The Control of Sulphate Activation in Bacteria

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1. ATP-sulphate adenylyltransferase (EC 2.7.7.4) and ATP-adenylyl sulphate 3'-phosphotransferase (EC 2.7.1.25) of *Escherichia coli* 9723, *E. coli* K₁₂ and *Bacillus subtilis* 1379 are each repressed by growth in the presence of cystine. Repression of the two enzymes in *E. coli* 9723 may be co-ordinate. 2. ATP-sulphate adenylyltransferase of *Desulphovibrio desulphuricans*, in which sulphate reduction is linked to the energy supply of the organism, is not repressed by growth in the presence of inorganic sulphite or cysteine. 3. *Leuconostoc mesenteroides* lacks all the enzymes between sulphate and cysteine whether grown on cysteine or glutathione.

Sulphate activation is the first stage in the biosynthesis of cysteine by micro-organisms (Gregory & Robbins, 1960; Wilson, 1962). In *Escherichia coli* and *Bacillus subtilis* the synthesis of the activating system that forms PAPS* from sulphate and ATP is repressed when cyst(e)ine is added to the growth medium (Pasternak, 1962). The two enzymes involved, ATP sulphurylase (ATP-sulphate adenylyltransferase, EC 2.7.7.4) and APS kinase (ATP-adenylyl sulphate 3'-phosphotransferase, EC 2.7.1.25), have now been examined separately (Pasternak, Crichton, Ellis, Jones-Mortimer & Wheldrake, 1964) to determine whether one or both are repressed.

In some strict anaerobes such as *Desulphovibrio desulphuricans* the reductive pathway from sulphate to sulphide is not merely used for the production of cysteine but is the means by which the organism oxidizes carbon substrates to obtain energy for growth (Postgate, 1959). Repression of the enzyme forming active sulphate, which in this case is APS, not PAPS (Peck, 1959, 1962), would therefore limit growth. To test whether the synthesis of ATP sulphurylase is accordingly insensitive to repression, the enzyme has been assayed in extracts of *D. desulphuricans* grown in the presence of added cysteine or sulphite.

A third class of organism cannot synthesize cysteine at all. *Leuconostoc mesenteroides*, for example, has been shown to lack the final enzyme (EC 4.2.1.22) in the biosynthetic sequence (Brüggemann, Schlossmann, Merckenschlager & Waldschmidt, 1962), but whether the other enzymes are also missing has not been determined. We have therefore measured ATP sulphurylase

and APS kinase, as well as PAPS reductase and sulphite reductase (EC 1.8.1.2), in *L. mesenteroides* grown on glutathione or cysteine.

MATERIALS AND METHODS

Chemicals. APS was prepared by the following method (Cherniak & Davidson, 1964). To 700 mg. of dry AMP (triethylammonium salt) were added 12 ml. of dioxan, 2 ml. of dimethylformamide and 1.1 ml. of pyridine, followed by 840 mg. of triethylamine-*N*-sulphonic acid, and the mixture was shaken overnight. Then 20 ml. of chloroform was added, the suspension extracted twice with 10 ml. of 0.05 *N*-ammonia and the pH of the combined extracts rapidly adjusted to 6.8. This solution (200 ml.) was chilled and adsorbed on a column (15 cm. × 3.5 cm. diam.) of Dowex 1 (X4; Cl⁻ form; 200–400 mesh), washed with water and eluted with a gradient achieved by allowing 2 l. of 2 *M*-LiCl to drip into a mixing vessel containing 3 l. of water at 2.5 ml./min. When 200 fractions (10 ml. each) had been collected on an LKB RadiRac fraction collector (Gallenkamp and Co. Ltd., London, E.C.2) at 3°, the reservoir was refilled with 4 *M*-NaCl and elution continued. The ultraviolet absorption of the effluent fractions was measured. The first peak (tubes 79–105) contained AMP and the second (tubes 250–285) APS. The pH of the combined APS fractions was adjusted to 6 with HCl and the solution adsorbed on a column (15 cm. × 2 cm. diam.) of acid-washed Nuchar C charcoal at 3°; the column was washed with water and eluted with 300 ml. of ethanol-aq. ammonia (sp. gr. 0.88)-water (20:1:20, by vol.). The eluate was concentrated to 100 ml. by rotary evaporation below 20° and freeze-dried. The yield (in terms of the AMP salt) was 40%. [³⁵S]APS was prepared by incubating 250 μmoles of ATP, 50 μmoles of Na₂³⁵SO₄ (2 mc), 100 μmoles of MgCl₂, 30 μmoles of EDTA, 5 m-moles of tris-HCl buffer, pH 8.5, and the supernatant fraction of an extract of *D. desulphuricans* (100–300 mg. of protein) or *E. coli* mutant 12C (50 mg. of protein) in a final volume of 50 ml. for 30 min. The reaction was stopped by heating at 100° for 1 min., protein removed by centrifugation and the chilled supernatant diluted to

