Some Properties of a Mutant Strain of *Escherichia coli* which Requires Lysine and Methionine or Lipoic Acid for Growth

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SUMMARY

The requirement of a mutant strain of *Escherichia coli* for lysine + methionine was due to its inability to make lipoic acid. Aerobic growth of the mutant in minimal medium + lipoic acid was equal to that of the wild-type organism. The factor was replaceable by acetate + succinate. When grown without lipoic acid, suspensions of this organism did not oxidize pyruvate but did so upon addition of the factor; they also accumulated pyruvate from glucose. Extracts from deficient organisms did not oxidize α -ketoglutarate with 3-acetyl-NAD as acceptor. The growth requirements were only exhibited aerobically when provision of acetate + succinate required the operation of the lipoic-dependent pyruvate and α -ketoglutarate oxidase systems, respectively. Anaerobically, these metabolites were formed by lipoic-independent mechanisms, such as fumarate reductase which is repressed by oxygen.

INTRODUCTION

Mutant strains of *Escherichia coli* have been described which respond to mixtures of methionine and lysine (Back & Westaway, 1962; Taylor & Thoman, 1964). One of these strains has been examined in the present work; it responded to lipoic acid in place of lysine + methionine and it appeared that the primary lesion was an inability to synthesize lipoic acid, which led to secondary effects on the metabolism of the organism. Lipoic acid is a growth factor for several micro-organisms and its function has been established as an electron acceptor in the oxidative decarboxylation of pyruvate and α -ketoglutarate (Reed, 1960). It has, therefore, an important role in the metabolism of substrates by the tricarboxylic acid cycle. In the present work the mutant was used to study the effect of deficiency of lipoic acid on the growth requirements of the organism under aerobic and anaerobic conditions, and to examine at the enzymic level the reasons for the observed growth responses.

METHODS

Organisms. *Escherichia coli* strain 15 was derived from *E. coli* strain 518 (Back & Westaway, 1962) and both strains were maintained by monthly subculture on Oxoid nutrient agar slopes, incubated for 18 hr at 37° and stored at 4°.

Growth tests. Minimal medium C containing inorganic salts with 0.2% (w/v) glucose was the basal medium (Roberts *et al.* 1957). The inoculum (equivalent 0.25 μ g.

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dry-wt organism/ml.) was prepared from washed organisms grown overnight on nutrient agar slopes. Anaerobic incubation was in completely filled tubes sealed with a rubber bung; aerobic cultures were grown in 5 ml. volumes in 15 mm. diam. tubes shaken on a reciprocating shaker at 120 oscillations/min. Growth was measured after incubation for 24 hr at 37° with an EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) and is recorded as equiv. mg. dry-wt organism/ml. (instrument reading 10 = 0.33 mg./ml.).

Experiments with suspensions of organisms. Organisms were grown at 37° in medium C, supplemented as required. The inoculum was derived from nutrient agar slopes and was equivalent to about 2 µg. dry-wt organism/ml. Aerobic cultures were in 300 ml. volumes in 2 l. flasks, incubated with shaking at 300 oscillations/min.; anaerobic growth was in completely filled flasks. The cultures were harvested by centrifugation after incubation for 10 hr while still in the logarithmic phase of growth. The organisms were washed in 0.04 M-phosphate buffer (pH 7.0) and resuspended in the same buffer to a concentration equiv. 10–15 mg. dry-wt. organism/ml.

Respiratory activity was determined manometrically at 37°. Formation of products from glucose utilization was determined with organisms incubated aerobically or anaerobically in reaction mixtures containing per ml.; organisms, equiv. 20 mg. dry-wt; potassium phosphate buffer (pH 7.0), 100 µmoles; glucose, 20 µmoles. Aerobic incubation was in 4 ml. volumes in 50 ml. flasks shaken at 300 oscillations/min. and anaerobic incubation was in tubes under an atmosphere of nitrogen. After incubation for 3 hr at 37° the suspensions were centrifuged at 0° and the supernatant fluids analysed for products.

