The Relationship of 4-Hydroxybenzoic Acid to Lysine and Methionine Formation in *Escherichia coli*

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(Received 26 September 1966)

1. A multiple aromatic mutant, *Escherichia coli* 156:53D2, required 4-hydroxybenzoic acid for rapid aerobic growth on a number of carbon sources. 2. In the absence of 4-hydroxybenzoic acid aerobic growth was stimulated by a mixture of lysine and methionine and by succinate. The influence of the amino acids is attributed to a sparing of succinyl-CoA. 3. Low activities of both α -oxoglutarate dehydrogenase and fumarate reductase were found in organisms grown aerobically without 4-hydroxybenzoate and consequently both mechanisms known for the formation of succinate were impaired. 4. The low fumarate-reductase activity in these organisms was due to repression of enzyme synthesis by aeration and not to enzyme inactivation. In contrast lactate dehydrogenase and ethanol dehydrogenase were induced. This is interpreted as the appearance of alternative routes of NADH oxidation when electron transfer to oxygen is impaired. 5. The activities of the other tricarboxylic acid-cycle enzymes tested were little influenced by 4-hydroxybenzoate deficiency, although anaerobiosis resulted in a fall in activity.

While studying the shikimic acid pathway of aromatic biosynthesis, Davis (1950) isolated a mutant of *Escherichia coli* which required 4-hydroxybenzoic acid as well as 4-aminobenzoic acid, tyrosine, phenylalanine and tryptophan for rapid growth in glucose-salts medium. The stimulatory action of 4-hydroxybenzoic acid was marginal with succinate, lactate or acetate as carbon sources or with glucose at pH 6.0 (Davis, 1952a). At pH 7.5, a response to 3,4-dihydroxybenzoic acid in addition to 4-hydroxybenzoic acid was observed.

The 4-hydroxybenzoic acid requirement of multiple aromatic auxotrophs could be largely replaced by a mixture of lysine and methionine (Davis, 1952b; 1955). Cystathionine and homocysteine replaced methionine, and diaminopimelate substituted for lysine in stimulating growth. Davis suggested that 4-hydroxybenzoic acid was involved in an early step in the biosynthesis of these amino acids.

The biosynthesis of lysine and methionine from aspartate by *E. coli* has been reviewed by Umbarger & Davis (1962). In methionine synthesis, the formation of cystathionine from cysteine and homoserine involves the succinylation of the latter to give *O*-succinylhomoserine; this compound condenses with cysteine to give cystathionine with the release of succinate (Rowbury & Woods, 1964; 1966; Kaplan & Flavin, 1965). A succinylation step also occurs in lysine synthesis giving rise to the *N*-succinyl

derivative of Δ -piperidine-2,6-dicarboxylate. This intermediate is further transformed to diaminopimelate with the release of succinate (Gilvarg, 1963; Yugari & Gilvarg, 1965).

These studies confirmed the role of succinate in lysine and methionine formation, first suggested on the basis of nutritional studies with a succinate auxotroph of *E. coli* (Davis, Kornberg, Nagler, Miller & Mingiolo, 1959). No evidence has, however, been provided for a direct involvement of 4-hydroxybenzoate in the formation of these amino acids.

The recent demonstration that 4-hydroxybenzoate is a precursor of ubiquinone (Parson & Rudney, 1964; 1965; Olsen, Dialameh, Bentley, Springer & Ramsey, 1965) led us to suppose that the replacement of the factor by lysine and methionine might be an indirect consequence of quinone deficiency. The present work provides evidence to support this. The influence of 4-hydroxybenzoic acid on the ubiquinone content and electron transport in the *E. coli* auxotroph is described in the following paper (Jones, 1967). A preliminary account of part of the work has been given by Jones & Lascelles (1966).

EXPERIMENTAL

Organisms. *E. coli* 150:53D2 was provided by Dr B. D. Davis. Stock cultures were grown on slopes of tryptic

casein-agar at 37° for 18 hr. Wild-type strains *E. coli* W (A.T.C.C. 9637) and 518 were also used.