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

100 ml. and the pH adjusted to 6 with HCl. The solution was adsorbed on a column (10 cm. $\times$ 1.5 cm. diam.) of acid-washed Nuchar C charcoal at 3°; the column was washed with 50 ml. of 0.1 M- Na_2SO_4 followed by water until the washings were free of radioactivity, eluted with 100 ml. of ethanol-aq. ammonia (sp. gr. 0.88)-water (20:1:20, by vol.) and the eluate concentrated to 5 ml. by rotary evaporation below 20°. The yield was 0.05–0.5 μmole . It was noticed during this work that neutral solutions of [^{35}S]APS, unlike [^{35}S]PAPS, are appreciably hydrolysed at temperatures above 0°. Reaction mixtures containing [^{35}S]APS were analysed immediately after incubation.

Organisms and conditions of growth. *E. coli* A.T.C.C. 9723, *E. coli* K₁₂ 701 and *B. subtilis* N.C.T.C. 1379 were grown as described by Pasternak, Ellis, Jones-Mortimer & Crichton (1965). *B. subtilis* N.C.T.C. 3610, obtained from the Chemical Microbiology Unit, University of Oxford, was grown in 1.3% (w/v) Oxoid nutrient broth CM1 or in the minimal medium of Pasternak (1962), each containing glucose (0.5%). *D. desulphuricans* (N.C.I.B. 8303), obtained from the Torry Research Station, Aberdeen, was maintained in stab culture as described by Starkey (1938). Cultures were grown statically for 3–4 days at 37° in medium C of Butlin, Adams & Thomas (1949), modified by the omission of Na_2SO_4 and the substitution of MgSO_4 by 0.5 g. of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ /l.; Na_2S was added before inoculation (Bornstein & Barker, 1948). *L. mesenteroides* P 60 (A.T.C.C. 7881), obtained from the Chemical Microbiology Unit, was grown statically overnight at 37° in the medium of Gibson & Woods (1960), except that cysteine was replaced by glutathione in some experiments. Mutants of *E. coli* 9723 and *E. coli* K₁₂, obtained by ultraviolet irradiation, were enriched by the penicillin technique (Davis, 1948) followed by replication from cystine-containing to sulphate-containing agar plates. Mutants, characterized as described by Ellis, Humphries & Pasternak (1964), were maintained on slopes of minimal medium containing cystine, and were grown in minimal medium (Pasternak, 1962) containing Difco yeast extract (0.004%). Bacteria were harvested and extracted as described by Ellis *et al.* (1964) and the supernatant fraction was used.

Enzyme assays. ATP sulphurylase could not be assayed satisfactorily. Molybdolysis (Wilson & Bandurski, 1958) gave spurious results in that *E. coli* 4–2 grown on glutathione and *E. coli* 9723 grown on 0.42 mM-L-cystine appeared to have up to 60% of the activity of *E. coli* 9723 grown on glutathione. However, when molybdate was added to extracts after incubation, the same amount of inorganic phosphate (60% of the glutathione-grown wild-type) was released. Further experiments showed that molybdate displaces inorganic phosphate adsorbed to proteins. The best assay found for ATP sulphurylase consisted in adding CuCl_2 (1.25 mM) to the incubation mixture of Pasternak (1962). Cu^{2+} inhibits the metabolism of APS (cf. Balasubramanian & Bachhawat, 1962), which therefore accumulates. However, ATP sulphurylase is also slightly inhibited (0.25 μmole of APS and 78 μmoles of PAPS formed in the absence of Cu^{2+} ; 22.2 μmoles of APS and 0.4 μmole of PAPS formed in its presence). The incubation mixture (Pasternak, 1962) without Cu^{2+} was used to assay ATP sulphurylase in *E. coli* 12C and *D. desulphuricans*, which lack APS kinase. APS kinase was assayed by incubating 5 μmoles of ATP, 5 μmoles of MgCl_2 , 0.5 μmole of [^{35}S]APS (0.01 μCi), 25 μmoles of tris-HCl buffer, pH 8.5, and enzyme extract in 0.45 ml. for

