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ORIGINAL ARTICLE

Bismuth-inhibitory effects on bacteria and stimulation of fungal growth *in vitro*

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Abstract Bismuth salicylate was found to inhibit the growth of a range of bacteria and yeast, "*Candida albicans*". In general the growth of bacteria did not result in increase in bismuth solubilisation, in contrast, bismuth solubilisation increased following the growth of *C. albicans*. A significant increase in the biomass (dry weight) of *Aspergillus niger* and *Aspergillus oryzae* occurred *in vitro* when these fungi were grown in the presence of bismuth salicylate. Biomass increase occurred over a range of bismuth compound additions, which in the case of *A. oryzae* was associated with increase in the solubilisation of the insoluble bismuth compounds.

1. Introduction

Since that bismuth is active against *Helicobacter pylori* it is widely used to treat gastric ulcers and active duodenal ulcers. Bismuth eliminates *H. pylori* in as many as 70% of treated patients when used alone, but when bismuth is added to amoxicillin the initial eradication rate approaches 95% at the end of four weeks (Lambert and Midolo, 1997). The increased use of bismuth in medicine is likely to lead to influencing the growth of other bacteria and fungi (both pathogens and saprophytes), particularly in the gut (Cornick et al., 1990; Manhart, 1990). It is important therefore that the effect of this element on bacteria and fungi be determined, especially when bacterial resistance to this element is increasing (Bierer, 1990; Domenico et al., 1996). Such increased medical use of bismuth has led to an increase in the concentration of the element in the environment (Mueller, 1989; Murata, 2002), a fact which provides another reason why its effect on individual bacteria and bacterial populations need to be determined. This is especially the case since bacteria are known to methylate bismuth and thereby increase its availability and toxicity in the environment (Feldmann et al., 1999).

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The aim of the work reported here was to investigate the effect of bismuth compounds on the growth of a range of bacteria and fungi (*in vitro*) and to determine the ability of these organisms to solubilise insoluble bismuth salicylate and accumulate it within their cells.

2. Materials and methods

The effect of bismuth compounds on the following was investigated: (A) Fungi: *Aspergillus niger*, *Aspergillus oryzae*, (Departmental stock). (B) Bacteria: *Escherichia coli*, and *Staphylococcus epidermidis* (Departmental stock). *Bacillus subtilis* (Medical School, Royal Hallamshire Hospital, Sheffield). (C) Yeast: *Candida albicans* 3153A (Departmental stock).

2.1. The effects of bismuth compounds on microbial growth and the solubilisation of insoluble bismuth were determined

2.1.1. Determination of soluble bismuth

Soluble bismuth was determined as described by Allport (1945). Filtrate (15 ml) was placed in a 25 ml volumetric flask, 5 ml of sulphuric acid (1 M), 1 ml of hypophosphoric acid (30% v/v), and 1 ml of potassium iodide (10% w/v) were then added and the mixture was then made up to 25 ml with distilled water. The intensity of the yellow colour formed was then measured at 460 nm using a spectrophotometer and the amount of soluble bismuth was determined by reference to a standard curve (0–100 μg bismuth ml^{-1}), prepared from a standard solution of soluble bismuth subnitrate.

2.2. In vitro effect of different amounts of bismuth compounds on the growth of bacteria

2.2.1. The following bacteria were used in these experiments: E. coli, B. subtilis and S. epidermidis

Bacteria were grown in (250 ml) Erlenmeyer flasks containing L-broth medium (100 ml). All flasks were autoclaved at 121 °C for 15 min. After autoclaving the bismuth compounds (2.63, 26.3, 262.8 mg) and (0.5, 1.5 g) were then added to the medium. The compounds were sterilized in a dry oven (160 °C) for overnight. The flasks were inoculated with (1 ml) of the (24 h) bacterial culture in liquid medium. Two types of controls were setup, one containing bismuth compounds without the bacterial inoculant and the other with bacteria, but lacking bismuth compounds. Three replicates were used, and all flasks were incubated at 37 °C with shaking for 24 h. After incubation (0, 5, 10, 24 h), 1 ml culture was added to 99 ml sterilized distilled water (the filtrate was then analysed for free bismuth). After further dilution, the bacterial suspension (1 ml) was then transferred to (99 ml) of sterilized distilled water. From the above solution, 0.1 ml was inoculated on Plate Count Agar (6 replicates) and incubated at 37 °C for 24 h. The final dilution used was 1×10^7 ml^{-1}. After incubation, bacterial counts were immediately determined.

