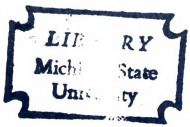

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LAWRENCE ALFRED DREYFUS

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Robert K Bruckley
Major professor

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CONSEQUENCES OF ASPARTASE DEFICIENCY IN *YERSINIA PESTIS*

By

Lawrence Alfred Dreyfus

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ABSTRACT

CONSEQUENCES OF ASPARTASE DEFICIENCY
IN YERSINIA PESTIS

by

Lawrence Alfred Dreyfus

Growing cells of Yersinia pseudotuberculosis but not closely related Yersinia pestis rapidly destroyed exogenous L-aspartic and L-glutamic acids thus prompting a comparative study of dicarboxylic amino acid catabolism. Rates of amino acid metabolism by resting cells of both species were determined at pH 5.5, 7.0, and 8.5. Regardless of pH, Y. pseudotuberculosis destroyed L-glutamic acid, L-glutamine, L-aspartic acid, and L-asparagine at rates greater than those observed for Y. pestis. Proline degradation by Y. pestis at pH 8.5 resulted in excretion of glutamic and aspartic acids. Yersinia pestis excreted aspartic acid when incubated with L-glutamic acid (pH 8.5) or L-asparagine. Aspartase activity was not detected in extracts of 10 strains of Y. pestis but was present in all of 11 isolates of Y. pseudotuberculosis which contained significantly more glutaminase, asparaginase, and L-glutamate-oxalacetate transaminase activity than Y. pestis. The observed differences in dicarboxylic amino acid metabolism are traceable to aspartase deficiency in Y. pestis.

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INTRODUCTION

Yersinia pestis, the causative agent of bubonic plague, is maintained in nature within a nutritionally enriched, protected, and fixed cycle formed by its mammalian host and insect vector. In contrast, cells of very closely related Yersinia pseudotuberculosis (53, 60) are usually transmitted orally and, like other Enterobacteriaceae, must exist at least transiently in nutritionally depleted environments which often require competition with saprophytes. Unlike Y. pestis, survival of Y. pseudotuberculosis may thus depend upon its maintaining broad biosynthetic and catabolic potential. Cells of Y. pestis, however, are unable to express many activities known to exist in Y. pseudotuberculosis including glucose-6-phosphate dehydrogenase, urease, abilities to synthesize 5 amino acids, and potential to ferment certain carbohydrates (11). Many of these determinants can be gained singly by meiotrophic mutation (14, 29, 30) and all are ancillary in the sense that their absence would not be expected to significantly reduce doubling times in nature or in conventional culture media. Nevertheless, typical generation times in enriched media for Y. pseudotuberculosis and Y. pestis are about 0.5 h and 2 h, respectively.

Although much is known about the intermediary metabolism of yersiniae, the reason for this difference in rates of growth is unknown. Aerobically grown cells of Y. pestis possess an operational Embden-Meyerhof pathway (65), tricarboxylic acid cycle (31, 64), and cytochrome system (31); amino acid transport (52, 68, 69), deamination (41, 49, 63), and incorporation into cellular components (41) have been described. Glycolytic and hexose monophosphate pathways exist in Y. pseudotuberculosis (9). This organism can catabolize a number of amino acids (8) presumably by conversion to tricarboxylic acid cycle intermediates (11).

In preliminary experiments it was noted that the two organisms could catabolyze common carbohydrates and organic acids at similar rates. However, significant differences were observed in rates of degradation of L-aspartate and L-glutamate. The purpose of this study is to define the basis of the lesion in dicarboxylic amino acid metabolism in Y. pestis and to compare the ability of yersiniae to degrade metabolically related amino acids.

LITERATURE REVIEW

The genus Yersinia (genus XI of the family Enterobacteriaceae) is currently recognized as having three members all of which are gram negative facultative intracellular parasites of rodents and man (3, 23, 59, 72). Two members of the genus, Y. pestis and Y. pseudotuberculosis, are antigenically and biochemically related to a high degree (11, 12). In fact, Y. pestis has been shown to share 85% of the deoxyribonucleic acid (DNA) sequence of Y. pseudotuberculosis (53). In this same study Y. enterocolitica, the third member of the genus, was demonstrated to possess 20% homology with Y. pseudotuberculosis (53). When less stringent conditions of hybridization were followed, Brenner et al. (6) found a slightly higher homology between Y. pseudotuberculosis and Y. enterocolitica than was observed by Moore and Brubaker (53). These data suggest a marginal intrinsic similarity within the genus as a whole and relatedness approaching identity between Y. pestis and Y. pseudotuberculosis.

Yersinia enterocolitica, a recently recognized member of the genus, occurs commonly in nature (33).

Clinical isolates are usually avirulent, however, human illness in the form of acute terminal ileitis has been reported (56). Y. pseudotuberculosis, like Y. enterocolitica, causes a relatively mild acute mesenteric lymphadenitis mimicking appendicitis in humans (26). When a comparative study was performed, both Y. pseudotuberculosis and Y. enterocolitica successfully produced experimental enterocolitis in rabbits resulting in necrobiotic centers in reticuloendothelial tissues of the intestine, mesenteric lymph nodes, liver and spleen (72). The author describes events occurring in infected HeLa cell and infected rabbit peritoneal macrophage model systems. These data correlated well with the pathology observed in the infected whole animal studies (72). The striking similarities observed may reflect the ability of both Y. pseudotuberculosis and Y. enterocolitica to gain entrance to and multiply within nonprofessional phagocytes and epithelial cells (3, 59, 72).

In spite of the marked similarities between Y. pseudotuberculosis and Y. pestis, the mild mesenteric lymphadenitis caused by the former species bears little resemblance to bubonic plague caused by the latter species. Data concerning the virulence determinants of yersiniae are largely if not solely centered on those determinants elaborated by Y. pestis. Much of this work was performed by T. W. Burrows and his colleagues and has been recently

reviewed (11, 12). In brief, Y. pestis possesses five recognized determinants of virulence. Loss of any one of the five results in varying degrees of avirulence (11, Table 1). It is noteworthy that upon discovery, these same virulence determinants were sought in Y. pseudotuberculosis. Two of the five determinants of virulence are shared by the two species, namely, the ability to produce V and W antigens (vwa^+) and purine independence (pur^+) (11). None of the five determinants possessed by Y. pestis have been reported to occur in Y. enterocolitica.

Fraction 1 (fra^+), a glycoprotein capsular antigen, is present in cells of Y. pestis cultivated at 37°C but not at 26°C (32). Fraction 1 antigen is an antiphagocytic capsule once thought to be obligately associated with virulence in human cases of fleaborne plague (44). Isolation of fra^- Y. pestis from a fatal case of human plague (76), however, suggests that virulence in man is not selected for by the presence of fraction 1 antigen. Thus, the role of the fra^+ phenotype in the pathogenesis of plague is somewhat confusing at this time. Mutation of fra^+ isolates to fra^- results in only marginal reduction in the virulence of Y. pestis for guinea pigs (19) and has no effect on the virulence in mice (11). Y. pseudotuberculosis does not express the fra^+ phenotype.

Purine independence (pur^+) has for some time been recognized to be associated with the virulence of a number

of bacterial pathogens including Y. pestis and Y. pseudotuberculosis (11, 17). This trait is therefore not unique to yersiniae but rather probably reflects the inability of the organism to scavenge purines from a purine deficient environment or inefficient transport of exogenous purines or purine precursors when available, thus overruling growth and therefore virulence.

