TECHNICAL MANUSCRIPT 118

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# EFFECTS OF BICARBONATE ON GROWTH OF *PASTEURELLA PESTIS*.

## II. CARBON DIOXIDE FIXATION INTO OXALACETATE BY CELL-FREE EXTRACTS

C.L. Baugh

J.W. Lanham

M.J. Surgalla

Medical Bacteriology Division
DIRECTOR OF BIOLOGICAL RESEARCH

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## ABSTRACT

Enzyme preparations from Pasteurella pestis will carboxylate phosphoenolpyruvate to form oxalacetate by two distinct reactions. The reactions are similar to those catalyzed by the enzymes phosphoenolpyruvic carboxylase and phosphoenolpyruvate carboxykinase. No significant differences in enzyme characteristics or enzyme content were found when virulent cells were compared with avirulent under the conditions of our experiments. The carboxykinase of P. pestis differs from that of animal origin, as it is dependent upon adenine derivatives rather than inosine or guanosine nucleotides. The latter two nucleotides can act indirectly by way of adenosine nucleotides, as nucleoside diphosphokinase and myokinase are present in the extract.

## I. INTRODUCTION

The virulent cells in an inoculum of Pasteurella pestis will not grow, or at least have a prolonged lag phase, when cultured aerobically at 37°C in a broth medium that supports the aerobic growth of virulent cells at 26°C.[1-3] The avirulent mutants invariably present in the inoculum initiate growth immediately at 37°C and soon predominate in the cultures grown at this temperature. This loss of virulence can be prevented, however, by the addition of sodium bicarbonate,[3,4] by the addition of calcium, strontium, or zinc ions,[5] by adjustment of initial pH to 7.8, or by the addition of spent culture filtrates.[6]

The present investigation is primarily a comparative study of carbon dioxide fixation by avirulent and virulent P. pestis utilizing cell-free extracts and partially purified enzyme preparations. The enzymes concerned with the carboxylation of phosphoenolpyruvate were identified and some properties of the reactions described.

## II. MATERIALS AND METHODS

Commercial preparations of oxalacetic acid (OAA), phosphoenolpyruvic acid (PEP),* nucleotides,** and $NaHC^{14}O_3$,*** were used.

The avirulent A-4 strain and the virulent Alexander strain of P. pestis were grown in heart infusion broth (HIB) containing various supplements at 26° or 37°C for 18 hours. The HIB was supplemented with 0.3 per cent magnesium gluconate for the growth of the A-4 strain and with 0.3 per cent sodium gluconate for the Alexander strain. When the Alexander strain was grown at 37°C, $CaCl_2$ (0.002 M) was also added to the medium.

The cells were harvested by centrifugation and washed twice with distilled water. Approximately 10 grams (wet weight) of washed cells were suspended in 50 ml of pH 7.2 buffer (0.1 M tris-HCl or 0.05 M potassium phosphate), treated for five minutes in a 10-kc Raytheon sonic oscillator, and centrifuged at 5°C to remove cellular debris.

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* and ** Obtained as tricyclohexylammonium and sodium salts respectively from California Corporation for Biochemical Research.

*** Obtained from New England Nuclear Corporation.

The $NaHC^{14}O_3$ fixation experiments were carried out in double-arm Warburg flasks at 26°, 31°, or 37°C for 15 minutes. The substrates and nucleotides were placed in one side arm and the $NaHC^{14}O_3$ in the other. The enzyme preparations and all other components were placed in the main chamber. The reactions were stopped by the addition of either HCl or trichloroacetic acid and the precipitate was removed by centrifugation. A sample of the supernatant was pipetted onto a ground-glass planchet, dried under a stream of air, and counted immediately with a gas flow counter. All of the radioactivity counted was in the organic form, as any residual $C^{14}O_2$ is lost in drying the acidified reaction mixture.

Radioactive oxalacetic acid was identified by paper chromatograms of the 2, 4-dinitrophenylhydrazone formed after the addition of five milligrams of carrier OAA and two milliliters of a saturated solution of 2, 4-dinitrophenylhydrazine in 2N HCl to two milliliters of the reaction mixture.

