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Molecular Events in the Growth Inhibition of *Bacillus subtilis* by D-Tyrosine

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The transformable strain of *Bacillus subtilis* strain 168 is extremely susceptible to growth inhibition by D-tyrosine. The molecular events associated with the inhibition of growth by D-tyrosine in this strain include the false feedback inhibition and probably the false repression of prephenate dehydrogenase. These effects were found to contribute to the formation of D-tyrosine-containing proteins by decreasing the intracellular concentration of L-tyrosine. Accordingly, growth inhibition of strain 168 by the D isomer of tyrosine was shown to be progressive, enduring, and delayed by prior growth on L-tyrosine. The synthesis of cellular macromolecules and viable cell count were progressively diminished in D-tyrosine-inhibited cultures. Several different enzyme activities were reduced after growth in the presence of D-tyrosine. Isotopic D-tyrosine was incorporated into cellular proteins without change of optical configuration. Long chains of cells with completed septa were observed microscopically, and therefore some cell wall effect may also be implicated.

The ability of structural analogues of natural metabolites to inhibit growth has been related to the ability of such compounds to mimic one or more of the cellular functions of the natural compound (23). In the case of amino acid antimetabolites, the D-isomers might be expected to fulfill adequately the requirement for structural similarity. Yet, few cases of growth inhibition by D-amino acids have been reported (23). Therefore, in microoranisms, stereospecific mechanisms are highly evolved to prevent the erroneous use of D-amino acids in cellular metabolism.

In strain 168 of *Bacillus subtilis*, however, D-tyrosine has been shown to be a powerful growth inhibitor (8). It was shown that D-tyrosine was a relatively poor inhibitor of prephenate dehydrogenase, mimicking L-tyrosine in this respect. Inhibition of growth by D-tyrosine was specifically and completely reversed by L-tyrosine, and we therefore concluded that D-tyrosine acts as an antimetabolite of L-tyrosine in the cell. This report describes the molecular events associated with the growth inhibition by D-tyrosine and indicates that the incorporation of this amino acid into the proteins of *B. subtilis* is the ultimate basis of its inhibitory action.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The characteristics of wild-type *B. subtilis* NP 40, a prototrophic derivative of strain 168 (4), and its sensitivity to inhibition by D-tyrosine have been described (8). Minimal glucose growth medium and routine culture conditions for *B. subtilis* strains were those described (8).

Macromolecular synthesis. The synthesis of macromolecular constituents of growing cells was analyzed by a modification of the method of Neidhardt and Magasanik (19). At appropriate intervals, 10-ml samples were removed from a growing culture of 200 ml of cells at 37 C. An equal volume of 10% trichloroacetic acid was added to each sample which was then left overnight at 4 C. Each sample of disrupted cells was filtered through a 25-mm filter (0.45-μm pore size; Millipore Corp., Bedford, Mass.) and washed with 10 ml of 5% trichloroacetic acid. Material trapped on the filter was resuspended in 5 ml of 5% trichloroacetic acid by agitation with a Vortex mixer for 3 min. The filter was dicarded and each sample was heated at 90 C for 20 min. After cooling, the samples were centrifuged, and the supernatant, containing the nucleic acids, was carefully decanted from the precipitated protein. The protein pellets were resuspended in 1.0 ml of 1 N NaOH.

The method of Lowry et al. (17) was used to determine protein concentration. Protein samples were diluted 10-fold in water and assayed in duplicate for protein concentration by using crystalline bovine serum albumin as a standard.

Ribonucleic acid (RNA) concentration was determined by the method of Dische (10). Supernatant samples were assayed in duplicate with adenosine in 5% acid as a standard.

A modification (13) of the Burton method (5) was used to determine deoxyribonucleic acid (DNA)

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concentration. Duplicate supernatant samples were reacted with the diphenylamine reagent and the concentration determined by reference to a standard solution of deoxyadenosine in 5% trichloroacetic acid.

Estimation of cell mass. Turbidity measurements were made by using a Klett-Summerson colorimeter equipped with a no. 66 (red) filter. Turbidity measurements in the range between 10 to 200 Klett units were corrected by relating them to a standard curve in which the relationship between cell mass and Klett units was linear. The standard curve was prepared by comparing turbidity readings from serial dilutions of cultures at various stages of the growth cycle. Under these conditions, our Klett value of 50 corresponded to a viable count of 1.6×10^8 cells per ml of culture and to an absorbance at 600 nm of 0.481.

Ribosome isolation. Cells of strain NP 40 were grown for 14 hr in 200 ml of minimal medium containing 0.4 μg of ^{14}C-D-tyrosine per ml (0.05 μCi/ml) and 0.6 μg of carrier ^{12}C-D-tyrosine per ml. The cells were removed by centrifugation at 5,000 $\times$ *g* for 20 min, and the pellet was resuspended in 0.01 M Tris (hydroxymethyl) aminomethane (Tris)-hydrochloride buffer (*p*H 7.8) containing 0.01 M $MgCl_2$. The cells were broken by a 30-min incubation at 37 C with 100 μg of lysozyme per ml and 10 μg of deoxyribonuclease per ml. The cell debris was removed by centrifugation at 12,000 $\times$ *g* for 20 min. The supernatant was centrifuged at 105,000 $\times$ *g* for 2 hr in a Beckman-Spinco model L2-65 ultracentrifuge at 4 C. The supernatant was decanted and saved, and the ribosome pellet was resuspended in 1.0 ml of Tris buffer. The ribosome preparation was dialyzed against 1 liter of distilled water for 4 hr (two changes). Absorbancy measurements of the dialyzed preparation were taken at 260 and 280 nm. The protein concentration was determined (17), and duplicate 0.1-ml samples were counted in 10 ml of dioxane-based scintillation counting fluor (3) in a Beckman CPM scintillation counting spectrophotometer.