The V and W or virulence antigens of Y. pestis, first described by Burrows and Burrows and Bacon (18, 20) have long been recognized to be obligately associated with the virulence of the plague bacillus (11). However, attempts to characterize the nature of their contribution to the pathogenicity of virulent Y. pestis have been frustrated by difficulty in their purification (48). Even with good preparations of the antigens their role in plague pathogenesis remains obscure. None-the-less, Y. pestis vwa^- cells are avirulent (11). V and W antigens have also been observed in Y. pseudotuberculosis (21) and are required for the virulence of this species in mice (11). However, here too the role of the vwa^+ gene products in regard to virulence is unknown.

Virulent (i.e., vwa^+) cells of Y. pestis possess an unusual temperature dependent requirement for substrate levels (2.5 mM) of Ca^{2+} (29, 47). Growth at 25°C or loss of the vwa^+ phenotype obviates the Ca^{2+} requirement which is manifest by vwa^+ cells at 37°C (12). The correlation

between V and W antigen production and calcium dependence was first noted by Brubaker and Surgalla (15). It was found that V and W antigens were produced only when cells pregrown in the absence of calcium at 25°C were shifted to 37°C. This shift resulted in the cessation of cell division and a high production of V and W antigens (15). Brubaker and Surgalla also described a rare phenotype termed "VW^+-avirulent" in which the requirement for Ca^{2+} but not the ability to produce V and W is lost. The finding suggests that the requirement for Ca^{2+} is directly associated with virulence and not subordinate to the production of V and W antigens. A redesignation of this virulence determinant from vwa^+ to cal^+ (representing the calcium dependence) to better describe the phenotype associated with virulence in Y. pestis has been proposed by Brubaker (12).

The consequences of shifting a growing Y. pestis cal^+ (virulent) culture from 26°C to 37°C in a medium containing no added Ca^{2+} and 20 mM Mg^{2+} (i.e., those concentrations found within host cell cytoplasm) are manifold. The effects described to date of such a temperature shift are turnoff of initiation of new rounds of chromosomal replication, cessation of stable ribonucleic acid synthesis, expression of the V and W antigens, reduction of nucleoside triphosphate pools, decrease in adenylate energy charge from 0.85 to 0.59 and bacteriostasis (77).

Avirulent cal^- (vwa^- or "VW^+-avirulent") isolates grow normally at 37°C in this medium. It was also noted in these studies that at pH 7.8 cal^+ cells grew without added calcium in the presence of any one of a number of nucleoside mono-, di-, or triphosphates (10.0 mM) including adenosine triphosphate (ATP). It should be noted, however, that stimulation was apparently not a result of uptake of the nucleotides since ^{14}C-ATP bound by cal^+ cells under these conditions could be dissociated from the cells by the addition of chelating agents (R.J. Zahorchak, personal communication).

It is of interest that the V and W antigens and the calcium restriction phenotype are expressed only under conditions which mimic an intracellular environment. For Y. pestis, a facultative intracellular parasite, these events which are seemingly required for its virulence may reflect a bioenergetic regulatory mechanism. Conceivably such a mechanism could serve as a signal for expression of intra- vs. extra-cellular modes of energy conservation. However, at this time the definitive role of these events in the virulence and success of Y. pestis as a facultative intracellular pathogen, remains highly speculative.

The fourth recognized virulence determinant of Y. pestis concerns the ability of the organism to produce pesticin (pst^+), a protein bacteriocin active against serotype I strains of Y. pseudotuberculosis, a few isolates

of Y. enterocolitica, and certain colicin-indicator strains of Escherichia coli (11). Pesticin production per se may not necessarily promote virulence of the organism since loss of pesticin activity (a means to measure its contribution to virulence) is concomitant with the loss of coagulase and fibrinolysin activities (2, 16). Intravenous injection of pst^- cells of otherwise fully virulent Y. pestis into lab animals results in death of the animals comensurate with wild type organisms injected subcutaneously (13). Thus pst^- cells are avirulent when administered via the natural port of entry (i.e., subcutaneous inoculation). These data support the necessity of coagulase and fibrinolytic activities for the expression of virulence in fleaborne plague, presumably by promoting dissemination of the organism from the initial foci to deep peripheral tissues (11, 13).

Cells of virulent Y. pestis grown on solid media containing either hemin or congo red can absorb the planar chromophores contained therein and grow as pigmented (pgm^+) colonies (42, 71). Avirulent pgm^- cells regain full virulence when injected intravenously with sufficient iron to saturate serum transferrin (43). However, whether restoration of virulence was due to the ability of the pgm^- strain to successfully acquire iron in the Fe^{3+} saturated system or to the compromising condition dealt the host upon such action (45) is not distinguishable

at this time. Differences in the iron metabolism of pgm^+ and pgm^- cells have yet to be observed. When finally elucidated, differences between pgm^+ and pgm^- isolates of Y. pestis may reflect a selective advantage of pst^+ over pst^- cells to bind complexed iron in vivo.

Yersiniae possess no unusual growth requirements and grow very well at 26°C in defined media containing a few L-amino acids, organic acids, inorganic salts and a source of energy, usually xylose and gluconate or glucose (10, 14, 22, 38, 40, 61). Prior to the demonstration of a chemically defined medium suitable for the growth of Y. pestis, Higuchi and Carlin obtained excellent growth in casein hydrolysate broth with no other additions (37). Subsequent defined medium studies focused on the identification of those factors which would allow growth of Y. pestis at 26°C as well as at 38°C while still maintaining the high yield obtained in casein hydrolysate broth (7, 38, 40, 61).

Though much discrepancy concerning the requirement of Y. pestis for various amino acids appears in the literature, it is generally accepted that at 26°C most strains require phenylalanine, methionine, glycine, and cysteine for growth (14, 22, 38). The requirement for cysteine may be obviated by the addition of thiosulfate or sulfite but not sulfate (28). The addition of L-threonine but not L-serine will satisfy the requirement

for glycine (42, 14). Valine and isoleucine stimulate growth at 26°C (28, 40). A medium containing glucose, inorganic salts, thiosulfate, valine, isoleucine, methionine, and phenylalanine will support excellent growth of Y. pestis at 26°C with final yields of ∿5 x 10^9 cells per ml (10, and unpublished observation), at 28°C strains of Y. pseudotuberculosis and Y. enterocolitica have either no requirements for growth (e.g., glucose and inorganic salts will support growth), or require either thiamine or Ca-pantothenate (22).

Growth of Y. pestis at 38°C is somewhat more restrictive than at 26°C. Brownlow and Wessman reported Y. pestis to require thiamine, Ca-pantothenate, biotin, isoleucine, valine, threonine, phenylalanine, cysteine, methionine and hemin at 38°C (7). These authors found that hemin could be replaced in the medium with α-ketoglutarate or high concentrations of glutamic acid. From these results they postulated that the exogenous addition of α-ketoglukarate or glutamic acid was necessary at 38°C for the synthesis of porphoryns by Y. pestis (7). However, these findings have not been substantiated. Burrows and Gillett more recently demonstrated reliable growth of Y. pestis at 37°C on solid media (glucose and inorganic salts) supplemented with cysteine, methionine, phenylalanine, glycine, valine, isoleucine, glutamic acid and thiamine under $C0_2$ enriched air (22). These same

authors found that the growth requirements of Y. pseudotuberculosis and Y. enterocolitica at 37°C, like those of Y. pestis, are more exacting. Most strains of Y. pseudotuberculosis required any three of the four factors, glutamic acid, thiamine, cystine, and pantothenate at 37°C, whereas Y. enterocolitica required thiamine and either cysteine or methionine at that same temperature (22).

