SOME INTERRELATIONSHIPS OF AMINO ACIDS IN THE NUTRITION OF *LEUCONOSTOC MESENTEROIDES*[1]

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In 1939 Gladstone clearly demonstrated the phenomenon of amino acid antagonism and showed its relationship to the nutrition of the pathogen *Bacillus anthracis*. Among the interrelationships involving specific amino acids was the ability of threonine to remove the toxic effects of serine and vice versa. Snell and Guirard (1943) noted the inhibition of growth of *Streptococcus lactis* by glycine, serine, threonine, or β-alanine and its removal by alanine or pyridoxine. Maas and Davis (1949) found that glycine and DL-alanine completely antagonized the inhibition of *Escherichia coli* by D-serine. Camien and Dunn (1950) studied the ability of glycine and serine to inhibit the utilization of L-, D-, and DL-alanine and the reversal of this inhibition by higher levels of alanine. An antagonistic effect of glycine upon L-alanine in the nutrition of *Lactobacillus casei* has been shown by Kihara and Snell (1952). The inhibitory nature of D- or L-alanine for L-serine has also been noted (Prescott *et al.*, 1953) with *Lactobacillus delbrueckii*.

With *Leuconostoc mesenteroides* strain P-60, it has been impossible to completely remove the lag in growth at low levels of threonine by lowering the serine content (Horn *et al.*, 1947), or the lag in growth at low levels of serine by lowering the threonine content (Meinke and Holland, 1948). Moreover, an induction period in the response of *L. mesenteroides* to glycine has been reported (Shankman *et al.*, 1947). In the present study an attempt has been made to evaluate the effect of antagonisms involving glycine, L-serine, L-threonine, and L-alanine upon the growth of *L. mesenteroides*.

EXPERIMENTAL METHODS

L. mesenteroides strain P-60 (ATCC-8042) was carried on yeast-extract-peptone-glucose agar and transferred weekly after passage through broth of similar composition at 37 C.[2] Inocula were prepared from 18-hr cultures grown at 37 C in transfer broth. Cells were spun down, washed once with saline, and diluted with saline to a reading of 70 per cent T at 550 mμ in a Beckman Model B spectrophotometer. One drop of this suspension from a 5-ml pipette served as the inocula. After autoclaving for 10 min at 115 C, tubes were inoculated and incubated at 37 C. Growth was measured turbidimetrically (Bausch and Lomb, Spectronic 20) after a suitable incubation period. The basal medium was that of Williams (1955) except that L-hydroxyproline, and L-cysteine were omitted while glycine, L-serine, L-threonine, and L-alanine were included at one of the three levels tested (0.01 mg/ml, 0.1 mg/ml, 1.0 mg/ml).[3] Total tube volume was 8 ml.

RESULTS

Typical data illustrating the response of *L. mesenteroides* to each of the 81 different media, necessary to test simultaneously all possible combinations of L-alanine, glycine, L-threonine, and L-serine at the three concentrations of 0.01

[1] The culture used in these investigations is catalogued as *Leuconostoc mesenteroides* strain P-60 (culture no. 8042) by the American Type Culture Collection. In not fermenting sucrose the culture fails to conform with one of the primary characteristics of *L. mesenteroides* as described in the sixth edition of *Bergey's Manual of Determinative Bacteriology*. Investigations are being sponsored by the American Type Culture Collection to properly classify the organism.

[2] Peptone (Difco), 1 g; yeast extract, 0.2 g; NaAcetate, 1.2 g; glucose, 2 g; salt A, 1 ml (50 g K_2HPO_4; 50 g KH_2PO_4; 500 ml H_2O); salt B, 1 ml (20 g $MgSO_4 \cdot 7H_2O$; 1 g NaCl; 1 g $FeSO_4 \cdot 7H_2O$; 1 g $MnSO_4 \cdot 4H_2O$; 500 ml H_2O); water, 200 ml.

[3] L-Glutamic at 0.5 mg/ml; L-arginine HCl, glycine, L-histidine HCl at 0.2 mg/ml; L-alanine, L-asparagine, L-aspartic, L-isoleucine, L-leucine, L-lysine HCl, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine at 0.1 mg/ml; L-cystine at 0.05 mg/ml.