Protein isolation and hydrolysis. Protein was isolated and purified from cells by the method described by Mandelstam (18). For radioactivity determinations, 1 mg of dried protein was dissolved in 1 ml of 1 N NH_4OH, and duplicate 0.1-ml samples were counted in 10 ml of dioxane-based scintillation counting fluor (3).

For acid hydrolysis of the purified protein, 20-mg samples were dissolved in 2 ml of 6 N HCl, and 10^{-3} M sodium thioglycolate was added to protect the tyrosine residues (24). The protein solution was sealed in evacuated glass tubing and heated at 110 C for 22 hr in a dry-air oven. Acid was removed from the hydrolysate by evaporation in an evacuated dessicator over anhydrous H_2SO_4 and pellets of NaOH. The hydrolysate was evaporated to dryness twice from water. Acid hydrolysis of D-tyrosine peptides was conducted exactly as described for protein samples except that the hydrolysis time was reduced to 16 hr.

For enzymatic hydrolysis of the purified protein, 10-mg samples were dissolved in 2 ml of 0.05 M potassium phosphate buffer (*p*H 7.8). Pronase, prepared by the method of Hotta and Bassel (15), was added to give a final concentration of 1 mg/ml, and the mixture was incubated at 50 C for 24 hr. Enzyme and undigested protein were removed from the free amino acids by passage through a Sephadex G-25 column (1.2 by 25 cm). The later column fractions, containing the bulk of the radioactivity, were pooled and concentrated by evaporation at 80 C for 4 hr.

Chromatography. Descending paper chromatography was conducted by using Whatman no. 4 paper. Samples (10 to 20 μliters) were applied with a capillary pipette, and each spot was dried with hot air. The solvent system used was butanol-acetic acid–water (4:1:5), and chromatograms were run for 6 to 8 hr. Tyrosine and *p*-hydroxyphenylpyruvate were observed on the dried papers by spraying with 0.3% diazotized *p*-nitroanaline (1).

For the location of radioactive material, consecutive 1.0-cm strips were cut from the paper, placed in vials containing 10 ml of toluene-based counting fluor (Omnifluor, New England Nuclear Co.), and counted as above. After counting, the dried strips were stained with diazo-*p*-nitroanaline for visualization of material.

Dowex-1 column chromatography of protein hydrolysates was conducted as described by Hirs et al. (14). Hydrolysates were made 0.5 N in acetic acid and were applied to a Dowex-1-X8 column (1.2 by 30 cm) in the acetate form. Fractions (1 ml) were collected, and the elution of tyrosine was detected fluorometrically. Corrections were made for acid quenching of tyrosine fluorescence. Radioactivity in each fraction was measured by counting 0.1-ml portions in 10 ml of dioxane-based scintillation counting fluor (3).

^{14}C-D-tyrosine incorporation. Two 10-ml cultures of cells were grown in parallel in 125-ml flasks with side arms. Turbidity was measured in a Klett-Summerson colorimeter (no. 66 red filter). After two doublings, 0.2 ml of ^{14}C-D-tyrosine (diluted 4:1 with carrier ^{12}C-D-tyrosine) was added to give a final concentration of 10 μg/ml (1.0 μCi/ml). The growth of each culture was monitored, and, at intervals, 0.1-ml samples were removed from the flask containing the D-tyrosine. Each sample was added to 0.9 ml of 5% trichloroacetic acid and maintained at ice temperature for at least 30 min. They were then filtered through 25-mm filters (0.45 μm pore size, Millipore) and washed with 10 ml of 5% acid containing 1 mg of D-tyrosine per ml. The samples were air-dried, placed in scintillation vials containing 10 ml of toluene-based scintillation counting fluor, and counted as before, at greater than 75% efficiency.

Tyrosine assay. The presence of tyrosine was determined fluorometrically in an Aminco-Bowman spectrophotofluorometer. Tyrosine fluorescence at 325 nm (uncorrected) was measured by excitation at 275 nm (uncorrected). Tyrosine concentration was related to fluorescence by a standard curve obtained by using authentic tyrosine.

Enzyme assays. The fluorometric assay for prephenate dehydrogenase activity has been described (8). Prephenate dehydratase was assayed as described by Coats and Nester (9). The method of Schmit and Zalkin (25) was used for the assay of chorismate mutase. Anthranilate synthetase was assayed by the method of Kane and Jensen (16).

Amino acid oxidase treatment. L-Amino and D-

amino acid oxidase solutions were prepared at 2 mg/ml in 0.05 M Tris buffer (*p*H 7.8) containing 0.025 M KCl (7). Catalase was dissolved in the same buffer at 1 mg/ml. Tyrosine from neutralized column fractions was reacted with 200 μg of either L- or D-amino acid oxidase and 100 μg of catalase at 37 C. The reaction was conducted in the Aminco-Bowman spectrophotofluorometer, and the disappearance of tyrosine was monitored by the diminishing fluorescence at 325 nm (excitation at 275 nm). Reactions were run from 1 to 3 hr and terminated by heating the reaction mixture at 100 C for 2 min. Denatured protein was removed by filtration of the material through a membrane filter (0.45 μm, Millipore Corp.). *p*-Hydroxyphenylpyruvate in the supernatant was assayed with 2,4-dinitrophenylhydrazine, as described below.

p-Hydroxyphenylpyruvate assay. This keto acid was determined by the method of Friedmann and Haugen (11). The assay volume was reduced 10-fold, and the 2,4-dinitrophenylhydrazone formed was measured at 450 nm. Concentration was determined by reference to a standard curve.

Chemicals. D-Tyrosine-*l*-^{14}C at 23.7 mCi/mmole was purchased from New England Nuclear Co. L-Tyrosine and D-tyrosine and Pronase were products of Calbiochem. Amino acid oxidases, catalase, and other biochemicals were purchased from Sigma Chemical Co. Chemicals and assay reagents were of the highest quality available commercially. Barium prephenate was isolated and purified as previously described (8).

