small, it could not be isolated for further spectral analysis. Therefore, the positions of hydroxy and methoxy substitution on the aromatic system were assigned, based on the following information: (*a*) the presence of the 163 and 164 pair in the mass spectrum precluded the possibility of any substitution located *ortho* to the diphenyl ether oxygen; (*b*) the product of enzymatic hydroxylation of *para*-substituted monohydric phenols is catechol, which undergoes *O*-methylation yielding *para*-substituted *ortho*-methoxy phenol (6); and (*c*) catechol *O*-methyltransferase does not *O*-methylate resorcinol derivatives (7).

***N*-Methyl-*N'*-*p*-(4-hydroxy-3-methoxyphenoxy)phenylsulfamide (VIII)**—Compound VIII is the *N*-demethylation product of VII. It was found in the urine of all three species in conjugated form. The amount was very small but was separated from the crude urine extract by repeated TLC. The mass spectrum of VIII of the trimethylsilyl derivative showed that the molecular ion was at m/e 540. Loss of the *N*-trimethylsilyl-*N*-methylsulfamoyl group (M − 166) and the appearance of the 4-hydroxy-3-methoxy-*N*-methylphenoxyaniline ion at 316 (M − 224) indicated a monomethyl sulfamide analog. Loss of 30 or 31 mass units suggested it was a methoxy phenol. Presence of the 163 and 164 pair indicated that the hydroxyl and methoxy substitutions were not *ortho* to the phenoxy oxygen.

The NMR spectrum of VIII was difficult to interpret because of the overlapping of aromatic protons. However, a set of *AA'BB'* multiplets indicated one phenyl ring had *para-para* symmetric disubstitution. This ruled out the possibility that the aniline ring was modified. Based on the same argument as for VII, VIII was assigned the structure of the *N*-demethyl-4-hydroxy-3-methoxy analog of the parent drug.

In conclusion, the results of these experiments indicated that the metabolic fate of the parent drug was different in these three species. Rats can only demethylate the drug at reduced efficiency, as shown by the presence of only trace amounts of II (the *N*-demethylated product) and the presence of VII in rat urine. The lack of IV and VI (*ortho*-hydroxylated metabolites) in human urine may indicate that humans can only hydroxylate I in the *para*-position. However, since the human subjects in this study were only administered a single dose of the drug while the animals were administered multiple doses, this apparent species variation may be caused by enzyme induction in animals.

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Microbiological Synthesis of L-Dopa

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Abstract ☐ The ability of microorganisms to convert *N*-carbobenzoxyl-L-tyrosine, *N-tert*-butyloxycarbonyl-L-tyrosine, and *N*-formyl-L-tyrosine into their respective *N*-substituted L-dopa derivatives was studied. These tyrosine derivatives were examined because of the ease with which the blocking groups may be removed. L-Tyrosine derivatives were incubated with *Aspergillus ochraceous* or *Gliocladium deliquescens*, and the resulting L-dopa products were isolated and characterized. L-Dopa was metabolized by *G. deliquescens* to 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenethyl alcohol. L-Ascorbic acid and hydrocinnamic acid increased yields of L-dopa when added to fermentation media.

Keyphrases ☐ L-Dopa—microbiological synthesis, L-tyrosine derivatives incubated with *Aspergillus ochraceous* and *Gliocladium deliquescens*, isolation and identification of L-dopa products ☐ *Aspergillus ochraceous*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives ☐ *Gliocladium deliquescens*—used for microbiological synthesis of L-dopa products from L-tyrosine derivatives ☐ Microbiology—synthesis of L-dopa from L-tyrosine by microorganisms ☐ Levodopa—microbiological synthesis of L-dopa from L-tyrosine derivatives

Three different types of enzymes are known to catalyze the formation of L-dopa from L-tyrosine:

1. Tyrosine hydroxylase is an enzyme associated with the biosynthesis of norepinephrine. It catalyzes the tetrahydropteridine-dependent hydroxylation of L-tyrosine to L-dopa (1).

2. Tyrosinase catalyzes the oxidation of tyrosine to melanin. It is generally accepted that the reaction proceeds through the formation of L-dopa and hallochrome, with the eventual formation of the polymeric pigment, melanin (2).

3. β-Tyrosinase from *Escherichia intermedia* catalyzes the synthesis of L-dopa from L-tyrosine and pyrocatechol *via* β-replacement (3).

