ON THE NITROGEN NUTRITION OF SILAGE STRAINS OF LACTIC ACID BACTERIA

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Summary

Consideration is given to the adequacy of the free amino acids in plant juices at the time of harvest as nitrogen substrate for strains of lactic acid bacteria isolated from silage. The requirements of several strains of the bacteria for free amino acids in synthetic media were compared with the concentration of these acids in the liquid phase of plants at the time of harvest; this comparison suggested that several amino acids, and particularly lysine, may at times be rate-limiting. Ethanolic extracts of plants, sampled before and after a period of post-harvest wilting, were assayed as nitrogen substrates for the bacteria. A marked response to additions of lysine, some response to arginine, and evidence of deficiency of other acids were noted. The importance of post-harvest proteolysis to the amino acid nutrition of the bacteria in the silage environment is discussed. Certain fractions of the plant extracts were found to promote early growth of the bacteria in the synthetic medium, and the distribution of this activity in different fractions is described.

I. Introduction

Studies of nitrogen redistribution during ensilage have emphasized the importance of plant enzymes in the hydrolysis of protein and in the metabolism of the released amino acids (Mabbitt 1951; Macpherson 1952; Kemble 1956; Macpherson and Slater 1959; Brady 1960, 1961). While the degradation of amino acids by species of *Clostridium* when the plant material is inadequately preserved is widely recognized (Barnett 1954), the metabolism and utilization of the nitrogen of the plant by the lactic-acid-producing bacteria has not been investigated.

Lactobacilli and pediococci of the types isolated from silage (Langston *et al.* 1958; Keddie 1959) require a range of amino acids for growth (Dunn *et al.* 1947; Jensen and Seely 1954; Scardovi and Bottazzi 1962) and, in the silage environment, are dependent on amino acids in the plant juices. In the absence of air infiltration, juices are released from herbage plants 2–3 hr after ensiling, and development of the lactic acid bacteria probably proceeds rapidly from this point (Greenhill 1964). At the same time, a rapid hydrolysis of the plant protein releases further amino acids, and changes their relative proportions.

In the present paper, the importance to the developing lactic acid bacteria of the changes in concentration of amino acids in the plant juice, which result from the action of the plant enzymes, is considered in relation to the developing lactic acid bacteria.

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II. Materials and Methods

(a) *Bacteria Studied*

The strains of *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Pediococcus* sp. studied have been isolated from local silage, and were preserved and cultured as described by Lanigan (1963). Before measuring the response to a nitrogenous substrate, bacteria from stock cultures were grown through two transfers in the basal assay medium containing an adequate supply of amino acids. All growth studies were with cultures incubated at 30 ± 1°C.

(b) *Plant Materials*

Short rotation ryegrass (*Lolium* sp., New Zealand H1 strain), white clover (*Trifolium repens*, Irrigation White strain), and lucerne (*Medicago sativa*, Hunter River strain) were grown under pure sward conditions in the laboratory grounds. Plants were cut about an inch above ground level, checked for botanical purity, and chopped to $\frac{1}{4}$–$\frac{1}{2}$ in. lengths by hand or with a mechanical green-feed chopper. After thorough mixing, chopped material was sampled for chemical analysis and preparation of extracts. When plants were wilted before extracts were prepared, wilting was in the dark in a forced-draught oven at 25°C.

(c) *Culture Media*

The composition of the basal medium was as follows:

Glucose	25·0 g	Nicotinamide	0·5 mg
$CH_3COONa.3H_2O$	24·0 g	Thiamine hydrochloride	0·5 mg
K_2HPO_4	1·25 g	Riboflavin	0·5 mg
KH_2PO_4	1·25 g	Calcium pantothenate	0·5 mg
Tween 80	1·0 g	*p*-Aminobenzoic acid	0·5 mg
Ascorbic acid	0·5 g	Folic acid	10 μg
Xanthine	20 mg	Biotin	4 μg
Adenine sulphate	20 mg	$MgSO_4.7H_2O$	0·8 g
Uracil	20 mg	NaCl	40 mg
Guanine hydrochloride	20 mg	$FeSO_4.7H_2O$	40 mg
Thymine	20 mg	$MnSO_4.4H_2O$	160 mg
Pyridoxine hydrochloride	1 mg	pH	6·5
Pyridoxamine dihydrochloride	0·5 mg	Distilled water	to 1000 ml

For addition to plant extracts, the inorganic salts and acetate were blended and added as a powder. The purine, pyrimidine, and growth factors, other than riboflavin, were added in solution. Glucose and riboflavin were added just before sterilization. Plant extracts were sterilized by filtration through sintered glass.

Media containing defined nitrogenous substrates were prepared by separately sterilizing the basal medium at double strength, and glucose and nitrogen source each at 10 times the final concentration, and completing the media by aseptic mixing just before inoculation. Sterilization was by filtration, or by autoclaving at 121°C for 2 min in previously sterilized glassware.

Amino acids were of the L-form, excepting DL-serine, and were chromatographically pure.

Non-protein nitrogen was extracted by blending chopped plant material with 3 volumes of aqueous ethanol to give an ethanol concentration of 80% (v/v). After filtration through cloth, the extract was concentrated *in vacuo* at 40°C till most of the ethanol was removed, and partitioned between chloroform and water. The water phase was partitioned against a second portion of chloroform, interphasal material was removed by filtration, and chloroform was removed by heating *in vacuo* at 40°C for 15 min.

(*d*) *Analytical Methods*

Methods used in the analysis of plant material were described by Brady (1960). Nitrogen in cells recovered by centrifuging was estimated by a Kjeldahl procedure (McKenzie and Wallace 1954) after the cells had been washed in 0·2 vol. cold 0·1M acetate buffer, pH 4·0. Acid production in culture media was determined by titration at 20°C to pH 6·5 with 0·2N sodium hydroxide.

(*e*) *Experimental Procedures*

Growth response was found not to be modified by the gas phase above the medium and was followed in 10 or 15 ml of medium in 6 by $\frac{3}{4}$ in. test tubes. Inoculation was usually with 0·03 ml of a logarithmic-phase culture but, on occasions, larger inocula or older inoculum cultures were used. Production of cell substance was measured as the decrease in light transmission at a wavelength of 600 mμ, and calibration curves relating optical density to cell nitrogen were prepared.

III. Results

In the basal medium, an acid digest of casein [Difco casamino acids, 1% (w/v)] required supplementation with tryptophan, tyrosine, phenylalanine, and histidine to give a growth response equivalent on a nitrogen basis with that given to an enzymic casein digest (Difco tryptone). This acid hydrolysate supplemented with the four amino acids (0·2 μmole/ml) was used for preparation of inoculum cultures and as a standard in assay experiments.