To date the most suitable defined liquid medium for the cultivation of Y. pestis at 37°C is that of Higuchi, Kupferberg, and Smith (39) as modified by Brubaker (10). This medium supports high yield growth ($\sim 10^{10}$ cells per ml) of Y. pestis at 37°C with a doubling time of ∿2 hours (unpublished observation). When cultured under the same conditions, Y. pseudotuberculosis doubles in approximately thirty minutes.

Both Y. pestis and Y. pseudotuberculosis possess an operational Embden-Meyerhoff pathway (65, 9). Y. pseudotuberculosis can also oxidize glucose via the hexose monophosphate pathway (9). Claims by Santer and Ajl (66) that Y. pestis also contains a functional pentose phosphate shunt could not be substantiated by other investigators, all of whom observed the lack of glucose-6-phosphate dehydrogenase in that organism (54, 55, 5). Gluconate is catabolyzed via the Entner-Douderoff pathway in both Y. pestis and Y. pseudotuberculosis

(54, 9). Pentose synthesis in Y. pestis presumably occurs via rearrangement of 3C and 6C fragments by the action of transketolase and transaldolase (11). Xylose isomerase has been purified from extracts of Y. pestis (67). Both Y. pestis and Y. pseudotuberculosis can oxidize a variety of sugars and organic acids including glucose, gluconate, ribose, xylose, pyruvate and lactate (11). Anaerobically Y. pestis ferments glucose primarily to lactate, ethanol, acetate and formate (31, 65).

Y. pestis possesses a functional tricarboxylic acid cycle (TCAC) (64, 31). The presence of the TCAC in Y. pseudotuberculosis has not been documented yet is assumed to be present (11). The presence of an ADP-dependent phosphoenolpyruvate carboxykinase and an irreversible phosphoenolpyruvate carboxylase serving to catalyze the fixation of CO_2 into oxalacetate has been reported in Y. pestis (1).

Y. pestis maintains the ability to transport, catabolyze, deaminate and/or incorporate into cellular material various amino acids (52, 68, 69, 57, 63, 41). Levine et al. (49) demonstrated the presence of serine dehydratase in Y. pestis which is apparently responsible for the rapid degradation of L-serine observed in other studies (57, 41). Other amino acids which have been reported to undergo degradation by Y. pestis are L-alanine and L-glutamate (41). Y. pseudotuberculosis degrades

L-serine and L-aspartate very rapidly followed more slowly by L-glutamate, L-proline, L-threonine and glycine (8).

The inability of Y. pestis to convert cysteine to cystathionine resulting in the nutritional requirement for methionine was documented by Englesberg (28). No such block exists in Y. pseudotuberculosis which can utilize methionine as its sole source of sulfur (11). No other unusual biochemical features have been reported for either of these yersiniae. The central anabolic pathways of these two organisms are presumably identical to those in other enteric bacteria (11).

Some of the phenotypic differences between wild type Y. pestis and Y. pseudotuberculosis have already been mentioned. These characteristics and others are listed in Table 2. Special emphasis should be given to those traits in Y. pestis which have been shown to become positive via mutation. Englesberg first used the expression meiotrophic mutation to describe the occurrence of rhamnose positive Y. pestis isolates which occurred naturally at the low frequency of 2.6×10^{-11} (29). Likewise, wild type Y. pestis which normally cannot ferment melibiose gives rise to melibiose meiotrophs when plated on selective media (11). Additional meiotrophic conversions recognized to date are the ability to synthesize glycine and assimilate low levels of ammonium salts (14) and the ability to synthesize phenylalanine and methionine

(30, 7). Judicious employment of novel selective methods will undoubtedly lead to the generation of additional meiotrophic markers in Y. pestis.

The ability of Y. pestis to undergo such meiotrophic conversions suggests a deficiency or lack of a specific mechanism for the excision of genetic information no longer in normal metabolic use. Further investigation into the mechanisms involved in the retrieval of these gene products may explain how the gene functions were initially rendered inactive and what role environmental factors play in this evolutionary pattern.

MATERIALS AND METHODS

Bacteria. Y. pestis EV76, Y. pseudotuberculosis PB1 and Escherichia coli K-12 were used in most experiments. All organisms were preserved in buffered glycerol as previously described (2).

Media and Cultivation. Upon removal from storage, cells were incubated at 26°C for 36 to 48 h on slopes of blood agar base (Difco). Organisms were removed in 0.033 M potassium phosphate buffer, pH 7.0 (phosphate buffer), washed by centrifugation at 48,000 x g for 10 min at 5°C, and inoculated at a density of about 10^8 cells/ml into 25 ml (per 250 ml Erlenmeyer flask) of the complex synthetic medium of Higuchi, Kupferberg, and Smith (39) as modified by Brubaker (10). After aeration at 26°C for 16 h at 170 rpm on a model G76 gyrotory shaker (New Brunswick Scientific Co., New Brunswick, NJ), the culture served as inoculum for 200 ml of the same medium contained in a 2 liter flask. The latter was similarly aerated at 37°C and, upon reaching late logorithmic growth, the bacteria were collected by centrifugation and suspended in 200 ml (per 2 liter flask) of preincubation mixture consisting of the inorganic salts

and vitamin components of the complex synthetic medium plus 5.0 mM concentrations of the 20 naturally occurring amino acids. This step was performed to insure induction of pertinent catabolic enzymes. After aeration for 3 h at 37°C the organisms were collected by centrifugation, and immediately used for determination of rates of amino acid degradation.

The same procedure was used to prepare cells for sources of enzymes except that the organisms were washed in phosphate buffer. Preincubation mixture supplemented with D-glucose (0.01 M) was also used to monitor by paper chromatography the degradation of amino acids by growing cells. For determination of aspartase activity the organisms were also grown in a minimal synthetic medium (10) and heart infusion broth (Difco) supplemented with L-aspartic acid (5.0 mM).

Degradation of Amino Acids. Destruction of amino acids by growing cells was assayed by sterilizing samples of culture by passage through 0.45 μm pore membrane filters (Millipore Corp., Bedfore, MA), exchange and elution with Dowex 50 W (H^+ form), and paper chromatography (14). Rates of degradation were determined in a reaction system containing distilled water-washed cells (∿5 mg), 33 μmoles of buffer, 1 μmole of $MgCl_2$, and 10 μmoles of L-[U-^{14}C] amino acid (0.1 μCi/μmole) in a volume of 1.0 ml contained

in a 25 ml Erlenmeyer flask. Buffers used were sodium citrate (pH 5.5), sodium morpholinopropane sulfonate (pH 7.0), or tris(hydroxymethyl)aminomethane·HCl (Tris·HCl buffer) (pH 8.5). Reactions were initiated by addition of radioactive amino acid and the flasks were aerated at 37°C as previously described. At appropriate intervals samples of 0.1 ml were removed, added to 0.1 ml of cold 10% trichloroacetic acid, stored for 30 min in an icebath, centrifuged, and 10 µl of the supernatant was applied to Whatman no. 1 paper. After chromatography in one dimension with either n-butanol:acetic acid:water (100:22:50 vol/vol), phenol:water (4:1 g/ml), or pyridine:acetic acid:water (50:35:15 vol/vol) the papers were cut into strips and examined with a model 7201 radiochromatogram scanner (Packard Instrument Co., Downers Grover, IL). Radioactive areas corresponding to those of ninhydrin-positive controls and of non-volatile products were removed, cut into squares of about 1 mm, and placed into scintillation vials with 10 ml of ACS counting fluid (Amersham-Searle Co., Arlington Heights, IL). Radioactivity was determined with a model 3320 Packard Tricarb liquid scintillation spectrometer. Appropriate controls were constructed to monitor nonspecific binding of radioactive amino acids to glassware and bacteria; such losses were never observed.