Washed cells were tested for $C^{14}O_2$ uptake by incubating the cells in 0.05 M potassium phosphate buffer, pH 7.2, with $NaHC^{14}O_3$ (one micromole per milliliter, $1 \times 10^7$ cpm*) for 30 minutes in Warburg flasks. The cells were then extracted with cold five per cent trichloroacetic acid. Samples of the extract were dried and counted with a gas flow counter. The radioactive compounds in the extract were identified by co-chromatography with known compounds and with radioautography, utilizing two solvents (ethanol, $NH_4OH$, $H_2O$: 80, 5, 20 and ethylacetate, formic acid, $H_2O$: 3, 1, 0.5).

## III. RESULTS

When washed cells of *P. pestis* strain A-4 were incubated with $NaHC^{14}O_3$, the majority of the incorporated radioactive carbon was found to be in aspartic and glutamic acids. Smaller amounts of radioactive alanine, lysine, succinic acid, and ureidosuccinic acid were detected. No significant difference was found in the amount of $C^{14}O_2$ incorporated or in the distribution of the $C^{14}$ when the cells were incubated at 26° or 37°C.

Oxalacetic acid, formed by the carboxylation of phosphoenolpyruvic acid (PEP), could be a common precursor for every compound identified. Therefore, a cell-free extract of *P. pestis* strain A-4 prepared in tris-HCl buffer was dialyzed for 18 hours at 5°C against 0.001 M tris-HCl, pH 7.2, and tested for the ability to carboxylate PEP with $NaHC^{14}O_3$ to form OAA (Table I). Radioactive bicarbonate is incorporated by the extract when PEP is used as the substrate with and without the addition of a nucleotide (adenosine diphosphate). When OAA is substituted for PEP in the system, $C^{14}O_2$ is fixed in an exchange reaction only if an adenosine nucleotide is added.

* Counts per minute.

Unless the dialyzed extract contains trace amounts of ADP, the results indicate the presence of two distinct carboxylation reactions: an irreversible reaction not dependent upon a nucleotide and a reversible reaction requiring a nucleotide. Treatment with charcoal and ion exchange resins to remove any residual nucleotides slightly reduced the activity of the enzymes but did not significantly change the fixation pattern. Attempts to separate two enzymes by ammonium sulfate fractionation after removal of nucleic acid with protamine sulfate were unsuccessful. Results were obtained, however, with different procedures, which demonstrate that the fixation pattern shown in Table I is due to the presence of two enzymes in the extract. The two activities were separated from each other and have been shown to be similar to the enzymes phosphoenolpyruvic carboxylase[7] and phosphoenolpyruvate carboxykinase.[8] These enzymes catalyze Reactions 1 and 2 respectively.

$$PEP + CO_2 \longrightarrow OAA + Pi \qquad (1)$$

$$PEP + CO_2 + ADP \longleftrightarrow OAA + ATP \qquad (2)$$

To obtain PEP carboxylase free of PEP carboxykinase activity, the following procedure was used. The pH of a cell-free extract prepared in phosphate buffer was adjusted to 6.0 by the addition of 2 M $K_2HPO_4$. Protamine sulfate (0.25 volume of a two per cent solution) was added dropwise with stirring. The extract was held at 5°C for 30 minutes and then centrifuged at the same temperature. The precipitate was extracted with 0.1 M tris-HCl (pH 7.2) for 30 minutes at room temperature and centrifuged. The supernatant solution contained almost all of the PEP carboxylase activity free from PEP carboxykinase.

Table II shows the results obtained when the enzyme preparation obtained with this procedure was tested for carboxylation activity. PEP was readily carboxylated without the addition of a nucleotide in a manganese-dependent reaction. OAA was the only radioactive compound detected in the reaction mixture. Inorganic phosphate does not stimulate the reaction and ADP is inhibitory. The reaction is irreversible, as $C^{14}O_2$ was not exchanged with the β-carboxyl group of OAA with or without the addition of ATP or pyrophosphate. PEP could not be replaced with pyruvate or pyruvate and ATP. These results were considered as evidence for the presence of PEP carboxylase.

