Metabolism of phenylacetic acid in *Aspergillus niger*

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Abstract

Phenylacetate metabolism in *Aspergillus niger* was studied. An alternate pathway, which is different from the one delineated earlier [Sugumaran, M., Ramanarayanan, M. and Vaidyanathan, C. S., *FEBS Letters*, 29, 69–72 (1973)] was found to operate for the catabolism of phenylacetate during the sporulation phase of the organism. Evidence has been presented for the formation of homogentisate, fumarylacetoacetate, maleylacetoacetate, fumarate and acetoacetate as the degradation products of phenylacetate. The activities of the three key enzymes of the pathway, *viz.*, homogentisate dioxygenase, maleylacetoacetate isomerase and fumarylacetoacetate hydrolase have been demonstrated. In addition, phenylacetate hydroxylase activity has also been demonstrated.

Key words: *Aspergillus niger*, Phenylacetate metabolism, homogentisate formation, ring cleavage.

1. Introduction

Phenylalanine and tyrosine are catabolized mainly *via* homogentisate in mammalian system and in some bacteria[1–5]. Homogentisate also assumes a key role during the degradation of phenylacetate and hydroxyphenylacetates by microorganisms[6–8]. However, there are conflicting reports on the involvement of homogentisate during the metabolism of phenylacetate by *Aspergillus* species. As early as 1951, Kluyver and van Zijp[9] observed the production of homogentisic acid out of phenylacetic acid by *Aspergillus niger*. Later, experiments carried out by Bocks[10] also provided evidence for the participation of homogentisate as a key intermediate in the catabolism of phenylacetate by *Aspergillus niger*. However, Faulkner and Woodcock[11] failed to observe formation of this compound in the Mulder strain of *Aspergillus niger*.

During our studies on the metabolism of phenylacetate by *Aspergillus niger*, we also failed to observe the formation of homogentisate from phenylacetate during the growth phase of the organism[12]. In contrast, Ueno *et al.* established the occurrence of homogentisate in an *Aspergillus* species during growth on phenylacetate[13]. This organism was subsequently identified to be *Aspergillus fumigatus*[14]. Most of the above studies, were aimed to test the formation of homogentisate. Nothing is known about the further metabolism of homogentisate in fungi. In order to clarify these points we carefully studied the metabolism of phenylacetate in *Aspergillus niger* and the results are presented in this paper.

2. Materials and methods

Phenylacetic acid was purchased from Fluka AG, Buchs SG, Switzerland. Protamine sulfate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 2-, 3- and 4-hydroxyphenylacetic acids, homogentisic acid, protocatechuic acid, homoprotocatechuic acid, $NADP^+$, NADPH and GSH were from Sigma Chemical Co., St. Louis, Mo., U.S.A. All other chemicals used were of analytical grade available commercially.

Aspergillus niger maintained in our laboratory was used throughout the studies. It was grown at 30° C for 40–60 hr on a Byrde's modified synthetic medium[15] containing 1 gm of sodium phenylacetate per litre of medium. The mycelia were harvested and washed thoroughly with distilled water. The excess water was squeezed out and the mycelial felts were directly used for the *in vivo* experiments and for the preparation of cell-free extracts. To obtain *A. niger* cells grown on glucose as the sole carbon source, phenylacetate was omitted from the medium and the organism was grown as mentioned above.

In vivo *experiments*

The *in vivo* experiments were carried out with mycelial felts, washed aseptically three times with sterile water and replaced in solutions containing 1 μ mole/ml of different substrates in 0·025 M potassium phosphate buffer, pH 5·4. The replacement cultures were kept on a rotary shaker at 30° C for 6 hr and the various products formed were analyzed.

Analysis of products from the growth medium and replacement culture medium

After removing the mycelial felts, the spent medium was acidified to pH 2 by the addition of 2N HCl and extracted thrice with equal volume of diethyl ether. The ether layers were pooled, concentrated dried over anhydrous sodium sulfate and subjected to both paper and thin layer chromatography. Uni- and two-dimensional chromatographic separations were carried out on Whatman No. 3 filter papers using the following solvent systems :

A : Isopropanol - ammonia - water (20 : 1 : 2; v/v/v)

B : Benzene - acetic acid - water (10 : 7 : 3; v/v/v; organic phase)

C : Formic acid-water (2 : 98; v/v).

Silica gel G plates were used for thin layer chromatography employing the first two solvent systems.

Identification of metabolites

Phenolic compounds were located by spraying either with diazotized 4-nitroaniline followed by alkali or with 1% ferric chloride-ferricyanide mixture (1 : 1). The individual compounds were isolated from paper as well as thin layer chromatograms by eluting with ether. The products were identified by comparing their

(*a*) R_f values in different solvent systems;

(*b*) UV absorption spectra; and

(*c*) IR spectra,

with those of authentic samples.

Growth of the organism

The growth of *A. niger* was followed by determining the dry weight of the mycelial felts after growth on Byrde's medium containing phenylacetate. Twenty-five ml of the medium were distributed in 250 ml conical flasks and the flasks were inoculated with a spore suspension of *A. niger*. At regular intervals, the mycelial felts were suction filtered through Whatman No. 1 filter paper and washed twice with water. The washed mats were then dried overnight in an oven at 110° C and cooled to room temperature before weighing. The spent medium was used for the quantitative determination of metabolites, after subjecting to chromatographic separation.

Preparation of cell-free extracts

The mycelial felts were washed with cold water and ground with an equal weight of glass powder in a chilled porcelain mortar. To the above broken cell paste, 0·025 M sodium phosphate buffer pH 7·0 (three ml per gm of wet mat) was added and mixed well. The slurry was passed through cheese cloth and centrifuged at 17,000 × *g* for 15 min. The supernatant obtained was designated as the crude enzyme preparation.

Preparation of particulate and supernatant fractions

The crude enzyme preparation was centrifuged at 100,000 × *g* for 1 hr in No. 40 rotor of a Spinco model L preparative ultracentrifuge. The dark red coloured pellet obtained was drained free from the supernatant fluid, washed with cold water and suspended uniformly in 0·025 M sodium phosphate buffer, pH 7·0. This preparation was designated as the particulate fraction and the 100,000 × *g* supernatant, as the supernatant fraction.

