THE REDISTRIBUTION OF NITROGEN IN SILAGE BY LACTIC-ACID-PRODUCING BACTERIA

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Summary

Consideration is given to the part played by the lactic-acid-producing bacteria in nitrogen redistribution during ensilage. In suitable media, silage strains of *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Pediococcus* sp. were shown to have a net uptake of ammonia during growth, but this uptake was considered to have small effect on the net production of ammonia during ensilage. Cell suspensions of *Lb. plantarum* produced ammonia from serine, and those of *Lb. brevis* and *Pediococcus* sp. ammonia from arginine. These reactions were studied particularly as regards the influence of pH and the physiological age of cells, and their likely contribution to ammonia production during ensilage is discussed. During growth of the lactic acid bacteria in media of defined amino acid composition, ornithine derived from arginine was the only ninhydrin-positive substance produced in significant amount.

I. Introduction

Most of the redistribution of nitrogen occurring during ensilage results from the activity of plant enzymes (Mabbitt 1951; Macpherson and Slater 1959; Brady 1960). However, the growth of the lactic-acid-producing bacteria under normal ensilage conditions necessarily has some influence on nitrogen distribution. The nitrogen of the cells of lactic acid bacteria held in the absence of air and at low pH remains in an insoluble particulate form for many months (Brady, unpublished data). This nitrogen represents a protein increment and its presence causes underestimation of the amount of plant protein hydrolysed during ensilage. No direct method of measuring the amount of bacterial nitrogen formed is available, but an estimate of about 2% of the total nitrogen of the plant has been made (Brady 1966). This is a small portion of the net change in protein content during ensilage, which is often 40–50% of the total nitrogen (Brady 1960).

This paper reports experiments with lactic-acid-producing bacteria isolated from silage, in which their capacity to modify nitrogen distribution in media by uptake of ammonia, or by catabolism of amino acids, was investigated. From these experiments an assessment of the contribution of these bacteria to nitrogen redistribution during ensilage is made.

II. Materials and Methods

The strains of *Lb. plantarum*, *Lb. brevis*, and *Pediococcus* sp. used were isolated from local silage and were cultured as described previously (Brady 1966). The basal medium was that listed in that paper, while the nitrogen source was Difco casamino

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acids (1·0% w/v), together with L-phenylalanine, L-tyrosine, L-tryptophan, and L-histidine, each added at the rate of 4·0 mg per 100 ml culture medium, or the amino acid substrate given in Table 1 of Brady (1966), but with doubled lysine and tyrosine content. Cultures were incubated in water-baths at 30±1°C. Cell suspensions were prepared by harvesting cells by centrifuging (5000 *g* for 20 min at 2°C), washing twice in acetic acid–sodium acetate buffer (pH 5·0, 0·074M acetate) and suspending in sodium acetate buffer (ionic strength 0·05) of appropriate pH. Suspensions were incubated in stoppered tubes containing a nitrogen atmosphere, generally at 30°C.

The analytical methods used were those described in Brady (1966). For two-dimensional paper chromatography, water-saturated phenol in an ammonia atmosphere was used in the first dimension and butanol–acetic acid–water (4 : 1 : 5 v/v) in the second.

TABLE 1

INFLUENCE OF INITIAL CONCENTRATION ON THE CHANGE IN THE CONCENTRATION OF AMMONIA WITH GROWTH

The initial concentration of ammonia was varied by additions of ammonium sulphate to a medium which contained amino acids to give 0·3 mg nitrogen/ml. Similar results were obtained when ammonia was added as ammonium citrate, and when grass juice was present in the medium

Time (days)	Ammonia Nitrogen (μg/ml)								
	Lb. plantarum Strain LP3			*Pediococcus* sp. Strain PI(1)			*Lb. brevis* Strain LB3		
0	5·8	14·1	28·4	5·7	14·9	27·0	5·3	14·7	27·1
1	1·0	5·4	16·0	10·8	18·9	29·1	11·1	17·3	28·9
4	1·0	5·2	16·0	10·1	18·0	29·9	11·9	19·0	29·7