Growth of strains of *Lb. brevis* in the basal medium with either the enzymic casein digest, or the supplemented acid digest of casein was slow in rate and limited in extent. Both growth rate and cell yield were substantially improved by L-arabinose. In the presence of 2·0% (w/v) glucose, the response to arabinose was proportional to its concentration and was saturated by a concentration of 0·30% (w/v). Ribose was as effective as arabinose in improving growth rate and cell yield, but xylose and fructose were about half as effective at equivalent concentration. Considerable growth occurred with arabinose alone as energy source, but both growth rate and cell yield were greater when arabinose and glucose were supplied together. In subsequent experiments, arabinose [0·50% (w/v)] and glucose [1·50% (w/v)] were added to media inoculated with *Lb. brevis* strains.

(*a*) *Amino Acid Requirements*

A mixture of amino acids, chosen to reflect the relative proportions of the free acids in the leaves of grasses and legumes (see Table 5), was added to the basal medium (Table 1). In this medium rapid and substantial growth of each of the bacterial strains investigated occurred. However, for strains of *Lb. brevis* and *Pediococcus* sp. the lysine concentration was found to be suboptimal, and for subsequent experiments was increased to 0·44 μmole/ml.

TABLE 1

AMINO ACID COMPOSITION OF BASAL MEDIUM

Total nitrogen in medium 300 μg/ml

Amino Acid	Amino Acid Group	Final Concn. (μmole/ml)	Amino Acid	Amino Acid Group	Final Concn. (μmole/ml)
L-Tyrosine	I	0·22	L-Cysteine	IV	0·32
L-Phenylalanine	I	0·24	Glycine	IV	0·54
L-Tryptophan	I	0·20	DL-Serine	IV	1·90
L-Threonine	II	0·31	L-Glutamic acid	V	1·36
L-Aspartic acid	II	0·75	L-Proline	V	1·74
L-Isoleucine	II	0·31	L-Arginine	V	0·48
L-Methionine	II	0·27	L-Lysine	VI	0·22
L-Leucine	III	0·76	L-Histidine	VI	0·21
L-Valine	III	0·86	L-Glutamine	V	0·40
L-α-Alanine	III	2·24	L-Asparagine	III	1·51
			γ-Aminobutyric acid	V	1·91

With this modified medium, the requirement for individual amino acids was investigated by omitting them one at a time. The response in terms of optical density was determined 16, 24, 48, and 72 hr from inoculation. For a strain of *Lb. plantarum*, glutamic acid, leucine, isoleucine, and valine were essential for growth, but either growth rate or cell yield was reduced when tryptophan, phenylalanine, methionine, glycine, alanine, proline, arginine, or serine were omitted. For each of two strains of *Lb. brevis*, each of the α-amino acids except alanine and serine was essential for growth. When serine was not added, cell yield was substantially reduced. Omission of asparagine or of γ-aminobutyric acid was without effect. With two strains of *Pediococcus* sp. growth was not affected by the omission of aspartic acid or asparagine but was reduced when neither was added. Reduced cell yields resulted when methionine, alanine, serine, or proline was omitted, while omission of γ-aminobutyric acid or of glutamine was without effect. All of the other amino acids were essential for growth.

The concentration of amino acids required for maximum growth was determined in the basal medium by reducing the concentration of all members of one group of acids (see Table 1), while the concentration of the other amino acids was held constant. When the concentration range in the group was large, the response to graded amounts of individual amino acids, while the supply of all the other amino acids remained the same, was measured. The amides and γ-aminobutyric acid were omitted from all

media. Limiting levels for each amino acid were not determined, but in Table 2 the minimum concentration of each amino acid which was observed to give maximum cell yield without change in growth rate is shown. In some cases, lower concentrations may suffice. Since with some amino acids, and particularly with glutamic and aspartic

TABLE 2

AMINO ACID REQUIREMENT (IN μMOLE/ML) OF SILAGE LACTIC ACID BACTERIA FROM SINGLE- OR GROUP-DEPLETION EXPERIMENTS

The response to each group of amino acids (see Table 1) was first determined; in many cases the response was defined further by varying the level of the individual acids; when the response to the group only was measured the results are bracketed

Amino Acid	*Lb. brevis* Strain LB2		*Lb. brevis* Strain LB3		*Pediococcus* sp.		*Lb. plantarum*	
	Half Max. Cell Yield	Max. Cell Yield	Half Max. Cell Yield	Max. Cell Yield	Half Max. Cell Yield	Max. Cell Yield	Half Max. Cell Yield	Max. Cell Yield
L-Tryptophan		0·04		0·06		0·04		0·04
L-Tyrosine		0·05		0·07	0·02	0·12		0·10
L-Phenylalanine		0·05		0·07	0·05	0·20		0·10
L-Methionine		0·08	0·02	0·08		0·04		
L-Threonine	0·11	0·20	0·11	0·20		0·13		
L-Aspartic acid	0·07	0·30	0·08	0·30	0·08	0·22		
L-Isoleucine	0·07	0·15	0·06	0·15		0·07	0·07	0·26
L-Leucine		0·27		0·23		0·08	0·06	0·20
L-Valine		0·27		0·23		0·06	0·13	0·29
L-Alanine		0·09		0·10	<0·08	0·33		
Glycine	0·06	0·27		0·21	0·04	0·12		
L-Cysteine		0·05		0·06		0·10		
DL-Serine		0·10		0·10		0·10		
L-Glutamic acid		0·82*	0·15	0·68*	0·05	0·30	0·15	0·45
L-Proline		0·20		0·17	<0·02	0·04		
L-Arginine	0·07	0·39	0·04	0·17†	0·06	0·52†		
L-Lysine	0·15	0·44	0·16	0·44	0·12	0·44		
L-Histidine		0·04		0·04		0·05		

* Interacts with arginine. † Interacts with glutamic acid.

acids and with arginine, continuing slight responses in cell yield occurred over a wide range of substrate level, the more readily measured level for half maximum cell yield is included in Table 2. With *Lb. plantarum* clearly defined minimum requirements were obtained only with the four essential amino acids. Responses were obtained to additions of each of the amino acids, but the level of the response depended markedly on the supply of the other amino acids. In this sense each of the amino acids was essential for maximum growth.