Isolation of enzymes of the homogentisate pathway

The nucleic acids from the crude extract were precipitated by the addition of 2% protamine sulfate (3 ml) to the crude extract (27 ml) and separated by centrifuging for

10 min at 12,000 × *g*. To the protamine sulfate supernatant was added solid ammonium sulfate, to obtain 70% saturation. The precipitate formed within 10 min was collected by centrifugation at 17,000 × *g* for 10 min, and was used for the assay of enzymes of the homogentisate pathway.

Isolation and identification of the products of homogentisic acid oxidation

To a reaction mixture containing 50 ml of 0·1 M sodium phosphate buffer, pH 7·0, and 100 mg of enzyme protein, were added 50 mg homogentisic acid and 5 mg of GSH. The reaction was allowed to proceed for 2 hr and then divided into two portions. One portion was treated with diazotized 4-nitroaniline according to the procedure of Walker[16]. The spectrum of the formazan formed was identical with that given by acetoacetate, similarly treated (λ_{max} in ethylacetate at 450 nm). The pH of the other portion was adjusted to 4·0 and sufficient absolute ethanol was added to give a final concentration of 95%. After removing the precipitated material, the clear solution was concentrated and subjected to thin layer chromatography on cellulose plates using the following solvent systems:

(A) Butanol - acetic acid - water (12 : 3 : 5; v/v/v), and

(B) Ethanol - ammonium hydroxide - water (16 : 1 : 3; v/v/v).

Fumaric acid was identified to be the product by comparison of R_f values (R_f value of fumaric acid is 0·8 in solvent A and 0·25 in solvent B).

Estimation of 2–and 3–hydroxyphenylacetic acids

2- and 3-hydroxyphenylacetic acids were estimated as their diazo derivatives.

Estimation of protein

The protein content of various preparations was estimated by the method of Lowry *et al.*[17] using bovine serum albumin as the standard.

Enzyme assays

Assay of phenylacetate monooxygenase activity

Phenylacetate monooxygenase activity was assayed by following the appearance of hydroxylated products in a 1 ml reaction mixture consisting of 1 mg of microsomal protein, 2 mM glucose-6-phosphate, 0·2 mM $NADP^+$, 0·2 units of glucose-6-phosphate dehydrogenase, 5 mM sodium phenylacetate and 0·5 ml of 0·2 M sodium phosphate buffer, pH 7·8. The reaction was carried out under aerobic conditions for 1 hr at 30° C. Hydroxylation was initiated by the addition of NADPH regenerating system and terminated by the addition of 0·1 ml of 50% trichloroacetic acid. After removing the denatured proteins by centrifugation, an aliquot from the supernatant solution was

pipetted out into a test-tube, made up to 1 ml. To this 1 ml of diazotized 4-nitroaniline (freshly prepared by mixing 10 ml of 0·138% 4-nitroaniline in 0·8 N HCl and 0·4 ml of 3·4% $NaNO_2$) and 1·5 ml of 10% NaOH were added in the given order and the red colour developed was immediately read against a reagent blank in Klett-Summerson photoelectric colorimeter using No. 54 filter (500–570 nm). The response to increasing concentration of 2-hydroxyphenylacetic acid in the test solution was linear up to 160 nmoles. A reaction mixture, to which substrate was added after TCA addition, served as the control.

Assay of the enzymes of the homogentisate pathway

Homogentisate-1, 2-dioxygenase activity was assayed in a 3 ml reaction mixture containing 1·0 ml of enzyme, 2·0 ml of 0·2 M sodium phosphate buffer, pH 7·0 and 0·4 μmoles of homogentisic acid. The increase in absorbance at 330 nm, due to the formation of maleylacetoacetate, was followed spectrophotometrically at room temperature. After the complete oxidation of homogentisate to maleylacetoacetate, 1 μmole of GSH was added and the decrease in absorbance at 330 nm was followed. The reaction mixture, in which substrate was absent, served as the control.

Spectrophotometry

All UV and visible spectrophotometric measurements were made in a Cary 14 double beam recording spectrophotometer. Ultraviolet absorption spectra of various compounds were determined in 95% ethanol. Infrared spectra were taken in Perkin-Elmer infrared spectrophotometer, model 700, on nujol mull.

3. Results

Growth of the organism

The growth curve of *Aspergillus niger* on sodium phenylacetate is shown in Fig. 1. Twenty hr after inoculation of spores, the organism enters the log phase and after 40 hr, it reaches the stationary phase. Sporulation starts from 55 hr onwards.

Analysis of the culture filtrate

The ether extract of the 40 hr old culture filtrate contained two major phenolic compounds. These two compounds were identified to be 2- and 3-hydroxyphenylacetic acids by comparison of the R_f values in three different solvent systems and UV absorption spectra with those of authentic sample. The IR spectra of authentic and isolated compounds are shown in Figs. 2 and 3.

The amount of 2- and 3-hydroxyphenylacetic acids excreted into the medium as a function of time is shown in Fig. 1 along with the growth curve. While the degradation of 3-hydroxyphenylacetate starts at around 38 hr, 2-hydroxyphenylacetate accumulates

