JOURNAL OF BACTERIOLOGY, Feb. 1969, p. 691-696
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Vol. 97, No. 2
Printed in U.S.A.

Phosphoenolpyruvate Carboxylation and Aspartate Synthesis in *Acetobacter suboxydans*[1]

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Received for publication 15 October 1968

Dialyzed extracts of *Acetobacter suboxydans* ATCC 621 catalyze $^{14}CO_2$ assimilation in the presence of phosphoenolpyruvate and a divalent cation. The formation of ^{14}C-oxalacetate was demonstrated and found not to be dependent upon the presence of orthophosphate or diphosphonucleotides. Oxalacetate synthesis was stimulated by orthophosphate and inhibited by aspartate. All attempts to demonstrate a reversible carboxylation mechanism have failed. ^{14}C-aspartate was synthesized when phosphoenolpyruvate, $H^{14}CO_3^-$, pyridoxal phosphate, and glutamate were added to dialyzed extracts. Chromatographic and spectrophotometric analyses and chemical degradation further demonstrate the presence of a reversible aspartate aminotransferase. The function of oxalacetate synthesis in a bacterium that reportedly lacks an operative tricarboxylic acid cycle is discussed.

In 1950, Frateur (15) divided the genus *Acetobacter* into four groups based on their "oxidizing power": peroxydans, oxydans, mesoxydans, and suboxydans. In contrast to other *Acetobacter* species, the suboxydans group is characterized (9, 32) as having no tricarboxylic acid cycle and utilizing the pentose cycle exclusively for terminal oxidation of a large number of substrates. Evidence supporting the absence of an active tricarboxylic acid cycle in *A. suboxydans* is based on the inability of whole cells or cell-free extracts (14, 16, 17, 18, 19, 32; M. R. R. Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955) to oxidize tricarboxylic acid cycle intermediates. Tricarboxylic acid cycle enzyme activities are either very slight (23, 39) or absent (M. R. R. Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955) in extracts of two *A. suboxydans* strains. Cells also fail to oxidize acetate (15, 23), to liberate $^{14}CO_2$ from radioactive acetate incubated in the presence of glucose (19), and to grow in media in which tricarboxylic acid cycle intermediates are the sole source of carbon and energy (17; M. R. R. Rao, Ph.D. Thesis, Univ. of Illinois, Urbana, 1955).

The reported lack of an active tricarboxylic acid cycle and the ability of this obligate aerobe to grow in the absence of aspatrate (26, 27) has prompted Rao (27) and Cheldelin (9) to question the synthetic mechanism by which *A. suboxydans* forms the C_4- and C_5-amino acids that normally arise from this cycle's intermediates. Cheldelin (9) also mentions unpublished results from his laboratory which demonstrated ability of cells to accumulate ^{14}C-aspartate in the presence of either ^{14}C-glucose or $^{14}CO_2$. Cheldelin and his associates have published indirect evidence (Federation Proc., p. 652, 1963) from which they suggest a scheme for the synthesis of aspartate in extracts of *A. suboxydans* ATCC 621 and suggest (29) that aspartate can be used as a substrate for oxalacetate formation.

In this report, we have demonstrated that *A. suboxydans* ATCC 621 extracts can readily catalyze the synthesis of ^{14}C-oxalacetate from phosphoenolpyruvate and $^{14}CO_2$ and the synthesis of ^{14}C-aspartate in the presence of phosphoenolpyruvate, $^{14}CO_2$, pyridoxal phosphate, and glutamate. Some characteristics of the phosphoenolpyruvate-carboxylating mechanism are presented, and the possible significance of these pathways to the organism's metabolism is discussed.

MATERIALS AND METHODS

Chemicals. $NaH^{14}CO_3$ was prepared from $Ba^{14}CO_3$ (4) obtained from Oak Ridge National Laboratory or obtained as an $NaH^{14}CO_3$ solution from Volk Radiochemical Co., Burbank, Calif. Malate dehydrogenase (EC 1.1.1.37, L-malate:NAD oxidoreduc-

[1] Preliminary studies were supported by a Public Health Service Fellowship (5-F1-GM-10,951) awarded to G.W.C. (1962-64) and were submitted to the Graduate College of Iowa State University, Ames, as part of the doctoral dissertation. Recent investigation was supported by PHS Training grant 5T1-GM-512 from the National Institute of General Medical Sciences, and reported to the 68th Annual Meeting of the American Society of Microbiology.

