Metabolism of Aromatic Compounds by Microbes

Part VI. Existence of Two Pathways, the Pathway *via* Protocatechuic Acid and the One *via* Gentisic Acid, in the Metabolism of *m*-Hydroxybenzoic Acid by Bacteria

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The metabolic products of *m*-hydroxybenzoic acid formed by certain *Pseudomonas*, *Micrococcus*, and *Bacterium* strains which possess oxidizing ability of this acid were detected by paperchromatography. It was recognized that protocatechuic acid or gentisic acid are intermediary metabolites of *m*-hydroxybenzoic acid by these bacteria and the both acids are not detected in one cultural broth.

The authors reported the metabolic pathways of *m*-hydroxybenzoic acid by various bacteria using the technique of sequential induction method and presumed that this acid was metabolized either *via* gentisic acid (2,5-dihydroxybenzoic acid) or *via* protocatechuic acid (3,4-dihydroxybenzoic acid)¹⁾.

In the previous reports, details in concern of the factors influencing adaptive formation of the enzymes which oxidatively split *m*-hydroxybenzoic acid (*m*-HBA) and gentisic acid (GA) by *Pseudomonas ovalis* S-5 have been described^{2,3)}. On the metabolism of *m*-HBA by microbes, N. Walker⁴⁾ and B.S. Roof et al.⁵⁾ reported on their experiments conducted on specificities and strength of metabolic activities of several bacterial strains on this acid. However, the metabolic pathway of *m*-hydroxybenzoic acid has so far not been elucidated.

In this report detection of the metabolic products of *m*-HBA by the fifteen strains of bacteria which possess oxidizing ability of this acid by paperchromatography is described.

EXPERIMENTS

Biological: Fifteen strains of *m*-HBA metabolizing bacteria which were identified taxonomically in the previous report⁶⁾ were used.

Method of culture: Stationary or shaking culture at 30°C were made on synthetic medium containing *m*-HBA as the sole carbon source. Stationary culture was carried out in 1,000 ml of the medium in a 2,500 ml Fernbach flask and the cells were harvested after 4 days. Shaking culture was carried out in 100 ml of the medium in a 500 ml Sakaguchi flask using reciprocal shaker (130~140 r. p. m. 7 cm) and the cells were harvested after 16~18 hours.

Cultural medium.

Nitrogen source: As nitrogen source, three compounds such as $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 and $(\text{NH}_4)_2\text{H}_2\text{PO}_4$, were tested for the accumulation of gentisic acid and protocatechuic acid and were proved to have no difference.

Influence of pH: Three levels of initial pH value such as 5.2, 7.2 and 8.5 were examined, and as shown

1) K. Arima, K. Komagata and N. Minoda, *J. Agr. Chem. Soc. Japan*, **28**, 629 (1954).

2) K. Arima, K. Komagata, S. Sugiyama, M. Kazama and K. Yano, *This Bulletin*, **19**, 51 (1955).

3) K. Arima, K. Komagata, M. Kazama, S. Sugiyama and K. Yano, *This Bulletin*, **19**, 61 (1955).

4) N. Walker and W. C. Evans, *Biochem. J.*, **52**, proc. XXXIII (1952).

5) B. S. Roof, T. J. Lannon and J. Turner, *Proc. Soc. Expt. Biol. Med.*, **84**, 38 (1953).

6) K. Arima, K. Komagata, S. Sugiyama and M. Kazama, *J. Agr. Chem. Soc. Japan*, **28**, 635 (1954).

TABLE I. THE EFFECT OF pH ON PRODUCTION OF GENTISIC ACID OR
PROTocatechuic ACID BY SEVERAL BACTERIA

	pH value		U.V. ray*	Detection		Pathway**
	Start.	After culture		FeCl ₃	Diazo-reaction	
<i>Micrococcus ureae</i> S-4	5.2	5.2	—	—	+	G
	7.2	7.2	+	—	±	
	8.5	7.8	+	—	+	
<i>Ps. ovalis</i> S-5	5.2	5.4	—	—	+	G
	7.2	7.2	++	—	+	
	8.5	8.0	++	—	+	
<i>Bact. sp.</i> Ca-1	5.2	5.4	—	—	+	G
	7.2	6.6	±	—	+	
	8.5	7.6	—	—	+	
<i>Ps. cruciviae</i> P-8-2	5.2	5.6	—	—	±	P
	7.2	7.0	—	+	±	
	8.5	7.6	—	±	±	
<i>Ps. dacunhae</i> R-10-2	5.2	5.4	—	—	+	P
	7.2	6.6	—	±	+	
	8.5	7.8	—	—	+	
<i>Ps. dacunhae</i> G1-7	5.2	5.6	—	—	+	P
	7.2	6.6	—	+	+	
	8.5	7.8	—	—	+	

