The Control of Sulphate Reduction in Bacteria

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1. An enzyme from *Escherichia coli* 9723 that reduces adenosine 3'-phosphate 5'-sulphatophosphate to inorganic sulphite is described. Extracts of *E. coli* K₁₂ and *Bacillus subtilis* 1379 contain a similar enzyme. 2. This reductase and sulphite reductase (EC 1.8.1.2) of *E. coli* 9723, *E. coli* K₁₂ and of *B. subtilis* are repressed by growth in the presence of L-cystine. Cysteine synthase (EC 4.2.1.22) is unaffected. 3. Growth of *E. coli* 9723 on inorganic sulphite represses the sulphate-activating enzymes (EC 2.7.7.4 and 2.7.1.25) almost completely but has little effect on sulphite reductase. Growth on 0.042–0.056 mM L-cystine gives a similar result. 4. Such differential repression by cyst(e)ine prevents *E. coli*, when growing on sulphite, from synthesizing unnecessary enzymes.

Escherichia coli is capable of growing on some of the intermediates of the biosynthetic sequence from sulphate to cysteine (Scheme 1) as sole source of sulphur (Roberts, Abelson, Cowie, Bolton & Britten, 1955). When sulphite, sulphide or cystine is added to growing cultures, the synthesis of the first two enzymes, ATP-sulphate adenylyltransferase (EC 2.7.7.4) and ATP-adenylyl sulphate 3'-phosphotransferase (EC 2.7.1.25), is repressed (Pasternak, 1962; Ellis, Humphries & Pasternak, 1964; Wheldrake & Pasternak, 1965). The next two enzymes, PAPS† reductase (Hilz, Kittler & Knape, 1959; Fujimoto & Ishimoto, 1961; Wilson, Asahi & Bandurski, 1961) and sulphite reductase (hydrogen sulphide-NADP oxidoreductase, EC 1.8.1.2), are repressed by L-cysteine in *Salmonella typhimurium* (Dreyfuss & Monty, 1963), and there are reports (Mager, 1960; Kemp, Atkinson, Ehret & Lazzarini, 1963) that sulphite reductase is repressed in *E. coli* also. We have therefore assayed PAPS reductase, sulphite reductase and the final enzyme in the sequence, cysteine synthase [L-serine hydro-lyase (adding hydrogen sulphide), EC 4.2.1.22], in extracts of *E. coli* grown on a variety of sulphur compounds.

The control of sulphate reduction in other bacteria appears to resemble that in *E. coli* since the enzymes catalysing PAPS formation in *Bacillus subtilis* are repressed by L-cyst(e)ine (Pasternak, 1962; Wheldrake & Pasternak, 1965). A study of

Scheme 1. Sulphate reduction in bacteria.

PAPS reduction, sulphite reduction and cysteine synthesis in *B. subtilis* has now been carried out to determine how far this similarity extends.

In *E. coli* the addition of an intermediate such as sulphite to the growth medium virtually completely represses the sulphate-activating enzymes. We have shown that sulphite is not itself a repressor but acts by metabolic conversion into cysteine (Ellis *et al.* 1964). Since cysteine also represses the enzyme utilizing sulphite, it is clear that co-ordinate repression (Ames & Garry, 1959) of the sulphur pathway would be detrimental to the cell. Instead one might envisage a mechanism whereby the synthesis of successive enzymes along the pathway is inhibited at increasingly higher concentrations of end product. This would require that added sulphate, sulphite or sulphide gives rise to increasingly higher concentrations of cysteine within the cell. This situation probably exists in *E. coli* because sulphide is a better repressor than sulphite, which is itself better than sulphate (Ellis *et al.* 1964). We now present evidence showing that sulphate reduction in *E. coli* is controlled by such a mechanism of 'differential repression'. A brief report of this work has appeared (Pasternak, Crichton, Ellis, Jones-Mortimer & Wheldrake, 1964).

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† Abbreviations: APS, adenosine 5'-sulphatophosphate; PAPS, adenosine 3'-phosphate 5'-sulphatophosphate.

