REQUIREMENT OF AN ALKALINE pH AND AMMONIA FOR SUBSTRATE OXIDATION BY *BACILLUS PASTEURII*

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ABSTRACT

WILEY, W. R. (Washington State University, Pullman) AND J. L. STOKES. Requirement of an alkaline pH and ammonia for substrate oxidation by *Bacillus pasteurii*. J. Bacteriol. **84**:730–734. 1962—Resting-cell suspensions of *Bacillus pasteurii* require a pH higher than 8.0 and also NH_4^+ for the oxidation of glycine, alanine, serine, glutamic acid, other amino acids, and compounds of the Krebs cycle. The optimal pH is 9.2. Maximal activity was obtained with 0.5% $(NH_4)_2SO_4$, the highest concentration tested. NH_4^+ can be supplied by a number of inorganic and organic ammonium salts. The effect of NH_4^+ is fairly specific. It cannot be replaced by Na^+, Cs^+, and Mg^{++}, and only partially replaced by Li^+, K^+, Rb^+, and Ca^{++}. Some possible metabolic functions of the required alkaline pH and NH_4^+ are discussed.

Bacillus pasteurii requires unusual conditions for growth. It develops only in alkaline media (pH 8 to 9 or higher) and in the presence of relatively high concentrations of ammonium salts (0.5 to 1.0%). Urea can substitute for ammonium salts because the organism is highly ureolytic and thus can produce its own ammonia from the decomposition of urea. These properties of *B. pasteurii* were established by the early investigations of Gibson (1934*b*) and confirmed more recently by Bornside and Kallio (1956*a*).

The present investigations were undertaken to determine the physiological basis for these unusual growth requirements. It has been found that an alkaline pH and ammonia are essential for the oxidation of a variety of organic compounds by cell suspensions of *B. pasteurii*.

MATERIALS AND METHODS

A pure culture of *B. pasteurii* was isolated from pasteurized soil by means of enrichment cultures in a 1% yeast extract-5% urea medium at pH 9.0 (Gibson, 1934*a*; Bornside and Kallio, 1956*a*). The culture was grown and maintained on a medium composed of 2% yeast extract, 1% $(NH_4)_2SO_4$, 0.13 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 9.0), and distilled water. The final pH of this medium, designated medium A, was 8.7. Each ingredient was sterilized separately in the autoclave. If all ingredients are sterilized together, the medium will not support growth. To obtain a solid medium, 1.5% agar was added.

Conventional manometric techniques were used to measure the rate and extent of oxidation of various substrates. Cell suspension (2 ml) and usually 4 to 10 μM of substrate dissolved in 0.1 to 0.25 ml of water were placed in each vessel. The gas phase was air and the bath temperature 30 C. KOH (10%) was present in the center well to absorb CO_2.

Cell suspensions were prepared in the following standardized manner to obtain consistently active cells. The growth from a 24-hr slant culture was removed with 5 ml of tris buffer (pH 9.0). This cell suspension (1 ml) was used to inoculate 500 ml of medium A contained in a 2-liter Erlenmeyer flask. The cultures were then grown for 12 to 15 hr at 30 C on a rotary shaker with vigorous agitation. The cells were harvested at 4 C in a refrigerated centrifuge and washed twice in the centrifuge with tris buffer containing 0.005 M $MgSO_4$. The washed cells were suspended in sufficient tris or phosphate buffer, with or without $(NH_4)_2SO_4$, to give a turbidity of 500 to 600 units in the Klett-Summerson photometer (red filter). Such suspensions contained 1.5 or 2.5 mg of cells (dry wt) per ml.

RESULTS

The aerobic sporeforming bacillus which was isolated from the urea enrichment culture had the characteristic properties of *B. pasteurii*. It

failed to grow in medium A below pH 8.0. To obtain these lower pH levels, tris buffer was replaced by phosphate buffer. Slight growth occurred at pH 8.0 and increased progressively as the pH was raised to the optimal level of 9.2. In the range of pH 9.2 to 10.0, growth decreased slightly.

The strain required also $(NH_4)_2SO_4$ for growth, since it failed to grow in medium A at pH 8.7 when $(NH_4)_2SO_4$ was omitted. There was slight growth with 0.1% $(NH_4)_2SO_4$ and maximal growth with 0.5% $(NH_4)_2SO_4$.