30 min. at 37°. The reaction was stopped by heating at 100° for 2 min., protein removed by centrifugation and a sample (0.05–0.1 ml.) of the supernatant assayed by paper electrophoresis ([^{35}S]APS, [^{35}S]PAPS and $^{35}\text{SO}_4^{2-}$ are well separated by 15 v/cm. for 2½ hr. in 0.1 M-ammonium acetate buffer, pH 5.6) and the radioactivity on the dried strips measured in a Radioactive Chromatogram Counter (Baird and Tatlock Ltd., Chadwell Heath, Essex) or a type 2029 chromatogram scanner operated automatically on stepwise drive (Isotope Developments Ltd., Beenham, Berks.).

Alternatively, unlabelled APS (1 μmole) was incubated with 7.5 μmoles of ATP, 7.5 μmoles of MgCl_2 , 200 μmoles of tris-HCl buffer, pH 8.5, and enzyme extract (3–4 mg. of protein) in a final volume of 2 ml. for 15 min. at 37°. The reaction was stopped by heating at 100° for 2 min., protein removed by centrifugation and a sample (0.1 ml.) of the supernatant analysed by paper electrophoresis. The area corresponding to PAPS was cut out, eluted with 1 M-ammonia and the extinction at 257 m μ measured. The formation of PAPS from sulphate and ATP was measured as described by Pasternak (1962). Protein was estimated by the method of Gornall, Bardawill & David (1957), with bovine plasma albumin as standard.

RESULTS AND DISCUSSION

Control of sulphate activation in *E. coli*. Two sulphiteless mutants proved useful in these studies. Mutant 4–2, derived from *E. coli* 9723, was found to lack ATP sulphurylase, and mutant 12C, derived from *E. coli* K₁₂, was devoid of APS kinase (Table 1). All other enzymes of the biosynthetic pathway from sulphate to cysteine (Pasternak *et al.* 1965) were intact in these mutants. By mixing extracts of the mutants grown on glutathione with an extract of *E. coli* 9723 or *E. coli* K₁₂ grown on cystine, it was shown that both the enzymes concerned with sulphate activation are repressed by cystine (Table 2). Since all *E. coli* extracts contain enzymes degrading APS and PAPS, mixing an active with an inactive extract decreases the expected yield. Similar results have been obtained by Monty (1964, and personal communication) with the sulphate-activating enzymes of *Salmonella typhimurium*. The two enzymes of *E. coli* 9723 were also assayed separately, as shown in Table 3. APS kinase appears to have a higher specific activity in crude extracts than has the overall conversion of sulphate into PAPS. This suggests that the first enzyme, ATP sulphurylase, is rate-limiting. Confirmation of this was obtained by adding an extract of mutant 12C grown on glutathione to extracts of *E. coli* 9723 grown on glutathione, sulphate, sulphite or a low concentration of cystine. In each case the specific activity of the overall conversion of sulphate into PAPS was increased to that of APS kinase. Table 3 shows that, within the limits of experimental error, the specific activity of ATP sulphurylase bears a constant ratio to that of APS kinase. Hence the two enzymes,

Table 1. *Characterization of two sulphiteless mutants of E. coli*

Extracts of the mutants grown (0.1–0.4 mg. dry wt. of cells/ml.) on glucose (0.1%) plus glutathione (0.21 mM) or L-cystine (0.42 mM) were incubated with 3 μ moles of ATP, 0.3 μ mole of Na₂³⁵SO₄ (10 μ C), 3.75 μ moles of MgCl₂ and 25 μ moles of tris-HCl buffer, pH 8.8, in 0.4 ml. for 15 min. and the products analysed by paper electrophoresis.

