JOURNAL OF BACTERIOLOGY, Dec. 1975, p. 1046-1051

Vol. 124, No. 3
Printed in U.S.A.

Effect of Growth Conditions on the Activation and Inactivation of Citrate Lyase of *Rhodopseudomonas gelatinosa*

F. GIFFHORN AND G. GOTTSCHALK*

Institut für Mikrobiologie der Universität Göttingen and Gesellschaft für Strahlen- und Umweltforschung mbH, München in Göttingen, D-3400 Göttingen, West Germany

Received for publication 3 June 1975

Cells of *Rhodopseudomonas gelatinosa* growing with citrate anaerobically in the light contained citrate lyase only in the acetylated, enzymatically active form of this enzyme. After exhaustion of citrate in the culture medium citrate lyase was deacetylated to yield the inactive sulfhydryl (HS) enzyme. Acetylation of HS-citrate lyase required light, anaerobic conditions and the availability of citrate as substrate. The acetylation reaction already in progress stopped immediately when the culture was placed in the dark. Deacetylation of citrate lyase occurred anaerobically in the light when citrate was exhausted and under aerobic conditions in the presence or absence of citrate. In cells of *R. gelatinosa* fermenting citrate in the dark neither the acetylating enzyme nor the deacetylating enzyme was active.

Rhodopseudomonas gelatinosa is one of the few phototrophic bacteria capable of growing on citrate (16). During anaerobic growth of this microorganism in the light, much more citrate is degraded than required by the cells for the synthesis of cellular material. Degradation products, acetate and a little malate, are excreted into the culture medium and utilized for growth after exhaustion of citrate (13).

The first enzyme involved in citrate breakdown was shown to be citrate lyase [EC 4.1.3.6; citrate (*pro*-3-*S*)-lyase]; it was purified from cell extracts of *R. gelatinosa* to homogeneity; electron microscopic and electrophoretic studies revealed that it consisted of six large and six small subunits arranged in a hexameric double-ring structure (2). Like citrate lyase from *Enterobacter aerogenes* (4), the enzyme from *R. gelatinosa* was shown to be an acetyl-*S* enzyme, and it was observed that after the exhaustion of citrate in the medium the citrate lyase of *R. gelatinosa* was inactivated by deacetylation (7).

In this publication we report the conditions under which citrate lyase of *R. gelatinosa* is activated and inactivated. Furthermore, we provide evidence that inactivation of the lyase by deacetylation is not brought about by reaction inactivation of the enzyme.

MATERIALS AND METHODS

Organism and growth conditions. *R. gelatinosa* strain DSM 149 was grown anaerobically in the light in a medium containing the following components: KH_2PO_4, 1 g; NH_4Cl, 0.4 g; NaCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; trace element solution (12), 10 ml; yeast extract, 0.5 g; sodium citrate, 10 mM; water to 1,000 ml. The pH was adjusted to 6.7 with NaOH. Cells were maintained in screw-capped bottles (500 ml), and batch cultures were grown in 5- or 10-liter flasks with magnetic stirring at approximately 3,000 lux and 30 C. Growth was followed by measuring turbidity at 650 nm using a Zeiss PM4 spectrophotometer.

Growth conditions for acetylation of sulfhydryl (HS)-citrate lyase. After 24 h of growth under the conditions and in the medium described above, cells of a 5- or 10-liter culture were harvested and resuspended in the same volume of the above medium without citrate. After the incubation of this suspension for approximately 1 h with magnetic stirring under anaerobic conditions in the light, citrate was added (final concentration, 5 or 10 mM) and samples (400 ml) were withdrawn at various times by means of N_2 pressure.

Conditions for deacetylation of citrate lyase. Growth conditions were as described above. After citrate was added (final concentration, 5 or 10 mM), the cell suspension was incubated in the light until the citrate concentration had decreased below 0.2 mM. Then the inactivation of citrate lyase was followed under the conditions specified in Results.

Citrate was determined according to Moellering and Gruber (11).