Surface culture at 30°C for 20 hrs was made on synthetic medium (see text).

* Blue fluorescence

** The results of investigation by sequential induction method.

G—Gentisic acid pathway

P—Protocatechuic acid pathway

in Table I, pH 7.2 was proved to be best for the accumulation of GA. The composition of the synthetic medium which will be used in this experiment is as follows;

<i>m</i> -hydroxybenzoic acid	0.5 per cent
(NH ₄) ₂ SO ₄	0.1 "
MgSO ₄ ·7H ₂ O	0.15 "
KH ₂ PO ₄	0.1 "
pH.....	7.2

Preparation of samples for chromatography from cultural broth.

The procedure is as follows;

1,000 ml cultural broth

Preparation of samples of chromatography from incubation mixture.

The bacteria were cultured on bouillon-peptone broth which contained 1 per cent *m*-HBA, and was harvested by centrifugation after 18 hours. Ten g of the wet cells were suspended in 100 ml of M/15 phosphate buffer at pH 7.2 with 300 mg GA or PCA, and were incubated at 30°C on the shaker. Sampling was carried out at an interval of each 2 hours, and the production of ketoacids were examined as their 2,4-dinitrophenylhydrazone.

Paperchromatography.

a) Filterpaper: The Tôyô filterpaper No. 50 (2×40 cm). Before development, filterpaper was saturated beforehand by the vapor of developing solvent in order to prevent the tailing of samples.

b) Developing solvents: After a survey was made for the purpose of detection of various intermediates, the following developing solvents were used;

- 1) Benzene: Acetic acid: Water (2:2:1)⁷⁾
- 2) Butanol; Ethanol: (NH₄)₂CO₃·NH₄OH buffer (4:1:2)^{8,9)}

7) H. G. Bray, W. U. Thorpe and K. White, *Biochem. J.*, **46**, 271 (1951).

8) D. Cavalini, N. Frontali and G. Toschi, *Nature*, **163**, 568 (1946).

9) M. E. Fewster and D. H. Hall, *Nature*, **168**, 78 (1951).

3) 3 per cent NH_4OH saturated butanol

4) Butanol: Formic acid: Water (4:1.5:1)

The solvents 1) and 2) were generally used for development of the aromatic compounds. The solvents 2) and 3) for development of 2,4-dinitrophenylhydrazones of keto acids, and the solvent 4) for fatty acids. Each solvent was applied from the mixing after 3 to 4 days.

c) Development was carried out at 20°C by the ascending method.

d) Detection of compounds: The indicators to detect each compound are follows;

1. *m*-hydroxybenzoic acid: the diazo reaction was used.

The first solution; 1 per cent *p*-aminoacetophenone in 1 N H_2SO_4

The second solution; 1 N HNO_3

After the developed papers were dried at room temperature, the first and then the second solution were sprayed and the reddish orange spots appeared.

2. Gentisic acid: This was detected by the blue fluorescence when the paper was exposed in ultra violet rays in the dark room or by the brown spots by reaction with FeCl_3 (1 per cent FeCl_3 alcoholic solution was sprayed). Among these, the most convenient and sensitive indicator was found to be its own blue fluorescence.

3. Protocatechuic acid: The detection of the acid was either due to the diazo reaction similar to the case of *m*-HBA, or due to its dark green spot by reaction with FeCl_3 . Usually, the dark green spot by spraying FeCl_3 was used.

4. Other phenolic compounds: These were mainly detected by the reaction with FeCl_3 , and when the coloration was weak, alkalic solution was used as the indicator. Salicylic acid has a specific fluorescence under the ultra violet rays, and was detected by such characteristics.