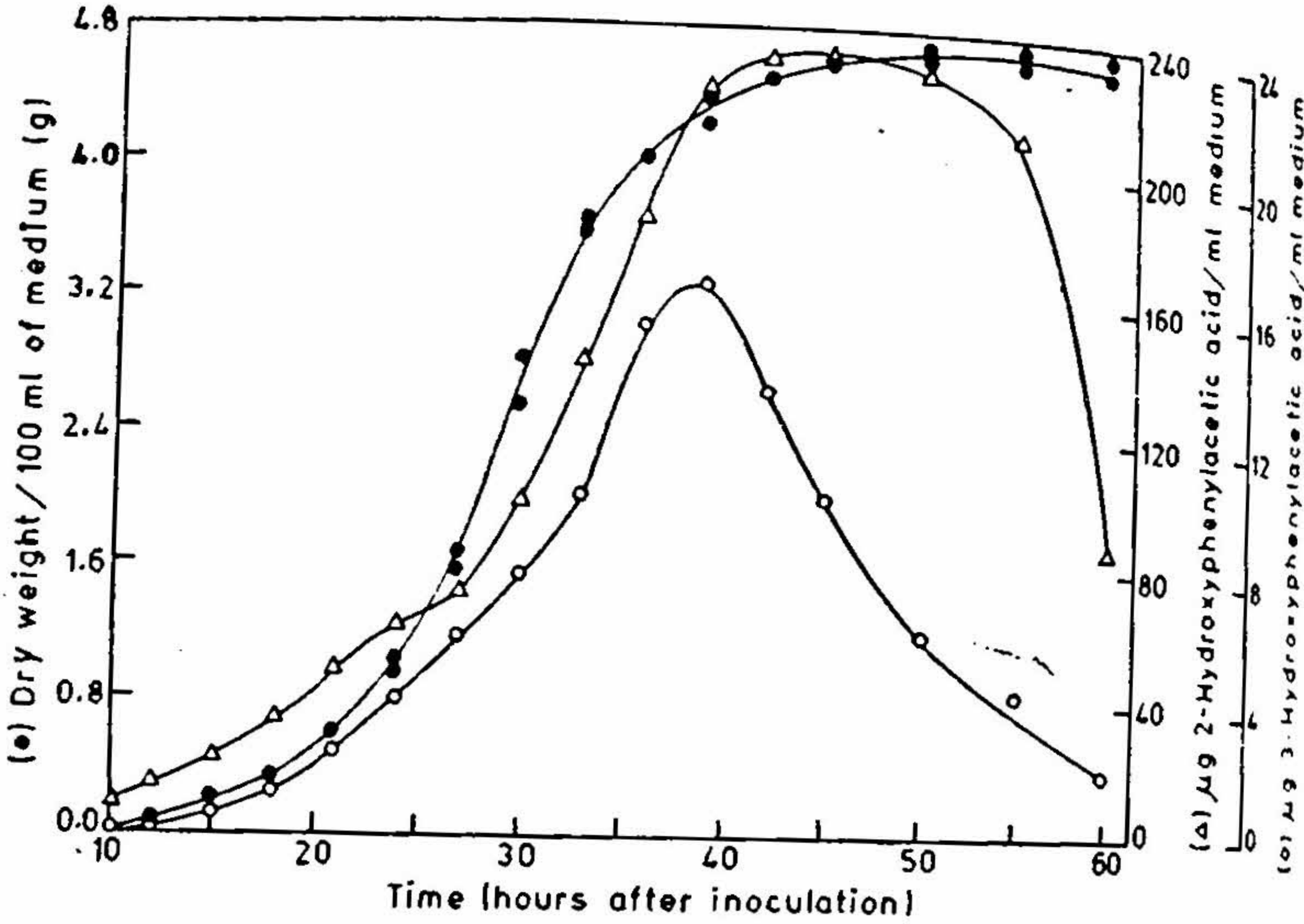

FIG. 1. Growth curve of *Aspergillus niger* on phenylacetic acid and time course of accumulation some metabolites.

in the medium till 55 hr. As 2-hydroxyphenylacetate was utilized rapidly after 55 h an attempt was made to isolate the metabolites from 55 hr old culture. This result in the isolation of a dihydroxyphenolic acid metabolite which could be identified homogentisic acid whose UV and IR spectra are given in Figs. 4 and 5.

Analysis of the replacement culture medium

The phenolic compounds that accumulate in the replacement culture medium, wh 40-hr grown mycelial felts of *A. niger* are incubated with the various metabolites phenylacetic acid, are shown in Table I. Even trace amounts of homogentisic ac homoprotocatechuic acid, mandelic acid, 4-hydroxyphenylacetic acid, 3- and 4-meth catechols or gentisic acid, could not be detected. However, when 55 hr old *A. ni* mats are used, homogentisic acid is formed rapidly (Table II). The R_f values of vari compounds isolated and the authentic samples are given in Table III.

Enzymes of the pathway

In order to get more insight into the mechanism of phenylacetate dissimilation *Aspergillus niger*, attempts were made to isolate the different enzymes involved in degradative pathway.

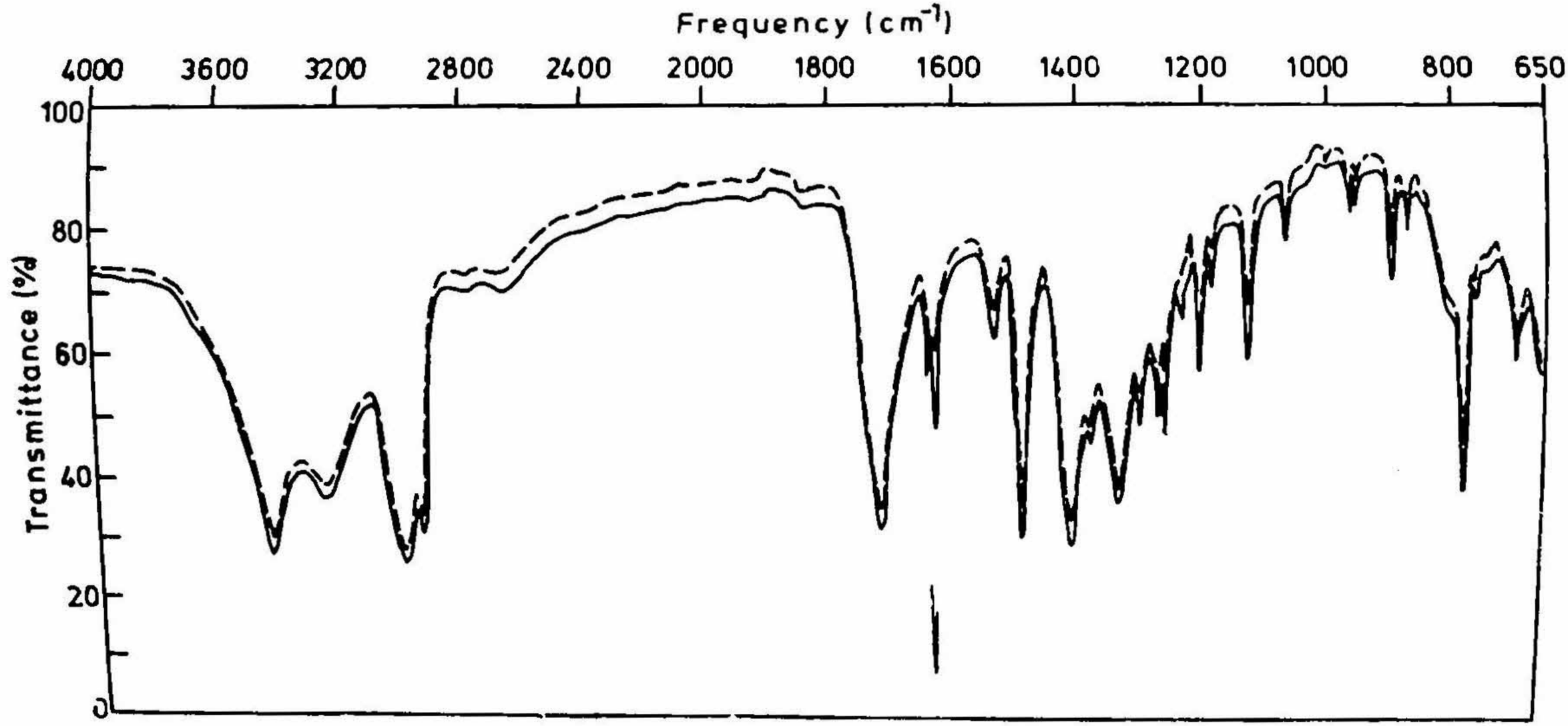

FIG. 2. Infrared spectrum of 2-hydroxyphenylacetic acid.