In view of the therapeutic importance of L-dopa (levodopa) in the treatment of Parkinsonism, the authors wish to record in detail the examination of reactions using microbial systems for the conversion

of L-tyrosine to L-dopa. A preliminary account was published previously (4).

EXPERIMENTAL

Materials—The following materials were purchased: *N*-carbobenzoxyl-L-tyrosine[1]; L-dopa[1]; D-tyrosine; 3,4-dihydroxyphenylacetic acid[1]; L-tyrosine[2]; *N-tert*-butyloxycarbonyl-L-tyrosine[3]; hydrocinnamic acid[4]; L-ascorbic acid[5]; ion-exchange resin[6], 200–400 mesh; silica gel G[7]; silicic acid[8], 100 mesh; and diatomaceous earth[9].

Gliocladium deliquescens (1086) and *Aspergillus ochraceous* (398) were maintained on malt-agar[10] slants[11].

Methods—*N*-Formyl-L-tyrosine and *N*-formyl-D-tyrosine were prepared by the method of Waley and Watson (5). *N*-Carbobenzoxyl-D-tyrosine was synthesized according to the procedure of Bergmann and Zervas (6). TLC was performed using 0.25-mm thick silica gel G plates with ethyl acetate–acetic acid–isooctane–water (11:2:5:10) as the developing solvent. After development, TLC plates were sprayed with diazotized sulfanilic acid reagent (7); tyrosine derivatives appeared yellow, whereas catechols appeared orange-brown on sprayed chromatograms. Mass spectra were taken on a mass spectrometer[12], operating at an ionization of 70 ev and a block temperature of 120°. IR spectra were recorded on a double-beam recording spectrophotometer[13].

L-Dopa and L-tyrosine were assayed quantitatively after their separation on an ion-exchange column[6]. A typical elution profile obtained in the separation of these compounds is illustrated in Fig. 1. L-Tyrosine has a UV absorption maximum at 274 nm (ϵ 13,300), whereas the absorption maximum for L-dopa occurs at 279 nm (ϵ 26,600). These extinction coefficients were used in the determination of L-dopa yields and L-tyrosine recoveries as shown in the tables.

Fermentations were conducted in cotton-plugged erlenmeyer flasks in a soybean-dextrose medium of the following composition: dextrose, 20 g; soybean meal, 5 g; yeast extract, 5 g; sodium chloride, 5 g; dibasic potassium phosphate, 5 g; and distilled water, 1000 ml; it was adjusted to pH 7.0 with hydrochloric acid. The medium was sterilized in an autoclave at 121° for 15 min prior to use.

A two-stage incubation procedure was used in the cultivation of microorganisms. In a typical fermentation, the surface growth from a 1-week-old malt-agar slant was suspended in 5 ml of sterile saline (0.85%) solution. Two-milliliter portions of the resulting spore suspension were aseptically transferred to inoculate 50 ml of soybean–dextrose medium held in 250-ml erlenmeyer flasks (F-1 stage). These F-1 stage flasks were incubated at 25° on a rotary shaker operating at 250 rpm [5.08-cm (2-in.) stroke] for 24–48 hr. The resulting thick F-1 stage cultures were used to inoculate F-2 stage cultures, which were also grown in erlenmeyer flasks containing the soybean-dextrose medium. The inoculation volume used was 10% of the volume of medium contained in F-2 flasks. Usually, after 24 hr of incubation on the rotary shaker, various L-tyrosine substrates, dissolved in dimethylformamide, were added aseptically to F-2 stage flasks. L-Ascorbic acid was dissolved in 0.2 *M* phosphate buffer, pH 6.8, when it was added to cultures. In the various experiments reported, substrates containing F-2 stage cultures were incubated on the rotary shaker for an additional 12–60 hr before being harvested and analyzed for L-dopa. In separate experiments, it was determined that both microorganisms had reached the stationary phase of the growth cycle after 24 hr in the F-2 stage. Thus, the potential for contaminating microorganisms to influence the outcome of fermentations was considered slight. To avoid the possibility that microbial contaminants might have been responsible for the observed conversions of L-tyrosine derivatives to L-dopa, controls consisting of sterile medium with substrates added as usual were examined. None of the controls gave evidence of bacterial or fungal contamination or of the possible artifactual conversions of L-tyrosine to L-dopa under the incubation conditions.