5. Keto acids: The detection of keto acid was due to yellow spots of their 2,4-dinitrophenylhydrazone.

6. Aliphatic acids: After development, the paper was put in a drier at 110°C for 10 min. Then bromocresolpurple solution (0.16 per cent in 5 per cent alcohol adjusted to pH 8.0) was sprayed and yellow spots were revealed on the blue back ground.

RESULTS

R_F values of several related compounds.

R_F values of various phenolic acid and several other acids which were related with the terminal oxidants of these acids were estimated by different solvents. Table II shows the R_F values

TABLE II. R_F VALUES OF THE RELATED COMPOUNDS OF MONOHYDROXYBENZOIC ACIDS WITH VARIOUS SOLVENTS

	Solvent			
	I	II	III	IV
<i>o</i> -hydroxybenzoic acid	0.81	0.62		0.95
<i>m</i> -hydroxybenzoic acid	0.55	0.40		
<i>p</i> -hydroxybenzoic acid	0.72	0.34		
2,3-dihydroxybenzoic acid	0.40	0.38		
β -resorcinic acid	0.27	0.40		
protocatechuic acid	0.12	0.18		0.85
gentisic acid	0.25	0.49		0.88
catechol	0.44	0.89		
resorcin	0.16	0.94		
hydroquinone	0.17	0.87		
2,4-dinitrophenylhydrazone of levulic acid		0.48		
pyruvic acid		0.41	0.61	0.34
α -ketoglutaric acid		0.092	0.06	
oxaloacetic acid		0.14	0.07	
levulic acid				0.76
succinic acid	0.28			0.74
fumaric acid				0.81
maleic acid	0.18			0.47
citric acid	0.22			0.35

Solvent I: Benzene 2: Acetic acid 2: Dist water 1

II: *n*-Butanol 4: Ethanol 1: 1 N $(\text{NH}_4)_2\text{CO}_3$ buffer 2

III: Butanol 3 per cent NH_3

IV: *n*-Butanol 4: Formic acid 1.5: Dist water 1.

of these compounds developed by different solvents. These values are the average of several experiments and pyruvate was generously shown to have two spots when developed with the solvent 2.

The detectable concentration of related compounds.

As shown in Table III, *m*-HBA was detectable from 3 gamma by the Diazo reaction, GA was detectable from 3 gamma by alkalic FeCl_3 solution, but from only 1 gamma by its specific blue fluorescence. PCA was detectable from 3 gamma with its green spot by FeCl_3 solution. Keto acids were detectable from 1 gamma in the form of their 2,4-dinitrophenylhydrazone. In the detection of fatty acids it was necessary to use at least 100 gamma of them for bromocresol-purple as an indicator.

Detection of aromatic compounds from stationary cultural broth.

R_F values obtained from the each Fraction I of the cultural broth of the several strains developed by the solvent 1 are shown in Table IV. Since the spots, of which the R_F value

TABLE III. THE DETECTABLE CONCENTRATION OF RELATED COMPOUNDS BY SEVERAL REAGENTS ON PAPERCHROMATOGRAPHY

	Concentration in spotting	Detection			
		FeCl ₃	FeCl ₃ +NH ₄ OH	U.V. ray	Diazo- reaction
gentisic acid	(r) 5	+	+	+	
	3	—	+	+	
	1	—	—	+	
protocatechuic acid	5	+	+	—	+
	3	+	+	—	+
	1	—	—	—	—
<i>m</i> -hydroxybenzoic acid	5				+
	3				+
	1				—

Developing solvent was the upper layer of a mixture of benzene 2, acetic acid 2, and water 1.

TABLE IV. R_F VALUES OF THE INTERMEDIATES OF *m*-HYDROXYBENZOIC ACID IN FRACTION 1 IN STATIONARY CULTURE

Strain	R_F Value	
<i>Micrococcus ureae</i> S-4	0.52	0.24
<i>Ps. ovalis</i> S-5	0.54	0.25
<i>Bact. sp.</i> Ca-1	0.57	0.27
<i>Bact. sp.</i> A-10-2		0.24
<i>Micrococcus varians</i> P-10-7	0.56	0.24
<i>Ps. dacunhae</i> G1-13	0.53	0.12
<i>Ps. dacunhae</i> G1-7	0.54	0.11
<i>Ps. dacunhae</i> R-10-2	0.55	0.11
<i>Ps. dacunhae</i> A-6-3	0.54	0.10
<i>Ps. dacunhae</i> A-7-2	0.54	0.12
<i>Ps. cruciviae</i> P-8-2	0.52	0.12
<i>m</i> -hydroxybenzoic acid	0.55	
gentisic acid		0.25
protocatechuic acid		0.11

developing solvent was the upper layer of a mixture of benzene 2, acetic acid 2, water 1.

