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# Effect of sub-lethal treatment with formalin on the germination of *Aspergillus fumigatus* spores

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SUMMARY

Sub-lethal exposure of *Aspergillus fumigatus* spore suspensions to formalin resulted in prolongation by 1–22 days of the period of less than one day normally needed by spores to produce visible growth in Sabouraud's liquid medium at 37° C.; the degree of delay depended on the concentration of formalin and the duration of exposure, and was due to an increase in the germination-time of spores. The formalin concentration could be adjusted so as to affect the germination-time of almost all spores in a suspension without reducing viability. The effect on germination was not abolished by thorough washing or treatment with sodium sulphite. The spores of four different strains of *A. fumigatus* and of cultures aged 3 to 14 days reacted similarly to formalin treatment. Although of greatly reduced virulence for mice, affected viable spores were still capable of producing infection and death following intravenous inoculation, provided they were not eliminated by the host before germination occurred.

## INTRODUCTION

Unlike many other germicides, formalin is capable, even when considerably diluted, of destroying all forms of microbic life (Williams, Blowers, Garrod & Shooter, 1966). Nordgren (1939) reviewed much of the earlier literature. Müller (1920) considered that the action of formalin on anthrax spores was partially reversible by ammonia, but Gegenbauer (1921) was unable to confirm this. Hailer (1921*a*, 1921*b*) concluded that sodium sulphite was able to reverse the action of formaldehyde solution on anthrax spores and certain vegetative bacteria, but Nordgren (1939) preferred to leave this question open, although his experiments led him to accept that objects exposed to formaldehyde should be treated with sodium sulphite before being subjected to sterility tests. In experiments on disinfection by gaseous formaldehyde (Report, 1956) the treatment of material with sodium sulphite solution or the incorporation of sodium sulphite into culture media was sometimes helpful in removing free formaldehyde. Schultz & Gebhardt (1935) reactivated formaldehyde-treated bacteriophage merely by dilution, and Ross & Stanley (1938) found that dialysis at pH 3 reactivated formalin-inactivated tobacco-mosaic virus. Englesberg (1952) suggested that the large differences which he found in viable counts of formaldehyde-treated *Pseudomonas fluorescens* indicated reactivation by certain media, but Nash & Hirch (1954) offered the

alternative explanation that damaged organisms might require a better growth medium than undamaged organisms. They found that a mixture of dimedone and morpholine reactivated formalin-treated bacteria and ascribed the effect to interference with the lethal process rather than to reversal of some action which had proceeded to completion, or to simple neutralization of free formaldehyde.

The literature contains little information on the action of formaldehyde on fungi. The present report arises from tests of formalin on *Aspergillus fumigatus* spores, made as a prelude to experiments on immunization with dead vaccines. Certain striking effects on germination were demonstrated and the virulence of spores treated with sub-lethal doses of formalin was examined.

## MATERIALS AND METHODS

### *Strains of* A. fumigatus

Purified seed cultures of strains $AF_1$, $AF_2$, $AF_3$, $AF_4$ and $AF_5$ (Smith, 1972) were stored at $-20°$ C. until used.

### *Preparation of spore suspensions*

Spores harvested from Sabouraud's dextrose-agar cultures (Oxoid, CM 41) grown for 3 days at 37° C. were triple-washed, suspended in nutrient broth (Oxoid, CM 67) and counted, as described by Smith (1972).

### *Formalin-treatment of spore suspensions*

Dilutions of formalin in distilled water were added to spore suspensions in the proportion 1:9 (v/v) to produce the required final concentration. A similar proportion of sterile distilled water alone was added to the control spore suspensions. The original formalin was stated by the manufacturers to contain 37 to 41 % w/v formaldehyde and 11 to 14 % methanol. Treatment with formalin was carried out at 4° C. with constant mechanical agitation.