tase; MDH) was obtained from C. F. Boehringer and Soehne. Tris(hydroxymethyl)aminomethane (Tris) was obtained from Fisher Scientific Co., Pittsburgh, Pa. Pyridoxal phosphate ($PyrPO_4$), reduced sodium nicotinamide adenine dinucleotide phosphate (NADPH), 2-phosphoenolpyruvic acid (PEP), pyruvic acid, and 2-oxoglutaric acid were purchased from the California Foundation for Biochemical Research. Oxalacetic acid, L-aspartic acid, and 2,4-dinitrophenylhydrazine were obtained from Mann Research Laboratories, New York, N.Y. Adenosine-, guanosine-, inosine-, thymidine-, uridine-, and cytidine-5'-di- and triphosphates, reduced disodium nicotinamide adenine dinucleotide (NADH), *S*-acetyl-coenzyme A (CoA) and L-glutamate decarboxylase (type II from *Escherichia coli*) were obtained from Sigma Chemical Co., St. Louis, Mo. Solutions were prepared with glass-distilled water having a resistance greater than 1.0 megohm per cm at 25 C.

Organism. A culture of *A. suboxydans* ATCC 621 was obtained from the American Type Culture Collection and was maintained on glucose *Acetobacter* agar slants (30) at 10 C. At the completion of the study, a fresh culture was obtained, and the results of this study were confirmed.

Bacterial growth. Cells were grown in the liquid medium described by Kitos et al. (20) except that GE-60 antifoam (80 μg/ml; General Electric Co., Waterford, N.Y.) was substituted for Dow Corning-AF. Subcultures were prepared by inoculating 50 ml of growth medium in 500-ml Erlenmeyer flasks and by incubating at 28 C for 24 hr on a reciprocating shaker. The entire subculture was used to inoculate a 14-liter New Brunswick Microferm fermentor containing 10 liters of growth medium aerated with a Ringer sparger at a rate of 8,000 cc/min. Cells were harvested by centrifugation at 5 C and 14,000 $\times$ *g* at the end of exponential growth and either used immediately or stored at -20 C.

Cell extracts. Cells were suspended in 0.1 M Tris buffer, *p*H 7.5, at a ratio of 1:2 (w/v). Portions (50 ml) were treated with a 10-kc Raytheon sonic oscillator for 5 min at an output current of approximately 1.3 amp and were centrifuged at 34,000 $\times$ *g* for 60 min to remove cellular debris. The resulting supernatant fluid was designated 34*s*60 following the terminology of Alexander and Wilson (1). The 34*s*60 extract was either (i) used without further treatment, (ii) dialyzed against three 30-volume changes of 0.001 M Tris buffer (pH 7.5) for 18 hr, or (iii) dialyzed; then two volumes of extract were treated with one part, by weight, of Dowex-1 resin (chloride form) while stirring in the cold for 15 min to remove endogenous nucleotides (31). Protein concentration of the extract was determined by biuret method using crystalline bovine gamma-globulin standards.

Assay for phosphoenolpyruvate carboxylation. Reaction mixture components were added to glass-stoppered test tubes, and a cell-free extract was added to initiate the reaction. After 30-min incubation at 28 C, the radioactive phenylhydrazones were formed and extracted according to the procedure of Maruyama and Lane (24).

Spectrophotometric detection of aspartate aminotransferase. The quantity of oxalacetate formed by transamination of aspartate to 2-oxoglutarate was determined in a manner similar to that described by Bergmeyer and Brent (7). Reaction mixtures consisted of extract, $PyrPO_4$, and Tris buffer (*p*H 7.5). To reduce endogenous oxidants in the cell extract, NADH was added to the reaction mixture until its oxidation could no longer be measured at 340 nm. Additional NADH was introduced, 2-oxoglutarate and MDH were added, and the transamination reaction was initiated by adding aspartate.