III. RESULTS

(*a*) *Change in Amino Acid Distribution*

After growth for 96 hr in the basal medium of defined amino acid composition, cells were removed by centrifuging, and the amino acids in the supernatant fluid were retained on an ion-exchange resin and then eluted with 2N ammonia solution. The concentrated eluates were examined by two-dimensional paper chromatography, and compared with similar preparations from uninoculated media. With each organism, a marked decrease of aspartic acid, alanine, and lysine was noted. This occurred equally with *Lb. plantarum*, for which none of these amino acids was essential for growth. With *Lb. brevis*, there appeared to be a decrease of asparagine. With *Lb. brevis* and *Pediococcus* sp., ornithine was detected in the medium after growth, while with *Lb. brevis* alone, a purple spot of low intensity appeared adjacent to proline. With both *Lb. plantarum* and *Pediococcus* sp., there appeared a zone adjacent to aspartic acid, which required heating before giving a faint purple colour with ninhydrin.

(*b*) *Change in the Ammonia Content of Media*

During growth, each of the three species of lactic-acid-producing bacteria caused some change in the ammonia concentration of the medium. In general, growth of strains

of *Lb. plantarum* caused a decrease in the concentration of ammonia, while growth of *Pediococcus* sp. and *Lb. brevis* caused an increase. When the initial concentration of ammonia was raised, though this medium change had no effect on the growth rate or

TABLE 2

INFLUENCE OF THE CONCENTRATION OF AMINO ACIDS IN THE MEDIUM ON THE CHANGE IN THE CONCENTRATION OF AMMONIA WITH GROWTH

The mixture of amino acids in the medium is that given in Table 1 of Brady (1966), but with the content of lysine and tyrosine doubled

Amino Acid Nitrogen in Medium (mg/ml)	Ammonia Nitrogen (μg/ml)					
	Lb. plantarum Strain LP3			*Pediococcus* sp. Strain PI(1)		
	0 hr	48 hr	Change	0 hr	48 hr	Change
0·3	4·2	1·0	−3·2	4·2	4·3	+0·1
0·6	5·3	2·8	−2·5	5·4	21·5	+16·1
0·9	6·5	4·4	−2·1	6·4	33·6	+27·2
1·5	8·8	6·8	−2·0	8·8	46·3	+35·5

on cell yield, the net uptake of ammonia by *Lb. plantarum* was increased, and the net production of ammonia by the other species was decreased (Table 1). With 28 μg of ammonia nitrogen per millilitre in the medium, *Lb. plantarum* derived about 10% of

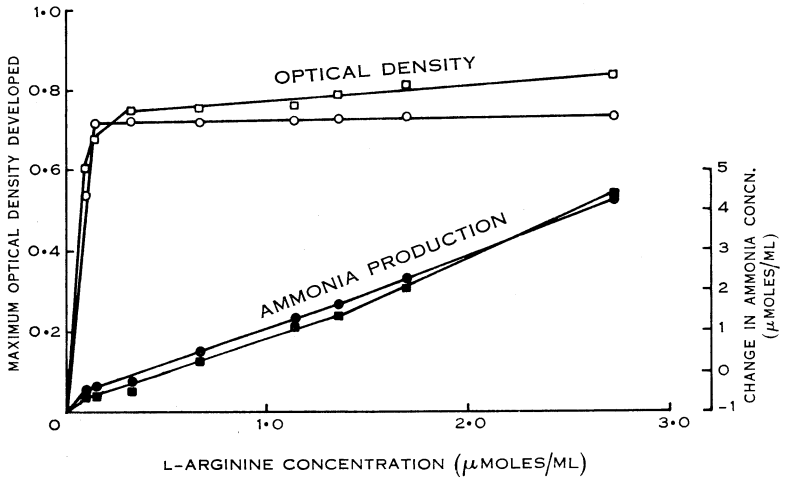

Fig. 1.—Ammonia production and cell yield during growth over 4 days by two strains of *Lb. brevis* as a function of the concentration of L-arginine in the medium. ○, ● Strain LB2. □, ■ Strain LB3.