Analytical methods. Glucose was determined by the method of Huggett & Nixon (1957). Pyruvate was estimated as the 2,4-dinitrophenylhydrazone (Friedemann & Haugen, 1943). Acetate was measured by the method of Rose (1955) which involves its conversion to acetyl phosphate with acetokinase and measurement of the acetyl phosphate by the hydroxamate method of Lipmann & Tuttle (1945). A crude extract of aerobically grown *Escherichia coli* 518 was used as the source of acetokinase.

Preparation of cell-free extracts. Suspensions of organisms (equiv. 20 mg. dry-wt/ml. in 0.04 M-phosphate buffer, pH 7.0) were disrupted by ultrasonic treatment for 4 min. with a Mullard E 7590B generator operating at 3.5 A. and producing 25 kc./sec. The preparations were centrifuged at 18,000 g for 15 min. at 4° and the supernatant fluids used for the determination of enzymic activities. Protein was measured spectrophotometrically (Warburg & Christian, 1941).

Assays of enzyme activity. α-Ketoglutarate dehydrogenase was measured spectrophotometrically by the method of Amarasingham & Davis (1965), in which 3-acetyl-NAD is the electron acceptor; it was also assayed with ferricyanide as acceptor in the system of Hager & Kornberg (1961), measurement being made at 420 mµ. Lipoic acid dehydrogenase was measured by the method of Hager & Gunsalus (1953), except that disappearance of sulphydryl groups was estimated by the procedure of Ellman (1959). Reduced lipoic acid, required for this assay, was prepared by the method of Wagner *et al.* (1956). Fumarate reductase was assayed anaerobically by following the rate of oxidation of reduced benzylviologen in the presence of fumarate; measurement was made with the EEL colorimeter, using a 625 filter (Wyn-Jones & Lascelles, 1967).

Special chemicals. DL-lipoic acid (L. Light and Co. Ltd., Colnbrook, Bucks., England) was used. 3-Acetyl-NAD was from Calbiochem (California, U.S.A.); 5,5'-

dithio-bis(2-nitrobenzoic acid), for the determination of sulphhydryl groups, was from the Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin, U.S.A.); benzylviologen was from British Drug Houses Ltd.

RESULTS

Growth requirements under aerobic and anaerobic conditions

Under aerobic conditions, growth of the mutant strain 15 of *Escherichia coli* was dependent on lipoic acid, and with this supplement it grew as well as the parent organism in the unsupplemented medium (Table 1). Lipoic acid was replaced by a mixture of acetate + succinate, though neither substance alone was effective and growth did not attain the degree given with lipoic acid. The low concentration of lipoic acid required for maximum growth (about 5 $m\mu M$) was of the same order as that required by other exacting micro-organisms. The requirement of acetate + succinate was higher by several orders of magnitude, which was to be expected if lipoic acid functioned catalytically in their synthesis. Anaerobically, the mutant grew on the unsupplemented minimal medium to the same extent as the parent, and the addition of lipoic acid or of acetate + succinate had no effect (Table 1).

Table 1. *Growth responses of Escherichia coli strains 15 and 518*

Cultures were grown as described in the Methods in medium C supplemented as shown.

Strain	Supplement to minimal medium (final conc.)			Growth in 24 hr (equiv. mg. dry-wt organism/ml.)	
	Acetate (mM)	Succinate (mM)	DL-lipoic acid ($m\mu M$)	Aerobic	Anaerobic
518	—	—	—	0.71	0.34
15	—	—	—	0	0.31
	1	—	—	0	0.26
	—	1	—	0	0.28
	1	1	—	0.35	0.31
	—	—	10	0.71	0.31

Utilization of glucose and pyruvate by suspensions of organisms

The difference in behaviour under aerobic and anaerobic conditions suggested that the mutant could make acetate + succinate anaerobically by lipoic-independent mechanisms, whereas aerobically these metabolites were provided by the lipoic-dependent oxidation of pyruvate and α -ketoglutarate. This was partially confirmed by experiments with suspensions of the mutant and parent strains grown with and without lipoic acid.

