THE ACTION OF HYDROXYLATED PHENYLALANINES ON THE GROWTH OF BACTERIA

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In recent years it has been found that for various microorganisms a number of substitution products of phenylalanine are competitively antagonistic to this amino acid. Compounds in which the phenyl group of phenylalanine was replaced by heterocyclic nuclei included β-2-thienylalanine (1-4), β-3-thienylalanine (5), β-2-furylalanine (6), and β-2-pyrrolealanine (7). A phenylalanine analogue substituted in the β-carbon position, β-hydroxyphenylalanine, was studied by Beerstecher and Shive (3), and ring halogenated phenylalanines and tyrosine were prepared by Mitchell and Niemann (8). These investigations have lately been reviewed by Winzler (9) and by Dittmer (10).

The present study deals with two hydroxy-substituted phenylalanines; viz., DL-β-hydroxyphenylalanine and DL-2,5-dihydroxyphenylalanine. Their effects on bacteria able to synthesize phenylalanine or tyrosine and on those requiring an extraneous supply of these amino acids are reported.

Methods and Materials

Cultures and Media—Leuconostoc mesenteroides P-60 was carried as a stab culture in an enriched basal medium described by Henderson and Snell (11). Inocula were grown by transfer from a 24 hour-old stab culture to 10 ml. of liquid medium of the same composition. After 24 hours incubation at 37°, the cells were centrifuged, resuspended in 10 ml. of sterile 0.9 per cent sodium chloride, and diluted 1:10 with sterile saline. Each experimental culture of 2 ml. was inoculated with 1 drop of this dilute suspension. For assay, the amino acid medium of Henderson and Snell (11), without phenylalanine or tyrosine, was used.

Parent wild type strain of Escherichia coli (ATCC 9637), E. coli phenylalanine-requiring mutant (M83-5), and E. coli tyrosine-requiring mutant (M83-9) were carried as slant cultures on minimum medium as described by Davis and Mingioli (12), supplemented with 0.5 per cent of casein enzymatic hydrolysate. For inoculum, a small amount of surface growth from an 8 hour culture was suspended to barely visible turbidity in sterile 0.9 per cent sodium chloride solution; 5 ml. of each experimental culture were inoculated with 1 drop of this suspension. The Davis and Mingioli...
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(12) basal medium was used for assay. From a 20 per cent sterile solution prepared in distilled water, 0.1 ml. of glucose was added aseptically to each test-tube.

E. coli (Shive) was carried as a slant culture on a basal medium, described by Shive and Macow (13), without hydrolyzed casein and supplemented with 1 mg. of DL-phenylalanine per 1 ml. of medium. For inoculum, a suspension was prepared from an 8 hour agar slant culture as described above. The same medium was used for assay.

Varying quantities of DL-o-hydroxyphenylalanine, L- or DL-phenylalanine, L-tyrosine, and DL-tryptophan were added to 1 ml. (for lactic acid bacteria) and 2.5 ml. (for E. coli strains) of the double strength medium in 18 X 150 mm. Pyrex culture tubes. The cultures were diluted with distilled water to 2 and 5 ml., respectively, capped, autoclaved at 15 pounds pressure for 10 minutes, cooled, and inoculated. E. coli cultures were incubated at 37° for 18 hours and lactic acid bacteria cultures for 48 hours; the latter were then diluted to 5 ml. with water and the turbidity was read in the Lumetron photoelectric colorimeter model 400-A with the 650 mmp filter. Turbidity readings of all E. coli cultures were made with the Klett-Summerson photoelectric colorimeter, red filter No. 64. All experiments were carried out in duplicate.

DL-o-Hydroxyphenylalanine—The compound was synthesized according to Dickinson and Marshall (14) with slight modifications. Glycine anhydride was prepared according to Schott et al. (15). After the reduction and hydrolysis of 2,5-diketo-3,6-di-o-methoxybenzylidene-piperazine, the filtered solution was evaporated in vacuo in a current of carbon dioxide. The brown solid obtained was dissolved in hot water with addition of a few ml. of glacial acetic acid. From this point, Neuberger’s procedure (16) for the preparation of DL-2,5-dihydroxyphenylalanine was followed. A 10 per cent solution of lead acetate was added until the pH was 1.8 to 2.0. Lead iodide was filtered, the filtrate was treated with H2S and evaporated to dryness in vacuo, and the residue dissolved in a minimum amount of hot water. After 3 volumes of alcohol were added, DL-o-hydroxyphenylalanine crystallized, which gave positive Millon and ninhydrin color reactions.

