Methyl Mercaptan and Dimethyl Disulfide Production from Methionine by *Proteus* Species Detected by Head-Space Gas-Liquid Chromatography

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Received for publication 4 August 1976

Head-space gas-liquid chromatography and mass spectrometry were used to detect and identify products formed by *Proteus vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri* from a defined medium supplemented with either phenylalanine, methionine, valine, leucine, histidine, lysine, ornithine, threonine, asparagine, aspartic acid, or tryptophan. In a detailed study of the products formed by 68 strains of *Proteus* spp. from L-methionine, the production of large amounts of both dimethyl disulfide and methyl mercaptan was found to be a characteristic of the genus. Both sulfur products appeared within a few hours of inoculation. Dimethyl disulfide was a more sensitive indicator of growth than the spectrometric determination of optical density. This suggests that it could be useful for the rapid, automated detection of any species of *Proteus*.

In recent years there has been increasing interest in rapid methods for detecting microorganisms, especially if they can be automated. Gas-liquid chromatography (GLC) is a sensitive, quantitative method of separating and distinguishing the components of mixtures of volatile substances. So much of microbiology depends on detecting chemicals, either from the organisms themselves or their metabolic products, that GLC would seem likely to be applicable for rapid techniques.

A variety of rapid, discriminatory microbiological tests using GLC have been described (3-5, 11, 15-18, 21, 24, 29, 31), but head-space sampling seems to have been overlooked and other methods of sample preparation preferred. However, head-space GLC appears to be particularly worthy of investigation as the basis of rapid techniques. Sample preparation is simple, and volatile compounds are fast to elute from the gas chromatograph. In addition, head-space GLC can be automated. In the present investigation the technique has been used to study metabolic products whose source has been indicated by using defined growth media.

MATERIALS AND METHODS

Proteus cultures. The 68 strains used in the investigation included 18 *Proteus vulgaris*, 15 *P. mi-*

rabilis, 14 *P. morganii*, 11 *P. rettgeri*, 6 *P. inconstans* subgroup A, and 4 *P. inconstans* subgroup B. Culture collections provided 32 strains, and 36 were freshly isolated. The identities of all strains were confirmed (9).

Culture media. The basal defined medium (LAS) was prepared from four solutions which were stored separately and mixed when required: 1 liter of LAS contained (i) 100 ml of a 2% (wt/vol) potassium lactate solution sterilized at 110°C for 25 min; (ii) a complex amino acid mixture (23) dissolved in 100 ml of water and sterilized by filtration through a Galenkamp Sinta glass filter of porosity 5; (iii) a salt mixture (23) dissolved in 780 ml, adjusted to pH 7.4, and sterilized at 121°C for 15 min; and (iv) 20 ml of a filter-sterilized solution of nicotinic acid (0.5 mg) and calcium pantothenate (0.5 mg).

Nutrient broth (Oxoid) and MacConkey agar (Oxoid) were prepared as directed by the manufacturers.

LAS medium or nutrient broth was supplemented with amino acids by dissolving them in the basal medium and sterilizing by filtration.

Incubation conditions. Samples (20 ml) of the liquid media were dispensed aseptically into sterile 125-ml Erlenmeyer flasks stoppered with cotton-wool plugs. All liquid cultures were incubated at 37°C either still or shaking on a rotary shaker at 160 rpm.

Measurement of bacterial growth. The optical densities (ODs) of bacterial suspensions were determined on a Bausch & Lomb Spectronic 20, at 500 nm for LAS cultures and at 600 nm for broth cultures, in a cell of 1-cm lightpath. A graph to convert OD to dry weight of cells was constructed from determinations on duplicate samples from six serial twofold dilutions each of eight *Proteus* cultures, two *P. vul-*

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garis, two *P. morganii*, and one each of *P. mirabilis*, *P. rettgeri*, *P. inconstans* A, and *P. inconstans* B. The results from all eight cultures lay on the same curve, which was linear for dry weights up to about 0.25 mg/ml.

Viable counts (14) were the mean from 9 drops (between 45 and 450 colonies), 3 on each of three well-dried MacConkey plates, incubated overnight at 30°C to restrict colony size and increase the number of well-isolated colonies for accurate viable counts.