[1] Sigma.
[2] Nutritional Biochemicals.
[3] Pierce.
[4] Eastman.
[5] Pfizer.
[6] Dowex 50W X4, Baker.
[7] Brinkmann.
[8] No. 2847, Mallinckrodt.
[9] Celite, Johns Manville.
[10] Difco.
[11] The microorganisms used in this study were supplied by Professor K. B. Raper, Department of Bacteriology, University of Wisconsin, Madison, Wis.
[12] Finnigan 1015.
[13] Beckman IR5A.

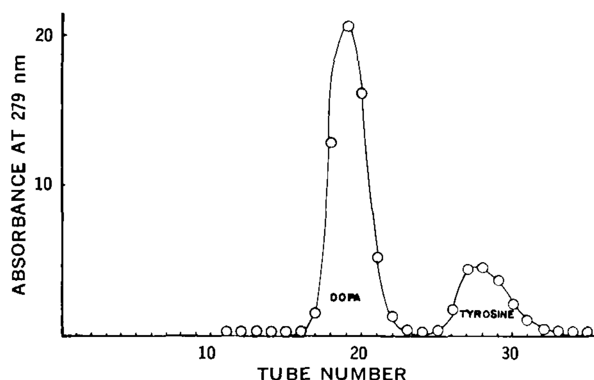

Figure 1—*Separation of L-tyrosine and L-dopa on a strong acid (H^+) ion-exchange column. The column (1.1 × 17.5 cm) was charged with 26 mg each of L-dopa and L-tyrosine and was eluted with 1.5* N *HCl at a rate of 6.4 ml/15 min. The absorbance at 279 nm was monitored as an estimation of their concentrations.*

***N*-Carbobenzoxyl-L-dopa**—*Fermentation*—*A. ochraceous* was grown according to the general fermentation procedure. The F-2 stage cultures were conducted in four 2-liter erlenmeyer flasks containing 500 ml each of the soybean–dextrose medium. After 24 hr of incubation on the rotary shaker, 250 mg of *N*-carbobenzoxyl-L-tyrosine, dissolved in 2 ml of dimethylformamide, was added to each flask. The F-2 stage flasks containing substrate were returned to the shaker and incubated for an additional 24 hr.

Isolation—Twenty-four hours after the addition of *N*-carbobenzoxyl-L-tyrosine, the mycelia were removed by filtration through cheesecloth. The filtrate was acidified to pH 2.8 with 6 *N* HCl and successively extracted with three 800-ml portions of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness *in vacuo*. The residue (1.95 g) was chromatographed on a silicic acid column, 2.54 × 20.32 cm (1 × 8 in.) in size. Elution of the column with benzene–ethyl acetate (3:1) afforded 523 mg of *N*-carbobenzoxyl-L-dopa, which was chromatographically identical to a sample prepared by acylation of L-dopa. The product gave orange-brown-colored spots on chromatograms sprayed with diazotized sulfanilic acid spray reagent and possessed a mobility on silica gel G TLC plates 0.9 that of *N*-carbobenzoxyl-L-tyrosine.

When the compound (200 mg) was dissolved in a 1:1 mixture of acetic acid and water (20 ml) and hydrogenated for 16 hr in the presence of 10% palladium-on-carbon, 63 mg of L-dopa, mp 286° dec., was obtained. The isolated compound was spectrally (IR and UV) and chromatographically identical to an authentic specimen of L-dopa.

***N-tert*-Butyloxycarbonyl-L-dopa**—*Fermentation*—*A. ochraceous* was incubated with *N-tert*-butyloxycarbonyl-L-tyrosine (500 mg) as previously described, except that the F-2 stage was incubated for a total of 60 hr after substrate addition.

Isolation—At 60 hr, the fermentation was terminated by filtration through cheesecloth to remove the mycelia. The filtrate was acidified with 6 *N* HCl to pH 2.5 and was successively extracted with three 600-ml portions of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness *in vacuo*. The residue was chromatographed over 22 g of silicic acid containing 15% diatomaceous earth. Elution of the column with 23% ethyl acetate in benzene gave 141 mg of *N-tert*-butyloxycarbonyl-L-tyrosine and 200 mg of *N-tert*-butyloxycarbonyl-L-dopa, which was identified by converting it into L-dopa by treatment with trifluoroacetic acid (8).