TABLE I. $C^{14}O_2$ FIXATION BY A DIALYZED CELL-FREE EXTRACT OF PASTEURELLA PESTIS STRAIN A-4

| Exp. | Additions | Activity Fixed, counts/min/0.1 ml |
|---|---|---|
| 1. | None | 28 |
| 2. | PEP | 1088 |
| 3. | PEP + ADP | 806 |
| 4. | OAA | 24 |
| 5. | OAA + ATP | 1420 |

The basic system contained: Tris-HCl (pH 7.4), 80 micromoles; $NaHC^{14}O_3$, 2 micromoles ($1 \times 10^6$ counts/min); cell-free extract, 0.5 ml; $MnCl_2$, 5 micromoles; water to make a total volume of 1.8 ml. The reaction was run for 15 minutes at 31°C. Reaction was stopped by the addition of 0.2 ml of 12N HCl. Additions: PEP, OAA, ADP, and ATP, 3 micromoles each.

TABLE II. PEP CARBOXYLASE ACTIVITY

| Exp. | Additions | Activity Fixed, counts/min/0.1 ml |
|---|---|---|
| 1. | None | 38 |
| 2. | PEP | 7508 |
| 3. | PEP + ADP | 370 |
| 4. | OAA | 42 |
| 5. | OAA + ATP | 70 |
| 6. | OAA + PP | 30 |

Experimental conditions were the same as in Table I, except for the enzyme preparation (see text). Addition: Pyrophosphate; 5 micromoles.

The reaction shows a variable response to glutathione; however, 0.002 M p-chloromercuribenzoate inhibits the reaction completely. Reduced glutathione (five micromoles) restores approximately 75 per cent of the activity. An apparent pH optimum was found at pH 6.8 in phosphate buffer, with the activity falling off rapidly on the acid side and more slowly on the basic side. The enzyme resembles PEP carboxylase from spinach,[7] wheat germ,[9] and Thiobacillus thiooxidans[10] as it functions at a relatively low concentration of bicarbonate.

PEP carboxykinase activity was obtained free from PEP carboxylase activity by adding dropwise with stirring one volume of two per cent protamine sulfate to four volumes of cell-free extract prepared in tris-HCl buffer. The mixture was held at 5°C for 30 minutes and then centrifuged at the same temperature. Twenty milliliters of the supernatant solution was placed on a DEAE-cellulose column (1.5 grams) that had been previously equilibrated with 0.05 M tris-HCl buffer, pH 7.2, and PEP carboxykinase was eluted from the column by the addition of 20 milliliters of 0.1 M tris-HCl buffer, pH 7.2, containing 0.2 M NaCl. The eluate was then stored at 5°C for 18 hours.

The fixation pattern obtained with the fresh eluate from the DEAE-cellulose column (Table III, Exp. 1 through 5) is essentially the same as with the crude dialyzed extract except that the carboxylation of PEP is slightly stimulated by the addition of ADP. When the eluate is held at 5°C for 18 hours, PEP carboxylase is apparently inactivated, as PEP is carboxylated only if ADP is added (Table III, Exp. 6 through 10).

The addition of NaCl to the protamine-treated extract before the DEAE-cellulose step did not inactivate PEP carboxylase after it was held at 5°C for the same time period.

The exchange reaction is completely dependent upon the addition of an adenosine nucleotide (ADP or ATP). Inosine diphosphate (IDP) or guanosine diphosphate (GDP) will not replace ADP, nor will inosine triphosphate (ITP), guanosine triphosphate (GTP), or pyrophosphate replace ATP in the exchange reaction. Manganese can be replaced with cobalt or magnesium. PEP cannot be replaced by pyruvate or pyruvate and ATP. The reaction is inhibited by p-chloromercuribenzoate and the inhibition can be reversed by the addition of reduced glutathione. The optimum pH for both the fixation and the exchange reactions was from 7.0 to 7.4 in tris-HCl buffer. The reaction is not influenced by avidin or supplemental biotin.

