The parasitic flagellates *Trichomonas vaginalis* and *Tritrichomonas foetus* produce indole and dimethyl disulphide: direct characterization by membrane inlet tandem mass spectrometry

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The use of a membrane inlet triple quadrupole mass spectrometer revealed indole as an end product in the growth medium of cultures of the cattle parasite *Tritrichomonas foetus* and the human parasite *Trichomonas vaginalis*: formation of indole is enhanced in the presence of added tryptophan. Two different clinical isolates of *Trich. vaginalis* also produce dimethyl disulphide. Electron impact ionization yielded complex fragmentation mixtures, but the facility for analysis of daughter ions enabled unequivocal assignments. Chemical ionization gave $[M + 1]^+$ species, and tandem mass spectrometry produced identification through daughter ions. The method provides a rapid single-step procedure for the characterization of microbial products without the need for preliminary separation and derivatization.

Introduction

The application of non-invasive physical methods to investigations of the metabolism of growing cultures of organisms or cells often provides new insights (Lloyd *et al.*, 1989): a recent example is the use of ^{13}C -NMR for the identification of glycerol as a major fermentation product of *Trichomonas vaginalis* (Chapman *et al.*, 1985). In the case of clinically important organisms, the identification of unusual metabolic characteristics may provide new avenues for chemotherapeutic attack (Linstead & Cranshaw, 1983; Thong & Coombs, 1987*b*). Ishiguro (1985) has used GC-MS to identify gaseous trichomonad products. Here we describe the use of a membrane inlet triple quadrupole mass spectrometer for the rapid identification of indole and dimethyl disulphide as unexpected metabolic end-products of trichomonads.

Methods

Maintenance and growth of organisms. *Tritrichomonas foetus* and *Trichomonas vaginalis* (strains 1910 and UCH-1) were obtained from Dr Alex Yule and Mr Bob Spice of the London School of Hygiene and Tropical Medicine. Cultures were frozen after adding 10% (v/v) dimethylsulphoxide by cooling in a cryostat at 1 °C per min and stored

at –80 °C. They were grown at 37 °C in screw-capped tubes (10 ml) or bottles (100 ml) in modified Diamond's (1957) medium containing ascorbate, cysteine, and 10% (v/v) heat-inactivated horse serum: organisms were subcultured daily. Recovery of frozen organisms was followed by serial subculture: at least 12 transfers (0.1 ml inocula in 10 ml media) preceded analyses of product formation. Organisms were counted in a modified Fuchs-Rosenthal haemocytometer (depth 0.2 mm, 1/16 mm²).

Treatment of samples. Culture supernatants were prepared by centrifugation at 4 °C in a Heraeus bench centrifuge at 1000 *g* for 2 min, carefully decanted and immediately frozen at –18 °C. Mass spectrometric analyses were performed directly on undiluted samples (3 ml).

Tandem mass spectrometry. The membrane inlet triple quadrupole mass spectrometer was based on the design of Yost & Enke (1979) and has been previously described in detail (Lauritsen *et al.*, 1990): it was constructed in collaboration with VG Quadrupoles, Cheshire, UK specially for process control using a membrane inlet. A VG 20-253T dual chemical ionization/electron impact ion source and quadrupole analysers (125 mm VG SX200 rods) were enclosed in two differentially-pumped stainless steel chambers evacuated by two 240 l s^{–1} turbomolecular pumps (Balzers, Lichtenstein). Edwards PE 505 Penning gauges were used to measure total pressure values. Three synchronized VG SX200 RF generators (0–880V) were used. Axial ion energy was variable between –50 and +50V in the collision cell, and between +12 and –12V in the mass-analysing quadrupole. Mass scanning used a PC with 14-bit A/D and D/A converters. Scan modes enabled computer-controlled scanning of first quadrupole only, third quadrupole only, or of first quadrupole with the third quadrupole delayed, enabling acquisition of normal mass spectra, daughter ion, parent ion or neutral loss spectra.

The thermostatted stainless steel reaction vessel (4 ml total volume) was interfaced with the vacuum system at a point as close as possible to

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the ion source. The liquid sample was separated from the vacuum by a 25 μm -thick silicone rubber membrane (Radiometer, Copenhagen) supported on a stainless steel porous plug. Stirring was by a vertically-mounted impellor in order to minimize effects of the unstirred liquid layer on the membrane.