Chromatography of reaction products. The 2,4-dinitrophenylhydrazones were separated on Whatman no. 3 filter paper by an ascending flow of either *n*-butyl alcohol, ethyl alcohol, 0.5 N NH_4OH (7:1:12), or *n*-butyl alcohol saturated with 3% (w/w) NH_4OH. Radioactive amino acids were separated on this paper by a descending flow of pyridine, acetic acid, water (10:7:3) and detected by exposure to Kodak No-Screen X-ray film. Nonradioactive reaction mixtures were first deproteinized with HCl; then the supernatant fraction was dried by exposure to a stream of nitrogen. Partial separation of amino acids from inorganic salts was achieved by overnight exposure of the dried reaction products to cold ethyl alcohol. The ethyl alcohol fraction was then exposed to two-dimensional paper chromatography using phenol-water (80:20) and butyl alcohol-propionic acid water (47:22:31).

RESULTS

$^{14}CO_2$ assimilation and product identification. Undialyzed 20*s*20 extracts catalyzed the fixation of $^{14}CO_2$ into the acid-soluble portion of reaction mixtures containing phosphoenolpyruvate to routinely yield ca. 5,000 counts per min per mg of protein when assayed according to the procedure of Baugh et al. (6). When samples of acid-deproteinized reaction mixtures were mixed with known oxalacetate and a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl, the resulting dinitrophenylhydrazone crystals were radioactive and corresponded to known oxalacetate-2,4-dinitrophenylhydrazone when subjected to chromatography and radioautography (Table 1).

Requirements for and inhibitor effect on oxalacetate synthesis. Phosphoenolpyruvate serves as a substrate for carboxylation by *A. suboxydans* extracts (Table 2). Pyruvate, pyruvate and guanosine triphosphate (GTP), pyruvate and NADPH, or pyruvate, GTP, and acetyl-CoA cannot substitute for phosphoenolpyruvate.

The effect of acetyl-CoA was found to depend upon the concentration used in reaction mixtures. Concentrations of less than 2 μmoles of acetyl-CoA per mg of protein were slightly stimulatory, and concentrations above this were inhibitory. For a given protein concentration, acetyl-CoA became inhibitory if the acetyl-CoA-phosphoenolpyruvate ratio was raised above 1:3. The addition

TABLE 1. *Identification of the radioactive phenylhydrazone formed by extracts*[a] *of Acetobacter suboxydans*

Solvent	Concentration	R_F of known phenylhydrazones[b]		R_F of unknown ^{14}C phenylhydrazone[c]
		Oxalacetic	Pyruvic	
Butyl alcohol-ethyl alcohol-NH_4OH (7:1:2)	Primary spot	0.23	0.37	0.23
	Secondary spots	0.49,0.65	0.60	
n-Butyl alcohol saturated in 3% NH_4OH	Primary spot	0.08	0.28	0.09
	Secondary spots	0.32,0.50	0.56	

[a] Reaction mixtures contained 3.0 mg of dialyzed 20*s*20 extract, 3 μmoles of phosphoenolpyruvate, 3 μmoles of magnesium chloride, 5 μmoles of $NaH^{14}CO_2$, 100 μmoles of Tris buffer (*p*H 7.7), adjusted to 2.0 ml with distilled water. The procedure of Baugh et al. (6) was used to isolate the ^{14}C phenylhydrazones after 30 min of reaction.

[b] Ascending chromatography on Whatman no. 3 filter paper.

[c] As determined by radioautography.

of 3 μmoles of DL-aspartate caused a 75 to 95% decrease in oxalacetate formation in these reaction mixtures (Table 2).

The addition of 3 μmoles of inorganic pyrophosphate to carboxylation assay mixtures lacking added inorganic phosphate (P_i) caused a 20% reduction in the quantity of ^{14}C-oxalacetate-phenylhydrazone formed. Since it was found that 34*s*60 extracts catalyzed the rapid hydrolysis of inorganic pyrophosphate, it seemed possible that the inhibitory effect of pyrophosphate on PEP-carboxylation might be due to the accumulation of hydrolyzed P_i. However, 6 μmoles of P_i stimulated rather than inhibited carboxylation.

Addition of P_i either had no effect or slightly stimulated $^{14}CO_2$ fixation depending upon the cation present. In the absence of added P_i, relatively large amounts of carboxylation activity were noted. Complete elimination of P_i was difficult because ca. 0.003 μmole of P_i contamination per 3.00 μmoles of phosphoenolpyruvate was routinely detected immediately upon placing the latter in solution. No P_i was detected in dialyzed 34*s*60 extracts. Addition of 34*s*60 extracts to phosphoenolpyruvate solutions adjusted to *p*H 7.7 caused no change in P_i hydrolysis unless bicarbonate and a cation were added.