TABLE 1

Response of Leuconostoc mesenteroides strain P-60 after incubation at 37 C for 18 hr to all possible combinations of 3 concentrations of L-serine, L-alanine, glycine, and L-threonine

Media Concentration of Glycine, L-Alanine, L-Threonine, L-Serine-mg/ml											Σ L-Alanine			Σ L-Threonine			Glycine-L-Serine*
	L-Alanine	0.01			0.1			1.0									
Glycine	L-Serine	L-Threonine									0.01	0.1	1.0	0.01	0.1	1.0	
		0.01	0.1	1.0	0.01	0.1	1.0	0.01	0.1	1.0							
		Optical Density															
	0.01	0.95	1.10	0.52	0.95	1.10	0.43	0.58	0.81	0.07	2.57	2.48	1.46	2.48	3.01	1.02	6.51
0.01	0.1	0.77	1.10	0.95	0.47	1.10	0.90	0.05	0.43	0.27	2.82	2.47	0.75	1.29	2.63	2.12	6.04
	1.0	0.00	0.25	0.11	0.00	0.24	0.08	0.00	0.11	0.04	0.36	0.32	0.15	0.00	0.60	0.23	0.83
	0.01	0.90	1.00	0.36	0.90	1.10	0.30	0.53	0.86	0.14	2.26	2.30	1.53	2.33	2.96	0.80	6.09
0.1	0.1	0.21	1.10	1.20	0.12	1.20	1.20	0.04	1.20	1.20	2.51	2.52	2.44	0.37	3.50	3.60	7.47
	1.0	0.00	1.10	1.20	0.00	1.20	1.20	0.01	1.20	1.20	2.30	2.40	2.41	0.01	3.50	3.60	7.11
	0.01	0.35	0.43	0.10	0.44	0.43	0.08	0.13	0.32	0.10	0.88	0.95	0.55	0.92	1.18	0.28	2.38
1.0	0.1	0.03	1.10	1.20	0.03	1.20	1.20	0.02	1.20	1.20	2.33	2.43	2.42	0.08	3.50	3.60	7.18
	1.0	0.00	1.10	1.20	0.00	1.20	1.20	0.01	1.20	1.20	2.30	2.40	2.41	0.01	3.50	3.60	7.11
											*L-Alanine-Glycine**			*L-Threonine-Glycine**			*Glycine†*
	0.01	1.72	2.45	1.58	1.42	2.44	1.41	0.63	1.35	0.38	5.75	5.27	2.36	3.77	6.24	3.37	13.38
ΣGlycine	0.1	1.11	3.20	2.76	1.02	3.50	2.70	0.58	3.26	2.54	7.07	7.22	6.38	2.71	9.96	8.00	20.67
	1.0	0.38	2.63	2.50	0.47	2.83	2.48	0.16	2.72	2.50	5.51	5.78	5.38	1.01	8.18	7.48	16.67
											*L-Alanine-L-Serine**			*L-Threonine L-Serine**			*L-Serine†*
	0.01	2.20	2.53	0.98	2.29	2.63	0.81	1.24	1.99	0.31	5.71	5.73	3.54	5.73	7.15	2.10	14.98
ΣL-Serine	0.1	1.01	3.30	3.35	0.62	3.50	3.30	0.11	2.83	2.67	7.66	7.42	5.61	1.74	9.63	9.32	20.69
	1.0	0.00	2.45	2.51	0.00	2.64	2.48	0.02	2.51	2.44	4.96	5.12	4.97	0.02	7.60	7.43	15.05
											L-Alanine†			*L-Threonine†*			
L-Alanine-L-Threonine*		3.21	8.28	6.84	2.91	8.77	6.59	1.37	7.33	5.42	18.33	18.27	14.12	7.49	24.38	18.85	

* First order interactions—summed values were divided by 9 to correspond to original data before plotting in figures 2, 5, 7, 9, and 11.