Contamination of commercial D-tyrosine by L-tyrosine was expected. Nonradioactive D-tyrosine purchased from Calbiochem was assayed by the manufacturer to be >99.9% pure. Isotopic D-tyrosine purchased from New England Nuclear Co. was claimed to contain <0.2% L-tyrosine. Corrections for L-tyrosine contamination have not been applied to the succeeding data.

RESULTS

When D-tyrosine was added to a culture of NP 40 growing in minimal medium, a lag characteristically preceded the inhibition of the growth rate (Fig. 1). Although the lag period was somewhat variable, it was clearly longer at lower temperatures and was influenced by the size of the inoculum used to begin the experiment. Continued growth in the presence of the analogue led to a progressive decline in the specific growth rate. A slow, perhaps linear increase in the turbidity of the inhibited culture was generally observed. In cultures inoculated to light turbidity, 10 μg of D-tyrosine/ml decreased the specific growth rate about 75%, independently of the growth temperature.

When D-tyrosine was removed from the growth medium of an inhibited culture by filtration, washing, and resuspension in minimal medium, a slow gradual recovery to the uninhibited growth rate ensued (Fig. 2). The rate of recovery from inhibition by D-tyrosine was not accelerated by the addition of 10 μg of L-tyrosine/ml.

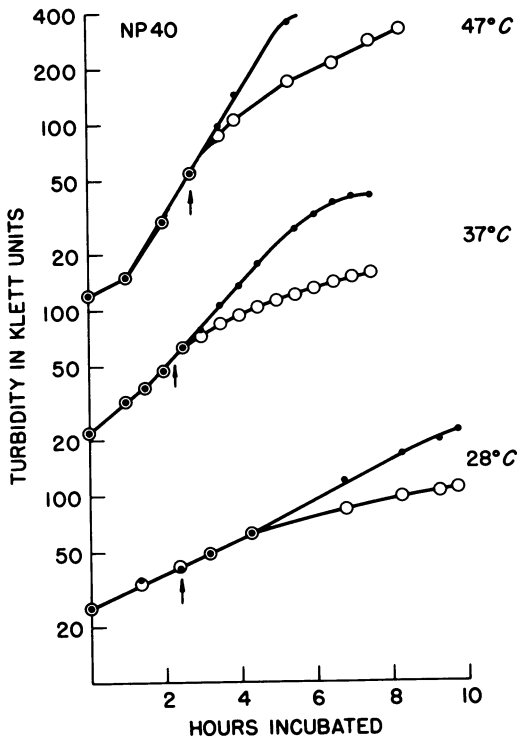

FIG. 1. *Effect of temperature on growth inhibition by D-tyrosine in NP 40. Cultures of NP 40 grown overnight at 37 C were used to inoculate duplicate flasks of minimal medium previously brought to the temperature indicated. The arrow indicates the time at which D-tyrosine at a final concentration of 10 μg/ml was added to one flask. Ordinate: culture turbidity expressed in Klett units. Note that the middle ordinate value of 20 represents a Klett value of 20 for the growth curve obtained at 37 C and a Klett value of 200 for the growth curve obtained at 28 C. Similarly the upper ordinate value of 20 represents a Klett value of 20 for the growth curve obtained at 47 C and a Klett value of 200 for the growth curve obtained at 37 C. Abscissa: growth time in hours at the temperature indicated.*

Growth of cells in minimal medium containing L-tyrosine influenced their subsequent sensitivity to inhibition by the D-isomer. The growth rate of cells grown on unsupplemented minimal medium was inhibited about 75% in the presence of 10 μg of D-tyrosine/ml (Fig. 3a). As Fig. 3b indicates, however, 10 μg of D-tyrosine/ml was not inhibitory to well-washed cells which had been previously grown on 50 μg of L-tyrosine/ml. It is clear from Fig. 3 that growth of the inoculum culture in the presence of L-tyrosine also altered the magnitude of growth inhibition observed subsequently in the presence of 100 μg of D-tyrosine/ml.

The synthesis of cellular macromolecules was analyzed in cultures growing with and without

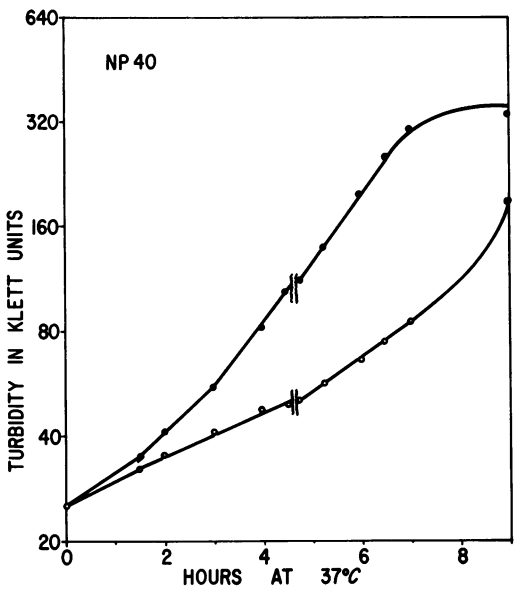

FIG. 2. *Recovery from* D-*tyrosine inhibition of growth in NP 40. A culture of NP 40 was grown in the presence of 10 μg of* D-*tyrosine/ml for 4.5 hr. The growth of a control cultured in the absence of* D-*tyrosine was followed. At the time indicated by the break, the cells in each culture were filtered through a membrane filter (0.45 μm pore size, Millipore), washed with 10 ml of prewarmed minimal medium, and resuspended in 10 ml of the same medium. The growth rate of the control culture was unaffected by this procedure. The lower growth curve shows the recovery of the* D-*tyrosine inhibited culture after resuspension in minimal medium. The recovery was similar in minimal medium containing 10 μg of* L-*tyrosine/ml. Ordinate and abscissa are as given in Fig. 1.*

D-tyrosine. The addition of D-tyrosine to a growing culture led to a gradual decline in the rate of synthesis of these macromolecules, approximately in parallel to one another. In the absence of the analogue, 510 μg of protein, 142 μg of RNA, and 17 μg of DNA were made per milligram of cell dry weight. A ratio of protein to RNA to DNA of 29:9:1 was obtained. The rate of increase of these macromolecules paralleled the increase in culture turbidity and viable cell count (*results not shown*).

