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The effect of benzoic acid, phenol and hydroxybenzoates on the oxygen uptake and growth of some lipolytic fungi

STELLA M. RIVERS* AND V. WALTERS†

Oxygen uptake by lipolytic fungi on arachis oil is in the order *Aspergillus flavus* (brown) > *A. niger* > *Rhizopus arrhizus* > *Rh. cohnii* > *A. flavus* (green). Respiration is stimulated by low concentrations of benzoic acid, hydroxybenzoates and phenol, but with higher concentrations it is inhibited. Concentrations which give relatively low oxygen uptake are also fungistatic. The factors governing partitioning in oil-water systems are used to calculate the inhibitory concentration of benzoic acid in the aqueous phase.

MICRO-ORGANISMS can multiply in emulsions containing preservatives (Atkins, 1950; Eggins & Walters, 1963). The preservation of emulsions has been reviewed by Wedderburn (1964) and the activity of antibacterials in two-phase systems by Bean, Heman-Ackah & Thomas (1965). We report the effect of toxic agents on the respiration and growth in agar medium of five lipolytic fungi, *Aspergillus flavus* Link (green), *A. flavus* Link (brown), *A. niger*, *Rhizopus arrhizus* Fischer and *Rh. cohnii.*

Experimental

The materials, suspensions containing 150×10^6 *Aspergillus flavus* (brown) spores per ml and 300×10^6 ml^{-1} of the other species, and the method of measuring oxygen uptake (Umbreit, Burris & Stauffer, 1964) were as described by Rivers (1965). Salts solution was a Czapek's solution modified as follows, $NaNO_3$ (0·4%), KH_2PO_4 (0·1%), NaCl (0·1%), $MgSO_4.7H_2O$ (0·1%), $FeSO_4.7H_2O$ (0·002%) in water. The control flasks contained 1·5 ml salts solution, 0·5 ml spore suspension, 0·15 ml arachis oil and water to 3 ml. Reaction flasks included suitable concentrations of benzoic acid A.R., sodium benzoate A.R., methyl hydroxybenzoate B.P., propyl hydroxybenzoate B.P., or phenol A.R., dissolved initially in the oil or salts solution; these substances were also included in the agar culture medium which consisted of salts solution 50%, arachis oil 10%, "Oxoid" agar 2% and water. The oil was added aseptically to the aqueous phase and 20 ml quantities of the well-shaken media poured, immediately before setting, into Petri dishes. The plates were inoculated centrally on their surfaces with one-drop volumes of *Rh. arrhizus* or *A. flavus* (brown) spore suspensions and incubated at 30°. The diameters of the zones of growth were measured after 7 and 14 days. Aseptic precautions and sterile materials were used, where appropriate, throughout.

From the Department of Pharmacy, University of Ife, Ibadan Branch, Ibadan, Nigeria.

* Present address: School of Pharmacy, Bristol College of Science and Technology, Bristol 7.

† Present address: School of Pharmacy, University of London, Brunswick Square, London, W.C.1.

Results

The percentages of antifungal agents refer to the overall concentrations unless otherwise stated, and all results are the means of replicate experiments.