its cell nitrogen from this net uptake of ammonia. When the concentration of amino acids in the medium was increased (Table 2), the net uptake of ammonia by *Lb. plantarum* decreased slightly; since cell yield increased with the substrate level of amino

acid, the proportion of cell nitrogen derived from ammonia decreased sharply. For the experiment recorded in Table 2, this decrease ranged from 3 to 1% of the cell nitrogen. With both *Lb. brevis* and *Pediococcus* sp. the ammonia content of the medium increased sharply as the level of the amino acid substrate was raised. This effect was shown to depend on the increased arginine concentration of the medium, and in Figure 1 the influence of L-arginine concentration on cell yield and on the net change in ammonia concentration is shown for *Lb. brevis*. While the dependency of ammonia production on the supply of L-arginine is obvious, a net uptake of ammonia is also shown for that part of the curve in which cell yield is a function of L-arginine supply.

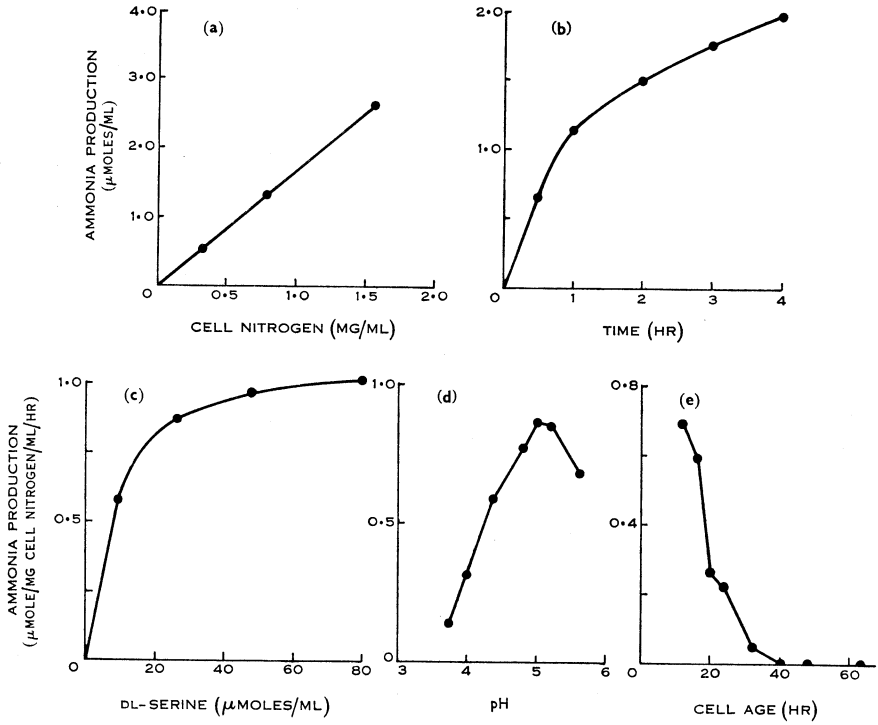

Fig. 2.—Release of ammonia from DL-serine by cell suspensions of *Lb. plantarum* strain LP3 incubated at 30°C, as a function of cell nitrogen, incubation period, serine concentration, pH, and cell age. (*a*) Cell age 16 hr, DL-serine concentration 93·5 μmoles/ml, incubation time 2 hr, and pH 5·0; (*b*) cell age 16 hr, cell nitrogen 0·67 mg/ml, DL-serine concentration 96·4 μmoles/ml, and pH 5·0; (*c*) cell age 16 hr, cell nitrogen 0·56 mg/ml, incubation time 2 hr, and pH 5·0; (*d*) cell age 16 hr, cell nitrogen 0·57 mg/ml, DL-serine concentration 62 μmoles/ml, and incubation time 2 hr; (*e*) cell nitrogen in the range 0·48–0·93 mg/ml, DL-serine concentration 96·4 μmoles/ml, incubation time 2 hr, and pH 5·0.

