Summary. A strain of hog cholera virus has been procured that produced CPE in tissue cultures of pig kidney cells. This tissue-cultured virus produced typical hog cholera when inoculated into susceptible swine and it was neutralized by immune sera.

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Response of Lactic Acid Bacteria to Amino Acid Derivatives. V. L- DL- and D-Valic Acids.* (26216)

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It was recognized as early as 1947(2) that either L- or D-phenyllactic acid, but not D-phenylalanine, is an effective substitute for L-phenylalanine in *Lactobacillus arabinosus* nutrition. Corresponding relationships between the optical forms of phenyllactic acid and phenylalanine were subsequently shown to hold additionally for a variety of other lactic acid bacteria(3). Utilization by *L. arabinosus, Leuconostoc mesenteroides* and *Streptococcus faecalis* of various hydroxy acid analogs in place of the corresponding amino acids was reported by Holden, *et al.* (4), but these authors' data do not include determinations of optical specificity. It was desirable, therefore, to secure more complete information on utilization of optically active hydroxy acids other than phenyllactic acid. The present report concerns utilization of the valine analogs, L-, DL- and D-valic (α-hydroxyisovaleric) acids.

Methods. L- and DL-valic acids were synthesized from L- and DL-valines essentially as described by Baker and Meister(5). D-Valic acid was produced by resolution of DL-valic acid with cinchonine†. Microbiological procedures and medium were the same as those used previously(3) except L-phenylalanine, final concentration 20 mg %, was included in the medium and DL-valine, DL-norleucine and DL-norvaline were omitted. Bacterial cultures have been described(1,6).

Results. Preliminary screening of 10 bacteria revealed that *Lactobacillus arabinosus*

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† DL-Valic acid 10 g, cinchonine 25 g and dry acetone 50 ml were stirred with a magnetic stirrer for 3 hours in a closed flask. Filtration of mixture yielded 13 g of essentially pure D-valic acid-cinchonine salt. The product was washed with cold acetone to remove residual traces of the highly acetone-soluble L-salt. It is of interest that although this procedure was consistently successful for resolution of DL-valic acid, it appeared to be relatively ineffectual in resolving DL-leucic acid.