Microscopic examination of both stained and unstained cells from the inhibited culture revealed long chains of 6 to 10 cells after 14 hr of growth in the presence of D-tyrosine (Fig. 4). The cells comprising the chains had fully formed septa and could be separated from one another by vigorous vortex action. The cultures used for the photographs shown in Fig. 5 all had grown beyond 100 Klett units after inoculation at a Klett value of 15. Under these conditions, 10 μg of D-tyrosine/ml did not induce chain formation nearly as well as 25 μg of D-tyrosine/ml. Similar experiments with cultures which were not allowed to grow beyond 10 Klett units starting with much smaller inocula showed that even more striking chain formation could be achieved at lower concentrations of D-tyrosine. Control cultures, grown in the absence of D-tyrosine, revealed a cell population consisting almost entirely of single and double cells after growth for 14 hr. Observations of cultures grown in the presence of other analogues of aromatic amino acids (5-methyltryptophan and β-2-thienylalanine) indicated that chain formation was not simply a general consequence of growth inhibition.

The influence of D-tyrosine on the synthesis of several enzymes in the pathway for aromatic amino acid biosynthesis was studied in NP 40. Extracts from cells grown in the presence and absence of 25 μg of D-tyrosine/ml were assayed for the activities of prephenate dehydrogenase, prephenate dehydratase, anthranilate synthetase, and chorismate mutase (Table 1). A three- to fourfold decrement in the specific activity of each enzyme was observed in extracts from D-tyrosine-grown cells.

The preceding results all were suggestive of the

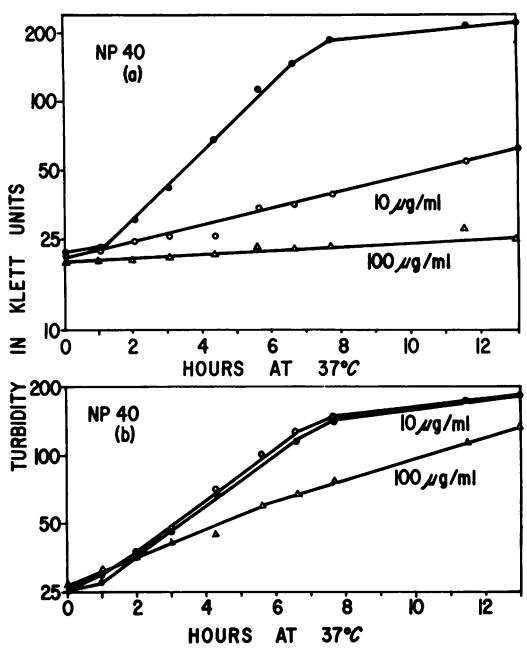

FIG. 3. *Effect of previous growth conditions on* D-*tyrosine inhibition of NP 40. (a) Cells grown overnight in minimal medium were washed and used to inoculate flasks containing minimal medium with* D-*tyrosine at 10 and 100 μg/ml. A control flask without* D-*tyrosine was also inoculated. (b) Cells grown overnight in minimal medium containing 50 μg of* L-*tyrosine/ml were washed and used to inoculate three flasks as in part a. Ordinate and abscissa are as given in Fig. 1.*