———— Isolated

— — — — Authentic

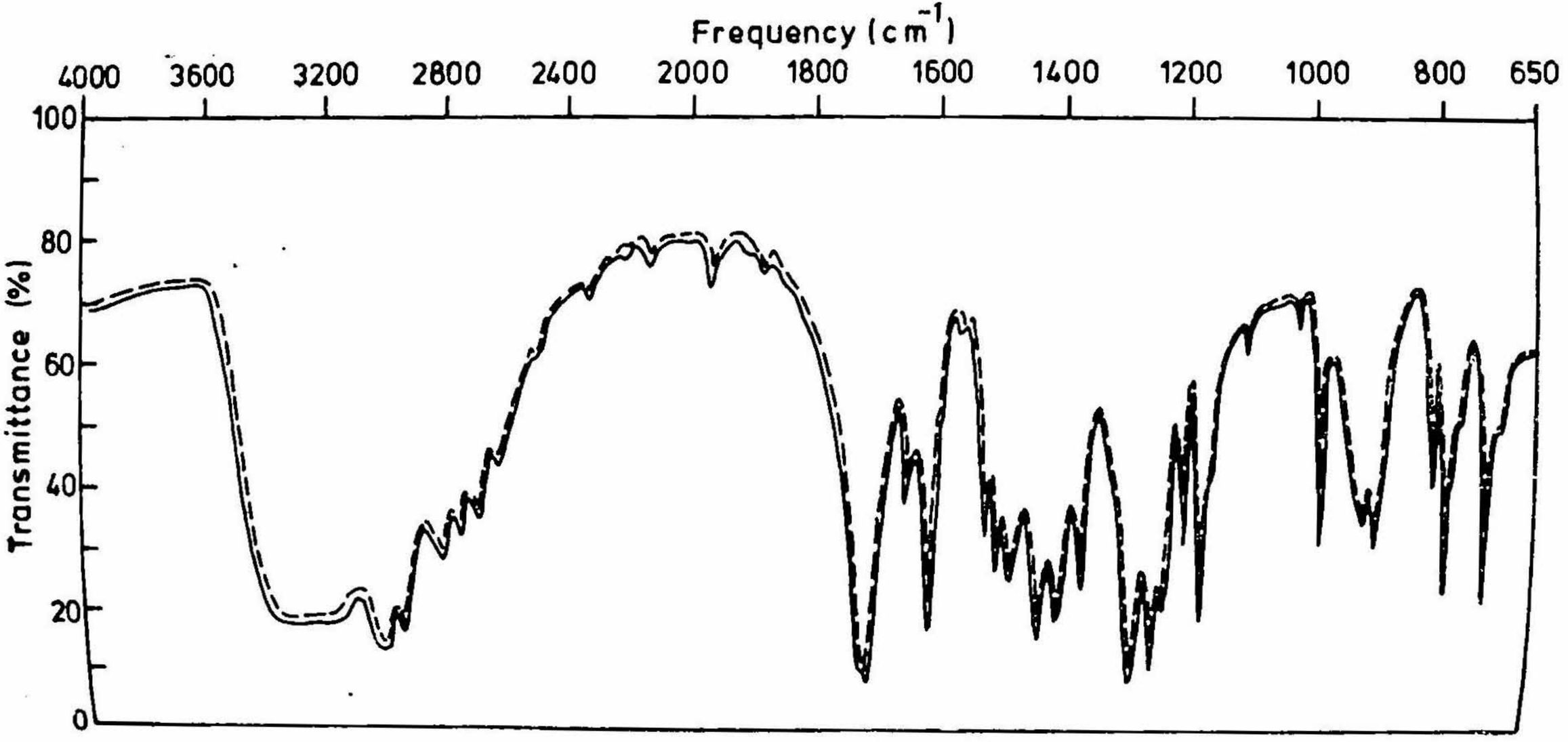

FIG. 3. Infrared spectrum of 3-hydroxyphenylacetic acid.