Growth of organisms. Wild-type *E. coli* was grown in mineral salts medium (Davis & Mingioli, 1950), supplemented with iron citrate (40 μ M), with glucose (1 mM) as carbon source. *E. coli* 156 was grown in the above medium supplemented with DL-phenylalanine, DL-tyrosine and DL-tryptophan (all 0.4 mM) and 4-aminobenzoic acid (0.1 μ M). Other compounds, including 4-hydroxybenzoic acid (1 μ M) were added as indicated in the test. Aerobic cultures were grown at 37° on a reciprocating shaker and the bacteria harvested during exponential growth by centrifuging at 2000g for 20 min. at 0°. Organisms were grown anaerobically in flasks completely filled with freshly autoclaved medium and tightly stoppered. The bacteria were harvested during exponential growth. The amount of growth was assessed with an EEL photoelectric colorimeter (Evans Electro-selenium Ltd., Halstead, Essex) with a neutral density filter.

Preparation and fractionation of cell-free extracts. Organisms, after washing in 10 mM-phosphate, pH 7.2, were suspended in 40 mM-phosphate buffer at 25–30 mg. dry wt./ml. The bacteria were disrupted by passage through a precooled French pressure cell (Aminco Instrument Co. Inc., Silver Spring, Md., U.S.A.) at 16 000 lb./in.² or by ultrasonic vibration as described by Porra & Lascelles (1965). Whole organisms and debris were removed by centrifugation at 16 000 g for 15 min. at 0°. The crude cell-free extract, which contained 15–20 mg. of protein/ml., was fractionated by centrifuging at 100 000 g for 80 min. at 0° in a Spinco model L ultracentrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.). The supernatant fraction was decanted and the pellet resuspended in half the original volume of 40 mM-phosphate buffer, pH 7.2.

Estimation of protein content. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard.

Assay of enzymic activities. Succinate dehydrogenase was measured with phenazine methosulphate and 2,6-dichlorophenol-indophenol as electron acceptors (Arrigoni & Singer, 1962). NADP-dependent isocitrate dehydrogenase and NAD-dependent malate dehydrogenase were measured by methods of Ochoa (1955a,b). Ethanol dehydrogenase was assayed as described by Dawes & Foster (1956), and lactate dehydrogenase was measured by the pyruvate-dependent oxidation of NADH in the presence of 2 mM-sodium cyanide at pH 6.5. Pyruvate and α -oxoglutarate-dehydrogenases were measured spectrophotometrically by the reduction of ferricyanide at 418 m μ by using an adaptation of the method of Hager & Kornberg (1961). Fumarate reductase was determined by the oxidation of reduced benzyl viologen in an atmosphere of helium (B. Paynter, personal communication). The dye was reduced with hydrogen and palladized asbestos and the reduced dye (1 μ mole) was transferred with a syringe to the reaction vessel. The vessel, which contained 500 μ moles of phosphate buffer, pH 7.2, and 100 μ moles of sodium fumarate, had been previously flushed with helium. After checking for non-specific oxidation of dye by oxygen leakage, the reaction was started by the addition of cell-free extract (0.1 ml.) from the side arm. The oxidation of dye was measured in an EEL photoelectric colorimeter with a 625 filter, maximum transmission 530–550 m μ [1 μ mole of dye in 6 ml. = 140 EEL units (B. Paynter, personal communication)].

Chemicals. 4-Hydroxybenzoic acid and 3,4-hydroxybenzoic acid were obtained from British Drug Houses, Ltd., Poole, Dorset, and were recrystallized before use. Benzyl viologen was also purchased from British Drug Houses Ltd.