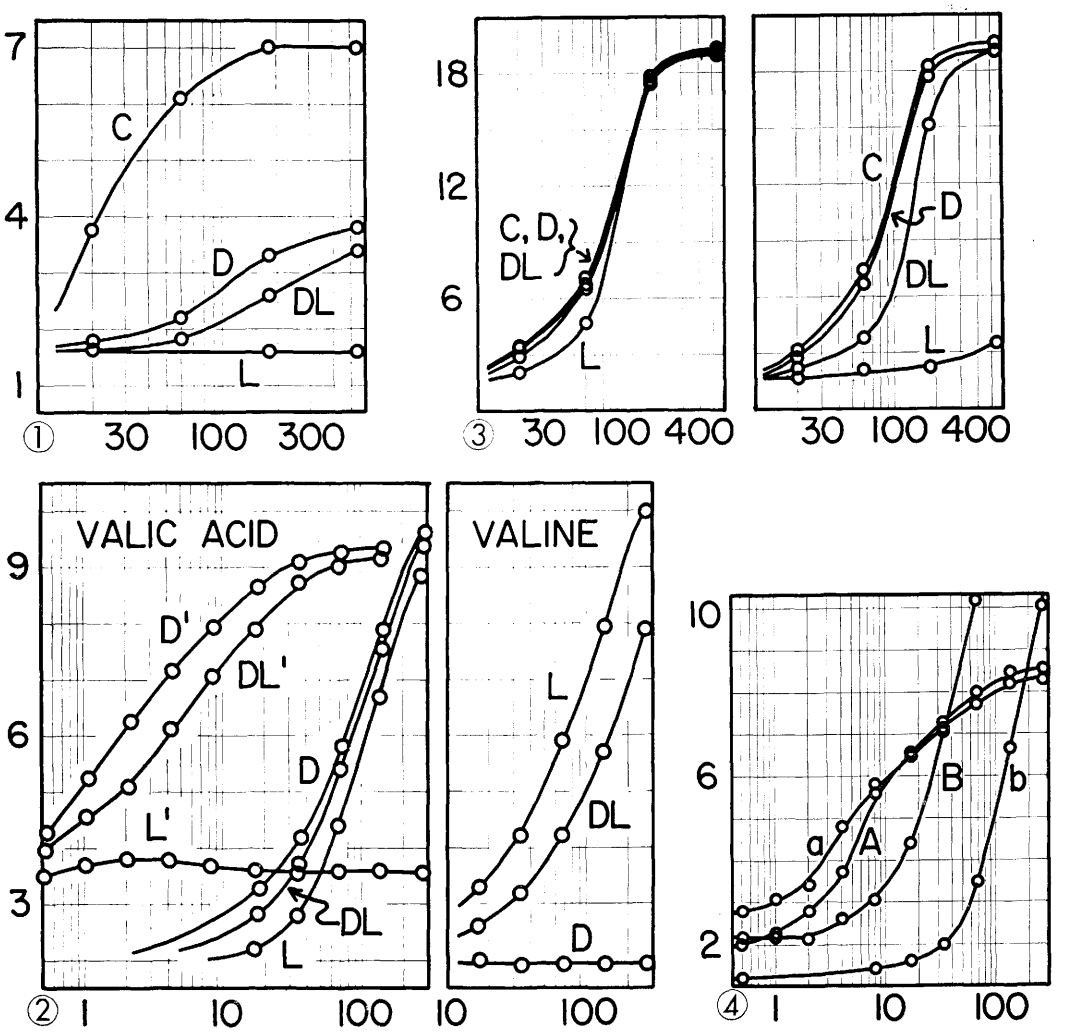

FIG. 1. Responses of *Streptococcus faecalis* 8043 to D-, DL- and L-valic acids (curves D, DL and L, respectively) and to the L-valine control (curve C). Incubation period was 72 hr. Response to L-valic acid remained negligible with incubation periods up to 10 days at test levels up to 4 μM/ml. D-Valic acid under the latter conditions yielded a maximal response nearly equal to that of the L-valine control. Values on the horizontal (logarithmic) scale represent valine and valic acid concentrations in mμM/ml; those on the vertical scale represent ml of 0.01 N sodium hydroxide required to titrate each ml of test culture.

FIG. 2. Responses of *Lactobacillus casei* 280-16A to D-, DL- and L-valic acids (left) and L-, DL- and D-valines (right). Curves D', DL' and L' were obtained when the described medium (see *Methods*) was supplemented with DL-valine; these responses therefore illustrate valic acid utilization as an α-hydroxy acid source rather than as a valine substitute. The remaining curves were obtained in the valine-free test medium supplemented with 1.5 μM of DL-lactic acid (as hydroxy acid source) per ml. The coordinates are on the same bases as in Fig. 1.

FIG. 3. Responses of *Lactobacillus mannitopoeus* to D-, DL- and L-valic acids (curves D, DL and L, respectively) and to the L-valine control (curves C) in the described medium (see *Methods*) with (right-hand figure) and without (left-hand figure) the addition of DL-α-hydroxybutyric acid at 34 μM/ml. The coordinates are on the same bases as in Fig. 1.

FIG. 4. Responses of *Lactobacillus casei* 280-16A to DL-valic acid in the described medium (see *Methods*) supplemented with DL-valine at 20 mg % (curves a, A) and in the same medium containing NZ-Case at 600 mg % in place of all the amino acids (curves b, B). Curves A and B were obtained when Tween 40 at 4 mg % was added to the test media. Curves a and b are the controls (no Tween 40). The coordinates are on the same bases as in Fig. 1.