Preparation of crude extracts. The 400-ml samples withdrawn from a culture of *R. gelatinosa* were immediately poured onto 100 g of crushed ice and then centrifuged for 20 min at 20,000 × *g* at 4 C. The sedimented cells (ca. 1 g [wet weight]) were resuspended in 5 ml of 0.1 M triethanolamine-

hydrochloride buffer, pH 7.2, containing 60 mM $MgCl_2$ and stored at 0 C up to 15 h. This suspension was quickly passed through a French press at a pressure of 80 kp/cm^2. Subsequently, cell debris was removed by centrifugation at 20,000 × *g* for 15 min at 4 C.

The protein content of the extracts was determined according to Beisenherz et al. (1) with crystalline bovine serum albumin as standard. Pigments were removed by extraction with acetone (5 ml per 0.1 ml of crude extract).

Enzyme assay. Citrate lyase activity was measured spectrophotometrically at 366 nm. The method of Dagley (5) was slightly modified. The assay mixture contained, in a final volume of 1 or 3 ml at 30 C: potassium phosphate buffer, pH 7.2, 0.1 M; $MgCl_2$, 3 mM; reduced nicotinamide adenine dinucleotide, 0.23 mM; lactate dehydrogenase, 11 U; malate dehydrogenase, 16 U; crude extract, approximately 0.3 mg of protein. The reaction was started by addition of 2 mM sodium citrate. The sum of active and inactive citrate lyase was determined after acetylation of the inactive enzyme. This was done with acetic anhydride (final concentration, 1.75 mM), which was added to the assay mixture 1 min before citrate was added (2).

Chemicals and enzymes. Reduced nicotinamide adenine dinucleotide, malate dehydrogenase (1,200 U/mg), lactate dehydrogenase (550 U/mg), and citrate lyase (8 U/mg of enzyme protein) were obtained from Boehringer Mannheim GmbH, Mannheim, Germany.

RESULTS

Stabilization of acetyl-S-citrate lyase in crude extracts. In crude extracts of *R. gelatinosa* citrate lyase activity decreased very rapidly. Curve a of Fig. 1 shows that during incubation of crude extracts at 30 C the enzyme had an average half-life of 2.5 min. It is evident from curve b that the product of this inactivation was HS-citrate lyase; enzyme activity could be fully restored by treatment of the samples with acetic anhydride. Prolonged incubation (more than 1 h) resulted in an additional breakdown of the HS-enzyme and irreversible loss of activity. Even during storage of crude extracts at 0 C the decrease of lyase activity was pronounced: 50% in 4 h. Consequently, unless the inactivation of the lyase in vitro was prevented it was not possible to determine the level of active (acetyl-*S*-) and inactive (HS-) citrate lyase in cells of *R. gelatinosa*.

It is known that $MgCl_2$ increases the stability of citrate lyase of *E. aerogenes* in dilute protein solutions (3). Moreover, $MgCl_2$ is essential for preserving the enzyme structure (9; T. J. Bowen and M. G. Mortimer, Biochem. J. **117**:71p, 1970) and its catalytic activity (6). Therefore, the effect of the $MgCl_2$ concentration on the stability of citrate lyase in crude extracts was studied. Figure 2 shows that increasing concentrations of $MgCl_2$ markedly delayed the inactivation at 30 C. In the presence of 60 mM $MgCl_2$ the enzyme was stable for almost 20 min

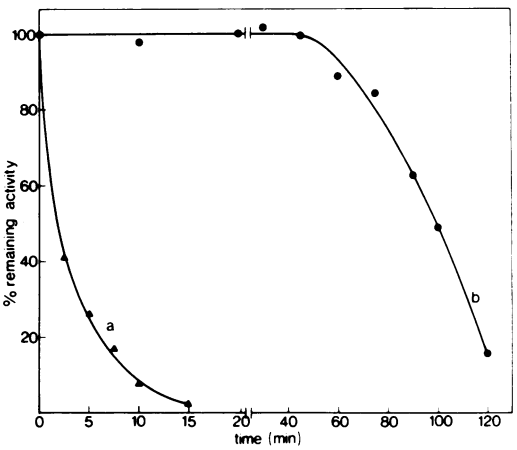

FIG. 1. *Inactivation of citrate lyase in crude extracts. Crude extracts were prepared from cells that contained a high level of acetyl-S-citrate lyase. Extract (0.5 ml), pH 7.2, contained 3 mM $MgCl_2$ and was incubated at 30 C. At zero time enzyme activity was 0.38 U without (a) and 0.72 U after (b) acetylation with acetic anhydride per 50 µl of extract.*

