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ECHNICAL MANUSCRIPT 450

ROWTH AND METABOLISM OF PASTEURELLA TULARENSIS LVS

Jeno M. Scharer
Frederick Klein
Ralph E. Lincoln

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DEPARTMENT OF THE ARMY
Fort Detrick
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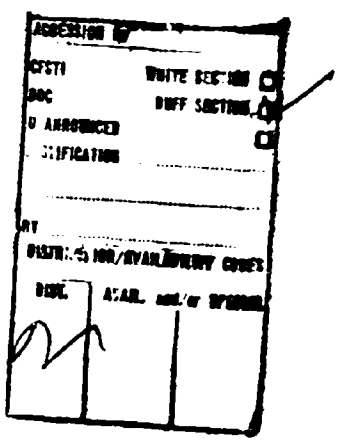

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TECHNICAL MANUSCRIPT 430

GROWTH AND METABOLISM OF *PASTEURELLA TULARENSIS* LVS

Jeno M. Scharer

Frederick Klein

Ralph E. Lincoln

Process Development Division
AGENT DEVELOPMENT AND ENGINEERING LABORATORY

Project 1B533001D426

April 1968

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ABSTRACT

The growth and metabolism of the live vaccine strain of Pasteurella tularensis in different media were investigated. Maximal growth was observed in a medium containing a sulfuric acid digest of casein as amino acid source. Amino acid metabolism produced considerable ammonia, and the rate of ammonia evolution was directly proportional to the growth rate. The most likely route for amino acid breakdown was nonspecific oxidative deamination.

I. INTRODUCTION

Most studies on nutritional requirements of Pasteurella tularensis have been restricted to the virulent strains. Early research on the virulent strain was conducted in peptone-blood medium with cysteine enrichment.[1] In blood-free medium, the organism required a generous supply of other amino acids in addition to cysteine. This requirement was satisfied by the incorporation of hydrolyzates of gelatin[2,3] or casein acid digest[3,4] into the medium.

Systematic study of amino acid requirement started with the development of chemically defined media. The first such medium was reported by Tamura and Fleming,[5] but they did not report any results obtained with their medium. Traub, Mager, and Grossowicz[6] listed 13 amino acids as essential for the propagation of several strains of P. tularensis. Amino acid metabolism during growth caused a rapid accumulation of ammonia; consequently, the pH increased until it became growth limiting. Direct dependence of growth on the total amino acid concentration in the medium was observed by Nagle, Anderson, and Gary.[7] When the amino acid concentration was increased 25 to 30 mg/ml, the cell yield was comparable to that in complex casein hydrolyzate medium.

A comprehensive report on sugar metabolism of 60 strains of P. tularensis was published by Francis.[8] All 60 strains utilized glucose, and 53 strains grew on glycerol. With some strains, Tamura and Gibby[2] obtained equivalent growth in media containing glucose or glycerol. An interaction between glucose and amino acid requirements with respect to growth was shown by Traub et al.[6] The glucose requirement was substantially reduced by increasing the total amino acid content of the medium.

The live vaccine strain (LVS) of P. tularensis is a derivative of a heterogeneous strain obtained from the Soviet Union.[9] Soviet publications on the live vaccine were reviewed extensively by Tiggert.[10] Production media in the USSR were based on hydrolyzates of gelatin, fish, liver, and meat, with glucose as the carbohydrate source.[11] Eigelsbach et al.[12] used a liquid medium containing casein hydrolyzate, cysteine, and thiamine to produce immunogenic live tularemia vaccine. In recent studies, Chamberlain[13] compared the growth supporting potential of a chemically defined medium with that of a complex undefined medium; the organism grew at somewhat lower viable cell populations in the defined medium, but otherwise appeared to retain its identity and to be equal in potency to cultures prepared in the undefined medium. Additions of uracil, adenine, and guanine to the synthetic medium did not enhance growth.

In the present study, the growth of P. tularensis LVS in different media was compared for the purpose of determining the optimal conditions for vaccine production. Particular emphasis was placed on amino acid, carbon, and oxygen requirements for growth.