***N*-Formyl-L-dopa**—*Fermentation*—*G. deliquescens* was grown according to the usual fermentation procedure, and the F-2 stage was conducted in a 125-ml erlenmeyer flask containing 25 ml of

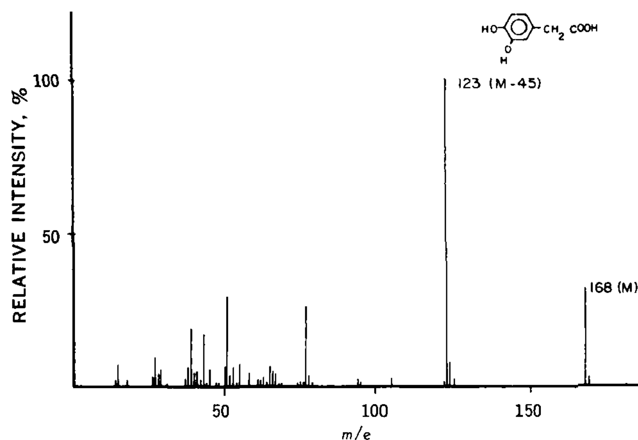

Figure 2—*Mass spectrum of Metabolite A which was isolated from* G. deliquescens *cultures containing* L-*dopa.*

the soybean-dextrose medium. After 12 hr of incubation on the shaker, 750 mg of *N*-formyl-L-tyrosine, dissolved in 2 ml of 1 *M* $KHCO_3$, was added to the flask. The substrate containing F-2 stage culture was incubated for an additional 42 hr under the prescribed conditions. L-Ascorbic acid was added intermittently to the F-2 stage flask in five separate portions at 0, 12, 18, 24, and 36 hr following substrate addition. A total of 500 mg of L-ascorbic acid was added. Because previous work had demonstrated that metabolic transformation was most active between 20 and 30 hr after substrate addition, most of the L-ascorbic acid was added during this time interval.

Isolation—Forty-two hours after the addition of the *N*-formyl-L-tyrosine, the mycelia were removed from the culture by filtration through cheesecloth. The filtrate was acidified with 6 *N* HCl to pH 2.5 and successively extracted with three 25-ml portions of *n*-butanol. The combined butanol extracts were evaporated to dryness *in vacuo*. The residue was dissolved in 20 ml of 5 *N* HCl and was allowed to stand at room temperature for 12 hr. After evaporation of the aqueous hydrochloric acid, the residue was dissolved in 5 ml of 0.75 *N* HCl and chromatographed on an ion-exchange column[6], 200–400 mesh, H^+ form, 2 × 28 cm in size. The column was eluted with 0.75 *N* HCl, and successive 9.5-ml fractions were collected. L-Dopa hydrochloride (186 mg) was found in fractions 35–46, and L-tyrosine hydrochloride (375 mg) was found in fractions 48–59. The isolated L-dopa, $[\alpha]_D^{25}$ −11° (c, 4% in 0.75 *N* HCl), was spectrally (IR) and chromatographically identical to authentic L-dopa.

Metabolism of L-Dopa by *G. deliquescens*—To a 24-hr F-2 stage culture of *G. deliquescens* in a 2-liter erlenmeyer flask containing 500 ml of the soybean-dextrose medium was added 1 g of L-dopa and 1 g of L-ascorbic acid. After incubating for 24 hr on a rotary shaker as previously described, the mycelia were removed by filtration through cheesecloth. The filtrate was acidified to pH 2.0 and extracted with *n*-butanol. The combined butanol extracts were evaporated to dryness *in vacuo*, and the residue was chromatographed on a silica gel column. The column was eluted with ethyl acetate containing 2% acetic acid. Two products were obtained from this column while residual L-dopa remained at the origin. These substances possessed R_f values of 0.5 and 0.35 on silica gel G TLC plates in a solvent system consisting of chloroform–methanol–acetic acid–water (80:17.5:2.4:0.1). The compounds were isolated by preparative TLC using this solvent system and were rechromatographed for further purification using benzene–propionic acid–water (2:2:1). The major product, A (R_f 0.5), was identified as 3,4-dihydroxyphenylacetic acid (200 mg); the minor product, B (R_f 0.35), was characterized as 3,4-dihydroxyphenylethanol (5 mg).