Cell-free Extracts. Organisms previously washed in phosphate buffer were suspended in 0.1 M Tris·HCl buffer (pH 7.8) containing 0.001 M 2-mercaptoethanol. After disruption at 20,000 psi in a French pressure cell or by treatment for 1 min with a sonic probe (MSE Ltd., London, England), debris was removed by centrifugation and the supernatant was dialyzed overnight at 5°C against 0.005 M Tris·HCl buffer (pH 7.8) containing 0.001 M 2-mercaptoethanol and 0.001 M $MgCl_2$. Protein was determined by the method of Lowry et al. (50) using bovine serum albumin as a standard.

Enzymes. The method of Reed and Willms (58) was used to determine α-ketoglutarate dehydrogenase where a unit of activity is defined as that amount of enzyme required to reduce 2 μmoles of ferricyanide per h. Specific activities of other enzymes are defined in terms of number of μmoles of substrate converted to product per min per mg of protein. L-Glutamate dehydrogenase was measured as described by Coolbaugh, Proger, and Weiss (25). L-Glutamate-oxalacetate transaminase and L-glutamate-pyruvate transaminase activities were determined by the methods of Hebeler and Morse (36).

Aspartase was assayed by measuring the release of ammonia from L-aspartate. The reaction mixture consisted of 50 μmoles of Tris·HCl buffer (pH 7.0), 50 μmoles of

sodium L-aspartate, 1 μmole of $MgSO_4$, 0.1 μmoles of ethylenediaminetetracetate, and bacterial extract in a volume of 1.0 ml. After starting the reaction by addition of aspartate, samples of 0.1 ml were added at intervals to 0.1 ml of cold 10% trichloroacetic acid. The resulting precipitate was immediately removed by centrifugation and 0.1 ml of the supernatant was analyzed with Nessler's reagent. Glutaminase activity was determined by radioassay in a reaction mixture containing 20 μmoles of potassium phosphate (pH 7.2), 20 μmoles of L-[U-^{14}C] glutamine (0.1 μCi/μmole), and bacterial extract in a volume of 1.0 ml. Samples of 0.1 ml of reaction mixture were added to 0.1 ml of 10% cold trichloroacetic acid at intervals and immediately clarified by centrifugation. The supernatant was chromatographed with phenol:water (4:1 g/ml) and radioactive glutamine and glutamic acid were determined as previously described. Asparaginase activity was measured by an identical procedure except that L-[U-^{14}C] asparagine was substituted for radioactive glutamine.

Reagents and Isotopes. All chemicals were commercial products of highest available purity. Radioactive compounds were purchased from Amersham-Searle.

RESULTS

Cells of Y. pseudotuberculosis and Y. pestis exhibited typical growth in preincubation mixtures plus glucose (see Materials and Methods) yielding maximum optical densities of 1.8, and 1.5, respectively. Prior to the termination of log phase growth essentially all of the L-aspartic acid and much of the L-asparagine and L-glutamic acid had disappeared from the culture medium of Y. pseudotuberculosis (Figure 1). Significant concentrations of these amino acids were detectable in comparable cultures of Y. pestis; at this time both organisms had eliminated L-serine from the medium. Chromatograms of stationary phase cultures showed that cells of both species could also degrade L-proline, glycine, L-alanine, and L-threonine. Having identified in these preliminary experiments those amino acids which underwent significant metabolism, individual rates of degradation were determined by radioassay. These results, shown in Table 3, suggested the existence of marked differences between the two species in abilities to catabolize dicarboxylic amino acids.

Degradation of L-glutamic Acid, L-glutamine, and L-proline. Cells of Y. pseudotuberculosis degraded L-glutamic acid at pH 5.5 and 7.0 about 5 times more rapidly than did preparations of Y. pestis. Metabolism of L-glutamic acid was markedly stimulated in both organisms at pH 8.5 where Y. pestis but not Y. pseudotuberculosis excreted aspartic acid (Figure 2). Furthermore, at pH 8.5 but not pH 5.5 or 7.0 both glutamic and aspartic acids were excreted by Y. pestis incubated with L-proline (Figure 3). Similar degradation products were not detected with Y. pseudotuberculosis. L-Glutamine was not significantly degraded by Y. pestis and only slow destruction occurred with Y. pseudotuberculosis; neither organism catabolized L-arginine.

Specific activities for L-glutamate-pyruvate transaminase and L-glutamate-oxalacetate transaminase for yersiniae and E. coli control are shown in Table 4. Cells of Y. pseudotuberculosis were somewhat deficient in the former enzyme whereas those of Y. pestis possessed reduced levels of the latter. The specific activities of L-glutamate dehydrogenase and α-ketoglutarate dehydrogenase in both yersiniae were comparable (Table 5). L-glutaminase activity was present in Y. pestis and Y. pseudotuberculosis at a specific activity of 0.003 and 0.012, respectively.

Degradation of L-aspartic acid and L-asparaginine. L-Aspartic acid was degraded slowly by cells of Y. pestis at pH 5.5; no detectable destruction occurred at pH 7.0 or pH 8.5. In contrast, L-aspartic acid was rapidly destroyed by Y. pseudotuberculosis over the pH range of 5.5 to 8.5 yielding fumarate, malate, succinate, and an unidentified product (Figure 4). Accumulation of these tricarboxylic acid cycle intermediates would be expected if the amino acid had undergone deamination via aspartase. L-Asparagine was degraded more rapidly by cells of Y. pseudotuberculosis than by those of Y. pestis (Table 3). The latter accumulated stoichiometric amounts of aspartic acid while catabolyzing L-asparagine under the 3 conditions of pH; results obtained at pH 8.5 are shown in Figure 5.

Aspartase activity was present in extracts of all of 11 isolates of Y. pseudotuberculosis but was not detected in similar preparations of 10 strains of Y. pestis (Table 5). Similarly, aspartase was not observed in Y. pestis grown in minimal synthetic medium (see Materials and Methods) or heart infusion broth supplemented with 5.0 mM L-aspartic acid. Extracts of Y. pseudotuberculosis and E. coli contained aspartase activity after growth in these media (Table 6). Significantly less L-asparaginase was present in Y. pestis than in comparable extracts of Y. pseudotuberculosis (Table 7).

DISCUSSION

Cells of Y. pestis are capable of actively transporting some (52, 68, 69) if not all naturally occurring amino acids. This process of accumulation probably had little influence on the rates of destruction reported here where favorable equilibria caused by catabolic enzymes were sufficient to have promoted entrance by passive mechanisms. In fact, in the low-potassium and carbohydrate-deficient environment that was chosen, an internal negative potential (created by alkaline pH) would have hindered active transport of neutral and acidic amino acids (24, 35). Since alkaline or neutral pH enhanced the destruction of L-glutamic acid and all tested neutral amino acids, degradation was not limited by the process of uptake. The role of alkaline pH in stimulating amino acid destruction was not resolved in this study. Elevated pH may promote turnover of critical catabolic enzymes including L-glutamate dehydrogenase (74) and can favor exit of acidic degradation products (75), especially carbonate and acetate ions.