II. MATERIALS AND METHODS

The morphological and immunological characteristics of P. tularensis LVS were described by Eigelsbach et al.[14-16] The primary cultures were grown in casein acid digest medium and stored in liquid nitrogen.

The basic liquid culture medium had the following composition: 5 g Basamine-Busch yeast/liter, 0.1 g cysteine HCl/liter, 0.01 g thiamine HCl/liter, 5 g NaCl/liter, 2.78 g KH_2PO_4/liter, and 1.16 g K_2HPO_4/liter. Either casein acid digest (CAD) or N-Z-Amine, Type A (NZAA)* was added to the medium in sufficient amounts to give a concentration of 0.85 to 1.05 mg amino nitrogen/ml after sterilization. CAD was prepared by autoclaving 20% casein solution in 1.25 M H_2SO_4 at 121 C for 2 hours. Our CAD and NZAA preparations contained 38 and 63% amino nitrogen, respectively.

Concentrations of 0.5% glucose (GLU) or 0.5% glycerol (GLY) were used as carbon sources. In medium containing glucose, the glucose was sterilized separately and added aseptically to the basal medium after sterilization. No separate sterilization was necessary with glycerol.

By using either GLY or GLU as carbon sources and CAD or NZAA as amino acid sources, four media combinations were obtained: CAD-GLY, CAD-GLU, NZAA-GLY, and NZAA-GLU.

Growth studies in these media were conducted in 8-liter New Brunswick fermentors** with a 6-liter liquid working volume. Agitation was maintained at 300 rpm with a single, flat-blade impeller, and aeration was at the rate of 6 liters of air per minute. Each fermentor was equipped with sterilizable pH electrodes*** and polarographic oxygen sensors.**** The oxygen probe was sterilized chemically and inserted aseptically into the fermentor. The initial pH of the medium was adjusted with 10 N NaOH; pH was controlled by automatic addition of 1 N HCL during the fermentation. Temperature was maintained at 37 C.

---

* Sheffield Chemical Co., Norwich, New York.
** New Brunswick Scientific Co., New Brunswick, N.J.
*** Leeds & Northrop, Inc., Fullerton, Calif.
**** Beckman Instruments, Inc., Fullerton, Calif.

Direct measurements of oxygen utilization rates were determined by the method of Hospodka.[17] After 6 hours of initial incubation, the flow of air to the fermentor was periodically interrupted for 2 to 3 minutes and the rate of depletion of oxygen from the liquid was recorded. From the slope of the curve, the oxygen utilization rate was determined.

The fresh inoculum consisted of 300 ml of an 18-hour culture grown in 1.5-liter Fernbach flasks. Care was taken to use an inoculum grown in the same medium as used in the fermentor run.

In some experiments, the mixed amino acid source was replaced with individual amino acids in concentrations of 2.5 g/liter. These runs were conducted in 250-ml Erlenmeyer flasks containing 20 ml of medium. The inoculum was 1 ml of a 16-hour culture. Flasks were incubated for 18 hours at 37 C on a 100-cpm reciprocal shaker with 2-inch strokes. The initial pH was adjusted to 6.2 with 10 N NaOH and was uncontrolled during the incubation period.

The viable count was determined by plating on glucose cysteine - blood agar.[12] Colonies were counted after 72 hours' incubation at 37 C. Optical density (OD) was on a Bausch & Lomb Spectronic 20 spectrophotometer* at 620 mμ. Amino nitrogen and ammonia nitrogen analyses were performed with a Technicon** autoanalyzer. The glucose and glycerol contents were determined by the methods of Somogyi[18] and of Bailey,[19] respectively.

III. RESULTS

A. EFFECT OF CARBON AND AMINO ACID SOURCE ON GROWTH

Growth studies were conducted in the New Brunswick fermentors at the controlled pH of 6.6. Exploratory experiments on the effect of pH had indicated that this pH level was the optimum for the propagation of P. tularensis LVS. Above this pH, the growth rate of the organism rapidly decreased.