† Main effects—summed values were divided by 27 to correspond to original data before plotting in figure 1.

mg/ml, 0.1 mg/ml, and 1.0 mg/ml, are presented in table 1. To obtain the main effects of amino acids as well as any interactions between individual amino acids, the data have been gathered in a form normally employed for an analysis of variance. Because of the complexity of the table the effects have been depicted in graphical form.

Main effects. Curves illustrating the main effect of glycine, L-serine, L-threonine, and L-alanine are shown in figure 1. All four of the amino acids were inhibitory at a level of 1.0 mg/ml, while L-serine, glycine, and L-threonine gave suboptimal growth at 0.01 mg/ml.

Interaction between L-serine and L-threonine. Figure 2 demonstrates the interaction between L-serine and L-threonine. When L-serine was present at 0.01 mg/ml, raising the concentration of L-threonine from 0.01 mg/ml to 0.1 mg/ml gave a slight increase in growth. A further tenfold increase in L-threonine concentration to 1.0 mg/ml became inhibitory. At the intermediate and highest levels of L-serine, the initial inhibition of growth, presumably due to the antagonism of L-threonine by L-serine, was overcome by the addition of more L-threonine. The position of the curve at the highest level of L-serine suggests that the reduced optical density is a reflection of other antagonistic relationships involving L-serine.

The effect of substituting glycyl-DL-threonine and/or glycyl-DL-serine for one of the antagonistic pairs of amino acids has been examined. In

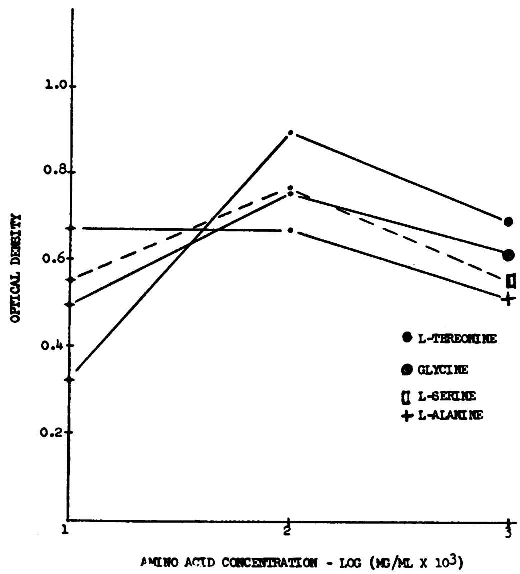

Figure 1. Main effects of individual amino acids in the nutrition of *Leuconostoc mesenteroides* strain P-60.

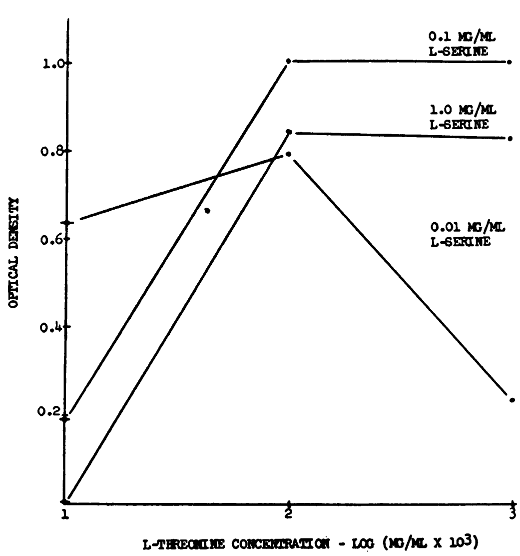

Figure 2. Interaction between L-serine and L-threonine in the nutrition of *Leuconostoc mesenteroides* strain P-60.

the presence of 1.0 mg/ml of L-serine, glycyl-DL-threonine was considerably more active in promoting growth of *L. mesenteroides* than L-threonine (figure 3). Similar tests made of the ability of glycyl-DL-serine to serve as a source of serine in the presence of 2.0 mg/ml of L-threonine gave comparable results (figure 4).