TABLE III. PEP CARBOXYKINASE ACTIVITY

| Exp. | Additions | Activity Fixed, counts/min/0.1 ml |
|---|---|---|
| 1. | None | 36 |
| 2. | PEP | 1400 |
| 3. | PEP + ADP | 1844 |
| 4. | OAA | 32 |
| 5. | OAA + ATP | 1090 |
| 6. | None | 28 |
| 7. | PEP | 70 |
| 8. | PEP + ADP | 2158 |
| 9. | OAA | 30 |
| 10. | OAA + ATP | 1618 |
| 11. | PEP + IDP | 76 |
| 12. | PEP + GDP | 48 |
| 13. | PEP + ADP + $MgCl_2$ | 1222 |
| 14. | PEP + ADP + $CoCl_2$ | 2246 |

Experimental conditions were the same as in Table I except for the enzyme preparation (Experiments 1-5: fresh DEAE cellulose eluate; experiments 6-14: DEAE cellulose eluate held at 5°C for 18 hours). $MnCl_2$ was omitted when $MgCl_2$ or $CoCl_2$ was added. Additions: IDP and GDP, 3 micromoles; $MgCl_2$ and $CoCl_2$, 5 micromoles.

Table IV shows the results of experiments designed to demonstrate the presence of nucleoside diphosphokinase and myokinase activities in the enzyme preparations.

TABLE IV. NUCLEOSIDE DIPHOSPHOKINASE AND MYOKINASE ACTIVITY

| Exp. | Additions | Activity Fixed, counts/min/0.1 ml |
|---|---|---|
| 1. | PEP | 70 |
| 2. | PEP + ATP | 44 |
| 3. | PEP + IDP | 62 |
| 4. | PEP + ATP + IDP | 942 |
| 5. | PEP + AMP | 56 |
| 6. | PEP + AMP + ATP | 860 |
| 7. | OAA + ADP | 1800 |

Experimental conditions were the same as in Table III, experiments 6 through 10. Additions: IDP and AMP, 3 micromoles each; ATP, 0.3 micromole.

PEP is not carboxylated by PEP carboxykinase when 0.3 micromole of ATP or 3 micromoles of IDP are added. When the same concentrations of ATP and IDP are added in combination, carboxylation equal to one-half that obtained with 3 micromoles of ADP occurs, indicating the presence of nucleoside diphosphokinase activity as shown in Reaction 3.

$$ATP + IDP \rightleftharpoons ADP + ITP \qquad (3)$$

Uridine diphosphate, GDP, and cytosine diphosphate will replace inosine diphosphate as a phosphate acceptor from ATP. The extract apparently contains myokinase as adenosine monophosphate and ATP will replace ADP in the carboxylation reaction only if both are added and, also, ADP will replace ATP in the exchange reaction. These results were considered indirect evidence for the presence of myokinase activity in the extract.

No difference in the enzymatic pattern was found when avirulent organisms were compared with virulent organisms. Both PEP carboxylase and PEP carboxykinase were found to be present in strain A-4 grown at 37°C and also in the virulent Alexander strain grown at 26° or 37°C. No evidence for thermal inactivation of either enzyme was found when the $CO_2$ fixation experiment with the cell-free extracts was performed at 37°C.

Resting cells and cell-free extracts were examined for reactions involving the incorporation of $CO_2$ (or equilibrium compounds) other than those forming OAA. Preliminary studies with the A-4 and Alexander strains have demonstrated carbamyl phosphokinase, ornithine transcarbamylase, and aspartic transcarbamylase activity. Experiments designed to demonstrate the presence of acetyl coenzyme A carboxylase or propionyl carboxylase were negative under the conditions of our experiments.

## IV. DISCUSSION

Considering the evidence presented here, it is unlikely that the differential growth response to sodium bicarbonate shown by virulent and avirulent strains of P. pestis is directly concerned with enzymatic differences in the formation of oxalacetate by carbon dioxide fixation reactions. Both types can carboxylate PEP to form OAA by two distinct reactions when grown at either 26° or 37°C. The reactions are similar to those catalyzed by PEP carboxylase and PEP carboxykinase.