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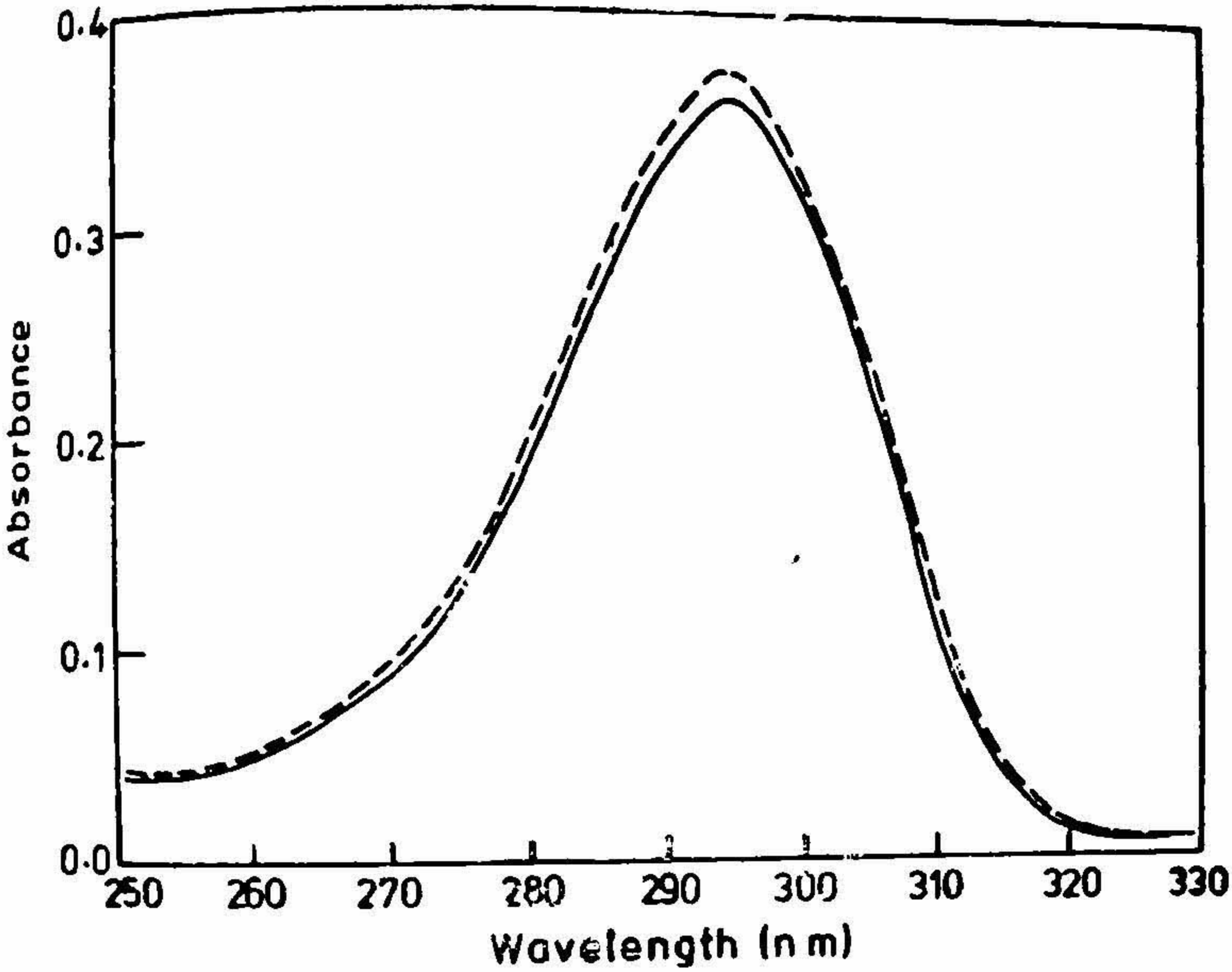

FIG. 4. Ultraviolet absorption spectrum of homogentisic acid.
——————— Isolated
— — — — — Authentic

Table I

Results of replacement culture studies with 40 *hr old* A. niger *mycelial felts*

Compounds added to the replacement culture medium	Phenolic compounds detected in the replacement medium		
	2-hydroxy-phenylacetic acid	3-hydroxy-phenylacetic acid	Homogentisic acid
Phenylacetic acid	+++	++	−
2-hydroxyphenylacetic acid	+++	−	−
3-hydroxyphenylacetic acid	−	+++	−

− = not detectable
+++ = very high yield
++ = good yield

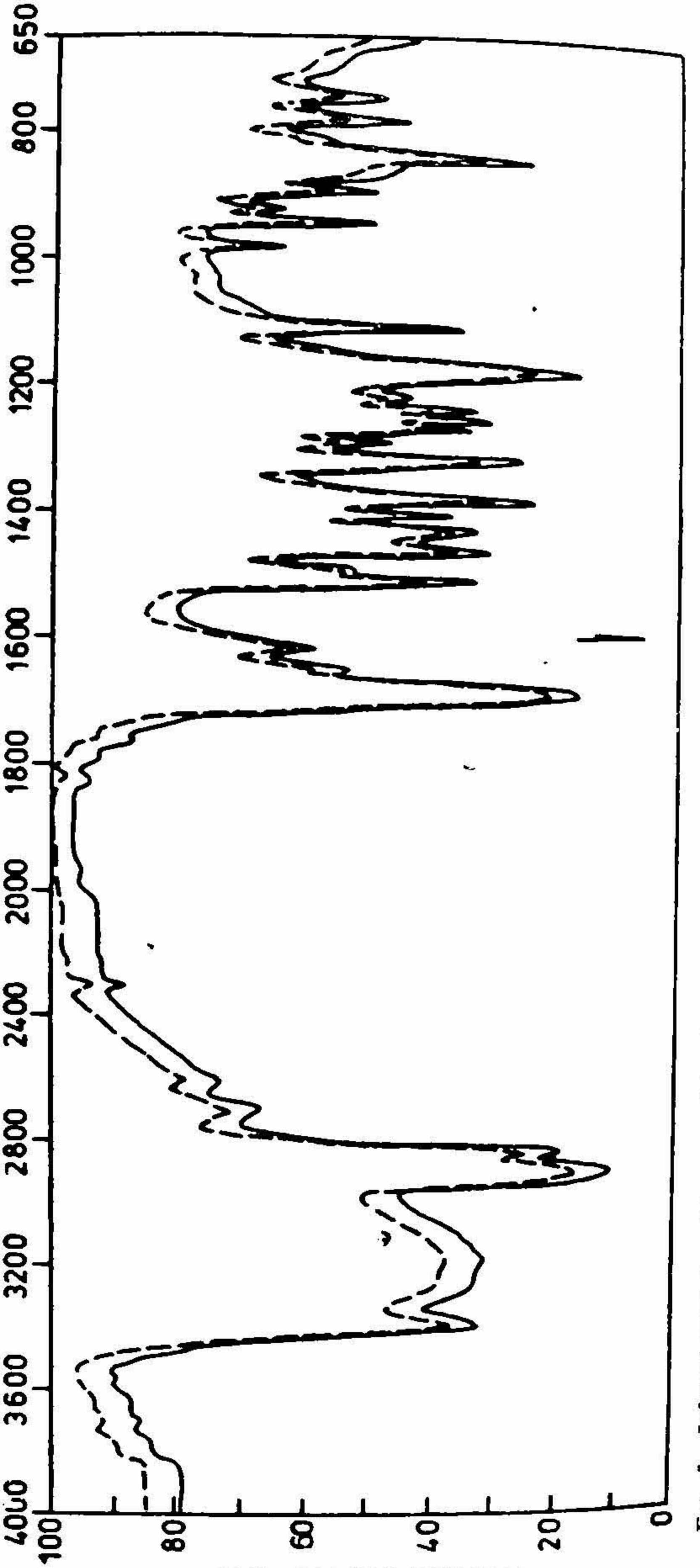

FIG. 5. Infrared spectrum of homogentisic acid.
——— Isolated
— — — — Authentic

Table II

Results of replacement culture studies with 55 hr old A. niger *mycelial felts*

Compounds added to the replacement culture medium	Phenolic compounds detected in the replacement medium		
	2-hydroxy-phenyl acetic acid	3-hydroxy-phenylacetic acid	homogentisic acid
Phenylacetic acid	++	+	++
2-hydroxyphenylacetic acid	+++	−	+++
3-hydroxyphenylacetic acid	−	+++	−

− = Not detectable
+++ = very high yield
++ = good yield.
+ = poor yield.

