The Ferredoxin-dependent Reduction of Chloramphenicol by Clostridium acetobutylicum

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SUMMARY

Chloramphenicol did not inhibit growth and protein synthesis when added (100 µg./ml.) to growing cultures of Clostridium acetobutylicum, even though the organism was sensitive to this antibiotic in plate assay. This paradox was explained by the finding that chloramphenicol was rapidly reduced and thus detoxified by actively growing cultures. Cell-free extracts of C. acetobutylicum reduced the aryl nitro group of chloramphenicol via a ferredoxin-dependent enzymic reaction for which pyruvate was the most effective primary electron donor. A number of other aryl nitro-compounds were similarly reduced.

INTRODUCTION

Chloramphenicol is an inhibitor of protein synthesis and halts the growth of a wide range of both Gram-positive and Gram-negative bacteria (Vazquez, 1966). Kiser, de Mello, Reichard & Williams (1950) reported that chloramphenicol (0.6 to 10 µg./ml.) prevented growth of eleven species of pathogenic Clostridium, while Mazurek (1950) found that at concentrations ranging from 0.1 to 100 µg./ml. the antibiotic was an effective inhibitor of the growth of some 30 Clostridium species.

During some studies on the effects of oxygen on Clostridium acetobutylicum (R. W. O'Brien & J. G. Morris, to be published) chloramphenicol was added to rapidly growing anaerobic cultures in an attempt to halt protein synthesis. Though the organism was quite sensitive to chloramphenicol in plate assay, its cultures continued to grow even when supplemented with relatively high concentrations of chloramphenicol (100 µg./ml.). In this paper we report that growing cultures of C. acetobutylicum rapidly reduced the nitro group of chloramphenicol (Fig. 1) via a ferredoxin-dependent reaction in which pyruvate served as the best electron donor. The reduced product at the same concentration was not toxic to the organism.

Fig. 1. Formula of chloramphenicol.

METHODS

Maintenance and growth of the organism. Clostridium acetobutylicum NCIB8052 was maintained as a spore culture in cooked meat medium, and the spores were germinated when required, by heat shocking at 80° for 5 min. Vegetative cultures were grown anaero-
bically under argon in 400 ml. quantities in 450 ml. jacketed vessels maintained at 35°. The medium contained (per l.): glucose, 10 g.; MgSO₄.7H₂O, 0·2 g.; MnSO₄.4H₂O, 0·01 g.; FeSO₄.7H₂O, 0·01 g.; casein hydrolysate (Oxoid), 4 g.; p-aminobenzoic acid, 1 mg.; thiamine. HCl, 1 mg.; and biotin, 2 μg. This mixture was sterilized by autoclaving (15 lb/in.² for 15 min.); K₂HPO₄ and KH₂PO₄ were then added aseptically as sterile solutions to final concentrations of 0·5 g./l., giving a final pH of 6·8. Growth was followed turbidimetrically at 680 nm. using a Unicam SP 600 spectrophotometer.

Preparation of cell extracts. Anaerobically grown organisms were harvested from cultures in their mid-exponential phase of growth (0·5 mg. dry wt organisms/ml.) and were then stored at -15° for 15 h. The organisms from 400 ml. of culture (200 mg. dry wt) were thawed in 20 mM-potassium phosphate buffer containing 20 mM-dithiothreitol and ruptured by passage through a chilled French pressure cell (Aminco, Silver Springs, Maryland, U.S.A.) at 12,000 lb/in.² Debris was removed by centrifuging at 25,000 g for 15 min. at 5°. Samples of extract (3 ml.) were depleted of ferredoxin by passage through a column of DEAE-cellulose (30 x 8 mm.; Whatman DE 11) which had been pre-equilibrated with 20 mM-potassium phosphate buffer at pH 7. The column (maintained throughout at 5°) was washed with the same buffer; the first yellow fraction (4 ml.) to be eluted contained the chloramphenicol-reducing enzyme. The column was then washed with 6 column vol. of the phosphate buffer followed by 10 column vol. of 0·2 M-KCl in the buffer. The ferredoxin (dark-brown band at the head of the column) was finally eluted (2 ml.) with 1 M-KCl in the phosphate buffer.