No CO_2 assimilation was observed in the absence of divalent cation. Magnesium stimulated as much as 10 times more activity than that observed in the presence of manganous ions.

The addition of adenosine diphosphate, guanosine diphosphate, inosine diphosphate, uridine diphosphate, or cytidine diphosphate consistently inhibited $^{14}CO_2$ assimilation in the presence of magnesium ion. Each nucleotide tested contained various amounts of contaminating P_i, although the concentration of this contaminant in nucleotide-containing flasks did not exceed that in flasks in which P_i and cation were added. Nucleotides had essentially no effect on the reaction in the presence of manganous ion.

All attempts to demonstrate reversible $^{14}CO_2$ assimilation by the "exchange" method (21) using varying concentrations of oxalacetate in the presence of pyrophosphate or triphosphonucleotides were unsuccessful.

Synthesis of aspartate. The ability of extracts to transaminate aspartate and 2-oxoglutarate to form oxalacetate and glutamate is demonstrated

TABLE 2. *Substrate specificity for and inhibitor effect on oxalacetate synthesis by extracts of Acetobacter suboxydans*[a]

No	Additions[b]	Radioactivity fixed (counts per min per mg of protein)
1	PEP	36,386
2	PEP, P_i	48,557
3	PEP, acetyl-CoA (1.5 μmoles)	59,000
4	PEP, acetyl-CoA (3 μmoles)	12,135
5	PEP, adenosine diphosphate	3,018
6	PEP, DL-aspartate	400
7	Pyruvate	101
8	Pyruvate, GTP	51
9	Pyruvate, NADPH	87
10	Pyruvate, GTP, acetyl-CoA	60
11	None	0

[a] The complete system contained 34*s*60 extract (dialyzed and resin-treated), 3 μmoles of magnesium chloride, 5 μmoles of $NaH^{14}CO_2$ (5.55×10^6 counts/min), 100 μmoles of Tris buffer (*p*H 7.7), adjusted to 1.5 ml with distilled water.

[b] Additions were 1.5 μmoles of NADH, 3 μmoles each of PEP, adenosine diphosphate, adenosine triphosphate, sodium pyruvate, and DL-aspartic acid, adjusted to *p*H 8.4 with KOH.

in Table 3. If the extract or aspartate and 2-oxoglutarate were not added to the assay system, oxalacetate was not formed. Some NADH was oxidized in the absence of aspartate by undialyzed extracts. The quantity of NADH oxidized in the presence of aspartate and 2-oxoglutarate varied from 0.6 to 1.9 μmoles/3 min when 3.0 mg of extract protein previously exposed to NADH was employed. The addition of $PyrPO_4$ stimulated the rate of this reaction.

Table 4 demonstrates chromatographic evidence for the reversibility of aspartate aminotransferase in extracts of this bacterium. When reaction mixtures similar to reactions 1 to 4 (Table 4) were stopped by boiling and the quantity of glutamate formed determined by the glutamate-decarboxylase manometric method (38), about 10% of the 2-oxoglutarate had been converted to glutamate; no glutamate was detected if the extract was boiled prior to reaction or if ammonium sulfate was substituted for aspartate.

TABLE 3. *Spectrophotometric measurement of aspartate aminotransferase in cell-free extracts of Acetobacter suboxydans*

No.	Deletions[a]	NADH oxidized (μmoles/3 min)
1	None	0.60
2	Aspartate, 2-oxoglutarate	0
3	Aspartate	0.20
4	Extract	0

[a] The complete system contained 3.0 mg of non-resin-treated, dialyzed, 20s20 extract; 10 μmoles of sodium-aspartate; 4 μmoles of 2-oxoglutarate; 0.5 μmole of $PyrPO_4$; 0.25 enzyme units (11) of malate dehydrogenase; 2 μmoles of NADH; 100 μmoles of Tris buffer (pH 7.5); and distilled water to 3.0 ml.

