The Oxidation of Cyst(e)ine by Mast-Cell Tumour P815 in Culture

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1. Mast-cell tumour P815 cells oxidize [^{35}S]cyst(e)ine to $^{35}\text{SO}_4^{2-}$. 2. Addition of cysteinesulphinatate or sulphite decreases the formation of $^{35}\text{SO}_4^{2-}$; at the same time [^{35}S]cysteinesulphinatate or $^{35}\text{SO}_3^{2-}$ accumulates. 3. Extracts of P815 cells form sulphite from cysteinesulphinatate and 2-oxoglutarate. The K_m for cysteinesulphinatate is 6.35 mM and that for 2-oxoglutarate is 0.165 mM. 4. Extracts oxidize sulphite to sulphate. 5. No formation of hydrogen sulphide from cyst(e)ine was detectable. 6. It is concluded that P815 cells oxidize cyst(e)ine to sulphate solely via cysteinesulphinatate and sulphite. 7. The concentration of the enzymes catalysing this sequence is unaltered by several variations in the conditions of growth.

Mammals oxidize cyst(e)ine to sulphate (Hele & Pirie, 1931; Lawrie, 1932; du Vigneaud, Craft & Loring, 1934); the possible routes (Meister, 1965) fall into two categories (Scheme 1). In the first (a) the sulphur atom is oxidized, probably as far as cysteinesulphinatate (β -sulphinyllalanine), while attached to the carbon skeleton. In the second (b) hydrogen sulphide is eliminated and oxidized, probably via thiosulphate.

Pirie (1934) observed the formation of sulphate from cysteine by rat liver slices and suggested that the pathway might be via cysteinesulphinatate and sulphite. This has become generally accepted (cf. Young & Maw, 1958), although Wainer (1964) and Ewetz & Sörbo (1966) have produced evidence to the contrary. The sulphur metabolism of a homogeneous cell population might be simpler than that of liver in which side reactions such as those leading to taurine (Blaschko, 1942) occur. We have studied the pathway of cysteine oxidation in cultured P815 cells and found it to be entirely by route (a). A preliminary account of the work has been presented (Wheldrake & Pasternak, 1967).

MATERIALS AND METHODS

Chemicals. [^{35}S]Cystine (30–150 $\mu\text{C}/\mu\text{mole}$) was obtained from either The Radiochemical Centre (Amersham, Bucks.) or Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). Its purity was checked by chromatography in solvent (2) and by chromatography as the *N*-ethylmaleimide–cysteine adduct (formed by incubation with excess of non-radioactive cysteine followed by addition of excess of *N*-ethylmaleimide) in solvents (1) and (2) (for details of the solvents see below). The lowest purity found was 95–96% (in a

batch from The Radiochemical Centre, after 3 months' storage as the solid at -20°). Most of the [^{35}S]cystine was obtained from Schwarz BioResearch Inc. and was found to be >99% pure, as claimed by the manufacturers. $\text{Na}_2^{35}\text{SO}_3$ was obtained from The Radiochemical Centre, GSH, cysteic acid, ATP, taurine, cortisone acetate, hydrocortisone acetate, thyroxine and tris were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.), NAD, NADH_2 and cysteinesulphinatate from Calbiochem (Los Angeles, Calif., U.S.A.), 2-oxoglutarate, L-aspartate–2-oxoglutarate aminotransferase ('glutamate–oxaloacetate transaminase'), lactate dehydrogenase, sodium pyruvate, NADH_2 , NADPH_2 and oxaloacetic acid from C. F. Boehringer Corp. Ltd. (London, W. 5) and cystamine, cycloheximide and *N*-ethylmaleimide from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Horse serum no. 5 was purchased from Wellcome Research Laboratories (Beckenham, Kent) and each batch was tested for its ability to support optimum growth of the cultured cells before use. Benzylpenicillin and streptomycin sulphate were obtained from Glaxo Research Laboratories Ltd. (Greenford, Middx.). Streptomycin sulphate was converted into its chloride by passing a solution of 2.5 g. of streptomycin sulphate in 50 ml. of water through a column (32 cm. long $\times$ 1.5 cm. diam.) of Dowex 1 (X4; Cl^- form) and collecting the eluate. The column was washed with 100 ml. of water and this wash combined with the original eluate. The solution was free of sulphate. All other chemicals were purchased from British Drug Houses Ltd. (Poole, Dorset).