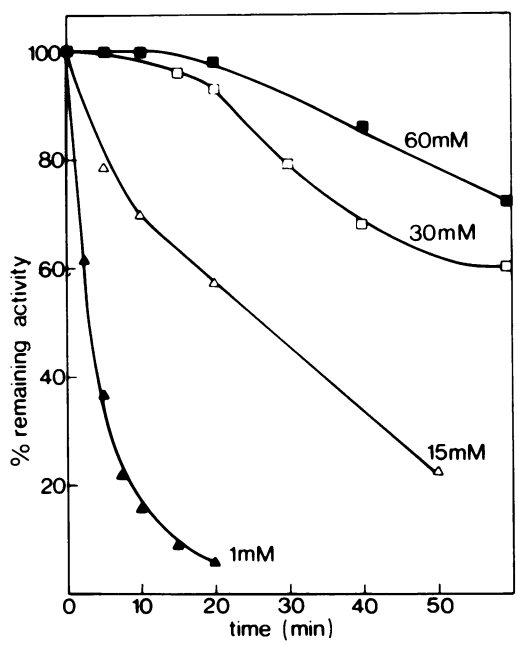

FIG. 2. *Effect of $MgCl_2$ concentration on the deacetylation of citrate lyase in crude extracts. Samples of a cell extract (0.5 ml), pH 7.2, were adjusted to the concentrations of $MgCl_2$ indicated and incubated at 30 C. At zero time enzyme activity was 0.54 U/50 µl of extract.*

at 30 C and for 3 h at 0 C. Thus, when $MgCl_2$ (60 mM) was added to cell suspensions before disrupting the cells, the actual level of active and inactive lyase could be determined. The optimal $MgCl_2$ concentration for the determination of citrate lyase activity was established by the appropriate dilution of the cell extract with assay mixture.

Acetylation of citrate lyase in vivo. Cells of *R. gelatinosa* which had been grown on 10 mM citrate for 24 h contained high levels of HS-citrate lyase and only small amounts of acetyl enzyme. When these cells were harvested, resuspended in fresh medium containing citrate, and incubated anaerobically in the light, rapid acetylation of the HS-lyase occurred (Fig. 3). After 1.5 h of incubation all the lyase was present in its active acetylated form. Then, de novo synthesis of citrate lyase proceeded as long as citrate was present in the medium. Exhaustion of the substrate initiated the inactivation of the enzyme by deacetylation. Between 1.5 and 3.5 h of incubation, the enzyme level determined after acetic anhydride treatment was lower than the one determined directly. This was due to an irreversible loss of enzyme activity in the order of 15% caused by the chemical acetylation procedure.

Cells with high levels of HS enzyme contained the acetylating enzyme. This could be deduced from the experiment shown in Fig. 4. In the presence of 10^{-5} M puromycin or 5×10^{-6} M chloramphenicol, de novo synthesis of citrate lyase was blocked completely. The acetylation of HS-enzyme, however, proceeded and citrate was degraded.

Neither acetylation of HS-citrate lyase nor de novo synthesis of the enzyme occurred anaerobically in the dark (Fig. 5). As soon as the light was switched on, a rapid acetylation of citrate lyase began, and citrate was degraded. This experiment showed that the acetylating enzyme was active only in the light. Darkness also prevented acetylation from progressing further (Fig. 6). Acetyl-*S*-citrate lyase, however, remained active in the dark and citrate was fermented. The results of Fig. 6 were important for two reasons. First, they explained why *R. gelatinosa* had only a very limited ability to grow with citrate in the dark (13); the active enzyme was diluted out under these conditions. Second, the constant level of acetyl-*S*-citrate lyase during citrate fermentation in the dark demonstrated that this enzyme was stable in vivo and not subject to a so-called reaction inactivation (15, 17).

Conditions of inactivation of citrate lyase.

