SHORT COMMUNICATIONS

A Relatively Rapid Procedure for the Preparation of Lysis-susceptible Forms of *Mycobacterium smegmatis*

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INTRODUCTION

Mycobacteria are difficult to lyse. Wayne & Gross (1968) described a method for the lysis of mycobacteria for isolation of DNA. However, it involves a total of 97 h treatment and so is of no value in metabolic studies. Mizuguchi & Tokunaga (1970) described a more rapid procedure and Asano *et al.* (1973) modified this so that osmotically sensitive forms of *Mycobacterium phlei* could be isolated and could subsequently be lysed. However, these procedures are still rather lengthy and, in our hands, have given disappointing results.

More recently, a report has appeared which describes the preparation of spheroplasts from lysozyme-sensitive mutants of *Mycobacterium smegmatis* (Yabu & Takahashi, 1977). However, apart from the use of special mutants, the method involves incubation with lysozyme and methionine for about 9 d before a high yield of spheroplasts is reported.

Here we describe a procedure which gives lysis-susceptible bodies relatively rapidly from *M. smegmatis* and describe some of their properties. This procedure has been used to permit the lysis of *M. smegmatis* on sucrose gradients for the study of damage to DNA in this organism (MacNaughton & Winder, 1977). It can also be used in the preparation of DNA in high yield from *M. smegmatis*, whereas the Mizuguchi & Tokunaga (1970) method has given us poor results.

RESULTS AND DISCUSSION

Figure 1 shows the changes in turbidity (as measured by apparent absorbance at 700 nm), and in susceptibility to lysis by sodium dodecyl sulphate (SDS) or by alkali, that occurred when cultures of *M. smegmatis* in late-exponential phase in nutrient broth/Tween 80 were treated by a modification of the procedure of Asano *et al.* (1973). Addition of sucrose and glycine caused an abrupt decrease in turbidity (mainly as a result of the change in refractive index of the medium) and almost complete cessation of growth. There was no change in susceptibility to lysis, as indicated by the fact that addition of SDS or alkali did not cause any further change in turbidity. Subsequent addition of lysozyme to the cultures led to the conversion of a substantial proportion of the bacteria to lysis-susceptible bodies, as shown by a marked increase in clearing on addition of SDS or alkali to samples. However, we found it necessary with *M. smegmatis* to add 500 μg lysozyme ml^{-1} instead of the 50 μg ml^{-1} employed by Asano *et al.* with *M. phlei* and, even with the higher concentration of lysozyme, the lysis-susceptible bodies did not rupture to a significant extent in response to osmotic shock. The formation of the lysis-susceptible bodies was accompanied by a marked increase in turbidity (Fig. 1). Presumably this increase was due to changes in the shape of the bacteria. Since their dimensions are of the same order as the wavelength of the light used, changes in their shape would be expected to have a distinct effect on light scattering (Koch, 1961; Wyatt, 1973).

Fig. 1. Effect of incubation in glycine/sucrose and treatment with lysozyme on the turbidity and susceptibility to lysis of *M. smegmatis* cultures. *Mycobacterium smegmatis* was grown in 250 ml nutrient broth, containing 0.06 % (v/v) Tween 80, in 1 l flasks on a rotary shaker at 37 °C. Growth was followed by removing samples and measuring turbidity as 'absorbance' at 700 nm (○). In late-exponential phase (15.5 h; arrow A), glycine was added to 0.2 M and sucrose to 0.5 M and incubation was continued. After a further 2 h (arrow B), lysozyme was added to 500 $\mu\text{g ml}^{-1}$ and incubation was again continued. At intervals throughout the experiment, samples were removed from the treated tubes for direct measurement of turbidity (●), and for measurement of turbidity in 1 % (w/v) SDS (□) or in 0.25 M-NaOH (■), as an indication of susceptibility to lysis.

Fig. 2. Effect of addition of lysozyme with various concentrations of sucrose on the turbidity and susceptibility to lysis of *M. smegmatis* cultures. *Mycobacterium smegmatis* was grown as described in the legend to Fig. 1. (a) In mid-exponential phase (arrow), lysozyme was added to 500 $\mu\text{g ml}^{-1}$, without sucrose (●, ○), or with sucrose to 0.1 M (■, □), 0.3 M (▲, △) or 0.5 M (▼, ▽). At intervals thereafter, samples were removed for direct measurement of turbidity (closed symbols) and for measurement of turbidity in 1 % (w/v) SDS (open symbols), as an indication of susceptibility to lysis. (b) Lysozyme was added in late-exponential phase (arrow). Sucrose concentrations were 0 (●, ○), 0.2 M (■, □) or 0.5 M (▼, ▽). Samples were removed as in (a).