Interaction of glycine with L-serine and L-threonine. Figure 5 shows the interaction of glycine with L-serine. Supplementing the medium with glycine depressed growth when L-serine was

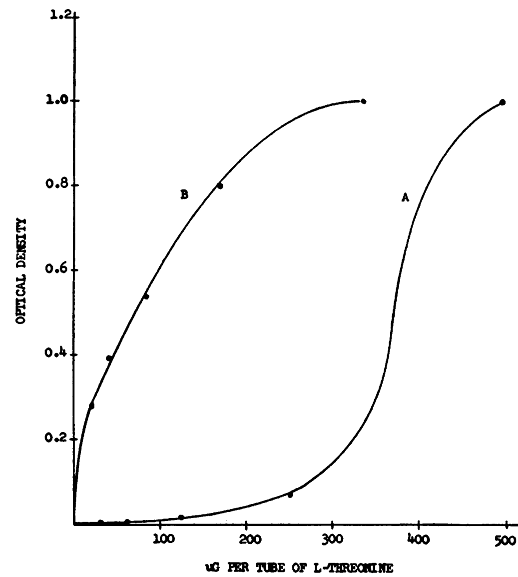

Figure 3. Response of *Leuconostoc mesenteroides* strain P-60 to L-threonine (curve *A*) and glycyl-DL-threonine (curve *B*) in the presence of 1.0 mg/ml of L-serine. Glycine and L-alanine are present at 0.37 mg/ml.

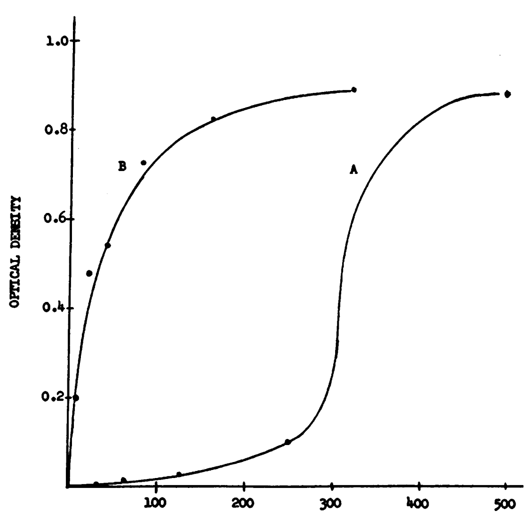

Figure 4. Response of *Leuconostoc mesenteroides* strain P-60 to L-serine (curve *A*) and glycyl-DL-serine (curve *B*) in the presence of 2.0 mg/ml of L-threonine. Glycine and L-alanine are present at 0.37 mg/ml.

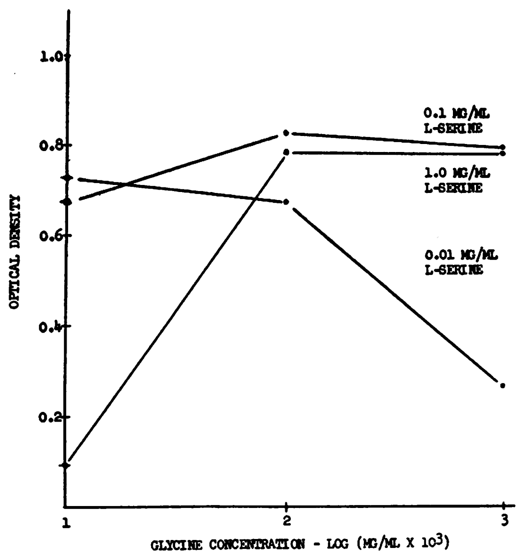

Figure 5. Interaction between glycine and L-serine in the nutrition of *Leuconostoc mesenteroides* strain P-60.