Preparation of head-space samples for GLC. A published technique (1) was modified. A 4.8-g portion of anhydrous potassium carbonate was transferred to a "10-ml" glass serum vial (capacity, 14 ml); 8 ml of standard solution or culture was added; and the rubber cap was fitted immediately. The vial was shaken mechanically for 5 min at 37°C to mix the salt in the liquid, but care was taken not to soil the cap with liquid. The vial was then incubated in a 60°C water bath for 3 min. While the vial remained immersed in the water, a 0.5-ml gas syringe fitted with a Teflon gas-sealing gland (Scientific Glass Engineering, no. 500A-RN-GSG) was rinsed by filling and emptying five times with the gas above the surface. The syringe was filled the sixth time with ≈ 0.6 ml of head-space gas and withdrawn from the vial. The needle was wiped with a tissue, and the sample was adjusted to exactly 0.5 ml and immediately injected into the gas chromatograph.

The reproducibility of results of head-space analysis is dependent on the care with which samples are prepared. In this investigation special precautions were taken to prevent contamination of the gas sample by splashing from the underlying liquid. Loss of gas either from around the large-bore needle of the gas syringe when it pierced the rubber cap of the vial or the rubber septum of the gas chromatograph or from around the sealing gland of the gas syringe itself was prevented by replacing caps, septa, and sealing glands frequently and regularly.

GLC. The instrument used was a Perkin-Elmer model 881 fitted with a flame ionization detector and a stainless-steel column (366 cm by 3.2 mm), packed with 5% Carbowax 20 M on Chromosorb G, acid washed, dimethyldichlorosilane treated, and of 60/80-mesh size. The instrument was operated at 100°C with a nitrogen carrier gas flow of 22 ml/min and hydrogen and air pressure of 114 kPa (16.5 lb/in²) and 255 kPa (37 lb/in²), respectively. The injector temperature was 150°C, and the detector temperature was 120°C.

Identification of products. Methyl mercaptan, dimethyl disulfide, diethyl disulfide, isoamyl alcohol, isovaleraldehyde, isobutanol, isobutyraldehyde, 2-phenylethanol, phenylacetaldehyde, and benzaldehyde were identified by using a GLC-mass spectrometry combination (27). Separated components were identified by comparing their mass spectra with a reference library file (8). This identification was confirmed by comparison of the GLC retention time of the unknown with that of an analytical-grade sample of the pure compound for all compounds except methyl mercaptan and isobutyraldehyde. In the case of methyl ethyl disulfide, neither pure sample nor library spectrum was available, but an al-

most certain identification was made from the mass spectrum alone. The molecule had molecular weight 108, and isotope ratios showed that it contained two sulfur atoms: the major fragments had molecular weights of 61 and 47, respectively, and each contained one sulfur atom. Ethyl mercaptan was identified by GLC retention time data alone.

The amounts of compounds are expressed as peak heights or areas and were used only to make comparisons.

Standard. An aqueous solution of dimethyl disulfide at a concentration of 0.01% (vol/vol) was analyzed with each day's samples to ensure that chromatographic conditions and the sensitivity of the detector remained constant. The limit of detection of dimethyl disulfide was about 1.06 μ g in 8 ml of solution (0.13 μ g/ml).

RESULTS

Selection of salt and comparison of head-space sampling with direct injection. Sodium sulfate has been used to increase the concentration of bacterial products in head-space gas samples (1) and potassium carbonate has been used for samples of blood gases (10). The effects of 4.8 g of K₂CO₃, anhydrous MgSO₄, or Na₂SO₄, more than enough to saturate 8-ml solutions, on some of the volatile compounds encountered in this study were compared (Table 1), and K₂CO₃ was chosen. A comparison was made between direct injections of 1.0 μ l of 0.01% (vol/vol) solutions of various classes of compounds(s) with boiling points of less than 200°C and injections of 0.5 ml of K₂CO₃ head-space gases from the same solutions. A 1.0- μ l amount of liquid approaches the practical limit of sample size, beyond which overloading occurs in a packed column of 3.2-mm diameter. Table 2 shows that the GLC response of the 0.5-ml head-space sample is greater than that of the 1.0- μ l liquid sample.

Accuracy and reproducibility of head-space analyses. When 0.5-ml samples of head-space gases were injected from 10 separate prepara-

TABLE 1. Effectiveness of salts for increasing the concentrations of compounds in head-space gas samples

Compound	Peak ht $\times$ attenuation factor (cm) ^a		
	K ₂ CO ₃	MgSO ₄	Na ₂ SO ₄
Ethanol	60	20	18
Acetone	409	107	57
Ethyl methyl ketone	88	27	9
Dimethyl disulfide	15	3	3
Ethyl acetate	90	123	
Acetaldehyde	42	42	
Diethylamine	700	0	

^a Average of duplicate determinations.