Cell culture. The medium was that described by Fischer & Sartorelli (1964) except that streptomycin was present as the chloride (see above). The medium was made up in 20 l. batches, sterilized by filtration through a microporous porcelain filter candle (Selas Flowtronics) of 0.3 μ absolute retention size and stored in sterile screw-capped bottles at 3° . Heat-labile compounds were sterilized by filtration through Oxoid membrane filters (0.45 μ pore size). Water-insoluble compounds were added in ethanol or dimethyl sulphoxide. Up to 0.5% of these solvents did not affect cell growth. P815 mouse mast-cell tumour (Dunn & Potter, 1957) strain Y was given by Dr G. A. Fischer and

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Scheme 1. Oxidation of cyst(e)ine to sulphate (solid arrows denote accepted reactions).

cultured (Schindler, Day & Fischer, 1959) in screw-capped tubes or, for larger cultures, in 250 ml. or 1 l. screw-capped Erlenmeyer flasks (James A. Jobling Ltd., Sunderland, Co. Durham). Stock cells were transferred weekly by using a 1:10 inoculum.

Preparation of cell extracts. Cells were harvested by centrifugation at 3000 rev./min. in an MSE Mistral 6L centrifuge, with a 6 × 250 ml. angle head, and the medium was removed as completely as possible. The pellet was resuspended in 3–4 vol. of 0.25 M-sucrose–1 mM-sodium phosphate buffer, pH 7.5, and homogenized in a cooled close-fitting glass homogenizer. High-speed supernatant was prepared by centrifuging this homogenate (usually diluted with 3 vol. of 0.25 M-sucrose–1 mM-sodium phosphate, pH 7.5) at 105 000g for 60 min. in a Spinco model L ultracentrifuge.

Mouse liver homogenate. DBA mice (obtained from K. Plant, Shenstone Animal Farm, St Albans, Herts.) were killed and the livers removed and chilled in ice. Livers were homogenized in 4 vol. of 0.25 M-sucrose–1 mM-sodium phosphate buffer, pH 7.5.

Assay methods

Cystine to sulphate. [³⁵S]Cystine (90 μmoles, 2–4 μCi), 1 μmole of GSH, 25 μmoles of tris-HCl buffer, pH 8.5, 20 μmoles of pyridoxal phosphate and cell homogenate (1–4 mg. of protein) in a final volume of 0.4 ml. were incubated in a shaking water bath at 37° for 45–90 min. The reaction was stopped by adding 0.05 ml. of 50% (w/v) trichloroacetic acid, chilling and centrifuging. A sample (40–60 μl.) of the supernatant was analysed for ³⁵SO₄²⁻ formation by paper electrophoresis. The amount of

sulphate formed was calculated from the specific radioactivity of the [³⁵S]cystine.

Oxidation of SO₃²⁻. Na₂SO₃ (150 μmoles), 50 μmoles of sodium phosphate buffer, pH 7.5, and cell homogenate (1–4 mg. of protein) in a final volume of 0.4 ml. were incubated in a shaking water bath at 37°. The reaction was stopped and SO₃²⁻ estimated as described by Grant (1947).

Cysteinesulphinatate degradation. This can be assayed by measuring SO₃²⁻ formation ('colorimetric assay') or by measuring pyruvate formation coupled to lactate dehydrogenase (which is present in excess in the extract) and following NADH₂ disappearance at 340 mμ ('enzyme assay'). In the colorimetric assay, 10 μmoles of cysteinesulphinatate, 50 μmoles of tris-HCl buffer, pH 8.5, 5 μmoles of 2-oxoglutarate, 20 μmoles of pyridoxal phosphate and cell homogenate were incubated in a final volume of 1 ml. for 10 min. No precautions were taken to exclude air, since SO₃²⁻ oxidation is relatively slow. The reaction was stopped and SO₃²⁻ estimated as described by Grant (1947). In the enzyme assay, 1 μmole of 2-oxoglutarate, 50 μmoles of sodium phosphate buffer, pH 7.5, 20 μmoles of pyridoxal phosphate, 0.2 μmole of NADH₂ and cell high-speed supernatant (0.05–0.3 mg. of protein) in a final volume of 1 ml. were added to each of two 1 cm. cells at 37° in a Beckman DB spectrophotometer coupled to a Sargent S.R. recorder. The components were pre-warmed for 5 min. and the reaction was started by adding 10 μmoles of cysteinesulphinatate to one of the cells.