Aspartic acid was another anionic degradation product that accumulated in suspensions of Y. pestis

containing added L-proline, L-glutamate or L-asparagine. Cells of Y. pseudotuberculosis, however, rapidly destroyed L-aspartic acid in a reaction yielding fumarate. Further work showed that extracts of 10 isolates of Y. pestis from diverse geographical sources lacked detectable aspartase activity when tested under conditions that yielded positive results for Y. pseudotuberculosis and an E. coli control.

This finding was not anticipated because Korobeinik and Domaradskii (46) had reported the presence of aspartase in Y. pestis of Russian origin. A recent search of the literature, however, revealed that Domaradskii (27) subsequently found 19 of 24 Y. pestis isolates to lack aspartase. Accordingly, this property may be variable among Russian strains, many of which resemble Y. pseudotuberculosis with respect to other properties (51, 62, 70). The results reported here indicate that aspartase is absent in typical Y. pestis.

As a consequence of this deficiency, significant conversion of L-glutamic acid to α-ketoglutarate can only occur in Y. pestis via the action of L-glutamic dehydrogenase. In addition to this mechanism, Y. pseudotuberculosis can form α-ketoglutarate by transamination of L-glutamic acid with a catalytic amount of oxalacetate to yield L-aspartate which can then be catabolized via aspartase, fumarase, and malic dehydrogenase,

consecutively, to regenerate oxalacetate. This second method of L-glutamic acid oxidation was originally proposed to account for the inability of L-glutamate decarboxylase-negative mutants of E. coli to utilize L-glutamate as a sole source of carbon (73). The importance of this pathway in normal metabolism was later underscored by isolation of aspartase-negative mutants of E. coli by selection for inability to utilize L-glutamic acid for growth (34).

Some consequences of aspartase-deficiency in Y. pestis are that the organisms are unable to catabolize exogenous L-aspartic acid or aspartic acid arising from L-asparagine. Likewise, oxidation of exogenous L-glutamic acid (or glutamic acid arising from L-glutamine or L-proline) via the tricarboxylic acid cycle is limited by the activity of L-glutamate dehydrogenase. This primary dehydrogenation, unlike that of its counterpart in Y. pseudotuberculosis (malic dehydrogenase), yields NADPH which must undergo transhydrogenation with NAD^+ before initiating oxidative phosphorylation (14). Furthermore, oxalacetate can undergo transamination with glutamic acid in Y. pestis yielding catabolically inert aspartate thus preventing the generation of citrate via the condensing enzyme. A block at this level of the tricarboxylic acid cycle resulting in the accumulation of acetate has been reported in Y. pestis (31). The occurrence of these

events in yersiniae result in marked differences in dicarboxylic amino acid metabolism and may contribute to the longer generation time of Y. pestis relative to that of Y. pseudotuberculosis.

Aspartase deficiency is uncommon in bacteria. Francisella tularensis is said to lack the enzyme (27) and Rickettsia prowaseki and Rickettsia mooseri excrete aspartic acid as a function of L-glutamate metabolism (4); the specific activity of aspartase in rickettsiae has not yet been reported. It may be significant that Yersinia, Francisella, and Rickettsia are mammalian intracellular parasites.

TABLE 1.--Established virulence determinants in Yersinia pestis (11).

Determinant[a]					Intraperitoneal LD_{50}		
Fra	Pur	Cal(vwa)	Pst	Pgm	Mouse	Guinea Pig	Mouse plus injected iron
+	+	+	+	+	<10	<10	<10
0	+	+	+	+	<10	10^4	--
+	0[b]	+	+	+	10^2	10^4	--
+	0[c]	+	+	+	$>10^7$	$>10^8$	--
+	+	0	+	+	$>10^7$	$>10^8$	$>10^7$
+	+	+	0	+	$\sim10^5$	$\sim10^6$	10^1
+	+	+	+	0	$\sim10^7$	$>10^8$	10^1

[a]See text for definition.

[b]Block before inosine monophosphate.

[c]Block after inosine monophosphate.

TABLE 2.--Some phenotypic differences between wild type Yersinia pestis and Yersinia pseudotuberculosis (11).

Determinant	Yersinia pestis	Yersinia pseudotuberculosis
Melibiose fermentation	0[a]	+
Rhamnose fermentation	0[a]	+
Aspartase	0	+
Assimilation of low levels of NH_3	0[a]	+
Urease	0[a]	+
Threonine/glycine biosynthesis	0[a]	+
Methionine biosynthesis	0[a]	+
Phenylalanine biosynthesis	0[a]	+
Isoleucine/valine biosynthesis	0[a]	+
Glucose-6-phosphate dehydrogenase	0	+
3,6-dideoxyhexoses in lipopolysaccharide	0	+[b]
Motility at 26°C	0	+
Fraction 1 (capsular) antigen	+[b]	0
Pesticin, coagulase, and fibrinolysin	+[b]	0
Murine toxin	+[b]	0
Absorption of exogenous chromatophores	+[b]	0

[a]Can become positive via meiotrophic mutation.

[b]Presumed or established virulence determinant.

TABLE 3.--Initial rates of destruction of some amino acids by resting cells of *Yersinia pestis* EV76 and *Yersinia pseudotuberculosis* PB1.[a]

Amino acid	*Yersinia pestis*			*Yersinia pseudotuberculosis*		
	pH 5.5	pH 7.0	pH 8.5	pH 5.5	pH 7.0	pH 8.5
L-Serine	153.7	243.8	402.9	31.6	53.1	111.6
L-Alanine	9.0	12.8	14.7	7.8	9.2	16.7
L-Cysteine	N.D.[b]	N.D.	N.D.	3.8	N.D.	N.D.
L-Glutamate	0.3	2.5	14.2[c]	1.9	10.2	25.9
L-Glutamine	0	0	0	1.3	2.2	3.0
L-Proline	7.7	11.9	25.7[c]	9.4	11.3	12.3
L-Aspartate	2.4	0	0	91.8	31.0	42.0
L-Asparagine	2.9[c]	6.4[c]	3.7[c]	46.1	46.6	54.0
L-Threonine	4.7	7.7	10.6	5.8	10.1	8.3
Glycine	9.7	18.1	5.6	3.2	5.8	6.7

[a]Results are expressed as nmoles of amino acid destroyed per min per mg of dry cell weight.

[b]Not determined.

[c]Condition that resulted in accumulation of aspartic acid.

TABLE 4.--Specific activities of L-glutamate-pyruvate transaminase and L-glutamate-oxalacetate transaminase in dialyzed extracts of cells of *Yersinia pestis* EV76, *Yersinia pseudotuberculosis* PB1, and *Escherichia coli* K12.[a]

Organism	L-Glutamate-pyruvate transaminase	L-Glutamate-oxalacetate transaminase
Yersinia pestis	0.007	0.001
Yersinia pseudotuberculosis	0.001	0.011
Escherichia coli	0.006	0.012

[a]Specific activities are given in terms of units (defined under Materials and Methods) per mg of protein.