Results

Volatile compounds accumulating in the growth medium of Trit. foetus

Electron impact ionization mass spectrometry (EI-MS) of those substances which are volatile at pH 6.2 and are able to penetrate the 25 μm -thick silicone membrane indicates that uninoculated Diamond's medium contains a variety of such compounds, and the fragmentation pattern is complex, with major maxima at $m/z = 58, 128, 72, 105, 106, 57, 55, 77, 56$ and 64 (Fig. 1*a*). After growth of *Trit. foetus* for 2 d (the late exponential phase of growth, 4×10^6 organisms ml^{-1}) a mass spectrum acquired under identical conditions (Fig. 1*b*) indicated that the volatiles giving rise to this fragmentation pattern had been utilized. The major components of the EI-MS spectrum were now at $m/z = 55, 117, 56, 70, 57, 71, 53, 58, 89, 90$ and 88 . At stationary phase (Fig. 1*c*, 6×10^6 organisms ml^{-1}) the intensities of peaks at $m/z 117, 70, 71, 89, 90$ and 88 had increased about two-fold over the corresponding features at 2 d, whereas those at $m/z = 55$ and 80 had declined.

When methane was used as reaction gas for chemical ionization (CI) a low-intensity background of symmetric sets of peaks is observed at low masses ($m/z < 150$). This background is caused by gas-phase reactions between reaction ions and the reaction gas itself and by impurities in the reaction gas (grade 3.5). Further, the CI-MS spectrum of the stationary phase sample (Fig. 2*a*) clearly shows two peaks at $m/z 118$ and 117 . A daughter ion spectrum of the $[M + 1]^+$ parent ion $m/z 118$ gave fragments at $m/z 91$ and 65 (Fig. 2*b*). Table 1 summarizes these data.

Fig. 3*a* shows the EI-MS spectrum from a stationary phase culture of *Trich. vaginalis* 1910 (3×10^6 organisms ml^{-1}). Peaks at mass 117, 90 and 89 are present, but in this case the dominant features of the mass spectrum are at 94 and 79. CI-MS showed peaks at $m/z 95, 117$ and 118 . A daughter-ion mass spectrum (EI) of the species at mass 94 gave fragments at 79 and 61 (Fig. 3*b*).

Comparison of the data with 'Eight Peak Index of Mass Spectra' (Anon, 1983) entries at $m/z 117$ and 94 indicates an excellent match with the fragmentation patterns for indole and dimethyl disulphide respectively (Table 2). Standard spectra of solutions of indole and dimethyl disulphide (Figs 4*a* and 4*b*, respectively) show excellent agreement with indole peaks (117, 90 and 89)

Fig. 1. Changes in ion fragments of volatile components in the growth medium of *Tritrichomonas foetus*. Mass spectra obtained after electron impact ionization of components from (a) uninoculated medium, (b) 2 d culture, and (c) 4 d culture.

Fig. 2. Tandem mass spectrometry of volatile components in the growth medium of *Trichomonas foetus*. (a) Chemical ionization mass spectrometry showing parent $[M + 1]^+$ superimposed on background spectrum of CH_4 . (b) Tandem mass spectrometry of the parent ion at $m/z = 118$.

Fig. 3. Mass spectrometry of volatile components in the growth medium of *Trichomonas vaginalis* strain 1910 after 4 d growth. (a) Fragment ions obtained by electron impact ionization. (b) Tandem mass spectrometry of the ion at $m/z = 94$.

Table 1. Mass spectrometric analyses of volatile components in the growth medium of *Trichomonas foetus*

Sample	Method	Mass spectra
Uninoculated medium	Electron impact ionization mass spectrometry	Peak (m/z) 58 128 72 105 106 77 76 64 Intensity 100 70 65 30 30 30 15 15
Medium from 2 d culture	Electron impact mass spectrometry	Peak (m/z) 55 117 70 71 90 89 88 80 Intensity 100 70 50 50 30 30 15 15 Peak 117 90
Medium from 4 d culture	Electron impact tandem mass spectrometry	
	Electron impact mass spectrometry	Peak (m/z) 117 55 70 71 90 89 88 94 Intensity 100 70 40 35 35 35 25 25
	Chemical ionization mass spectrometry	Peak 118 117
	Chemical ionization tandem mass spectrometry	Peak (m/z) 118 91 65 Intensity 100 2-3

Fig. 4. Electron impact ionization mass spectrum of (a) indole and (b) dimethyl disulphide.