Radioactive aspartate was formed by cell extracts when 5 μmoles of $PyrPO_4$ and 3 μmoles of glutamate were added to reaction mixtures containing phosphoenolpyruvate and $H^{14}CO_2^-$. When reaction mixtures were deproteinized and analyzed by paper chromatography and radioautography, the presence of radioactive aspartate was clearly demonstrated (Fig. 1). Some radioactive aspartate was formed by undialyzed extracts in the absence of phosphoenolpyruvate, but this synthesis was eliminated when dialyzed extracts were used.

DISCUSSION

The ability of *A. suboxydans* ATCC 621 extracts to catalyze ^{14}C-oxalacetate formation in the presence of pyruvate + NADH, pyruvate + GTP, or pyruvate + acetyl-CoA indicates either the absence or inactivity of the "malic enzyme" (EC 1.1.1.38 or 39) and pyruvate carboxylase [EC 6.4.1.1 (28)]. Consistent failure of either purine- or pyrimidine-nucleosidediphosphates to stimulate assimilation and the lack of reversibility of the reaction under conditions similar to those used with other bacterial extracts (3, 4, 5, 25, 35) suggest that an enzyme similar to PEP-carboxykinase (EC 4.1.1.32) is not present in our extracts.

Nucleotide inhibition of CO_2 assimilation has been reported in reactions catalyzed by plant

TABLE 4. *Chromatographic identification of products formed by aspartate aminotransferase from Acetobacter suboxydans*

Reaction[a]	Additions[b]	R_F of ninhydrin-positive areas[c]	
		Phenol-water (80:20)	Butyl alcohol-propionic acid-water (47:22:31)
1	2-OG + asp + CFE	0.12, 0.80	0.24, 0.75
2	2-OG + asp + boiled CFE	0.80	0.75
3	asp + CFE	0.80	0.75
4	2-OG + NH_4^+ + CFE	None	None
5	glu + OA + CFE	0.12, 0.80	0.24, 0.80
6	glu + OA + boiled CFE	0.12	0.24,
Known	L-Glutamate	0.12	0.24,
Known	L-Aspartate	0.80	0.75

[a] The complete system (reactions 1–6) contained 5.2 mg of dialyzed 34s60 extract (CFE), 2 μmoles of pyridoxal phosphate, 100 μmoles of Tris buffer (pH 7.5), plus additions to a final volume of 1.5 ml. Reaction mixtures were incubated at 30 C for 3 hr and the products were extracted.

[b] Additions were 100 μmoles of 2-oxoglutarate (2-OG), 100 μmoles of L-aspartate (asp), 50 μmoles of ammonium sulfate (NH_4^+), and 100 μmoles of oxalacetate (OA).

[c] Separation of ninhydrin-positive spots was routinely accomplished by two-dimensional paper chromatography. The R_F for each dimension is listed separately.

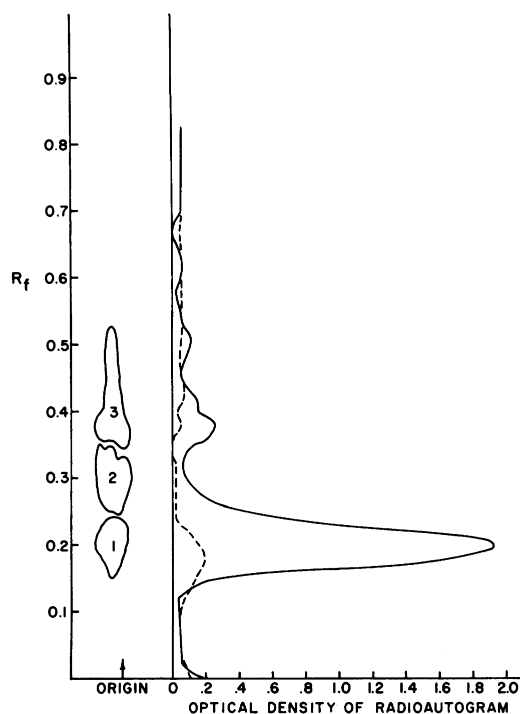

FIG. 1. *Chromatographic identification of radioactive aspartate formed by undialyzed cell-free extracts of A. suboxydans. Spots 1 and 2 represent known aspartate and glutamate, respectively. Spot 3 is an unknown amino acid. Adjacent densitometer tracing represents superimposed radioautogram formed after chromatography of reaction mixtures when PEP is present* (*solid line*) *or absent* (*broken line*).