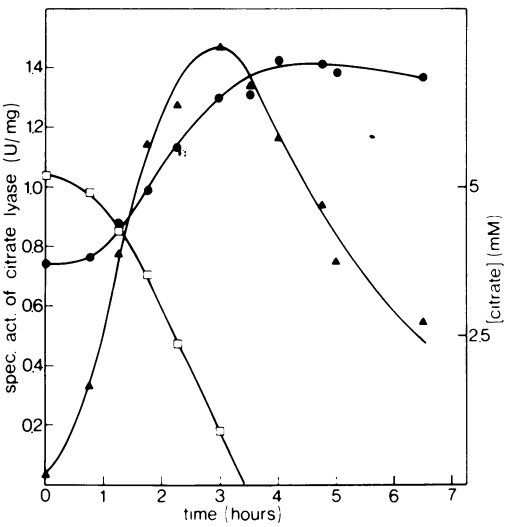

FIG. 3. *Acetylation and deacetylation of citrate lyase in cells growing with citrate anaerobically in the light. Ten liters of a batch culture was grown as described in Materials and Methods. After centrifugation the cells were resuspended in 10 liters of growth medium without citrate. After incubation for 1 h in the light, citrate was added to give a final concentration of approximately 5 mM. Samples (400 ml) were withdrawn anaerobically. Enzyme activities were determined as described in Materials and Methods. Symbols:* □, *concentration of citrate;* ▲, *citrate lyase activity;* ●, *citrate lyase activity after acetylation with acetic anhydride.*

As a consequence of the results of Fig. 6, it became apparent that the inactivation of citrate lyase observed under certain conditions was catalyzed by a special enzyme. Inactivation took place when *R. gelatinosa* growing anaerobically in the light had consumed citrate completely (see Fig. 3). The rate of inactivation was not affected by the addition of chloramphenicol or puromycin (concentration as in Fig. 4); it was, however, increased when the cultures were aerated after the exhaustion of citrate (Fig. 7). Under these conditions 50% of the lyase was deacetylated within 30 min. The HS enzyme formed was unstable in the presence of oxygen. Similar results were obtained when the aerobic incubation was carried out in the dark. Even in the presence of citrate, inactivation started immediately after aeration of the culture had begun (Fig. 8).

DISCUSSION

Citrate lyase exists in cells of *R. gelatinosa* in two chemically defined forms, inactive as HS enzyme and active as acetyl-*S* enzyme. Both

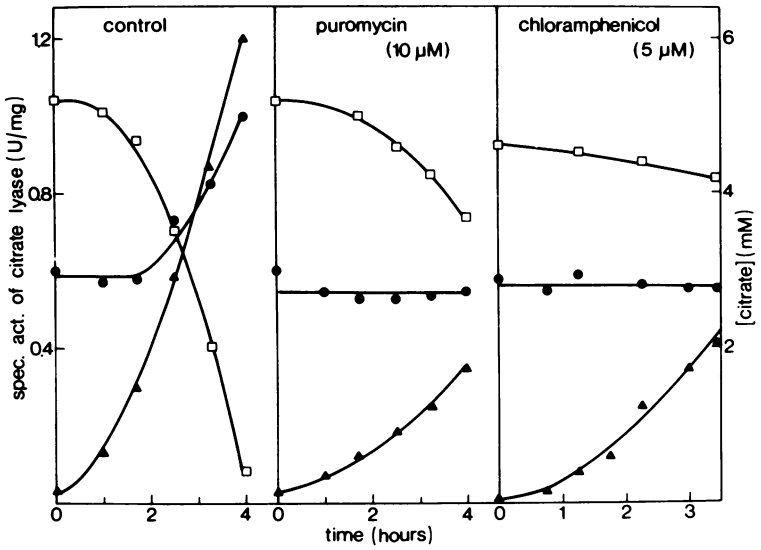

FIG. 4. *Acetylation of HS-citrate lyase anaerobically in the light in the presence of puromycin and chloramphenicol. Cell suspensions were prepared as described in Fig. 3. Fifteen minutes before the addition of citrate, the antibiotics were added up to the concentrations indicated. Symbols:* □, *concentration of citrate;* ▲, *citrate lyase activity;* ●, *citrate lyase activity after acetylation with acetic anhydride.*

