# THE FUNGISTATIC ACTIVITY OF METHYL AND PROPYL HYDROXYBENZOATES AND A MIXTURE OF THESE AGAINST *PENICILLIUM SPINULOSUM*

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The fungistatic activity of the methyl and propyl esters of *p*-hydroxybenzoic acid and of a mixture of these esters has been investigated. A mucilaginous substance is formed by fungal spores in contact with low concentrations of hydroxybenzoates in liquid medium. A difficulty in reading fungistatic end points from this cause was resolved by using a microscope.

The use of the esters of *p*-hydroxybenzoic acid as preservatives has long been established in the food and cosmetic industries (Sabalitschka, 1924, 1929). In recent years these esters marketed commercially as "Nipagin M" (methyl ester) and "Nipasol M" (propyl ester), have found increasing use as preservatives for pharmaceutical and related preparations (Gershenfeld and Perlstein, 1939; Lawrence, 1955; Schimmel and Husa, 1956; Boehm and Jones, 1957).

The British Pharmaceutical Codex of 1934 included references to the methyl and propyl esters of *p*-hydroxybenzoic acid, but it was not until the edition of 1949, that these compounds were formulated in a solution for eyedrops. The Codex of 1959 recommends the use of the propyl ester at a concentration of 0·05 per cent w/v for aqueous preparations and 0·1 per cent w/v as a preservative in creams and emulsions and the methyl ester at a concentration of 0·1 to 0·2 per cent w/v for aqueous preparations. The Codex solution for eyedrops contains 0·023 per cent w/v of methyl ester and 0·011 per cent w/v of propyl ester, a mixture thought to show potentiation (Schimmel and Husa, 1956).

The fungistatic action of methyl and propyl hydroxybenzoates, alone, and in admixture has been evaluated in both liquid maltose medium and in solid agar medium using roll tube counts. *Penicillium spinulosum* was chosen as a test organism since its suitability for such purposes has been established (Gerrard, Harkiss and Bullock, 1960).

## Experimental

*The test organism. Penicillium spinulosum.* Strain 42237 of the Commonwealth Mycological Institute.

*Preparation of spore suspensions.* Suspensions of single spores in sterile water were prepared from 21 day old cultures grown on malt agar slopes by the method previously described by Gerrard, Harkiss and Bullock (1960).

*Double strength liquid maltose medium.* A 10 per cent w/v solution of maltose in water, distributed in 5 ml. volumes into tubes capped with metal caps and sterilised by autoclaving.

*Solid roll tube medium.* Malt extract (6 per cent w/v), rose bengal (0·025 per cent w/v) and agar (3 per cent w/v). The medium was distributed into tubes (4 ml. volumes) and sterilised by autoclaving.

*Preparation of media containing the esters.* Stock solutions in sterile water were prepared with methyl ester 0·15 per cent w/v, propyl ester 0·04 per cent w/v, ester mixture; methyl ester 0·057 per cent and propyl ester 0·029 per cent w/v (combined ester concentration 0·086 per cent w/v). The esters used were of B.P.C. standard.

*Final liquid media* for use were prepared by mixing suitable volumes of ester stock solution, double strength maltose medium (5 ml.) and sterile water to give 10 ml. volumes containing the following ester concentration ranges; methyl ester 0·07 to 0·007 per cent w/v, propyl ester 0·02 to 0·002 per cent w/v, ester mixture 0·04 to 0·004 per cent w/v combined concentration. *Final solid media* for use were prepared by incorporating the esters into the solid medium to give the following concentration ranges, methyl ester 0·05 to 0·015 per cent w/v, propyl ester 0·042 to 0·015 per cent w/v, ester mixture 0·045 to 0·015 per cent w/v combined concentration.

*Fungistatic Activity*

*Liquid medium.* To each tube of medium containing one of the various concentrations of ester was added 0·1 ml. of spore suspension (containing about 7,000 spores per ml.). Each ester concentration was replicated five times and the tubes incubated at 25° and examined for growth every day for 21 days. A viability control on the spore suspension was made simultaneously.

