THE ENZYMATIC OXIDATION OF *p*-HYDROXYMANDELIC ACID TO *p*-HYDROXYBENZOIC ACID[1]

SHIRLEY E. GUNTER[2, 3]

Department of Bacteriology, University of California, Berkeley, California

Received for publication March 9, 1953

During an investigation of the relationship between the adaptive enzymes of *Pseudomonas fluorescens* catalyzing the oxidation of mandelate and *p*-hydroxymandelate, it became evident that establishment of the pathway of the breakdown of *p*-hydroxymandelate was necessary to an understanding of the problem. On the basis of structural similarity it seemed likely that the oxidation of *p*-hydroxymandelate might proceed in a manner analogous to that of mandelate. The degradation of the latter compound to benzoate with the formation of benzoylformate and benzaldehyde as intermediates was postulated by Stanier (1948) and has been confirmed by Gunsalus, Stanier, and Gunsalus (1953).

The oxidation of *p*-hydroxymandelate would be expected to occur in the following series of steps:

(1) $HOC_6H_4CHOHCOOH + \frac{1}{2}O_2 \rightarrow HOC_6H_4COCOOH + H_2O$
(*p*-hydroxymandelic acid → *p*-hydroxybenzoylformic acid)

(2) $HOC_6H_4COCOOH \rightarrow HOC_6H_4CHO + CO_2$
(*p*-hydroxybenzoylformic acid → *p*-hydroxybenzaldehyde)

(3) $HOC_6H_4CHO + \frac{1}{2}O_2 \rightarrow HOC_6H_4COOH$
(*p*-hydroxybenzaldehyde → *p*-hydroxybenzoic acid)

Further oxidation of *p*-hydroxybenzoic acid would proceed via protocatechuic and β-ketoadipic acids as shown by Sleeper and Stanier (1950) and Stanier *et al.* (1950). This paper presents the results of an investigation of the validity of this postulated metabolic scheme.

The general sequence of reactions in the oxidation of *p*-hydroxymandelate via *p*-hydroxybenzoate and protocatechuate was determined by the technique of "simultaneous adaptation". In order to establish the individual reactions in the conversion of *p*-hydroxymandelate to *p*-hydroxybenzoate, the intermediates were isolated from reaction mixtures and characterized. Enzyme extracts which were capable of catalyzing only a portion of the reactions in the pathway were employed in the accumulation of the intermediates.

MATERIALS AND METHODS

P. fluorescens, strain A.3.12, or enzymatic extracts prepared from this strain were used throughout this investigation. Yeast extract agar was used for the maintenance of the culture and for the growth of unadapted cells. Cells adapted to mandelate were obtained by growing the organisms on mandelate mineral medium composed of 0.1 per cent ammonium nitrate, 0.1 per cent dipotassium phosphate, 0.05 per cent magnesium sulfate, 0.01 M mandelic acid, and 2 per cent agar. All media were adjusted to pH 7 prior to sterilization. In order to conserve the small supply of *p*-hydroxymandelic acid available, cells were adapted to this substrate by shaking 50 ml of a suspension of unadapted organisms in the presence of 100 to 200 μM of the compound at 30 C for a period of about nine hours.

Cell-free enzymatic solutions were prepared by the alumina grinding technique of McIlwain (1948), starting with freshly grown cells in some cases, but more frequently with lyophilized, mandelate grown cells. One gram of lyophilized cells

[1] This work was supported by a grant-in-aid made to Dr. R. Y. Stanier and Dr. M. Doudoroff by the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

[2] The data presented in this paper are included in a thesis submitted to the Graduate School of the University of California in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[3] Present address: Radiological Laboratory, School of Medicine, University of California Medical Center, San Francisco 22, California.

was moistened with 1.5 ml of the extracting liquid and ground with six grams of levigated alumina. Following grinding, the mixture was extracted with a chilled solution at a ratio of 20 ml of liquid per gram of dried cells. Three solutions were used: 0.017 M Sorenson's phosphate buffer of pH 7, 0.1 M Na_2HPO_4 solution, and 0.013 M $NaHCO_3$. The extracts were separated from the alumina by centrifugation at low speed. All procedures were carried out in the cold.