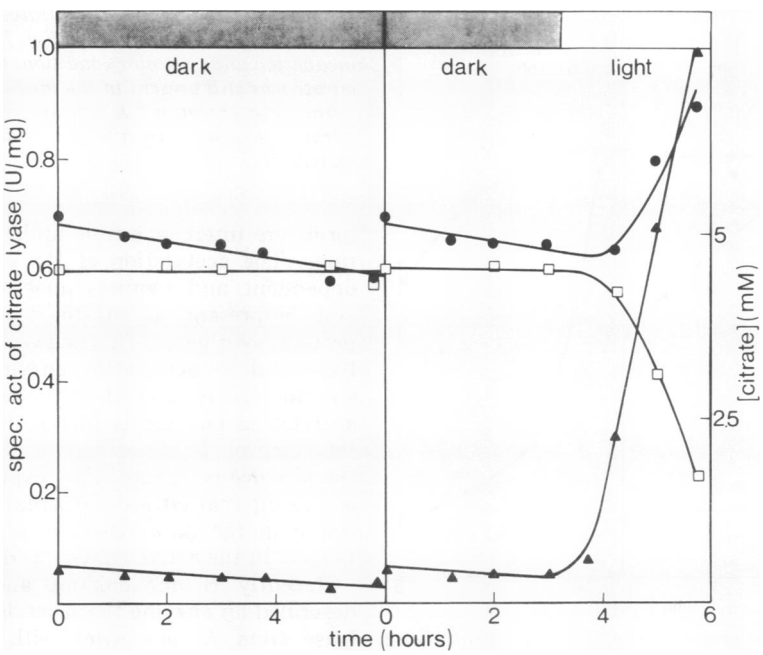

FIG. 5. *Light dependence of the conversion of HS-citrate lyase to acetyl-S-citrate lyase. Cells of a 5-liter culture were resuspended in 5 liters of growth medium and incubated anaerobically in the dark. After 3.25 h an aliquot of the suspension (2 liters) was transferred anaerobically into a 2-liter flask and incubated further under light conditions. Samples were taken, and enzyme activity was determined as described in Materials and Methods. Symbols:* □, *citrate concentration;* ▲, *citrate lyase activity;* ●, *citrate lyase activity after acetylation with acetic anhydride.*

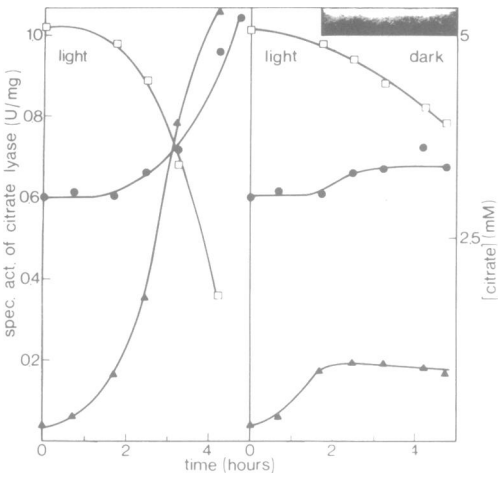

FIG. 6. *Discontinuation of the acetylation reaction by the change from anaerobic light to anaerobic dark conditions. For experimental details see Fig. 5 and Materials and Methods. After incubation in the light for 1.75 h, an aliquot of the suspension (2 liters) was transferred to a 2-liter flask and further incubated in the dark. Symbols: □, citrate concentration; ▲, citrate lyase activity; ●, citrate lyase activity after acetylation with acetic anhydride.*