According to Knight and Proom (1950) and Bornside and Kallio (1956*b*), *B. pasteurii* requires thiamine and amino acids for growth, and some strains also need biotin and nicotinic acid. Similarly, our strain requires thiamine, biotin, and a large number of amino acids for growth. In addition, its inability to metabolize carbohydrates conforms with the description of *B. pasteurii* by Smith, Gordon, and Clark (1946).

Oxidation of substrates. Initial attempts to obtain appreciable oxidation of amino acids with cell suspensions of *B. pasteurii* in pH 7.0 or 9.0 buffer were uniformly unsuccessful. However, when $(NH_4)_2SO_4$ was added to the suspensions at pH 9.0 an immediate and relatively rapid oxidation of the amino acids was obtained. Representative data are given in Table 1. Appreciable oxidation of glutamic acid, isoleucine, and threonine occurred only at pH 9.0 with $(NH_4)_2SO_4$, and there was little or no oxidation at pH 9.0 without $(NH_4)_2SO_4$, or at pH 7.0 with or without $(NH_4)_2SO_4$. Thus, the conditions necessary for growth of *B. pasteurii* are essential also for the oxidative metabolism of the organism.

The kinetics of the oxidation of glutamic acid are shown in Fig. 1. There is an almost immediate and rapid oxidation of glutamic acid, which proceeds until 63% of the acid has been oxidized; the remainder is presumably assimilated.

The results of experiments with additional amino acids, including some D-amino acids, and with members of the tricarboxylic acid cycle are presented in Table 2. Most of the L-amino acids were oxidized at a low rate in the absence of $(NH_4)_2SO_4$, and the rate was greatly increased by $(NH_4)_2SO_4$. Of the D-isomers of glutamic acid, threonine, and isoleucine, only threonine was metabolized, and its oxidation also was stimulated by $(NH_4)_2SO_4$. This salt also markedly increased the rate of oxidation of pyruvic, fumaric, and oxalacetic acids. At pH 7.0, there was little or no oxidation of any of the compounds, with or without $(NH_4)_2SO_4$.

A variety of carbohydrates, including erythritol, xylose, arabinose, glucose, fructose, galactose, lactose, sucrose, trehalose, mellibiose, and inositol, and also the fatty acids, butyric, β-hydroxybutyric, and valeric, were not attacked by *B. pasteurii* under any of the above conditions. Also, Smith et al. (1946) noted that *B. pasteurii* cannot ferment carbohydrates. Other members of the Krebs cycle, namely, acetate, citrate, α-ketoglutarate, and malate, were not oxidized or only slightly oxidized. Glycerol, lactic acid, glutamine, and asparagine were not oxidized. Apparently, the organism has a sharply limited oxidative capacity.

Effect of pH. The oxidation of glutamic acid in the range of pH 7 to 10 was investigated. For pH 7.0, 7.5, and 8.0, 0.15 M phosphate buffer was used and for pH 8.5, 9.0, 9.2, 9.5, and 10.0, 0.15 M tris buffer. These are the initial pH values of the mixtures in the vessels. Each vessel contained 4 μmoles of glutamic acid and 0.2%

TABLE 1. *Effect of pH and $(NH_4)_2SO_4$ on substrate oxidation by Bacillus pasteurii*

Substrate*	Q_{O_2}†			
	pH 9.0		pH 7.0	
	Without $(NH_4)_2SO_4$	With 0.2% $(NH_4)_2SO_4$	Without $(NH_4)_2SO_4$	With 0.2% $(NH_4)_2SO_4$
L-Glutamic acid	5	55	4	0
L-Isoleucine	0	30	0	3
L-Threonine	0	24	0	0

* Each vessel contained 4 μmoles.

† Endogenous oxidation subtracted.

$(NH_4)_2SO_4$ (0.015 M) in addition to the cell suspension.

The results are plotted in Fig. 2. The lack of oxidation of glutamate below pH 8.5 is not due

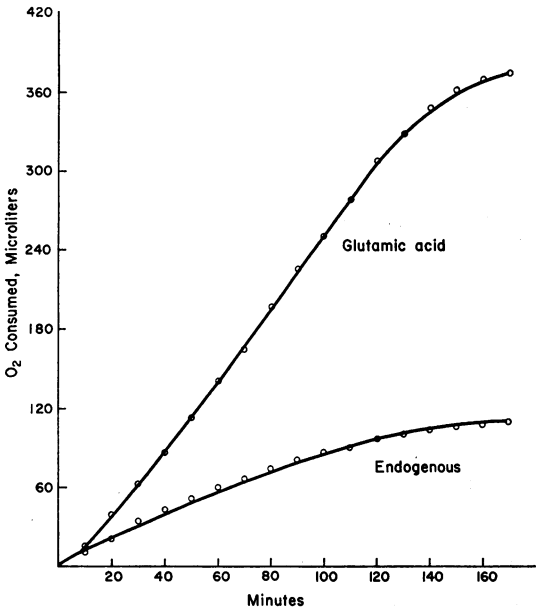

FIG. 1. *Oxidation of 4 μmoles of glutamic acid by Bacillus pasteurii at pH 9.0 with 0.2% $(NH_4)_2SO_4$.*