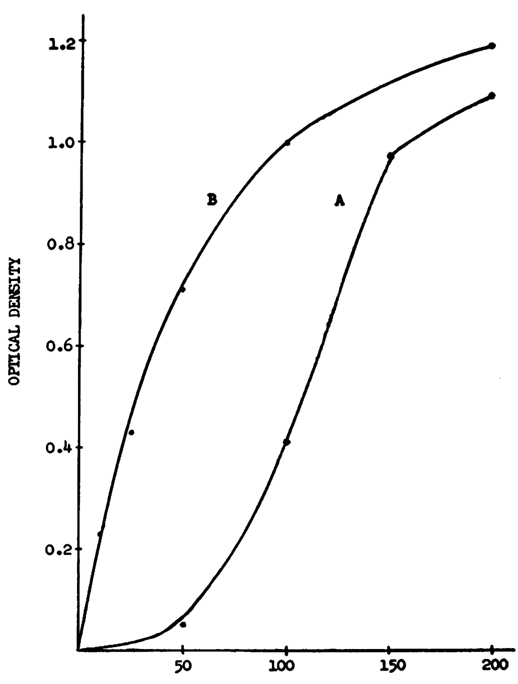

Figure 6. Response of *Leuconostoc mesenteroides* strain P-60 to glycine (curve *A*) and glycylglycine (curve *B*) in the presence of 1.0 mg/ml of L-serine. L-threonine and L-alanine are present at 0.37 mg/ml.

present at 0.01 mg/ml. The inhibitory effect of glycine was readily overcome by increasing the level of L-serine. Moreover, inhibition of glycine by L-serine was absent at higher glycine concentrations. Thus, it would appear that glycine and L-serine are mutually antagonistic.

A comparison was made of the growth promoting ability of glycylglycine and glycine in the presence of 1.0 mg/ml of L-serine. The pronounced lag in the response of *L. mesenteroides* to increasing increments of the free amino acid was absent when the peptide was substituted for glycine (figure 6).

The relationship of glycine to L-threonine was found to be very similar to that of glycine and L-serine. As shown in figure 7, glycine and L-threonine antagonized each other. In addition, glycylglycine was much more efficient than glycine in promoting growth of *L. mesenteroides* strain P-60 in the presence of 2.0 mg/ml of L-threonine (figure 8). Again, half maximum growth was obtained with 50 μg of glycylglycine or 125 μg of glycine. Since the amount of glycine required for half maximum growth in the presence of 1.0 mg/ml of L-serine or 2.0 mg/ml of L-threonine was the same, L-serine was quantitatively more effective in inhibiting glycine than L-threonine.

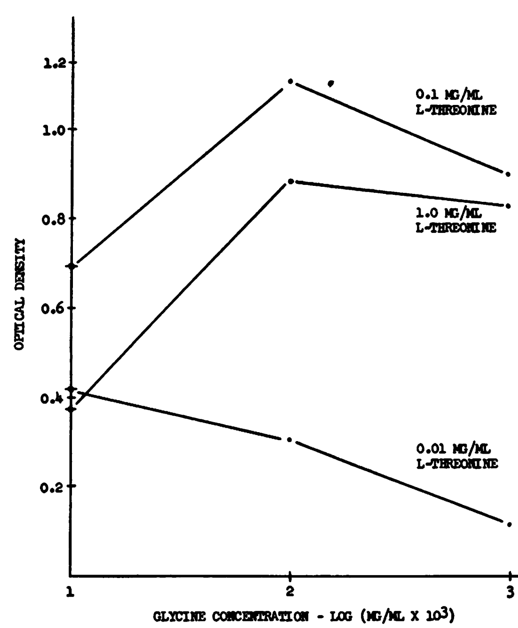

Figure 7. Interaction between glycine and L-threonine in the nutrition of *Leuconostoc mesenteroides* strain P-60.

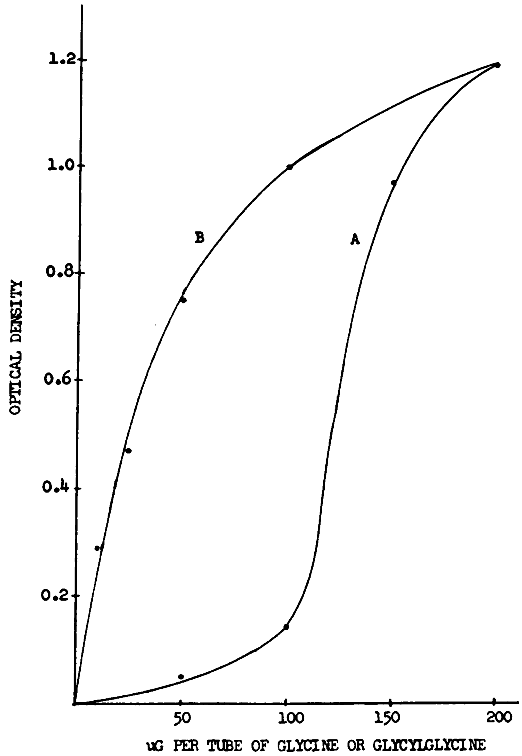

Figure 8. Response of *Leuconostoc mesenteroides* strain P-60 to glycine (curve *A*) and glycylglycine (curve *B*) in the presence of 2.0 mg/ml of L-threonine. L-serine and L-alanine are present at 0.37 mg/ml.