The dependency of the growth response on the balance of the different amino acids was also apparent, when the response to different levels of amino acid mixtures was measured. Figure 1 shows the response by strain LB3 of *Lb. brevis* and strain LP3 of *Lb. plantarum* to different amino acid mixtures. For LB3, when each of the

amino acids was presented at the concentration which had been found adequate by the single-deletion technique in the amino acid mixture of Table 1, growth was only 50% maximal (84 μg/ml nitrogen, Fig. 1), and the concentrations had to be increased threefold for growth to be maximal. With this amino acid mixture, isoleucine was limiting for LP3 till the nitrogen level was 120 μg/ml. Beyond this level no specific deficiency could be demonstrated.

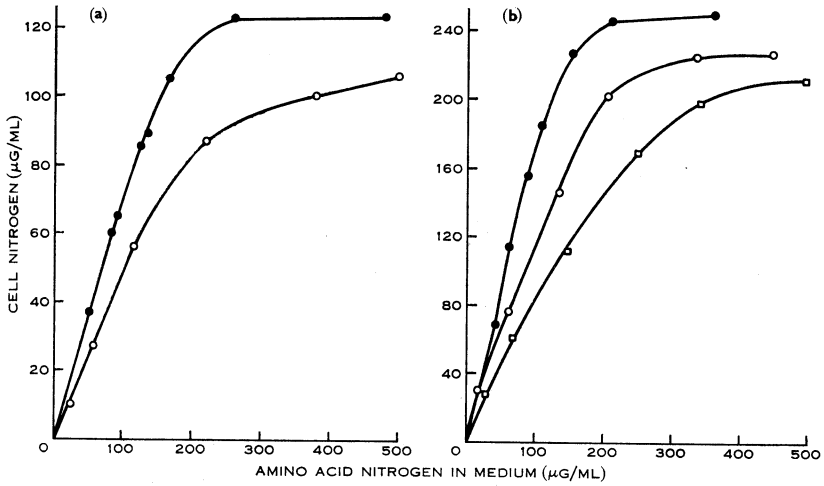

Fig. 1.—Response of (*a*) *Lb. brevis* strain LB3 and (*b*) *Lb. plantarum* strain LP3 to a varied supply of amino acid mixtures in which the proportions of the different amino acids varied. In both cases the more-efficient mixture (●) is that shown in Table 2 as satisfying the requirements of *Lb. brevis* strain LB3. The less-efficient mixture (○) is as in Table 1, but with a doubled content of lysine, arginine, and tyrosine, and with the amides and γ-amino-butyric acid omitted. With strain LP3 the least-efficient mixture (□) is as in Table 1.

(*b*) *Requirements for Maximum Growth*

In the basal medium provided with an adequate supply of amino acids and with arabinose present, each of 9 isolates of *Lb. plantarum*, 2 isolates of *Lb. brevis*, and 7 of 12 isolates of *Pediococcus* sp. gave rapid and extensive growth. Additions of thymidine (4 μg/ml) had no influence on the growth of these strains, but allowed three of the five strains of *Pediococcus* sp., which did not grow well in the basal medium, to develop. The remaining two strains of *Pediococcus* sp. grew well when folinic acid (1 μg/ml) was included in the medium.

The rate of growth of each species was increased when ammonium citrate (60 μg/ml) was added to the medium, but cell yield was not changed. Addition of L-glutamine (1 μmole/ml) improved cell yield of *Lb. plantarum* and *Lb. brevis*.

When small additions of filter-sterilized grass juice were made to the basal medium, supplemented with ammonium citrate, glutamine, and folinic acid, and including an excess of each of the amino acids, a marked response in terms of early

growth was observed (Fig. 2). A variety of substances which have been reported to improve early growth of lactic acid bacteria, namely D-alanine, D-glutamic acid, spermine, and peptides of histidine, leucine, or glycine had no similar effect in this medium.

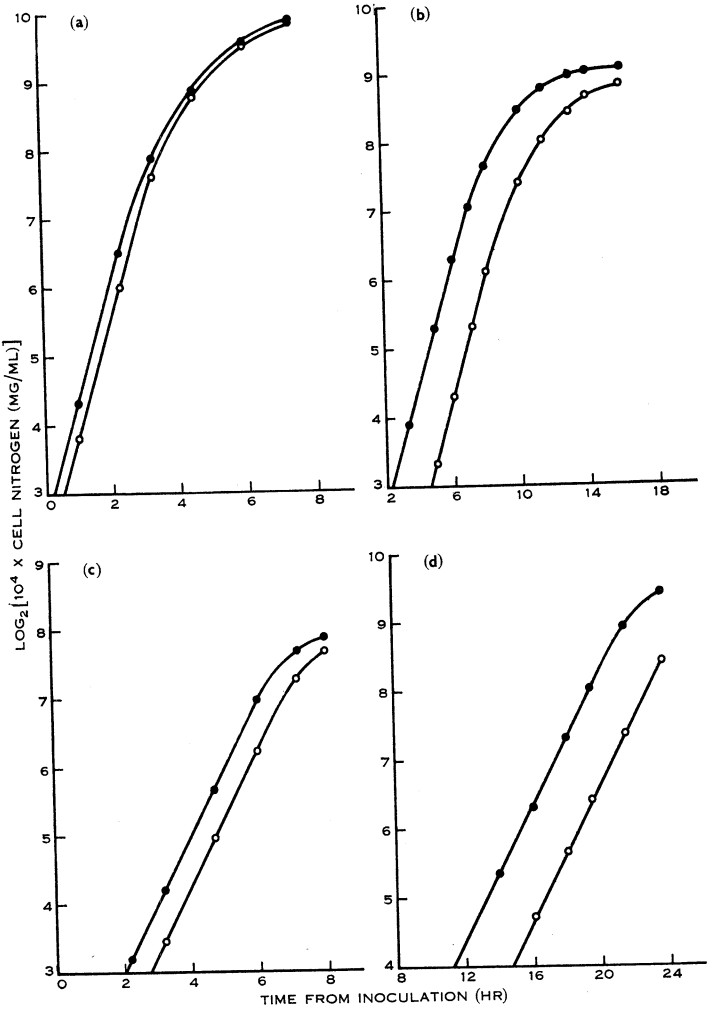

Fig. 2.—Change in growth performance when a small portion (10 μg nitrogen/ml) of an 80% (v/v) ethanol extract of ryegrass juice (●) was added to the synthetic medium (○). The inoculum was (*a*) *Lb. plantarum*; (*b*) *Lb. brevis*, (*c*) and (*d*) *Pediococcus* sp. In (*d*) a very small portion of a stationary-phase culture was used as inoculum; in (*a*)–(*c*) the inoculum culture was in logarithmic phase.