TABLE 2. *Influence of $(NH_4)_2SO_4$ on the oxidation of D and L amino acids and tricarboxylic acid cycle compounds by Bacillus pasteurii at pH 9.0*

Substrate*	Q_{O_2}†	
	Without $(NH_4)_2SO_4$	With 0.2% $(NH_4)_2SO_4$
Glycine	11	62
L-Alanine	24	67
L-Serine	18	85
L-Cysteine	6	17
L-Aspartic acid	0	24
L-Proline	10	31
D-Glutamic acid	0	0
D-Threonine	10	23
D-Isoleucine	0	0
Pyruvic acid	11	30
Fumaric acid	3	23
Oxalacetic acid	12	47

* Each vessel contained 4 μmoles.
† Endogenous oxidation subtracted.

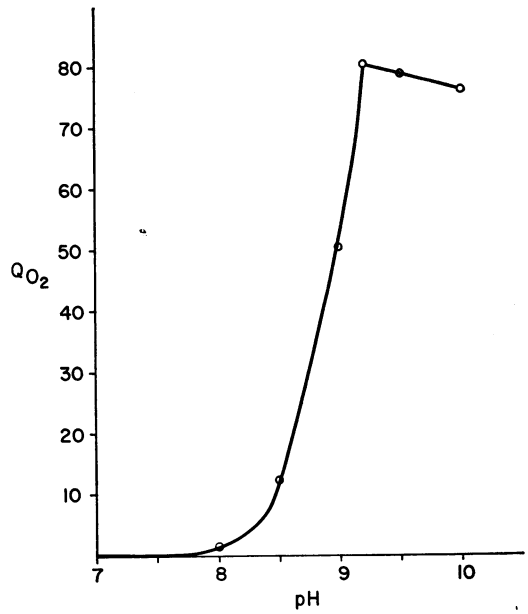

FIG. 2. *Effect of pH on the oxidation of glutamic acid.*

to toxicity of the phosphate buffer, since oxidation also did not take place with tris buffer at pH 8.0. Oxidation was maximal at pH 9.2 and decreased only slightly at pH 9.5 and 10.0. The effect of pH on oxidation is very similar, therefore, to its effect on growth. The optimal pH range for both oxidation and growth is optimal also for the formation of free NH_3 from $(NH_4)_2SO_4$.

In the absence of $(NH_4)_2SO_4$, there was no oxidation of glutamic acid at any of the pH levels.

Activity and specificity of NH_4^+. As previously indicated, growth increases progressively as the $(NH_4)_2SO_4$ concentration is raised from 0.1 to 0.5%. Similarly, the rate and extent of oxidation of glutamic acid increases with increase in $(NH_4)_2SO_4$ in the same range of concentration (Fig. 3). Maximal oxidation occurred with 0.5% $(NH_4)_2SO_4$; higher concentrations were not tested. Endogenous oxidation also was stimulated by the ammonium salt but to a lesser extent than that of glutamic acid.

The need for NH_4^+ or, rather, free NH_3 for oxidation is fairly specific. As shown in Table 3, various inorganic and organic ammonium salts promote oxidation, although some are more active than others. Thus, ammonium molybdate was

