Formation of methylglyoxal by bacteria isolated from human faeces

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Summary. Bacteria present in the human gut may produce methylglyoxal—a cytotoxic substance in mammals. This was investigated by studying the activity of methylglyoxal synthase, which produces methylglyoxal from dihydroxyacetone phosphate, and methylglyoxal concentration in growth medium of various bacteria isolated from human faeces. Facultative and strictly anaerobic bacteria isolated from faeces were able to produce methylglyoxal in both defined and complex media. *Proteus* spp. produced large amounts of methylglyoxal and had the greatest methylglyoxal synthase activity. Supplementing defined medium for facultative anaerobes with glucose 1% w/v did not significantly alter enzyme activity or methylglyoxal production. Inclusion of short chain fatty acids or bile acids in the medium reduced methylglyoxal synthase activity and methylglyoxal production by *Proteus* spp. None of the organisms examined had amine oxidase activity which could have contributed to methylglyoxal production from aminoacetone.

Introduction

Enterobacteria are capable of producing methylglyoxal (Cooper, 1975*a*), a cytotoxic substance which interferes with cell division in prokaryotes and eukaryotes (Szent-Gyorgyi, 1968). Two important sources suggested for methylglyoxal are dihydroxyacetone phosphate, an intermediate of glycolysis, and aminoacetone, an intermediate of glycine and threonine metabolism. Formation of methylglyoxal from dihydroxyacetone phosphate is catalysed by methylglyoxal synthase (E.C. 4.2.99.11) (Cooper, 1975*a*) and from amino acetone by amine oxidase (E.C. 1.4.3.4) (Elliott, 1960*a*). Methylglyoxal is detoxified by the glyoxalase system (Dakin and Dudley, 1913; Neuberg, 1913), which consists of glyoxalases I and II. These enzymes convert methylglyoxal to D-lactic acid, with reduced glutathione as cofactor (Racker, 1951). Glyoxalase-I is present in mammalian intestine (Baskaran and Balasubramanian, 1987) and may have a protective function against methylglyoxal produced by enterobacteria in the mammalian gut (Aronsson and Mannervik, 1977). As the lumen and mucosa of the gastrointestinal tract of normal southern Indians is heavily colonised by a variety of bacteria (Bhat *et al.*, 1972; Albert *et al.*, 1978), we thought that it was important to document

methylglyoxal production by these organisms. Several different groups of bacteria isolated from the faeces of southern Indians were tested for methylglyoxal synthase activity, amine oxidase and the quantity of methylglyoxal produced *in vitro* in defined and nondefined culture media.

Materials and methods

Chemicals

Bovine serum albumin, dihydroxyacetone phosphate, catalase, maleic acid, methylglyoxal (40% w/v), reduced glutathione and Tris were purchased from Sigma Chemical Co., USA. 2,4-Dinitrophenylhydrazine was obtained from British Drug House (BDH) Ltd, London, and semicarbazide hydrochloride from Eastman Kodak Co., New York. Aminoacetone was a gift from Dr S. Ray, Jadavpur University, Calcutta.

Bacteria and media

Facultative and strict anaerobes and microaerophilic organisms belonging to the following genera were isolated from stool samples: facultative anaerobes—*Escherichia*, *Klebsiella*, *Proteus*, *Morganella*, *Salmonella*, *Shigella* and *Aeromonas*; anaerobes—*Bacteroides*, *Bifidobacterium*, *Fusobacterium* (isolated from saliva) and *Veillonella*; microaerophilic organism—*Lactobacillus*. Facultative anaerobes were grown separately in Minimal Essential Medium of Davis and Mingioli (Cruickshank *et al.*, 1975), peptone water (Cruickshank *et al.*, 1975), peptone

water supplemented with glucose, short chain fatty acids or bile acids, and Evans medium (Evans *et al.*, 1975) for 24 h at 37°C. Anaerobes were grown in Thioglycollate Medium (Difco) except for *Veillonella* sp. which was grown in Brucella Medium (Difco) for 48 h at 37°C. Bacterial quantitation was by the serial dilution method (Bhat *et al.*, 1972) and cultures with $>10^8$ cfu/ml were used for enzyme assay and methylglyoxal estimation.