RESULTS

Growth of E. coli 156:53D2

4-Hydroxybenzoic acid requirement under various growth conditions. *E. coli* 156 required phenylalanine, tyrosine, tryptophan and 4-aminobenzoic acid for growth. In the presence of these compounds slow aerobic growth was observed on glucose; this was greatly stimulated by 4-hydroxybenzoic acid (1 μ M) (Fig. 1a). A similar requirement for 4-hydroxybenzoic acid was found with gluconate, lactate, succinate or pyruvate as carbon source or, anaerobically, with lactate and nitrate. In contrast, little stimulation of anaerobic growth on glucose by 4-hydroxybenzoic acid was observed (Fig. 1b). An additional improvement of growth was found under some conditions with 3,4-hydroxybenzoic acid (1 μ M). This effect was greatest at pH 7.6 on glucose but was also found at pH 7.0. In the absence of 4-hydroxybenzoate, 3,4-hydroxybenzoic acid did not influence growth.

Replacement of 4-hydroxybenzoic acid. A mixture of lysine and methionine partially replaced the requirement for 4-hydroxybenzoic acid although there was a lag before growth occurred with these supplements (Table 1). Methionine was replaced by homoserine and cystathionine but not by homoserine and cysteine, and diaminopimelate substituted for lysine.

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Fig. 1. Effect of 4-hydroxybenzoic acid on (a) aerobic and (b) anaerobic growth of *E. coli* 156. Organisms were grown in glucose-salts medium supplemented with phenylalanine, tyrosine, tryptophan and 4-aminobenzoic acid as described in the Experimental section. The mutant was grown without (○) and with (●) 1 μ M-4-hydroxybenzoic acid.

Table 1. Growth of *Escherichia coli* 156 in the absence of 4-hydroxybenzoic acid

The auxotroph was grown in glucose-salts medium with the supplements described in the Experimental section. The extent of growth was measured in an EEL photoelectric colorimeter after the times shown.

Additions to medium	Growth (EEL reading)			
	Aerobic		Anaerobic	
	24hr.	48hr.	24hr.	48hr.
None	0	9	2	9
4-Hydroxybenzoic acid (1 μ M)	14	28	8	9
DL-Lysine hydrochloride and DL-methionine (0.1 mM)	0	20	2	9
Sodium succinate (0.1 mM)	0	17	2	9
Sodium L-glutamate (0.3 mM)	0	13	9	10

carboxylic acid cycle and related compounds were examined. Of these, succinate promoted growth almost as effectively as a mixture of lysine and methionine; α -oxoglutarate and glutamate were less active than succinate (Table 1).

Anaerobic growth on glucose either with or without 4-hydroxybenzoic acid was not affected by succinate or by lysine and methionine but was stimulated by glutamate (Table 1).

These observations suggest that one lesion in organisms growing aerobically without 4-hydroxybenzoic acid is an inability to form sufficient succinate to promote lysine and methionine synthesis.

Effect of 4-hydroxybenzoic acid and aeration on enzymic activities

Enzymes of the tricarboxylic acid cycle. The growth studies showed that the requirement for 4-hydroxybenzoic acid was exhibited only under aerobic conditions. The mutant grew anaerobically without this factor and neither succinate nor lysine and methionine were required. This suggested that an alternative route for succinate occurred anaerobically, which was not influenced by 4-hydroxybenzoic acid deficiency.

α -Oxoglutarate dehydrogenase is known to be important for the aerobic formation of succinate since an auxotroph lacking this enzyme required succinate for aerobic growth (Davis *et al.* 1959; Hager & Kornberg, 1961). Fumarate reductase, which catalyses the synthesis of succinate from fumarate, is present in *E. coli* growing anaerobically and can provide succinate under these conditions

(Hirsch, Rasminsky, Davis & Lin, 1963). The activities of these enzymes and others associated with the tricarboxylic acid cycle were measured in the mutant and wild-type organisms grown under various conditions.

The α -oxoglutarate-dehydrogenase activity of the mutant grown aerobically with 4-hydroxybenzoate was similar to that of the wild-type organism (Table 2). In contrast, cells grown without the factor had only about 25% of the normal activity. The enzyme was not detectable in organisms grown anaerobically; this applied both to the wild type and to the mutant grown with or without 4-hydroxybenzoate (Table 2).

