THE ENZYMATIC CONVERSION OF MANDELIC ACID TO BENZOIC ACID

I. Gross Fractionation of the System into Soluble and Particulate Components[1]

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Mandelic acid can serve as sole source of carbon and energy for the growth of certain strains of *Pseudomonas fluorescens*. Analysis of the enzymatic patterns of cells grown on various substrates (Stanier, 1947, 1948; Sleeper and Stanier, 1950) has led to formulation of the following sequence for the initial steps in its dissimilation: Mandelic acid → benzoylformic acid → benzaldehyde → benzoic acid → catechol. All the relevant enzymes are substrate induced. Living cells consume approximately 11 atoms of oxygen per mole of mandelate oxidized with an R.Q. of slightly less than one. After slow vacuum drying, mandelate grown cells yield preparations which consume only two atoms of oxygen per mole of mandelate—sufficient for oxidation to benzoic acid (Sleeper, Tsuchida, and Stanier, 1950). Cells harvested from a medium which contains sodium DL-mandelate, or dried cells prepared from them, oxidize both isomers at equal rate.

In this work the series of enzymes which convert DL-mandelic acid to benzoic acid has been separated and some of their properties determined. In this paper the properties of the overall system, together with the primary separation of the relevant enzymes into particulate and soluble fractions, are reported. The properties of the particle fractions are described in paper two of the series (Stanier *et al.*, 1953), and the further fractionation and characterization of the soluble enzymes, in paper three (Gunsalus *et al.*, 1953).

METHODS

Pseudomonas fluorescens, strain A.3.12, was used throughout this work. Cells containing the enzymes of the mandelate-benzoate reaction

sequence were prepared by growth at the expense of mandelic acid as previously described (Sleeper *et al.*, 1950). Early in the work, slow vacuum-dried or lyophilized cells were used as a source of enzymes. Subsequently, freshly harvested cells (5 g wet weight per 50 ml phosphate buffer, 0.02 M, pH 7.0) were treated for 10 to 15 minutes in a Raytheon 10 kc, 200 watt sonic oscillator. After treatment, the extract was centrifuged in the cold at 7,000 G for 15 minutes to remove unbroken cells. Dried cells, cell-free extracts, and enzymatic preparations were stored at −20 C. Some batches of freshly harvested cells also were frozen and stored at −20 C for several weeks prior to sonic treatment, without apparent effect on their enzymatic activity.

Manometric measurements were made in the customary way (Umbreit *et al.*, 1949) with the Warburg apparatus. Unless otherwise specified, manometric experiments were conducted at 30 C in an atmosphere of air. Studies on the pyridine nucleotide-linked dehydrogenases were conducted with a Beckman model DU ultraviolet spectrophotometer at room temperature.

The protein content of crude extracts and of the first fractions derived from them was estimated colorimetrically using the Folin-Ciocalteu reagent (Hawk *et al.*, 1949). Protein estimations on purified enzymatic fractions which contained little nucleic acid were made spectrophotometrically by the method of Warburg and Christian (1941).

RESULTS

Experiments with whole dried cells. Dried cells of *P. fluorescens* resuspended in phosphate buffer usually oxidize sodium DL-mandelate with the uptake of two atoms of oxygen and formation of one mole of carbon dioxide per mole of substrate. With many preparations (especially those prepared by slow vacuum drying) the rate of oxygen uptake declines sharply after one atom has been consumed (figure 1, left). The addition

of diphosphopyridine nucleotide (DPN) to such preparations maintains the initial rate of oxidation until the second atom has been consumed. Cocarboxylase (DPT) and Mg^{++} slightly increase the initial rate of oxidation but do not affect the rate of uptake of the second atom of oxygen. Simultaneous addition of diphosphopyridine nucleotide, cocarboxylase, and Mg^{++} gives