Table III

R_f values and λ_{max} of the phenolic compounds

Compound	R_f values in solvent systems						λ_{max} in ethanol (nm)	
	A		B		C			
	a	*i*	*a*	*i*	*a*	*i*	*a*	*i*
2-hydroxy-phenylacetic acid	0·52	0·52	0·43	0·44	0·88	0·88	274	274
3-hydroxy-phenylacetic acid	0·29	0·28	0·31	0·31	0·82	0·82	273	273
Homogentisic acid	Trailing with oxidation		0·02	0·02	0·88	0·88	295	295

A = Isopropanol - ammonia - water (20 : 1 : 2; v/v/v)
B = Benzene - acetic acid - water (10 : 7 : 3; v/v/v; organic phase)
C = Formic acid - water (2 : 98; v/v)
a = authentic; *i* = isolated.

Phenylacetate monooxygenase, occurs in the particulate fraction. Fig. 6 shows the time course of hydroxylation of phenylacetate. The products of the hydroxylation were isolated from a bulk reaction mixture and identified to be 2- and 3-hydroxyphenylacetates. The ratio of 2-hydroxyphenylacetate to 3-hydroxyphenylacetate was found to be approximately 93 : 7. Enzyme requires, in addition to the substrate, both NADPH and molecular oxygen for exhibiting the hydroxylase activity.

2-Hydroxyphenylacetate accumulates in the growth medium, till the onset of sporulation (Fig. 1). During the sporulation phase, it was rapidly utilized and the product of the oxidation was identified to be homogentisate (Table II). However, 2-hydroxyphenylacetate-5-monooxygenase activity could not be demonstrated in the cell-free extracts. The terminal dioxygenase, *viz.*, homogentisate-1, 2-dioxygenase occurs in the supernatant fraction and oxidizes homogentisate to maleylacetoacetate. Fifty-five hr.

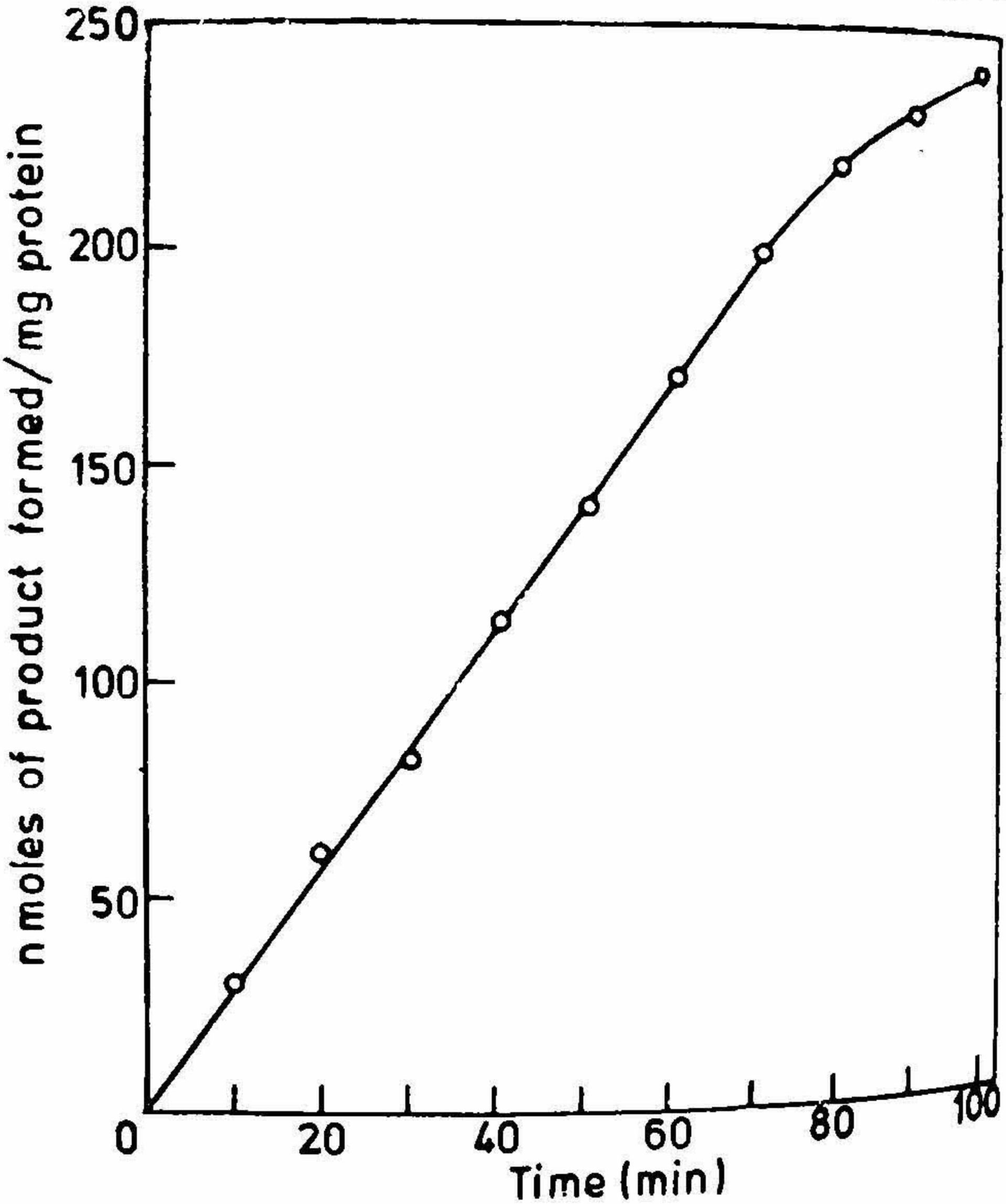

FIG. 6. Time course of phenylacetate hydroxylation reaction.