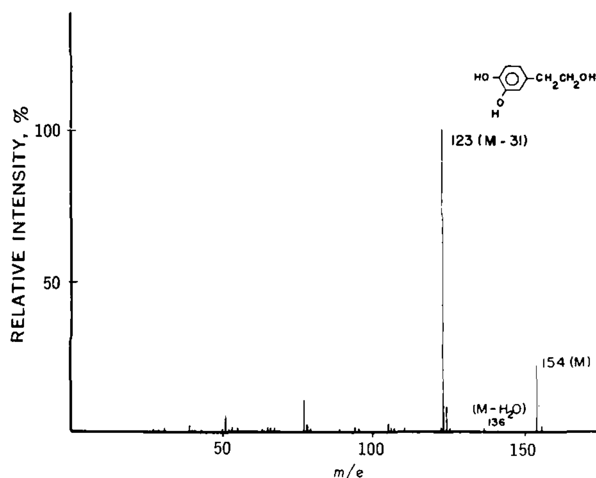

Figure 3—*Mass spectrum of Metabolite B which was isolated from* G. deliquescens *cultures containing* L-*dopa.*

Table I—Effect of L-Ascorbic Acid on Yield of L-Dopa[a]

Ascorbate, mg/ml	L-Dopa, % Yield	L-Tyrosine, % Recovered
None	10.0	13.2
2.1	11.2	14.7
5.3	23.1	63.5

[a] Fermentation conditions using *G. deliquescens* were the same as described under *Experimental*. *N*-Formyl-L-tyrosine concentration was 10 mg/ml, and incubations were terminated 12 hr after substrate addition. L-Ascorbic acid was added at the time of substrate addition.

Table II—Effect of Substrate Concentration on Yield of L-Dopa[a]

Substrate, mg/ml	L-Dopa, % Yield	L-Tyrosine, % Recovered
3	22.3	42.2
10	23.1	63.5
30	10.6	76

[a] *G. deliquescens* was incubated with substrate for 12 hr under the same conditions as described under *Experimental*.

RESULTS AND DISCUSSION

Among the wide variety of microorganisms that metabolize L-tyrosine, the majority appears to degrade this amino acid *via* homogentisic acid with the initial formation of *p*-hydroxyphenylpyruvic acid, a route characteristic of mammals (9–12). An alternative route for the dissimilation of L-tyrosine *via p*-coumaric acid, *p*-hydroxybenzoic acid, and protocatechuic acid was shown to be widespread among the *Basidiomycetes* (13). Although protocatechuic acid and catechol (14) were identified as metabolites of L-tyrosine, no L-dopa was detectable, suggesting that deamination of L-tyrosine may be the first and major degradative reaction.

If deamination and aromatic hydroxylation do not follow a compulsory sequence, it should be possible to inhibit deaminase activity selectively by the introduction of suitable *N*-blocking groups, resulting in the accumulation of the desired *N*-substituted L-dopa derivatives. As additional features, *N*-substituted tyrosine substrates should be resistant to the action of racemases and also should be more soluble in organic solvents. Thus, substrates and products are more easily extracted from aqueous fermentation media. For this study, *N*-carbobenzoxyl, *N*-formyl, and *N*-*tert*-butyloxycarbonyl derivatives of L-tyrosine were selected because they are easily removed, either by acid hydrolysis or by hydrogenolytic cleavage (8).

In initial experiments, several microorganisms were screened for their capacities to convert *N*-formyl-L-tyrosine and *N*-carbobenzoxyl-L-tyrosine into their L-dopa derivatives. As previously reported (4), not every microorganism examined accomplished this conversion, but *A. ochraceous, Penicillium duclauxi, G. deliquescens, Stemphylium solani, Scopulariopsis constantini, Memnoniella echinata, Trichoderma viride, Corynespora cassicola, Fusarium solani, Stysanus fimetarius,* and others did. *A. ochraceous* and *G. deliquescens* appeared to accomplish the transformation efficiently, and these cultures were selected for further work.

Table III—Effect of Time on Yield of L-Dopa by *G. deliquescens*[a]

Hours	L-Dopa, % Yield	L-Tyrosine, % Recovered
12	10.6	76
39	16.0	51.0
46	18.5	57.3

[a] Fermentations were conducted as described under *Experimental*. L-Ascorbate (5.3 mg/ml) was added to the cultures every 12 hr.

When *N*-carbobenzoxyl-L-tyrosine and *N-tert*-butyloxycarbonyl-L-tyrosine were added to growing cultures of *A. ochraceous*, the corresponding L-dopa derivatives were formed in about 30% yield. This organism gave poor yields of *N*-formyl-L-dopa when incubated with *N*-formyl-L-tyrosine. Conversely, *G. deliquescens* preferentially converted *N*-formyl-L-tyrosine into *N*-formyl-L-dopa, while giving poorer yields with *N*-carbobenzoxyl- and *N-tert*-butyloxycarbonyl-L-tyrosine substrates. Qualitative studies in which these organisms were incubated with *N*-formyl-D-tyrosine and *N*-carbobenzoxyl-D-tyrosine resulted in the formation of the respective catechol derivatives as judged by TLC. Thus, it appears that hydroxylase activity is not dependent on the symmetry of the amino acid derivative used.

