Biosynthesis of Ethylene from Methionine

ISOLATION OF THE PUTATIVE INTERMEDIATE 4-METHYLTHIO-2-OXOBUTANOATE FROM CULTURE FLUIDS OF BACTERIA AND FUNGI

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Methods are described for identifying the 2,4-dinitrophenylhydrazones of 4-methylthio-2-oxobutanoate by means of t.l.c., n.m.r. and mass spectroscopy. By using these methods 4-methylthio-2-oxobutanoate, a putative intermediate in the biosynthesis of ethylene from methionine, has been identified in culture fluids of *Aeromonas hydrophila* B12E and a coryneform bacterium D7F grown in the presence of methionine. Relative to 4-methylthio-2-oxobutanoate, the yield of 3-(methylthio)propanal (methional) from the same cultures was less than 1%. Because 4-[²H]methylthio-2-oxobutanoate was obtained from cultures grown on [Me-²H]methionine, the 4-methylthio-2-oxobutanoate must be derived from methionine. By means of t.l.c. alone, 4-methylthio-2-oxobutanoate was identified in the culture fluids of a range of bacteria, the yeast *Saccharomyces cerevisiae* and the fungus *Penicillium digitatum*. A photochemical assay developed for 4-methylthio-2-oxobutanoate shows it to be a product of the metabolism of methionine by *Escherichia*, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Aeromonas*, *Rhizobium* and *Corynebacterium* species.

Methionine is known to be the precursor of ethylene in higher plants, some fungi and a number of aerobic heterotrophic bacteria (Lynch, 1972; Primrose, 1976a,b; Primrose & Dilworth, 1976; Yang, 1974), but there is disagreement concerning the metabolic pathway utilized. Two derivatives of methionine, 3-(methylthio)propanal (methional) and 4-methylthio-2-oxobutanoate, are possible biosynthetic intermediates, for they are readily converted into ethylene in model systems involving either peroxidase or flavin and light (Yang, 1967; Yang *et al.*, 1967). Bauer & Yang (1969) claimed that these two compounds were not biological precursors because methional was not converted into ethylene in apple tissues and the efficiency of the conversion of 4-methylthio-2-oxobutanoate into ethylene was less than that of methionine. Furthermore, a known antagonist of peroxidase, catalase, failed to inhibit the formation of ethylene from methionine. By contrast, Baker *et al.* (1977) found that 4-methylthio-2-oxobutanoate was more effective than methionine in stimulating ethylene production by both mature green and pink tomato slices. Production of [¹⁴C]ethylene from L-[¹⁴C_{3,4}]methionine by such tomato slices was strongly inhibited by 4-methylthio-2-oxobutanoate, suggesting that unlabelled 4-methylthio-2-oxobutanoate dilutes the radioactive 4-methylthio-2-oxobutanoate produced from [¹⁴C]methionine.

Support for a role for either 4-methylthio-2-oxobutanoate or methional in ethylene biosynthesis comes from work with micro-organisms. Cultures of *Mucor heimalis* and *Escherichia coli* grown in the presence of methionine accumulate a substance that is converted into ethylene, either photochemically in the presence of a flavin (Lynch, 1974; Primrose, 1976a, 1977) or enzymically by peroxidase (Primrose, 1977). Evidence favouring the identity of this substance as 4-methylthio-2-oxobutanoate, rather than methional, was provided by comparing the pH-dependence of the photochemical conversion of methional, 4-methylthio-2-oxobutanoate and the accumulating metabolite into ethylene (Primrose, 1977).

In the present paper we describe methods for identifying 4-methylthio-2-oxobutanoate and show that it is a common metabolic product of micro-organisms growing in the presence of methionine. The significance of this finding is discussed in relation to the biosynthesis of ethylene.

Materials and Methods

Dinitrophenylhydrazine reagent

This was a 0.4% (w/v) solution of 2,4-dinitrophenylhydrazine (twice recrystallized from AnalaR ethyl acetate) in aq. 2M-H₂SO₄.

Preparation of dinitrophenylhydrazones of 4-methylthio-2-oxobutanoate

Two methods were used for the preparation of a mixture of isomers of the dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate.