Subsequent treatment of the extracts varied depending upon the type of determination in which the material was to be used. Preliminary experiments revealed that centrifugation at 20,000 rpm for 20 minutes caused a sedimentation of the particulate debris leaving a water-clear supernatant. The latter apparently contained only the dehydrogenases inasmuch as it would not oxidize mandelate with the uptake of oxygen unless a small amount of the debris was added back. However, substrate oxidation was catalyzed by the clear supernatant if the reaction was carried out in the presence of a suitable electron acceptor such as potassium ferricyanide under anaerobic conditions. The whole cell extract was used during the R.Q. determinations. If intermediates were to be isolated from the reaction mixtures, only the clear enzymatic solution was added.

The usual techniques were employed in the manometric experiments. In most cases, the suspending medium was 0.017 M Sorenson's phosphate buffer, pH 7, or 0.025 M sodium bicarbonate solution. The substrate solutions were 0.02 M in concentration and were prepared in 0.017 M phosphate buffer. In the case of the aromatic acids, the solutions were always neutralized with 1 N NaOH and adjusted to pH 7.0 to 7.2. The method of Haas (1937), as modified by Quastel and Wheatley (1938), was followed in those experiments in which potassium ferricyanide was employed as the electron acceptor during substrate oxidation.

Bacterial suspensions which were to be irradiated with ultraviolet light were prepared according to the usual procedures and dispensed into petri dishes, 12 ml per dish. A 30 watt GE sterilizing lamp served as the source of the ultraviolet light, and the plates were placed on a rotary shaking machine to provide thorough mixing of the cells during the irradiation. An exposure time of 1 to 3 minutes was adequate to stop adaptation in a suspension adjusted to a density of 1.3 to 2.6×10^9 bacteria per ml.

The spectrophotometric analyses of the reaction mixtures were carried out in the following manner: The samples were deproteinized by the addition of sufficient 10 per cent trichloroacetic acid to give a final concentration of 1 per cent and the supernatants extracted at pH 3 five times with equal volumes of ether. The ether extract was evaporated to dryness *in vacuo*, and the residue was taken up in a small volume of 0.017 M phosphate buffer of pH 7. The samples were diluted to give a final substrate concentration of approximately 0.0001 M before determining the absorption spectra in a Beckman spectrophotometer, Model DU. The results were always corrected for the absorption of an extract of a control sample containing only enzymes or cells without added substrate.

Mandelic, benzoic, *p*-hydroxymandelic, *p*-hydroxybenzoylformic, and *p*-hydroxybenzoic acids were separated by means of ascending paper chromatography, using the method of Williams and Kirby (1948). The solvent mixture was composed of methanol, 24 ml; *n*-butanol, 12 ml; propionic acid, 2 ml; benzene, 12 ml; and water, 4 ml. The method of Lugg and Overell (1948) employing an alcoholic solution of brom-phenol-blue was used for the detection of spots on the chromatograms, which usually were steamed for a short time after they were sprayed with the indicator solution.

Keto acids were identified by the color reaction resulting from the treatment of the 2,4-dinitrophenylhydrazone with sodium hydroxide following extraction with ethyl acetate and sodium carbonate solution, according to the procedure of Friedemann and Haugen (1943).

RESULTS

The technique of "simultaneous adaptation" provided evidence for the role of *p*-hydroxybenzoate and protocatechuate as intermediates in the oxidation of *p*-hydroxymandelate. Washed suspensions of whole cells, unadapted and adapted to *p*-hydroxymandelate, were irradiated with ultraviolet light to prevent subsequent adaptation and tested manometrically for their ability to oxidize *p*-hydroxymandelate, *p*-hydroxybenzoate, and protocatechuate. The results presented in table 1 show that cells adapted to *p*-hydroxymandelate oxidized not only that compound but

also p-hydroxybenzoate and protocatechuate at high rates. None of these compounds was oxidized actively by the unadapted cells. The data indicate that adaptation to p-hydroxymandelate induces formation of the enzymes catalyzing the oxidation of p-hydroxybenzoate and protocatechuate and suggest that these compounds are intermediates in the degradation of p-hydroxymandelate.