To measure the effectiveness of different fractions of grass juice, dilutions of the fractions were added to the medium and inoculated with a very small portion

CORRIGENDUM

p. 108, Table 1: *For* Group III (opposite L-Asparagine) *read* Group II.

Table 3. When the phenol-rich phase of a phenol–water countercurrent distribution (see Table 3) was passed through a second column of Sephadex G-25, the distribution of

TABLE 3

RELATIVE ACTIVITY OF FRACTIONS OF GRASS JUICE FACTOR

Treatment of Grass Juice	Fraction Tested	Relative Activity of Fractions	
		Lb. brevis	*Pediococcus* sp.
1. Expressed juice (untreated)		100	100
2. Extraction of juice with 80% (v/v) ethanol	Whole extract	94	80
3. Absorption of juice on Dowex-50 and elution with 2N ammonia	Fraction not retained on column	0	0
	Eluted fraction	94	80
4. Fractionation of juice on Sephadex G-25 (solvent 0·05M acetic acid)	S1 (fast)	0	0
	S2	6	10
	S3	66	45
	S4	22	25
5. Countercurrent distribution of fraction S3 in phenol–water	Phenol-rich phase	60	5
	Water-rich phase	6	40

* Fractions S1 and S2 contained no free amino acids; the bulk of the free amino acids was in fraction S3, but some acids, and particularly the aromatic acids, were in fraction S4.

activity for *Lb. brevis* relative to the elution pattern of free amino nitrogen was that shown in Figure 3. The effect of the "juice factor" was completely destroyed by

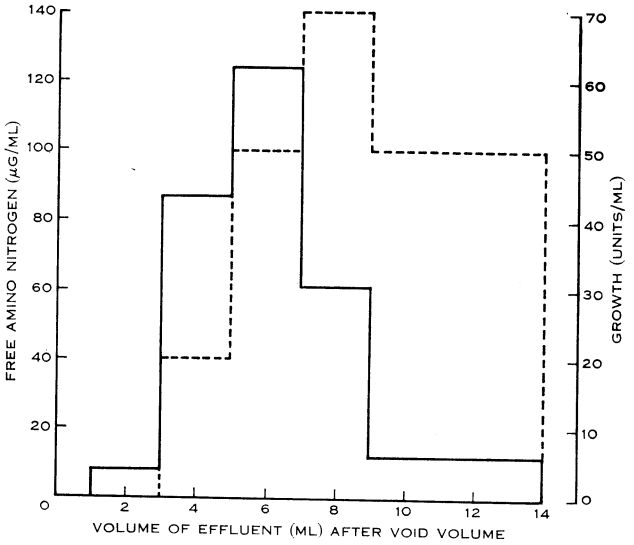

Fig. 3.—Elution pattern of amino nitrogen (——) and of growth-stimulating activity for *Lb. brevis* strain LB3 (– – – –) after passage of a phenol-rich phase fraction (see Table 3) through a column (25 by 1 cm) of Sephadex G-25 (solvent 0·05M acetic acid).

treatment with 6N hydrochloric acid (6 hr, 105°C) but was retained when autoclaved with the carbohydrate-free medium.

The distribution of juice factor activity in a number of plant samples was measured by assaying ethanolic extracts after absorption on Dowex-50 and elution with 2N ammonia. Assuming uniform distribution of activity through the plant moisture, the least active of six samples of ryegrass gave a saturated response when the dilution was 1 : 30, and the least active of three white clover samples and of three lucerne samples, when the dilution was 1 : 12.

TABLE 4

GROWTH AND ACID FORMATION BY SILAGE LACTIC ACID BACTERIA IN MEDIA WITH AND WITHOUT GRASS JUICE FRACTION

Amino* Acid Nitrogen (μg/ml)	Grass Juice Fraction†	*Lb. plantarum* Strain LP 3			*Lb. brevis* Strain LB 3			*Pediococcus* sp. Strain PI (1)		
		O.D.‡ (16 hr)	O.D. (10 days)	Acid§ (10 days)	O.D. (16 hr)	O.D. (10 days)	Acid (10 days)	O.D. (16 hr)	O.D. (10 days)	Acid (10 days)
348	+	0·87	1·12	25·4	0·29	0·56	9·3	0·13	0·80	18·4
348	−	0·73	1·13	25·7	0·18	0·56	9·3	0·03	0·80	18·5
139	+	0·58	0·78	22·5	0·19	0·26	7·1	0·09	0·54	12·1
139	−	0·49	0·78	21·8	0·08	0·25	6·9	0·03	0·56	12·2
95% confidence interval				0·85			0·48			0·44

* Amino acid mixture as in Table 1, but with proportions of L-lysine, L-tyrosine, and L-arginine doubled.

† Fraction S4 (Table 3), adding 4 μg nitrogen/ml to the medium.

‡ Optical density at 600 mμ with uninoculated medium as zero.

§ Milliequivalents of acid produced per 100 ml of medium.

The observations made on the properties and the distribution of the juice factor suggested that its concentration in plant extracts would not influence assays of these extracts as a nitrogen source, if the response was measured as acid produced after prolonged incubation. That the presence or absence of the juice factor has no effect on acid yield when nitrogen substrate is limiting was confirmed in the experiments summarized in Table 4.

(c) *Relation between Cell Yield and Acid Production*

In experiments in which the response to extracts of plants was to be measured, optical methods lacked sensitivity and the response of the bacteria was measured as acid production. To investigate the possibility that a limited cell population could, by prolonged activity, match the acid productivity of a much larger population, response to graded amounts of an amino acid mixture was measured as the change of optical density with time, and as the acid produced during incubation for 10 days. The results are shown in Figure 4.

With *Pediococcus* sp. *and Lb. plantarum* response in terms of cell production or acid production was very similar, and the capacity of cells to produce acid under these conditions was approximately proportional to the cell concentration. With *Lb. brevis*, the amount of acid produced was almost independent of cell concentration over quite a wide range.