(a) Dinitrophenylhydrazone reagent (30ml) was added to 4-methylthio-2-oxobutanoate (45mg) in a minimum amount of water and the mixture was incubated at room temperature (23°C) for 30min. The precipitated dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate was collected by filtration and was washed with water and dried. This procedure gave the dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate (yield 56mg; approx. 1:1 mixture of isomers, m.p. 121–123°C).

(b) The second procedure was that of Cahill & Rudolph (1942). DL-Methionine was converted into its *N*-2-bromopropionyl derivative, which gave, on treatment with sodium acetate/acetic anhydride followed by acidic hydrolysis, 4-methylthio-2-oxobutanoate isolated as its 2,4-dinitrophenylhydrazone (m.p. 122–124°C; approx. 1:1 mixture of isomers).

T.l.c.

Dinitrophenylhydrazones were separated on Merck 5554 Kieselgel plates containing fluorescent indicator (BDH, Poole, Dorset, U.K.), with the following solvent systems: (1) dichloromethane/methanol (7:3, v/v); (2) propan-2-ol; (3) pentane/diethyl ether (2:1, v/v).

N.m.r.

Dinitrophenylhydrazone concentrations of 100 mg/ml of [²H]dimethyl sulfoxide were used.

Preparation of isomer (I) of the dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate

In one preparation of the dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate by the procedure of Cahill & Rudolph (1942) hot (approx. 70°C) dinitrophenylhydrazine reagent was added to a hot (approx. 70°C) solution of 4-methylthio-2-oxobutanoate. After cooling, the precipitate was collected and examined by t.l.c., which showed it to contain mainly isomer (I) (*R_F* 0.58 in solvent system 1, *R_F* 0.23 in solvent system 2). This product was purified further by chromatography on a column (27cm × 1.5cm) of silica gel (Fisons, Loughborough, Leics., U.K.) eluted with solvent system (1). This procedure yielded 45mg of isomer (I) as yellow crystals, m.p. 148.5–149°C [Cahill & Rudolph (1942) report an m.p. of 148–149°C].

Isolation of dinitrophenylhydrazones from culture fluids

Dinitrophenylhydrazine reagent (150ml) was added to the supernatant fluid obtained by low-speed

centrifugation of two cultures (each 500ml). After stirring the mixture for 30min at room temperature, the precipitates were isolated by low-speed centrifugation, followed by washing with water and drying.

Photochemical production of ethylene

Methionine, or a methionine analogue (20μmol), in 200mm-sodium acetate or potassium phosphate buffer (20ml) was sealed in a 250ml Erlenmeyer flask with a rubber serum cap (Subaseal; William Freeman, Barnsley, Yorks., U.K.). Riboflavin (50mm) in water (1ml) was injected through the serum cap and the flask was incubated with illumination in a shaking water bath at 30°C. Illumination was provided by either a 100W tungsten bulb placed at a distance of 30cm (about 120lx) or 3 fluorescent tubes placed at a distance of 18cm (about 420lx).

Assay of ethylene

Gas samples (1 ml) were withdrawn from the sealed flasks and analysed by using a Pye–Umicam series 104 chromatograph with a flame-ionization detector, a column (1.6m × 6mm) of Porapak R. at 85°C, and a nitrogen-carrier gas flow of 40 ml/min. For a standard, a known volume of pure ethylene was injected into an identical flask containing the same volume of sterile growth medium.

Chemicals

L-Methionine and its analogues were purchased from Sigma Biochemicals, Kingston upon Thames, Surrey, U.K. L-[Me-²H]Methionine was obtained from Prochem, Deer Park Road, London, U.K. and [³⁵S]methionine was from The Radiochemical Centre, Amersham, Bucks., U.K.

Isolation of the dinitrophenylhydrazones of 4-methyl-³⁵S-thio-2-oxobutanoate

Cultures were grown for 2 days at 30°C in the defined medium (20ml) containing methionine (500μg/ml, 67μmol) and [³⁵S]methionine (50μCi, sp. radioactivity 300Ci/mmol). After centrifugation of the cultures at low speed the supernatant fraction was removed and treated with unlabelled 4-methylthio-2-oxobutanoate (10mg, 68μmol) and dinitrophenylhydrazine reagent (10ml). After stirring for 10min at room temperature the precipitate was collected by centrifugation and washed with water and dried.

Radioautography

Kodak X-Omat H film was overlaid with the t.l.c. chromatogram and stored at room temperature in the dark for 5–7 days. The film was then developed in Kodak DX 80 and fixed with Kodak FX 40.