### *Examination of viability and delayed germination of formalin-treated spores*

Unless stated otherwise, Sabouraud's dextrose liquid medium (Oxoid, CM 147), pH 5·7 approximately, was dispensed in 10 ml. volumes in 25 ml. McCartney bottles and inocula consisted of small standard loopfuls of spore suspension; the standard loop used throughout the experiments was made from 23 SWG platinum and had an internal diameter of approximately 2 mm. Thus, any residual free formalin was diluted to an ineffective level. To permit the entry of sufficient air for complete growth of *A. fumigatus*, the bottles were closed either with loose metal caps or cotton-wool stoppers. The incubator atmosphere was humidified during protracted incubation at 37° C. to reduce the rate of evaporation of culture medium. In one experiment sodium sulphite was added to autoclaved Sabouraud's liquid medium, which was then re-sterilized by filtration. In any experiment, cultures were examined at least once a day for the first appearance of visible growth, and incubation of negative cultures was continued for at least 2 weeks after the last positive

culture was recorded. As shown later, delays in the time taken for visible growth to occur were due to increases in germination time.

### *Mice*

Females weighing 20 g. were obtained from the outbred, closed colony of Swiss white mice referred to by Smith (1972) as the SA colony. Spores suspended in nutrient broth were inoculated intravenously in 0·25 ml. doses.

## RESULTS

### *Effect on germination of exposing spore suspension to different concentrations of formalin for different times*

A spore suspension prepared from strain $AF_1$ was treated for 11 days with concentrations of formalin ranging from 0·05 to 1·8% (equivalent to approximately 0·02 to 0·72% formaldehyde). After each day of treatment, a small standard loopful from each bottle including that containing untreated control suspension was cultured in Sabouraud's liquid medium. Viable counts of the control suspension made at the beginning and end of the 11-day period of treatment and on one intermediate occasion showed that the number of living spores remained constant throughout at 139–178 million per ml.

Table 1 shows that each daily subculture of the control suspension first produced visible growth within 24 hr. incubation, but formalin induced a delay the length of which usually increased as the concentration of formalin and the duration of exposure increased. The longest delay produced in this experiment was 19 days. Once visible growth appeared, it proceeded at the normal rapid rate, and in similar experiments repeated microscopical examination of formalin-treated suspensions revealed only ungerminated spores up to the day before the first appearance of visible growth. Thus, the effect of the formalin was on the germination-time of the spores.

Treatment with 1·8% formalin for 24 hr. failed to sterilize, but 0·4% produced sterility after 7 days' treatment. Although some evaporation of the medium had occurred by the time the experiment was terminated, each negative bottle readily supported growth from an inoculum of 0·1 ml. medium containing approximately eight spores. This showed that concentration of the constituents of the medium, including the small amount of free formalin, which occurred during prolonged incubation had no appreciable inhibitory effect on growth.

A further experiment showed that the action of formalin on washed, saline suspensions of spores was similar to that on nutrient broth suspensions.

### *Quantitative examination of the effect on germination-time and viability of exposing $AF_1$ spore suspension to different concentrations of formalin for 24 hr.*

A preliminary experiment is briefly described, as it assists in interpreting certain aspects of the main experiment. Approximately 18 spores produced normal growth in 4 ml. Sabouraud's liquid medium containing 0·0016% formalin, but no macro-

Table 1. *Effect of formalin on the germination of strain $AF_1$ spores*

| Duration of exposure to formalin (days) | Time taken (days) for visible growth to occur from subcultures of spore suspension exposed to formalin in concentrations (%) of | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0·05 | 0·1 | 0·2 | 0·4 | 0·6 | 0·8 | 1·0 | 1·2 | 1·4 | 1·6 | 1·8 |
| 1 | 1 | 1 | 1 | 2 | 3 | 5 | 6 | 7 | 10 | 12 | 14 | 15 |
| 2 | 1 | 1 | 1 | 2 | 4 | 7 | 11 | 10 | N | N | N | N |
| 3 | 1 | 1 | 2 | 3 | 6 | 12 | N | N | N | N | N | N |
| 4 | 1 | 1 | 2 | 3 | 8 | N | N | N | N | N | N | N |
| 5 | 1 | 1 | 2 | 4 | 11 | N | N | N | N | N | N | N |
| 6 | 1 | 1 | 3 | 6 | 20 | N | N | N | N | N | N | N |
| 7 | 1 | 2 | 3 | 6 | N | N | N | N | N | N | N | N |
| 8 | 1 | 2 | 3 | 8 | N | N | N | N | N | N | N | N |
| 9 | 1 | 2 | 4 | 9 | N | N | N | N | N | N | N | N |
| 10 | 1 | 2 | 3 | 10 | N | N | N | N | N | N | N | N |
| 11 | 1 | 2 | 2 | 13 | N | N | N | N | N | N | N | N |

N = no growth.