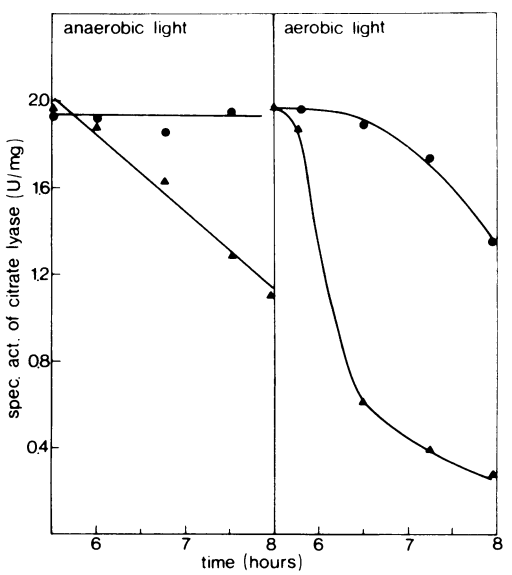

FIG. 7. *Comparison of the course of citrate lyase inactivation anaerobically and aerobically in the light. A 10-liter culture was used for this experiment. After the exhaustion of citrate in the medium, 4 liters of the suspension was withdrawn and distributed to 1-liter Erlenmeyer flasks (200 ml/flask). The flasks were incubated in the light (ca. 2,000 lux) on a rotary shaker (150 rpm). Symbols: ▲, citrate lyase activity; ●, citrate lyase activity after acetylation with acetic anhydride.*

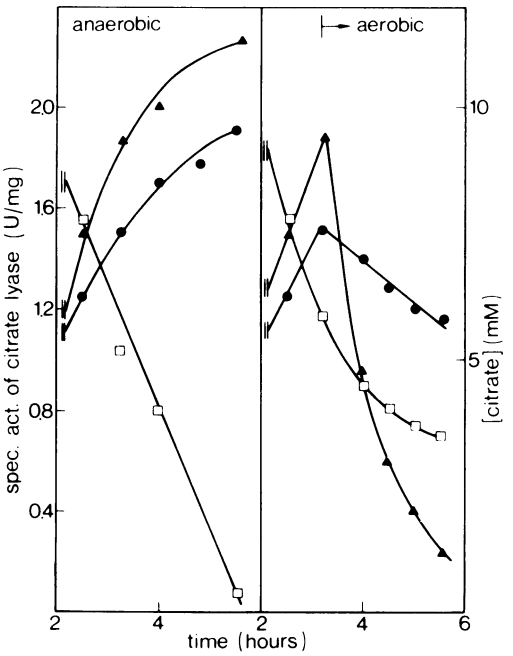

FIG. 8. *Inactivation of citrate lyase under aerobic conditions in the presence of citrate. The experiment was performed as described in Fig. 7, except that the incubation under aerobic conditions was started when citrate was still present in the medium. Symbols: □, citrate concentration; ▲, citrate lyase activity; ●, citrate lyase activity after acetylation with acetic anhydride.*

forms are interconvertible under certain conditions. The acetylation of HS enzyme is light dependent and requires anaerobic conditions and the presence of citrate; it does not require protein synthesis. This suggests that citrate lyase and its acetylating enzyme are induced simultaneously and that the activity of the acetylating enzyme is under stringent regulatory control. It is not active in the dark or in the absence of citrate. This explains our previous result that citrate fermentation by *R. gelatinosa* in the dark ceases after a few generations (13); the active enzyme is diluted out.

Recently, Schmellenkamp and Eggerer (14) described an enzyme that acetylates HS-citrate lyase from *E. aerogenes* with adenosine 5'-triphosphate and acetate as substrates. A similar enzyme activity is associated with purified citrate lyase from *Streptococcus diacetilactis* (9). In vitro studies on the acetylating enzyme of *R. gelatinosa* have not yet been performed. The factors that affect its activity are not yet known.

The finding that acetyl-*S*-citrate lyase is not deacetylated during citrate fermentation ex-

cludes the possibility that reaction inactivation is involved in the process of deacetylation. The reaction inactivation of citrate lyase is well known from studies on the enzymes isolated from *E. aerogenes* (15) and *Escherichia coli* (17). Purified citrate lyase from *R. gelatinosa* also shows this type of inactivation (2). However, it is suppressed by increasing concentrations of Mg^{2+}. This might indicate how the enzyme is stabilized inside the cells.

It is noteworthy, in this connection, that purified citrate lyases from *S. diacetilactis* and *Leuconostoc citrovorum* show only a very weak reaction inactivation and that these enzymes remain active in vivo in the absence of citrate (9). The latter bacteria, however, need not inactivate their citrate lyase in the absence of citrate because they lack the antagonistic enzyme, citrate synthase. The situation of *R. gelatinosa* is different. Unlike the above-mentioned lactic acid bacteria, it does not require glutamate for growth and contains citrate synthase (7). Consequently, after the exhaustion of citrate as substrate, metabolism must be converted from citrate degradation to citrate synthesis. This can only be effectively accomplished if citrate lyase is inactivated. Such an inactivation is precisely what has been observed. Since the reaction inactivation is excluded as a possible mechanism for the deacetylation of the *R. gelatinosa* lyase, a special inactivating enzyme must be responsible for this process in this organism; its properties are described in the accompanying paper (8).