Sulphide formation. Sodium pyruvate (20 μmoles), 50 μmoles of sodium phosphate buffer, pH 7.5, 25 μmoles of cysteine, 0.2 μmole of pyridoxal phosphate and mast-cell homogenate (4 mg. of protein) or mouse liver homogenate (8 mg. of protein) in a final volume of 2 ml. were incubated

in tubes sealed with rubber serum caps. The tubes had previously been gassed with 99.9% N₂ for 5 min. Sulphide formation was assayed by a modification of the method of Fogo & Popowsky (1949). A portion (2.5 ml.) of a 1% (w/v) solution of zinc acetate was injected through the serum cap, followed by 0.1 ml. of 3N-NaOH. The solution was thoroughly mixed and a few minutes later the cap was removed and 0.5 ml. of a solution containing 0.5 g. of *p*-aminodimethylaniline sulphate in 500 ml. of 5.5N-HCl was added. After the solution had been mixed and centrifuged, 1 ml. of 0.025M-FeCl₃ in 1.2N-HCl acid was added, and the solutions were mixed and recentrifuged. The extinction of the supernatant was read against a water blank at 675 mμ. This method eliminates the necessity of separating sulphide from other compounds by distillation. Cysteine, S₂O₃²⁻ and SO₃²⁻ do not give a colour under these conditions.

Cell number. A 25-fold diluted cell suspension was counted in a Coulter model A counter (Coulter Electronics Ltd.) with a 100μ orifice. The gain (*I*) was set at 5 and threshold (*T*) at 50. This gave a cell count within 10% of that obtained by direct counting on a haemocytometer.

Protein. This was determined by the biuret method of Gornall, Bardawill & David (1957) with crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) as standard.

Paper chromatography. This was carried out by using conventional descending techniques, normally on Whatman no. 3 paper. Solvents were: (1) ethanol-2-methylpropan-2-ol-aq. NH₃ (sp.gr. 0.88)-water (300:100:4:75, by vol.) (Margolis & Mandl, 1958); (2) 2-methylpropan-2-ol-formic acid (90%, w/v)-water (14:3:3, by vol.) (Margolis & Mandl, 1958); (3) butan-1-ol-acetic acid-water (12:3:5, by vol.) (Smith, 1958); (4) 2-methylpropionic acid-aq. NH₃ (sp.gr. 0.88)-water-0.05M-EDTA (144:9.5:90:5, by vol.) (Suzuki & Strominger, 1960). Amino acids were detected by spraying with 0.2% ninhydrin in ethanol. Radioactive compounds were located by radioautography for 1-6 weeks with Kodirex X-ray film and assayed as described below.

Paper electrophoresis. This was carried out on 2.5 cm. × 30 cm. strips of Whatman no. 3 paper in 0.1M-ammonium acetate, pH 5.6, at 13.5 v/cm. and 50 ma for 1½ hr. Under these conditions cystine moves approx. 1 cm., taurine 7 cm., cysteinesulphonate and cysteic acid 9 cm. and sulphate 18 cm. towards the anode. After electrophoresis the strips were dried and the distribution of radioactivity was determined as described below.

Radioactivity measurements. Radioactive areas on chromatograms were assayed with an end-window probe fitted to a Panax D657 Scaler (efficiency 1.3%). Electrophoresis strips were fed through an IDL automatic strip scanner on ½ in. stepwise drive (Isotope Developments Ltd.,

Table 1. Oxidation of cyst(e)ine to SO₄²⁻

The standard reaction mixture (see the Materials and Methods section), with 0.5 μmole of 2-oxoglutarate, was incubated for 60 min. except where noted otherwise, and ³⁵SO₄²⁻ formation was assayed as described.