Preparation of bacterial extract

The cells were harvested by centrifugation, growth medium was collected separately, and the cells were washed with and resuspended in 10 mM Tris/HCl buffer, pH 7.4. The cell suspension was sonicated at 0°C in an MSE ultrasonic disintegrator. The sonicated suspension was centrifuged at 12 000 g for 20 min at 4°C and the supernate was used for enzyme assay.

Enzyme assays

Methylglyoxal synthase. Formation of methylglyoxal was measured spectrophotometrically with either 2,4-dinitrophenylhydrazine (Cooper and Anderson, 1970) or semicarbazide (Alexander and Boyer, 1971). One unit was defined as the activity of enzyme catalysing the formation of 1 nmol of methylglyoxal/min in the standard assay procedure.

Amine oxidase. Amine oxidase activity was measured as described by Ray and Ray (1983). The assay mixture contained 50 µmol of sodium phosphate buffer, pH 6.9, one unit of catalase, 0.2 µmol of aminoacetone, bacterial extract and water to a total volume of 1 ml. After incubation for 30 min at room temperature, the methyl-

glyoxal formed was measured colorimetrically with 2,4-dinitrophenylhydrazine-alkali reagent (Cooper and Anderson, 1970). One unit of amine oxidase activity was defined as the amount of enzyme that catalysed the formation of 1 nmol of methylglyoxal from aminoacetone/min under standard assay conditions.

Glyoxalase-I. Increase in absorbance at 240 nm due to thiolester (S-D-lactoylglutathione) formation from methylglyoxal and reduced glutathione was measured spectrophotometrically (Racker, 1951). One unit of glyoxalase-I was defined as the amount of the enzyme catalysing the formation of 1 nmol of S-D-lactoylglutathione/min in the standard assay system.

Determination of protein concentration

Protein was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results

Table I shows the activity of methylglyoxal synthase in the bacterial cell extract and the concentration of methylglyoxal in the culture fluid of various facultative anaerobes grown in different culture media. Compared with other facultative anaerobes, *Proteus* spp. had higher activity of methylglyoxal synthase in minimal essential medium and peptone water with a correspondingly high concentration of methylglyoxal in the medium. All these facultative anaerobes showed glyoxalase-I activity in the range 10–90 units/mg of protein.

Table I. Methylglyoxal synthase activity in bacterial extract and methylglyoxal concentration in culture fluid of various facultative anaerobes

Bacteria	Methylglyoxal synthase specific activity (u/mg protein)*					Methylglyoxal concentration (nmoles/ml of culture fluid)*		
	A	B	C	D	E	A	B	C
<i>A. hydrophila</i>	15.0 ± 4.3	12.8 ± 3.0	13.9 ± 2.3	12.4 ± 2.0	8.1 ± 1.4	ND	ND	25 ± 4
<i>E. coli</i>	20.0 ± 2.0	5.7 ± 1.0	4.7 ± 1.2	43.2 ± 3.0	49.7 ± 3.6	ND	ND	55 ± 5
<i>Klebsiella</i> sp.	7.7 ± 1.9	6.4 ± 1.6	7.0 ± 1.0	9.9 ± 2.1	19.7 ± 3.2	ND	ND	ND
<i>Salmonella</i> sp.	9.6 ± 2.0	6.0 ± 1.0	6.5 ± 1.5	37.5 ± 3.8	8.0 ± 1.0	ND	ND	82 ± 7
<i>Shigella</i> sp.	5.7 ± 2.1	4.6 ± 1.1	4.0 ± 1.0	6.6 ± 1.5	9.7 ± 2.0	ND	ND	ND
<i>M. morganii</i>	77.0 ± 5.0	40.5 ± 8.7	36.9 ± 3.0	NG	NG	25 ± 5	117 ± 6	142 ± 7
<i>P. mirabilis</i>	57.6 ± 2.0	29.0 ± 4.0	31.4 ± 3.5	20.0 ± 3.0	43.9 ± 6.7	27 ± 3	105 ± 9	62 ± 8
<i>P. vulgaris</i>	54.7 ± 6.9	39.5 ± 5.0	28.6 ± 2.0	12.6 ± 1.2	18.5 ± 2.2	30 ± 5	120 ± 10	120 ± 8

