhibition of this phase of CO_2 fixation in extracts of the photosynthetic bacterium *Chromatium* and in the green plant *Spinacia oleracea* (6). The apparent widespread inhibition of CO_2 fixation by these nucleotides has led to the postulation (6, 7) that AMP may be universally active as a regulator of ATP-dependent CO_2 fixation in autotrophic organisms, and to the suggestion of a possible role for ADP in the photosynthetic autotrophs. The data presented in this paper extend the inhibitory role of AMP to another species of the thiobacilli, namely, *Thiobacillus neapolitanus* (X), and show that ADP is equally as inhibitory as AMP in this nonphotosynthetic autotrophic bacterium.

[1] Presented in part at the 66th Annual Meeting of The American Society for Microbiology, Los Angeles, Calif., 1966.

[2] National Academy of Sciences—National Research Council Postdoctoral Resident Research Associate at Ames Research Center. Present address: Department of Microbiology, Colorado State University, Fort Collins 80521.

[3] Present address: Bruce Lyon Memorial Research Laboratory, Oakland, Calif.

MATERIALS AND METHODS

Cultural procedures and preparation of extracts. T. neapolitanus was grown in the medium of Santer, Boyer, and Santer (10) with the following modifications: NH_4Cl, 1.0 g, and $Na_2S_2O_3 \cdot 5H_2O$, 10 g; bromo-

cresol purple was added as a *p*H indicator at 0.001 g/liter. The cultures were either incubated at 30 C on a rotary shaker in 2-liter Erlenmeyer flasks (500 ml/flask) with intermittent neutralization, or were grown in 10-liter batches in a Microferm Laboratory Fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) at 30 C with forced aeration (5 liters/min) and stirring at 400 rev/min. In the latter case, the *p*H was continuously maintained at 6.6 by the addition of 50% K_2CO_3 with a metering pump (Leeds & Northrup Co., Philadelphia, Pa.). Sodium thiosulfate was added at intervals until 20 to 30 g/liter had been utilized by the cells (cell yield, approximately 2 g/liter, wet weight, with the fermentor and 0.5 to 1 g/liter on the shaker). The cells were harvested with a refrigerated Sharples supercentrifuge and washed with 0.1 M tris-(hydroxymethyl)aminomethane (Tris) chloride buffer (*p*H 7.45). A 20 to 50% (wet weight/volume) cell paste was prepared in 0.1 M Tris and broken with a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 18,000 psi. The homogenate was then treated with 20 μg of deoxyribonuclease (Calbiochem, Los Angeles, Calif.) per 10 ml of homogenate and was allowed to stand at 4 C up to 1 hr. The homogenate was centrifuged at 20,000 × *g* for 30 min. The supernatant fluid from this centrifugation was passed over a column (1.5 by 30 cm) of G-25 coarse Sephadex beads equilibrated with 0.1 M Tris chloride buffer (*p*H 7.45). This effluent extract, when stored frozen, maintained its carboxylative activity for at least 2 months.

Carbon dioxide fixation and radioactivity measurements. The incorporation of ^{14}C from $Na_2{}^{14}CO_3$ into the acid-stable fraction and the radioactivity measurements were carried out according to the procedure described by Johnson and Peck (7). Specific activity for the CO_2-fixing process is defined as micromoles of CO_2 fixed per milligram of protein per minute. $Na_2{}^{14}CO_3$ was purchased from Volk Radio Chemical Co., Burbank, Calif., and was diluted with unlabeled sodium carbonate to a specific activity of 2.5×10^5 counts per min per μmole.

Chromatography. The products of CO_2 fixation were determined by two-dimensional thin-layer chromatography essentially as described by Bieleski (3), but with the following modifications: MN cellulose-300 powder (Machery-Nagel & Co., Düren, West Germany) was used with *n*-propanol-ammonia (specific gravity, 90)-water (6:3:1, v/v) plus 2 g of sodium ethylenediaminetetracetate (EDTA)/liter run twice (3 hr each) in the first dimension and *n*-propyl acetate-90% formic acid-water (11:5:3, v/v) run twice (80 min) in the second dimension.