Suspensions of the mutant grown aerobically without lipoic acid (with acetate + succinate present) did not oxidize pyruvate but did so upon addition of the factor (Fig. 1). Suspensions of such deficient organisms accumulated pyruvate when incubated aerobically with glucose, in contrast to organisms grown with lipoic acid which behaved like the parent strain (Table 2). Surprisingly, acetate was also found as a product of glucose utilization by lipoic-acid-deficient organisms. This acetate might have been formed via the pyruvate oxidase system which is induced by pyruvate and which is formed in the later stages of growth of *Escherichia coli* (Gounaris & Hager, 1961). The reaction does not appear to be quantitatively significant for the growing

Fig. 1. Oxidation of pyruvate by suspension of *Escherichia coli* strain 15. Each manometer vessel contained in 2.5 ml.: organisms (equiv. 10 mg. dry-wt) suspended in 50 mM-phosphate buffer (pH 7.0) with sodium pyruvate (20 μmoles) added at zero time from the side arm; lipoic acid (1 μmole) was present in B. The centre well contained 5 N-NaOH. Incubation was at 37° in air. The endogenous respiration (not more than 12 $\mu\text{l./mg. dry-wt organism/hr}$) has been subtracted. Curves A and B contained lipoic-deficient organisms, grown with mM-acetate + succinate; curve C contained organisms grown with 10 mM-lipoic acid.

Table 2. Formation of acetate and pyruvate from glucose by suspensions of *Escherichia coli* strains 15 and 518

Organisms were grown in the basal medium supplemented as indicated, and the washed suspensions incubated aerobically or anaerobically in 4.0 ml. mixture containing: organisms, equiv. 80 mg. dry-wt; potassium phosphate buffer (pH 7.0), 400 μmoles ; glucose, 80 μmoles . Incubation was for 3 hr, by which time the glucose was completely utilized. After removal of the organisms by centrifugation acetate and pyruvate were determined in the supernatant fluids (see Methods).

Strain	Growth conditions	Products found			
		Aerobically		Anaerobically	
		Acetate	Pyruvate	Acetate	Pyruvate
		(μmoles/ml.)			
15	Aerobic with 10 mM-lipoic acid	2.0	1.6	11	0.7
	Aerobic with mM acetate+ succinate	7	8	14	0.7
	Anaerobic without supplement	14	0.4	8	1.3
518	Aerobic without supplement	3	0.6	13	0.5
	Anaerobic without supplement	15	2.2	13	0.4

organism since the mutant requires exogenous acetate when grown aerobically without lipoic acid.

Under anaerobic conditions, suspensions of the lipoic acid deficient mutant behaved like the parent strain; only small amounts of pyruvate were detected and there was considerable accumulation of acetate (Table 2). These observations confirmed the existence of a lipoic-independent mechanism for acetate formation, which operated under anaerobic conditions.

Enzymic activities

Lipoic dehydrogenase. The activity of this enzyme was examined in extracts of the mutant and parent strain grown under various conditions (Table 3). The enzyme was present in the mutant grown either with or without lipoic acid and was, indeed, significantly higher in organisms grown aerobically with acetate and succinate in place of the factor. The level of activity in both parent and mutant organisms grown anaerobically was about one third of that found in aerobic organisms.

Table 3. *Enzymic activities in extracts of Escherichia coli strains 15 and 518*

Organisms were grown in the salts medium supplemented as shown. Activities were determined in extracts of the organisms as described in the Methods.

Strain	Growth conditions	Lipoic dehydrogenase (μ moles substrate or product/hr/mg. protein)	α -Ketoglutarate dehydrogenase		Fumarate reductase
			NAD*	Ferricyanide	
15	Aerobic with 10 mM-lipoic acid	1.4	0.07	1.7	0.85
	Aerobic with mM acetate + succinate	2.4	0	1.26	0.85
	Anaerobic without supplement	0.4	0	0	7.8
518	Aerobic without supplement	1.4	0.06	1.26	0.45
	Anaerobic without supplement	0.6	0	0	4.3

* 3-acetyl-NAD.