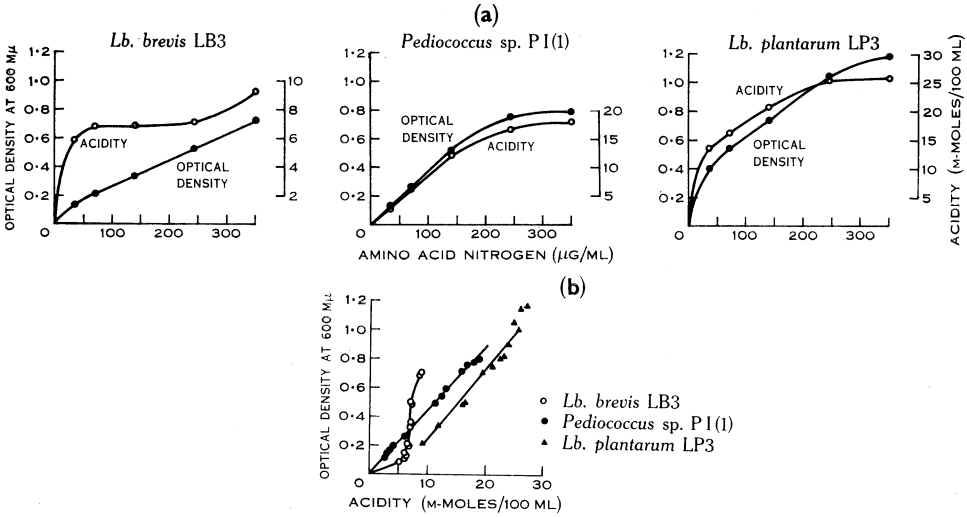

Fig. 4.—(*a*) Response of *Lb. brevis*, *Pediococcus* sp., and *Lb. plantarum* to a varied supply of the amino acid mixture (Table 1), modified to double the proportions of lysine, arginine, and tyrosine. Optical density was the maximum observed; acidity was titrated after cultures had been incubated for 10 days. (*b*) Culture acidity plotted against optical density for different cultures of *Lb. brevis*, *Pediococcus* sp., and *Lb. plantarum*.

(*d*) *Quality of Plant Extracts as Nitrogen Substrate*

The concentrations of the free amino acids in the juice of herbage plants have been studied by a number of investigators. In Table 5 are listed the concentrations observed in a number of pasture species. The values are drawn from reports in which the data presented permit the concentrations in the juice, assuming uniform distribution through the plant moisture, to be calculated. These data indicated that the dicarboxylic acids and their amides and alanine, serine, and proline are often present in much higher concentrations than the other amino acids, while the basic amino acids and methionine may be present in small amount.

The concentrations of the acids in plant juices are very much influenced by the moisture content of the plant. Moisture loss from plants either before or after harvest will cause a substantial increase in concentration. Even allowing for this, a comparison with the amino acid requirements of the bacteria for maximal growth (Table 2) presents the possibility that the supply of some amino acids, and particularly of the basic acids may be marginal. However, this neglects the contribution which amino acids in bound form may make to the nutrition of the bacteria.

To measure more directly the quality of the nitrogen in small-molecule form as a nitrogen substrate for the lactic acid bacteria, 80% (v/v) ethanol extracts of plants were added to the basal medium containing folinic acid, and the response measured as the acid produced after 10 days incubation at 30°C. When *Lb. brevis* was used in the assay, arabinose (0·7%) and fructose (1·5%) replaced glucose as the carbon and energy source. Each sample was assayed with at least one strain of *Lb. plantarum*, *Lb. brevis*, and *Pediococcus* sp.

TABLE 5

FREE AMINO ACID CONCENTRATION IN THE WATER PHASE OF FRESH PLANTS

Concentrations given as μmoles/ml, assuming uniform distribution in the whole of the plant moisture

Amino Acid	Smooth Brome	Perennial Ryegrass				Short Rotation Ryegrass	Red Clover 60% "Grass" 40%
Moisture content (% dry matter)	400	610	425	418	340	730	400
Reference	1*	2†	3‡	4§	4§	5‖	6¶
Tyrosine	0·96	0·28	0·28			0·15	
Phenylalanine	0·89	0·28	0·35				
Methionine		0·11					
Threonine	0·75	1·43	0·35				
Aspartic acid	5·59			0·53	1·12		1·07
Isoleucine	0·86	0·17	0·14				
Leucine		0·28	0·20				
Valine	0·61	0·44	0·35				
α-Alanine	0·71	1·49	1·11			1·08	2·72
Glycine	0·04	0·44	0·35				0·64
Serine	1·39	2·20	1·04				0·36
Glutamic acid	3·68			4·29	4·70		0·21
Proline		0·61	0·86			0·68	
Arginine	0·13					0·08	
Lysine	0·35					0·10	
Histidine	0·12					0·08	
Glutamine	1·57	8·29		1·07	2·39		
Asparagine	0·59	5·54		1·07	1·34		3·71
γ-Aminobutyric acid				1·07	0·70	1·26	1·57

* Maslowski, Minakowski, and Ho (1962); nitrogen content of 2% of dry matter assumed.

† Kemble (1956). ‡ Kemble and Macpherson (1954). § Macpherson and Slater (1959).

‖ Brady (unpublished data). ¶ Landis (1960).

The general pattern of response is shown for strains of *Pediococcus* sp. and *Lb. brevis* in Figures 5(*a*) and 5(*b*), showing in one case the response to extracts of white clover harvested at different times, and in the other to samples of ryegrass, white clover, and lucerne harvested at the same time. In Figures 5(*c*) and 5(*d*), the influence of plant maturity with ryegrass during the spring season is shown with assays with *Lb. plantarum* and *Pediococcus* sp. In each of four seasons, the lower quality of the nitrogen in extracts of leafy immature ryegrass has been observed. The difference is

significant but less marked with *Lb. brevis*, and appears again but is less marked when the nitrogen content of the grass is lower.

In each of two seasons, wilting of early harvest leafy grass improved nitrogen quality to that found in later harvest plants. At the later harvests, no change in nitrogen quality resulted from wilting. In grass grown in the 1964 spring, post-harvest wilting of early-harvest grass had no influence on the quality of nitrogen in the extracts.