TABLE 5.--Specific activities of aspartase, α-ketoglutarate dehydrogenase, and L-glutamate dehydrogenase in dialyzed extracts of cells of Yersinia pestis and Yersinia pseudotuberculosis.[a]

Strain	Phenotype or serotype[b]	Aspartase	α-Ketoglutarate dehydrogenase	L-Glutamate dehydrogenase
		Yersinia pestis		
A4	0	0.001	1.02	0.09
A16	A	0.001	1.00	0.13
D1	A	0.001	1.05	0.11
EV76	0	0.001	1.07	0.11
G32	0	0.001	1.14	0.12
KIM	M	0.001	0.90	0.14
Kuma	A	0.001	1.13	0.10
Salazar	0	0.001	1.25	0.24
TS	0	0.001	1.27	0.22
Yokohoma	A	0.001	1.40	0.12
		Yersinia pseudotuberculosis		
1	IA	0.86	1.08	0.15
IB	IB	0.56	1.06	0.16
7	IIA	0.4	0.96	0.11
1779	IIB	0.70	1.02	0.07
43	III	0.57	1.04	0.17
32	IVA	0.42	0.70	0.19
Ikegaki	IVB	1.01	0.65	0.15
25	VA	0.44	0.87	0.20
R2	VB	0.80	0.97	0.15
3	VI	0.60	0.68	0.15
PB1	IIB	0.83	0.58	0.13

[a]Specific activities are given in terms of units (defined under Materials and Methods) per mg of protein.

[b]Phenotypes of Yersinia pestis are the varieties orientalis (O), antiqua (A), and mediaevalis (M); serotypes of Yersinia pseudotuberculosis are listed.

TABLE 6.--Specific activity of aspartase in dialyzed extracts of Yersinia pestis EV76, Yersinia pseudotuberculosis PB1, and Escherichia coli K12 prepared after growth in complex synthetic medium (CSM), heart infusion broth containing added 5.0 mM sodium L-aspartate (HIB), and minimal synthetic medium (MSM).[a]

Organism	Temperature of growth (C)	Medium		
		CSM	MIB	MSM
Yersinia pestis	26°	N.D.[b]	<0.001	<0.001
	37°	<0.001	<0.001	<0.001
Yersinia pseudotuberculosis	26°	N.D.	N.D.	0.05
	37°	0.83	0.31	0.07
Escherichia coli	26°	N.D.	N.D.	0.01
	37°	0.16	0.16	0.03

[a]Specific activity is given in units (defined in Materials and Methods) per mg of protein.

[b]Not determined.

TABLE 7.--Specific activities of asparaginase in dialyzed extracts of *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Escherichia coli*.

Organism	Asparaginase[a]
Yersinia pestis EV76	0.004
Yersinia pestis KUMA	0.009
Yersinia pestis KIM	0.005
Yersinia pestis A1122	0.007
Yersinia pseudotuberculosis PB1	0.024
Escherichia coli K12	0.180

[a]Specific activity is given in terms of units (defined in Materials and Methods) per mg of protein.

Figure 1.--Degradation of L-amino acids by Yersinia pestis EV 76 (middle) and Yersinia pseudotuberculosis (bottom) chromatogramed at culture optical densities of 0.85 and 0.91, respectively, during aeration at 37°C in preincubation mixture plus 0.01 M D-glucose. Position of amino acids (top) correspond to: lysine (1), histidine (2), arginine (3), asparagine (4), glutamine (5), methione sulfoxide (6), proline (7), threonine (8), alanine (9), glycine (10), serine (11), glutamic acid (12), aspartic acid (13), tyrosine (14), valine, tryptophan and methionine (15, 16, 17), phenylalanine (18), leucine and isoleucine (19, 20); solvent 1 was phenol:water (4:1 g/ml) and solvent 2 was n-butanol:acetic acid:water (100:22; 50 v/v).

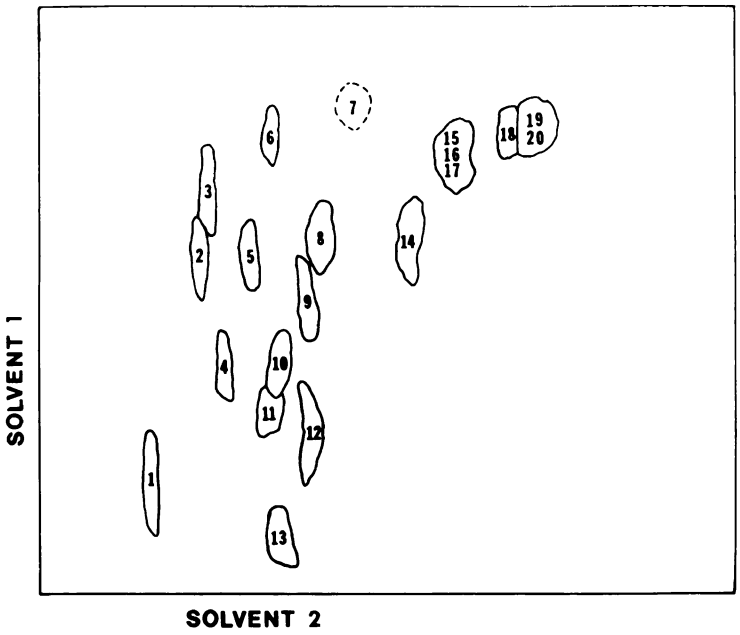
SOLVENT 1
SOLVENT 2
1
2
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Figure 2.--Degradation of L-glutamic acid by resting cells of Yersinia pseudotuberculosis PB1 (◑) and Yersinia pestis EV76 (●) with accumulation of aspartic acid (○) by the latter.