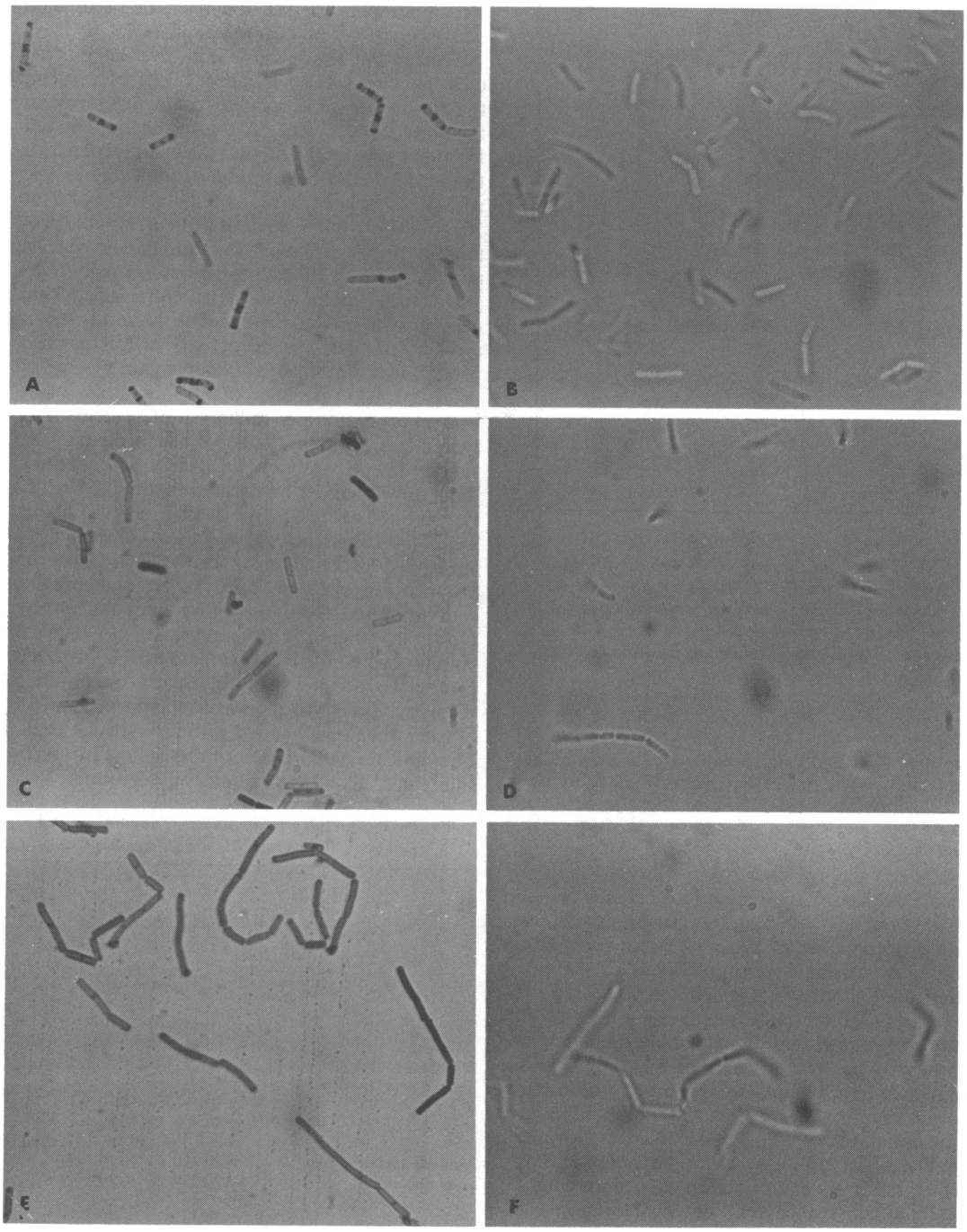

FIG. 4. *Photomicrographs of B. subtilis cells cultured in the presence and absence of* D-*tyrosine.* × *1,600.* (*A*) *Photograph of cells of NP 40 after 14 hr of growth at 37 C in the absence of* D-*tyrosine. Cells were stained with methylene blue.* (*B*) *Photograph of cells of NP 40 after 14 hr of growth at 37 C in the absence of* D-*tyrosine. Unstained.* (*C*) *Photograph of cells of NP 40 after 14 hr of growth at 37 C in the presence of 10 μg of* D-*tyrosine/ml. Cells were stained with methylene blue.* (*D*) *Photograph of cells of NP 40 after 14 hr of growth at 37 C in the presence of 10 μg of* D-*tyrosine/ml. Unstained.* (*E*) *Photograph of cells of NP 40 after 14 hr of growth at 37 C in the presence of 25 μg of* D-*tyrosine/ml. Cells were stained with methylene blue.* (*F*) *Photograph of cells of NP 40 after 14 hr of growth at 37 C in the presence of 25 μg of* D-*tyrosine/ml. Unstained.*

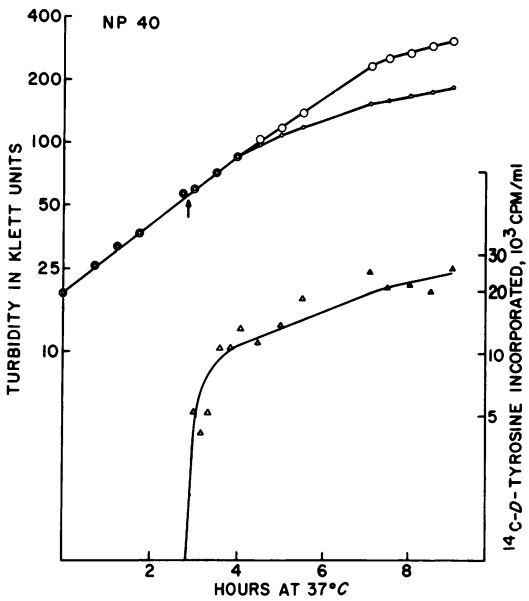

FIG. 5. *Incorporation of ^{14}C-D-tyrosine into proteins of growing cultures of NP 40. Two cultures of NP 40, started from the same inoculum, were grown in parallel. After 3 hr, ^{14}C-D-tyrosine, diluted by 25% with carrier ^{12}C-D-tyrosine to give a final concentration of 10 μg/ml (1.0 μCi/ml), was added to one culture. Samples were removed at intervals and the incorporation of isotope into protein was measured as described in the text. The growth of NP 40 with and without 10 μg of D-tyrosine/ml is indicated at the top of the figure. Left ordinate: culture turbidity expressed as Klett units. Right ordinate: ^{14}C-D-tyrosine incorporated, 10³ counts per min per ml of culture. Abscissa: growth time in hours at 37 C.*

incorporation of D-tyrosine into cellular protein. This possibility was tested in growth experiments using isotopic ^{14}C-D-tyrosine. The incorporation of ^{14}C-D-tyrosine into the proteins of NP 40 is illustrated in Fig. 5. Growth curves for the D-tyrosine-inhibited culture and for a control culture growing in the absence of the analogue are shown in the top portion of Fig. 5. After an immediate rapid uptake, the incorporation followed the growth curve of the inhibited culture. The initial rate of uptake is identical to the rate of uptake of isotopic L-tyrosine (*unpublished data*). Thus, the initial rate undoubtedly represents the preferential scavenging of contaminating L-tyrosine from the isotope preparation. Exhaustion of the small amount of L-tyrosine present then allows the rate of incorporation to be seen that is characteristic of D-tyrosine. A graph of the differential incorporation of ^{14}C-D-tyrosine (counts per minute incorporated versus Klett units, *not shown*) indicated the uptake of 1.5 × 10^4 counts per min per 100 Klett units, or 6.66 × 10^4 counts per min per mg of protein.

To rule out the possible exclusive entry of D-tyrosine into some specialized class of protein, the ribosomes were isolated from cells grown in the presence of 1 μg of ^{14}C-D-tyrosine/ml. The cell fractions were analyzed, and the characteristics of the purified ribosomes are given in Table 2. The ribosomes isolated had a specific activity of 3 × 10^3 counts per min per mg of protein. Soluble protein in the supernatant fraction had a specific activity of 5.8 × 10^3 counts per min per mg.