ACKNOWLEDGMENTS

This work was supported by a grant of the Deutsche Forschungsgemeinschaft and by Forschungsmittel des Landes Niedersachsen.

LITERATURE CITED

1. Beisenherz, G., H. J. Bolze, T. Bücher, R. Czok, R. K. Garbade, E. Meyer-Ahrendt, and E. Pfleiderer. 1953. Diphosphofructose-Aldolase, Phosphoglycerinaldehyd-Dehydrogenase aus Kaninchenmuskulatur in einem Arbeitsgang. Z. Naturforsch. Teil B **8**:555-557.
2. Beuscher, N., F. Mayer, and G. Gottschalk. 1974. Citrate lyase from *Rhodopseudomonas gelatinosa*: purification, electron microscopy and subunit structure. Arch. Microbiol. **100**:307-328.
3. Blair, J. M., S. P. Datta, and S. S. Tate. 1967. Reversible inactivation of citrate lyase: effect of metal ions and adenine nucleotides. Eur. J. Biochem. **1**: 26-28.
4. Buckel, W., V. Buschmeier, and H. Eggerer. 1971. Der Wirkungsmechanismus der Citrat-Lyase aus *Klebsiella aerogenes*. Hoppe Seyler's Z. Physiol. Chem. **352**: 1195-1205.
5. Dagley, S. 1969. Citrate lyase, p. 160-163. *In* J. M. Lowenstein (ed.), Methods in enzymology, vol 13. Academic Press Inc., New York.
6. Dagley, S., and E. A. Dawes. 1955. Citridesmolase: its properties and mode of action. Biochim. Biophys. Acta **17**:177-184.
7. Giffhorn, F., N. Beuscher, and G. Gottschalk. 1972. Regulation of citrate lyase activity in *Rhodopseudomonas gelatinosa*. Biochem. Biophys. Res. Commun. **49**:467-472.
8. Giffhorn, F., and G. Gottschalk. 1975. Inactivation of citrate lyase from *Rhodopseudomonas gelatinosa* by a specific deacetylase and inhibition of this inactivation by L-(+)-glutamate. J. Bacteriol. **124**:1052-1061.
9. Kümmel, A., G. Behrens, and G. Gottschalk. 1975. Citrate lyase from *Streptococcus diacetilactis*. Association with its acetylating enzyme. Arch. Microbiol. **102**: 111-116.
10. Mahadik, S. P., and C. Siva Raman. 1968. Citrate lyase: molecular weight and subunit structure. Biochem. Biophys. Res. Commun. **32**:167-172.
11. Moellering, H., and W. Gruber. 1966. Determination of citrate with citrate lyase. Anal. Biochem. **17**:369-376.
12. Pfennig, N., and D. Lippert. 1966. Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. **55**:245-256.
13. Schaab, C., F. Giffhorn, S. Schoberth, N. Pfennig, and G. Gottschalk. 1972. Phototrophic growth of *Rhodopseudomonas gelatinosa* on citrate; accumulation and subsequent utilization of cleavage products. Z. Naturforsch. Teil B **27**:962-967.
14. Schmellenkamp, H., and H. Eggerer. 1974. Mechanism of enzymic acetylation of des-acetyl citrate lyase. Proc. Natl. Acad. Sci. U.S.A. **71**:1987-1991.
15. Singh, M., and P. A. Srere. 1971. The reaction inactivation of citrate lyase from *Aerobacter aerogenes*. J. Biol. Chem. **246**:3847-3850.
16. Weckesser, J., G. Drews, and H. D. Tauschel. 1969. Zur Feinstruktur und Taxonomie von *Rhodopseudomonas gelatinosa*. Arch. Mikrobiol. **65**:346-358.
17. Wheat, R. W., and S. J. Ajl. 1955. Citritase, the citrate-splitting enzyme from *E. coli*. II. Reaction mechanism. J. Biol. Chem. **217**:909-920.