Assay methods. Both chloramphenicol and reduced chloramphenicol were assayed by the colorimetric method of Bessman & Stevens (1950). Plate assays of the inhibitory activity of chloramphenicol and reduced chloramphenicol were performed by the agar-diffusion cup method using reinforced clostridial medium agar (Oxoid) thickly seeded with Clostridium acetobutylicum; the plates were incubated anaerobically at 37° for 24 h. and the diameters of the zones of inhibition were then measured. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Phosphoroclastic activity was measured by determining the rate of anaerobic production of acetyl phosphate from pyruvate (Buchanan, Lovenberg & Rabinowitz, 1963).

Chemicals. Chloramphenicol was purchased from Sigma Chemical Co. Ltd (London) and reduced chloramphenicol was prepared by reduction of chloramphenicol with titanous trichloride (Glazko, Wolf & Dill, 1949). Nitrofurantoin was obtained from Smith, Kline & French, Ltd (Welwyn Garden City, Hertfordshire). The remaining aryl nitro compounds were from British Drug Houses, Ltd (Poole, Dorset).

RESULTS

Reduction of chloramphenicol by growing cultures

Chloramphenicol at 100 μg./ml. (a tenfold greater concentration than that previously reported by Mazurek (1950) to inhibit Clostridium acetobutylicum) had no effect on the growth of Clostridium acetobutylicum NCIB 8052 when added to a logarithmically growing culture containing 0·39 mg. dry wt of organisms/ml. (Fig. 2). Yet chloramphenicol was decidedly growth-inhibitory when tested by the plate assay procedure (Methods); 50 μg. chloramphenicol produced a zone of growth inhibition 1·7 cm. in diameter. In rapidly growing liquid cultures of C. acetobutylicum, chloramphenicol was quickly reduced (Fig. 3) at an initial rate of 30 μg./min./mg. dry wt of organisms. The product of reduction was identified by paper chromatography (Smith & Worrel, 1950a) as the arylamine formed by
complete reduction of the nitro group. This ‘reduced chloramphenicol’ (100 μg.) produced no zone of growth inhibition of *C. acetobutylicum* in the plate assay.

**Reduction of chloramphenicol by cell-free extracts**

Anaerobic cell-free extracts of *Clostridium acetobutylicum* reduced chloramphenicol when pyruvate was supplied as the electron donor (Table 1). The rate of enzymic reduction of chloramphenicol was almost doubled by methyl viologen (\(E'_o\) at pH 7 is \(-440\) mV), and was enhanced by either purified spinach ferredoxin or by crude ferredoxin from *C. acetobutylicum*. There was no reduction of chloramphenicol when either extract or pyruvate was omitted from the reaction mixture. Extracts depleted of ferredoxin by passage through a column of DEAE-cellulose (Methods) had only slight chloramphenicol-reducing activity (Table 1). The activity of this ferredoxin-depleted extract was again enhanced by the addition of methyl viologen or spinach ferredoxin or ferredoxin from *C. acetobutylicum* (Table 1). No reduction of chloramphenicol was obtained when FMN and FAD (0.1 mM each) were provided as potential electron carriers (with pyruvate supplied as primary electron donor), or when NADH and NADPH (2 mM each) were supplied as potential electron donors in place of pyruvate.