*The values are means ± SD of four separate experiments.

A. Minimum essential medium.

B. Peptone water.

C. Peptone water containing glucose 1% w/v.

D. Grown aerobically in Evans medium.

E. Grown anaerobically in Evans medium.

ND = Not detected; NG = no growth.

Table II. Methylglyoxal synthase activity in bacterial extract and methylglyoxal concentration in the medium of *Proteus* spp. grown in peptone water containing short chain fatty acids and bile acids

Medium	Methylglyoxal synthase specific activity (u/mg protein) of			Methylglyoxal concentration (nmol/ml of culture fluid) from		
	<i>M. morganii</i>	<i>P. mirabilis</i>	<i>P. vulgaris</i>	<i>M. morganii</i>	<i>P. mirabilis</i>	<i>P. vulgaris</i>
Peptone water	80.5	77.6	65.1	105	125	105
+ 5 mM propionic acid	37.0	66.2	43.6	80	110	90
+ 10 mM propionic acid	47.5	76.0	37.6	70	115	85
+ 5 mM butyric acid	63.9	40.0	56.9	85	120	75
+ 10 mM butyric acid	57.7	56.4	24.2	90	105	65
+ 5 mM cholic acid	62.6	68.5	17.9	105	130	55
+ 5 mM deoxycholic acid	71.9	65.3	26.0	130	130	70

The values are means of two separate experiments.

These facultative anaerobes grown in peptone water supplemented with glucose 1% w/v showed no significant change in enzyme activity, but methylglyoxal was detected in the growth medium of *Aeromonas*, *E. coli* and *Salmonella*.

Facultative anaerobes were grown in Evans medium and *E. coli* and *Salmonella* exhibited greatest activity of methylglyoxal synthase (table I). Methylglyoxal concentration in this culture fluid could not be measured due to the interference of the assay by the medium. *Proteus* spp. grown in minimal essential medium showed higher methylglyoxal synthase activity than those grown in peptone water or Evans medium. Methylglyoxal synthase activity by *Proteus* spp. was less in Evans medium than in peptone water (table I). *Salmonella* and *Aeromonas* had decreased methylglyoxal synthase activity whereas other facultative anaerobes had increased activity when grown in anaerobic conditions on Evans medium (table I). The methylglyoxal synthase activity was decreased in *Morganella morganii*, *P. mirabilis* and *P. vulgaris* grown in peptone water containing short chain fatty acids; methylglyoxal concentration in the medium was correspondingly decreased. Inclusion of bile acids in the growth medium slightly decreased the enzyme activity of *M. morganii* and *P. mirabilis* but it did not significantly alter the concentration of methylglyoxal in the medium (table II). Conversely, bile acids significantly decreased *P. vulgaris* methylglyoxal synthase activity and the subsequent concentration of methylglyoxal in the medium.

All anaerobes tested also showed both methylglyoxal synthase activity and the presence of methylglyoxal in the culture medium except *Veillonella*, with which methylglyoxal could not be detected due to the assay interference by the

brucella medium. Among the anaerobes, *Veillonella* and *Lactobacillus* had comparatively high methylglyoxal synthase activity (table III). Glyoxalase-I activity in these anaerobes was in the range 10–20 units/mg of protein.

None of these facultative anaerobes and anaerobes showed any amine oxidase activity.