buffer (pH 7.0) with 1 mM of sodium DL-mandelate and incubated at 30 C for 60 minutes with mechanical agitation to provide the necessary aeration. The mixture then was deproteinized with sulfuric acid, clarified by centrifugation, and extracted with ether. The ether was evaporated, and the residue sublimed *in vacuo* to yield 70 mg of a white crystalline solid. After one recrystal-
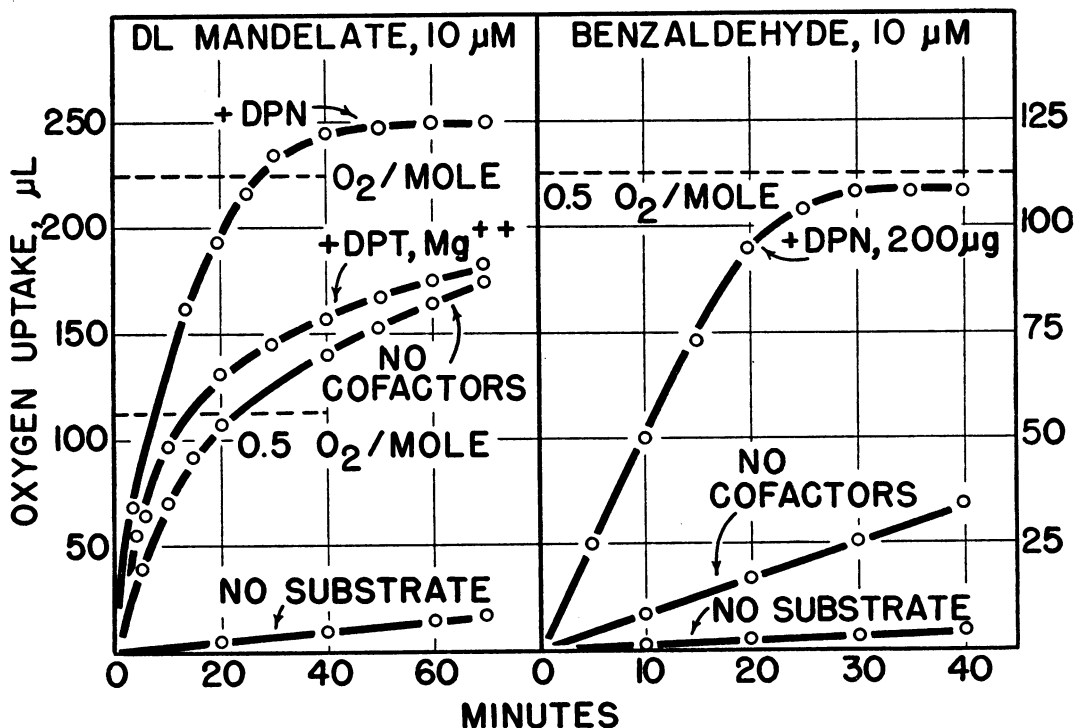

Figure 1 (left). Influence of cofactors on oxidation of DL-mandelic acid by dried cells of *Pseudomonas fluorescens*. Each vessel contained: 10 μM sodium DL-mandelate, 5 mg dried cells, 2 ml M/10 phosphate buffer, pH 7.0. Where added diphosphopyridine nucleotide (DPN), 200 μg; cocarboxylase (DPT), 100 μg; 10 μM Mg^{++}.

Figure 1 (right). Influence of cofactors on benzaldehyde oxidation. Each vessel contained 10 μM benzaldehyde, 10 mg dialyzed dried *Pseudomonas fluorescens*, 2 ml M/10 phosphate buffer, pH 7.0.

the same result as addition of diphosphopyridine nucleotide alone. In the light of earlier work (Stanier, 1947, 1948) the slow uptake of the second atom of oxygen (figure 1, left) should reflect a low rate of oxidation of benzaldehyde, capable of being increased by addition of diphosphopyridine nucleotide; the data in figure 1 (right) show this prediction to be correct.

The formation of benzoic acid as the end product of the oxidation by dried cells was established as follows. One gram of lyophilized cells was suspended in 200 ml of 0.02 M phosphate

lization from water, this substance melted at 121.5 C (uncorrected), and the melting point was not depressed by admixture with synthetic benzoic acid. The absorption spectrum between 210 and 300 mμ was virtually superimposable on that of pure benzoic acid measured under the same conditions (1 cm cell, pH 7.0, calculated molarity 0.0001 M). Both spectra showed a maximum at 225 mμ with molar extinction coefficients of 8,400 (synthetic benzoic acid) and 8,300 (isolated material).