Omissions (—) and additions (+)	Buffer	³⁵ SO ₄ ²⁻ formed (mμmoles/mg. of protein)
None*	Tris-HCl, pH 8.5	0.04
None	Tris-HCl, pH 8.5	0.88
None†	Tris-HCl, pH 8.5	1.38
Homogenate boiled	Tris-HCl, pH 8.5	0.07
None‡	Tris-HCl, pH 8.5	0.05
— GSH, — pyridoxal phosphate, — 2-oxoglutarate	Tris-HCl, pH 8.5	0.18
— GSH, — 2-oxoglutarate	Tris-HCl, pH 8.5	0.36
— GSH	Tris-HCl, pH 8.5	0.66
— 2-Oxoglutarate	Tris-HCl, pH 8.5	0.66
— 2-Oxoglutarate, + sodium pyruvate (1 μmole)	Tris-HCl, pH 8.5	0.36
— 2-Oxoglutarate, + sodium oxaloacetate (1 μmole)	Tris-HCl, pH 8.5	0.32
— 2-Oxoglutarate, — GSH, + GSSG (0.5 μmole)	Tris-HCl, pH 8.5	0.21
— 2-Oxoglutarate, — GSH, + β-mercaptoethanol (1 μmole)	Tris-HCl, pH 8.5	0.39
— 2-Oxoglutarate, — GSH, + NADH ₂ (0.1 μmole)	Tris-HCl, pH 8.5	0.23
— 2-Oxoglutarate, — GSH, + NAD (0.1 μmole)	Tris-HCl, pH 8.5	0.33
— 2-Oxoglutarate, — GSH, + NADPH ₂ (0.1 μmole)	Tris-HCl, pH 8.5	0.19
+ Fe ²⁺ (0.1 μmole)	Tris-HCl, pH 8.5	0.83
+ NADPH ₂ (0.1 μmole), + Fe ²⁺ (0.1 μmole)	Tris-HCl, pH 8.5	0.76
+ <i>N</i> -Ethylmaleimide (10 μmoles)	Tris-HCl, pH 8.5	0.06
None	Sodium phosphate, pH 6.0	0.17
None	Sodium phosphate, pH 7.0	0.29
None	Sodium phosphate, pH 7.5	0.50
None	Tris-HCl, pH 8.0	0.84
None	Tris-HCl, pH 9.0	0.29

* Not incubated.

† Incubated in a sealed tube with air replaced by 99.9% nitrogen.

‡ Incubation time 120 min.

Table 2. *Effect of possible intermediates on the formation of $^{35}\text{SO}_4^{2-}$ from [^{35}S]cystine*

The standard reaction mixture (see the Materials and Methods section) was incubated with 0.5 μmole of 2-oxoglutarate for 1 hr. and $^{35}\text{SO}_4^{2-}$ formation assayed as described.

Additions	Omissions	$^{35}\text{SO}_4^{2-}$ formed ($\mu\text{moles/mg. of protein}$)
None	None	1.03
None	2-Oxoglutarate	0.79
None	2-Oxoglutarate	0.85†
Na_2S (5 μmoles)	None	0.62*
Na_2SO_3 (5 μmoles)	None	0.25*
$\text{Na}_2\text{S}_2\text{O}_3$ (5 μmoles)	None	0.83*
Cysteinesulphinate (5 μmoles)	2-Oxoglutarate	0.05
Cysteinesulphinate (5 μmoles)	2-Oxoglutarate	0.28†
Cysteate (5 μmoles)	2-Oxoglutarate	0.40
Taurine (5 μmoles)	2-Oxoglutarate	0.52
Cystamine (5 μmoles)	2-Oxoglutarate	0.06
Cystine disulphoxide (5 μmoles)	2-Oxoglutarate	0.64

* 10 μmoles of *N*-ethylmaleimide in 0.1 ml. of ethanol were added immediately after the reaction was stopped.

† The reaction was stopped by boiling for 2 min., then 10 μmoles of 2-oxoglutarate and 3–6 units of L-aspartate–2-oxoglutarate aminotransferase were added to a 0.2 ml. sample, and the mixture was incubated again at 37° for 30 min.