grown cells possessed a three fold higher dioxygenase activity than the 40-hr grown cells. A typical assay of homogentisate pathway enzymes is shown in Fig. 7. Due to the formation of maleylacetoacetate in the experimental cuvette, where homogentisate undergoes oxidation, there is an increase in absorbance at 330 nm. α, α'-dipyridyl, the known inhibitor of homogentisate dioxygenase, inhibits this increase in absorbance (curve B). Glutathione, the cofactor for maleylacetoacetate isomerase, causes a rapid decrease in the absorbance at 330 nm, due to the isomerization of maleylacetoacetate to fumarylacetoacetate and the rapid hydrolysis of the latter. The formation

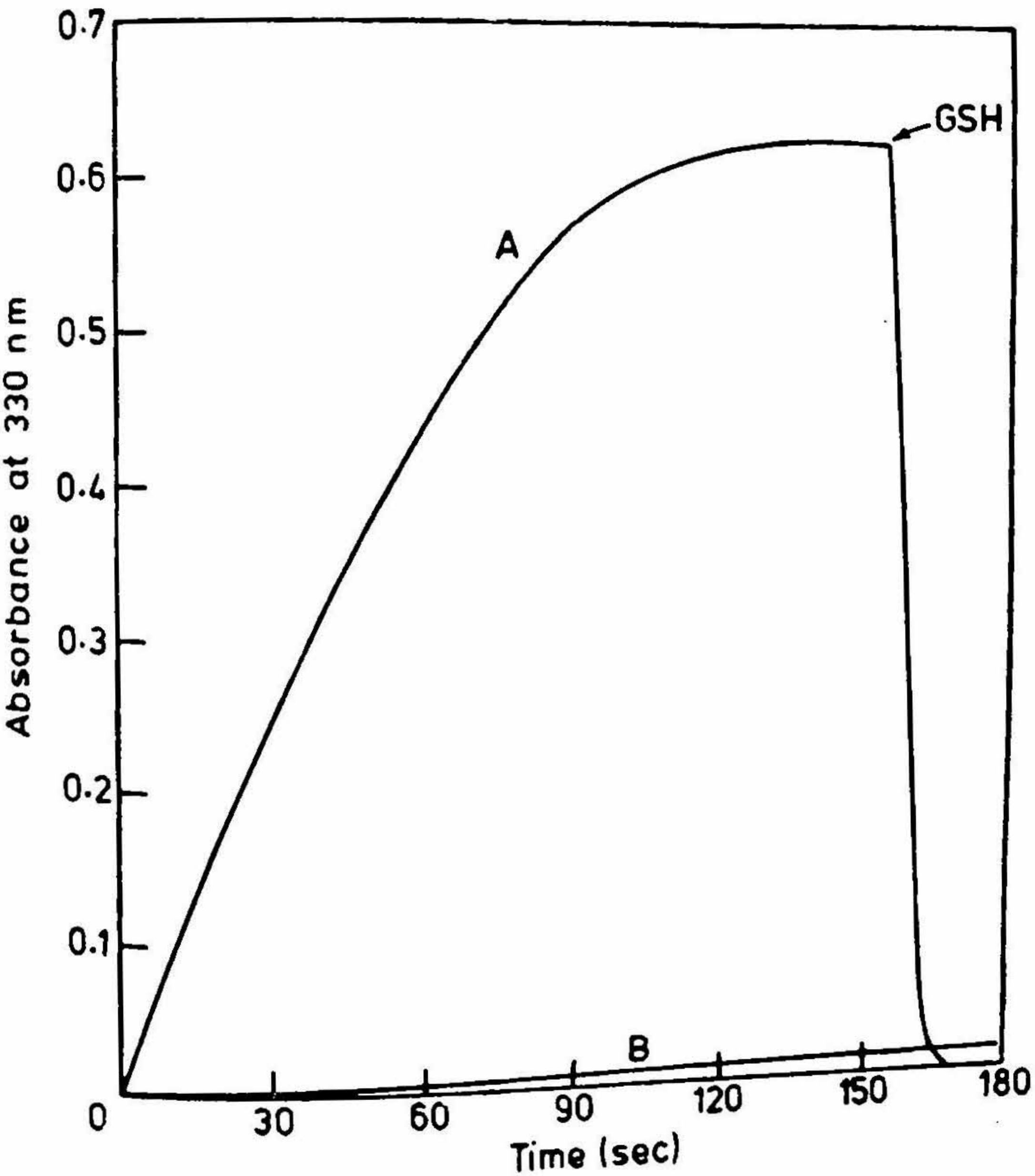

FIG. 7. Spectral changes during oxidation of homogentisic acid.

of fumarate and acetoacetate, as the end products of homogentisate pathway, has also been confirmed by isolating and identifying them following the methods detailed earlier.

All the enzymes studied were inducible in nature and were present only in phenyl-acetate-grown cells.

4. Discussion

The isolation and identification of 2- and 3-hydroxyphenylacetic acids, and homogentisic acid suggest that these compounds are intermediates in the catabolism of phenylacetic acid by *A. niger*. An examination of Fig. 1 reveals that both 2- and 3-hydroxyphenyl-acetates start accumulating in the medium; the former several times more than the latter. While 3-hydroxyphenylacetic acid is utilized rapidly during the stationary phase, 2-hydroxyphenylacetic acid continues to accumulate till late stationary phase, by which time the sporulation starts. When 3-hydroxyphenylacetate has nearly disappeared, utilization of 2-hydroxyphenylacetate starts. This might be due to the inhibition of 2-hydroxyphenylacetate degradation by 3-hydroxyphenylacetate and might be a strategy adopted by the organism to keep substrates in reserve for utilization at a later period.

In order to establish the pathway, some of the key enzymes of phenylacetate metabolism were isolated and characterized. Phenylacetate monooxygenase, which occurs in the particulate fraction of *A. niger*, converts the substrate to 2- and 3-hydroxyphenyl-acetates. Several investigators have reported the hydroxylation of phenylacetate to 2-hydroxyphenylacetate in fungi[10, 11, 18–20], bacteria[6] and plants[21]. However, the enzyme catalyzing this conversion has not yet been isolated.

Hydroxylation of phenylacetic acid to 3-hydroxyphenylacetic acid, occurs in *Rhizoctonia solani*[20], *A. niger*[10, 11] and *Penicillium*[20]. The enzyme effecting this hydroxylation has been recently isolated from *R. solani*[22] and shown to be a tetrahydropteridine-dependent monooxygenase requiring NADH as the external electron donor. However, in *A. niger*, such an enzyme activity could not be demonstrated either in the particulate or in the supernatant fractions.