Micro-organisms

All bacteria and fungi used in the present study were from the culture collection maintained in the Department of Biological Sciences, University of Warwick.

Growth of micro-organisms

All organisms were grown in the defined medium of Brown & Dilworth (1975) supplemented with L-methionine (500 µg/ml). Cultures were incubated with shaking until the stationary phase of growth was reached (2 days at 30°C). To prevent photochemical degradation of 4-methylthio-2-oxobutanoate, light was excluded from all cultures.

Results

Characterization of the dinitrophenylhydrazones of 4-methylthio-2-oxobutanoate

Dinitrophenylhydrazones of 4-methylthio-2-oxobutanoate were prepared from commercial 4-methylthio-2-oxobutanoate and 4-methylthio-2-oxobutanoate synthesized by the method of Cahill & Rudolph (1942) by treatment with cold acidic dinitrophenylhydrazine reagent. In each case the precipitated dinitrophenylhydrazone was found to be an approximately 1:1 mixture of *E* and *Z* isomers (I and II respectively).

As with paper chromatography (Meister & Abendschein, 1956), t.l.c. of these precipitates

(I)

showed two spots with R_F values of 0.38 and 0.58 (with dichloromethane/methanol as solvent) or 0.07 and 0.23 (with propan-2-ol as solvent). Their ^1H n.m.r. spectra (Table 1) revealed singlets at δ (p.p.m.) 2.12 and 2.17 corresponding to the methylthio groups in the isomers (I) and (II) (cf. Karabatsos *et al.*, 1962, 1963). Additional features indicative of the presence of isomers were two NH signals [δ (p.p.m.) 11.1 and 11.5], overlapping quartets around δ (p.p.m.) 8.45 (H-5') and doublets at δ (p.p.m.) 8.02 and 8.08 (H-6'). ^1H n.m.r. spectroscopy showed that heating a solution of the approximate 1:1 isomeric mixture in $[\text{D}_2]\text{dimethyl sulphoxide}$ at 100°C for 30 min gave a mixture containing approx. 90% (I) and 10% (II). Evidently, under these conditions equilibration of the isomers takes place to give a mixture containing predominantly the more stable

isomer. By comparison with the isomeric dinitrophenylhydrazones of α -oxo acids (Isherwood & Jones, 1955; Stewart, 1953; Katsuki *et al.*, 1971, 1972), the intramolecular hydrogen-bonded structure (I) can be assigned to this isomer.

A pure sample of isomer (I) was obtained by treating 4-methylthio-2-oxobutanoate with hot acidic dinitrophenylhydrazine reagent to give an approx. 9:1 mixture of isomers (I) and (II) followed by chromatography on silica gel. The ^1H n.m.r. spectrum (Table 1) of pure isomer (I) showed a signal at δ (p.p.m.) 2.12 (CH_3S), but not δ (p.p.m.) 2.17, and a single NH resonance at δ (p.p.m.) 11.5. The i.r. spectrum of this isomer in dichloromethane showed an intense band at 1629 cm^{-1} assigned to an intramolecular H-bonded $\text{C}=\text{O}$ stretch, but no significant $\text{C}=\text{O}$ absorption at higher wave numbers. The mass spectra of the isomeric mixture of dinitrophenylhydrazones and purified isomer (I) were very similar, showing M^+ 328 and also fragments at m/e 281 and 237 amongst others. Metastable ions were observed in each spectrum at m/e 240.7 ($328 \rightarrow 281$, calc. m^* 240.7) and 200.0 ($281 \rightarrow 237$, calc. m^* 199.9).

Identification of 4-methylthio-2-oxobutanoate in growth media of strains B12E and D7F

The precipitates obtained after treating culture fluids of *Aeromonas hydrophila* B12E and the coryne-

(II)

form organism D7F with dinitrophenylhydrazine reagent were examined by t.l.c. and ^1H n.m.r., i.r. and mass spectroscopy. T.l.c. showed two spots corresponding in R_F values to the isomers of authentic dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate. The ^1H n.m.r. spectra were similar to the spectrum of a 1:1 isomeric mixture of authentic dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate. Heating the dinitrophenylhydrazone from a culture of D7F at 100°C for 30 min in $[\text{D}_2]\text{dimethyl sulphoxide}$ gave a new spectrum almost identical with the spectrum of equilibrated dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate. The i.r. spectra of culture fluid dinitrophenylhydrazones were similar to that of the 1:1 isomeric mixture of the dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate. Mass spectra of dinitrophenylhydrazones from

Table 1. ¹H n.m.r. data for the 2,4-dinitrophenylhydrazones of 4-methylthio-2-oxobutanoate derived from natural sources and authentic materials

Spectra were of solutions in [²H₆]dimethyl sulphoxide run at 90 MHz. The chemical shifts of aromatic protons are especially sensitive to the conditions.