Table 3. *Accumulation of radioactivity as [^{35}S]cysteinesulphinate*

The standard reaction mixture (see the Materials and Methods section) was incubated for 1–2 hr. and $^{35}\text{SO}_4^{2-}$ formation assayed as described. [^{35}S]Cysteinesulphinate and [^{35}S]cysteate were assayed by subjecting 0.1 ml. samples to chromatography [solvent (2) for 60 hr., followed by solvent (4) for 24 hr.] with unlabelled markers (1–2 μmoles), followed by radioautography. The radioactivity of [^{35}S]cysteinesulphinate coincided with the marker but that of [^{35}S]cysteate did not. N.D., Not determined.

Additions (+) and omissions (–)	Yields ($\mu\text{moles/mg. of protein/hr.}$)		
	$^{35}\text{SO}_4^{2-}$	[^{35}S]Cysteine-sulphinate	[^{35}S]Cysteate
None	1.09	0.32	< 0.54
+ Cysteinesulphinate (5 μmoles)	0.08	1.27	< 0.51
+ Cysteate (5 μmoles)	0.58	0.33	< 0.35
+ Hydroxylamine (1.25 μmoles), – pyridoxal phosphate	0.50	1.22	N.D.

Table 4. *Accumulation of radioactivity as $^{35}\text{SO}_3^{2-}$*

The standard reaction mixture (see the Materials and Methods section) with 0.5 μmole of 2-oxoglutarate was incubated for 1 hr. *N*-Ethylmaleimide (10 μmoles in 0.1 ml. of ethanol) was added immediately after the reaction had been stopped, and $^{35}\text{SO}_4^{2-}$ was assayed as described. The *N*-ethylmaleimide adducts of $^{35}\text{SO}_3^{2-}$ and [^{35}S]sulphide were assayed by subjecting 0.1 ml. samples to two-dimensional chromatography [solvent (1) for 16 hr. followed by solvent (2) for 16 hr.], followed by radioautography.

Additions	Yield ($\mu\text{moles/mg. of protein}$)		
	$^{35}\text{SO}_4^{2-}$	<i>N</i> -Ethylmaleimide adduct of $^{35}\text{SO}_3^{2-}$	<i>N</i> -Ethylmaleimide adduct of [^{35}S]sulphide
None	1.03	0.41	< 0.08
Na_2SO_3 (5 μmoles)	0.25	0.99	< 0.08
Na_2S (5 μmoles)	0.62	1.26	< 0.08

Table 5. SO_3^{2-} formation from cysteinesulphinate

The standard reaction mixture for the colorimetric assay (see the Materials and Methods section) was used.

Additions (+) and omissions (-)	SO_3^{2-} formed ($\mu\text{moles/mg. of protein}$)
None	1.78, 0.08*
- 2-Oxoglutarate	0.11
- Cysteinesulphinate	0.08
- Pyridoxal phosphate	1.41
- Supernatant fraction	0.00
Supernatant fraction boiled	0.07
- Supernatant fraction, + cell homogenate (1-2mg. of protein)	1.24
+ Oxaloacetate (10 μmoles)	1.39
+ Pyruvate (10 μmoles)	1.67
+ Oxaloacetate (10 μmoles), - 2-oxoglutarate	1.74
+ Pyruvate (10 μmoles), - 2-oxoglutarate	0.23
+ Sulphate (10 μmoles)	1.78
+ Cysteate (10 μmoles)	1.64
+ Taurine (10 μmoles)	1.51
+ Thiosulphate (10 μmoles)	1.98
+ Hydroxylamine (2.5 μmoles), - pyridoxal phosphate	0.53
+ Alanine (10 μmoles)	1.64
+ Glutamate (10 μmoles)	1.46
+ Aspartate (10 μmoles)	0.44
+ Glutamate (10 μmoles), + oxaloacetate (10 μmoles), - 2-oxoglutarate	1.43
+ Aspartate (10 μmoles), + oxaloacetate (10 μmoles), - 2-oxoglutarate	0.66

* Zero time.

Table 6. Oxidation of SO_3^{2-} to SO_4^{2-}

The standard reaction mixture (see the Materials and Methods section) was incubated for the times shown and residual SO_3^{2-} estimated. The value was subtracted from that obtained with boiled homogenate to give the values shown. In the last row 1 μmole of $\text{Na}_2^{35}\text{SO}_3$ (0.6 μC) was incubated with the standard reaction mixture and 10 μmoles of *N*-ethylmaleimide in 0.1 ml. of ethanol were added before boiling for 2 min. [^{35}S]Sulphite oxidized was determined by measuring [^{35}S]sulphate electrophoretically. Boiling the homogenate at zero time gave the value shown in parenthesis.