MATERIALS AND METHODS

Chemicals. [^{35}S]APS was prepared as described by Wheldrake & Pasternak (1965) and freed of other nucleotides (Robinson, 1965). [^{35}S]PAPS was prepared by incubating an extract (42–54 mg. of protein) of *E. coli* A.T.C.C. 9723 (grown on glutathione) with 90 μmoles of ATP, 110 μmoles of MgCl_2 , 9 μmoles of $\text{Na}_2^{35}\text{SO}_4$ (0.1–0.3 mc) and 0.75 m-mole of tris in a final volume of 12 ml., adjusted to pH 8.8 with HCl, for 15 min. at 37°. After heating for 2 min., the reaction mixture was chilled, protein removed by centrifugation and the supernatant diluted to 200 ml. and adjusted to pH 6.1 with H_2SO_4 . It was applied to a column (10 cm. $\times$ 1 cm. diam.) of acid-washed Norit A or Nuchar C charcoal at 4°, washed with 100 ml. of 0.01M- Na_2SO_4 followed by 100 ml. of water and eluted with 40 ml. of ethanol–water–aq. ammonia (sp.gr. 0.88) (20:20:1, by vol.). The volume of the eluate was reduced to 2–3 ml. by rotary evaporation below 30°. The yield of [^{35}S]PAPS, which contained less than 1% of inorganic [^{35}S]sulphate, was 1–4 μmoles . *N*-Ethylmaleimide was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Growth of organisms. The organisms and media used were those described by Pasternak (1962) and by Ellis *et al.* (1964). Cultures (600 ml. in 21. flasks) were incubated overnight at 37° on a Gyrotary shaker (New Brunswick Scientific Co. Inc., N.J., U.S.A.). Cells were harvested towards the end of the exponential phase, washed with 100 mM-tris–5 mM-EDTA buffer adjusted to pH 8 with HCl, suspended in 5 ml. of buffer and disrupted by passage through a French pressure cell (American Instrument Co. Inc., Silver Spring, Md., U.S.A.). The supernatant fraction obtained by centrifuging at 105000g for 1 hr. in a Spinco model L ultracentrifuge was used.

Assay of enzymes. The enzyme system catalysing the synthesis of PAPS from sulphate and ATP was assayed as described by Pasternak (1962). PAPS reductase was assayed by incubating 12 m μmoles of [^{35}S]PAPS (0.07 μC), 2.2 μmoles of Na_2SO_4 , 9 μmoles of Na_2SO_3 , 25 μmoles of sodium phosphate buffer, pH 7, 0.045 μmole of NADP, 0.45 μmole of MgCl_2 , 4.5 μmoles of glucose 6-phosphate, 0.7 unit of glucose 6-phosphate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) and enzyme extract (0.2–4.0 mg. of protein) in a final volume of 0.5 ml. in centrifuge tubes, which were closed with rubber serum caps, at 37°. The reaction was stopped by injection of 0.2 ml. of 0.2 M-*N*-ethylmaleimide, reincubating for 10 min. and heating at 100° for 1 min. after removal of the caps. Protein was removed by centrifugation and a sample (0.2 ml.) of the supernatant applied to Whatman no. 3 paper and subjected to descending chromatography in solvent *A* (see below). The dried papers were cut into strips and radioactivity was measured with a type 2029 chromatogram scanner operated automatically on stepwise drive (Isotope Developments Ltd., Beenham, Berks.). $\text{Na}_2^{35}\text{SO}_4$ and [^{35}S]PAPS remain near the origin, the *N*-ethylmaleimide derivative of sulphite (which turns pink after 1–2 months) moves with R_F 0.4 and the *N*-ethylmaleimide derivative of sulphide moves just behind the solvent front. The last-named compound becomes labelled only when Na_2SO_3 is omitted from the reaction mixture. The *N*-ethylmaleimide derivative of [^{35}S]sulphite was identified by co-chromatography with unlabelled material in solvent *C* followed by solvent *A* (see below) and spraying