Typical growth curve and ammonia formation in CAD-GLY medium are shown in Figure 1. During growth, ammonia formation was considerable and the rate of production was directly proportional to the growth rate of the organism. Although not shown, the amino nitrogen utilization curve exactly paralleled the ammonia curve. Of the total available amino nitrogen, 27% was utilized during the growth cycle.

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* Bausch & Lomb, Rochester, N.Y.
** Technicon Instruments, Ardsley, N.Y.

With an aeration rate of 1 volume air per volume medium per minute, the dissolved oxygen concentration fell to a minimum of 63% saturation level after 9 hours' incubation (Fig. 2). The minimum level of dissolved oxygen corresponded to the end of the logarithmic growth phase. The oxygen utilization rate was 2.8 mg moles per liter-hour at this time. After completion of growth, air utilization rapidly decreased. Oxygen utilization, as well as amino acid utilization, was directly proportional to the growth rate of the organism.

The hourly interruption of air for the determination of oxygen utilization rates had no significant effect on the growth rate or final cell yield. Consequently, this was a rapid and simple method for indicating the metabolic state of the culture.

The same growth parameters are shown for NZAA-GLU media in Figures 3 and 4. The organism displayed similar growth and metabolic patterns. However, the cell yield, growth rate, ammonia formation, and oxygen utilization were considerably lower than in the CAD-GLY system. Only 15% of the available amino nitrogen was utilized during the fermentation.

The fermentation characteristics of P. tularensis LVS in the four types of medium are summarized in Table 1. Glucose was interchanged with glycerol without significant effect on the growth rate or cell yield. However, CAD proved to be a significantly better amino acid source than NZAA. In CAD medium, the final cell concentration was consistently above 1.2×10^{10} organisms/ml; organisms grew faster, and the total fermentation time was 2 to 3 hours shorter than in NZAA medium. The total amino acid utilized was dependent on the cell yield. Higher terminal cell numbers were associated with more pronounced amino acid metabolism during the fermentation. The ratio of amino nitrogen utilized to ammonia formed was almost 1:1, indicating that the major pathway of amino acid utilization was deamination.

B. REPLACEMENT OF MIXED AMINO ACID SOURCE WITH SINGLE AMINO ACIDS

In order to determine the exact function of CAD or NZAA in the medium, the mixed amino acid sources were replaced by single amino acids. Thirteen amino acids were tested (Fig. 5). Amino acid utilization was estimated from amino nitrogen and ammonia nitrogen balances before and after the 18-hour incubation period; the extent of growth was measured by the usual optical density method.

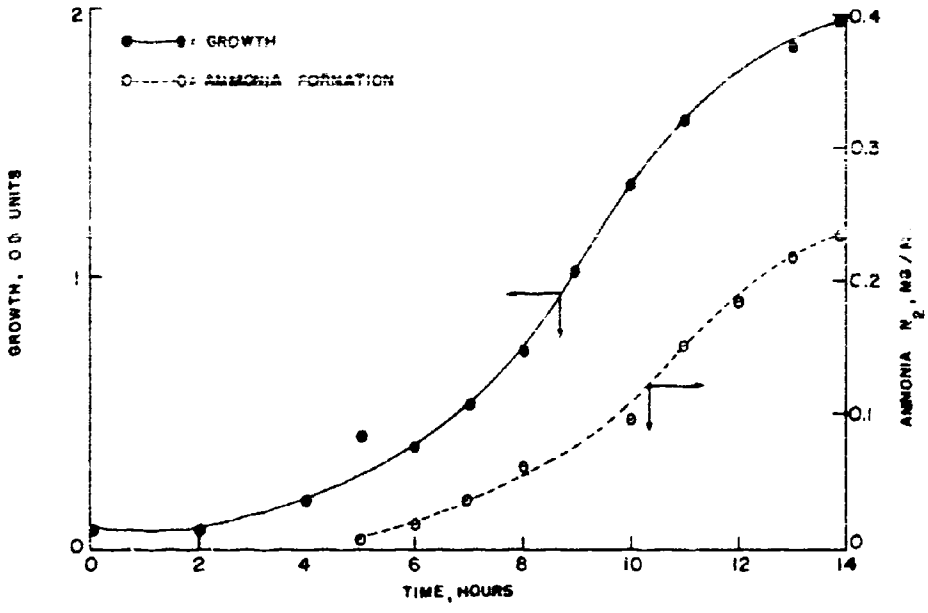

Figure 1. Growth of Pasteurella tularensis LVS and Ammonia Formation in CAD-GLY Medium, pH 6.6.