In experiments to be reported in a subsequent paper, cells adapted to mandelate were found to possess the ability to oxidize p-hydroxymandelate. Extracts of lyophilized cells grown on mandelate medium also actively attacked p-hydroxymandelate. However, some of the enzymes, including that catalyzing the degradation of p-hydroxybenzoate, were inactivated partially or wholly by the lyophilization process, resulting in the accumulation of certain intermediates during the oxidation of the primary substrate. Such extracts were well suited to the investigation of the individual steps of the degradation of p-hydroxymandelate to p-hydroxybenzoate and eliminated the necessity of using large quantities of the former compound for the preparation of adapted cells.

TABLE 1

The effect of adaptation to p-hydroxymandelate on the oxidation of p-hydroxybenzoate and protocatechuate

SUBSTRATE	RATE OF OXIDATION IN MICROLITERS OXYGEN CONSUMPTION PER 20 MINUTES	
	Unadapted cells	Cells adapted to p-hydroxymandelate
None	2.2	2.3
p-Hydroxymandelate	11.2	52.1
p-Hydroxybenzoate	5.9	48.7
Protocatechuate	2.6	22.7

Tentative identification of the intermediates was made by the spectrophotometric analysis of ether extracts of samples removed at intervals during the oxidation of p-hydroxymandelate. The reaction was carried out under anaerobic conditions in a number of respirometer vessels using limiting amounts of substrate so that the degradation might be followed manometrically. Samples were removed when a change in the rate of carbon dioxide evolution indicated that a given reaction had ended. The absorption spectra of the extracts of these samples were compared with those of solutions of p-hydroxymandelic and p-hydroxybenzoic acids and p-hydroxybenzaldehyde. The manometric data are presented in figure 1 and the absorption spectra in figures 2 and 3.

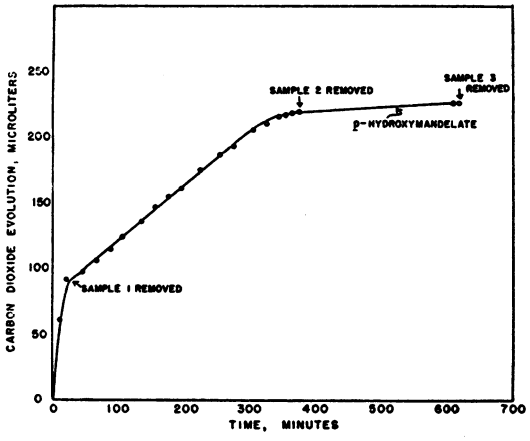

Figure 1. The anaerobic oxidation of p-hydroxymandelate by an extract of alumina ground, lyophilized, mandelate grown cells.

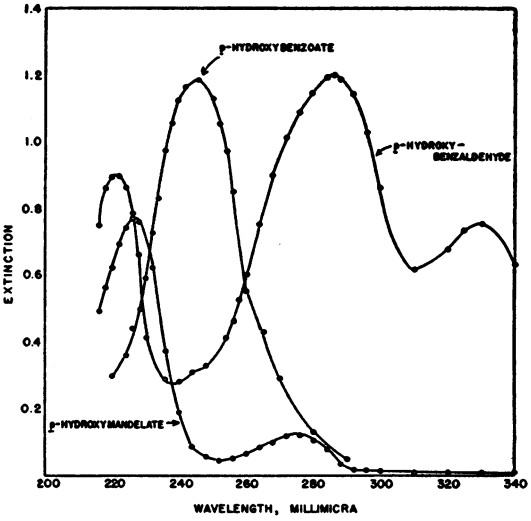

Figure 2. The ultraviolet absorption spectra of p-hydroxymandelate, p-hydroxybenzaldehyde, and p-hydroxybenzoate measured at pH 7.0.