(*c*) *Catabolism of Amino Acids by Resting Cell Suspensions*

When resting cell suspensions of *Lb. plantarum* were incubated at pH 5·0 with amino acids, individually or in mixtures, a net production of ammonia occurred only when serine was present in the medium. The release of ammonia from serine by strain LP3 of *Lb. plantarum* is shown in Figure 2 as a function of cell nitrogen and

serine concentration, of incubation time and pH, and of the physiological age of the cells. Five other strains of *Lb. plantarum* isolated from silage displayed the same activity. Additions of pyridoxine or pyridoxamine to the suspensions had no influence on the rate of ammonia release. When other amino acids were present together with serine, the rate of ammonia release decreased. No specific effect of any individual amino acid could be demonstrated in this. The rate of release of ammonia from serine in the presence of 12 other amino acids each at 25 μmoles per millilitre was 60% of what

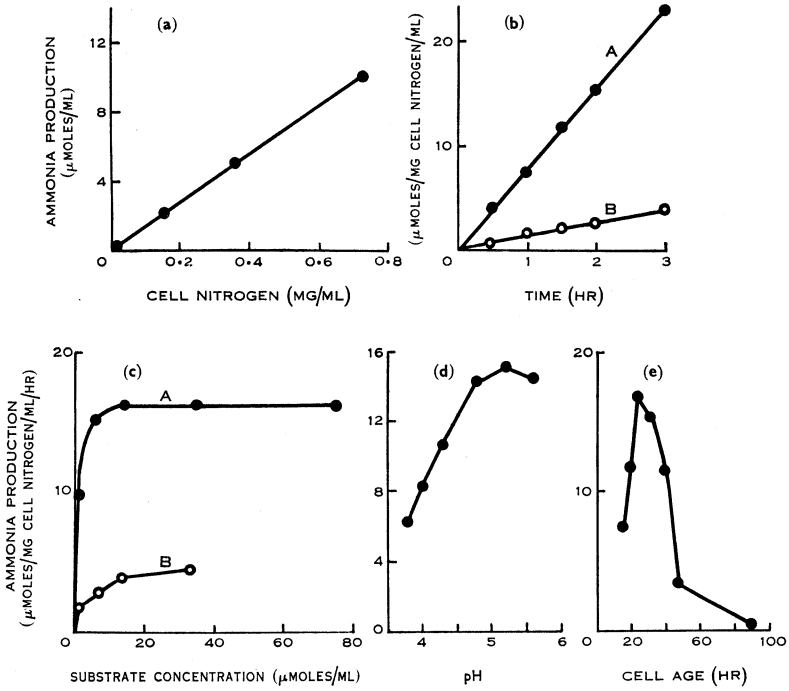

Fig. 3.—Release of ammonia from L-arginine (and L-citrulline) by cell suspensions of *Pediococcus* sp. PI(1) incubated at 30°C as a function of cell nitrogen, incubation period, substrate concentration, pH, and cell age. (*a*) Cell age 24 hr, L-arginine concentration 66 μmoles/ml, incubation time 2 hr, and pH 5·0; (*b*) cell age 21 hr and cell nitrogen 0·24 mg/ml for curve *A* and 41 hr and 0·41 mg/ml, respectively, for curve *B*, L-arginine concentration 55 μmoles/ml, and pH 5·0; (*c*) substrate L-arginine for curve *A* and L-citrulline for curve *B*, cell age 16 hr, cell nitrogen 0·18 mg/ml, incubation time 2 hr, and pH 5·0; (*d*) cell age 24 hr, cell nitrogen 0·77 mg/ml, L-arginine concentration 66 μmoles/ml, and incubation time 2 hr; (*e*) cell nitrogen within the range 0·11–0·40 mg/ml, L-arginine concentration 66 μmoles/ml, incubation time 1 hr, and pH 5·0.

it was when serine alone was present at this concentration. This same level of inhibition resulted when glycine and cysteine, or when alanine, proline, and asparagine (each of these at 50 μmoles per millilitre) were present with serine. When glucose (1% w/v) was added to the suspension the net yield of ammonia over a 2-hr period decreased by 22%. When the reaction was studied within the range at which rate varied with DL-serine concentration, ammonia yield from DL-serine was 85–92% of that from L-serine at the same concentration in a series of four experiments. Experiments with D-serine were not undertaken. No action on L-threonine could be demonstrated.