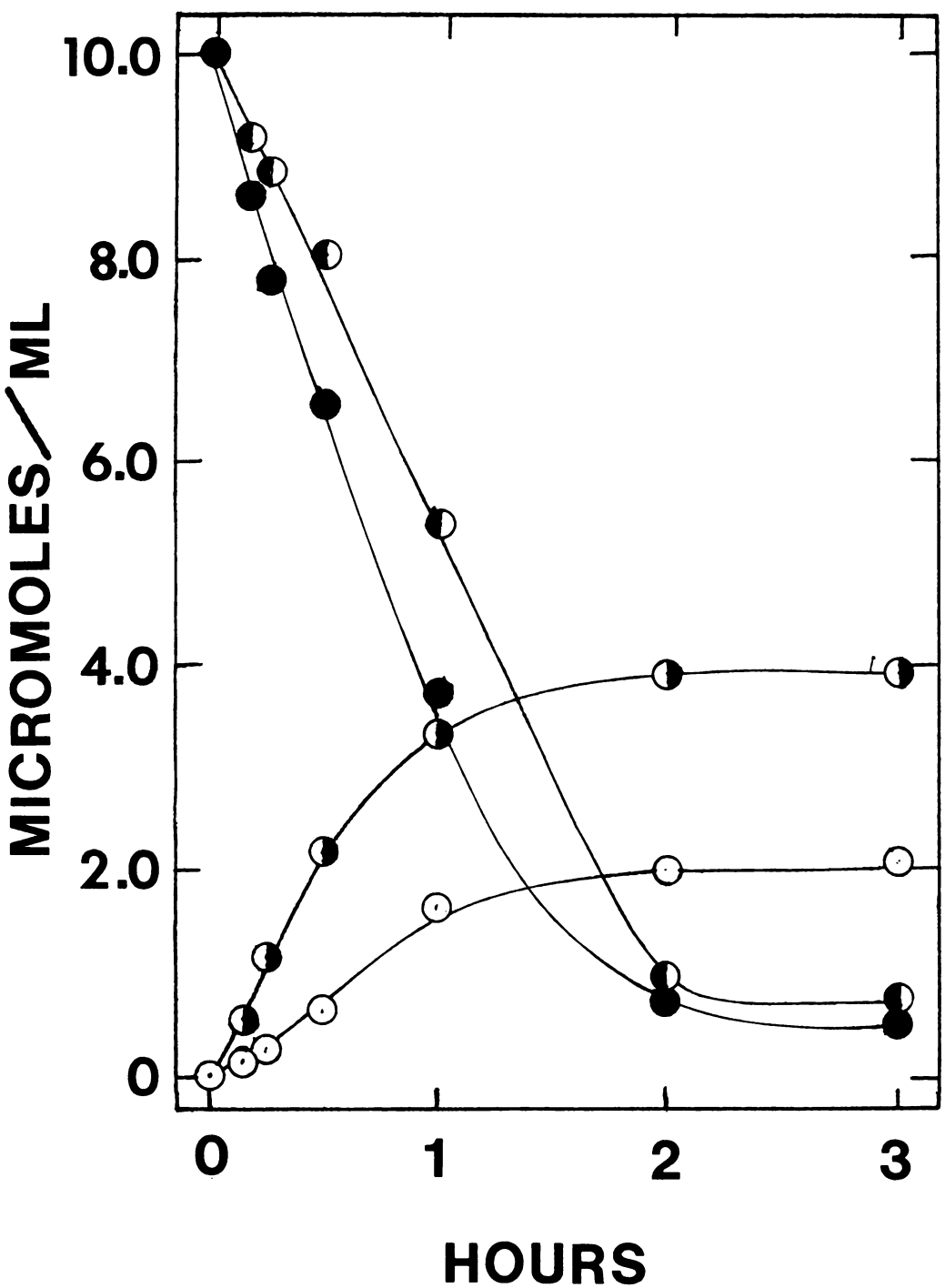

10.0
8.0
6.0
4.0
2.0
0
MICROMOLES / ML
0
1
2
3
HOURS

Figure 3.--Degradation of L-proline by resting cells of Yersinia pseudotuberculosis PB1 (●) and Yersinia pestis EV76 (◐) with accumulation of glutamic acid (◑) and aspartic acid (○) by the latter.

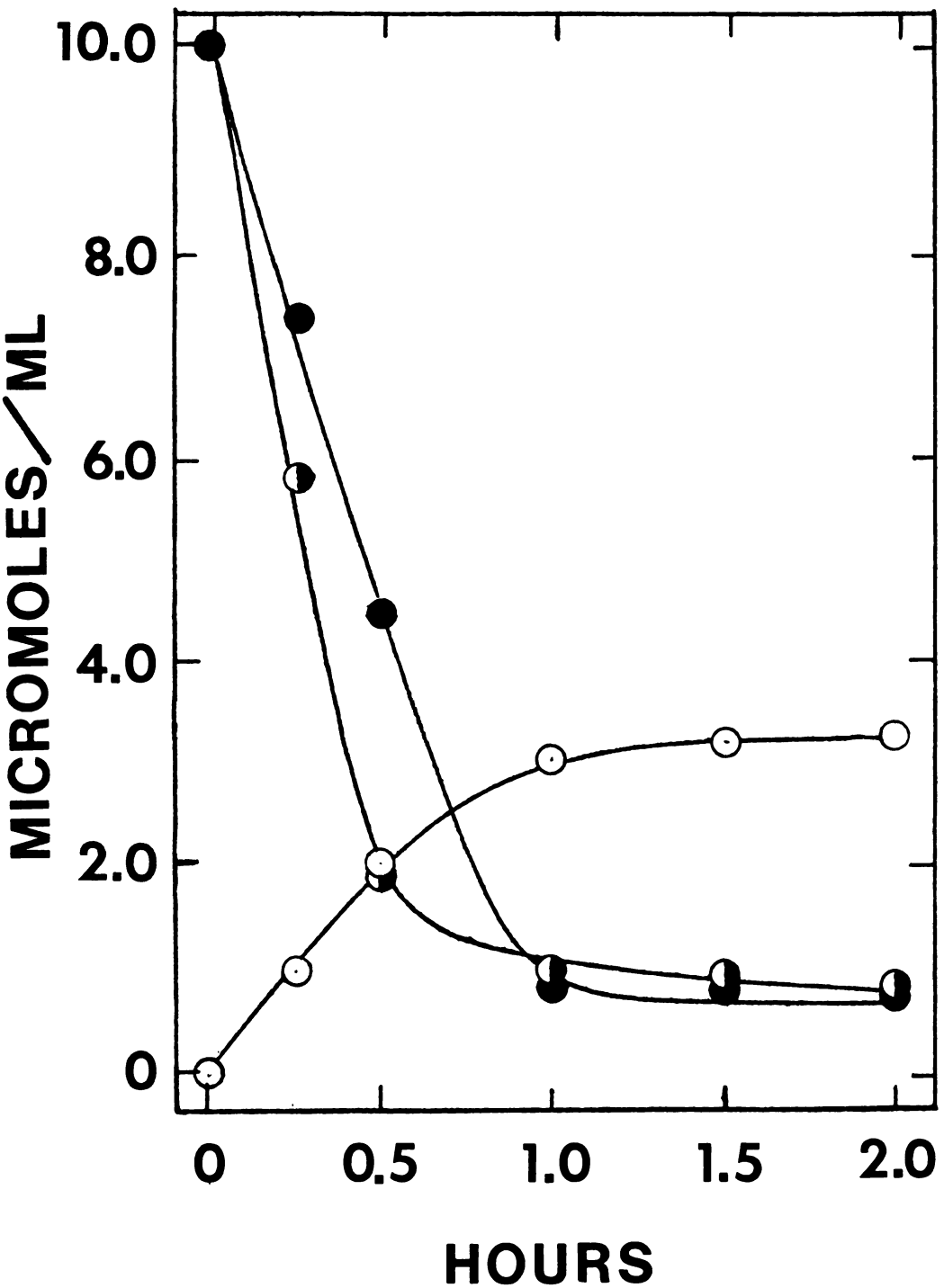

10.0
8.0
6.0
4.0
2.0
0
MICROMOLES/ML
0
0.5
1.0
1.5
2.0
HOURS

Figure 4.--Degradation of L-aspartic acid by resting cells of Yersinia pestis EV76 (◑) and Yersinia pseudotuberculosis PB1 (●) with accumulation by the latter of fumarate (○), malate (◒), succinate (◓), and an unidentified non-volatile product (◐).