Chemicals. AMP, ADP, ATP, ribose-5-phosphate (R-5-P), ribulose-1,5-diphosphate (Ru-D-P) and reduced nicotinamide adenine dinucleotide ($NADH_2$) were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Figure 1 presents data depicting the influence of the sodium carbonate concentration on the activity of the carboxylative-phase enzymes. Previously, investigators have used carbonate concentrations as low as 0.05 μmole/ml while studying the carboxylative-phase enzymes (1). Although high counts can be obtained by using carbonate of high specific activity, very little CO_2 is actually incorporated under these conditions.

In searching for the optimal reaction conditions, we found that increasing the amount of R-5-P to 10 μmoles, at the optimal carbonate concentration shown in Fig. 1, provided at least a twofold increase in the amount of CO_2 fixed. Similar increases in activity had been found while determining the optimal $MgCl_2$ and ATP concentrations.

Data in Table 1 show that AMP and ADP inhibit ATP-dependent CO_2 fixation by the extracts of cells of *T. neapolitanus*. An AMP–ATP ratio of 1:1 gave 18% inhibition of the CO_2 incorporated into the acid-stable fraction, whereas 5:1 AMP-ATP inhibited by 47%. Adding ADP to ATP in a 1:1 ratio inhibited by 23%. At a ratio of 3:1, ADP inhibited CO_2 fixation by 36%, and at a 5:1 ratio of ADP to ATP the inhibition was 53%. Complete inhibition was not obtained with either AMP or ADP when up to 100 μmoles was added (10:1 AMP or ADP to ATP), nor were we able to reverse the inhibition by increasing the

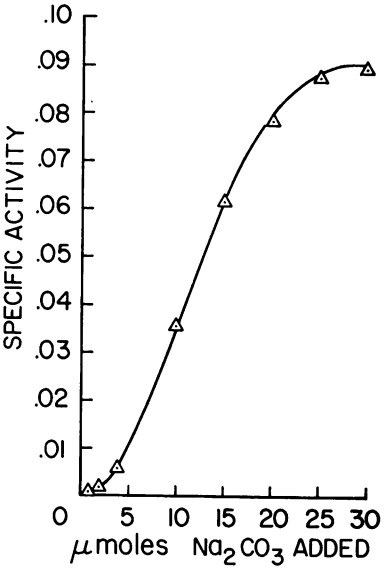

FIG. 1. *Effect of the carbonate concentration on total carbon dioxide incorporated by extracts of Thiobacillus neapolitanus.* [*The assay system contained, in μmoles: Tris (pH 7.45), 100; R-5-P, 5; ATP, 10; $MgCl_2$, 100; $Na_2{}^{14}CO_3$ (250,000 counts per min per μmole), as indicated. Total volume, 2.7 ml; reaction time, 10 min; helium atmosphere; cell extract, 3.84 mg of protein (Sephadex-treated).*]

Mg^{++} concentration. Earlier experiments with the reactants ATP, R-5-P, and CO_2 at less than optimal concentration had provided inhibition of up to 94% with an AMP addition of 50 μmoles.

The inhibition of ATP-dependent CO_2 fixation by a combination of AMP and ADP was examined (Table 2). A combination of 10 μmoles of ADP and AMP exhibited essentially the same degree of inhibition as did 30 μmoles of AMP or ADP alone. The degree of inhibition by each nucleotide was approximately additive at the lower concentration, whereas the combined inhibition by the nucleotides at the higher concentration (30 μmoles) was slightly less than the added values of each alone. ADP added in place of ATP was able to support CO_2 fixation, although much less than ATP. The ADP was probably being converted to ATP by myokinase activity.

TABLE 1. *Inhibition of ATP-dependent CO_2 fixation in extracts of Thiobacillus neapolitanus by added AMP and ADP*

Assay system[a]	AMP	ADP	CO_2 fixed	Specific activity	Inhibition
	μmoles	μmoles	μmoles		%
Complete			3.01	0.22	
Minus R-5-P			0.063	0.004	
Minus ATP			0.074	0.005	
Minus $MgCl_2$			0.14	0.010	
Minus ATP	10		0.086	0.006	
Minus ATP		10	0.51	0.037	
Complete	10		2.48	0.18	18
Complete	30		2.04	0.15	32
Complete	50		1.59	0.12	47
Complete		10	2.25	0.17	23
Complete		30	1.83	0.14	36
Complete		50	1.42	0.11	53

[a] The complete system contained, in μmoles: Tris (*p*H 7.45), 100; R-5-P, 10; ATP, 10; $MgCl_2$, 100; and $Na_2{}^{14}CO_3$, 30 (2.5×10^5 counts per min per μmole). Cell extract, 1.35 mg of protein (Sephadex-treated); reaction time, 10 min; total volume, 2.7 ml; temperature, 30 C; helium atmosphere.