with *N*-ethylmaleimide (Margolis & Mandl, 1958). Radioactivity and pink colour coincided. Sulphite reductase was measured by the method of Ellis (1964). Serine sulphhydrase was estimated by the method of Brüggemann, Schlossmann, Merckenschlager & Waldschmidt (1962), modified as follows: 0.1 ml. of 0.5 M-L-serine, 0.05 ml. of pyridoxal phosphate (50 $\mu\text{g.}/\text{ml.}$), 0.1 ml. of 10 mM-KCN (Brüggemann & Waldschmidt, 1962), 0.2 ml. of 0.1 M-tris–0.1 N-NaOH–1.3 mM-EDTA into which H_2S had been bubbled until the pH was 8, enzyme extract (1–4 mg. of protein) and enough 0.1 M-tris–HCl buffer, pH 8, to bring the volume to 0.65 ml. were incubated in small stoppered tubes at 37°. The reaction was halted by the addition of 0.35 ml. of 10% (w/v) trichloroacetic acid, protein removed by centrifugation and oxygen-free nitrogen bubbled for 10 min. through the supernatant to which a drop of octan-1-ol had been added. A sample was assayed by the method of Grunert & Phillips (1951). Zero-time values were subtracted from those obtained after incubation. The amount of cysteine formed during incubation corresponded to the amount of radioactivity moving in the area of the *N*-ethylmaleimide derivative of cysteine after two-dimensional chromatography in solvents *C* and *A* (Margolis & Mandl, 1958) when [^{14}C]serine was used as substrate. The desulphurylation of cysteine (Brüggemann & Waldschmidt, 1962) was assayed by incubating 50 μmoles of L-cysteine hydrochloride, 5 $\mu\text{g.}$ of pyridoxal phosphate, enzyme extract (0.02–0.2 mg. of protein) and 120 μmoles of sodium phosphate buffer, final pH 8, in 1.7 ml. in serum-capped tubes under nitrogen. The reaction was stopped by injecting 0.3 ml. of 25% (w/v) trichloroacetic acid. Nitrogen was next bubbled through the incubation mixture to remove H_2S , which was trapped in 5 ml. of 0.1 M-sodium phosphate buffer, pH 8, containing 15 μmoles of bis-(3-carboxy-4-nitrophenyl) disulphide (Ellman, 1959), and the extinction at 412 m μ measured against a reagent blank. Protein was measured as described by Wheldrake & Pasternak (1965).

Paper chromatography. Descending chromatography was carried out with the following solvents: *A*, 2-methylpropan-2-ol–formic acid (90%, w/v)–water (14:3:3, by vol.); *B*, propan-1-ol–aq. ammonia (sp.gr. 0.88)–water (6:3:1, by vol.); *C*, ethanol–2-methylpropan-2-ol–aq. ammonia (sp.gr. 0.88)–water (300:100:4:75, by vol.). Some breakdown of [^{35}S]PAPS to [^{35}S]sulphate occurred during incubation and this was estimated by paper electrophoresis (Pasternak, 1962) or by paper chromatography in solvent *B*, which separates sulphate, PAPS and the *N*-ethylmaleimide derivative of sulphite. The last-named area contained the same amount of radioactivity as the area of R_F 0.39 after chromatography in solvent *A*.

RESULTS

Control of sulphate reduction in *E. coli*. Table 1 shows some of the properties of PAPS reductase in an extract of *E. coli* grown on glutathione. The system differs from that described by Mager (1960) and Fujimoto & Ishimoto (1961) in preferring NADPH to NADH as reducing agent. It is stimulated by a thermostable cofactor, but whether this is a metal activator, a coenzyme such as FAD or a low-molecular-weight protein (Torii & Bandurski, 1964) was not established. The enzyme, like that of