Suspensions of *Pediococcus* sp. at pH 5 and 30°C rapidly released ammonia from an amino acid mixture, and L-arginine was shown to be the sole amino acid involved. The presence of other amino acids had no effect on the rate of ammonia release from arginine. Figure 3 shows results of experiments relating ammonia release to cell nitrogen, substrate concentration, incubation time, pH, and the physiological age of the cells. As shown in Figures 3(*b*) and 3(*c*), L-citrulline served as a substrate for the reaction but the rate of ammonia release from L-citrulline was very much less than

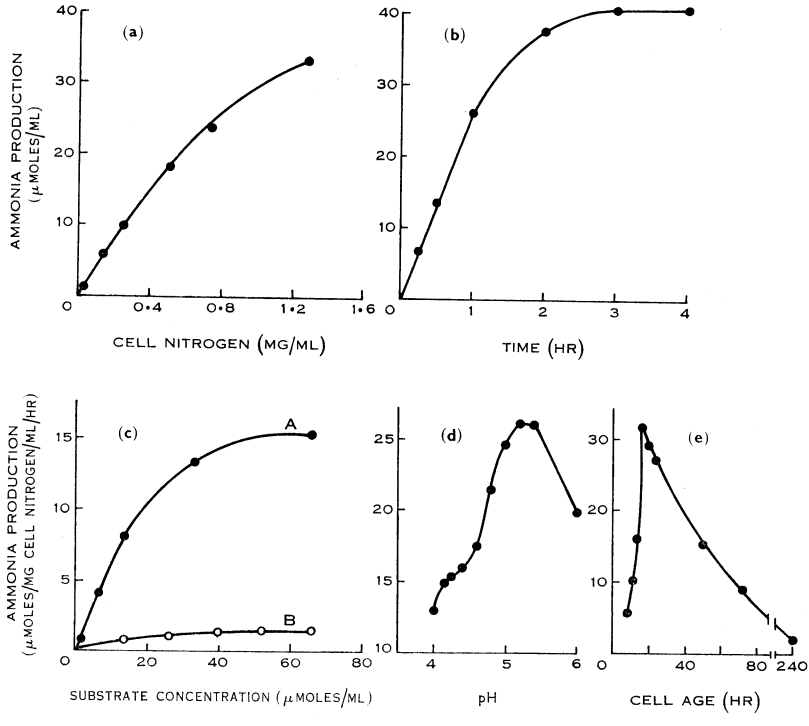

Fig. 4.—Release of ammonia from L-arginine (and L-citrulline) by cell suspensions of *Lb. brevis* strain LP3 incubated at 30°C as a function of cell nitrogen, incubation period, substrate concentration, pH, and cell age. (*a*) Cell age 16 hr, L-arginine concentration 67 μmoles/ml, incubation time 2 hr, and pH 5·0; (*b*) cell age 16 hr, cell nitrogen 0·65 mg/ml, L-arginine concentration 66·2 μmoles/ml, and pH 5·0; (*c*) substrate L-arginine for curve *A* and L-citrulline for curve *B*, cell age 16 hr, cell nitrogen 0·73 mg/ml, incubation time 1 hr, and pH 5·0; (*d*) cell age 16 hr, cell nitrogen 0·27 mg/ml, L-arginine concentration 53 μmoles/ml, and incubation time 1 hr; (*e*) cell nitrogen in the range 0·25–0·44 mg/ml, L-arginine concentration 53 μmoles/ml, incubation time 1 hr, and pH 5·0.

that from L-arginine. Nonetheless, when the medium with L-arginine as substrate was examined by paper chromatography in water-saturated phenol with and without ammonia, and by electrophoresis at pH 4 and pH 10, ornithine but no citrulline was detected. When glucose was added to the suspension, the rate of ammonia release decreased by about 35%. The action on L-arginine was demonstrated in each of six isolates of *Pediococcus* sp. from silage.

Strains of *Lb. brevis* as cell suspensions at pH 5 and 30°C also catalysed a rapid release of ammonia from L-arginine and a less rapid release from L-citrulline (Fig. 4).