Mutant	S source for growth	Amount of extract added (mg. of protein)	Yield of APS (m μ moles)	Yield of PAPS (m μ moles)
<i>E. coli</i> 4-2	Glutathione	1.2	< 0.15	< 0.15
<i>E. coli</i> 4-2	L-Cystine	1.6	< 0.15	< 0.15
<i>E. coli</i> 12C	Glutathione	1.0	11.4	< 0.15
<i>E. coli</i> 12C	L-Cystine	2.6	< 0.15	< 0.15
<i>E. coli</i> 4-2	Glutathione	1.2	< 0.15	5.25
<i>E. coli</i> 12C	Glutathione	1.0		
<i>E. coli</i> 4-2	L-Cystine	1.6	4.5	< 0.15
<i>E. coli</i> 12C	Glutathione	1.0		
<i>E. coli</i> 4-2	Glutathione	1.2	< 0.15	< 0.15
<i>E. coli</i> 12C	L-Cystine	2.6		

Table 2. *Repression of sulphate-activating enzymes in E. coli 9723 and K₁₂*

Extracts of *E. coli* 9723 or K₁₂ grown on glucose (0.067%) plus glutathione (0.21 mM) or L-cystine (0.42 mM) were harvested (0.2–0.4 mg. dry wt. of cells/ml.) and assayed as described in Table 1. An extract of *E. coli* 4-2 or 12C (each 1.1 mg. of protein) grown on glutathione (0.21 mM) was added as indicated.

Organism	S source for growth	Amount of wild-type extract added (mg. of protein)	Mutant extract added	Yield of APS (m μ moles)	Yield of PAPS (m μ moles)
<i>E. coli</i> 9723	Glutathione	1.4	—	< 0.15	98.4
<i>E. coli</i> 9723	L-Cystine	1.7	—	< 0.15	< 0.15
<i>E. coli</i> 9723	L-Cystine	1.7	<i>E. coli</i> 4-2	< 0.15	< 0.15
<i>E. coli</i> 9723	L-Cystine	1.7	<i>E. coli</i> 12C	6.0	< 0.15
<i>E. coli</i> K ₁₂	Glutathione	0.6	—	< 0.15	4.3
<i>E. coli</i> K ₁₂	L-Cystine	0.55	—	< 0.15	< 0.15
<i>E. coli</i> K ₁₂	L-Cystine	0.55	<i>E. coli</i> 4-2	< 0.15	< 0.15
<i>E. coli</i> K ₁₂	L-Cystine	0.55	<i>E. coli</i> 12C	3.0	< 0.15

unlike the subsequent ones involved in sulphate reduction (Pasternak *et al.* 1965), may be repressed in a co-ordinate manner (Ames & Garry, 1959).

Control of sulphate activation in B. subtilis. It is probable that ATP sulphurylase and APS kinase are each repressed by growth on cystine (Table 4). The reason for the lower activity of sulphate-grown *B. subtilis* 1379 compared with that reported by Pasternak (1962) may be due to different compounds present in the current batch of broth. The composition of broth may also be the cause of the repressive effect of glutathione, which does not occur in *E. coli* growing in minimal medium. *B. subtilis* 3610, which grows well on minimal medium, was therefore investigated. Table 5 shows that in this strain glutathione represses to a variable extent in broth and actually de-represses in minimal medium. This result is compatible with the suggestion (Pasternak, 1962) that glutathione represses in *B. subtilis* 1379

because it is converted into cysteine faster than in *E. coli*.

Control of sulphate activation in D. desulphuricans and L. mesenteroides. Growth of *D. desulphuricans* in the presence of cysteine had little significant effect on ATP sulphurylase (Table 6). Sulphite, which theoretically might repress since the organism can be grown on it instead of sulphate (Table 6), was without effect.

It is generally accepted that organisms which require preformed amino acids for growth lack the necessary enzymes for their synthesis, though this is not always easy to demonstrate since most amino acids repress their own synthesis (e.g. Pardee, 1962). In cysteine biosynthesis it is possible to investigate this point with an organism whose cysteine requirement can be met by glutathione, which in minimal medium does not repress the enzymes of sulphate reduction (e.g. Tables 1, 2, 3 and 5; and Pasternak

Table 3. *Concentrations of ATP sulphurylase and APS kinase in E. coli 9723*

Extracts of *E. coli* grown (0.16–0.46 mg. dry wt. of cells/ml.) on glucose (0.075%) and the compounds indicated were assayed as described in the Materials and Methods section. ATP sulphurylase was measured in the presence of CuCl_2 (1.25 mM). The values quoted in the first row are the means of four or more experiments.