Bulk cell protein was isolated and purified from cells grown in the presence of 1 μg of ^{14}C-D-tyrosine/ml. The specific activity of this purified protein was 2.3 × 10^3 counts per min per mg of protein. It was calculated that 0.2 μg of D-tyrosine was incorporated per milligram of protein. If L-tyrosine is normally 2.7% of the protein of *B. subtilis* (27; Champney, *unpublished data*), these data show that 10 hr of growth on 1 μg of D-tyrosine/ml results in the replacement of about 1% of L-tyrosine molecules by the D-isomer.

Purified protein was hydrolyzed by acid or enzymatic procedures and the tyrosine present was separated from the other amino acids by Dowex-1 column chromatography. A typical column profile for tyrosine from acid-hydrolyzed protein is shown in Fig. 6. Nearly quantitative recovery of the tyrosine was accomplished and all of the radioactivity eluted with the tyrosine peak. The optical configuration of the radioactive tyrosine eluted from the column was determined by

TABLE 1. *Activity of aromatic enzymes of NP 40 grown with and without* D-*tyrosine*

Enzyme	Growth on D-tyrosine[a]	Activity	Specific activity	Specific activity ratio of −D-tyr to +D-tyr
		μmoles/min	*μmoles/min/mg*	
Prephenate dehydrogenase	−	3.4 × 10^{-3}	24.0 × 10^{-3}	
	+	0.9 × 10^{-3}	6.4 × 10^{-3}	3.75
Prephenate dehydratase	−	2.3 × 10^{-3}	1.60 × 10^{-3}	
	+	0.3 × 10^{-3}	0.42 × 10^{-3}	3.80
Anthranilate synthetase	−	0.35 × 10^{-3}	0.24 × 10^{-3}	
	+	0.09 × 10^{-3}	0.06 × 10^{-3}	3.90
Chorismate mutase	−	8.8 × 10^{-3}	18.5 × 10^{-3}	
	+	4.0 × 10^{-3}	6.3 × 10^{-3}	3.00

[a] D-Tyrosine was used at a final concentration of 25 μg/ml; the growth rate was reduced 80%. Cultures were inoculated to a Klett reading of about 15 and were harvested after a 10-fold increase in cell mass. The NP 40 extracts used contained 14.4 and 7.3 mg of protein/ml, for cells grown in the absence and presence of D-tyrosine (D-tyr), respectively.

the use of L-amino acid oxidase. The tyrosine fractions were pooled, neutralized, and treated with this enzyme for 3 hr at 37 C. After this procedure, 50 μg of tyrosine was converted to 45 μg of *p*-hydroxyphenylpyruvate, determined as the 2,4-dinitrophenylhydrazone (11). The products of this reaction were concentrated by evaporation and applied to chromatography paper. The developed chromatogram is illustrated in Fig. 7. None of the radioactivity was associated with the keto acid after the enzyme treatment, and all of it migrated with tyrosine. On the other hand, similar treatments with D-amino acid oxidase did not yield radioactive *p*-hydroxyphenylpyruvate.

Enzymatically digested protein showed a different Dowex-1 elution profile for tyrosine than did acid hydrolyzed protein. Figure 8 indicates the location of tyrosine and of radioactivity in column fractions of enzymatically degraded protein. The interpretation of this profile was that D-tyrosine in peptide linkage with other amino acids was poorly susceptible to the L-directed hydrolytic activity of the proteolytic enzyme. The first peak of tyrosine eluted was ^{14}C-labeled and was assumed to represent D-tyrosine in dipeptide linkage with other amino acid residues. The second peak of tyrosine eluted was unlabeled L-tyrosine which was released from the protein by the enzyme activity.

These interpretations were substantiated by the two following experiments. In the first L-tyrosine was mixed with a dipeptide containing an L-tyrosine residue, L-leucyl-L-tyrosine, and chromatographed on the Dowex-1 column as before. As expected, the dipeptide eluted first and was clearly separated from free L-tyrosine (Fig. 9). In a second experiment, the two leading fractions of the first peak shown in Fig. 8, assumed to contain the D-tyrosine dipeptide, were pooled and subjected to acid hydrolysis for 16 hr at 110 C. This hydrolyzed material was chromatographed on a Dowex-1 column and gave the profile indicated in Fig. 10. In this case, the majority of the tyrosine and the radioactivity migrated together, in the position expected for free tyrosine.

TABLE 2. *Characteristics of fractions from ribosome isolation in NP 40*

Material	Vol	A_{260}[a]	A_{280}[a]	Ratio of A_{260} to A_{280}	Protein	Counts per min per ml
	ml				*mg/ml*	
Growth supernatant.....	200					33,250
Cell debris...	1					14,000
High-speed supernatant.....	5				3.0	17,350
Ribosomes (dialyzed)..	1	0.767	0.403	1.9	0.4	1,200

[a] Dilution in buffer, 1/100.

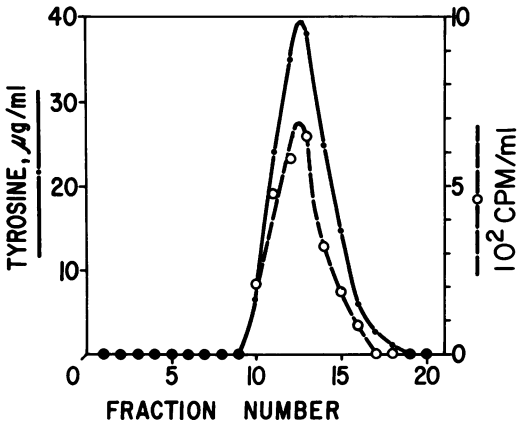

FIG. 6. *Elution profile of tyrosine from Dowex-1 column. A dried protein hydrolysate, dissolved in 2.0 ml of 0.5 N acetic acid, was applied to a Dowex-1-X8 column in the acetate form (1.2 by 30 cm). The amino acids were eluted with 0.5 N acetic acid, and 1.1-ml fractions were collected. Tyrosine in each fraction was measured fluorometrically and 0.1-ml portions were counted in 10 ml of dioxane scintillation fluor (3). Left ordinate: tyrosine, μg/ml. Right ordinate: 10² counts per min per ml. Abscissa: eluate fraction number.*