Contrary to the findings of Osteux & Laturaze (1952), with *Clostridium welchii* we were
unable to show any inhibition of the phosphoroclastic reaction by chloramphenicol; instead, we observed that the antibiotic would act as an electron acceptor in this reaction. Crude extracts of *C. acetobutylicum* assayed under semi-aerobic conditions (Methods)

Table 1. Enzymic reduction of chloramphenicol by extracts of Clostridium acetobutylicum

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Crude cell extract</th>
<th>Ferredoxin-depleted extract</th>
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<tbody>
<tr>
<td>Complete</td>
<td>14.5</td>
<td>1.1</td>
</tr>
<tr>
<td>+ methyl viologen</td>
<td>33.6</td>
<td>8.6</td>
</tr>
<tr>
<td>+ ferredoxin (<em>C. acetobutylicum</em>)</td>
<td>24.3</td>
<td>6.3</td>
</tr>
<tr>
<td>+ ferredoxin (spinach)</td>
<td>18.9</td>
<td>3.9</td>
</tr>
<tr>
<td>- pyruvate</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Table 2. Reduction of aryl nitro compounds by cell-free extracts of Clostridium acetobutylicum

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rate of reduction (µmoles/mg. protein/h.)</th>
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<tbody>
<tr>
<td>3-Nitrophenol</td>
<td>27.6</td>
</tr>
<tr>
<td>2-Nitrobenzoic acid</td>
<td>19.0</td>
</tr>
<tr>
<td>2-Nitrobenzaldehyde</td>
<td>15.1</td>
</tr>
<tr>
<td>4-Nitrobenzoic acid</td>
<td>12.7</td>
</tr>
<tr>
<td>3-Nitrobenzoic acid</td>
<td>10.2</td>
</tr>
<tr>
<td>2-Nitrophenol</td>
<td>10.0</td>
</tr>
<tr>
<td>'Nitrofurantoin'</td>
<td>8.4</td>
</tr>
<tr>
<td>3-Nitroaniline</td>
<td>5.6</td>
</tr>
<tr>
<td>2,4-Dinitrophenol</td>
<td>4.4</td>
</tr>
</tbody>
</table>

normally did not show any phosphoroclastic activity unless methyl viologen was added to the assay mixture. Yet when chloramphenicol was used in place of methyl viologen there was an identical yield of acetyl phosphate from pyruvate. For example, extract
Chloramphenicol reduction by C. acetobutylicum

(0.65 mg. protein/ml.) which in 10 min. at 30\(^\circ\) in the presence of 5 mM-methyl viologen produced 3.0 mm-acetyl phosphate from 10 mM-pyruvate formed 3.1 mm-acetyl phosphate when the methyl viologen was replaced by 5 mM-chloramphenicol.

Reduction of other aryl nitro compounds

In the presence of methyl viologen, and when supplied with pyruvate, the extracts rapidly reduced 2- and 3-mononitrophenols, 2-, 3- and 4-mononitrobenzoates and 2-nitrobenzaldehyde (Table 2). The rate of reduction of 3-nitrophenol was almost as great as that of chloramphenicol, while 3-nitroaniline and 2,4-dinitrophenol were reduced at about one-sixth this rate. Though not listed in Table 2, 4-nitrophenol, 2-nitroaniline and 4-nitroaniline were also reduced, but even more slowly.

Like chloramphenicol, the antibacterial agent ‘nitrofurantoin’, i.e. N-(5-nitro-2-furfurylidine)-1-aminohydantoin, was an effective inhibitor of growth of Clostridium acetobutylicum in plate assay, but was ineffectual when added (180 \(\mu\)g./ml.) to a growing culture. Both growing cultures and cell-free extracts of C. acetobutylicum reduced the nitro group of nitrofurantoin (Table 2).

Discussion

The ferredoxin-dependence of the chloramphenicol-reducing system in Clostridium acetobutylicum was shown by: (a) stimulation of the activity of crude cell extracts by ferredoxin (spinach or C. acetobutylicum), or by the low potential, one-electron carrier methyl viologen; (b) the loss of reducing ability following removal of ferredoxin from the extracts; (c) partial restoration (25\%) of the activity of the ferredoxin-depleted extracts by addition of ferredoxin or methyl viologen; and (d) failure of other electron carriers and donors to replace ferredoxin or methyl viologen. The electrons required for the reduction of the chloramphenicol were supplied by pyruvate, which in C. acetobutylicum is metabolized via the phosphoroclastic reaction to yield acetyl CoA, CO\(_2\) and H\(_2\). Electrons normally transferred in this reaction (via ferredoxin) to hydrogenase (Valentine, 1964) were here diverted to reduce chloramphenicol or some other aryl nitro-compound. The inability of ferredoxin to restore full activity to a ferredoxin-depleted extract was most probably due to inactivation of the enzyme during treatment with DEAE-cellulose; crude extracts irreversibly lost 30\% of their chloramphenicol-reducing activity over a 6 h. period even when stored under H\(_2\).