Dinitrophenyl-hydrazone	Chemical shifts from tetramethylsilane (p.p.m.)					
	CH ₃ S	CH ₂ CH ₂	H-6'	H-5'	H-3'	NH
Synthetic 4-methylthio-2-oxobutanoate 2,4-dinitrophenylhydrazone (mixture of isomers I and II)*	2.12 (s), 2.17 (s)	2.82 (s)	8.01 (d), 8.08 (d)	8.40 (q), 8.50 (q)	8.86 (d)	11.09 (s), 11.50 (s)
Single isomer of 4-methylthio-2-oxobutanoate 2,4-dinitrophenylhydrazone (I)†	2.12 (s)	2.81 (s)	7.95 (d)	8.37 (q)	8.81 (d)	11.53 (s)
4-Methylthio-2-oxobutanoate 2,4-dinitrophenylhydrazone from coryneform D7F‡	2.12 (s), 2.17 (s)	2.83 (s)	8.03 (d), 8.09 (d)	8.39 (q), 8.48 (q)	8.86 (d)	11.10 (s), 11.49 (s)

* Synthesized by the method of Cahill & Rudolph (1942).

† Synthesized by a modification of the method of Cahill & Rudolph (1942).

‡ Material was obtained by addition of 2,4-dinitrophenylhydrazine reagent to culture fluids of coryneform D7F.

cultures of strains B12E and D7F showed all the main peaks, including metastable ions, in the spectrum of the dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate. Finally, the dinitrophenylhydrazone of 4-[²H]methylthio-2-oxobutanoate was obtained from the growth medium of strain B12E supplemented with L-[Me-²H]methionine. The structure of this product was confirmed by its mass spectrum, which showed *M*⁺ 331. There was no significant peak at *m/e* 330. The peak at *m/e* 328 in this spectrum (about 20% of the intensity of the peak at *m/e* 331) presumably pertains to the dinitrophenylhydrazone of unlabelled 4-methylthio-2-oxobutanoate derived from endogenous methionine. It is concluded that cultures of B12E and D7F produce 4-methylthio-2-oxobutanoate, the source of which is methionine.

Production of 4-methylthio-2-oxobutanoate by bacteria

T.l.c. of dinitrophenylhydrazone precipitates from culture fluids of *A. hydrophila* B12E and B19E and coryneform bacteria D7F and D19E showed two spots with *R_F* values corresponding to the two isomers of authentic 4-methylthio-2-oxobutanoate. Other bacteria failed to produce sufficient 4-methylthio-2-oxobutanoate to be detected by this technique. However, after growth on [³⁵S]methionine and treatment with dinitrophenylhydrazine reagent, the dinitrophenylhydrazone of 4-methyl[³⁵S]thio-2-oxobutanoate could be detected by radioautography of

thin-layer chromatograms. By this means 4-methylthio-2-oxobutanoate but not methional was shown to be a product of *Pseudomonas aeruginosa* PAT, *E. coli* C603, *E. coli* SPAO, *Saccharomyces cerevisiae* DD1 and *Penicillium digitatum*.

Attempts to detect methional, as its dinitrophenylhydrazone, utilized t.l.c. with solvent system 3. In a typical experiment the precipitated dinitrophenylhydrazone from culture fluids of strain B12E did not contain detectable methional dinitrophenylhydrazone. The filtered culture fluid was extracted with dichloromethane to give an estimated yield of methional relative to 4-methylthio-2-oxobutanoate of less than 1%. Detectable amounts of methional dinitrophenylhydrazone were not obtained from other 4-methylthio-2-oxobutanoate-producing cultures in which a greater than 1% yield would easily have been detected.