*Solid medium.* For each ester concentration six roll tubes were prepared each inoculated with 1 ml. of a spore suspension containing approximately 40 spores per ml. The roll tubes were incubated at 25°, examined daily for 21 days and the highest colony count recorded. Early experiments had shown that no formation of new colonies was apparent after 21 days incubation. Replicate experiments, three in all, were made.

## Results

*Fungistatic Action in Liquid Maltose Medium*

The fungistatic action of the esters against the test organism was assessed by their inhibition of macroscopic, visible growth attributable to mycelium formation. It was found difficult to determine the precise ester concentration range at which no growth occurred. At the lower concentrations full and typical mould growth, comparable with that in the viability control, occurred. As the concentration increased, visible growth decreased but a uniform turbidity developed. At higher concentrations no visible growth occurred. The contents of the media tubes were deposited by centrifuging (3,150 R.C.F. for 15 min.) and examined microscopically. Three conditions were seen. (i) In those tubes which showed no macroscopic visible growth, denoted in Table I by —, the deposit contained mould spores only. (ii) In those tubes which showed a uniform turbidity rather than a typical mould growth, the deposit contained mould spores, irregular in shape and associated with a mucilaginous

material. These tubes are denoted in Table I by ±. (iii) In those tubes which showed macroscopic visible growth, denoted in Table I by +, the deposit consisted of typical mycelium. The source of mucilaginous material would seem to be the mould spores themselves. This material evidently appears as a result of the action of low concentrations of the esters upon the germinating spores.

TABLE I

THE RECOVERY IN MALTOSE BROTH CONTAINING DIFFERENT CONCENTRATIONS OF ESTERS

| | | | Concentration (per cent w/v) | Result |
|---|---|---|---|---|
| Methyl hydroxybenzoate .. .. .. .. .. .. | | | 0·052 | − |
| | | | 0·045 | ± |
| | | | 0·030 | ± |
| | | | 0·022 | + |
| | | | 0·015 | + |
| Propyl hydroxybenzoate .. .. .. .. .. .. | | | 0·016 | − |
| | | | 0·014 | − |
| | | | 0·012 | − |
| | | | 0·010 | ± |
| | | | 0·008 | ± |
| | | | 0·006 | + |
| Ester mixture .. | Me 0·0114 | Pr 0·0058 | Combined | |
| | | | 0·0172 | − |
| | | | 0·0129 | ± |
| | | | 0·0086 | + |

\+ Presence of mycelium.
± Uniform turbidity.
− No growth, undeveloped spores only present.

The results show that the methyl ester inhibits mycelial growth at a concentration of 0·03 per cent although turbidity is present. A concentration of 0·052 per cent is required to give a clear solution with no turbidity. The propyl ester inhibits mycelial growth at a concentration of 0·008 per cent, and turbidity is absent at a concentration of 0·012 per cent. The mixed esters inhibit mycelial growth and turbidity at a combined concentration of 0·0172 per cent.

*Fungistatic Action in Solid Medium*

In the control tubes an average of 40 colonies per roll tube developed and maximum colony formation was attained within 21 days incubation. The methyl ester prevented colony formation at a concentration of 0·05 per cent, and allowed 50 per cent colony formation at a concentration of 0·0425 per cent and was ineffective at a concentration of 0·035 per cent. The propyl ester prevented colony formation at a concentration of 0·024 per cent, allowed 50 per cent colony formation at 0·021 per cent, and was ineffective at a concentration of 0·018 per cent. The ester mixture prevented colony formation at a concentration of 0·036 per cent, combined esters, and was ineffective at a concentration of 0·031 per cent.

## DISCUSSION

The methyl and propyl esters of *p*-hydroxybenzoic acid and mixtures of these effectively inhibit the growth of *P. spinulosum* in both liquid and solid media.

A mutual potentiating effect is evident in mixtures of the esters investigated (Tables I and II) as has been reported by others (Schimmel and Husa, 1956; Boehm and Jones, 1957). In general the esters are more active in liquid medium than in solid medium and the Codex recommendations allow for this factor.