The effect of growth conditions on fumarate-reductase activity was similar in the mutant and wild-type strains (Table 2). Only low activity was exhibited by cells grown aerobically, whereas 10–20 times more enzyme was found in anaerobically grown organisms. The absence of 4-hydroxybenzoate during growth did not influence the fumarate-reductase activity of the mutant strain.

Upon transfer of cultures from anaerobic to aerobic conditions, the synthesis of fumarate reductase ceased immediately. This was apparently due to repression rather than to inactivation since the total amount of enzyme in the culture (0.2–0.3 m-mole of benzyl viologen reduced/5 ml. of extract/hr.) remained constant for at least three generations of aerobic growth. The α -oxoglutarate dehydrogenase was similarly repressed upon transfer from aerobic to anaerobic conditions; the amount of enzyme per ml. remained at 30–40 μ moles of α -oxoglutarate oxidized/5 ml. of extract/hr. for two generations of anaerobic growth. Conversely, a chloramphenicol-sensitive increase in fumarate-reductase and α -oxoglutarate-dehydrogenase activities was observed immediately on transfer to, respectively, anaerobic and aerobic conditions.

The effect of aerobic growth with and without 4-hydroxybenzoate on other enzymes of the tricarboxylic acid cycle was also examined. No significant difference was found in the activity of pyruvate, isocitrate, succinate and malate dehydrogenases (Table 2). These enzymes were less active in cells grown anaerobically but under all conditions the activities were similar to those found in the wild-type organism (Table 2).

Similar observations of the effects of anaerobiosis on the activities of tricarboxylic acid-cycle enzymes in *E. coli* have been made (Gray, Wimpenny, Hughes & Mossman, 1966; Gray, Wimpenny & Mossman, 1966).

Lactate and ethanol dehydrogenases. In some organisms the activity of the NAD-linked lactate and alcohol dehydrogenases is higher in anaerobically grown cells, in keeping with their function in the recycling of NAD during fermentation (Dawes

Table 2. *Effect of 4-hydroxybenzoic acid deficiency and of aeration on enzymic activities*

E. coli 156 was grown in glucose-salts medium with the supplements described in the Experimental section and the addition of succinate (0.1 mM). Wild-type *E. coli* was grown as described in the Experimental section. Enzyme assays were carried out with unfractionated cell-free extracts, except for isocitrate and malate dehydrogenases, which were localized in the 100000g supernatant. Values given are means of similar values obtained from three separate experiments. 4-OB, 4-hydroxybenzoic acid (1 μ M).

		Enzymic activities (μ moles of substrate/mg. of protein/hr. at 25°)		
		<i>E. coli</i> 156		
		Wild-type <i>E. coli</i>	With 4-OB	Without 4-OB
(a) Organisms grown aerobically	α -Oxoglutarate dehydrogenase	0.8	0.7	0.2
	Fumarate reductase	0.6	0.4	0.5
	Isocitrate dehydrogenase	42	52	51
	Succinate dehydrogenase	0.7	0.8	0.8
	Malate dehydrogenase	33	30	22
	Pyruvate dehydrogenase	3	4	3.5
	Lactate dehydrogenase	15	28	75
	Ethanol dehydrogenase	< 1	1	10
	α -Oxoglutarate dehydrogenase	< 0.05	< 0.05	< 0.05
(b) Organisms grown anaerobically	Fumarate reductase	9	8	8
	Isocitrate dehydrogenase	6	5	6
	Succinate dehydrogenase	0.4	0.5	0.4
	Malate dehydrogenase	8	15	12
	Pyruvate dehydrogenase	0.9	1.5	1.6
	Lactate dehydrogenase	45	80	75
	Ethanol dehydrogenase	8	8	7

& Foster, 1956; McPhedran, Sommer & Lin, 1961; Collins & Lascelles, 1962). Mutant 156 when grown without 4-hydroxybenzoate cannot oxidize NADH by the normal respiratory electron-transport chain (Jones, 1967) and may rely on the NAD-linked hydrogenases to regenerate NAD under both aerobic and anaerobic conditions. Assay of these enzymes showed them to be elevated in cells grown aerobically without 4-hydroxybenzoate, their activities reaching those found in anaerobically grown organisms (Table 2). The activities of the dehydrogenases in wild-type organisms and in the mutant grown aerobically with 4-hydroxybenzoate were one-third to one-tenth those of the anaerobically grown organisms (Table 2).