2.3. In vitro effect of bismuth compounds on the growth of fungi (A. niger, A. oryzae and Penicillium sp.), and solubilization of bismuth compounds

The fungi were initially grown on Czapek Dox agar medium at 25 °C for 7 days.

To Czapek Dox liquid medium (100 ml), in 250 ml Erlenmeyer flasks (in triplicates), were added, (50 and 100 mg ml^{-1}) of bismuth salicylate compound, which had been sterilised in a dry oven at (160 °C) overnight. All flasks were autoclaved at 121 °C for 15 min. Each flask was inoculated with a fungal disc (4 mm). Two types of controls were set-up, one containing bismuth compounds without the fungal inoculant and the other with fungi, but lacking bismuth compounds. All flasks were incubated for 7, 14, 21 and 28 days at 25 °C with shaking (150 rpm). The contents of the flasks were filtered each week using Whatman No. 1 dried (at 60 °C overnight) filter papers. The filtrate was then analysed for biologically available bismuth. Mycelial dry weight was determined by weighing the filter papers (after washing the contents with 4% HCl to remove any remaining bismuth compounds) and drying at 60 °C overnight. The pH of the culture was determined as mentioned above.

2.4. In vitro effect of different amounts of bismuth compounds on pathogenic yeast

The yeast used in this experiment was: *C. albicans* 3153 A.

The above procedure (as bacteria) was repeated using Yeast Extract Peptone Dextrose (YEPD) broth; incubation was at 30 °C with shaking for 24 h.

2.5. Transmission electron microscope (TEM) studies

The culture of *A. niger* was incubated at 25 °C with shaking (150 rpm) for two weeks, before the mycelium was prepared for electron microscope study. Samples received primary fixation in 3% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for a period of 3 h at 4 °C. The samples were then washed three times at 30 min intervals in 10% sucrose in 0.1 M phosphate buffer at 4 °C followed by secondary fixation in 2% aqueous OsO_4 at room temperature for 1 h. Dehydration was carried out using the series of (75%, 95%, and 100% dried over anhydrous $CuSO_4$) ethanol with 15 min between changes. The samples were then embedded in Araldite resin and ultra thin sections were cut on a Reichert ultramicrotome. The section thickness was approximately 70–90 nm. Sections were captured in copper grids and stained with 3% uranyl acetate in 50% ethanol followed by staining with Reynolds lead citrate solution. The sections were examined on a Phillips EM.301 transmission electron microscope at accelerating voltage of 80 kV.

3. Results and discussion

In general, bismuth salicylate has significantly inhibited the growth of all of the bacteria tested as well as the pathogenic yeast, *C. albicans*, with inhibition increasing with increasing bismuth concentration and length of exposure. Of the three bacteria tested, *E. coli* was the least affected, followed by *B. subtilis* and *S. epidermidis* (Table 1).

Tables 2 and 3 show the concentrations of biologically available bismuth in media containing bismuth subsalicylate inoculated with the individual bacteria and *C. albicans*. Growth of *E. coli* had no affect on the amount of soluble bismuth, whilst in the medium with the presence of *B. subtilis* and *S. epidermidis* leads to reduction in available bismuth. The growth of *C. albicans* in contrast, leads to increase in the concentration of available bismuth, which were statistically significant after the addition of 100 mg^{-1} (Table 1).

Table 1 Percentage reduction in bacterial growth following addition of bismuth salicylate.