Analysis of the data indicated that the preparation rapidly degraded p-hydroxymandelate to p-hydroxybenzaldehyde, which then was oxidized slowly to p-hydroxybenzoate. Since the enzyme responsible for the oxidation of the latter compound was inactivated completely by lyophili-

zation, the reaction never proceeded beyond this stage.

The first two steps in the oxidation of *p*-hydroxymandelate were postulated to be: (1) the dehydrogenation of *p*-hydroxymandelate to form *p*-hydroxybenzoylformate and (2) the decarboxylation of the latter compound to *p*-hydroxybenzaldehyde. From the spectrophotometric data it was apparent that, if the hypothetical scheme were correct, the second reaction was proceeding at least as rapidly as the first. Fortunately, dialysis of the bacterial extract against 0.1 M Na_2HPO_4 for four days at 3 C inactivated the enzyme catalyzing the decarboxylation reaction but not that carrying out the initial dehydrogenation of the substrate, making it possible to separate the two reactions.

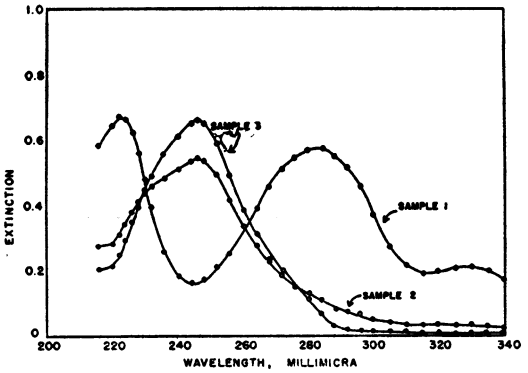

Figure 3. The ultraviolet absorption spectra of extracts of samples removed during the anaerobic oxidation of *p*-hydroxymandelate by a cell-free enzyme preparation. Measurements made at pH 7.0. Time of sampling is shown in figure 1.

Evidence for the inactivation of the decarboxylase as well as for the occurrence of the two postulated reactions was obtained from R.Q. determinations, carried out during the oxidation of *p*-hydroxymandelate by dialyzed and undialyzed preparations. An undialyzed enzymatic solution ordinarily oxidized the substrate to *p*-hydroxybenzaldehyde with an R.Q. of approximately two. Following dialysis of the whole enzyme, the reaction proceeded with the usual uptake of oxygen but with no evolution of carbon dioxide, indicating that the initial oxidation step still occurred, but that decarboxylation had ceased.

Information concerning the chemical nature of the first intermediate was obtained in the following manner. *p*-Hydroxymandelate was oxidized with a dialyzed preparation until the oxygen uptake had ceased. An ether extract of the deproteinized reaction mixture was concentrated to a small volume and analyzed by means of paper chromatography. Solutions of *p*-hydroxymandelic and *p*-hydroxybenzoic acids, treated in a similar fashion, served as controls.

The supernatant liquid from the reaction vessel contained both unreacted substrate and another acid which occupied a position different from that of *p*-hydroxybenzoic acid.

The reaction mixture was chromatographed a second time in the same manner, except that the mixture was applied to the paper in a series of spots, and only two narrow areas of the paper were sprayed during the final step. Sections of the paper corresponding to the position of the unknown acid were cut from the portions of the sheet which had not been sprayed with indicator. The unknown compound was eluted from the paper with phosphate buffer and tested for the presence of keto acids by the Friedemann and Haugen method. A very strong positive keto acid test resulted. A control solution prepared from a blank section of paper gave a negative reaction as did a sample of *p*-hydroxymandelic acid.

The above results indicated that the first step in the degradation of *p*-hydroxymandelate was an oxidation which resulted in the production of a keto acid. All evidence was in favor of the formation of *p*-hydroxybenzoylformate as the first intermediate; however, technical difficulties were such that it was not feasible to prepare and purify a derivative in sufficient quantities for chemical analysis, and the pure compound was not available for comparative tests.