Control were prepared as acetone solution.

was about 0.52 gave a negative reaction with FeCl₃ while by the Diazo reaction gave a clear reddish orange, this substance was considered to be *m*-HBA. The spot with a R_F value of 0.24~0.25 was proved to be GA, since their R_F and specific color reaction were the same as the synthetic samples GA was detected from the cultural broth of the strains S-4, S-5, A-10-2, and P-10-7. The strains G1-7, G1-13, R-10-2, A-6-3, A-7-2 and P-8-2 produced spots which were identical with PCA.

The spot with a R_F value of 0.27 produced by strain Ca-1 was most probably GA due to its fluorescence and to the results of coloring reactions, although the R_F value of 0.27 also corresponded to β -resorcilic acid.

Detection of aromatic compounds from shaking culture broth.

Table V shows the results of detection of the intermediate from shaking cultural broth. As well as in the stationary culture, GA was recognized in the cultural brothes of strains S-4, S-5, while PCA was detectable in those of strains G1-7, R-10-2 and P-8-2.

Summarizing the above results, as seen in Table VI, the same conclusion from the experiment with the sequential induction method was obtained. That is, *m*-HBA was oxidized *via* gentisic acid or protocatechuic acid according to bacterial strain, respectively.

Detection of keto acids.

As described above, the distinction between

TABLE V. R_F VALUES OF THE INTERMEDIATES OF *m*-HYDROXYBENZOIC ACID IN FRACTION 1 IN SHAKING CULTURE

Strain	R_F Value	
<i>Micrococcus ureae</i> S-4	0.51	0.25
<i>Ps. ovalis</i> S-5	0.52	0.24
<i>Ps. dacunhae</i> G1-7	0.57	0.12
<i>Ps. dacunhae</i> R-10-2	0.52	0.11

Developing solvent was the upper layer of a mixture of Benzene 2, acetic acid 2, water 1.

TABLE VI. SUMMARIZING THE PRODUCTION OF GENTISIC ACID OR PROTOCATECHUIC ACID BY *m*-HYDROXYBENZOIC ACID ASSIMILATING BACTERIA

Strain	Gentisic acid	Protocatechuic acid
<i>Micrococcus ureae</i> S-4	+	—
<i>Ps. ovalis</i> S-5	+	—
<i>Bact. sp.</i> Ca-1	+	—
<i>Bact. sp.</i> A-10-2	+	—
<i>Micrococcus varians</i> P-10-7	+	—
<i>Ps. dacunhae</i> G1-7	—	+
<i>Ps. dacunhae</i> G1-13	—	+
<i>Ps. dacunhae</i> R-10-2	—	+
<i>Ps. dacunhae</i> A-6-3	—	+
<i>Ps. dacunhae</i> A-7-2	—	+
<i>Ps. cruciviae</i> P-8-2	—	+

TABLE VII. R_F VALUES OF THE INTERMEDIATES OF *m*-HYDROXYBENZOIC ACID IN FRACTION 3 IN SHAKING CULTURE

Time (culture)	<i>Ps. ovalis</i> S-5		<i>Ps. dacunhae</i> G1-7	
	Spot I	Spot II	Spot I	Spot II
2	0.12	0.46		
4	0.16	0.49	0.14	
6	0.13	0.49	0.12	
8	0.13	0.49	0.13	
10	0.14	0.41	0.14	0.41
12	0.17	0.47	0.21	0.50
14	0.16	0.42	0.18	0.46
16	0.16	0.44	0.18	0.48
18	0.17	0.44	0.18	0.48
24	0.16	0.44	0.21	0.44

Developing solvent was the upper layer of a mixture of *n*-butanol 4, ethanol 1, water 2.