Analysis—C8H11O3N. Calculated, N 7.74; found, N 7.76

DL-2,5-Dihydroxyphenylalanine was obtained through the courtesy of Dr. J. P. Lambooy. It was added aseptically in a solution freshly prepared in distilled water filtered through a glass filter.

Results

Effect of DL-o-Hydroxyphenylalanine (OHPA) on L. mesenteroides—The organism was grown in a basal medium containing 6 γ per ml. of L-phenyl-
alanine (yielding 50 per cent of full growth) to which varying amounts of OHPA up to 5 mg. per ml. were added. Growth decreased as the amounts of OHPA were increased; half maximum growth was reached at 1100 \( \gamma \) per ml. and complete inhibition was found at 3000 \( \gamma \) per ml.

As shown in Fig. 1, L-phenylalanine alleviated this inhibition. The ratio of OHPA to L-phenylalanine which permits half maximum growth is 29:1, 55:1, and 71:1 at increasing levels of OHPA.

When \( L. \) mesenteroides was grown in basal medium supplemented with 3 \( \gamma \) per ml. of tyrosine (yielding 40 per cent of full growth), addition of OHPA up to 5 mg. per ml. failed to affect the growth.

\[ \text{\% of light transmitted} \]
\[ \text{L- PHENYLALANINE, \( \gamma \) PER ML} \]
\[ 0 \quad 20 \quad 40 \quad 60 \quad 80 \quad 100 \]

\[ 1 \quad 2 \quad 3 \quad 4 \]

**Fig. 1.** Reversal of OHPA inhibition of the growth of \( L. \) mesenteroides by L-phenylalanine. Incubated 48 hours at 37\. Curve 1, no OHPA; Curves 2, 3, and 4 obtained with 500, 1500, and 2500 \( \gamma \) of OHPA, respectively, per ml.

**Effect of OHPA on Wild Type \( E. \) coli and Its Phenylalanine- and Tyrosine-Requiring Mutants**—Growth of wild type \( E. \) coli was not affected by OHPA in amounts up to 2 mg. per ml.\(^1\) Unlike the parent strain, the phenylalanine-requiring mutant of \( E. \) coli is inhibited by OHPA.

Fig. 2 shows the effect of simultaneous addition of D\( L \)-phenylalanine and OHPA on the growth of the phenylalanineless mutant. Here, as with \( L. \) mesenteroides, the inhibition is counteracted by phenylalanine. The antagonistic ratio between OHPA and D\( L \)-phenylalanine which permits half maximum growth is 27:1, 43:1, 64:1, and 66:1 at increasing levels of the inhibitor.

Like the \( E. \) coli parent strain, the tyrosine-requiring mutant grown in various concentrations of L-tyrosine was not affected by OHPA in amounts up to 600 \( \gamma \) per ml.

\(^1\) Similar results were obtained when the organism was grown in the Shive and Macow medium (13). Nor was another wild type strain of \( E. \) coli ATCC 9723, grown under identical conditions in the Davis and Mingioli medium (12), affected by o-hydroxyphenylalanine.
Effect of OHPA on E. coli (Shive Strain)—Beerstecher and Shive (3, 4) report that the E. coli strain of Shive, which was repeatedly grown on a phenylalanine-containing medium, and which had been observed to grow as well in the absence of that amino acid, is inhibited by \(\beta\)-2-thienylalanine, \(\beta\)-hydroxyphenylalanine, and by tyrosine. Therefore, it seemed of interest to study the response of this organism to \(\alpha\)-hydroxyphenylalanine.

When the organism was grown in a basal medium containing various amounts of OHPA, growth was gradually inhibited with increasing concentrations of OHPA, reaching half maximum in the presence of 6 \(\gamma\) per ml., 90 per cent inhibition at 30 \(\gamma\) per ml., and complete inhibition at 300 \(\gamma\) per ml.\(^2\)

As shown in Fig. 3, full growth is restored by addition of 1 \(\gamma\) per ml. of DL-phenylalanine. The antagonistic ratio between OHPA and DL-phenylalanine which permits half maximum growth is 8:1, 18:1, and 50:1 at

\(^2\)Growth was also inhibited when the organism was grown in the Davis and Mingioli medium (12). Somewhat higher concentrations of the antagonist were needed to produce inhibition, probably due to the heavier growth of the organism in this medium.
increasing levels of the inhibitor. \textit{dL}-Tryptophan was one-twentieth as effective as phenylalanine.