Development of an assay for 4-methylthio-2-oxobutanoate

Although a method is available for the determination of α-oxo acids (Ariga, 1971), it is not specific for 4-methylthio-2-oxobutanoate and in our hands was subject to interference by constituents of culture media. A better approach to the assay of 4-methylthio-2-oxobutanoate would be to convert it into ethylene and to quantify the ethylene produced. This conversion could be effected either enzymically by peroxidase (Yang, 1967) or photochemically in the

Fig. 1. Effect of pH and light source on the rate of flavin-mediated ethylene production from methionine and its derivatives. Production of ethylene was from: 4-methylthio-2-oxobutanoate, A; methional, B; methionine, C; methioninol, D; 2-hydroxy-4-(methylthio)butanoic acid, E. (a) Tungsten light, pH 3.5; (b) tungsten light, pH 8.0; (c) fluorescent light, pH 3.5; (d) fluorescent light, pH 8.0.

presence of a flavin (Yang *et al.*, 1967). There are disadvantages with both methods. The peroxidase Vol. 182

assay could be subject to interference by constituents of culture media, whereas methionine is

Table 2. *Optimum pH for the photochemical conversion of methionine and methionine analogues into ethylene*

The photochemical production of ethylene from methionine and analogues of methionine was measured over the pH range 3–10, as described in the Materials and Methods section.

Compound	Optimum pH for photochemical conversion
Methionine	9.0
4-Methylthio-2-oxobutanoate	3.5
Methional	6.0
2-Hydroxy-4-(methylthio)butanoate	3.5
N-Acetylmethionine	3.5
N-Formylmethionine	3.5
Homocysteine	9.0
2-Amino-4-(methylthio)butan-1-ol (methioninol)	9.0
Cysteine	No reaction
Norleucine	No reaction

Fig. 2. *Standard curve for the assay of 4-methylthio-2-oxobutanoate in the presence of excess methionine*

Symbols: ▲, ethylene produced from 4-methylthio-2-oxobutanoate; ○, ethylene produced from 4-methylthio-2-oxobutanoate in the presence of 40 μmol of methionine.

reactive in the photochemical assay. However, we have found that the reaction with methionine can be eliminated by careful choice of conditions.

The two most important factors for the photochemical conversion of methionine into ethylene are the pH value of the reaction mixture and the type of light source. Yang *et al.* (1967) reported that a pH value of 8–9 was optimal for the reaction and this is confirmed by the data in Table 2. In contrast the pH

optimum for 4-methylthio-2-oxobutanoate is 3.5 (Primrose, 1977) and the values for other methionine analogues are shown in Table 2. More significantly, the photochemical reaction with methionine only occurs if a fluorescent light source is used, whereas conversion of 4-methylthio-2-oxobutanoate can be effected by either a tungsten or a fluorescent light source. Fig. 1 shows the relative reaction rates of 4-methylthio-2-oxobutanoate and the various methionine analogues at pH values of 3.5 and 8 with both tungsten and fluorescent light. It is noteworthy that even at pH 8, 4-methylthio-2-oxobutanoate is still the most reactive of the methionine derivatives tested.

Fig. 2 illustrates a typical standard curve obtained when 4-methylthio-2-oxobutanoate in 10 mM-sodium acetate buffer (pH 3.5) was illuminated with tungsten light in the presence of 5 mM-riboflavin. Linearity was achieved with 4-methylthio-2-oxobutanoate concentrations up to 1 mM, which was the highest concentration tested. No change in the slope of the line was observed when the assay mixture was made

Table 3. *Ethylene and 4-methylthio-2-oxobutanoate production by various isolated bacteria*

Bacteria were inoculated into 250 ml Erlenmeyer flasks with 20 ml of defined medium containing methionine and 0.1%, 0.15%, 0.2%, 0.25%, 0.3% or 0.4% glucose. Cultures were incubated with shaking at 30°C for 2 days in the absence of light. Samples (1 ml) of the gas phase were assayed for ethylene as described in the Materials and Methods section. Samples (1 ml) of the liquid phase were assayed photochemically (pH 3.5, tungsten light) for 4-methylthio-2-oxobutanoate after removal of cells by centrifugation. Control cultures were incubated for the same time in the absence of methionine that was added before the assays for ethylene and 4-methylthio-2-oxobutanoate. The ranges shown are the minimum and maximum values found for different glucose concentrations.