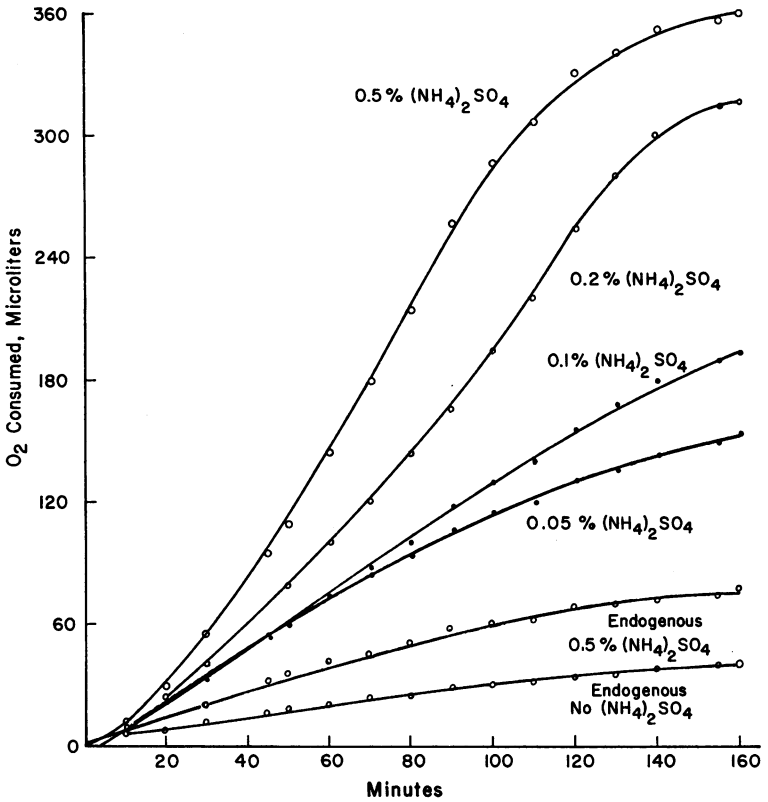

FIG. 3. *Effect of various concentrations of $(NH_4)_2SO_4$ on the oxidation of 4 μmoles of glutamic acid by Bacillus pasteurii at pH 9.0.*

TABLE 3. *Specificity of the NH_4^+ requirement for the oxidation of glutamic acid*

Compound*	Relative activity†	Compound*	Relative activity†
$(NH_4)_6Mo_7O_{24}$	160	LiCl	38
$(NH_4)_2$ tartrate	105	KCl	30
$(NH_4)_2SO_4$	100	K_2SO_4	18
NH_4Cl	100	RbCl	15
$(NH_4)_2HPO_4$	94	CsCl	0
NH_4NO_3	61	NaCl	0
$(NH_4)_2CO_3$	55	Na_2MoO_4	0
$(NH_4)_2$ citrate	30	Na_2 tartrate	0
		$CaCl_2$	44
		$MgSO_4$	0

* Tested at 0.015 M concentration.

† Based on 100 for the rate of oxidation with $(NH_4)_2SO_4$. Glutamic acid (4 μmoles) and tris buffer (pH 9.0) were used. Endogenous oxidation was subtracted.

considerably more active, and ammonium citrate less active, than $(NH_4)_2SO_4$. Although some activity was exhibited by Li^+, K^+, Rb^+, and Ca^{++}, the effect persisted only for a relatively short time. The oxidation of glutamate ceased after only 12 to 36% of the theoretical amount of O_2 necessary for complete oxidation was consumed, compared to 60 to 70% with NH_4^+. However, Na^+, Cs^+, and Mg^{++} were completely inactive.

DISCUSSION

Our data indicate that an alkaline pH and ammonium salts are essential for the oxidative metabolism of *B. pasteurii*. The specific roles of these two factors, however, remain to be elucidated. It may be that the cells are impermeable to the positively charged ammonium ion but permeable to free NH_3. The principal function of the alkaline pH, therefore, may be to form

NH_3 from NH_4^+. Once inside the cell, the NH_3 may be reconverted to NH_4^+, which may function there as an essential cofactor for various enzyme systems.

It is well known that NH_4^+, Na^+, K^+, Ca^{++}, Mg^{++}, and other mono- and divalent cations are essential for the activity of many enzyme systems. Tomlinson and MacLeod (1957) reported that a marine bacterium which requires Na^+, K^+, and Mg^{++} for growth also requires both Na^+ and K^+ for the oxidation of alanine and succinate. Similarly, Payne (1960) has shown that a marine pseudomonad which requires Na^+ or K^+ for growth also requires either of these cations for optimal rate of induction of glucuronate-oxidizing enzymes. Barker et al. (1959) demonstrated a relatively nonspecific monovalent cation requirement for β-methylaspartase activity of purified extracts of *Clostridium tetanomorphum*. K^+ and NH_4^+ were most active. Campbell et al. (1961) reported that exogenous NH_4^+ stimulated growth of *Micrococcus sodonensis* in media containing amino acids, and that, in the absence of NH_4^+, metabolism was altered and large quantities of α-ketoglutarate accumulated in the cultures.

Further experiments are underway to determine the specific metabolic functions of high pH and NH_4^+ in *B. pasteurii*.

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