Conditions	Time (min.)	SO_3^{2-} oxidized ($\text{m}\mu\text{moles/mg. of protein}$)
Sodium phosphate, pH 7.5	0	0
Sodium phosphate, pH 7.5	30	29
Sodium phosphate, pH 7.5	60	73
Sodium phosphate, pH 7.5	120	150
Sodium phosphate, pH 6.0	60	44
Sodium phosphate, pH 7.0	60	59
Tris-HCl, pH 7.5	60	55
Tris-HCl, pH 8.0	60	76
Tris-HCl, pH 9.0	60	76
Sodium phosphate, pH 7.5	60	(21), 118

Table 7. Sulphide formation by mouse liver and mast-cell homogenates

The standard reaction mixture (see the Materials and Methods section) was incubated for 30 min. For the results given in parentheses, 60 m μmoles of [^{35}S]cystine (3 μC), 1 μmole of GSH, 25 μmoles of tris-HCl buffer, pH 8.5, 10 m μmoles of pyridoxal phosphate and 1 μmole of 2-oxoglutarate in a final volume of 0.4 ml. were incubated for 30 min. under N_2 (as described in the Materials and Methods section). *N*-Ethylmaleimide (10 μmoles in 0.1 ml. of ethanol) was added and the mixture was boiled for 2 min. The *N*-ethylmaleimide adduct of [^{35}S]sulphide was assayed by subjecting a 0.1 ml. sample to chromatography in solvent (2) for 16 hr., followed by radioautography.

Homogenate	Sulphide formed ($\text{m}\mu\text{moles/mg. of protein/hr.}$)
Mast cell	< 8 (< 0.02)
Mast cell + boiled mouse liver	< 8
Mouse liver	65 (5.9)
Mouse liver + boiled mast cell	63

Beenham, Berks.; efficiency 6.9%). Radioactive solutions were evaporated on aluminium planchets and assayed in a low-background automatic sample-changing counter (Isotope Developments Ltd.; efficiency 5%). Self-absorption was negligible for the sample sizes used. A standard solution of sodium [^{14}C]carbonate (The Radiochemical Centre) was used to determine the efficiency of the counters.

RESULTS

Capacity of cells to oxidize cyst(e)ine to sulphate.

It was shown that $^{35}\text{SO}_4^{2-}$ was formed from [^{35}S]cystine by mast cells incubated for 6 hr. in medium containing salts only. The $^{35}\text{SO}_4^{2-}$ formed was separated by paper electrophoresis and identified by (i) co-precipitability with barium sulphate, (ii) co-electrophoresis with authentic $^{35}\text{SO}_4^{2-}$, (iii) co-chromatography with authentic $^{35}\text{SO}_4^{2-}$ in solvents (1), (2) and (3), and (iv) activation to form adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate when incubated with ATP, Mg^{2+} and *Escherichia coli* extract (Pasternak, 1962). Cell homogenates also oxidize [^{35}S]cystine to $^{35}\text{SO}_4^{2-}$. The optimum conditions are shown in Table 1. There is a requirement for pyridoxal phosphate, 2-oxoglutarate and GSH. The 2-oxoglutarate requirement is not satisfied by other ketonic acids such as pyruvate or oxaloacetate, nor is the GSH requirement satisfied by NADH_2 , NADPH_2 or mercaptoethanol. The optimum pH is 8.5. The absence of an effect of Fe^{2+} or NADPH_2 is of interest since Ewetz & Sörbo (1966) showed these compounds to be cofactors for cysteinesulphinate formation by rat liver supernatant.

Effect of possible intermediates. The effect of

Fig. 1. Lineweaver-Burk plots for cysteinesulphinate degradation. Initial rates were determined enzymically as described in the Materials and Methods section. (a) Reaction mixture contained 1mM-2-oxoglutarate. (b) Reaction mixture contained 10mM-cysteinesulphinate.