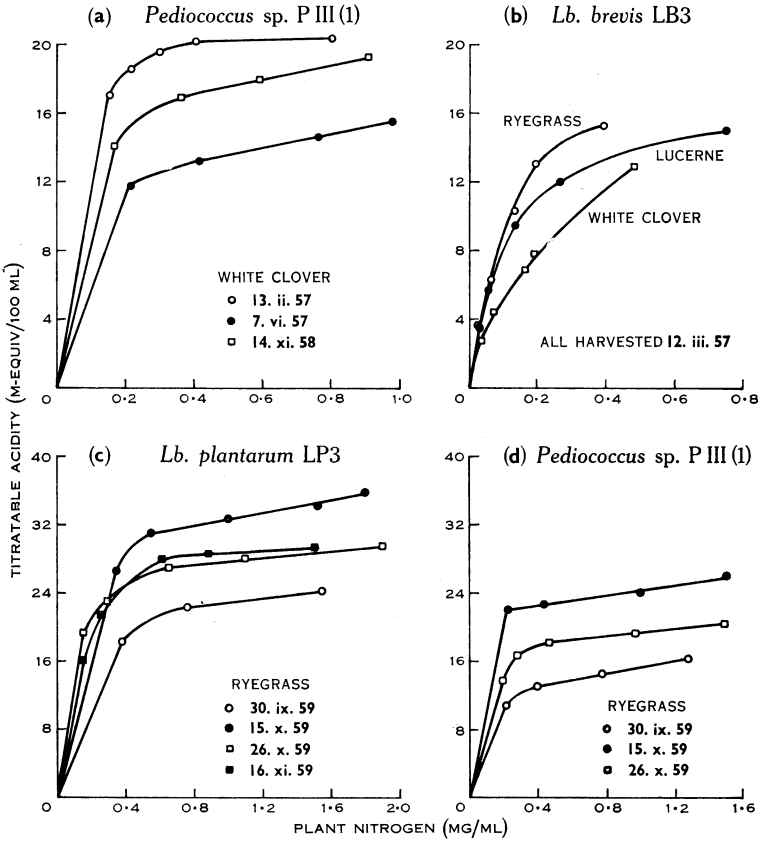

Fig. 5.—(*a*)–(*d*) Acid yield in basal medium with plant extracts as nitrogen source. Assay organisms, plants from which extracts were obtained, and dates of harvesting are indicated.

Wilting the tops of later harvests considerably reduced the nitrogen quality. In this later harvest, ryegrass was grown without nitrogen fertilizers and compared to earlier harvests its nitrogen content was low. When wilting caused a change in nitrogen quality, the change was apparent with each of the assay organisms. In Figure 6 the response of *Pediococcus* sp. to extracts of wilted and unwilted ryegrass in each of two seasons is shown.

Analyses of plant extracts when compared with the amino acid requirements of the lactic acid bacteria suggest that lysine and arginine, in particular, may be limiting. Supplementation of the extracts with lysine (2 μmoles/ml) has indeed always led to an improved titre when *Lb. brevis* or *Pediococcus* sp. strains were the assay

organisms, but not with *Lb. plantarum*. No response to arginine (2 μmoles/ml) alone has been noted, but a response to arginine in the presence of additional lysine was noted with some samples. Figure 7 shows the response to lysine and arginine supplementation for *Lb. brevis* strain LB3 and spring-growth ryegrass; for comparison the response for *Pediococcus* sp. PI(1) at one harvest is included. The marked response to lysine with the wilted grass may be noted.

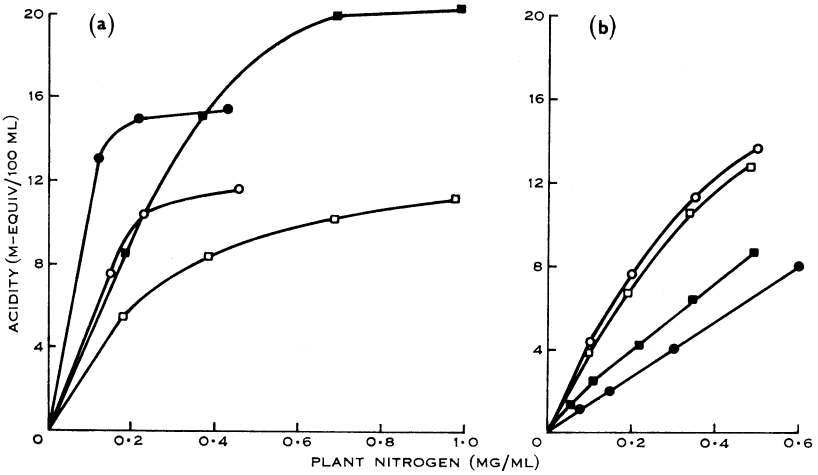

Fig. 6.—Response of *Pediococcus* sp. to extracts of ryegrass with (solid symbols) and without (open symbols) post-harvest wilting for 24 hr. Other data as follows:

		Date of Harvest	Moisture Content (%)	Nitrogen Content (%)	Non-protein Nitrogen (%)
(*a*)	○	7.x.58	624	3·36	0·74
	●	7.x.58	320	3·29	0·91
	□	30.ix.59	538	4·27	0·77
	■	30.ix.59	137	4·31	0·95
(*b*)	○	26.x.64	302	1·60	0·16
	●	26.x.64	98	1·60	0·30
	□	11.xi.64	259	1·53	0·17
	■	11.xi.64	120	1·50	0·31

In these samples, free lysine concentration in the juice was found to be 0·25, 0·18, and 0·25 μmole/ml for the first, second, and third harvests respectively. Related to the initial moisture contents of the samples, these concentrations had changed to the equivalents of 0·26, 0·21, and 0·41 μmoles/ml respectively after a 24-hr period of aerobic wilting.

In Table 6 are summarized the acid yields obtained for four strains of lactic acid bacteria when grown with extracts of plants as nitrogen source. In this table the concentration of plant nitrogen in assay media is related to the concentration of "non-protein nitrogen" calculated to exist in the plant moisture with the assumption that the nitrogen is uniformly spread through the plant water. Acid yields were

TABLE 6

ACID PRODUCTION BY LACTIC ACID BACTERIA GROWN WITH PLANT EXTRACTS AS NITROGEN SOURCE

Plant Extract Used	Harvest Date	Moisture Content (as % of dry matter)	Nitrogen Content (as % of dry matter)	Non-protein Nitrogen in Plant Moisture (μg/100 g)	Assay Concn. (as % of concn. in plant moisture)	Titratable Acidity (m-equiv/100 ml)				Acid Yield as Lactic Acid* (as % of plant dry matter)			
						Lb. plantarum Strain LP3	*Lb. brevis* Strain LB3	*Pediococcus* sp.		*Lb. plantarum* Strain LP3	*Lb. brevis* Strain LB3	*Pediococcus* sp.	
								Strain PI (1)	Strain PIII (1)			Strain PI (1)	Strain PIII (1)
White clover	13.ii.57	428	3·70	820	50	20·1		11·5	20·7	7·6		4·3	7·8
					20	17·4		9·3	17·0	6·6		3·5	6·4
	12.iii.64	483	3·43	720	67		12·8				5·4		
					20		7·9				3·3		
Lucerne	12.iii.64	336	3·82	1120	72	18·4	15·1			5·4	4·4		
					24	14·6	12·1			4·3	3·5		
Ryegrass	30.ix.59	538	4·27	1500	100	24·0	11·6	11·6	15·5	11·6		5·6	7·5
					50	21·9		10·1	15·3	10·6		4·9	7·4
	26.x.59	510	2·37	600	100	27·2		8·6	18·1	12·0		3·8	8·0
					50	23·3		7·0	13·4	10·3		3·1	5·9
	16.xi.59	305	1·59	1000	100	29·2		8·7	18·9	7·7		2·3	5·0
					50	25·0		6·8	15·9	6·6		1·8	4·2
	14.x.64	292	2·01	714	54	23·8	14·2	9·7		6·0	3·6	2·4	
					33	21·3	12·5	4·4		5·4	3·2	1·1	
	26.x.64	302	1·60	530	93	22·9	15·6	12·3		6·0	4·1	3·2	
					37	20·2	11·8	6·6		5·3	3·1	1·7	
	11.xi.64	259	1·53	656	72	23·2	16·9	13·7		5·2	3·8	3·1	
					30	20·8	12·5	7·6		4·7	2·8	1·7	