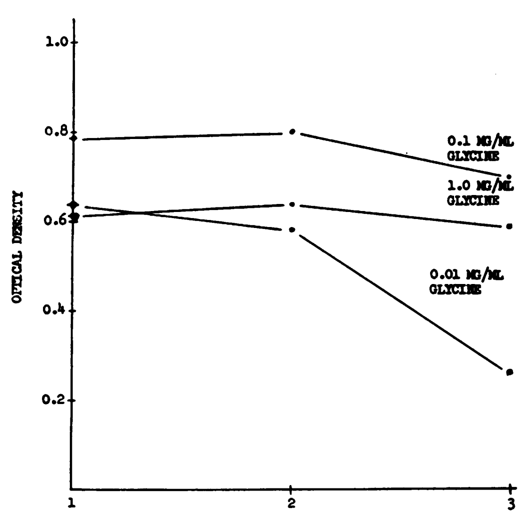

Figure 9. Interaction between glycine and L-alanine in the nutrition of *Leuconostoc mesenteroides* strain P-60.

Interaction of L-alanine with glycine and L-serine. When glycine was included in the medium at 0.01 mg/ml, L-alanine effectively inhibited the growth of *L. mesenteroides* strain P-60 (figure 9). This effect of L-alanine was essentially absent at the second and third levels of glycine. Unlike the serine-threonine, glycine-threonine, and glycine-serine interactions, glycine had little effect upon the utilization of L-alanine. The response of *L. mesenteroides* to glycylglycine and glycine in the presence of 1.0 mg/ml of L-alanine showed the peptide to be much more efficient (figure 10). Half maximum growth was obtained with 50 μg of peptide as compared to 125 μg of glycine.

Figure 11 illustrates the interaction of L-alanine with L-serine. It is apparent that the inhibition of L-serine by L-alanine was completely overcome by the addition of more L-serine. This relationship is similar to that of L-alanine and glycine in that L-alanine did not reverse growth inhibition due to L-serine.

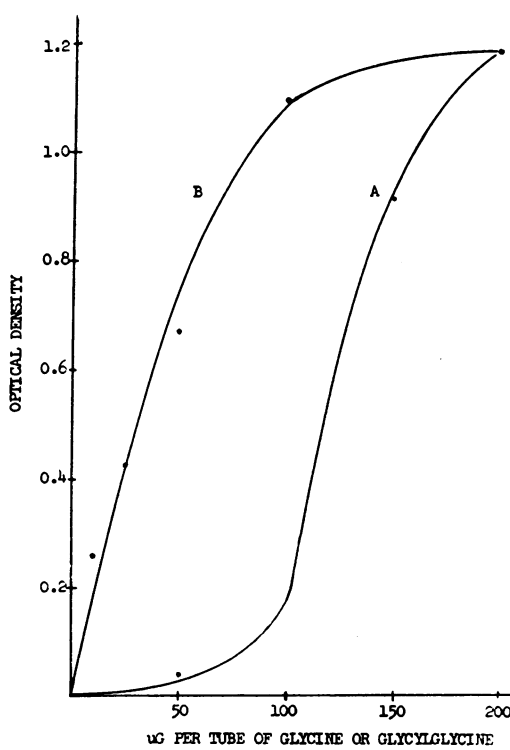

Figure 10. Response of *Leuconostoc mesenteroides* strain P-60 to glycine (curve *A*) and glycylglycine (curve *B*) in the presence of 1.0 mg/ml of L-alanine. L-serine and L-threonine are present at 0.37 mg/ml.