the strains having GA pathway and the other having the PCA pathway, was recognized. The detection of keto acids was carried out from incubation mixtures of *Ps. ovalis* S-5 and *Ps. dacunhae* G1-7. The former was oxidized *m*-HBA via GA, the latter oxidized *m*-HBA via PCA. An amount of 20 ml of samples was taken every 2 hours, and 2,4-dinitrophenylhydrazone was prepared and then developed in solvent 2. As seen in Table VII, different acids were detected depending upon the bacterial strain. In the case of *Ps. ovalis* the composition of acids was constant irrespective of the time of culture. Spot 1 of the former R_F value of 0.12

corresponds to α -ketoglutaric acid but assumed an orangish red color which is quite different from that of 2,4-dinitrophenylhydrazone of α -ketoglutaric acid. In the chromatogram of the mixture with the authentic α -ketoglutaric acid, the keto acid in question gave different spots hence the possibility that this keto acid is α -ketoglutaric acid was excluded. As for the spot 1 of *Ps. dacunhae*, from the R_F value it seemed to be 2,4-dinitrophenylhydrazone of α -ketoglutaric acid from paperchromatography mixture with the authentic samples. In the other case, spot 11 was not detectable between 2 to 8 hours' incubation, and was considered to be 2,4-dinitrophenylhydrazone of pyruvate.

DISCUSSION

These results do not clearly indicate successive steps of the metabolic pathway of *m*-HBA. However, they agreed with the assay of sequential induction method, which concluded that two metabolic pathways exist in the oxidation of *m*-HBA and its pathways are specific by bacterial strain. Namely, the existence of a common precursor is considered in the induced enzyme formation between analogous compounds, but against one inducer the bacterial metabolic activity differs between bacterial strains. R. Y. Stanier and N. Tsuchida¹⁰⁾ in bacterial metabolism of tryptophan, previously demonstrated that there were two pathways, one the aromatic path *via* anthranilic acid, the other the quinoline path *via* quinolic acid, but both pathways did not exist together in one strain.

The production of lower keto acids are intermediary steps to terminal oxidation as well as the metabolism of aromatic compounds investigated by S. Dagley¹¹⁾ and M. Isono¹²⁾. An unknown keto acid produced by *Ps. ovalis* S-5 is considered to be a direct intermediate cleaving benzene ring of GA. As will be described in the

10) R. Y. Stanier and M. Tsuchida, *Biochem. J.*, **53**, 45 (1949).

11) S. Dagley, M. E. Fewster and F. C. Happold, *Biochem. J.*, **63**, 327 (1952).

12) M. Isono, *J. Agr. Chem. Soc. Japan*, **27**, 260 (1953).

latter report¹³⁾, the present authors assumed 7 carbons diketo-dicarboxylic acid, probably fumarylpyruvic acid, from the results obtained by manometrical experiment of gentisic acid oxidase. Therefore, considering the spot of its hydrazone not having appeared in developing Fraction 3 from cultural broth, it seems that metabolic decomposition of it had already been carried out or decomposition during prolonged hot ether extraction and other treatment had occurred.

SUMMARY

Oxidation of *m*-HBA by the eleven bacterial strains which were able to metabolize this compound as a sole source of carbon was carried out. The cultural broths were examined by paperchromatography for detection of intermediates.

Gentisic acid was identified in the culture

filtrate of the five strains, while protocatechuic acid by the remaining six strains. Protocatechuic acid was not detected in all the cultural broth of bacteria which belong to the gentisic acid path type, and *vice versa*.

Strains which possess the gentisic acid path were *Pseudomonas ovalis* (two strains), *Micrococcus ureae*, and an unidentified *Bacterium* and a strain of *Aerobacter*. Strains which have the protocatechuic acid path were *Pseudomonas dacunhae* (five strains) and *Pseudomonas cruciviae*.

An unknown keto acid was detected as its 2,4-dinitrophenylhydrazone from the incubation mixture of the resting cell of *Pseudomonas ovalis* S-5 and gentisic acid. This new keto acid was most probably the direct intermediate after the cleavage of gentisic acid.

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13) S. Sugiyama, K. Yano, H. Tanaka and K. Arima. This Bulletin, in press.