It is possible that the finding by Osteux & Laturaze (1952) that chloramphenicol inhibited the phosphoroclastic activity of Clostridium welchii is attributable to their assaying this activity by following evolution of H\(_2\). Should C. welchii, like C. acetobutylicum, be capable of reducing chloramphenicol, production of H\(_2\) by the phosphoroclastic reaction would be diminished in proportion to the amount of chloramphenicol reduced. This would mean that when gas evolution was employed as the sole criterion of phosphoroclastic activity, an artefactual inhibition by chloramphenicol must be observed. Again, while Osteux & Laturaze (1952) reported that 2,4-dinitrophenol (20 mM) inhibited the phosphoroclastic activity of C. welchii, del Campo, Ramirez, Paneque & Losada (1966) observed ferredoxin-dependent reduction of this compound by the hydrogenase of C. pasteurianum. In our hands, 2,4-dinitrophenol (20 mM) caused no inhibition of the phosphoroclastic activity of crude extracts of C. acetobutylicum when this was measured as the rate of production of acetyl phosphate from pyruvate.

Detoxification of chloramphenicol by reduction could also explain the finding of...
Altermeier, McMurrin & Alt (1950) that chloramphenicol could not combat a *Clostridium welchii* infection in guinea pigs if definite gas production was demonstrable before antibiotic treatment was started. The incidence of death was also higher in animals whose treatment with chloramphenicol was delayed for four or more hours after infection with *C. welchii*, than in those in which antibiotic treatment coincided with experimental infection. Presumably in the 4 h period the organisms had become established and had started to multiply after generating a low $E_h$ in their environment (Bullen, Cushnie & Stoner, 1966).

Other bacteria, including strains of *Escherichia coli* and species of Bacillus, reduce the nitro group of chloramphenicol (Smith & Worrel, 1949, 1950b, 1953; Merkel & Steers, 1953). However, the ability to reduce chloramphenicol is inducibly acquired by these organisms (Merkel & Steers, 1953) and, at least in strains sensitive to chloramphenicol, its reduction was a very slow process. Indeed, the chloramphenicol resistance of *E. coli* $R^+$ (Shaw, 1967) and of a resistant strain of *Staphylococcus aureus* (Shaw & Brodsky, 1968) was due not to reduction of the antibiotic but to its acetylation by a specific acetyl transferase. Smith & Worrel (1949) suggested that in *E. coli* chloramphenicol was reduced by ‘dehydrogenases’ of which formate and lactate dehydrogenases were most effective, while Egami, Ebata & Sato (1951) suggested that chloramphenicol was reduced by the nitrite reductase of *Streptococcus haemolyticus*, though once again the measured rate of reduction was slow. This latter explanation is certainly not tenable in the case of *Clostridium acetobutylicum* since we have been unable to detect any nitrite or nitrate reductase activities in extracts of this organism. Instead we found that *C. acetobutylicum* constitutively possessed the means of reducing numerous aryl nitro-compounds (Table 2).

Our findings emphasize the need for caution in assessing the growth-inhibitory effectiveness of antibacterial agents that are subject to detoxification by reduction. Particular care should be taken when testing the toxicity of such substances to notoriously reducing organisms, such as species of Clostridium. In such cases the efficacy of the antibiotic should be assayed using proliferating organisms (growing cultures) as well as freshly inoculated media.

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REFERENCES


Chloramphenicol reduction by C. acetobutylicum