TABLE 2. *Inhibition of ATP-dependent CO_2 fixation by AMP and ADP in combination*[a]

ATP	AMP	ADP	CO_2 fixed	Inhibition
μmoles	μmoles	μmoles	μmoles	%
10	0	0	5.84	—
0	10	0	0.11	—
0	0	10	1.56	—
10	10	0	5.17	12
10	0	10	4.88	16
10	10	10	4.40	25
10	30	0	4.56	22
10	0	30	4.22	28
10	30	30	3.46	41

[a] Conditions were as listed in Table 1 with the exception that Sephadex-treated cell extract (3.84 mg of protein) was used.

The inhibition of ATP-dependent CO_2 fixation by AMP and ADP apparently occurs at some point other than the Ru-D-P carboxydismutase enzyme site, as the data in Table 3 show no inhibition by either ADP or AMP. The site of inhibition appears to be phosphoribulokinase (E. J. Johnson, Bacteriol. Proc., p. 96, 1966). Some inhibition of the carboxydismutase was obtained only when the magnesium ion concentration was limiting.

Recently published data (1) imply that $NADH_2$ is required for the carboxylative phase of autotrophic CO_2 fixation, since $NADH_2$ appeared to be required for $^{14}CO_2$ fixation, when over 90% of the CO_2 fixed was recovered in 3-phosphoglyceric acid (3-PGA). Although reducing power is needed for further reducing the primary fixation product 3-PGA to 3-phosphoglyceraldehyde, present concepts do not show a need for reducing power during the actual carboxylative phase of the carbon reduction cycle. Table 4 presents data which show the influence of $NADH_2$ on ATP-dependent CO_2 fixation in extracts of *T. neapolitanus* with limiting amounts of carbonate, as was the case in the earlier report of $NADH_2$ stimulation (1). No apparent change of activity was observed by the addition of $NADH_2$, not even when the acceptor R-5-P was omitted from the system. The results were the same whether or not the extracts were passed over G-25 Sephadex gel. These data again show that any increase in Na_2CO_3 content stimulates the rate at which CO_2 is incorporated by the enzyme system, as evidenced by increases in the amount of fixation associated with increases in the carbonate concentration. This experiment was conducted under

TABLE 3. *Influence of AMP and ADP on ribulose-1, 5-diphosphate carboxydismutase*

Assay system[a]	AMP	ADP	CO_2 fixed
	μmoles	μmoles	μmoles
Complete			1.50
Minus $MgCl_2$			0.045
Complete	10		1.48
Complete	30		1.55
Complete	50		1.55
Complete		10	1.48
Complete		30	1.48
Complete		50	1.49

[a] The complete system contained, in μmoles: Tris (*p*H 7.45), 100; Ru-1,5-P, 2; $MgCl_2$, 150; $Na_2{}^{14}CO_3$, 40 (2.5×10^5 counts per min per μmole). Cell extract, 1.50 mg of protein (Sephadex-treated); reaction time, 10 min; volume, 2.7 ml; temperature, 30 C; helium atmosphere.

aerobic conditions; the results were the same, however, in the absence of air. In fact, if any conclusions can be drawn from the data with carbonate at 0.05 μmole, it is that R-5-P, ATP, and $NADH_2$ are inhibitory at their optimal concentrations when other reactants (CO_2 in this case) are limiting. This becomes obvious when the complete system is compared with the controls without added ATP or R-5-P.