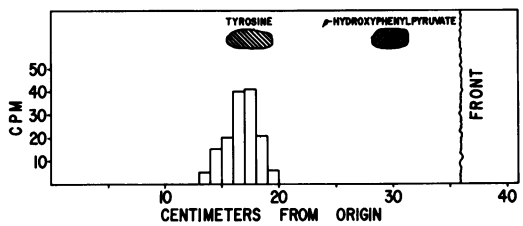

FIG. 7. *Paper chromatogram of L-amino acid oxidase reaction products. About 10 μliters of deproteinized supernatant from the treatment of tyrosine with L-amino acid oxidase was spotted in duplicate on Whatman no. 4 chromatography paper. Appropriate standards were also applied to the paper. The chromatogram was run in the solvent butanol-acetic acid-water (4:1:5) for 7 hr, descending. The dried paper was then cut into two halves. Tyrosine and p-hydroxyphenylpyruvate were visualized on one half by spraying with 0.3% diazotized-p-nitroanaline. The other half of the chromatogram was sectioned lengthwise into 1-cm strips, and these were counted in 10 ml of toluene scintillation counting fluor.*

DISCUSSION

The antimetabolite activity of D-tyrosine in *B. subtilis* is a consequence of its close structural similarity to L-tyrosine. D-Tyrosine, mimicking the

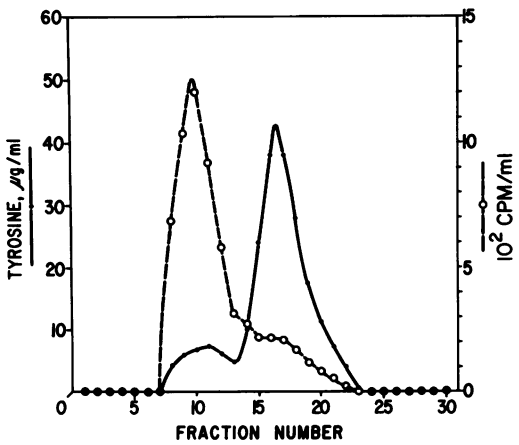

FIG. 8. *Elution profile of tyrosine from Dowex-1 column. Amino acids, resulting from the enzymatic digestion of protein labeled with* ^{14}C-D-*tyrosine, were dissolved in 2 ml of 0.5* N *acetic acid and applied to the Dowex-1-X8 column. The amino acids were eluted with 0.5* N *acetic acid, and 1.2-ml fractions were collected. Tyrosine in each fraction was measured fluorometrically and 0.1-ml portions were counted in 10 ml of dioxane scintillation counting fluor (3). Ordinate and abscissa are as given in Fig. 6.*

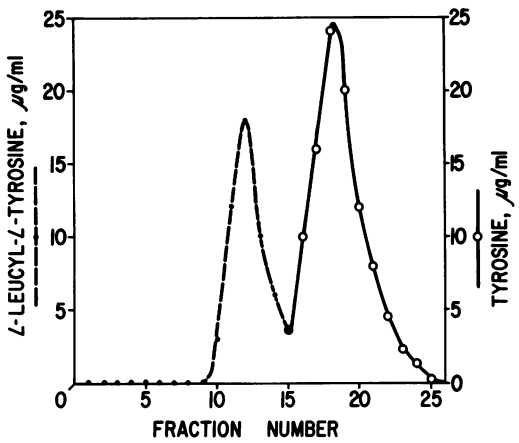

FIG. 9. *Elution profile of* L-*leucyl-*L-*tyrosine +* L-*tyrosine mixture from Dowex-1 column. A mixture of 90 μg of* L-*leucyl-*L-*tyrosine and 270 μg of* L-*tyrosine was dissolved in 2 ml of 0.5* N *acetic acid and applied to the Dowex-1-X8 column. The mixture was eluted with 0.5* N *acetic acid and 1-ml fractions were collected.* L-*Leucyl-*L-*tyrosine and* L-*tyrosine in each fraction were measured fluorometrically. The presence of* L-*leucyl-*L-*tyrosine in the first peak was confirmed by its location on a paper chromatogram, in a position distinct from* L-*tyrosine. Left ordinate:* L-*leucyl-*L-*tyrosine, μg/ml. Right ordinate:* L-*tyrosine, μg/ml. Abscissa: eluate fraction number.*

action of L-tyrosine in several of its biochemical roles, promotes a complex pattern of effects similar to those discussed by Richmond (23) for an antimetabolite which mimics many or all of the activities of a natural compound.

Evidence from this and the previous study of D-tyrosine in *B. subtilis* (8) implicate the following complex sequence of events in the inhibition of growth by this compound. D-Tyrosine, when added to growing cells, is transported into the cell pool of amino acids. Its dilution by endogenous L-tyrosine probably contributes to the lag period observed before the onset of growth inhibition. Within the cell D-tyrosine inhibits the activity of prephenate dehydrogenase (8), curtailing the production of L-tyrosine. As the amount of intracellular L-tyrosine decreases, activation of D-tyrosine by tyrosyl-transfer RNA synthetase occurs (6) with an increasing probability, and incorporation of the analogue into protein occurs. As the amount of L-tyrosine in the cell decreases further, due to the effects of false feedback inhibition and the preferential utilization of this amino acid for protein synthesis (6), increasingly greater amounts of D-tyrosine are activated for entry into protein. Continued growth in the presence of the analogue results in the formation of false proteins containing D-tyrosine, and a reduction in the catalytic efficiency