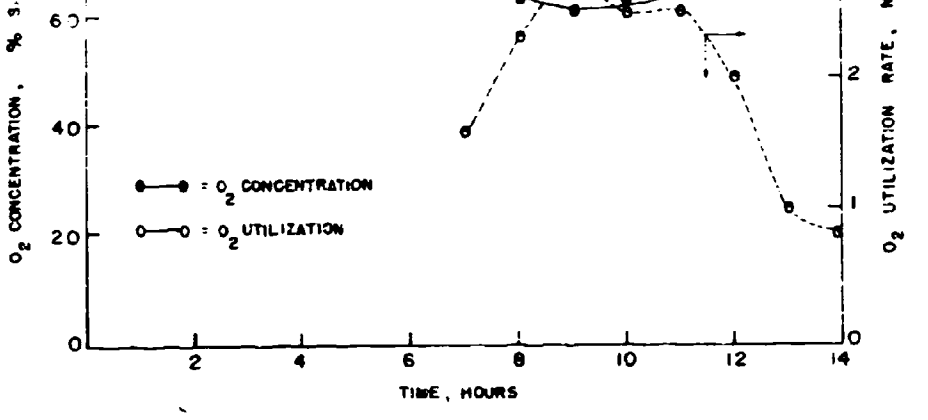

Figure 2. O_2 Concentration and O_2 Utilization vs. Time in CAD-GLY Medium, pH 6.6.

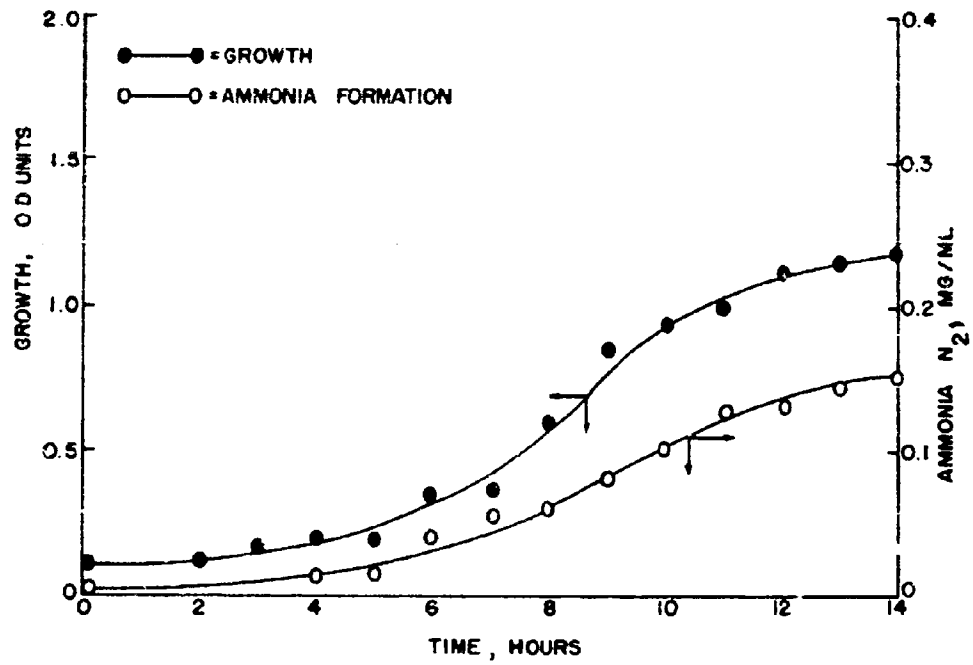

Figure 3. Growth of Pasteurella tularensis LVS and Ammonia Formation in NZAA-GLU Medium, pH 6.6.

