A Rapid Method for Preparing Single-cell Suspension of *Aspergillus* Conidia Depleted in Germination Inhibitor

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INTRODUCTION

Conidia which are not 'wetttable' in water, such as those of *Aspergillus nidulans*, stick together in clusters of irregular shapes in liquid suspension due to the presence of lipids on their outer walls. In order to prepare a homogeneous single-celled suspension, a 'wetting agent' such as Tween-80 is used. Such agents can break down the lipid barrier to diffusion of water-soluble compounds and so allow self-inhibitors of spore germination to diffuse out of the cell, as has been demonstrated in *Puccinia graminis* (Allen, 1955) and *Aspergillus nidulans* (Scott, Alderson & Papworth, 1972). In the latter organism progressive loss of the germination inhibitor from the conidium results in a corresponding increase in sensitivity to radiation; constant and maximal lethality is exhibited only after the inhibitor has been effectively removed (Scott *et al.* 1972). Since the Tween-80 cannot be removed before conducting assays for radiation effects, an alternative method of preparing a homogeneous single-celled suspension of inhibitor-depleted spores was sought.

The germination inhibitor of *Puccinia graminis* has been identified as the *cis* and *trans* isomers of methyl ferulate (Macko, Staples, Allen & Renwick, 1972). This inhibitor was extracted from an aqueous suspension of the spores by diethyl ether, a solvent used by other workers to obtain lipid-free spores of *Aspergillus* and *Penicillium* for physiological studies (Koval, Sivers & Redchits, 1968). Diethyl ether was therefore tested as a possible alternative agent to Tween-80 for studies of radiation response.

METHODS

Preparation of conidial suspensions. Conidia from a single colony of the *meth G1 bi A1* strain of *Aspergillus nidulans*, grown for 5 to 6 days at 37 °C on complete medium, were suspended in ether water (1 %, v/v, solution of diethyl ether in distilled water), vortexed for 15 min to break up conidial chains and filtered through sterile cotton wool to remove mycelial debris. Conidia were concentrated on a 0.45 µm Millipore filter, washed with 20 ml ether water and resuspended at a density of approximately 1×10^7 conidia/ml in 30 ml ether buffer (1 %, v/v, solution of diethyl ether in buffer, without Tween-80) in a 20 oz medical flat (screw cap). This flat was then subjected to a rotating washing action at 42 rev./min at room temperature (approx. 20 °C). At various times samples were withdrawn from the flat, reconstituted on a Millipore filter, washed with 20 ml of buffer without Tween-80 or diethyl ether, and resuspended in the same fluid at a density of approximately 2×10^6 conidia/ml. This method of preparation, where the volatile diethyl ether which is present during the early stages of preparation is removed by washing and evaporation before the suspension is irradiated, contrasts with the Tween-80 method (Scott *et al.* 1972), where the Tween-80 was present throughout the preparation, and in the irradiated suspension.

Media and techniques. With the exception of Difco purified agar replacing Oxoid No. 3 agar, the media and techniques used to assess conidial survival (Scott *et al.* 1972) and to reveal mutagenic effects (Scott, Alderson & Papworth, 1973) were as previously described. Conidia were plated before and immediately after irradiation of the ether-free samples. The methionine revertants observed after 5 to 6 days' incubation on the plates without methionine were classified as A (large green colonies), B (heavily-pigmented brown colonies) or C (small green colonies with a white hyaline edge) in accordance with the system described by Lilly (1965).

Ultraviolet irradiation. The necessary thin uniform films were easily obtained when suspensions contained the surfactant Tween-80. In the absence of Tween-80, only incomplete films of variable thickness are formed on surfaces such as smooth plastic, but sufficiently uniform films of surfactant-free suspensions could be obtained by confining the liquid in a space of the desired dimensions.

Conidial suspensions (approx. 7 ml) were positioned beneath a 7 cm diam quartz disc, supported by a polythene washer 0.15 cm above the surface of a Petri dish, and irradiated for 60 s at a distance of 35.5 cm from a low-pressure mercury vapour lamp where the incident flux was 2 J/m²/s (95 % of the incident u.v. flux was at 254 nm). All manipulations were carried out under a low level of illumination by yellow light (blue minus) in a darkened room.

RESULTS

The germination of diethyl ether-prepared conidia, observed by time-lapse cinematography, was indistinguishable from that of inhibitor-depleted conidia prepared with Tween-80 (Scott & Alderson, 1974).

Samples of conidia were withdrawn at various times from an ether buffer suspension rotating in a medical flat and after removal of the ether they were u.v. irradiated in ether-free buffer. In duplicate experiments, using a suspension of single colonies of the same strain exposed to the same fixed dose of u.v. radiation, survival was constant for periods of rotation varying from 5 to 420 min (Fig. 1). However, the survival of samples withdrawn from Tween-80 suspension and given the same u.v. dose progressively decreased during the first 300 min of rotation, after which a constant value was obtained (Fig. 1). The final value of the weighted mean value for the surviving fraction was 0.53 ± 0.07 for the Tween-80 suspension and 0.50 ± 0.07 for the ether-prepared spores.

The induction of revertants to methionine independence by the same u.v. dose was determined in inhibitor-depleted conidial samples prepared either by 20 min ether treatment or 420 min treatment in Tween-80. Each experiment was duplicated, with the same result. For the fixed dose of u.v. radiation used, the weighted mean value of the induced mutation frequency per 10⁶ survivors for A, B and C mutants added together for the ether buffer-prepared conidia was 144 ± 30 ($\chi^2_1 = 0.31$, $P = 0.8$) and that for the Tween-80 prepared conidia was 143 ± 31 ($\chi^2_1 = 0.005$, $P = 0.9$). The frequencies of A, B and C mutants, considered separately, were also the same, irrespective of the method of preparation of the spores.

With γ -irradiation in anoxic condition, survival and induced mutation frequencies were also independent of the method of spore preparation, but when the irradiation was in oxygen there was a nearly two-fold difference both in level of survival and induced mutation frequency, which can be attributed to an interaction between irradiation and the presence of Tween-80 (unpublished observation).

Fig. 1. The survival of conidial samples withdrawn from a population at various times after the start of rotation in a buffer containing Tween-80 (Δ , $\blacktriangle$) or diethyl ether ($\circ$, $\bullet$) and then irradiated with an u.v. dose of 120 J/m^2 . The vertical lines represent plus or minus one standard error on the log scale, and the open and solid symbols represent duplicate experiments.

DISCUSSION

The results demonstrate that diethyl ether can be used as an alternative agent to Tween-80 for the preparation of single-cell suspensions of *Aspergillus* conidia depleted of their germination inhibitor. One important advantage of preparing inhibitor-depleted spore suspensions with diethyl ether instead of Tween-80 is that preparation takes 5 min instead of 5 h. Moreover, diethyl ether, unlike Tween-80, can be removed from the conidial suspension before exposure to radiation. In some circumstances Tween-80 and perhaps other surfactants can modify radiation response.

Dr R. J. Munson and Mr P. Gray kindly performed the dosimetry of the u.v. lamp using an N.P.L. calibrated thermopile. The radiation cell was built, to exacting engineering standards, by Mr C. F. Wright and Mr G. W. J. Wheeler.

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