Table 1. *Reduction of PAPS by E. coli 9723*

An extract (5.6 mg. of protein/ml.) of *E. coli* grown (0.7 mg. dry wt. of cells/ml.) on glutathione (0.21 mM) plus glucose (0.075%) was incubated with 12 mμmoles of [³⁵S]PAPS (0.07 μC), 2.2 μmoles of Na₂SO₄, 9 μmoles of Na₂SO₃, 0.45 μmole of MgCl₂, 25 μmoles of sodium phosphate buffer, pH 7, NADPH-generating system and the compounds indicated in a final volume of 0.5 ml. for 30 min. and assayed as described in the Materials and Methods section. NADPH-generating system: 4.5 μmoles of glucose 6-phosphate, 0.045 μmole of NADP and 0.7 unit of glucose 6-phosphate dehydrogenase. NADH-generating system: 18 μmoles of ethanol, 0.09 μmole of NAD and 5 units of yeast alcohol dehydrogenase.

Incubation mixture		Enzyme extract added (ml.)	Yield of <i>N</i> -ethylmaleimide derivative of [³⁵ S]sulphite (mμmoles)
Addition	Omission		
None	None	0.05	0.7
None	None	0.1	3.2
None	None	0.2	10.1
Supernatant from boiled enzyme (0.05 ml.)	None	0.05	3.4
Supernatant from boiled enzyme (0.1 ml.)	None	0.05	4.1
None	NADPH-generating system	0.1	< 0.03
NADH-generating system	NADPH-generating system	0.1	0.5
NADH-generating system	NADPH-generating system	0.2	2.2
None	None	None	< 0.03
None	None	0.1*	< 0.03
None	None	0.1†	< 0.03
[³⁵ S]APS (21 mμmoles)	[³⁵ S]PAPS	0.1	0.8
[³⁵ S]APS (21 mμmoles) + ATP (3.3 μmoles) + MgCl ₂ (4 μmoles)	[³⁵ S]PAPS	0.1	1.5

* Zero time.

† Boiled enzyme.

Table 2. *Repression of sulphate-reducing enzymes in E. coli 9723*

Cultures were grown in the presence of glucose (0.05%) and the compounds indicated, harvested at 0.15–0.25 mg. dry wt./ml. and the enzymes assayed as described in the Materials and Methods section. The values quoted in the first row are means of several experiments, the numbers performed being given in parentheses.

S source for growth	Specific activity (mμmoles/mg. of protein/min.)			
	SO ₄ ²⁻ → PAPS	PAPS → SO ₃ ²⁻	SO ₃ ²⁻ → S ²⁻	S ²⁻ → cysteine
	3.4 (11)	0.62 (8)	13.5 (11)	0.81 (5)
Relative specific activity				
Glutathione (0.21 mM)	(100)	(100)	(100)	(100)
Na ₂ SO ₄ (0.85 mM)	40	47	39	240
Na ₂ SO ₄ (4.2 mM)	26	60	49	220
Na ₂ SO ₃ (0.85 mM)	2.5	29	31	370
Na ₂ SO ₃ (4.2 mM)	0.9	56	25	380
Na ₂ S (0.2 mM)	< 0.1	2.5	< 0.1	300
Na ₂ S (0.85 mM)	< 0.1	0.9	< 0.1	220
L-Cystine (0.042 mM)	5.5	12	23	200
L-Cystine (0.056 mM)	< 0.1	15	10	160
L-Cystine (0.069 mM)	< 0.1	10	< 0.1	270
L-Cystine (0.85 mM)	< 0.1	3.7	< 0.1	230

yeast (Wilson *et al.* 1961), has some activity with APS. Extracts of *E. coli* K₁₂ contain a similar reductase. Tables 2 and 3 show that PAPS reductase and sulphite reductase, in addition to the enzyme system catalysing the synthesis of PAPS (Pasternak,

1962), are repressed by growth on cystine. Fujimoto & Ishimoto (1961) found less PAPS reductase in *E. coli* grown on a medium of meat extract and peptone than on one containing glucose and salts. Both reductases are de-repressed by glutathione.