Hours incubation	5	10	24
B. subtilus			
50 mg ml^{-1}	81	92	100
100 mg ml^{-1}	95	100	100
E. coli			
50 mg ml^{-1}	66	68	100
100 mg ml^{-1}	72	74	84
S. epidermidis			
50 μg ml^{-1}	90	85	95
100 μg ml^{-1}	98	98	100
Candida			
50 mg ml^{-1}	68	90	84
100 mg ml^{-1}	72	88	91

Table 2 Percentage increase in fungal growth following addition of bismuth salicylate.

Days	7	14	21	28
A. niger				
50 mg ml^{-1}	10	25	10	50
100 mg ml^{-1}	403	420	512	550
A. oryzae				
50 mg ml^{-1}	10	25	10	14
100 mg ml^{-1}	502	423	500	549

Table 3 Concentration of free bismuth in media in which bacteria and yeast were grown in the presence of bismuth salicylate (μg 15 ml^{-1}).

Bacteria/yeast	Control	0.5 g	Control	1.5 g
Bacillus subtilis	48	0.1*	106	1.6*
Escherichia coli	48	45	106	104
Staphylococcus epidermidis	48	5*	106	11*
Candida albicans 3153 A	113	143*	218	271

Means of triplicate, significant difference from control value, $^{*}P \leqslant 0.05$.

The addition of the bismuth compounds to media did not immediately effect medium pH however over the 24 h incubation period, the pH of media in which bacteria grew declined, often markedly (Table 1). In the case of *S. epidermidis* cultures (containing bismuth subsalicylate), the pH fell from pH 7.8 to pH 4.9 whilst in the case of media supporting *C. albicans* the pH increased from pH 4.6 to pH 5.4; the fact that the growth inhibition occurred independent of these pH changes suggests that such changes did not cause the observed growth inhibitions.

4. Effect of bismuth compounds on the growth of fungi (*A. niger*, *A. oryzae* and *Penicillium* sp.), and the ability of fungi solubilise insoluble bismuth compounds

Bismuth subsalicylate caused a marked and statistically significant increase in fungal biomass (dry weight) in all three fungi, over the full 28 days incubation period (Table 1). Fungal dry weight generally increased with increasing amount of added

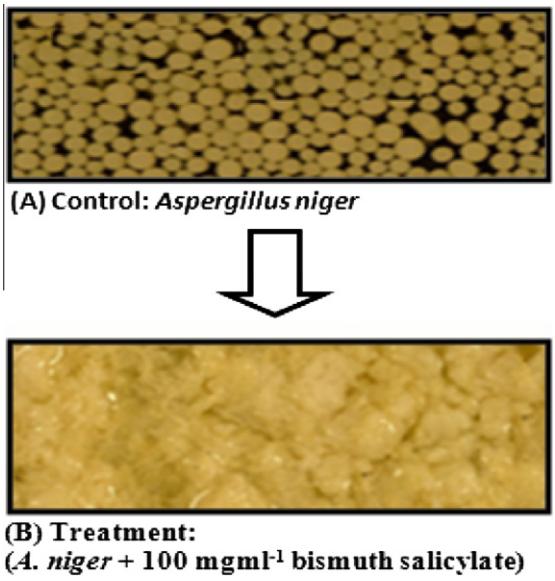

Figure 1 Fungal growth in Czapek Dox liquid medium.

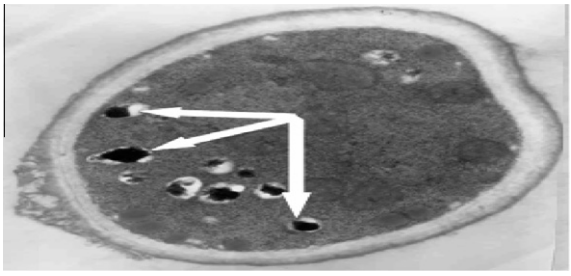

Figure 2 Electron micrograph of electron dense bodies (elemental bismuth, arrowed) located in the mycelium of *A. niger* grown in bismuth salicylate (Magnification ×15,500).