Table 2. *Quantitative examination of the effect of formalin on germination-time in relation to viability of strain $AF_1$ spores*

| Number of living or dead spores in 9 ml. liquid medium | Time taken (days) for visible growth to occur from decimal dilutions of a spore suspension exposed for 24 hr. to formalin in concentrations (%) of | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0·2 | 0·6 | 1·0 | 1·4 | 1·8 |
| $152 \times 10^6$ | 1 | 3 | N* | N† | N† | N† |
| $152 \times 10^5$ | 1 | 2 | 4 | 12 | N | N |
| $152 \times 10^4$ | 1 | 2 | 4 | 3 | 15 | N |
| $152 \times 10^3$ | 1 | 2 | 5 | 9 | 17 | N |
| $152 \times 10^2$ | 1 | 2 | 5 | 10 | 23 | N |
| $152 \times 10$ | 1 | 2 | 5 | 10 | N | N |
| 152 | 1 | 2 | 5 | 11 | N | N |
| 15 | 1 | 2 | 6 | N | N | N |
| 1–2 | 1 | N | 6 | N | N | N |

N = no growth, but at the end of the experiment, inoculation with 0·1 ml. medium containing nine *A. fumigatus* spores gave normal growth on incubation.

N* = no growth, and medium produced mycelial growth only from nine spores inoculated at the end of the experiment.

N† = no growth, and medium failed to produce growth from nine spores inoculated at the end of the experiment.

scopic growth in the same medium containing 0·04% formalin. Mycelium and surface spores were produced in the presence of 0·008% formalin, but growth was slightly retarded.

In the main experiment (Table 2) a spore suspension prepared from strain $AF_1$ was treated for 24 hr. with five different concentrations of formalin ranging from 0·2 to 1·8%. A series of decimal dilutions, ranging from $10^{-1}$ to $10^{-9}$ and prepared by transferring 1 ml. volumes of suspension into 9 ml. volumes of Sabouraud's

liquid medium, was made from each of the five bottles containing formalin-treated spores, and also from a control bottle containing untreated spores. In addition, a viable count of the control portion of the spore suspension was made by the plate method (Smith, 1972), thus enabling the number of spores, alive or dead, in the limiting dilution of the six series to be calculated. The 54 dilution bottles were incubated at 37° C., and the times of first appearance of visible growth as revealed by daily examination are shown in Table 2.

Even 1–2 spores from the control suspension produced visible growth within 24 hr. Formalin in low concentrations induced only a delay in germination, but in higher concentrations it also had a sporicidal effect; a concentration of 1·8 % produced complete sterilization.

Treatment with 0·6 % formalin had no effect on viability, but the delays in production of visible growth from spores were distributed as follows: not more than 1 in 10 spores was affected for less than 5 days; not more than 1 in $10^5$ for less than 4 days; not more than 1 in $10^6$ for less than 3 days. It is more than likely that the number of spores unaffected by 0·6 % formalin – if such spores existed at all – was considerably less than 1 in $10^6$, but a firm statement cannot be made because the bottle containing $152 \times 10^5$ spores (Table 2) may have contained enough free formalin to exert some slight effect. Between 1 in $10^3$ and 1 in $10^4$ spores survived treatment with 1·4 % formalin, but of the survivors at least 99 % had a germination-time which was prolonged by 14–22 days.

Table 2 shows that at least four and probably five bottles containing $10^{-1}$ dilutions of spore suspension ($152 \times 10^6$ spores) possessed sufficient free formalin to prevent growth or affect it adversely. It also appears that free formalin carried over into the $10^{-2}$ dilution of spore suspension treated with 1·0 % formalin played some part in delaying the appearance of growth; this should not be confused with the phenomenon with which this paper is mainly concerned – i.e. delayed germination in the absence of an effective concentration of free formalin.