TABLE II

EFFECT OF ESTER CONCENTRATION IN THE RECOVERY MEDIUM UPON THE COLONY COUNT

| | Concentration (per cent) | | | Viability (per cent) |
|---|---|---|---|---|
| Methyl hydroxybenzoate .. | 0·05 | | | Nil |
| | 0·045 | | | 3·0 |
| | 0·0425 | | | 49·2 |
| | 0·040 | | | 73·9 |
| | 0·0375 | | | 90·0 |
| | 0·035 | | | 100·0 |
| Propyl hydroxybenzoate .. | 0·024 | | | Nil |
| | 0·021 | | | 50·0 |
| | 0·018 | | | 100·0 |
| Mixture of esters .. .. | Me | Pr | Combined | |
| | 0·024 | 0·0120 | 0·036 | Nil |
| | 0·021 | 0·0105 | 0·0315 | 100·0 |

The fungistatic end points recorded in liquid maltose medium (Table I) may be compared with those of Neidig and Burrell (1944), who reported the methyl ester to be fungistatic against *Penicillium glaucum* in senna syrup at a concentration of 0·07 per cent and the propyl esters at a concentration of 0·03 per cent. Boehm and Jones (1957), reported that neither of the esters, when incorporated singly in glucose broth, to a concentration of 0·02 per cent, was active against *Penicillium brevicaule*, whereas a combined ester concentration of 0·02 per cent was fungistatic.

The type of procedure described, in which the fungistat is placed in contact with mould spores in nutrient liquid medium has proved difficult to interpret. The usual method in which visual growth is looked for after adequate periods of incubation is complicated by a turbidity developing in those tubes containing concentrations of ester approaching that which totally inhibits growth. This turbidity is not comparable with the full mould growth seen in the viability control and in those tubes containing totally ineffective concentrations of esters, but is such that it would not normally be recorded as no growth. A turbidity effect has not been reported by other workers referred to here and in comparing their values with those in the present work this factor must be considered. Thus, the actual criterion taken in reporting a fungistatic concentration in this work, has been the presence or absence of mycelium as checked microscopically.

The measurement of fungistatic concentration of the esters incorporated in solid medium gave results which concur with those of Schimmel and Husa (1954). They incorporated the esters in agar medium which was inoculated with *P. glaucum* and plated out. Colony formation was prevented by the methyl ester at a concentration of 0·05 per cent and by the propyl ester at a concentration of 0·025 per cent.

An observation of special interest has been the fall in the percentage of spores developing with small increase in concentration of ester. This was particularly evident in the case of the propyl ester and the ester mixture, where difference in concentration of 0·006 and 0·0045 per cent, respectively represented the difference required to bring sub-fungistatic concentration up to a fully fungistatic concentration. With the methyl ester an increase in concentration of 0·015 per cent was required to give the full fungistatic effect to a sub-fungistatic concentration. This phenomenon is related to the relative efficacy of the esters as fungistatic agents and may represent the presence of different mechanisms of action.

## REFERENCES

Boehm, E. and Jones, E. (1957). *J. Soc. Cosmetic Chem.*, Jan., 1–12.
Gerrard, H. N., Harkiss, A. V. and Bullock, K. (1960). *J. Pharm. Pharmacol.*, **12**, 127*T*–133*T*.
Gershenfeldt, L. and Perlstein, D. (1939). *Amer. J. Pharm.*, **111**, 227–287.
Lawrence, C. A. (1955). *J. Amer. pharm. Ass., Sci. Ed.*, **45**, 457–464.
Neidig, C. P. and Burrell, H. (1944). *Drug. Cosmetic Ind.*, **54**, 408–410.
Sabalitschka, T. H. and Boehm, E. (1929). *Arch. Pharm. Berl. dtsch. pharmaz. ges.*, 267–272. Through *Chem. Abstr.*
Sabalitschka, T. H. (1924). *Pharm. Monat.*, **5**, 235–255. Through *Chem. Abstr.*
Schimmel, J. and Husa, W. J. (1956). *J. Amer. pharm Ass., Sci. Ed.*, **45**, 204–208.