Organism	Ethylene (nmol/mg of cell protein)	4-Methylthio-2-oxobutanoate (equivalents/mg of cell protein)
<i>E. coli</i> K12	1–26	33–864
<i>E. coli</i> SPAO	174–2000	936–1470
<i>E. coli</i> C	20–86	186–282
<i>Rhizobium trifolii</i> N2P7	1–22	750–1680
<i>Acinetobacter calcoaceticus</i>	2–49	17–582
<i>Pseudomonas aeruginosa</i>	0–4	42–900
<i>Pseudomonas pisi</i>	0–19	39–1200
<i>Bacillus mycoides</i>	90–1860	744–4200
<i>Aeromonas hydrophila</i>	15–1032	2400–5100
<i>Corynebacterium</i> D7F	1380–10800	6600–10680
<i>Aeromonas</i> B12E	210–7230	8280–9840
<i>Azotobacter</i> sp.	0–2	15–65
Control cultures	0–3	1–17

Fig. 3. Induction of ethylene and 4-methylthio-2-oxobutanoate formation by strain B12E

Ten 20ml cultures of strain B12E were grown overnight (17h) at 30°C in defined medium lacking methionine. The ten cultures were pooled and dispensed in 20ml portions in 250ml Erlenmeyer flasks. After addition of chloramphenicol as shown and methionine to final concentrations of 500 µg/ml where appropriate, the cultures were capped with Suba-seals and incubated with shaking at 30°C in the absence of light. Samples (1 ml) of the gas phase were assayed for ethylene as described in the Materials and Methods section. Samples (1 ml) of the liquid phase were photochemically assayed (pH 3.5, tungsten light) for 4-methylthio-2-oxobutanoate after removal of the bacterial cells by centrifugation. (a) Ethylene formation. Symbols: ●, cells + methionine; ○, cells + methionine + chloramphenicol. Addition of methionine to boiled cells or culture filtrates gave identical results with that shown for cells + methionine and chloramphenicol. (b) 4-Methylthio-2-oxobutanoate formation. Symbols: ●, cells + methionine; ○, cells + methionine + chloramphenicol (150 µg/ml); □, culture filtrates + methionine; ■, boiled cells + methionine.

2mm with respect to methionine, confirming that methionine does not interfere with the assay. Kinetically, the reaction was completed in 30–60 min, but did not always yield equimolar amounts of ethylene. The reason for this is unknown, but in all assays a 4-methylthio-2-oxobutanoate standard was included. Although methionine analogues other than 4-methylthio-2-oxobutanoate could react with riboflavin to yield ethylene, only 4-methylthio-2-oxobutanoate has been detected in culture fluids. However, because of this fact, all concentrations are expressed in terms of 4-methylthio-2-oxobutanoate equivalents.

Production of 4-methylthio-2-oxobutanoate by strains D7F and B12E

The amounts of 4-methylthio-2-oxobutanoate accumulated by 2 litre cultures of D7F and B12E were

determined by means of the 4-methylthio-2-oxobutanoate assay described above, as well as by gravimetric estimation of dinitrophenylhydrazones. The gravimetric method would be expected to give an underestimate and this is confirmed by the observations that organisms B12E and D7F converted administered methionine into 4-methylthio-2-oxobutanoate 2,4-dinitrophenylhydrazones in 42% and 43% yield respectively when estimated gravimetrically, and 48% and 49% yield when assayed photochemically.

Although considerable quantities of 4-methylthio-2-oxobutanoate accumulated in the growth medium of strains D7F and B12E it is conceivable that these were produced, not enzymically, but by strictly chemical reactions involving low-molecular-weight coenzymes. Results previously presented for *E. coli* (Primrose, 1977) strongly suggest that 4-methylthio-2-

Fig. 4. Conversion of 4-methylthio-2-oxobutanoate into ethylene by strain B12E

Ten 20 ml cultures of strain B12E were grown overnight (17 h) at 30°C in defined medium lacking methionine. The ten cultures were pooled and 20 ml portions dispensed in 250 ml flasks. Culture filtrates were produced by removing cells by centrifugation and filtering the supernatant fraction through a 0.2 µm Millipore filter. (a) Symbols: ●, cells + 4-methylthio-2-oxobutanoate (200 µg/ml) + light; ○, cells + 4-methylthio-2-oxobutanoate (200 µg/ml) in dark; □, culture filtrate + 4-methylthio-2-oxobutanoate (200 µg/ml) + light; ■, boiled cells + 4-methylthio-2-oxobutanoate (200 µg/ml) in dark. (b) Symbols: ○, cells + 4-methylthio-2-oxobutanoate (200 µg/ml); □, cells + 4-methylthio-2-oxobutanoate (200 µg/ml) + catalase (100 µg/ml); ■, cells + 4-methylthio-2-oxobutanoate (200 µg/ml) + catalase (1 mg/ml); ●, cells + 4-methylthio-2-oxobutanoate (200 µg/ml) + boiled catalase (1 mg/ml). All these reactions were carried out in the absence of light.