\textbf{Effect of DL-2,5-Dihydroxyphenylalanine on Wild Type \textit{E. coli} and Its Phenylalanine- and Tyrosine- требующие Мутанты.} Growth of wild type \textit{E. coli} was not affected by the presence of 2,5-dihydroxyphenylalanine up to 200 \(\gamma\) per ml. But complete inhibition occurred at 600 \(\gamma\) per ml. and could not be alleviated by addition of \textit{dL}-phenylalanine or \textit{l}-tyrosine in quantities up to 600 \(\gamma\) per ml.

\textbf{DISCUSSION}

Neither the phenylalanine nor the tyrosine mutants, grown in the presence of 1 to 12 \(\gamma\) per ml. of the respective amino acids (yielding 16 to 100 per cent growth), showed any change of growth when 2,5-dihydroxyphenylalanine was added in amounts up to 100 \(\gamma\) per ml. Complete inhibition was obtained at 200 \(\gamma\) per ml.; it could not be reversed by adding \textit{dL}-phenylalanine or \textit{l}-tyrosine, up to 1 mg. per ml., to the respective mutants.
the phenylalanine-requiring bacteria. Growth of tyrosine requiring organisms and of two wild type strains of *E. coli* which synthesize phenylalanine was not affected by this compound in concentrations up to 2 mg. per ml. This behavior recalls the observation of Snell (17) and Shive and Snell (18) that pantothenic acid antagonists are effective only for organisms which are unable to synthesize that metabolite. However, the effect reported here is even more striking, as it is demonstrated not only on different organisms, but also in both the parent strain and the mutants of the *same* bacterium.

Unlike the resistant *E. coli* strains, Shive’s strain, which does not require phenylalanine for growth, resembled the phenylalanine-requiring bacteria in its response to o-hydroxyphenylalanine. That cultural procedures were not responsible for the difference observed is supported by the finding in a single experiment that, both in the Davis and Mingioli (12) and in the Shive and Macow media (13), only the Shive strain of *E. coli* was inhibited by the antagonist, while the growth of the wild type strain of *E. coli* was not affected. Tryptophan also antagonized the o-hydroxyphenylalanine inhibition of the "phenylalanine-modified" *E. coli* strain of Shive; this has been observed by Beerstecher and Shive (3) for the same organism inhibited by β-hydroxyphenylalanine. Since this organism is also inhibited by tyrosine (19), the effect of this compound as an antagonist to o-hydroxyphenylalanine could not be studied.

Unlike o-hydroxyphenylalanine, 2,5-dihydroxyphenylalanine was found to be equally toxic to wild type *E. coli* and to its phenylalanine- and tyrosine-requiring mutants; in no case could this inhibition be reversed by either phenylalanine or tyrosine.

The data reported here are insufficient to correlate the chemical structure of the various hydroxylated phenylalanines with their biological activity, although it seems not unlikely that the toxicity of the 2,5-dihydroxy compound is due to its ready conversion to the corresponding quinone. It remains to be seen, however, what structural variations of phenylalanine affect its synthesis or utilization. Wild type and amino acid-requiring mutants of *E. coli* appear to be useful test organisms for studying this and similar effects with other amino acid analogues.

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Experiments to be reported elsewhere, however, revealed that wild type *E. coli* showed an inhibition by β-hydroxyphenylalanine similar to that of Shive’s strain.
**SUMMARY**

Dl-α-Hydroxyphenylalanine inhibits the growth of the phenylalanine-requiring bacteria: *Leuconostoc mesenteroides*, *Escherichia coli* phenylalanine-requiring mutant, and a “phenylalanine-modified” strain of *E. coli*. This inhibition is reversed by phenylalanine; tryptophan also reverses the inhibition of the “modified” strain of *E. coli*, but less effectively.

Wild type *E. coli*, its tyrosine-requiring mutant, and *L. mesenteroides*, grown in the presence of suboptimum amounts of tyrosine, are not inhibited by α-hydroxyphenylalanine.

Dl 2,5-Dihydroxyphenylalanine is equally toxic to *E. coli* and to its phenylalanine- and tyrosine-requiring mutants; the inhibition is not antagonized by either phenylalanine or tyrosine.

**BIBLIOGRAPHY**

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