Ornithine was identified as a product in each case. The presence of other amino acids did not decrease the reaction rate, but in the presence of glucose, the net release of ammonia was less rapid. *Lb. brevis* strains showed no capacity to release ammonia from other amino acids, but ammonia was released from asparagine and glutamine. At pH 5, the rate of ammonia production with a saturated substrate supply was 0·55 μmole/mg cell nitrogen/ml/hr for L-asparagine and 0·10 μmole/mg cell nitrogen/ml/hr for L-glutamine. In contrast to *Lb. plantarum* and *Pediococcus* sp., suspensions of *Lb. brevis* showed a significant rate of release of ammonia from endogenous subtrate. At pH 5·0 with early stationary-phase cells, this endogenous rate was about 0·1 μmole/mg cell nitrogen/ml/hr when the incubation time of the suspensions was 1½ hr or less but rose to about three times this value when more extended incubation times were used. The rate was a little lower with older cells and decreased when the suspensions were at a lower pH.

IV. Discussion

Of the amino acids, only serine and arginine appear to be extensively catabolized and only ornithine formed in significant amount by the silage lactic acid bacteria. The appearance of ornithine in low-moisture silage at the same stage as when lactic acid production became apparent (Brady 1965) is consistent with this conclusion. Net uptake of the other amino acids by the lactic acid bacteria appears to reflect the amino acid composition of the cells and for most of the acids will be a small factor in their redistribution during ensilage.

While the production of volatile basic nitrogen, which is mostly ammonia (Jackson 1964), is usually attributed to anaerobic bacteria, the results of Mabbitt (1951) indicate a considerable release of volatile base in "silage" made in the absence of microorganisms. Kemble (1956), however, found little volatile base in microbe-free silage, while the less convincing evidence from ensilage in the presence of volatile bacteriocides (Brady 1960) shows an increment in volatile basic nitrogen which is less than that arising when bacteria develop. The conditions of growth of plants in aseptic conditions may influence their composition, and information, for example, on the amount of amide in the harvested material may suggest a reason for the conflicting evidence. Neumark (1962) from experiments with clover treated with volatile bacteriocides or with antibiotics concluded that aromatic amines are formed from amino acids in "ensiled" plants in the absence of bacterial development.

The results in Tables 1 and 2 and in Figure 1 demonstrate that not only are the lactic acid bacteria able under suitable conditions to produce ammonia from amino acid substrates, but also to take up ammonia and convert this to cell nitrogen. Having regard, however, to the proportion of plant nitrogen converted to bacterial nitrogen (Brady 1966), to the proportion of bacterial nitrogen which may be derived from ammonia, and to the amount of ammonia formed during ensilage, the conclusion is apparent that incorporation of ammonia into cell nitrogen has a very small influence on net ammonia production.

The release of ammonia from serine has been described in organisms related to *Lb. plantarum* (Kristoffersen and Nelson 1954; Kristoffersen 1956). This reaction, however, probably makes little contribution to the release of ammonia during ensilage,

for the cells lose the activity very rapidly as they age, while a pH near 4·0 limits the reaction rate which even at its optimum is not particularly rapid. The fact that no net ammonia production could be shown during growth of *Lb. plantarum* cultures also suggests that serine catabolism by *Lb. plantarum* is of limited importance to nitrogen redistribution during ensilage.

The release of ammonia from arginine has been described for *Lb. brevis* (Briggs 1953; Naylor and Sharpe 1958) and for some of the pediococci (Jensen and Seeley 1954; Günther and White 1961). The reaction has been studied in detail in related lactic acid bacteria (Oginsky and Gehrig 1953; Bibb and Straughn 1959) and shown to proceed by hydrolysis of arginine by arginine desaminase to citrulline, and of citrulline to ornithine by citrullinase. The relative impermeability of cells to L-citrulline has been noted and was studied in detail in *Streptococcus faecalis* by Bibb and Straughn (1964). The activity of the strains of *Lb. brevis* and *Pediococcus* sp. studied was high, was well maintained at pH levels likely to be encountered in silage, and, particularly for *Lb. brevis*, persisted as the cells aged. Release of ammonia from arginine can then be expected to contribute to nitrogen redistribution during ensilage when *Pediococcus* sp. and *Lb. brevis* form a significant part of the bacterial flora. Calculations based on the arginine content of leaf protein (Chibnall, Rees, and Lugg 1963) suggest that the total contribution of ammonia production from arginine released by proteolysis, could be of the order of 2% of the total nitrogen.

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