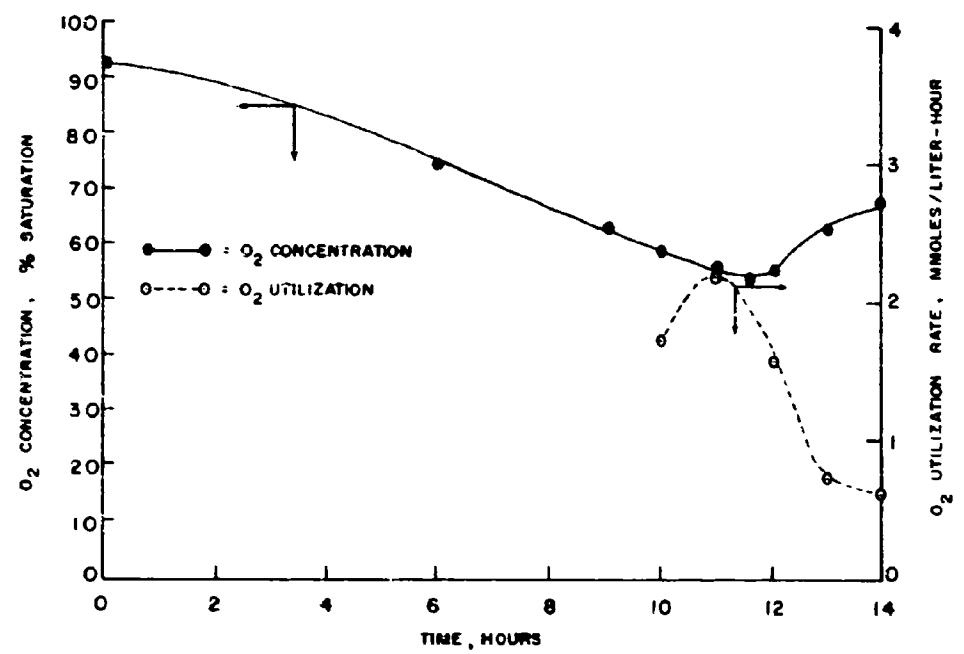

Figure 4. O_2 Concentration and O_2 Utilization vs. Time in NZAA-GLU Medium, pH 6.6.

TABLE 1. FERMENTATION CHARACTERISTICS OF *PASTEURELLA TULARENSIS* LVS AT CONTROLLED pH 6.6

Amino Acid Source	Carbon Source	No. of Runs	Viable Count, 10^9/ml	Specific Growth Rate, OD Units, hours^{-1}	Ammonia N_2 Formed, mg/ml	Amino N_2 Utilized, mg/ml	Glycerol or Glucose Utilized, mg/ml	Max O_2 Utilized, mmoles per liter-hour
CAD	GLY	4	14.4	0.382[a]	0.234[a]	0.240[a]	1.3[a]	2.70[a]
NZAA	GLY	4	11.0	0.267	0.209	0.213	1.1	2.57
CAD	GLU	2	16.0	0.360	ND[b]	ND	2.48	ND
NZAA	GLU	1	8.16	0.268	0.156	0.150	1.00	1.34

a. Significant differences in growth rates and metabolic activities.
b. No data.