Table 2. Mass spectra of indole and dimethyl disulphide after electron impact ionization (Eight Peak Index)

Indole $C_8H_7N = 117$							
Peak (m/z)	117	90	89	63	39	116	118
Intensity	100	40	24	14	9	8	8
Dimethyl disulphide $C_2H_6S_2 = 94$							
Peak (m/z)	46	79	45	94	49	44	61
Intensity	100	99	99	98	71	36	33

found in both cultures and with dimethyl disulphide peaks (m/z 94, 79, 64 and 61) found in *Trich. vaginalis* strain 1910. Standard daughter ion mass spectra of the molecular ions $[M + 1]^+$ (indole; CI) and $[M]^+$ (dimethyl disulphide; EI) showed excellent agreement with the spectra observed from these ions from the cultures. The

concentrations of indole and dimethyl disulphide in the 4-d-old sample of *Trich. vaginalis* strain 1910 were estimated to be $20 \mu M$ and $4 \mu M$, respectively. *Trich. vaginalis* strain UCH-1 gave similar results to those shown for strain 1910: when grown for 24 h in the presence of 5 mM-tryptophan, the former strain gave an indole/dimethyl disulphide ratio twice that of organisms grown in its absence.

Discussion

Both indole and dimethyl disulphide (Table 2) are known end-products of amino acid catabolism. The bacterial conversion of tryptophan to indole has been known since the work of Hopkins & Cole (1903); tryptophanase, a pyridoxal-phosphate-requiring enzyme, cleaves tryptophan to give indole, pyruvate and ammonia (Wood *et al.*, 1947; Gooder & Happold, 1954). This reaction can occur anaerobically, and aminoacrylic acid is the immediate precursor of pyruvate and ammonia.

The major nitrogenous excretory product of many parasitic protozoa is ammonia (Gutteridge & Coombs, 1977). In some trypanosomatids, tryptophan- α -ketoglutarate aminotransferase yields glutamate and indole-pyruvate; the latter compound is then reduced to indole-lactate or decarboxylated to indole-acetate and tryptophol (Stibbs & Steed, 1975). Trichomonads have a great capacity to digest proteins (Lockwood *et al.*, 1987; Lockwood & Coombs, 1989); although critical experiments have not been performed, it seems likely that they utilize amino acids as sources of energy when available in excess, especially if carbohydrates are not available. In the experiments reported here, the growing organisms have a plentiful supply of maltose, and the trace amounts of indole detected may not be representative of the full potential for utilization of the tryptophan degrading pathway that would be only manifest under conditions of carbohydrate starvation. No detailed studies of tryptophan catabolism in trichomonads have been reported; that indole accumulation in the growth medium is enhanced when organisms were incubated in the presence of added tryptophan, indicates a precursor-product relationship.

By comparison, the interconversions involved in the trichomonad metabolism of sulphur-containing amino acids are much better understood (Thong *et al.*, 1987a, b; Thong *et al.*, 1987). Methionine, required for the production of *S*-adenosyl methionine, the central metabolite for transmethylation reactions, can be rapidly hydrolysed by methionine γ -lyase to methanol in rumen ciliate protozoa (Merricks & Salisbury, 1973, 1974; Onodera & Migita, 1985) and in a pseudomonad (Tanaka *et al.*, 1985). Methanethiol, first detected as a product of trichomonad metabolism by Gobert *et al.* (1971), is easily

autooxidized in solution to dimethyl disulphide. Our results confirm those of Thong & Coombs (1987*b*) who showed that of five trichomonad-like organisms, only *Trich. vaginalis* produces significant quantities of methanethiol.

The rapid and direct identification of metabolic end-products without prior purification or derivatization described here provides an example of the usefulness of membrane inlet tandem mass spectrometry in the recognition of previously unsuspected metabolic pathways and transformations.

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