* Calculated from the expression $[(A \times 0{\cdot}09 \times M)/(S \times 100)]$, where A = titratable acidity (m-equiv/100 ml), M = plant moisture content (as a percentage of plant dry matter), and S = specific gravity of the plant juice.

calculated in terms of plant weight by taking all the titrated acid to be lactic, and by using observed values within the range 1·02–1·04 for the specific gravity of the plant juice. This method of calculation gives high yields of acid for the more moist samples; in ensilage practice much of this moisture would be lost as effluent.

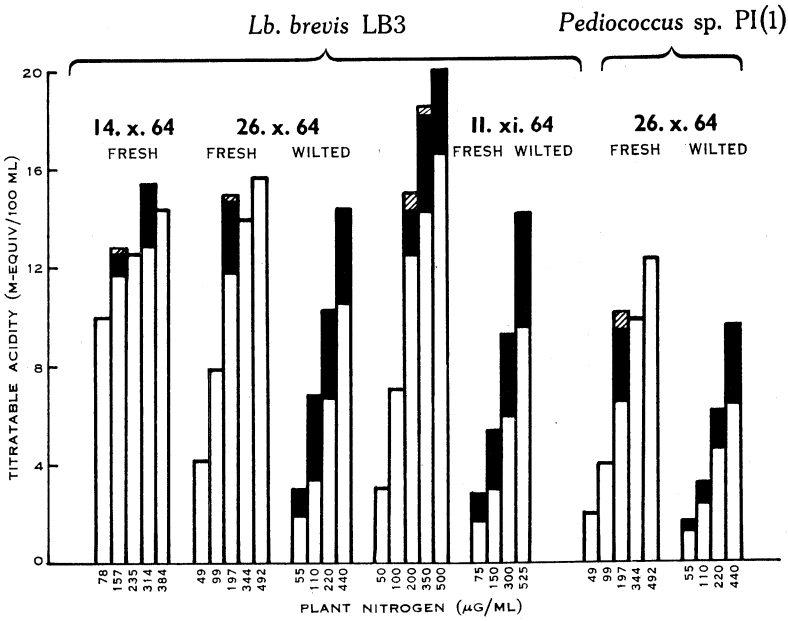

Fig. 7.—Response to supplementation of ryegrass extract with lysine (shaded) and to arginine in addition to lysine (cross-hatched). Amino acids were added to a final concentration of 2 μmoles/ml. Where no response to lysine is shown, the lysine treatment was not given. The arginine treatment was not given to the extracts of wilted grass but was given to each of the extracts of fresh grass which were treated with lysine.

IV. Discussion

The amino acids found to be essential for the strains of lactic-acid-producing bacteria studied show close similarities to those reported as essential for related organisms (Jensen and Seeley 1954; Deshpande and Desai 1962; Scardovi and Bottazzi 1962). In this regard, then, it is likely that the strains studied are representative of silage types.

The quantitative requirements of the bacteria for the various amino acids cannot be defined in general terms but only in terms of the medium used in the assay. For this reason the amino acid substrate in the basal medium was designed to reflect the type of amino acid balance commonly encountered in the free amino acid pool in plant leaves. The fact that the quantitative requirement for an amino acid tends to reflect the proportion of this amino acid in the bacterial protein (Spector 1956) is noteworthy. Phenylalanine which appears to be used very efficiently, lysine which is required for the cell walls, and arginine which is rapidly catabolized by the cells

(Brady 1966) depart from this correlation. For *Lb. plantarum*, which is able to synthesize many of the amino acids, it was not possible to define quantitative requirements for each of the acids. Though this organism may be better able to cover individual deficiencies, nonetheless there is a favourable response to a liberal and balanced supply of the amino acids [Fig. 1(*b*)].

A comparison of the requirements of the bacteria with the likely substrate concentrations (Table 5) indicates that many of the amino acids will be in excess, while others and especially the basic amino acids may well be deficient. The importance of amino acid balance in bacterial nutrition has often been stressed (cf. Dunn and McClure 1950) and the results of Figure 1 and Figure 5 demonstrate that neither with amino acid mixtures nor with plant extracts does improved quantity readily compensate for poor quality.

The occurrence of bound amino acids in plant leaves (Synge 1951; Synge and Wood 1958; Carnegie 1961) and the ability of bacteria to use some peptides more efficiently than free amino acids (Kihara and Snell 1960) makes inadequate a consideration of amino acid nutrition only in terms of the free amino acids required and those available. Direct assays of plant extracts have been made only with acid yield as an index of growth. The results of Figure 4 indicate that with *Lb. brevis* the acid yield may bear little relation to the cell population, but it remains to be demonstrated that this conclusion is valid under the mixed culture conditions prevailing during ensilage.

The results in Figure 7 clearly demonstrate that lysine may be a primary deficiency in plant juice, and this deficiency may be increased by a period of post-harvest wilting. When the lysine deficiency has been overcome other amino acids appear to be limiting and in certain cases the supply of arginine was shown to be suboptimal. The decrease in quality after a wilting period recorded in this experiment contrasted with improvements in quality after wilting noted in some earlier experiments (Fig. 6). An experimental difference lay in the nitrogen content of the respective crops, this being 1·5–2·0% when the decrease in quality occurred, and 2·4–4·0% when improved quality resulted from the wilting treatment.