Fig. 2. Effect of pH on cysteinesulphinate degradation. The standard reaction mixture for the colorimetric assay of sulphite formation (see the Materials and Methods section) was used. $\circ$, Sodium phosphate buffer; $\square$, tris-HCl buffer; $\bullet$, sodium borate buffer.

adding various possible intermediates to the reaction mixture is shown in Table 2. Cysteinesulphinate, cystamine and, to a lesser extent, SO_3^{2-} lower the formation of $^{35}\text{SO}_4^{2-}$. When excess of non-radioactive cysteinesulphinate is present in the

incubation mixture, or when the degradation of cysteinesulphinate is prevented by the omission of pyridoxal phosphate and the addition of hydroxylamine, accumulation of radioactivity as [^{35}S]cysteinesulphinate occurs (Table 3). Similarly $^{35}\text{SO}_3^{2-}$ accumulates in the presence of excess of non-radioactive sulphite, but no [^{35}S]sulphide accumulation is found in the presence of excess of non-radioactive sulphide (Table 4). The accumulation of $^{35}\text{SO}_3^{2-}$ in the presence of non-radioactive sulphide is probably attributable to the oxidation of sulphide to sulphite. No accumulation of radioactivity as [^{35}S]cysteic acid occurs in the presence of excess of non-radioactive cysteic acid (Table 3), nor does radioactivity accumulate as [^{35}S]cystamine in either the presence or the absence of cystamine. In the presence of cystamine most of the radioactivity moves towards the cathode on electrophoresis and a new radioactive compound, moving just behind cystamine in solvent (2) and solvent (4), is formed. It seems likely that this is the product of a disulphide exchange, i.e. $\text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{S} \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{CO}_2\text{H}$, since it is also formed non-enzymically. No [^{35}S]taurine could be detected (cf. Green & Day, 1963).

Degradation of cysteinesulphinate by high-speed supernatant. The high-speed supernatant fraction has the capacity to degrade cysteinesulphinate to sulphite and pyruvate (Table 5). This activity requires 2-oxoglutarate and is stimulated by pyridoxal phosphate. Oxaloacetate but not pyruvate can be substituted for 2-oxoglutarate. There is no feedback inhibition by sulphate or pyruvate, but hydroxylamine and aspartate are inhibitors of this reaction, which is in accord with the view that the enzyme under study is actually L-aspartate-2-oxoglutarate aminotransferase (EC 2.6.1.1), with cysteinesulphinate acting as an analogue of aspartate (Singer & Kearney, 1956). The apparent K_m for this reaction is 6.35mM for cysteinesulphinate and 0.165mM for 2-oxoglutarate at 1mM-2-oxoglutarate and 10mM-cysteinesulphinate respectively (Figs. 1a and 1b). The pH-activity relationship for this enzyme is shown in Fig. 2.

Oxidation of SO_3^{2-} . The cell homogenate oxidizes SO_3^{2-} to SO_4^{2-} (Table 6). This activity is heat-labile (2min. in boiling water); the pH optimum in tris is between 8.0 and 9.0.

Sulphide formation from cysteine. No formation of sulphide by P815 cells was demonstrable. A liver extract from DBA mice (the strain from which the tumours were originally derived), on the other hand, was active (Table 7). Mixing experiments eliminated the possibility that the cells lack a cofactor or contain an inhibitor.

Control of cysteine oxidation. The ability to degrade cysteinesulphinate and to oxidize SO_3^{2-} was measured in cells grown under a variety of

Table 8. *Control of cystine oxidation*

Cells were grown as shown for 3–4 days. Final cell counts ($6-9 \times 10^5$ cells/ml.) were unaffected by the growth conditions except where indicated. Cysteinesulphinate degradation (the enzymic assay for pyruvate formation being used), SO_3^{2-} oxidation and oxidation of cystine to sulphate were measured as described in the Materials and Methods section. Values are the means of three experiments except where indicated in parentheses. S.E.M. of control values are shown. N.D., Not determined.