Noninhibitory nature of glycylglycine as compared to glycine. Table 2 presents data comparing the effect of glycine and glycylglycine upon growth of *L. mesenteroides* under conditions found to be most applicable for demonstration of glycine inhibition. Glycine almost completely suppresses growth at 1.0 mg/ml while supplements of the peptide up to 2.0 mg/ml give equal or greater growth than the control.

Hydrolysis of peptides by resting cells of L. mesenteroides. Figure 12 shows a paper chromatogram of incubation mixtures containing washed cells of *L. mesenteroides* plus the appropriate peptide. The mixtures, which contained 0.5 ml

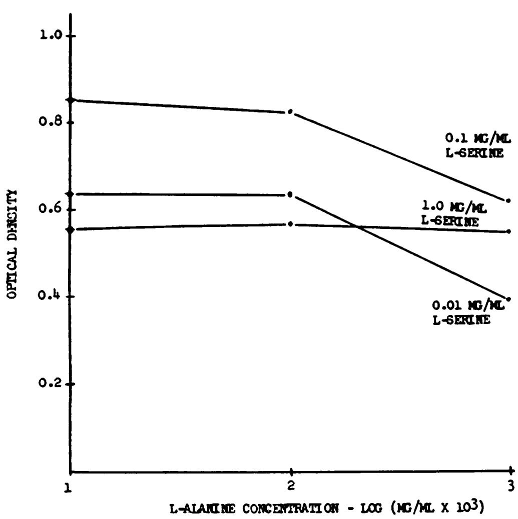

Figure 11. Interaction between L-alanine and L-serine in the nutrition of *Leuconostoc mesenteroides* strain P-60.

TABLE 2

Growth of Leuconostoc mesenteroides strain P-60 in the presence of glycine and glycylglycine

Tube	Additions	Optical Density
1	Control*	0.66
2	0.1 mg/ml glycine	0.12
3	1.0 mg/ml glycine	0.02
4	0.1 mg/ml glycylglycine	0.67
5	1.0 mg/ml glycylglycine	0.74
6	2.0 mg/ml glycylglycine	0.75

* L-Alanine, L-threonine, Glycine (0.01 mg/ml); L-serine (0.1 mg/ml). Additional amino acids present at levels employed in the basal medium.

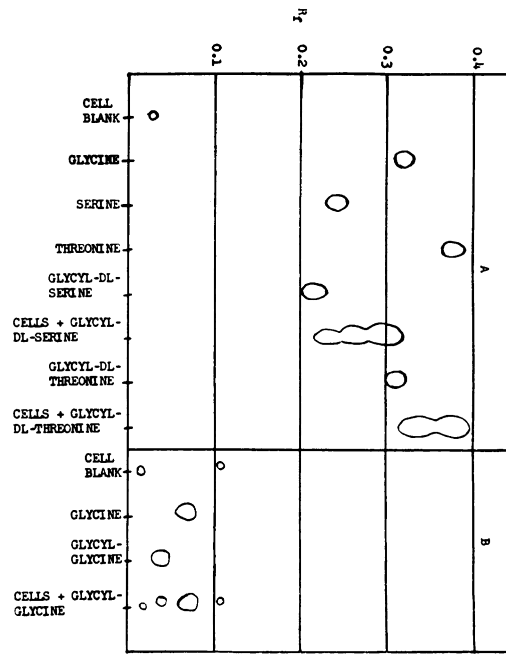

Figure 12. Paper chromatogram showing hydrolysis of peptides by resting cells of *Leuconostoc mesenteroides* strain P-60. (*A*) Whatman no. 1 filter paper and water saturated phenol. (*B*) Whatman no. 1 filter paper and 80 ml *n*-butanol, 20 ml 95 per cent ethanol, 20 ml water. Chromatograms were sprayed with 0.2 per cent ninhydrin in water saturated *n*-butanol.

of cell suspension (18-hr culture washed 3 times with saline and resuspended in 5 ml of saline + 0.5 ml of M/50 phosphate buffer pH 7 containing 1 mg/ml of peptide), were incubated 18 hr at 37 C.