Table 3. *Repression of sulphate-reducing enzymes in E. coli K₁₂*

Cultures were grown in the presence of glucose (0.067%) and the compounds indicated, harvested at 0.18–0.28 mg. dry wt./ml. and the enzymes assayed as described in the Materials and Methods section. The values quoted in the first row are means of two experiments.

S source for growth Glutathione (0.21mm)	Specific activity (mμmoles/mg. of protein/min.)			
	SO ₄ ²⁻ → PAPS	PAPS → SO ₃ ²⁻	SO ₃ ²⁻ → S ²⁻	S ²⁻ → cysteine
	3.1	0.185	6.6	0.74
Glutathione (0.21mm)	Relative specific activity			
	(100)	(100)	(100)	(100)
Na ₂ SO ₄ (4.2mm)	74	82	68	120
Na ₂ SO ₃ (4.2mm)	<0.3	41	38	120
L-Cystine (0.42mm)	<0.3	4.3	<0.9	140

Table 4. *Repression of sulphate-reducing enzymes in B. subtilis*

Cultures were grown in the presence of 1.3% nutrient broth containing glucose (0.5%) and the compounds indicated harvested at 0.2–0.35 mg. dry wt. of cells/ml. and the enzymes assayed as described in the Materials and Methods section. The values quoted are the means of two or more experiments.

Sulphur compound added to growth medium	Specific activity (mμmoles/mg. of protein/hr.)			
	SO ₄ ²⁻ → PAPS	PAPS → SO ₃ ²⁻	SO ₃ ²⁻ → S ²⁻	S ²⁻ → cysteine
Na ₂ SO ₄ (0.85 mm)	3.00	1.08	13.4	78
Glutathione (0.85 mm)	0.59	0.11	11.4	62
L-Cystine (0.42 mm)	0.33	0.08	4.3	115

The specific activities quoted in Tables 2 and 3 do not represent maximum catalytic activity in the extracts because the enzymes were not all assayed at saturating substrate concentrations. Mixing experiments eliminated the possibility that the lowered activity of repressed extracts was due to the presence of inhibitory substances. At high concentrations of cystine there is still appreciable activity of PAPS reductase. Some non-leaky sulphiteless mutants, obtained from *E. coli* 9723 by ultraviolet irradiation (Wheldrake & Pasternak, 1965), that are blocked at PAPS reductase (they contain ATP sulphurylase and APS kinase) still have up to 20% of the activity of wild-type extracts. It is therefore possible that some reduction of PAPS is catalysed by non-specific enzymes. The final enzyme in the biosynthesis of cysteine, cysteine synthase, is not repressed by cystine. This could be because *in vivo* the enzyme actually catalyses the reverse reaction (Brüggemann & Waldschmidt, 1962) under such conditions. The formation of hydrogen sulphide from cysteine, which is catalysed by cysteine desulphydrase (EC 4.4.1.1) in addition to cysteine synthase, was therefore measured. However, activity in extracts of *E. coli* 9723 was 19 mμmoles of hydrogen sulphide formed/mg. of

protein/min. when grown on 0.21mm-glutathione and 14 mμmoles/mg. of protein/min. on 0.42mm-L-cystine.

The point to note is that repression of the enzymes leading from sulphate to sulphide is not co-ordinate. This is most clearly seen with cells grown on sulphite. Whereas the activation of sulphate is almost completely repressed, sulphite reductase is only partly repressed and PAPS reductase even less so. Since sulphite is not itself a repressor of PAPS formation but acts as a result of metabolic conversion into cysteine (Ellis *et al.* 1964) a similar situation should exist during growth on low concentrations of cystine. This was confirmed by growth on an initial concentration of 0.042–0.056mm (Table 2). It is thus apparent that the synthesis of each of the enzyme systems that is repressible by cystine is separately controlled by having a differing sensitivity to intracellular cysteine.

Control of sulphate reduction in B. subtilis. Extracts contained a PAPS reductase similar to that in *E. coli*. PAPS reduction was almost as rapid with NADH as with NADPH. The activity of the enzyme was less than that of *E. coli* 9723, which may explain Peck's (1961) failure to detect PAPS reductase in *B. subtilis* or related species.