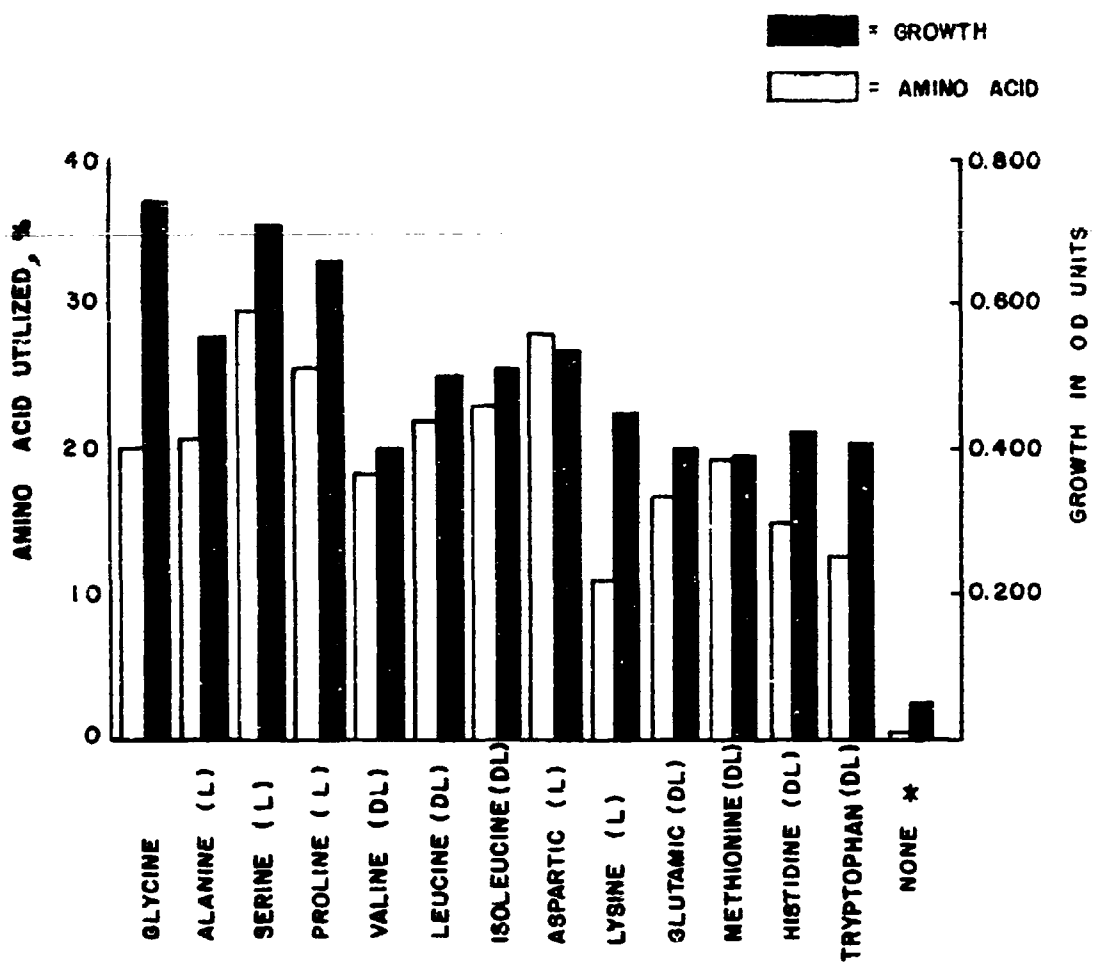

Figure 5. Per Cent Amino Acid Utilization by Pasteurella tularensis LVS and Growth in Optical Density Units. *Trace amounts of amino acids are present in the yeast supplement.

All the amino acids tested were utilized; after the incubation period, decreases in amino nitrogen content and equivalent ammonia formation were detected. On a percentage basis, serine utilization was the highest (29%) and lysine the lowest (12%). Generally, growth was proportional to amino acid utilization. One notable exception was lysine, which, although not utilized to any great degree, produced good growth. Maximal growth was observed in glycine medium. The optical density of 0.740 units was somewhat lower than that obtained in the NZAA-GLU medium. In the absence of amino acid supplement, however, very little of the amino acid introduced by the yeast supplement was utilized, and virtually no increase in cell population occurred. Again, higher terminal cell numbers were associated with more pronounced amino acid metabolism during the fermentation.

C. MECHANISM OF DEAMINATION

During growth experiments, ammonia formation was proportional to the oxygen demand; consequently, oxidative deamination appeared to be a logical choice for the pathway of amino acid metabolism.