### *Effect of formalin on four additional strains of* A. fumigatus

Spore suspensions from strains $AF_2$, $AF_3$, $AF_4$ and $AF_5$ were adjusted to the opacity of Brown's tube 20, treated with 0·6 % formalin for 3 days, and subcultured in Sabouraud's liquid medium with a small standard loop after each day of treatment. Untreated control suspensions were also subcultured. Table 3 shows that the effect of 0·6 % formalin on germination of spores from these four additional strains was similar to that already found with strain $AF_1$.

### *Effect of formalin on spores from cultures of different ages*

Spore suspensions from 7-day-old and 14-day-old cultures of strain $AF_1$ were prepared, formalinized and subcultured by the method described in the previous experiment. After treatment with 0·6 % formalin for 1, 2 and 3 days, the spores from 7-day culture took 5, 6 and 13 days respectively to produce visible growth in subcultures, as compared with less than 1 day for control spores. The corresponding results for spores from 14-day culture were 5, 7 and 13 days. These figures were similar to those obtained with spores from 3-day-old cultures (Tables 1 and 3).

Table 3. *Effect of formalin on the germination of spores of strains $AF_2$, $AF_3$, $AF_4$ and $AF_5$*

| Duration of exposure to formalin (days) | Time taken (days) for visible growth to occur from subcultures of spore suspensions prepared from four strains of *A. fumigatus* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $AF_2$ | | $AF_3$ | | $AF_4$ | | $AF_5$ | |
| | C | F | C | F | C | F | C | F |
| 1 | 1 | 3 | 1 | 6 | 1 | 5 | 1 | 5 |
| 2 | 1 | 7 | 1 | 10 | 1 | 9 | 1 | 10 |
| 3 | 1 | 11 | 1 | 18 | 1 | 13 | 1 | 13 |

Spore suspensions with the opacity of Brown's tube 20 were treated with 0·6% formalin. C, control suspension; F, formalin-treated suspension.

### *Failure of thorough washing to abolish the effect of formalin on germination-time of spores*

Spore suspension prepared from strain $AF_1$ and adjusted to the opacity of Brown's tube 20 was treated with 0·6% formalin for 24 hr., and an equal volume was used as control suspension. Both the formalinized and control suspensions were washed six times by centrifugation and resuspension in 10 ml. volumes of nutrient broth with thorough agitation. Subculture showed that the production of visible growth from control and formalin-treated spores took 1 and 5 days respectively. Comparison with the data in Tables 1 and 3 indicates that the washing procedure had no significant effect.

### *Failure of sodium sulphite treatment to abolish the effect of formalin on germination-time of spores*

A suspension of strain $AF_1$ spores was made to the opacity of Brown's tube 28. Control spore suspension and suspension treated with 0·6% formalin for 24 hr. were subcultured by means of a standard loop in 10 ml. volumes of Sabouraud's liquid medium, and in the same medium containing five doubling concentrations of sodium sulphite ranging from 0·25 to 4·0%. Cultures of the formalin-treated spores in each of the six media were made in groups of six replicates.

Subcultures of control spores invariably gave visible growth within 24 hr., although sodium sulphite had an obvious inhibitory effect, varying from just appreciable to almost complete in 0·5% and 4·0% concentrations, respectively. Formalin-treated spores in medium without sodium sulphite gave rise to visible growth in 3 days. This time was increased by the presence of sodium sulphite; it varied from 4–6 days in a 0·25% concentration to 7–9 days in a 4·0% concentration.

Thus the experiment gave no evidence that sodium sulphite could reverse the effect of formalin on spores. If any such tendency existed, it was too slight to reveal itself in the presence of the anti-fungal effect of the sodium sulphite.

Table 4. *Reduction in virulence of spores by sub-lethal treatment with formalin*

| Days after inoculation | Deaths in mice following intravenous inoculation with: control spores in doses of $51{\cdot}2 \times 10^6$ | control spores in doses of $12{\cdot}8 \times 10^6$ | control spores in doses of $3{\cdot}2 \times 10^6$ | formalinised spores in doses of $51{\cdot}2 \times 10^6$ | formalinised spores in doses of $12{\cdot}8 \times 10^6$ | formalinised spores in doses of $3{\cdot}2 \times 10^6$ |
|---|---|---|---|---|---|---|
| 7 | 12/12 | 12/12 | 1/12 | 0/6 | 0/6 | 0/6 |
| 16 | — | — | 3/12 | 2/6 | 0/6 | 0/6 |
| 28 | — | — | 5/12 | 2/6 | 1/6 | 1/6 |
| | | | (5/7) | (2/4) | (1/5) | (0/5) |

Suspension containing $205 \times 10^6$ spores per ml. was treated with 0·6 % formalin for 24 hr. Figures in parentheses indicate number of survivors showing active renal aspergillosis when killed 28 days after inoculation.