The sulphite-reductase activity of extracts was also lower than in *E. coli*, but the cysteine-synthase activity (Brüggemann *et al.* 1962) was the same (Table 4). The low activity of enzymes that are repressible by cysteine may be a reflection of components in the particular batch of broth used for growth of the organisms (see Wheldrake & Pasternak, 1965).

The type of control that regulates the reduction of sulphate is the same in *B. subtilis* (Table 4) as in *E. coli* (Tables 2 and 3). Thus the enzymes of sulphate activation, PAPS reduction and sulphite reduction are all repressed by growth in the presence of cysteine, but cysteine synthase is unaffected. As in *E. coli*, the repression appears not to be co-ordinate, since glutathione, which in *B. subtilis* 1379 grown on broth represses sulphate activation (Pasternak, 1962; and Table 4), has little effect on sulphite reductase (Table 4).

DISCUSSION

When bacteria are grown on the intermediates of a biochemical pathway the synthesis of some of the enzymes of the sequence may be repressed, induced or unaffected. For example, in the catabolism of mandelic acid to β -oxoadipic acid by *Pseudomonas fluorescens*, growth on benzoic acid causes repression of the enzymes between mandelic acid and benzoic acid and induction of the enzymes converting benzoic acid into β -oxoadipic acid (Mandelstam, 1964). Also, the addition of histidinol, an intermediate in the biosynthesis of histidine, to certain *S. typhimurium* mutants causes de-repression of all the enzymes of that pathway (Ames, Hartman & Jacob, 1963). Growth of *E. coli* on sulphite represses two of the enzymes between sulphate and sulphite but has little effect on the enzyme converting sulphite into sulphide (Tables 2 and 3). All these effects of intermediates are explicable in terms of the operon hypothesis (Jacob & Monod, 1961). In histidine biosynthesis one operon regulates the synthesis of all the enzymes in a co-ordinate manner and exogenous histidinol de-represses them by being converted into histidine slowly. The degradation of mandelic acid to β -oxoadipic acid is controlled by three operons, of which the first is repressed and the next two are induced by benzoic acid. This intermediate is itself the repressor since it is effective in mutants blocked beyond benzoic acid (Mandelstam, 1964). In the reduction of sulphate to cysteine, also, there are probably several operons, since the synthesis of at least four enzymic sequences (sulphate activation, PAPS reduction, sulphite reduction and cysteine synthesis) is controlled in a non-co-ordinate way (Tables 2 and 3). The two enzymes synthesizing PAPS may be co-ordinately repressed (Wheldrake & Pasternak,

1965). The regulation differs from that of mandelic acid oxidation in that an intermediate such as sulphite is not itself a repressor but acts by conversion into the end product, cysteine (Ellis *et al.* 1964).

Repression by cystine of the first three steps (sulphate activation, reduction and sulphite reduction) is 'coincident' (Dreyfuss & Monty, 1963) at high concentrations of cystine, but at low concentrations only the first step is significantly repressed (Table 2). Thus the rate at which an intermediate is converted into cysteine determines whether all or only some of the enzymes are repressed. Such 'differential' repression results in a more effective control of enzyme synthesis than is possible with co-ordinate repression. When the organism grows on sulphite, only the enzymes synthesizing PAPS are repressed. When sulphide is the sulphur source, synthesis of sulphite reductase is prevented also (Table 2). It must be admitted that the synthesis of PAPS reductase appears not to conform to this pattern. Two enzymes in the biosynthesis of methionine (Foster, Rowbury & Woods, 1963) appear to be under differential control. One may conclude that the biosynthesis of cysteine resembles that of arginine (Gorini, 1963) in two main respects. First, the synthesis of the enzymes catalysing the various steps is not co-ordinate, and, secondly, the genes that control the synthesis of the enzymes are scattered, at least in *S. typhimurium*, along the chromosome (Mizobuchi, Demerec & Gillespie, 1962).

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