Thus, the Mizuguchi & Tokunaga (1970) procedure as modified by Asano *et al.* (1973) did, after slight further modification, convert this organism to lysis-susceptible forms, though not into osmotically fragile forms. However, further investigation showed that, with *M. smegmatis*, the 2 h incubation with glycine prior to addition of lysozyme played no useful role in the conversion. Figure 2 shows the results when lysozyme, with or without the addition of sucrose as an osmotic stabilizer, was added directly to growing cultures. Addition of SDS or alkali to samples showed that the bacteria were converted more rapidly and more completely to lysis-susceptible bodies than was the case when they had been preincubated with glycine (compare Fig. 2 with Fig. 1). The conversion to lysis-susceptible bodies appeared to be most rapid when 0.2 to 0.3 M-sucrose was added with the lysozyme, though it did not appear to affect the final degree of conversion (Fig. 2).

As in the experiment shown in Fig. 1, the conversion to susceptible bodies was accompanied by a marked increase in turbidity. This increase was smaller the higher the molarity of the sucrose. The effect of the concentration of sucrose can be accounted for quantitatively in terms of its effect on the refractive index of the medium, if one assumes that any osmotic swelling of the lysis-susceptible bodies is only slight, or slow, and if one assumes that the bodies have a refractive index of about 1.4. Thus, the results provide some evidence for both of these assumptions. For osmotic swelling of spheroplasts or mitochondria, the absorbance varies nearly *inversely* with the volume (Koch, 1961). The slow decrease in absorbance, which followed the initial increase when young cultures were used in the absence of sucrose and which is referred to below, was due to slow swelling under these conditions.

Conversion to the lysis-susceptible bodies occurred only when growing cultures were used, but occurred almost equally well with late-exponential as with mid-exponential cultures (Fig. 2). However, the properties of the bodies were somewhat different in the later cultures, as will be referred to below.

Examination, by phase contrast microscopy, of the cultures which had been converted to lysis-susceptible forms showed that very few unchanged bacteria remained. Most had been converted to swollen corrugated rods or rods with spherical ends. A proportion were converted to spherical bodies, and this proportion was higher the younger the cultures used. Thus, the lysozyme treatment removed sufficient of the wall structure for the protoplasts to swell or to escape partially and to become susceptible to lysis by detergent or alkali, but only a minority escaped fully from the walls under the conditions which we have described. Hence, the term 'spheroplasts' cannot be applied to the majority of these lysis-susceptible bodies.

The stability of the lysis-susceptible bodies varied with the age of the cultures. The increase in turbidity, presumably due to a change in the shape of the bacteria, which accompanied the conversion was greater the younger the cultures (Fig. 2) and in very young cultures a fivefold increase in turbidity was observed. Further, when conversion took place in the absence of sucrose, the initial increase in turbidity was followed by a decline in the case of the younger cultures [visible in Fig. 2(a) but more noticeable in still younger cultures], while this decline did not take place in older cultures (Fig. 2b). In the absence of sucrose, the spherical bodies which were released by the younger cultures were seen under the microscope to swell slowly and become vacuolated. However, bodies even from young cultures showed moderate stability, while with the older cultures the lysis-susceptible bodies were quite stable to osmotic shock, even if this were followed by heat treatment (to 50 °C), or by addition of isobutanol (to 20 %, v/v) or of β -mercaptoethanol (to 10 mM), though they were lysed readily by alkali or SDS.

In view of their osmotic stability, various attempts were made to culture the lysis-susceptible bodies, both in the presence and absence of osmotic stabilizers and using various growth conditions. These did not appear to be successful, nor was there any outgrowth of normal bacteria, indicating that the conversion was complete. Millipore filtrates from the preparations were also inoculated on to slopes of nutrient agar or Löwenstein-Jensen medium.

After prolonged incubation, slight growth occurred on these slopes and when the organisms were examined microscopically they resembled bacterial L-forms. However, further investigations would be necessary before it could be concluded definitely that these bodies from *M. smegmatis* can grow as L-forms.

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