Table 4. Effect of glucose concentration on the production of ethylene and 4-methylthio-2-oxobutanoate by three bacterial isolates

Abbreviation used: KMBA, 4-methylthio-2-oxobutanoate. Concentrations of ethylene and 4-methylthio-2-oxobutanoate are in nmol of compound/mg of cell protein. Control cultures were incubated in the absence of methionine and 500 µg of methionine/ml was added before ethylene and 4-methylthio-2-oxobutanoate determination. Further details of experimental conditions are described in the legend to Table 3 and control cultures were incubated for the same time as experimental cultures.

Glucose concn. (% w/v)	<i>E. coli</i> SPAO		<i>Corynebacterium</i> D7F		<i>A. hydrophila</i> B12E	
	Ethylene	KMBA	Ethylene	KMBA	Ethylene	KMBA
0.10	179	936	1090	6600	7200	8880
0.15	600	1320	4200	7470	6720	9480
0.20	1350	1470	6060	9600	7320	8280
0.25	1760	1410	10800	10680	1440	9840
0.30	2010	1380	9360	10200	1170	8760
0.35	780	1370	1920	—	210	—
0.40	228	1470	1380	8040	354	9480
Control	1	7	3	17	15	15

oxobutanoate is produced enzymically and from the results presented in Fig. 3 a similar conclusion can be

drawn for strain B12E. The formation of both 4-methylthio-2-oxobutanoate and ethylene was in-

duced by methionine, but induction did not occur if chloramphenicol was added simultaneously with the methionine, indicating a requirement for protein synthesis. Boiled cells failed to convert methionine into 4-methylthio-2-oxobutanoate or ethylene, as did culture filtrates, indicating that the oxidative deamination of methionine is not a purely chemical process.

In the case of strain B12E the conversion of 4-methylthio-2-oxobutanoate into ethylene is also enzymic (Fig. 4). The rate of conversion of 4-methylthio-2-oxobutanoate into ethylene is similar regardless of the presence or absence of light, indicating that photochemical reactions are not involved (Primrose, 1977). This conclusion is supported by the observation that culture fluids do not degrade 4-methylthio-2-oxobutanoate. Peroxidase has already been implicated in the conversion of 4-methylthio-2-oxobutanoate into ethylene mediated by *E. coli* (Primrose, 1977), and probably plays a similar role in strain B12E, as shown by the effects of catalase on ethylene formation (Fig. 4).

Production of ethylene and 4-methylthio-2-oxobutanoate by different strains of bacteria

Primrose (1977) has suggested that the failure of many strains to produce significant quantities of ethylene is due to an inability to degrade 4-methylthio-2-oxobutanoate rather than a failure to synthesize 4-methylthio-2-oxobutanoate. This hypothesis has now been tested directly for a number of bacteria by using the 4-methylthio-2-oxobutanoate assay described above. From the results shown in Table 3, it is clear that this hypothesis is correct: bacteria such as *Rhizobium trifolii* NZP7, *P. aeruginosa* PAT and *E. coli* K12 produce much more 4-methylthio-2-oxobutanoate than ethylene. With many of the bacteria tested the formation of ethylene, unlike the accumulation of 4-methylthio-2-oxobutanoate, was greatly influenced by the concentration of glucose in the medium (Tables 3 and 4).