Table 4 Concentration of bismuth in media in which *Aspergillus niger* were grown in the presence of bismuth salicylate (100 mg ml^{-1}).

	Aspergillus niger			
Days	7	14	21	28
Control 0.5 g	221	219	260	248
Treatment 0.5 g	222	120*	102*	117*
Control 1.5 g	230	259	272	268
Treatment 1.5 g	186	89*	68*	262*

Means of triplicate, significant difference from control value, $^{*}P \leqslant 0.05$.

bismuth. In the case of *A. niger*, growth in bismuth salicylate led to a marked change in fungal morphology in shake culture. This change in morphology and growth stimulation was associated with bismuth uptake by the *A. niger*, a fact that was evidenced by the presence of electron-dense bodies in the mycelium after growth in the presence, but not in the absence, of bismuth salts. EDAX analysis showed that these electron dense bodies were comprised of elemental bismuth (Figs. 1 and 2).

Uptake of bismuth is confirmed by the fact that concentrations of soluble bismuth in the medium decreased in the presence of *A. niger*, generally significantly (Table 4).

5. Conclusion

Bismuth compounds are used in medicine to inhibit the growth of *H. pylori*, in the gut, so it is not surprising that they were shown here to inhibit the growth of the pathogenic bacteria (notably *S. epidermidis)*. Bismuth compounds also inhibited the growth of the pathogenic yeast, *C. albicans*. This may be of some clinical significance as *Candida* species are amongst a range of yeasts and bacteria, which inhabit in the human stomach and may, like *H. pylori*, play a role in the formation of gastric ulcers and even cancer (Wainwright, 2002).

The most surprising result to appear from these studies is the marked stimulatory effect that bismuth compounds have on the growth of filamentous fungi. The effect of bismuth compounds on these organisms appears not to have been previously reported. Clearly however, such a stimulatory response may again be of clinical importance, as filamentous fungi have also been reported to be present in the human stomach (Wainwright, 2002). One could imagine a scenario where treatment for *H. pylori* with bismuth compounds might lead to an overgrowth of filamentous fungi on the gastric mucosa. Again, this is of potential clinical significance because like yeasts, filamentous fungi have been implicated in the aetiology of stomach ulcers and cancer (Wainwright, 2002).

It could be argued however, that the observed increase in fungal biomass following growth in media containing the bismuth compounds was due to the ability of these organisms to adsorb particles to their surface. The ability of fungi to achieve such adsorption is well documented (Wainwright et al., 1986). Such surface adsorption of insoluble particles would contribute to the apparent dry weight of the fungus, making it appear as if bismuth compounds had stimulated mycelial growth. To eliminate such potential artefacts, the mycelium was washed well with HCl prior to drying and subsequent dry weight determination; thereby removing the problem of particulate-bismuth adsorption. The fact that the stimulatory effect of bismuth compounds on fungal growth is real was confirmed by visual observations (i.e. the biomass was clearly seen to be greater in the bismuth amended flasks than in the controls); such growth stimulation could obviously not have been due to anomalous increases in dry weight due to the adsorption of insoluble bismuth. It is not clear however, why bismuth compounds should stimulate fungal growth. Some of the increase in fungal biomass following growth in bismuth might have resulted from the accumulation of bismuth and its deposition within the mycelium; this would not however, explain the visible increase in biomass induced by bismuth. It is possible that bismuth may in some way facilitate the absorption of nutrients, or to enhance the removal of inhibitory waste products, thereby allowing growth to continue in excess of the values achieved in its absence. It is notable that whilst growth of the fungi and *C. albicans* led to an increase in the solubilisation of the bismuth compounds, this was not true of bacteria. It is possible therefore that the ability of fungi to solubilise these insoluble bismuth compounds is in some way linked to the resultant increases in biomass. Other than these suggestions, there appears to be no obvious reason why this element should stimulate fungal growth. Nadeau et al. (1992) showed similar deposition of electron dense bodies of bismuth in *Yersinia enterocolitica* incubated in the presence of bismuth subsalicylate.

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