8014, *Lactobacillus buchneri*,‡ *Lactobacillus casei* 7469, *Lactobacillus fermenti* 9338 and *Lactobacillus mannitopoeus*‡ were able to substitute DL-valic acid for L-valine with little or no loss of efficiency. *Leuconostoc citrovorum* 8081,§ in some experiments but not in others, gave a delayed response to valic acid at the usual levels, suggesting a capacity of this organism to utilize the hydroxy acid through a readily occurring mutation. *Streptococcus faecalis* 8043 required exceptionally high concentrations of valic acid in substitute for L-valine and was unique in its specificity for D-valic acid in this respect (Fig. 1). *Lactobacillus brevis* 8287,† *Lactobacillus gayonii* 8289† and *Lactobacillus lycopersici* 4005† did not utilize DL-valic acid at test levels up to 3.2 μM per ml in place of L-valine.

Optical specificity of valic acid utilization was not marked except for *S. faecalis* as already mentioned and in the special case of *L. casei* 280-16A to be considered further on. D-Valic acid was slightly preferred by *L. buchneri*, *L. casei* 7469 and *L. mannitopoeus*, whereas L-valic acid was somewhat better for *L. arabinosus* and *L. fermenti*. *L. casei* 280-16A has a special requirement for D-α-hydroxy acids(1) which prompted the following modifications of testing procedure: a) the described medium (see *Methods*) was supplemented with 1.5 μM of DL-lactic acid (as D-α-hydroxy acid source) per ml to permit tests of valic acid as a valine substitute (Fig. 2-left, curves D, DL and L; corresponding valine controls in Fig. 2-right), and b) the lactic acid supplement was omitted and DL-valine was added at 20 mg % to permit tests of valic acid as a D-α-hydroxy acid source (Fig. 2-left, curves D′, DL′ and L′). It is apparent from these data (Fig. 2) that the valine requirement of *L. casei* 280-16A is L-specific, that utilization of valic acid as an hydroxy acid source for this organism is D-specific, and that utilization of valic acid by this strain as a valine substitute is essentially lacking in optical specificity.

DL-α-Hydroxybutyric acid,‖ α-hydroxyisobutyric acid,‖ DL-α-hydroxy-α-methylbutyric acid,‖ DL-α-aminobutyric acid and α-aminoisobutyric acid were screened as potential antagonists of L-valine and DL-valic acid utilization because of their apparent structural similarities to the latter compounds. The most impressive inhibitory effects were produced by DL-α-hydroxybutyric acid, while DL-α-hydroxy-α-methylbutyric acid and DL-α-aminobutyric acid appeared to be similar but less potent inhibitors. α-Hydroxyisobutyric acid did not effectively inhibit any of the test organisms, whereas α-amino isobutyric acid strongly inhibited *L. casei, L. buchneri* and *L. mannitopoeus*, but not *L. arabinosus* and *L. fermenti*. The α-aminoisobutyric acid inhibition of *L. casei* was exceptional in that its reversal was effected by alanine peptides (data to be presented later) but not by either L-valine or DL-valic acid. Data representative of final tests with DL-α-hydroxybutyric acid are shown in Fig. 3. These response curves (Fig. 3) show that the inhibitor at 36 μM/ml suppresses utilization by *L. mannitopoeus* of L-valic acid but not that of either D-valic acid or L-valine. Data analogous to those presented in Fig. 3 were either interpolated or extrapolated to provide the condensed summary of DL-α-hydroxybutyric acid effects listed in Table I. It is apparent from these data (Table I) that L-valine is generally less susceptible than valic acid to antagonism by DL-α-hydroxybutyric acid and that L-valic acid is often markedly less potent than D-valic acid in reversing the effects of this antagonism.

Additions of Tween 40 (Atlas Powder Co. polyoxyethylene sorbitan monopalmitate) to the present experimental medium did not enhance valic acid utilization, although marked increases in growth-promoting potencies of various α-hydroxy acids for *L. casei* 280-16 had previously been observed in media supplemented with this non-ionic detergent(8). The apparent discrepancy was traced to a

‡ Three-fourths of the glucose was replaced with separately sterilized L-arabinose in media for the indicated organisms, for which a pentose supplement is highly stimulatory(7).

§ The medium for this organism was supplemented with 15 mμg of calcium leucovorin per ml.