Typical CO_2-fixing data are presented in Table 5 for the extracts not treated with Sephadex which were assayed at the optimal carbonate concentration. The assay was usually conducted under anaerobic conditions to eliminate oxygen-requiring reactions which might interfere with the quantitative assay of the autotrophic carboxylative system. In the complete assay system, approximately 4 μmoles of CO_2 were incorporated during the 10-min incubation period. The addition of $NADH_2$ to the complete system, or to the ATP or magnesium controls, failed to stimulate CO_2 fixation. The only system stimulated by $NADH_2$ was the one without added CO_2 acceptor (i.e., the system lacking R-5-P), which was stimulated 10%. In fact, in most instances, adding $NADH_2$ to a system deficient in one of the components necessary for optimal enzyme activity reduced endogenous CO_2 fixation.

TABLE 4. *Effect of $NADH_2$ on CO_2 fixation in the presence of limiting amounts of carbonate*

Assay system[a]	Total counts/min incorporated	CO_2 fixed (mμmoles)	Specific activity (10^{-6} μmole per mg of protein, per min)
Complete (0.05 μmole of $^{14}CO_3$)	975	0.171	8.9
+ $NADH_2$	855	0.150	7.8
Minus R-5-P (0.05 μmole of $^{14}CO_3$)	1,260	0.222	11.5
+ $NADH_2$	1,185	0.208	10.8
Minus ATP (0.05 μmole of $^{14}CO_3$)	4,635	0.815	42.4
+ $NADH_2$	3,420	0.601	31.3
Minus $MgCl_2$ (0.05 μmole of $^{14}CO_3$)	1,125	0.197	10.2
+ $NADH_2$	1,110	0.195	10.1
Complete (0.15 μmole of $^{14}CO_3$)	3,165	0.557	29.0
+ $NADH_2$	1,965	0.345	17.9
Complete (0.30 μmole of $^{14}CO_3$)	6,450	1.135	59.1
+ $NADH_2$	6,255	1.100	57.2

[a] Conditions were the same as for Table 1 except as indicated: R-5-P, 5 μmoles; $Na_2{}^{14}CO_3$, 2.5×10^5 counts per min per 0.05 μmole; $NADH_2$, 5 μmoles; cell extract, 1.92 mg of protein (not Sephadex-treated); atmosphere, air.

TABLE 5. *Effect of $NADH_2$ on ATP-dependent CO_2 fixation in extracts of Thiobacillus neapolitanus (anaerobic assay)*

Assay system[a]	Total counts/min incorporated	CO_2 fixed (μmoles)	Percentage difference with $NADH_2$
Complete	308,836	3.98	
+ $NADH_2$	295,936	3.82	−4.2
Minus R-5-P	7,192	0.09	
+ $NADH_2$	8,107	0.10	+10
Minus ATP	9,102	0.12	
+ $NADH_2$	7,796	0.10	−16
Minus $MgCl_2$	8,539	0.11	
+ $NADH_2$	6,098	0.08	−28

[a] Conditions were the same as for Table 1 except where indicated: $Na_2{}^{14}CO_3$, 26 μmoles (2.5×10^5 counts per min per μmole); $NADH_2$, 5 μmoles; cell extract, 1.95 mg of protein (not Sephadex-treated).

Table 6 shows the same system incubated under aerobic conditions. The slight increases in CO_2 fixation by $NADH_2$ in the complete system, although probably not significant, could be due to heterotrophic reactions being stimulated by the presence of oxygen. On other occasions, a 7% increase in CO_2 incorporation by the aerobic system has been observed. Under aerobic conditions, in the absence of R-5-P, $NADH_2$ stimulated CO_2 fixation 21%. The most significant difference between the anaerobic and aerobic incubation of the reaction mixtures was that aerobic incubation caused an approximately fivefold increase in the endogenous fixation compared with the anaerobic system when the extracts were not passed over a column of Sephadex (Table 5). No stimulation of the complete system was observed when $NADH_2$ was added to the extracts treated with Sephadex.

It has been reported (1) that a stimulation of autotrophic CO_2 fixation by $NADH_2$ occurred in experiments incubated for 30-min periods. Table 7 presents results from a similar 30-min experiment, conducted both aerobically and anaerobically with the ATP, Mg^{++}, R-5-P, and $Na_2{}^{14}CO_3$ at the optimal concentrations determined by titration. After 30 min of incubation, no significant stimulation was caused by the addition of $NADH_2$. The endogenous fixation was again five times higher in the aerobic system. A duplicate set of flasks removed after the 10-min incubation period had the same specific activity as was obtained for the 30-min period.