DISCUSSION

4-Hydroxybenzoate is a precursor of ubiquinone in *E. coli* and the following paper shows that deficiency of the factor results in the inability of the mutant organism to respire by the normal electron-transport chain, which includes a quinone component. In the present paper we have sought reasons to account for the increased ability of the mutant to grow without 4-hydroxybenzoate when either (a) succinate or lysine and methionine are provided during aerobic growth or (b) anaerobically even without these supplements.

The original observation of Davis that lysine and methionine substitute for 4-hydroxybenzoic acid in promoting the rapid growth of the mutant was confirmed although in modified form; the effect was only found aerobically and the amino acids did not completely restore the growth rate to the value found with 4-hydroxybenzoic acid. The influence of the amino acids may be attributed to a sparing of succinyl-CoA, which is involved in cystathionine and diaminopimelate formation, as succinate alone was almost as effective as the amino acids in stimulating growth. Further evidence of succinate deficiency is the low haem content of organisms grown in the absence of 4-hydroxybenzoic acid or succinate (Jones, 1967). No evidence was obtained to support the suggestion that 4-hydroxybenzoic acid was directly involved in an early step in lysine and methionine biosynthesis.

The alternative growth response to succinate or to lysine and methionine resembles the behaviour of an α -oxoglutarate dehydrogenase mutant of *E. coli* (Davis *et al.* 1959; Kaplan & Flavin, 1964). A similar requirement has been shown with a lipolic acid mutant of *E. coli* (Back & Westaway, 1962; Vise & Lascelles, 1967). It may be noted that the alternative requirement occurs despite the lack of net utilization of succinate in the formation of the amino acids.

The need for exogenous succinate in aerobically

grown deficient cells appears to be due to the low activities of both α -oxoglutarate dehydrogenase and fumarate reductase. Under anaerobic conditions fumarate reductase is highly active and probably provides adequate amounts of succinate. It is not clear why the α -oxoglutarate-dehydrogenase activity should be influenced by deficiency of 4-hydroxybenzoate, since the enzyme complex does not appear to contain a quinone component (Koike, Reed & Carroll, 1963; Mukherjee, Matthews, Horney & Reed, 1965). Amarasingham & Davis (1965) have suggested that the dehydrogenase may be induced by endogenous α -oxoglutarate. This possibility is consistent with the present observations. The low NADH oxidase in the deficient cells necessitates regeneration of NAD by the alcohol- and lactate-dehydrogenase systems. The products of glucose metabolism, acetate and pyruvate, may therefore be diverted into ethanol and lactate, whereas under normal aerobic conditions they would have been oxidized by the tricarboxylic acid cycle with consequent generation of α -oxoglutarate.

Even if the α -oxoglutarate dehydrogenase were not repressed such a diversion of glucose carbon atoms into fermentation products and away from the tricarboxylic acid cycle could lead to a requirement for succinate in the absence of an active fumarate reductase.

Low activities of fumarate reductase were found under all conditions of aerobic growth in both mutant and wild-type organisms, whereas the enzyme was highly active in anaerobically grown cells. Since the enzyme was low in the mutant when grown without 4-hydroxybenzoate under aerobic conditions, repression occurs in the absence of a functional respiratory chain. This is in contrast with the behaviour of the lactate and alcohol dehydrogenases, which exhibited anaerobic levels of activity when the mutant was grown aerobically without 4-hydroxybenzoate. The present work does not clarify the mechanism of oxygen repression of fumarate reductase or of the regulation of synthesis of the NAD-linked dehydrogenases.

Much of the work was done while the authors were associated with the Microbiology Unit, Department of Biochemistry, University of Oxford, and they are indebted to the late Professor D. D. Woods for his advice and encouragement. R.G.W.J. thanks Jesus College, Oxford, for a Meyricke Senior Research Scholarship.

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