(2, 24) and *Ferrobacillus ferroxidans* (13) extracts both of which appear to contain PEP-carboxylase (EC 4.1.1.31) as the sole PEP-carboxylating enzyme. Although these previous reports do not consider the presence of pyruvic kinase, the addition of nucleotides would permit competition between PEP-carboxylase and pyruvic kinase for the substrate. Such competition would lower the apparent activity of PEP-carboxylase in the presence of nucleosidediphosphates. It is likely that the apparent effect of nucleotides in *A. suboxydans* catalyzed PEP-carboxylation is an indirect effect caused by the presence of pyruvic kinase. If the nucleosidediphosphates are listed in the order of their decreasing inhibitory effect on *A. suboxydans* catalyzed PEP-carboxylation, the list closely parallels the order of decreasing nucleotide activity listed for rabbit muscle pyruvic kinase (37).

The stimulatory effect of P_i and the inhibitory effect of aspartate on the oxalacetate-synthesizing capacity of *A. suboxydans* are similar to the characteristics of PEP-carboxylase from plants (33, 36) and bacteria (8, 13, 22). The ability of *A. suboxydans* to catalyze oxalacetate synthesis and P_i evolution in the absence of measurable quantities of P_i suggests that PEP-carboxylase is primarily responsible. However, it is also possible that an undetectable quantity of P_i is present and serves as a substrate for the initial PEP-carboxytransphosphorylase reaction; the resulting pyrophosphate may then be hydrolyzed regenerating the P_i needed for additional carboxylation. The inorganic pyrophosphatase, known to be present in our extracts, may also inhibit the exchange reaction by catalyzing the rapid hydrolysis of one of the reaction products (34). Although many previous investigators have employed unfractionated extracts in an attempt to characterize PEP-carboxylating enzymes from a wide variety of cells, the possible presence and competitive effect of enzymes such as pyruvic kinase, inorganic pyrophosphatase, and citrate synthetase may have altered these apparent characteristics. Accurate identification of the enzymes responsible for PEP-carboxylation will result only when the active fraction is shown to be free of enzymes competing for reaction substrates or products.

The function of PEP-carboxylation for this tricarboxylic acid cycle-deficient bacterium is suggested by the apparent irreversibility of the reaction and the inhibitory effect of aspartate. The reported effect of aspartate on bacterial PEP-carboxylase (8, 13, 22) and the generalization proposed for allosteric inhibitors (12) both suggest that aspartate inhibition of *A. suboxydans* catalyzed PEP-carboxylation is the expression of an allosteric inhibitor affecting an anabolic pathway.

The extract-catalyzed formation of ^{14}C-aspartate demonstrates that aspartate synthesis in *A. suboxydans* can occur by carboxylation of phosphoenolpyruvate and subsequent transamination of oxalacetate with an organic amino donor. The reversible transamination between aspartate and 2-oxoglutarate appears to be an exception to the glycophile generalization made by Cooksey and Rainbow (10).

It has previously been shown (23) and confirmed in our laboratory (R. T. Belly and G. W. Claus, Bacteriol. Proc. p. 27, 1968) that growth of *A. suboxydans* ATCC 621 in chemically defined medium is not dependent upon the addition of aspartate. These observations are in agreement with the general nutritional characteristics proposed for the glycophiles (10). Cheldelin et al. (Federation Proc. p. 652, 1963) have published preliminary evidence which suggests aspartate synthesis resulting from an initial acetate-glyoxylate condensation, although Cheldelin had

previously reported the presence of a very active glyoxylate reductase and suggested that glyoxylate has only a transient existence in this bacterium (9). The reported synthesis of ^{14}C-aspartate in the presence of ^{14}C-glucose or $^{14}CO_2$ but not in the presence of ^{14}C-acetate (9) supports our proposal that PEP-carboxylation and subsequent transamination of the oxalacetate with an organic amino donor is an important mechanism for the synthesis of aspartate in *A. suboxydans* ATCC 621.

ACKNOWLEDGMENTS

We express our gratitude to the late C. H. Werkman, Department of Bacteriology, and to D. J. Graves, Department of Biochemistry and Biophysics, Iowa State University, for advice and encouragement during the initial phases of this study.

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