TABLE 2. Peak areas of responses to 1.0- μ l liquid samples and 0.5-ml head-space gas samples

Compound	Peak area (cm ²)		
	Liquid sample (A)	Head-space gas sample (B)	Relative response (B/A)
Ethanol	18	82	4.6
Isoamyl alcohol	24	542	22.6
Isovaleraldehyde	28	3,342	119.4
Ethyl methyl ketone	19	1,062	55.9
Ethyl acetate	15	497	33.2
Dimethyl sulfide	26	2,880	110.0
Dimethyl disulfide	28	957	34.2
Diethylamine	22	600	27.3

tions of a standard solution of dimethyl disulfide, the standard error of the mean of the peak areas was 5.4%. When 10 replicate cultures of *P. morganii* in LAS medium supplemented with 0.1 M L-methionine were analyzed by head-space, the mean peak areas and their standard errors were 593 ± 20 (3.4%) cm² for methyl mercaptan and 472 ± 27 (5.7%) cm² for dimethyl disulfide, which is the same order of reproducibility as for the head-space technique itself.

Head-space products formed by *Proteus* spp. from 11 selected amino acids. The amino acids, 0.1 M L-methionine, L-leucine, L-valine, L-phenylalanine, L-histidine hydrochloride, L-lysine, L-ornithine hydrochloride, L-threonine, and L-asparagine, and 0.05 M L-aspartic acid and L-tryptophan, were tested as individual supplements in LAS medium with one strain each of *P. vulgaris*, *P. mirabilis*, *P. morganii*, and *P. rettgeri*. Inocula were grown in LAS medium and added to give approximately 10^8 organisms per ml both in still cultures incubated for 24 h and in shaken cultures incubated for 18 h. All species grew well, with *P. mirabilis* cultures reaching the highest OD.

In general, all four species yielded the same major head-space products (Fig. 1): from phenylalanine, benzaldehyde; from methionine, methyl mercaptan and dimethyl disulfide; from valine, isobutyraldehyde and isobutanol; and from leucine, isovaleraldehyde and isoamyl alcohol. Some of the products were detected in smaller amounts from control cultures in unsupplemented LAS medium since it contains some leucine, valine, and methionine, but the increase in products on adding any of these three amino acids was clear-cut. No additional head-space products were detected in cultures grown on the other amino acids, and tryptophan suppressed the products normally produced in LAS medium. However, some cultures had distinctive odors that suggested there were

other volatile products not detected by this head-space technique; when culture filtrates from phenylalanine medium were examined by direct injection, phenylacetaldehyde and 2-phenylethanol were detected. In general, the still cultures formed lower yields of the same products as the shaken cultures.

Head-space products formed by several strains of all species of *Proteus* from methionine. Sixty-seven of the 68 strains of *Proteus* yielded similar products from shaken cultures in LAS medium supplemented with 0.1 M L-methionine after incubation for 18 h. The two chromatograms shown in Fig. 1 for *P. mirabilis* and *P. morganii* are typical. The remaining strain, *P. morganii*, grew very poorly and yielded only traces of a few products. Methyl mercaptan was detected only when cultures were grown in LAS medium supplemented with methionine, whereas small amounts of dimethyl disulfide were produced by cultures in unsupplemented LAS medium. Various other products, including isobutyraldehyde, isovaleraldehyde, and isoamyl alcohol, were produced in similar amounts from both methionine-supplemented and unsupplemented LAS medium. It was concluded that methyl mercaptan and dimethyl disulfide arose with methionine. This was confirmed when shaken cultures of one strain each of *P. vulgaris*, *P. mirabilis*, and *P. morganii* failed to form either methyl mercaptan or dimethyl disulfide in 15 h in a simplified LAS medium consisting of lactate, salts, vitamins, and only glutamic acid and cystine. The addition of 0.1 M L-methionine to this medium resulted in the production of high yields of both methyl mercaptan and dimethyl disulfide.

The yields of methyl mercaptan and dimethyl disulfide from LAS medium supplemented with 0.1 M L-methionine expressed both as GLC peak areas per dry weight per milliliter of culture and as GLC peak areas are shown in Table 3. When yields are correlated with degree of growth (dry weight per milliliter), small species differences are apparent. *P. mirabilis*, *P. rettgeri*, and all but two strains of *P. morganii* reached high OD values and gave lower yields in relation to dry weight per milliliter than *P. vulgaris*, *P. inconstans* A, and *P. inconstans* B, which reached only moderate OD values. Two strains of *P. morganii* grew poorly so that their yields related to dry weight per milliliter were exceptionally high, although in terms of GLC peak areas the yields were similar to those of other *Proteus* spp. These apparent species differences, related to degree of growth, do not imply any inherent difference in the organisms' ability to decompose methionine. GLC peak areas of sulfur products formed by all 67 strains