3-hydroxyphenylacetate is utilized by the organism employing the pathway published earlier[12]. At present it is not clear why the organism uses 3-hydroxyphenylacetate first and then 2-hydroxyphenylacetate. As stated earlier, one possible explanation could be the inhibition of enzymes of 2-hydroxyphenylacetate pathway by 3-hydroxy-phenylacetate and its metabolites. In fact, 3-hydroxyphenylacetate and protocatechuate inhibit homogentisate dioxygenase activity drastically (unpublished results). However, 2-hydroxyphenylacetate also inhibits this enzyme. Perhaps, studies on 2-hydroxyphenyl-acetate-5-monooxygenase might throw more light on this aspect.

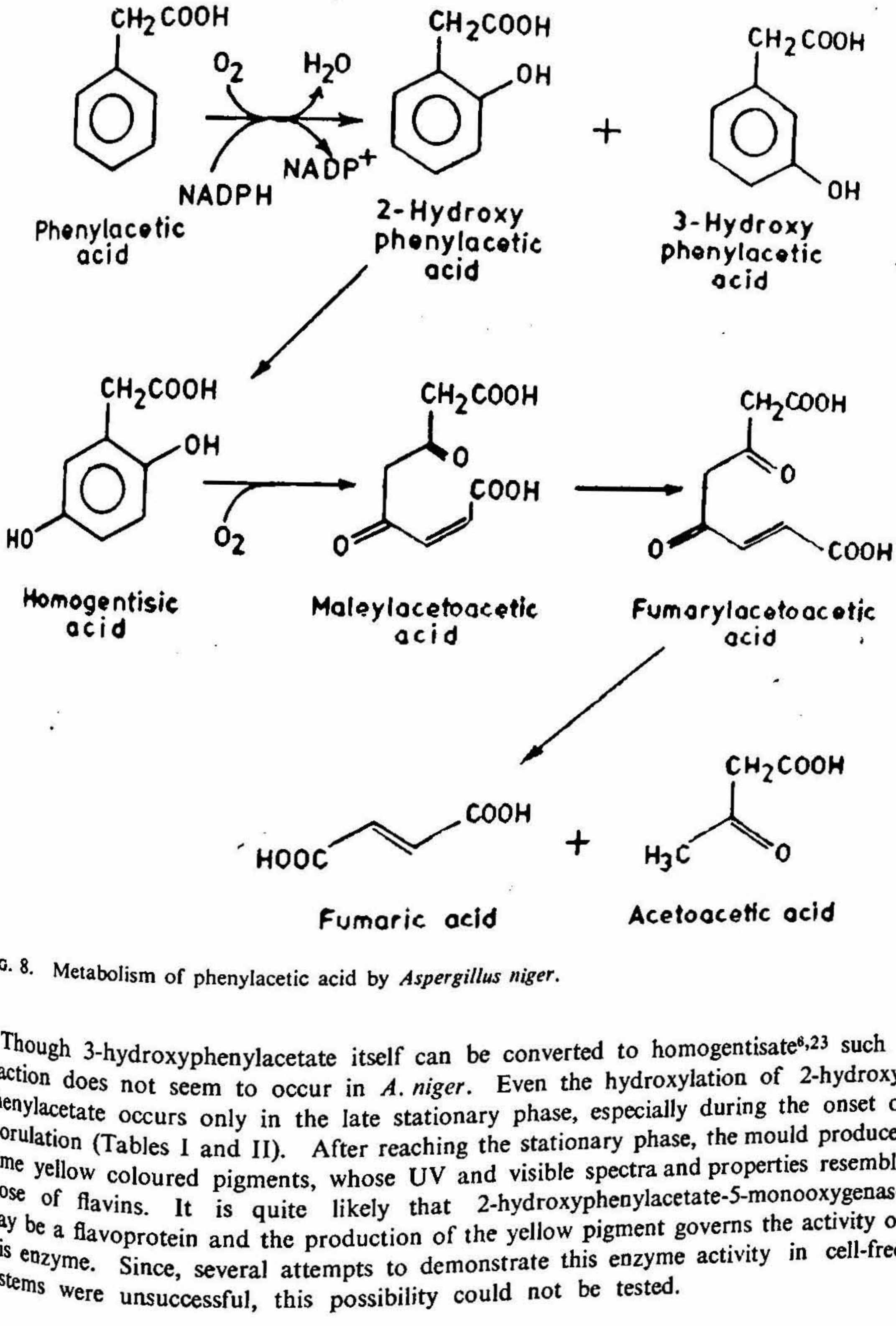

FIG. 8. Metabolism of phenylacetic acid by *Aspergillus niger*.

Though 3-hydroxyphenylacetate itself can be converted to homogentisate[6,23] such a reaction does not seem to occur in *A. niger*. Even the hydroxylation of 2-hydroxyphenylacetate occurs only in the late stationary phase, especially during the onset of sporulation (Tables I and II). After reaching the stationary phase, the mould produces some yellow coloured pigments, whose UV and visible spectra and properties resemble those of flavins. It is quite likely that 2-hydroxyphenylacetate-5-monooxygenase may be a flavoprotein and the production of the yellow pigment governs the activity of this enzyme. Since, several attempts to demonstrate this enzyme activity in cell-free systems were unsuccessful, this possibility could not be tested.

As 2-hydroxyphenylacetate is not utilized before the onset of sporulation, even if it is exogenously added to the medium, it is likley that its catabolic reactions are triggered only during the pre-sporulation phase. Perhaps, the high rate of anabolism, occurring during sporulation, may necessitate the utilization of available secondary metabolites in the medium. Apart from this, there is a three-fold increase in the specific activity of homogentisate dioxygenase, if it is isolated from 55-hr old cells rather than from 40-hr old cells. This finding clearly indicates that homogentisate pathway is operating only in the later stages of growth of *A. niger*.

The evidence presented above clearly shows the operation of the pathway shown in Fig. 8 for the catabolism of phenylacetate by *A. niger*. The pathway seems to operate during the sporulation phase. Probably, due to this reason, homogentisate formation could not be observed during growth phase. Secondly, if the rate of degradation is higher than the rate of synthesis, homogentisate would not accumulate to any detectable amount in the medium. Due to any of these two reasons Faulkner and Woodcock[11] have failed to observe homogentisate accumulation in the medium.

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