Dried cells, after dialysis overnight at 5 C

TABLE 1

Effect of cofactors on DL-mandelic acid oxidation by a dialyzed dried cell suspension

Per Warburg Cup:
M/20 phosphate buffer, pH 7
5 mg (dry weight) cells
10 μM sodium DL-mandelate

ADDITIONS	OXYGEN CONSUMED		CARBON DIOXIDE EVOLVED		R.Q.
	μM	M/Mole sub-strate	μM	M/Mole sub-strate	
(1) None	4.2	0.42	0	0	0
(2) DPT, 50 μg; + Mg++, 10 μM	5.2	0.52	9.8	0.98	1.90
(3) (2), + DPN, 20 μg	11.0	1.10	10.6	1.06	1.04

hyde; but addition of cocarboxylase activates the decarboxylation of the former compound, and addition of diphosphopyridine nucleotide activates the oxidation of the latter. Illustrative data are given in table 1.
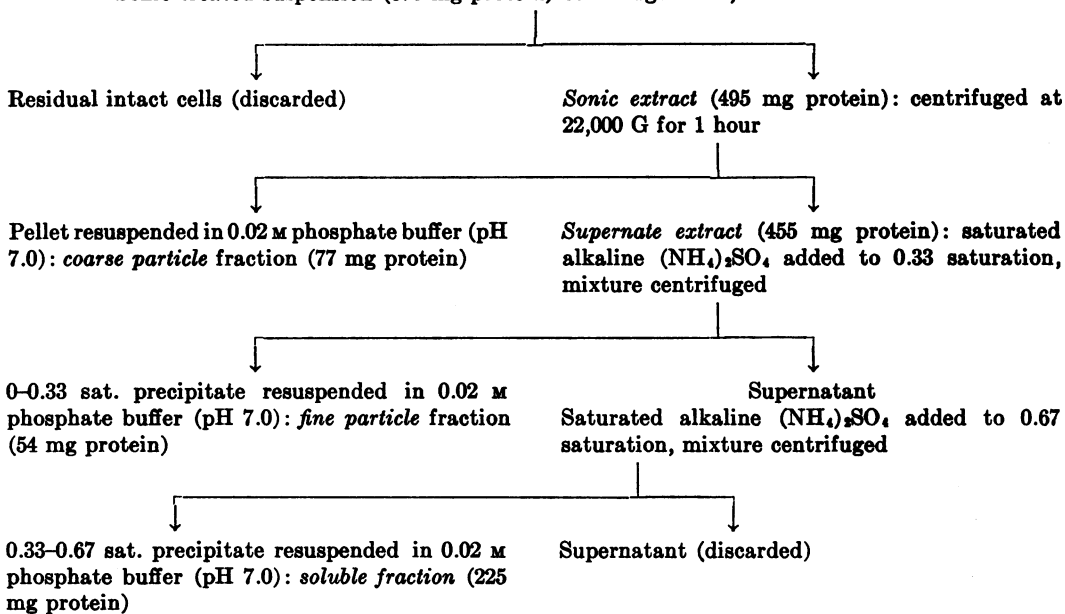
These findings confirm the conclusions arrived at on the basis of earlier *in vivo* studies (Stanier, 1947, 1948) and indicate that dried cells convert mandelic acid to benzoic acid by the following reactions: (a) primary dehydrogenation of mandelic acid to benzoylformic acid, (b) decarboxylation of benzoylformic acid to benzaldehyde, requiring cocarboxylase, (c) dehydrogenation of benzaldehyde to benzoic acid, requiring diphosphopyridine nucleotide.

Attempts were then made to extract the en-

DIAGRAM 1

Fractionation of sonic extract of mandelate grown cells (5 g wet cells in 50 ml 0.02 M phosphate buffer)

Sonic treated suspension (670 mg protein) centrifuged at 7,000 G for 15 minutes

Residual intact cells (discarded)

Sonic extract (495 mg protein): centrifuged at 22,000 G for 1 hour

Pellet resuspended in 0.02 M phosphate buffer (pH 7.0): *coarse particle* fraction (77 mg protein)

Supernate extract (455 mg protein): saturated alkaline (NH₄)₂SO₄ added to 0.33 saturation, mixture centrifuged

0–0.33 sat. precipitate resuspended in 0.02 M phosphate buffer (pH 7.0): *fine particle* fraction (54 mg protein)

Supernatant
Saturated alkaline (NH₄)₂SO₄ added to 0.67 saturation, mixture centrifuged

0.33–0.67 sat. precipitate resuspended in 0.02 M phosphate buffer (pH 7.0): *soluble fraction* (225 mg protein)