Discussion

Primrose (1977) reported that *E. coli* SPAO, grown in the presence of methionine, secretes a compound into the growth medium that has the properties of 4-methylthio-2-oxobutanoate. In the present paper we have reported the characterization of the dinitrophenylhydrazone of 4-methylthio-2-oxobutanoate by t.l.c., i.r. and n.m.r. spectroscopy and mass spectrometry and used these methods to identify 4-methylthio-2-oxobutanoate conclusively in the growth medium of ethylenogenic isolates D7F and B12E. T.l.c. of dinitrophenylhydrazones isolated from cultures of other organisms, including *E. coli* SPAO, indicate that these cultures also secrete 4-methylthio-2-oxobutanoate. In contrast, significant quantities of

methional have never been detected in the growth medium of any of the cultures so far examined. Interestingly, 4-methylthio-2-oxobutanoate is a more reactive substrate than methional for the photochemical reaction, even at high pH values (Fig. 1), for the peroxidase-mediated reaction (Yang, 1969) and for the Fe(II)-catalysed degradation (S. B. Primrose, unpublished work).

Bauer & Yang (1969) have suggested that in apple tissue, at least, neither 4-methylthio-2-oxobutanoate nor methional is a precursor of ethylene, although Primrose (1977) has questioned this conclusion. Adams & Yang (1977) now favour a pathway involving *S*-adenosylmethionine. However, even if there is a pathway to ethylene that involves *S*-adenosylmethionine, the fact that 4-methylthio-2-oxobutanoate was found in all culture fluids examined (Table 3), and is easily converted into ethylene (Primrose, 1977), indicates that it is a precursor for at least some of the ethylene produced. Indeed, the observation that up to half the added methionine was converted into 4-methylthio-2-oxobutanoate by cultures of D7F and B12E indicates that 4-methylthio-2-oxobutanoate is the major, if not the sole, precursor of ethylene in these organisms.

Like *E. coli* SPAO, other organisms secrete a compound into the growth medium that can be converted into ethylene in a flavin-mediated photochemical reaction (Table 3). The controls indicate that this compound cannot be methionine. Although a number of methionine derivatives and analogues (Table 2) can be degraded photochemically to yield ethylene, Fig. 1 shows that under the experimental conditions of the assay only 4-methylthio-2-oxobutanoate and methional react to any significant extent. Because methional has not so far been detected in culture fluids it is assumed that 4-methylthio-2-oxobutanoate is being assayed. The results presented in Table 3 indicated that the ability to convert methionine into 4-methylthio-2-oxobutanoate is widespread among heterotrophic bacteria and may indeed be a property of all of them, particularly because it is found even in ethylene-oxidizing bacteria (S. B. Primrose, unpublished work).

Until recently it was thought that the ethylene produced by *P. digitatum* was not derived from methionine, but from glutamate or δ -oxoglutarate (for review see Yang, 1974). However, Primrose (1977) reported that methionine stimulated ethylene production by *P. digitatum*, and Chalutz *et al.* (1977) have recently shown, by using shake cultures instead of static cultures, that this organism synthesizes ethylene from methionine as well as from glutamate. Our finding that 4-methylthio-2-oxobutanoate can be detected in culture fluids of *P. digitatum* confirms the results of Chalutz *et al.* (1977) and suggests that the pathway from methionine in this organism is identical with that found in other micro-organisms. Why *P.*

digitatum should evolve two different biosynthetic routes to ethylene is not immediately apparent.

When a metabolite accumulates in a cell or the growth medium its synthesis must be proceeding faster than its degradation. Despite the fact that three routes for converting 4-methylthio-2-oxobutanoate into ethylene are known, its accumulation is not surprising. Although chemical degradation of 4-methylthio-2-oxobutanoate can be mediated by flavins or Fe(II) neither of these species is normally present in high enough concentrations in the growth medium, except in the case of exceptional flavin over-producers (Primrose, 1976a). Clearly, in most organisms the peroxidase-mediated reaction is the only biologically significant route to ethylene. Even if peroxidase were constitutive in most ethylene producers, its activity is moderated by catalase whose synthesis is known to be regulated by the concentration of glucose in the medium (Ishida, 1976). This observation may explain the effect of glucose concentration on the production of ethylene shown for a number of bacteria (Table 4). However, there are other explanations for the effect of glucose, e.g., the amount of cell lysis, the final pH of the medium etc. Nevertheless, the important point is not why glucose affects the yield of ethylene, but the need to screen organisms in media of different compositions. Failure to do so explains the inability of previous workers to detect ethylene production by their isolates.

Note Added in Proof (Received 20 July 1979)

Adams & Yang (1979) have provided evidence that in apple tissue *S*-adenosylmethionine is converted into ethylene via 1-aminocyclopropane-1-carboxylic acid (see also Boller *et al.*, 1979).

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