The PEP carboxykinase reaction differs from that of animal tissue[8] and of other bacteria studied,[10-14] as adenosine nucleotides are required rather than inosine or guanosine nucleotides. PEP carboxykinase from yeast has been reported to be specific for adenosine nucleotides.[15]

Studies presented in another paper[16] demonstrate that a deficiency in carbamyl phosphate necessary for pyrimidine biosynthesis is probably a contributing factor in the failure of virulent cells to grow in aerated broth cultures at 37°C. Carbamyl phosphate is formed from $CO_2$ and ammonia in an ATP-dependent reaction.[17] In this respect, it is of interest that the PEP carboxylase found in P. pestis is unusual in that adenosine nucleotides are involved. At increased bicarbonate concentrations PEP carboxykinase and carbamyl phosphokinase could possibly couple, as shown in Figure 1. When $CO_2$ reacts with PEP to form OAA, ADP is phosphorylated to form ATP. The ATP formed would be available to fix $CO_2$ (or carbamate) into carbamyl phosphate and the resulting ADP would be available for the fixation of $CO_2$ into OAA once again. The coupled reactions would result in a greater production of carbamyl phosphate and aspartate. Both carbamyl phosphate and aspartate are pyrimidine precursors and are needed in larger quantity for the increased ribonucleic acid that occurs during the initial stage of growth.

Wheat germ,[9] Thiobacillus thiooxidans,[10-18] Nocardia corallina,[20] and Mycobacterium pheli[13] have been reported to contain both PEP carboxylase and PEP carboxykinase. It is of interest that this is the first study in which each enzyme has been obtained completely free from the other. This is also the first report of obtaining both of these enzymes from a pathogenic organism, although PEP carboxykinase has been studied in extracts of Neisseria gonorrhoeae,[14] and PEP carboxylase has been demonstrated in Salmonella typhimurium.[19] Fixation of $C^{14}O_2$ by the extract prepared from N. gonorrhoeae was found to be stimulated by inosinic acid and inhibited by adenylic acid.

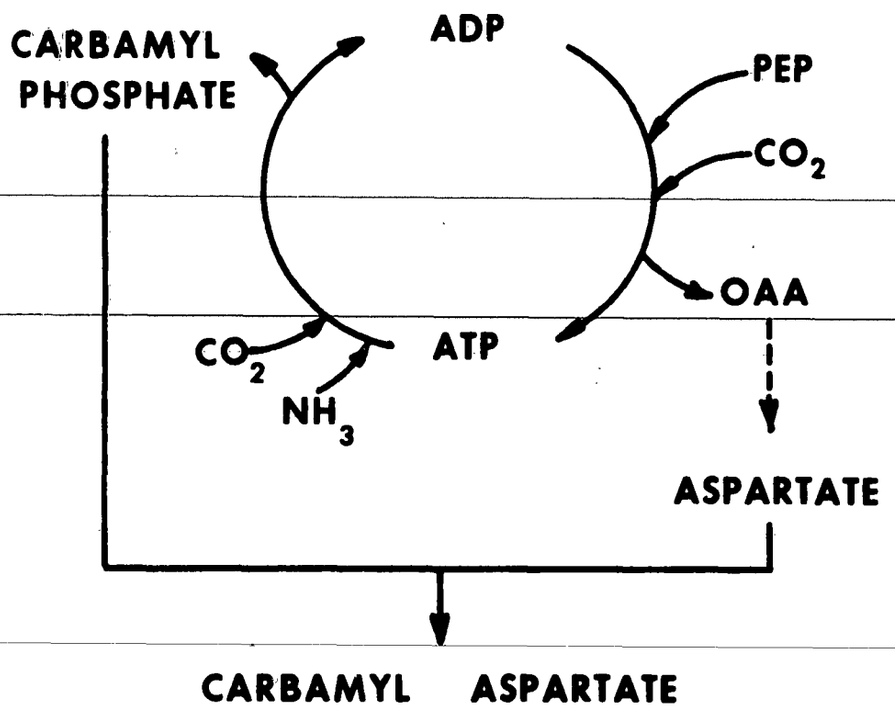


Figure 1. Proposed Interrelationship of PEP Carboxykinase and Carbamyl Phosphokinase.

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