Supernatant (discarded)

against 0.1 M phosphate buffer (pH 8.5), consume one atom of oxygen per mole of DL-mandelate with an R.Q. of zero. Addition of cocarboxylase to such a preparation causes formation of one mole of CO₂ without a change in oxygen uptake; addition of diphosphopyridine nucleotide together with cocarboxylase restores the oxygen uptake to its original value of 2 atoms per mole of substrate oxidized. The dialyzed cells do not attack either benzoylformic acid or benzalde-

zymes from suspensions of dried cells. Repeated freezing and thawing liberated the benzoylformic carboxylase and benzaldehyde dehydrogenase, but the mandelate oxidizing system always remained exclusively in the debris. Although the soluble benzaldehyde dehydrogenase no longer connected to molecular oxygen, its activity could be measured spectrophotometrically by diphosphopyridine nucleotide reduction or manometrically with ferricyanide as an electron ac-

ceptor (Haas, 1937). Our inability to solubilize the mandelic dehydrogenase led us to try other methods of breaking the cells, and it was found that concentrated suspensions of living cells could be almost completely disintegrated by brief sonic oscillation to yield a cell-free extract which catalyzed all steps of the mandelate-benzoate reaction sequence. Subsequent experiments were conducted with these preparations.

Preparation and fractionation of sonic extracts. A suspension of living cells is largely disrupted (75 to 90 per cent) by sonic treatment for 15

of NH_4OH to pH 7.9, as measured with the glass electrode). The particulate material is aggregated completely at 0.33 saturation, whereas most of the dissolved proteins ("soluble protein fraction") precipitate between 0.33 and 0.70 saturation. The flow sheet for an actual fractionation is shown in diagram 1, and the distribution of some enzymatic activities in the various fractions is presented in table 2.

The data of table 2 reveal that this treatment effects a primary separation of the enzymes of the mandelate-benzoate reaction sequence. The oxi-

TABLE 2

Enzymatic activities of sonic extract fractions on mandelate and benzoyl formate (see diagram 1)

FRACTION	PROTEIN	ENZYMATIC ACTIVITIES[*]			ACTIVITY/MG PROTEIN			RATIO OF ACTIVITIES $D(-)/L(+)$- MANDELATE
		Mandelate		Benzoyl-formate	Mandelate		Benzoyl-formate	
		L(+)	D(−)		L(+)	D(−)		
	%	%		%	Q_{O_2}		Q_{CO_2}	$\dfrac{Q_{O_2}D(-)}{Q_{O_2}L(+)}$
(1) Sonic extract	100†	100†	100†	100†	77	61	415	0.8
(2) Coarse particles	15	72	5	3	315	32	86	0.1
(3) Supernate, after 22,000 G, 60 min	92	22	28	102	18	18	455	1.0
(4) Fine particles	11	18	5	6	133	29	210	0.22
(5) Soluble proteins	62	0	0	94	0	0	760	
(6) Sum of (2), (4), (5)‡	88	90	10	103	—	—	—	—

  * Activity with the 2 isomers of Na mandelate measured as rate of oxygen intake at 30 C in air: activity with Na benzoylformate measured as rate of CO_2 evolution at 30 C in nitrogen.

  † Expressed as per cent of values for sonic extract (= 100).

  ‡ Per cent recovery.

minutes (Ratheon 10 kc, 200 watt oscillator). After sedimentation of unbroken cells, a reddish-brown extract which shows a marked Tyndall effect is obtained. Centrifugation for one hour at 22,000 G in the high-speed attachment of an International refrigerated centrifuge sediments most of the light scattering material as a translucent, gelatinous amber pellet. Resuspension of the pellet in a small volume of buffer yields an opaque suspension whose brown color is largely masked by intense light scattering ("coarse particle fraction").

The supernate after high-speed centrifugation still contains some particulate material of relatively large dimensions ("fine particle fraction") which can be separated from the dissolved proteins by fractionation with alkaline saturated ammonium sulfate (made alkaline by addition

dation of L(+)-mandelic acid is associated exclusively with the particle fractions; in this experiment 90 per cent of the L-mandelate oxidizing activity of the crude extract was recovered in them. Since the coarse particle fraction contains relatively little protein, separation by high-speed centrifugation increases considerably the specific activity of the L-mandelic acid oxidizing system.