Chromatography of the reaction mixtures confirmed the fact that $NADH_2$ does provide reducing power for further reduction of the primary fixation product, 3-PGA. Experiments

TABLE 6. *Effect of $NADH_2$ on ATP-dependent CO_2 fixation in extracts of Thiobacillus neapolitanus (aerobic assay)*

Assay system[a]	Total counts/min incorporated	CO_2 fixed (μmoles)	Percentage difference with $NADH_2$
Complete.....	322,526	4.61	
+ $NADH_2$...	328,469	4.70	+1.9
Minus R-5-P.....	35,984	0.51	
+ $NADH_2$...	43,675	0.62	+21.5
Minus ATP.....	41,038	0.59	
+ $NADH_2$...	37,546	0.54	−8.5
Minus $MgCl_2$.....	36,362	0.52	
+ $NADH_2$...	34,137	0.49	−5.8

[a] Conditions were the same as for Table 5 except that an air atmosphere was used in place of the helium atmosphere.

TABLE 7. *Effect of $NADH_2$ on CO_2 fixation in extracts of Thiobacillus neapolitanus incubated for 30 min*

Assay system[a]	CO_2 fixed (μmoles)		Specific activity	
	Anaerobic	Aerobic	Anaerobic	Aerobic
Cell extract....	0.15	0.59	0.004	0.019
+ ATP.....	0.14	0.62	0.004	0.020
+ ATP + $NADH_2$...	0.17	0.61	0.005	0.020
+ R-5-P.....	0.20	0.70	0.006	0.023
+ R-5-P + ATP.....	6.76	6.60	0.22	0.22
+ R-5-P + ATP + $NADH_2$...	6.41	6.46	0.21	0.21

[a] Assay conditions were the same as for Table 1 except that $NADH_2$ was added at 5 μmoles and the reaction time was increased to 30 min.

conducted with Sephadex-treated extracts showed only two radioactive compounds when the extracts were chromatographed and exposed to X-ray film. The two components in the extracts incubated in the absence of $NADH_2$ were 3-PGA and phosphoenolpyruvate, with the radioactivity divided about 95 and 5%, respectively. Adding $NADH_2$ to the Sephadex-treated extracts caused the appearance of a third radioactive spot which has not been identified but is in the region of phosphorylated trioses. Use of extracts which had not been desalted on Sephadex gel but to which $NADH_2$ was not added exogenously also gave more than two radioactive compounds, suggesting that endogenous reducing power is available.

DISCUSSION

The initial observation that AMP inhibited ATP-dependent CO_2 fixation in extracts of *T. thioparus* led Johnson and Peck (7) to suggest that this might represent a basic control mechanism in autotrophic metabolism. The extension of this observation to the autotrophs *Chromatium* and *S. oleracea* (6) strengthened the hypothesis, although certain differences exist among the various organisms studied.

In extracts of *T. thioparus* (7) an AMP-ATP ratio of 2 completely inhibited ATP-dependent CO_2 fixation, whereas with *S. oleracea* extracts (6), an AMP-ATP ratio of 2 caused 60% inhibition of CO_2 fixation. AMP caused complete inhibition in *Chromatium* at a ratio of 0.05. The system for *T. neapolitanus* resembles the spinach system and the system in *T. ferrooxidans* (5) more than it does that of *T. thioparus*, since only partial inhibition was obtained with AMP. An AMP-ATP ratio of 5 caused a 47% inhibition in *T. neapolitanus*, whereas Gale and Beck (5) obtained 10% inhibition at a 1:1 ratio with nonlimiting ATP and 50% at a 10:1 ratio with limiting ATP.