Insight into this microbial conversion was sought so that fermentation conditions might be optimized to obtain higher yields of L-dopa from various L-tyrosine derivatives. For this purpose, efforts were concentrated on *G. deliquescens* using *N*-formyl-L-tyrosine as the substrate. Initially, it was observed that the fermentation medium rapidly turned black following addition of substrate. The appearance of pigment could be correlated with the hydroxylation of *N*-formyl-L-tyrosine, but the amount of L-dopa and L-tyrosine recoverable from these fermentations was low. This observation suggested that melanin formation was taking place at the expense of L-dopa as it was being formed. To counteract this undesirable effect, L-ascorbic acid was included in the medium. Table I clearly shows that the recovery of both L-dopa and L-tyrosine was improved and that the yield of L-dopa was significantly increased when L-ascorbic acid was added.

An experiment was conducted to determine the effect of substrate concentration on the yield of L-dopa with *G. deliquescens*. The results in Table II indicate that the optimum substrate concentration level is 10 mg/ml of *N*-formyl-L-tyrosine. Higher substrate levels at 30 mg/ml appeared, on the basis of TLC, to inhibit side reactions and also delayed the formation of melanin-like pigments in the medium.

To take advantage of this finding, the kinetics of L-dopa formation were investigated utilizing 30 mg/ml of *N*-formyl-L-tyrosine as substrate. Table III shows that the maximum yield of L-dopa was obtained at 46 hr in this experiment.

The level of *N*-formyl-L-dopa decreased on prolonged incubations with *G. deliquescens*. This finding suggested that the organism may possess an enzyme capable of cleaving the formyl group, albeit slowly. When *G. deliquescens* was incubated in a medium containing L-dopa, two metabolites were detected. The major product (A), obtained in 20% yield, was identified as 3,4-dihydroxyphenylacetic acid on the basis of the following data. The mass spectrum of Metabolite A is shown in Fig. 2. The molecular ion of this metabolite occurred at m/e 168, while the base peak occurred at m/e 123, corresponding to an $M^+ - COOH$ ion. Both the IR and mass spectra of the metabolite were identical to an authentic specimen sample of 3,4-dihydroxyphenylacetic acid. The mass spectrum of the minor metabolite, B, is shown in Fig. 3. The molecular ion of this metabolite occurred at m/e 154, while the base peak occurred at m/e 123, consistent with the loss of a fragment of mass 31, or $M^+ - CH_2OH$. The mass spectral evidence suggested that Metabolite B is 3,4-dihydroxyphenylethanol.

The previous experiment demonstrated that *G. deliquescens* possesses the enzymatic capacity to degrade L-dopa to 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylethanol. The reaction sequence giving rise to these metabolites is probably analogous to the decomposition of other amino acids in yeasts and other organisms (15). On the basis of these results, it is suggested that the presence of an acylase capable of cleaving the formyl group from the formyl amino acids would lower the yields of L-dopa produced in fermentations. Hydrocinnamic acid has been reported to be a powerful inhibitor of carboxypeptidase activity (16). In an attempt to inhibit the hydrolytic activity of *G. deliquescens*, hydrocinnamate was added to the medium. The results of two experiments are shown in Table IV. An increase in the yield of L-dopa was obtained by utilizing only small quantities of hydrocinnamate. Although yields of L-dopa were improved, the exact mechanism by which hydrocinnamate exerts its influence remains uncertain. It was observed that the metabolic transformation of *N*-formyl-L-tyrosine to *N*-formyl-L-dopa proceeded at a more rapid rate in the presence of hydrocinnamic acid. Thus, it is possible that hydrocinnamate may be functioning as an inducer of aromatic hydroxylase rather than as an inhibitor of acylase activity.

Table IV—Effect of Hydrocinnamate on Yield of L-Dopa by *G. deliquescens*[a]

	L-Dopa, % Yield	L-Tyrosine, % Recovered
Experiment I (32-hr fermentation)		
Control (no hydrocinnamate)	10.6	65
1×10^{-4} *M* (hydrocinnamate)	17.0	60
Experiment II (62-hr fermentation)		
Control (no hydrocinnamate)	14.0	30.5
1×10^{-4} *M* (hydrocinnamate)	18.5	37.7

[a] Fermentation conditions are described under *Experimental*.

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