The isolation of *p*-hydroxybenzaldehyde, the product of the second reaction, was relatively simple since this compound accumulated during the oxidation of *p*-hydroxymandelate by an undialyzed enzymatic solution. The reaction was carried out in several respirometer vessels under anaerobic conditions, using potassium ferricyanide as the electron acceptor. As soon as the first sharp decrease in the rate of carbon dioxide evolution occurred, the vessels were removed and their contents pooled and deproteinized with trichloroacetic acid. The supernatant liquid was adjusted to pH 7.5 to 8.0 and extracted with ether.

The resulting ether extract was evaporated to dryness *in vacuo*, and the residue was taken up in distilled water.

A saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl was added to the aqueous solution of the intermediate, and the 2,4-dinitrophenylhydrazone which formed was collected by filtration and recrystallized from hot ethanol. The melting point of this derivative was determined and compared with that of the 2,4-dinitrophenylhydrazone prepared from a known sample of *p*-hydroxybenzaldehyde. The 2,4-dinitrophenylhydrazone of the "product" melted at 271 to 274 C. The derivative of the known *p*-hydroxybenzaldehyde melted at 270 to 274 C. The mixed melting point was found to be 271 to 274 C. All values are uncorrected. These data show conclusively that *p*-hydroxybenzaldehyde is formed during the oxidation of *p*-hydroxymandelate.

p-Hydroxybenzoic acid had been identified tentatively as an intermediate by its absorption spectrum. Paper chromatography was used to obtain further evidence as to the identity of this compound.

A solution of *p*-hydroxymandelate was oxidized anaerobically until the second decrease in the rate of oxidation had occurred. The material was collected, deproteinized with trichloroacetic acid, and extracted with ether at pH 3. Then the ether extract was concentrated to a small volume and analyzed by paper chromatography according to the method outlined above.

The reaction mixture was found to contain two acids, the Rf of one of which corresponded to *p*-hydroxymandelic acid, while that of the other corresponded to that of *p*-hydroxybenzoic acid. The presence of the primary substrate was to be expected since the gas exchange in such experiments usually corresponded to about 50 per cent of the theoretical value, indicating that only a portion of the substrate was oxidized to completion. The results obtained by paper chromatography confirm the spectrophotometric data and show that *p*-hydroxybenzoate is one of the intermediates formed during the oxidation of *p*-hydroxymandelate.

ACKNOWLEDGMENTS

The author wishes to express her appreciation to Dr. M. Doudoroff and Dr. R. Y. Stanier for their interest and advice during this investigation.

SUMMARY

Employing the technique of "simultaneous adaptation", evidence has been obtained which indicates that whole cells of *Pseudomonas fluorescens*, strain A.3.12, oxidize *p*-hydroxymandelate with the formation of *p*-hydroxybenzoate and protocatechuate as intermediates.

Extracts of alumina ground, lyophilized, mandelate adapted cells were shown to degrade *p*-hydroxymandelate only as far as *p*-hydroxybenzoate. Prolonged dialysis of the enzymatic extracts against dibasic sodium phosphate solution rendered the preparations incapable of carrying the reaction beyond the initial dehydrogenation of the substrate.

The dialyzed enzymatic preparation catalyzed the oxidation of *p*-hydroxymandelate with the formation of a keto acid believed to be *p*-hydroxybenzoylformic acid. The degradation of *p*-hydroxymandelate by undialyzed enzymatic extracts proceeds rapidly through the dehydrogenation and decarboxylation steps, giving rise to a compound identified as *p*-hydroxybenzaldehyde by means of its absorption spectrum and the formation of the 2,4-dinitrophenylhydrazone. The isolation and identification of *p*-hydroxybenzoic acid as the end product of the oxidation of *p*-hydroxymandelate by the enzymatic preparations have been described.

An analysis of the oxidation of *p*-hydroxymandelate by an enzymatic extract has demonstrated that this compound is degraded to *p*-hydroxybenzoate by a series of reactions parallel to those by which mandelate is oxidized to benzoate.

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