Enzymes concerned in succinate formation. The dependence of the mutant upon succinate for aerobic growth without lipoic acid could be attributed to its inability to form succinate from α -ketoglutarate via the α -ketoglutarate oxidase system. Extracts of the mutant grown aerobically without lipoic acid contained no detectable α -ketoglutarate dehydrogenase activity with 3-acetyl-NAD as electron acceptor, but the activity was found with ferricyanide, which bypasses lipoic acid as electron acceptor. Preparations from the mutant grown aerobically with lipoic acid had both activities at degrees similar to that found in the parent (Table 3). α -Ketoglutarate dehydrogenase with 3-acetyl-NAD or ferricyanide as acceptor was detectable only in organisms grown aerobically; this applied to both parent and mutant strains and confirmed recent observations of Amarasingham & Davis (1965).

The ability of the mutant to grow anaerobically without added succinate could be attributed to the synthesis of succinate by fumarate reductase. This enzyme catalyses fumarate reduction more rapidly than succinate oxidation, whereas succinic dehydrogenase favours the formation of fumarate from succinate (Hirsch, Rasminsky, Davis & Lin, 1963). Fumarate reductase was high in mutant and parent organisms grown anaerobically (Table 3). In both strains it was repressed by oxygen, and in the case of the mutant the degree of activity was low in organisms grown with or without lipoic

acid. Repression of this enzyme by oxygen accounts for the dependence of the mutant on added succinate when the normal mechanism for its aerobic formation from α -ketoglutarate is prevented by deficiency of lipoic acid.

DISCUSSION

The mutant strain 15 of *Escherichia coli* is apparently blocked at a stage in the synthesis of lipoic acid; there is no knowledge of this biosynthetic pathway. The consequences of lipoic acid deficiency only become evident when the cultures are grown aerobically and are dependent upon the operation of the tricarboxylic acid cycle for the biosynthesis of intermediates. Under these conditions the organism apparently forms acetate + succinate predominantly by the lipoic-mediated pyruvate and α -ketoglutarate oxidase complexes. Other enzymic mechanisms which provide these metabolites by lipoic-independent steps are repressed or inhibited by oxygen, whereas they can function anaerobically. Consequently, the mutant grows anaerobically without supplementation.

Acetate can be formed anaerobically by *Escherichia coli* by the phosphoroclastic reaction, which is not detectable in organisms grown aerobically (Henning, 1963). The pyruvate-induced pyruvate oxidase described by Gounaris & Hager (1961) does not apparently provide sufficient acetate to support growth of the mutant. The alternative anaerobic mechanism for succinate formation is by the fumarate reductase enzyme, which is repressed by oxygen in both mutant and parent strain. Repression of this enzyme by aerobiosis was previously found by Hirsch *et al.* (1963).

The original observations of the response of the *Escherichia coli* mutant 15 to lysine + methionine may be partly explained as follows. Succinylation reactions occur in the biosynthesis of both amino acids (Davis *et al.* 1959; Rowbury & Woods, 1964) and their addition to the medium would therefore spare the requirement for succinate. However, the fact that acetate is no longer necessary for growth when these amino acids are present is more difficult to understand. Possibly, lipoic-independent reactions for acetate formation, such as the system described by Gounaris & Hager, (1961) may provide sufficient amounts of acetate when other growth requirements have been met. A similar pattern of growth responses has been observed with a mutant strain of *E. coli* which requires 4-hydroxybenzoic acid, a precursor of quinones (Wyn-Jones & Lascelles, 1967). Aerobic growth occurs without the factor in the presence of methionine + lysine or succinate; under anaerobic conditions, however, the organism grows without supplementation. Thus, with both mutant strains, a deficiency in the aerobic electron transport chain results in an impairment in the tricarboxylic acid cycle as a source of intermediates.

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