FIG. 1. Chromatograms of head-space products from shaken cultures of *P. mirabilis* and *P. morganii* in LAS medium alone or supplemented with 0.1 M amino acid incubated for 18 h at 37°C. Stationary phase, Carbowax 20 M; column temperature, 100°C; attenuation setting, $\times 5$ unless otherwise marked, $\times 50$ being 10 times less sensitive than $\times 5$. Peak a, Benzaldehyde; b, methyl mercaptan; c, dimethyl disulfide; d, isobutyraldehyde; e, isobutanol; f, isovaleraldehyde; g, isomyl alcohol.

from methionine do not show any apparent species differences and do not vary widely, most of those for mercaptan falling between 200 and 600 cm^2 and, for disulfide, between 300 and 900 cm^2 (Fig. 2).

Product yields from methionine in relation to incubation time. Analyses at 2-h intervals for products from shaken cultures of *P. mirabilis* in nutrient broth supplemented with 0.1 M L-methionine with initial inocula of 3×10^2 and 3×10^5 organisms per ml are shown in Fig. 3. A moderate amount of dimethyl disulfide was detectable at 2 h from an inoculum of 3×10^5 organisms per ml and increased to an apparent limiting value at 6 h, when growth had reached the early stationary phase (3×10^9 organisms per ml). Methyl mercaptan was first detectable at 4 h and quickly increased in yield. A large

amount of dimethyl disulfide was detectable after 6 h from an inoculum of 3×10^2 organisms per ml, although there was no apparent change in OD at this time. When LAS medium replaced nutrient broth the lag phase was longer, and the growth rate was slower. Dimethyl disulfide again appeared first and methyl mercaptan later. The detection of dimethyl disulfide from either medium was found to be a more sensitive indicator of growth than the spectrometric determination of OD. The fact that the formation of sulfur products from methionine starts in the earliest stages of growth and is complete by the time the early stationary phase is reached explains why yields of sulfur products are unrelated to degree of growth after incubation for 18 h (Table 3). It appears that all *Proteus* spp. are equally efficient in decompos-

TABLE 3. Yields of sulfur products from LAS medium supplemented with 0.1 M methionine by all species of *Proteus*

Organism	No. of strains	Peak area/dry wt per ml (cm ² /mg per ml)		Peak area (cm ²)	
		Methyl mercaptan	Dimethyl disulfide	Methyl mercaptan	Dimethyl disulfide
<i>P. vulgaris</i>	18	511 ± 21	640 ± 58	450 ± 30	599 ± 59
<i>P. mirabilis</i>	15	354 ± 18	427 ± 39	428 ± 22	526 ± 62
<i>P. morganii</i>	11	234 ± 27	356 ± 37	323 ± 31	488 ± 34
	1	2,400	7,474	228	710
	1	550	1,316	105	250
<i>P. rettgeri</i>	11	467 ± 24	498 ± 39	477 ± 26	512 ± 45
<i>P. inconstans</i> A	6	715 ± 54	566 ± 106	479 ± 36	380 ± 69
<i>P. inconstans</i> B	4	478 ± 46	684 ± 52	515 ± 36	739 ± 36

FIG. 2. GLC peak areas of sulfur products formed by strains of *Proteus* from shaken cultures in 0.1 M L-methionine-supplemented LAS medium incubated for 18 h at 37°C.

ing methionine and, since most of the growth occurs after methionine decomposition has ceased, yields of sulfur products are unrelated to it.

Head-space products from ethionine. The synthetic amino acid ethionine was investigated to enlarge the results from methionine.

Concentrations of DL-ethionine in LAS medium greater than 0.01 M were shown to inhibit the growth of *P. mirabilis*, although some cells remained viable. Tests for head-space products from 18-h shaken cultures of eight strains of *Proteus* in LAS medium supplemented with 0.01 M DL-ethionine yielded ethyl mercaptan, diethyl disulfide, and another compound, probably methyl ethyl disulfide, which were not

formed from similar cultures in LAS medium alone (Table 4).

Analyses at various times up to 24 h of duplicate shaken cultures of *P. mirabilis* in LAS medium supplemented with 0.01 M DL-ethionine are shown in Fig. 4. Diethyl disulfide was detected after incubation for 4 h when the culture was in the early exponential phase of growth, 2 h before the first detection of ethyl mercaptan. The detection of mercaptan coincided with a leveling off in the production of disulfide, and thereafter the yield of ethyl mercaptan increased rapidly. The results from methionine are supported because analogous products are formed in the same order and at the same stage of growth from ethionine.