S source for growth	Specific activity (m μ moles/mg. of protein/min.)		
	ATP sulphurylase	APS kinase	$\text{SO}_4^{2-} \rightarrow \text{PAPS}$
Glutathione (0.21 mM)	2.9	11.3	5.5
Glutathione (0.21 mM)	Relative activity		
	(100)	(100)	(100)
Na_2SO_4 (4.2 mM)	26	25	26
Na_2SO_3 (4.2 mM)	4	4	2
L-Cystine (0.042 mM)	4	10	5
L-Cystine (0.046 mM)	2	5	2
L-Cystine (0.052 mM)	0.6	0.4	0.6
L-Cystine (0.056 mM)	< 0.2	< 0.2	< 0.1
L-Cystine (0.42 mM)	< 0.2	< 0.2; < 0.4*	< 0.1

* Assayed spectrophotometrically with unlabelled APS.

Table 4. *Repression of sulphate-activating enzymes in B. subtilis 1379*

Extracts of *B. subtilis* grown in broth containing glucose (0.5%) and the compounds indicated were harvested (0.16–0.25 mg. dry wt. of cells/ml.) and assayed as described in Table 1. An extract of *E. coli* 4–2 (1.0 mg. of protein) or *E. coli* 12C (1.1 mg. of protein) was added as indicated.

S compound added to growth medium	Amount of wild-type extract added (mg. of protein)	Mutant extract added	Yield of APS (m μ moles)	Yield of PAPS (m μ moles)
Na_2SO_4 (0.85 mM)	2.45	—	< 0.05	2.09
Glutathione (0.85 mM)	2.6	—	< 0.05	0.28
L-Cystine (0.42 mM)	2.05	—	< 0.05	0.06
L-Cystine (0.42 mM)	2.05	<i>E. coli</i> 4–2	< 0.05	< 0.05
L-Cystine (0.42 mM)	2.05	<i>E. coli</i> 12C	2.38	0.06

Table 5. *Sulphate activation in B. subtilis 3610*

Cultures of *B. subtilis* were grown in broth or minimal medium as described by Pasternak (1962), harvested (0.1–0.8 mg. dry wt. of cells/ml.) and assayed for ability to synthesize PAPS. Different batches of broth were used for Expts. 1 and 2. The values quoted for minimal medium are the means of two experiments.

Growth medium	S compound added to growth medium	$\text{SO}_4^{2-} \rightarrow \text{PAPS}$ specific activity (m μ moles/min./mg. of protein)	
		Expt. 1	Expt. 2
Broth	Na_2SO_4 (0.85 mM)	0.09	1.12
Broth	Glutathione (0.85 mM)	0.07	0.47
Broth	L-Cystine (0.42 mM)	0.14	0.18
Minimal	Na_2SO_4 (0.85 mM)	2.28	
Minimal	Glutathione (0.85 mM)	9.38	
Minimal	L-Cystine (0.42 mM)	0.32	

et al. 1965). Such an organism is *L. mesenteroides*, and the sulphate-activating enzymes, PAPS reductase, sulphite reductase and serine sulphhydrase were accordingly assayed (Pasternak *et al.* 1965) in

extracts of cysteine-grown and glutathione-grown cultures. None of the enzymes could be detected, though other enzymes [cysteine desulphhydrase (EC 4.4.1.1) and glucose 6-phosphate dehydrogenase

Table 6. *Concentration of ATP sulphurylase in D. desulphuricans*

Extracts of *D. desulphuricans* grown in the presence of the compounds indicated were assayed as described in the Materials and Methods section. The values quoted are the means of two experiments.

S compounds added to growth medium	Extent of growth (mg. dry wt. of cells/ml.)	Specific activity (m μ moles/mg. of protein/min.)
Na ₂ SO ₄ (21 mm)	0.23	0.67
Na ₂ SO ₃ (24 mm)	0.11	0.92
Na ₂ SO ₄ (10.5 mm) + Na ₂ SO ₃ (12 mm)	0.11	0.79
Na ₂ SO ₄ (21 mm) + L-cysteine (12 mm)	0.03	0.34

(EC 1.1.1.49)] were present. The possibility that glutathione breaks down rapidly enough to yield an intracellular concentration of cysteine which represses cannot, however, be discounted.

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