This method of fractionation also dissociates the oxidation of the two stereoisomers of mandelic acid, as indicated in the last column of table 2, which lists the ratios of the Q values for the two isomers obtained with various fractions. Crude sonic extracts usually oxidize the L(+)-isomer slightly faster than the D(−)-isomer; in this experiment there was a disparity of 20 per cent. The disparity is enhanced greatly in the coarse

particle fraction which, after resuspension in buffer, barely oxidizes the D-isomer. The supernate after high-speed centrifugation, which contains only about 20 per cent of the total mandelate oxidizing capacity of the crude extract, oxidizes the two isomers at equal rates. The fine particle fraction, separated from the supernate by precipitation with ammonium sulfate, carries all the residual mandelate oxidizing capacity. Like the coarse particle fraction, it shows a markedly greater ability to oxidize the L-isomer. The combined recovery of D-mandelate oxidizing activity in the separate fractions is always very low; table 2 shows a recovery of 10 per cent, all in the two particulate fractions, as contrasted with 90 per cent recovery of L-mandelate oxidizing activity. From these findings one can infer that the mandelate oxidizing system associated with the particles is specific for the L-isomer, and that another enzyme, required for the metabolism of the D-isomer and not connected to molecular oxygen, is present in the soluble fraction. As we shall show later (Gunsalus et al., 1953), this enzyme is a racemase.

The enzyme which decarboxylates benzoylformic acid is recovered almost quantitatively (94 per cent) in the soluble protein fraction with negligible activity in the two particle fractions (table 2). The dehydrogenation of benzaldehyde as measured by diphosphopyridine nucleotide reduction proceeds rapidly with crude extracts and with the supernate from high-speed centrifugation, but cannot be quantitated readily because of reoxidation of the reduced diphosphopyridine nucleotide; therefore, data for the distribution of this system have not been included in table 2. It may be stated, however, that the benzaldehyde oxidizing enzyme is located almost entirely in the soluble fraction.

SUMMARY

Dried, mandelate grown cells of *Pseudomonas fluorescens* oxidize both isomers of mandelic acid to benzoic acid. Studies with dried cells show that this sequence involves: a mandelate oxidizing enzyme which converts mandelic acid to benzoylformic acid; a benzoylformic carboxylase which requires cocarboxylase for activity; and a benzal-

dehyde dehydrogenase, activated by diphosphopyridine nucleotide.

By differential centrifugation and ammonium sulfate fractionation of sonic extracts prepared from living cells, the enzymes of the sequence have been separated into particulate and soluble fractions. The particles contain the L-mandelate oxidizing system, while the soluble fraction contains benzoylformic carboxylase, benzaldehyde dehydrogenase, and an enzyme which is required for the metabolism of the D(−)-isomer of mandelic acid.

REFERENCES

GUNSALUS, C. F., STANIER, R. Y., AND GUNSALUS, I. C. 1953 The enzymatic conversion of mandelic acid to benzoic acid. III. Fractionation and properties of the soluble enzymes. J. Bact., **66,** 548–553.

HAAS, E. 1937 Manometrische Mikrotitration mit Ferricyanid. Biochem. Z., **291,** 79–80.

HAWK, P. B., OSER, B. L., AND SUMMERSON, W. H. 1949 *Practical physiological chemistry.* 12th edition. Blakiston Co., Philadelphia, Pa.

SLEEPER, B. P., AND STANIER, R. Y. 1950 The bacterial oxidation of aromatic compounds. I. Adaptive patterns with respect to polyphenolic compounds. J. Bact., **59,** 117–127.

SLEEPER, B. P., TSUCHIDA, M., AND STANIER, R. Y. 1950 The bacterial oxidation of aromatic compounds. II. The preparation of enzymatically active dried cells and the influence thereon of prior patterns of adaptation. J. Bact., **59,** 129–133.

STANIER, R. Y. 1947 Simultaneous adaptation: A new technique for the study of metabolic pathways. J. Bact., **54,** 339–348.

STANIER, R. Y. 1948 The oxidation of aromatic compounds by fluorescent pseudomonads. J. Bact., **55,** 477–493.

STANIER, R. Y., GUNSALUS, I. C., AND GUNSALUS, C. F. 1953 The enzymatic conversion of mandelic acid to benzoic acid. II. Properties of the particulate fractions. J. Bact., **66,** 543–547.

UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F. 1949 *Manometric techniques and tissue metabolism.* 2nd edition. Burgess Publishing Co., Minneapolis, Minn.

WARBURG, O., AND CHRISTIAN, W. 1941 Isolierung und Kristallisation des Gärungsferments Enolase. Biochem. Z., **310,** 384–425.