‖ Test solutions were adjusted to pH 6.5 with sodium hydroxide.

TABLE I. Effects of DL-α-Hydroxybutyric Acid on Responses of Five Lactic Acid Bacteria to L-Valine and L-, DL- and D-Valic Acids.

Organism	DL-α-Hydroxybutyric acid conc. (μM/ml)	Substrate concentration required to yield half maximal response			
		L-valine	Valic acid		
			L-	DL-	D-
		(mμM/ml)			
L. casei 7469	0	49	79	62	55
	4	54	116	68	60
	12	54	436	96	82
	36	57	470	180	158
	108	60	1600	690	690
L. arabinosus	0	48	54	81	85
	4	47	62	93	120
	12	48	76	106	139
	36	46	115	160	166
	108	53	240	250	290
L. buchneri	0	134	220	174	148
	4	125	1000	167	134
	12	137	2600	240	148
	36	140	>3000	350	200
	108	166	>3000	630	380
L. mannitopoeus	0	76	94	84	82
	4	84	570	94	75
	12	84	1600	116	88
	36	88	>3000	230	88
	108	83	>3000	400	186
L. fermenti	0	89	104	116	171
	4	89	145	145	184
	12	71	190	152	200
	36	75	280	290	320
	108	88	840	540	440

marked inhibition of α-hydroxy acid utilization by enzymatically digested casein, which was included in earlier media(8). Antagonism of this inhibition is readily effected by Tween 40, which thus appears to potentiate the growth-promoting properties of α-hydroxy acids in media containing enzymatically digested casein, but not in media lacking such an inhibitor. These relationships are illustrated (Fig. 4) by the effects of Tween 40 on response of *L. casei* 280-16A to DL-valic acid in the present test medium (supplemented with DL-valine) and in a medium containing NZ-Case (Sheffield Chemical Company's enzymatic digest of casein). Further data concerning inhibition of α-hydroxy acid utilization by peptide derivatives from casein and other proteins are to be presented later.

Discussion. The present findings that whereas valine utilization is markedly L-specific, utilization of the valine substitute, valic acid, is either D-specific or relatively lacking in optical specificity closely parallel those of earlier investigations(2,3) of phenylalanine and its nutritional substitute, phenyllactic acid. In either case it seems probable that the hydroxy acid is utilized through conversion to the corresponding amino acid essentially as proposed by Holden *et al.*(4), *i.e. via* dehydrogenation to the α-keto acid and subsequent transamination. Lack of optical specificity toward the α-hydroxy acids when these are used in place of the corresponding α-amino acids suggests that some of the organisms may possess appropriate α-hydroxy acid racemases, but this does not appear to be true of *L. casei* 280-16A, which is evidently incapable of racemizing either L-valic acid (curve L′, Fig. 2) or phenyllactic acid (1). It seems likely, therefore, that *L. casei* 280-16A possesses separate L-specific and D-specific dehydrogenases which account for utilization of either optical form of these hydroxy acids in place of the corresponding L-amino acids. Optically selective antagonisms

of hydroxy acid utilization (Table I) are of considerable interest, but since various metabolic steps (*e.g.* cell penetration, racemization, dehydrogenation) might be involved in such antagonisms, these results are not unequivocally informative.

Summary. Substitution of L-, DL- and D-valic acids in place of valine in the nutrition of 10 lactic acid bacteria has been investigated. Five of the bacteria were able to utilize either optical form of valic acid efficiently in place of L-valine, even though the requirement for this amino acid is highly L-specific. Three of the latter 5 bacteria, on the other hand, could be made to exhibit a marked preference for D-valic acid by selective antagonism of L-valic acid with the inhibitor, DL-α-hydroxybutyric acid. One other organism showed a similar preference for D-valic acid without use of the inhibitor, but in this case utilization of D-valic acid was relatively inefficient. The α-hydroxy acid-dependent organism, *L. casei* 280-16A, was D-specific in its utilization of valic acid as an α-hydroxy acid source, but was not optically specific in its utilization of valic acid as a valine substitute.

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