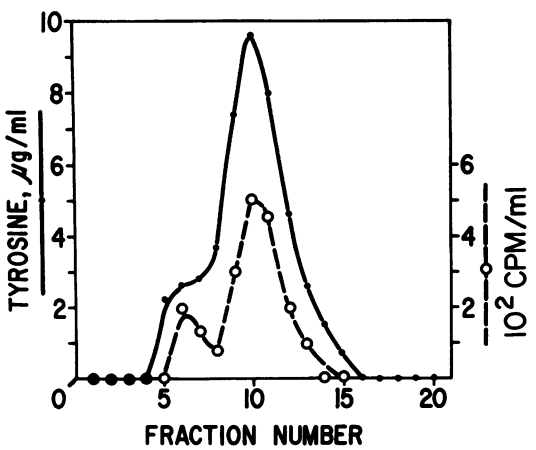

FIG. 10. *Elution profile of tyrosine from Dowex-1-X8 column. The acid hydrolysate of fractions 8 and 9 of Fig. 8 was dissolved in 1 ml of 0.5* N *acetic acid along with 40 μg of carrier* ^{12}C-D-*tyrosine, and applied to the Dowex column. The tyrosine was eluted with 0.5* N *acetic acid, and 1.2-ml fractions were collected. Tyrosine in each fraction was measured fluorometrically, and 0.05-ml portions were counted in 10 ml of dioxane scintillation counting fluor (3). Ordinate and abscissa are as given in Fig. 6.*

of some (or all) enzyme proteins was made. D-Tyrosine may also act to repress the synthesis of those enzymes in the aromatic amino acid pathway susceptible to repression by L-tyrosine (21), thus further diminishing the precursors available for the biosynthesis of L-tyrosine.

The preceding series of events is consistent with the progressive, accelerating degree of growth inhibition observed after the addition of D-tyrosine to growing cultures of *B. subtilis*. D-Tyrosine is dramatically more potent as an inhibitor of growth as the size of the initial inoculum is decreased. For example, it is extremely inhibitory on plates in which many generations intervene between a cell localized in situ on the plate and the subsequent visible colony. On the other hand, in liquid cultures in which the experiment only spans a few generations, even high concentrations of D-tyrosine may be relatively ineffective.

The ultimate mechanism of antimetabolite action by D-tyrosine is its erroneous incorporation into proteins. This was shown by demonstrating the uptake of isotopic D-tyrosine into isolated *B. subtilis* protein. By using ion-exchange column chromatography to isolate the tyrosine from acid hydrolysates, it was demonstrated that all of the radioactivity associated with the protein eluted with the tyrosine. The label was not associated with *p*-hydroxyphenylpyruvate after L-amino acid oxidase treatment, although 90% of the tyrosine was converted to the keto acid by this treatment. D-Amino acid oxidase treatment of isolated tyrosine was presumably ineffective in causing the formation of labeled *p*-hydroxyphenylpyruvate because of the inhibition of this enzyme by aromatic compounds (12, 22) and because of the relatively smaller amounts of D-tyrosine than L-tyrosine present in the hydrolysate mixture.

Enzymatic hydrolysis of D-tyrosine-containing proteins, followed by ion-exchange chromatography, gave a separation of free L-tyrosine and of D-tyrosine as a di-(or poly-) peptide. This result was consistent with the action of a proteolytic enzyme with an L-amino acid-directed specificity (27). Subsequent acid hydrolysis of the D-tyrosine peptide gave rise to free D-tyrosine.

The available evidence suggests that the fundamental basis of the metabolic inhibition of growth by D-tyrosine is its incorporation into the proteins of strain NP 40. The incorporation of D-tyrosine into protein is consistent with the findings of Calendar and Berg (7), who showed that D-tyrosine could be incorporated into peptide linkages by using an in vitro protein synthesizing system, including a purified tyrosyl-transfer RNA synthetase from *B. subtilis*. A similar conclusion was arrived at by Aronson and Wermus (2), in analyzing the growth inhibition of *B. subtilis* by *m*-tyrosine, a phenylalanine antimetabolite.

The following observations also support the finding that D-tyrosine residues enter protein structures. (i) The onset of growth inhibition is not immediate, (ii) recovery from growth inhibition after removal of D-tyrosine is not immediate, and (iii) the specific activities of four enzymes were reduced three- to fourfold in extracts of a culture grown in the presence of D-tyrosine. The kinetics of uptake of isotopic D-tyrosine into protein showed that the incorporation of the label paralleled the growth curve of the inhibited culture. Ribosomes and soluble proteins isolated from such cultures contained ^{14}C-D-tyrosine in amounts suggesting the substitution of about 1% of the L-tyrosine molecules by the D-isomer. Prior growth of cells on L-tyrosine afforded good protection against the inhibitory effects of D-tyrosine, implicating the L-tyrosine pool of the cell as an important barrier to the potential inhibitory action of the D-isomer. The inverse relationship of growth temperature and the duration of the lag period preceding the onset of growth inhibition by D-tyrosine is in agreement with the observation that less L-tyrosine is made and excreted by cells growing at elevated temperatures (8*a*). After approximately one generation of growth in the presence of the analogue, the synthesis of protein, RNA, and DNA decreased, approximately in parallel with one another. D-Tyrosine also exerted a similar decrease in the rate of viable cell count increase of inhibited cultures.

An effect of this compound on cell wall synthesis was suggested by the observation of long chains of cells with completed septa in inhibited cultures. Since the rate of viable count increase paralleled the rate of macromolecule synthesis, the chains were probably disrupted by the mechanical force of vortex action. Long chains of undivided *B. subtilis* cells have been described for *m*-tyrosine-inhibited cultures (2).

D-Amino acid-containing proteins are of considerable interest in view of the expected alteration in the chemical and biological features of such proteins. The incorporation of D-tyrosine into *B. subtilis* proteins would be expected to lead to alterations in enzyme configurations (with drastic effects in alpha-helical regions), resulting in diminished, if not abolished catalytic activities. The general lack of D-amino acids in the natural environment and the L-directed stereospecificity of most biological processes should preclude the incorporation of such "foreign" amino acids into proteins. Our demonstration of D-tyrosine in the proteins of *B. subtilis* suggests a defect in the

cellular ability to exclude or sequester this D-amino acid and implies a divergent evolutionary development from other *Bacillus* species known to be resistant to growth inhibition by this compound (8).

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