The overall equation for oxidative deamination is:

$$\begin{array}{l} NH_2 \\ | \\ R\text{-}C\text{-}COOH \\ | \\ H \end{array} + \tfrac{1}{2}O_2 = \begin{array}{l} \\ \\ R\text{-}C\text{-}COOH \\ \| \\ O \end{array} + NH_3 \qquad (1)$$

Thus, for each mole of NH_3 formed, ½ mole of O_2 is utilized, i.e.:

$$\frac{P(O_2)}{P(NH_3)} = 0.5 \qquad (2)$$

Equation (2) can be rewritten in differential (rate) form:

$$\frac{dP(O_2)}{dt} = 0.5\,\frac{dP(NH_3)}{dt} \qquad (3)$$

During growth, oxygen may be utilized by pathways other than oxidative deamination. One may assume that the additional oxygen demand is proportional to the cell mass. Thus, a better approximation of the oxygen balance, Equation (3), is:

$$\frac{dP(O_2)}{dt} = 0.5\,\frac{dP(NH_3)}{dt} + RX \qquad (4)$$

where B = proportionality constant, and X = cell mass in OD units. Rearranging Equation (4), one obtains:

$$\left(\frac{1}{X}\frac{dP(O_2)}{dt}\right) = 0.5\left(\frac{1}{X}\frac{dP(NH_3)}{dt}\right) + B \qquad (5)$$

In Figure 6, rate of oxygen utilization per unit cell mass (OD units) is plotted against rate of ammonia production per unit cell mass. The data are the pooled values of five experimental runs in CAD-GLY and NZAA-GLY media. The rate of oxygen utilization was obtained by the Hospodka[17] method, and the rate of ammonia production by graphical differentiation of the total ammonia curve. By regression analysis, the slope of the curve was found to be 0.61. This value was not significantly different from the theoretical value of 0.5 at the 95% significance level. The line did not intersect the Y axis at the origin; therefore, not all the oxygen was associated with the deamination reaction.

IV. DISCUSSION

The amino acid utilization of P. tularensis LVS is similar to that of the virulent strains, inasmuch as deamination causes pronounced ammonification of the medium during growth.[6] If the pH is uncontrolled, the culture fluid turns alkaline, and the growth rate of the organism decreases. The most likely route of amino acid metabolism is oxidative deamination. Because oxidative deamination does not change the basic organic structure, the amino acids and their keto acid analogs may serve as energy sources as well as carbon skeletons for the organisms. Whether amino acids can completely replace glucose as a carbon source is not certain.

The primary function of both CAD and NZAA is to supply free amino acids in the medium, as it is unlikely that either contain any additional growth factors. Analysis of NZAA showed 40% protein nitrogen present; therefore the significantly lower cell yield in NZAA medium may have been caused by the presence of some undigested protein that inhibited growth. After sulfuric acid hydrolysis, casein was almost completely decomposed to amino acids and ammonium salts with less than 5% protein nitrogen. The mixed amino acid source can be replaced, to a great extent, by single amino acids.