*Virulence of spores after sub-lethal treatment with formalin*

A suspension containing $205 \times 10^6$ viable spores per ml. was prepared from strain $AF_1$. A portion was treated for 24 hr. with 0·6 % formalin whilst a second portion was used as control suspension. The formalin-treated and control suspensions were then thoroughly washed six times and resuspended to the original volumes in nutrient broth. Decimal dilutions of control and treated suspensions ranging from $10^{-1}$ to $10^{-10}$ were made in Sabouraud's liquid medium and incubated at 37° C.; the time of first appearance of growth in each bottle was recorded. Even 1–2 control spores produced visible growth within 24 hr. The formalin treatment was shown not to have killed any spores, but delays in production of visible growth from spores were distributed as follows: not more than 1 in $10^2$ spores, 1 in $10^4$ spores and 1 in $10^6$ spores were affected for less than 5 days, 4 days and 1 day respectively. Less than 1 in $10^6$ but more than 1 in $10^7$ were not appreciably affected by the formalin.

Six groups of mice were inoculated intravenously with doses of (millions) 51·2, 12·8 and 3·2 of the freshly prepared formalin-treated or control spores. The mortality patterns and the number of survivors found to be infected when killed 28 days after inoculation are shown in Table 4. It is clear that spores whose germination-time was increased by formalin treatment were of greatly reduced virulence. Nevertheless, reference to the data given by Smith (1972) on the MID and MLD of strain $AF_1$ for SA mice leads to the following conclusion: the small number of infections and deaths which did occur in mice given formalin-treated spores could not have resulted from the minute proportion of the inocula ($< 1$ in $10^6$ spores) which remained unaffected by the formalin. Thus, spores whose germination-time was increased by formalin treatment were still capable of producing disease, provided they were not eliminated by the host's defence mechanisms before germination occurred.

## DISCUSSION

In relating these findings to earlier work on formaldehyde the important differences between bacterial endospores and the phialospores (conidia) of *A. fumigatus* should be borne in mind.

Fungal spores can remove large quantities of certain fungicides from dilute solutions, often accumulating up to 1·0 % of their own weight (Miller, McCallan & Weed, 1953; Byrde, 1966; Somers, 1966). Müller & Biedermann (1952) showed that germination of *Alternaria tenuis* spores was retarded by sub-lethal doses of copper, but that the spores later recovered; retained copper could be removed by exchange and chelation, with restoration of germination potential. Fildes (1940) found that the anti-bacterial action of mercury was reversible by -SH compounds.

The delaying effect of certain sub-lethal concentrations of formalin on the germination of *A. fumigatus* spores was a phenomenon quite distinct from the inhibition of germination and growth which may result from the carry-over of free formalin into subcultures. It was noted not only when subcultures were made by a method which diluted any free formalin to an ineffective level, but also in formalin-treated spores that had been thoroughly washed. It could not be reversed by treatment with sodium sulphite, but protracted incubation at 37° C. in Sabouraud's liquid medium eventually restored the ability to germinate after delays which varied from 1 to 22 days according to the duration of exposure to formalin and the concentration used. A particular degree of formalin treatment was capable of delaying the germination of almost all spores in a suspension, without reducing viability. In testing for sterility of formalin-treated suspensions of *A. fumigatus* spores, it is important to appreciate that subcultures should be incubated for at least a month. Although fungal spores of different ages may vary in their susceptibility to fungicides (McCallan, 1930), *A. fumigatus* spores from cultures aged 3, 7 and 14 days did not differ significantly in respect of the effect of formalin on germination-time.

*A. fumigatus* spores whose germination-time had been prolonged by treatment with sub-lethal concentrations of formalin were of greatly reduced virulence for mice, yet were still capable of producing infection and death. It seems possible that their ability to produce disease was completely unaffected by formalin, provided that they were not eliminated by the host's defence mechanisms before germination occurred.

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