DISCUSSION

All previous studies of production of sulfur compounds from methionine by microorganisms or their enzymes have been undertaken with biochemical objectives so that single strains, often incompletely identified, have sufficed (2, 13, 19, 20, 22, 25, 26, 30). The only information about any *Proteus* species is that three strains of *P. vulgaris* produced methyl mercaptan from methionine (19, 26). The present investigation has been concerned with the microbiological significance of methionine breakdown and has shown that 67 strains of the

six species *P. vulgaris*, *P. mirabilis*, *P. morganii*, *P. rettgeri*, *P. inconstans* A, and *P. inconstans* B were alike in possessing powerful enzymes for the production of methyl mercaptan and dimethyl disulfide from methionine. Both sulfur products were formed early in the exponential phase of growth and therefore could be ideal markers for the rapid detection of *Proteus* spp. by head-space GLC.

It is most probable that *Proteus* spp. pro-

FIG. 3. Time course of formation of sulfur products from 0.1 M L-methionine-supplemented nutrient broth by shaken cultures of *P. mirabilis* with an initial inoculum of 3×10^2 organisms per ml (small) or 3×10^8 organisms per ml (large). Symbols: ●—●, dimethyl disulfide, large inoculum; ○—○, methyl mercaptan, large inoculum; ●---●, dimethyl disulfide, small inoculum.

FIG. 4. Time course of growth and formation of sulfur products from 0.01 M DL-ethionine-supplemented LAS medium by shaken cultures of *P. mirabilis*: mean values of duplicate cultures. Symbols: ●, viable count; □, diethyl disulfide; ○, ethyl mercaptan; △, methyl ethyl disulfide.

TABLE 4. Growth and product formation by *Proteus* spp. in LAS medium supplemented with 0.01 M DL-ethionine

Species	Growth (mg, dry wt/ml)	Peak area (cm²) of products		
		Ethyl mercaptan	Diethyl disulfide	Methyl ethyl disulfide ^a
<i>P. vulgaris</i>	0.005	0	6	0
	0.066	110	55	3
<i>P. mirabilis</i>	1.135	223	209	5
<i>P. morganii</i>	0.013	0	11	1
	0.045	16	75	6
<i>P. rettgeri</i>	0.895	156	162	5
<i>P. inconstans</i> A	0.485	344	144	5
<i>P. inconstans</i> B	1.135	182	388	8

^a Mass spectrometric identification only (see text).

duce mercaptans from methionine and ethionine by fission of the bond between the alkyl sulfide group and its adjoining carbon atom and that the mercaptans are then oxidized by dissolved oxygen to dialkyl disulfides (6), a reaction shown to be very fast (12). Disulfides were always detected before mercaptans, their supposed precursors. It was possible that failure of early detection of methyl mercaptan was due either to its low boiling point or to insensitivity of the flame ionization detector to one-carbon compounds, but the identical results with ethyl mercaptan make this unlikely. However, the detection of a product before its precursor always occurs in a reaction mechanism in which the rate of formation of the successor is very much faster than the rate of formation of its predecessor; for example, in any chain reaction the free radical intermediates are normally undetectable. Whether either one or both mercaptan and disulfide are found in a culture is determined by the time of sampling relative to demethiolase activity and to access of oxygen to the medium; disulfide alone is found when diffusion of oxygen is sufficient to oxidize mercaptan as fast as it is formed, mercaptan alone under anaerobic conditions, and both sulfur products under aerobic conditions when demethiolase activity yields more mercaptan than can be oxidized immediately.

Dimethyl sulfide has been reported as a microbial metabolic product (7, 28). However, it was not detected by us as a breakdown product from methionine. The GLC-mass spectrometry technique would have detected it and distinguished it from methyl mercaptan and dimethyl disulfide if it had been present in amounts comparable to them.

Apart from benzaldehyde, the aldehydes and alcohols from phenylalanine, valine, and leucine can be postulated to have arisen after an initial step of decarboxylation and oxidative deamination of their amino acid groups. The aldehyde and alcohol that would have arisen from a similar breakdown of the amino acid group of methionine and ethionine, propionaldehyde and propanol, were not detected. Their boiling points, 97 and 49°C, respectively, are not low enough for them to have been lost during incubation, particularly from still cultures. It must be concluded that *Proteus* spp. do not decompose the amino acid groups of methionine and ethionine in the same way as those of the other three amino acids. It is probable that 2-ketobutyric acid was formed as described by Segal and Starkey (26).

ACKNOWLEDGMENTS

We thank the curators of the National Collection of Type Cultures, London, the Queensland University Culture col-

lection, the Institute of Medical and Veterinary Science, Adelaide, and the International Shigella Centre, Central Public Health Laboratory, London, for cultures from their collections and Jennifer Spragg for her capable technical assistance.

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