Discussion

Intestinal bacteria may be one of the most important sources of methylglyoxal, a toxic compound which is detoxified by glyoxalase. We have earlier shown the presence of glyoxalase activity in monkey intestinal mucosa. This enzyme has been purified and characterised (Baskaran and Balasubramanian, 1987). Methylglyoxal has antimitotic activity, and if it is produced by intestinal bacteria, it may have an adverse effect on the mucosal lining

Table III. Methylglyoxal synthase activity in bacterial extract and methylglyoxal concentration in culture fluid of various anaerobic organisms

Bacteria	Methylglyoxal synthase specific activity (u/mg of protein)*	Methylglyoxal concentration (nmoles/ml of culture fluid)*
<i>Bifidobacterium</i>	9.4 ± 1.0	44 ± 9
<i>Bacteroides</i>	9.1 ± 1.0	35 ± 5
<i>Fusobacterium</i>	7.0	45
<i>Veillonella</i>	37.4 ± 2.7	ND
<i>Lactobacillus</i>	27.5 ± 5.6	40 ± 4

* The values are means ± SD of four separate experiments. ND = Not detected due to the interference of brucella medium components.

of the gastrointestinal tract, which has a high rate of cell proliferation. The present work has shown that various bacterial species isolated from human faeces have methylglyoxal synthase activity and are capable of producing methylglyoxal *in vitro*. Presence of methylglyoxal synthase activity in certain enterobacteria has also been reported (Cooper, 1975a). The enzyme has been purified and characterised from various bacteria and mammalian liver (Hopper and Cooper, 1972; Cooper, 1974; Tsai and Gracy, 1976; Ray and Ray, 1981). In the present study, *Proteus* spp. showed highest methylglyoxal synthase activity, producing more methylglyoxal in the culture medium. The concentration reported in the present study are similar to some earlier reports (Cooper, 1975b). However, higher levels of methylglyoxal synthase in *E. coli* and *P. vulgaris*, have been previously reported (Hooper and Cooper, 1971). This difference may reflect the source of organisms or growth conditions.

Supplementation of peptone water with glucose 1% w/v did not alter methylglyoxal synthase activity significantly but production of methylglyoxal by *Aeromonas*, *E. coli* and *Salmonella* was detected in this medium. Metabolism of unabsorbed sugars by colonic bacteria results in the production of short chain fatty acids which may influence their growth. Inclusion of two of these fatty acids, propionic and butyric acids, in the culture medium for *Proteus* spp. decreased methylglyoxal synthase activity and methylglyoxal production. Similarly, inclusion of cholic or deoxycholic acid in the medium also reduced the enzyme activity and methylglyoxal production by *P. vulgaris*. These findings suggest

that conditions prevailing in the gut could influence the production of methylglyoxal by bacteria colonising the gut.

Aminoacetone is produced from threonine by threonine dehydrogenase and also from glycine and acetylCoA by aminoacetone synthase. Formation of aminoacetone by these enzymes has been reported in mammals (Urata and Granick, 1963; Fubara *et al.*, 1986), birds (Aoyama and Motokawa, 1981), and bacteria (Elliott, 1960b; Boylan and Dekker, 1981). As aminoacetone is formed by micro-organisms, the conversion of aminoacetone to methylglyoxal was investigated. The enzyme amine oxidase, responsible for this conversion, was not detected in any of the bacteria tested, suggesting that aminoacetone does not serve as a precursor for methylglyoxal in the bacteria isolated from faeces. Although there are reports of amine oxidase activity in mammalian tissues (Elliott, 1960a; Ray and Ray, 1983) and *Pseudomonas* (Higgins and Turner, 1966), this activity was not demonstrated in faecal bacteria.

The present work has shown that various bacteria which colonise the human gastrointestinal tract, have methylglyoxal synthase activity and are capable of producing methylglyoxal. The presence of glyoxylase-I in the intestinal epithelial cells of mammals is, therefore, a potentially important protective mechanism.

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