Growth conditions	Cysteinesulphinate degradation ($\mu\text{moles/mg. of protein/hr.}$)	SO_3^{2-} oxidation ($\text{m}\mu\text{moles/mg. of protein/hr.}$)	Overall oxidation of cystine to SO_4^{2-} ($\text{m}\mu\text{mole/mg. of protein/hr.}$)
Normal (0.083 mM-cystine)	16.25 ± 4.25 (10)	96.5 ± 24.5 (9)	0.812 ± 0.180 (13)
Cystine changed to 0.415 mM	14.8	105	0.92
Cystine changed to 0.0415 mM	11.6	99	0.81
Transferred to cystine-free medium*	9.0	71	0.27
Transferred to valine-free medium†	7.5	118	0.66
Cycloheximide ($10 \mu\text{g./ml.}$) added‡	1.1	6.6	0.22
Na_2SO_4 (10 mM) added	18.0 (2)	N.D.	0.71 (2)
Insulin ($50 \mu\text{g./ml.}$) added	12.9	102	0.75
Thyroxine ($10 \mu\text{M}$) added	13.4	114	0.90
Cortisone acetate ($10 \mu\text{M}$) added	8.5	141	0.83
Hydrocortisone acetate ($10 \mu\text{M}$) added	14.1	128	0.95

* Grown for 4 days in normal medium (0.083 mM-cystine) followed by 3 days in cystine-free medium. Cell counts declined from $5-7 \times 10^5$ cells/ml. to $3-5 \times 10^5$ cells/ml. during the final 3 days.

† Grown for 4 days in normal medium (0.61 mM-valine) followed by 3 days in valine-free medium. Cell counts increased from $4-6 \times 10^5$ cells/ml. to $7-9 \times 10^5$ cells/ml. during the final 3 days.

‡ Grown for 3 days in normal medium, followed by 3 days in the presence of cycloheximide. Cell counts increased from 8×10^5 cells/ml. to 1×10^6 cells/ml. during the final 3 days.

conditions chosen to reveal possible control mechanisms (Table 8). Lack of cystine appears to decrease all three activities. However, a similar decline occurs when another amino acid, valine, is omitted from, or cycloheximide added to, the medium. This suggests that the effect of cystine lack is not specific but reflects a general consequence of enzyme inactivation or degradation in cells that are not synthesizing protein. None of the hormones tested affected either growth rate or the concentration of the enzymes studied.

DISCUSSION

Cysteinesulphinate fulfils four criteria necessary to implicate it as an intermediate in cysteine oxidation by P815 mast cells. (i) Addition of cysteinesulphinate to a cell homogenate metabolizing [^{35}S]cystine to $^{35}\text{SO}_4^{2-}$ results in a decrease of radioactivity appearing in SO_4^{2-} (Table 2). (ii) At the same time [^{35}S]cysteinesulphinate accumulates (Table 3). (iii) Cysteinesulphinate is degraded to SO_3^{2-} (Table 5). (iv) Inhibition of this reaction (by hydroxylamine) leads to accumulation of cysteinesulphinate (Table 3). If cysteinesulphinate is an intermediate, then it is likely that SO_3^{2-} is one also (Scheme 1). This is confirmed by the fact that SO_3^{2-} satisfies the three criteria of (i) isotope dilution (Table 2), (ii) isotope accumulation

(Table 4) and (iii) further metabolism (Table 6). Of the other possible intermediates tested (Table 2), only cystamine and cysteic acid fulfil criterion (i). However, radioactivity from [^{35}S]cystine does not accumulate [criterion (ii)] in either compound (Results section and Table 3). Sulphide is clearly not an intermediate (Tables 2, 4 and 7) and hence pathway (b) (Scheme 1) is ruled out.

The absence of cysteine desulphhydrase (Table 7) from P815 cells may be compared with its distribution in the rat; it, or cystathionase, with which it is thought to be identical (Binkley & Okeson, 1950; Matsuo & Greenberg, 1959), is present in liver, kidney and pancreas (Greenstein, 1954; Mudd, Finkelstein, Irreverre & Laster, 1965), but low in intestinal mucosa and adipose tissue and absent in brain, spleen, adrenal, lung, testis, heart and skeletal muscle (Mudd *et al.* 1965). In the last-mentioned group of tissues, as in P815 cells, pathway (a) for the formation of SO_4^{2-} may be the only operative one.

Lack of cysteine desulphhydrase, and hence of cystathionase making cysteine from methionine, may explain the failure of P815 cells to grow on methionine as sole sulphur amino acid (Table 8). Since P815 cells are probably diploid, they fit into the generalization (Eagle, Washington & Friedman, 1966) that such cells, in contrast with polyploid ones, cannot grow in the absence of cystine.

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