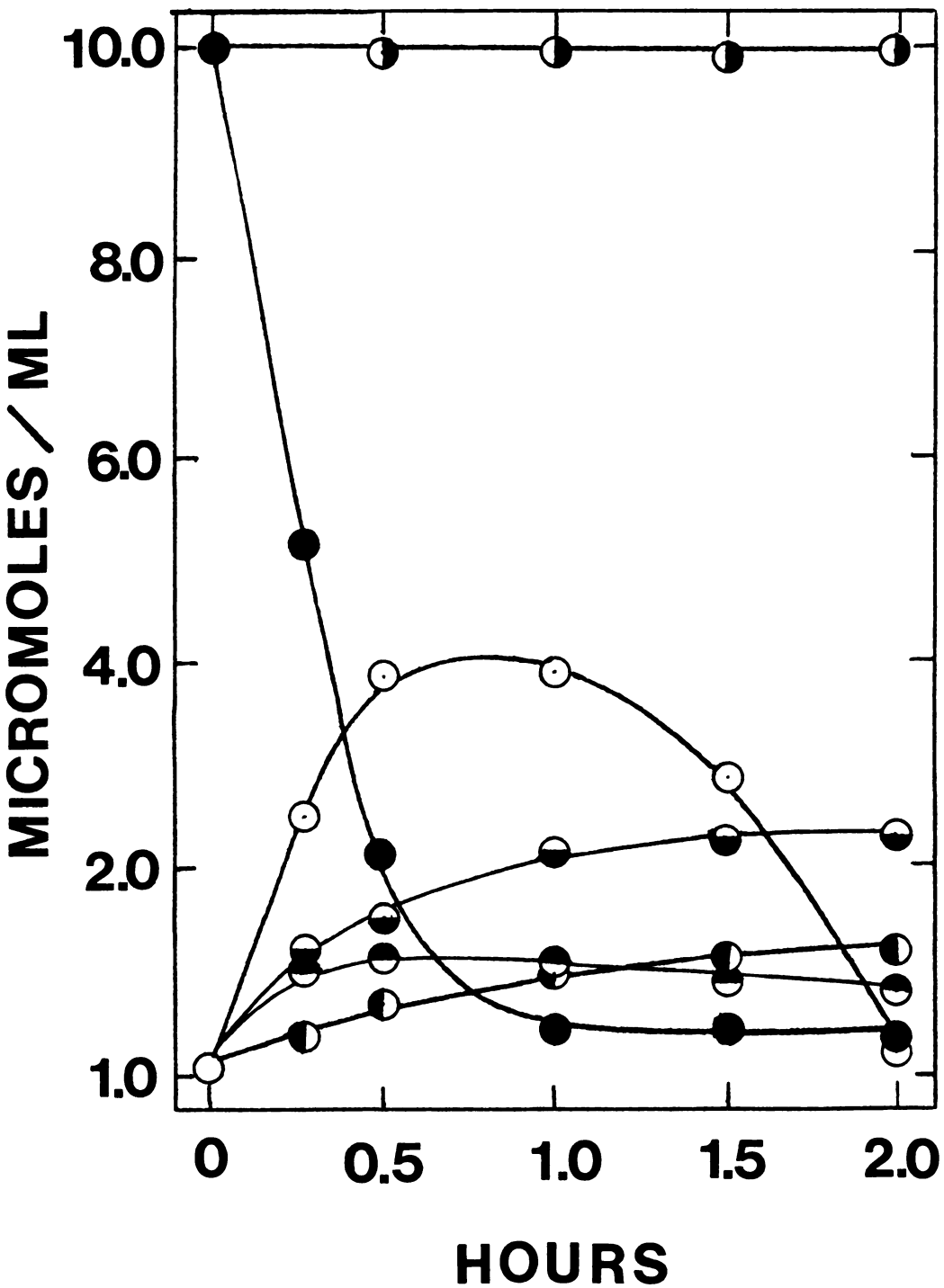
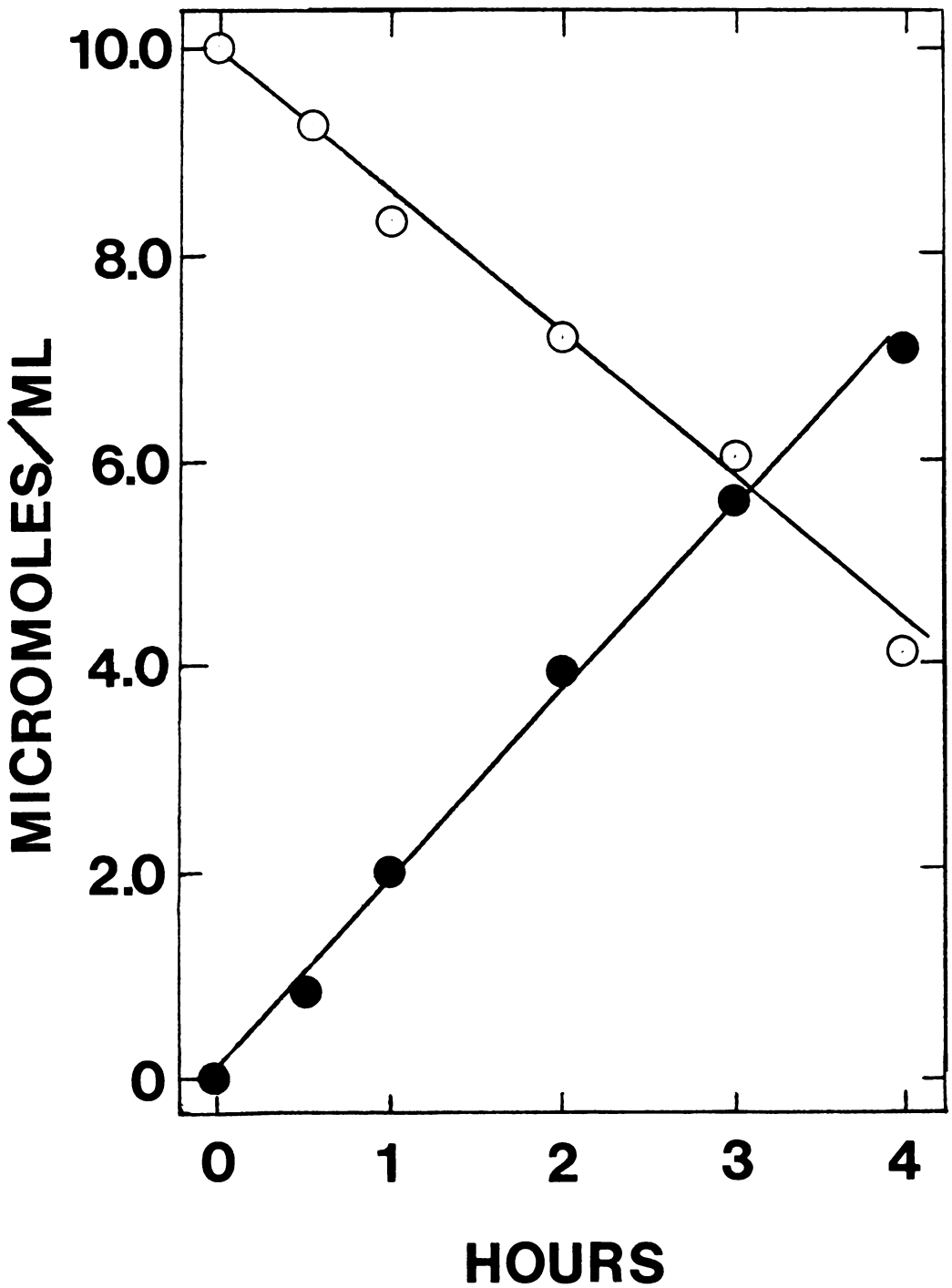

10.0
8.0
6.0
4.0
2.0
1.0
MICROMOLES / ML
0
0.5
1.0
1.5
2.0
HOURS

Figure 5.--Degradation of L-asparagine (○) by resting cells of Yersinia pestis EV76 with accumulation of aspartic acid (●).


10.0
8.0
6.0
4.0
2.0
0
MICROMOLES/ML
0
1
2
3
4
HOURS

LIST OF REFERENCES

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