As was the case with *T. thioparus* (7), in extracts of *T. neapolitanus* ADP was able to support about 50% of the CO_2 fixation observed with ATP, probably through the action of myokinase. In addition to supporting CO_2 fixation in the absence of ATP, ADP inhibits CO_2 fixation in the presence of ATP with extracts of *T. neapolitanus*, but not with *T. thioparus* extracts. ADP has also been shown to inhibit ATP-dependent CO_2 fixation in *Chromatium* and in spinach leaf extracts (6). Whereas ADP was less inhibitory than AMP in the *Chromatium* extracts, and was more inhibitory than AMP in extracts of *S. oleracea*, it was at least as inhibitory as AMP in extracts of *T. neapolitanus*.

We have shown that extracts of *T. neapolitanus* behave similarly to extracts of *S. oleracea* (6) with regard to inhibition by ADP or AMP of the carboxylative system of enzymes. It is apparent that *T. neapolitanus* behaves quite unlike *T. thioparus* in this respect; its behavior is more like that of the photosynthetic plant *S. oleracea* and somewhat like that of the photosynthetic bacterium *Chromatium*. As a result of these observations, an attempt was made to determine the effect of light on the growth of *T. neapolitanus*. Light was found to stimulate the growth rate of the organism approximately twofold. Although growth yield on thiosulfate in the light did not exceed growth in the dark, maximal yield was achieved in about one-half the time if the organism was incubated in light.

Evidence (6; E. J. Johnson, Bacteriol Proc., p.

96, 1966) suggests that inhibition of ATP-dependent CO_2 fixation is probably due to direct action of AMP on phosphoribulokinase, and that AMP may be a negative allosteric effector. Although phosphoriboisomerase is inhibited slightly under certain conditions, it is not inhibited under the same conditions that CO_2 fixation is inhibited. Also, as was shown earlier and as we have shown here, neither ADP nor AMP inhibits carboxydismutase when an adequate magnesium ion concentration is provided to the system.

Gale and Beck (5) recently reported that kinetic studies conducted on cell-free extracts of *T. ferrooxidans* indicate that the inhibition of CO_2 fixation by AMP is of a competitive nature between AMP and the substrate ATP for the phosphoribulokinase enzyme. Preliminary studies in our laboratory on cell-free extracts of *T. neapolitanus* indicate that there is some competitive inhibition, but that the total inhibition observed involves more than the competitive inhibition of AMP for the ATP site on phosphoribulokinase.

The $NADH_2$ stimulation of ATP-dependent CO_2 fixation in *T. neapolitanus* which has been reported (1) could not be demonstrated in the complete system with these extracts under either aerobic or anaerobic conditions with either limiting or optimal carbonate concentrations. In the absence of adequate CO_2 acceptor (R-5-P), $NADH_2$ caused a 10% stimulation of CO_2 fixation under anaerobic conditions and a 21% stimulation under aerobic conditions in the presence of an optimal carbonate concentration. With a limiting carbonate concentration, no such stimulation was observed.

It is possible that the stimulation reported upon addition of $NADH_2$ (1) was due to heterotrophic CO_2-fixing reactions, since the system was apparently aerobic and the extracts had not been dialyzed or treated with Sephadex. This view is supported by the fact that endogenous fixation in our system increased approximately fivefold when incubation was under aerobic conditions and the extracts were not chromatographed on a Sephadex column. The addition of $NADH_2$ to cell-free enzyme systems of autotrophic organisms may allow the synthesis of certain CO_2 acceptors needed for heterotrophic reactions or, in the case where stimulation of autotrophic CO_2 fixation is noted, may allow for the synthesis of R-5-P.

The importance of CO_2 fixation as a primary metabolic event in autotrophic organisms has been well documented (2, 4), as has the importance of control mechanisms in biological processes (8, 9, 11). Observations made earlier in a chemosynthetic bacterium (7) and a photosynthetic bacterium and green plant (6) suggested that AMP may play a regulatory role in the primary events of the CO_2-fixing system of autotrophic metabolism. In addition, a possible role was suggested for ADP in the photosynthetic organisms. It appears from the data presented in this paper that ADP and AMP may both be involved in regulating ATP-dependent CO_2 fixation in chemoautotrophic organisms, some being regulated by AMP, others by AMP and ADP, and, possibly, although yet to be demonstrated, other autotrophs may be influenced by ADP only.

ACKNOWLEDGMENTS

The excellent technical assistance of Joann Stevenson and Henry M. Mack is gratefully acknowledged.

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