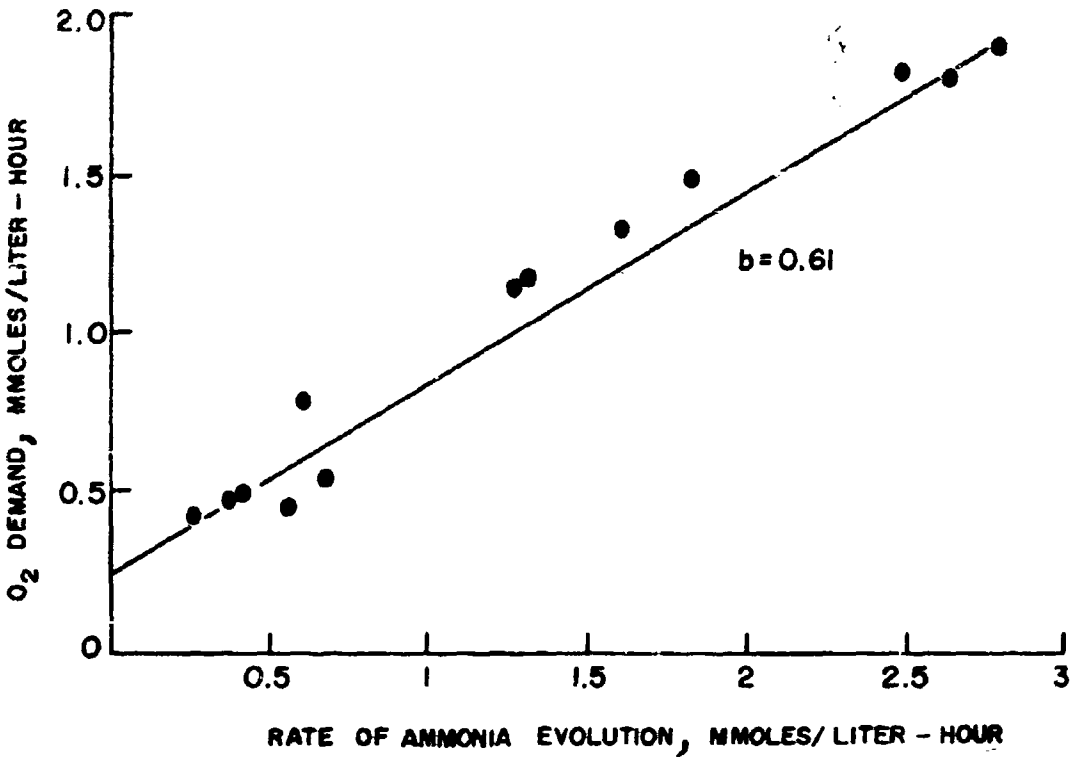

Figure 6. Oxygen Demand vs. Rate of Ammonia Evolution per Unit Cell Mass (OD Units).

It is somewhat difficult to assess the roles of individual amino acids in the medium. Unlike Chamberlain,[13] we were unable to grow the live vaccine strain in purely synthetic medium. The Basamine-Busch yeast, an essential medium component, contains some free amino acids.

Oxidative deamination of serine, proline, and aspartic acid is greater than that of the other amino acids. All three have been reported to be essential for the propagation of virulent strains. The live vaccine strain appears to have very little or no proteolytic activity. In the absence of a significant amount of free amino acids, very limited ammonia formation and growth occur.

Although both glucose and glycerol are utilized by the live vaccine strain, the amounts required for growth are considerably less than for virulent strains. Comparable initial concentrations of glucose have been reported to be completely exhausted during the growth of the virulent SCHU strain.[7]

The organism required vigorous aeration for growth. The oxygen demand is maximum at the end of the logarithmic growth phase and rapidly declines in final stationary cultures. The major part of oxygen consumption is attributable to deamination activity. The oxygen uptake rate of the organism is a function of its growth rate, being highest in the most actively growing system. Working with virulent strains, Traub et al.[8] observed oxygen toxicity for P. tularensis at low population levels. We have observed no ill effect of relatively high oxygen concentrations on the live vaccine strain. Indeed, growth started in a logarithmic manner soon after inoculation at oxygen saturations of near 100%. On the other hand, under microaerophilic conditions, such as inadequately stirred shake flasks, growth tended to be scanty or, in some cases, absent. For some reason, this was especially true in shake flasks containing glycerol as a carbon source.

Nutritionally, the live vaccine strain is perhaps even more fastidious than the highly virulent strains. The concentrations of vitamins, amino acids, and oxygen and the temperature and pH must be carefully controlled. Unless adequate control measures are taken during medium preparation and fermentation, cell yield will fall far below the maximal or average level.

The immunogenicity of live vaccines produced in four different types of production media discussed in this paper is presently being investigated. Early results indicate that each medium produces immunologically active vaccine. Colonies produced on peptone-cysteine agar by cultures from each of the four vaccines are all of the immunogenic, smooth type.

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13. ABSTRACT

The growth and metabolism of the live vaccine strain of Pasteurella tularensis in different media were investigated. Maximal growth was observed in a medium containing a sulfuric acid digest of casein as amino acid source. Amino acid metabolism produced considerable ammonia, and the rate of ammonia evolution was directly proportional to the growth rate. The most likely route for amino acid breakdown was nonspecific oxidative deamination.

14. Key Words

*Pasteurella tularensis LVS
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Ammonia
Growth
*Metabolism
Vaccines

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