In some samples assayed, and notably with immature ryegrass, and the low-nitrogen ryegrass of 1964, the quality of the nitrogen substrate provided was inadequate to allow the achievement of high acid yields. However, in most samples assayed, the strains that produced most acid in the medium gave acid yields sufficient to ensure good ensilage (Table 6), with concentrations of nitrogen substrate much lower than those in the plant juice. On the other hand, improvement in the acid yield always resulted from an increased substrate level. Interpretation is dependent on (1) whether conditions in the silo are such as to allow expression of the improved yield, and (2) to what extent the competing facultative flora immobilizes the substrate nitrogen.

The buffering of the assay medium is greater than the buffering of plant juices in the ensilage environment, so that high acid yields do not cause the same pH fall. On the other hand, the homofermentative bacteria at least are tolerant of low pH. However, acid yields improve considerably when extracted grass juices are buffered, and it seems likely that in the ensilage environment some of the improved acid yields with high levels of nitrogen substrate will find no expression.

There appears to be no information available on the amount of nitrogen converted to bacterial nitrogen in silage. In the assay media, considerably less than 20% of the nitrogen supplied is converted to bacterial nitrogen. In grass silage, bacterial numbers reach a maximum in 24–48 hr (Stirling 1951; Kroulik *et al.* 1955). The major increase in bacterial nitrogen would be at the same time. A considerable increase in soluble non-protein and amino nitrogen occurs in ensilage during this period (Kemble 1956). This increase is not greater in the presence of bacteriocides (Brady 1960), and appears to proceed evenly without lag or inversion phases. Thus uptake of nitrogen would not seem to account for any considerable proportion of the nitrogen released unless protein hydrolysis by the bacteria balanced their nitrogen uptake.

The peak value of bacterial numbers found in silage is of the order of 10^9 per gram dry weight. Since this value is obtained very early during ensilage, the viable count will be a high proportion of the total count (Stirling 1951). Assuming the high value of $2{\cdot}5\times10^{-10}$ mg nitrogen per cell (Stephenson 1949), the bacterial nitrogen will be $2{\cdot}5\times10^{-1}$ mg per gram dry weight, or less than 2% of the total nitrogen of mature ryegrass, and about 10% of the initial non-protein nitrogen of such grass. Growth of lactic acid bacteria occurs early in ensilage (Allen *et al.* 1937; Kempton and San Clemente 1959) so that they would be assimilating nitrogen before any large part of the available nitrogen was bound by other organisms. It seems likely then that the lactic acid bacteria will have a high proportion of the amino acids in the plant juice available to them.

From the available evidence, it would seem a valid conclusion that non-protein nitrogen of ryegrass, clover, and lucerne tops at the maturities harvested for ensilage is usually an adequate nitrogen source for the production of the amounts of acid normally found in silage. Ryegrass of low nitrogen content may not, however, provide an adequate substrate. Information on such factors as the performance of lactic acid bacteria in mixed culture, and the possible need for the development in the silo of organisms other than those most productive of acid in laboratory media may indicate some modification of this conclusion.

V. References

Allen, L. A., Harrison, J., Watson, S. J., and Ferguson, W. S. (1937).—*J. Agric. Sci.* **27**: 271–93.

Barnett, A. J. G. (1954).—"Silage Fermentation." (Butterworths Scientific Publications: London.)

Brady, C. J. (1960).—*J. Sci. Fd. Agric.* **11**: 276–84.

Brady, C. J. (1961).—*Biochem. J.* **78**: 631–40.

Brady, C. J. (1966).—*Aust. J. Biol. Sci.* **19**: 123–30.

Carnegie, P. R. (1961).—*Biochem. J.* **78**: 697–707.

Deshpande, V. G., and Desai, D. B. (1962).—*Ind. J. Med. Res.* **50**: 453–9.

Dunn, M. S., Shankman, S., Camien, M. N., and Block, Harriette (1947).—*J. Biol. Chem.* **168**: 1–22.

Dunn, M. S., and McClure, L. E. (1950).—*J. Biol. Chem.* **184**: 223–33.

Greenhill, W. L. (1964).—*J. Brit. Grassl. Soc.* **19**: 30–7.

Jensen, E. M., and Seeley, H. N. (1954).—*J. Bact.* **67**: 484–8.

Keddie, R. M. (1959).—*J. Appl. Bact.* **22**: 403–16.

Kemble, A. R. (1956).—*J. Sci. Fd. Agric.* **7**: 125–30.

KEMBLE, A. R., and MACPHERSON, H. T. (1954).—*Biochem J.* **58**: 46–9.
KEMPTON, A. G., and SAN CLEMENTE, C. L. (1959).—*Appl. Microbiol.* **7**: 362–7.
KIHARA, H., and SNELL, E. E. (1955).—*J. Biol. Chem.* **212**: 83–94.
KIHARA, H., and SNELL, E. E. (1960).—*J. Biol. Chem.* **235**: 1409–14.
KROULIK, J. T., BURKEY, L. A., GORDON, C. H., WISEMAN, H. G., and MELIN, C. G. (1955).—*J. Dairy Sci.* **38**: 263–72.
LANDIS, J. (1960).—Proc. 8th Int. Grassld. Congr. Reading, 1960. pp. 625–8.
LANGSTON, C. W., ET AL. (1958).—Tech. Bull. U.S. Dep. Agric. No. 1187.
LANIGAN, G. W. (1963).—*Aust. J. Biol. Sci.* **16**: 606–15.
MABBITT, L. A. (1951).—*Proc. Soc. Appl. Bact.* **14**: 147–50.
MACPHERSON, H. T. (1952).—*J. Sci. Fd. Agric.* **3**: 362–5.
MACPHERSON, H. T., and SLATER, J. S. (1959).—*Biochem. J.* **71**: 654–60.
MASLOWSKI, P., MINAKOWSKI, W., and HO, K. G. (1962).—*Roczn. Nauk Roln.* A**87**: 99–106.
MCKENZIE, H. A., and WALLACE, H. S. (1954).—*Aust. J. Chem.* **7**: 55–70.
SCARDOVI, V., and BOTTAZZI, V. (1962).—*Ann. Microbiol.* **12**: 85–98.
SPECTOR, W. S. (ED.) (1956).—"Handbook of Biological Data." p. 90. (W. B. Saunders Company: Philadelphia.)
STEPHENSON, MARJORY (1949).—"Bacterial Metabolism." 3rd Ed. (Longmans, Green & Co.: London.)
STIRLING, ANNA C. (1951).—*Proc. Soc. Appl. Bact.* **14**: 151–6.
SYNGE, R. L. M. (1951).—*Biochem. J.* **49**: 642–50.
SYNGE, R. L. M., and WOOD, J. C. (1958).—*Biochem. J.* **70**: 321–39.