DISCUSSION

The above findings serve to re-emphasize the complex nature of the interrelationships among essential nutrients. Through application of factorially designed experiments, it has been possible to demonstrate the simultaneous action of five different antagonisms involving glycine, L-serine, L-threonine, and L-alanine, upon growth of *L. mesenteroides*. With a clearer picture of the antagonisms involved, considerable insight has been gained into the cause of the previously noted induction period in the response of *L. mesenteroides* to glycine (Shankman *et al.*, 1947) and the inability to remove completely the lag in the serine and threonine response curves by lowering the threonine and serine, respectively (Horn *et al.*, 1947; Meinke and Holland, 1948).

The greater activity of glycyl-DL-serine, glycyl-DL-threonine, and glycylglycine, as compared to L-serine, L-threonine and glycine in the several antagonisms tested, may be cited as additional examples of a phenomenon which appears to be quite general (Kihara and Snell, 1952, 1955). Since the peptides tested in this investigation were readily hydrolyzed by resting cells of *L. mesenteroides*, they have uniqueness only outside the cell. Therefore, it is not unreasonable to suppose that in these antagonisms the free amino acid is antagonized during the absorption process, whereas the peptide passes uninhibited. The noninhibitory nature of glycylglycine under conditions where glycine is markedly inhibitory adds support to this idea and indicates an additional condition under which appropriate peptides may surpass their component amino acids in promoting the growth of bacteria.

SUMMARY

A method has been described for simultaneously examining the effect of variation in the concentration of essential nutrients upon growth of a test organism. By this procedure it has been possible to show the influence of five different amino acid antagonisms upon growth of *Leuconostoc mesenteroides* strain P-60. These antagonisms include the amino acid pairs; L-threonine-L-serine; L-serine-glycine; L-threonine-glycine; L-alanine-glycine; and L-alanine-L-serine. In every case examined, peptides containing one of the antagonistic pairs proved more effective than the free amino acid in relieving these antagonisms.

REFERENCES

CAMIEN, M. N. AND DUNN, M. S. 1950 Antagonisms in the utilization of D-amino acids by lactic acid bacteria. II. Influence of DL-serine and glycine on the utilization of D-alanine. J. Biol. Chem., **185**, 553–559.

GLADSTONE, G. P. 1939 Interrelationships between amino acids in the nutrition of *Bacillus anthracis*. J. Exptl. Pathol., **20**, 189.

HORN, M. J., JONES, B. D., AND BLUM, A. E. 1947 Microbiological determination of threonine in proteins and food. J. Biol. Chem., **169**, 739–743.

KIHARA, H. AND SNELL, E. E. 1952 Peptides and bacterial growth. II. L-Alanine peptides and growth of *Lactobacillus casei*. J. Biol. Chem., **197**, 791–800.

KIHARA, H. AND SNELL, E. E. 1955 Peptides and bacterial growth. VII. Relation to inhibitions by thienylalanine, ethionine, and canavanine. J. Biol. Chem., **212**, 83–94.

MAAS, W. K. AND DAVIS, B. D. 1949 D-Serine inhibition of *E. coli* and its reversal by other amino acids and by mutation. Federation Proc., **8**, 221.

MEINKE, W. W. AND HOLLAND, B. R. 1948 Threonine-serine antagonisms in some lactic acid bacteria. J. Biol. Chem., **173**, 535–545.

PRESCOTT, J. M., PETERS, V. J., AND SNELL, E. E. 1953 Peptides and bacterial growth. V. Serine peptides and growth of *Lactobacillus delbrueckii* 9649. J. Biol. Chem., **202**, 533–540.

SHANKMAN, S., CAMIEN, M. N., AND DUNN, M. S. 1947 The determination of glycine in protein hydrolysates with *Leuconostoc mesenteroides* P-60. J. Biol. Chem., **168**, 51–59.

SNELL, E. E. AND GUIRARD, B. M. 1943 Some interrelationships of pyridoxine, alanine, and glycine and their effect on certain lactic acid bacteria. Proc. Natl. Acad. Sci. U. S., **29**, 66–77.

WILLIAMS, H. H. 1955 Essential amino acid content of animal feeds. Memoir 337, Cornell University, Agricultural Experiment Station, Ithaca, New York.