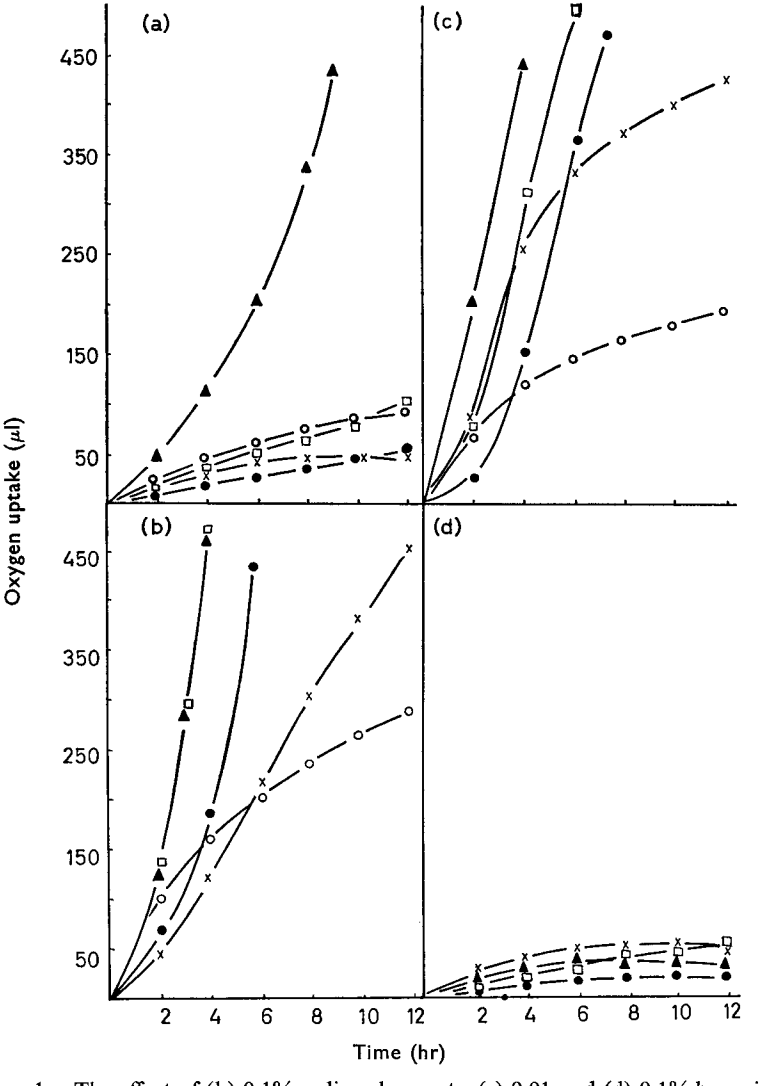

FIG. 1. The effect of (b) 0·1% sodium benzoate, (c) 0·01 and (d) 0·1% benzoic acid on the oxygen uptake of *A. flavus* (brown) — ▲ — ▲ —. *A. niger* — □ — □ —. *Rh. arrhizus* — ○ — ○ . *Rh. cohnii* — ● — ●—. *A. flavus* (green) — X — X —. *Rh. arrhizus* had no uptake in the presence of 0·1% benzoic acid. Controls (a) contain: 0·15 ml arachis oil, 0·5 ml spore suspension, 1·5 ml salts solution, water to 3 ml. Reaction suspensions include the test substance at overall concentration stated.

Sodium benzoate 0·1% and benzoic acid 0·01% stimulated oxygen uptake, but benzoic acid 0·1% suppressed respiration, immediately with *Rh. arrhizus* and after some hours with the other species except *A. flavus* (green) (Fig. 1). The effect on *Rh. arrhizus* and *A. flavus* (brown) of benzoic acid 0·1%, obtained by dissolving it in either the oil or the aqueous phase, was the same, indicating equilibrium distribution. After 12 hr there were no viable spores remaining in these two reaction mixtures; subsequent inocula in Sabouraud medium gave no growth on incubation at 30°.

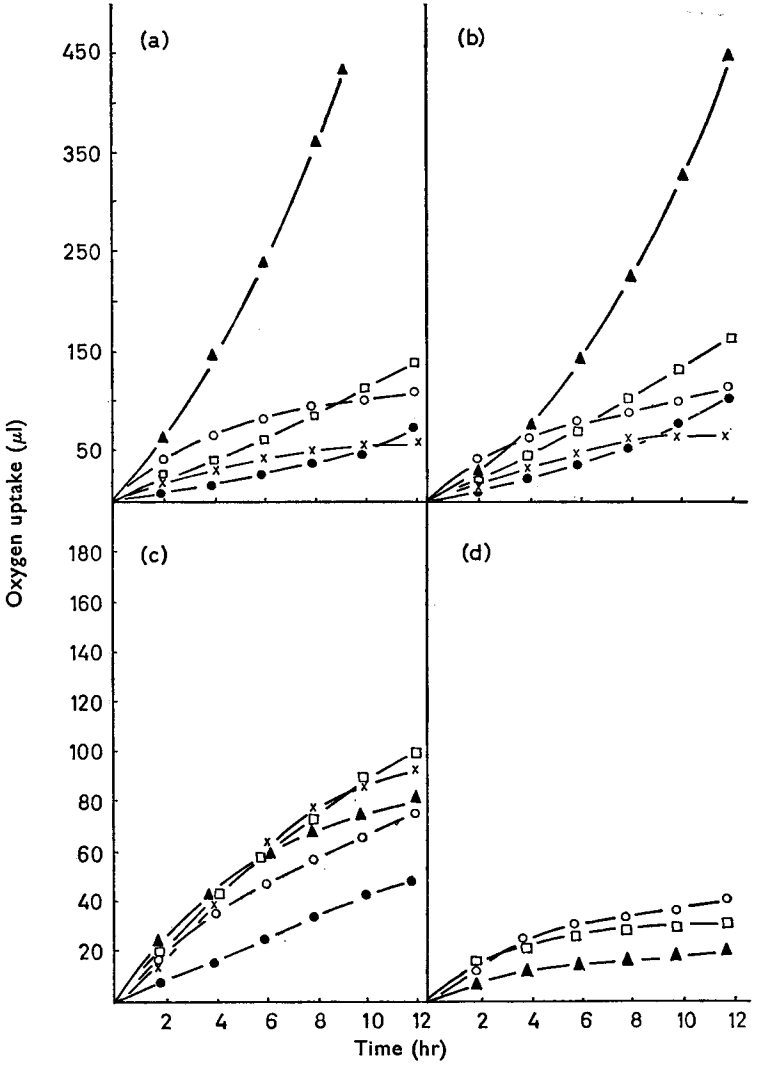

FIG. 2. The effect of concentration of phenol (a) 0·025%, (b) 0·05%, (c) 0·25% and (d) 0·5%, on oxygen uptake. Symbols as in Fig. 1. No experiments were made with *Rh. cohnii* and *A. flavus* (green) with 0·5% phenol.

Phenol in low concentration, 0·025 and 0·05%, had no effect on the oxygen uptake of *Rh. arrhizus* and *A. flavus* (green), whereas that of *Rh. cohnii* and *A. niger* was slightly increased and *A. flavus* (brown) decreased. With 0·25% the uptake of all organisms except *A. flavus* (green) was depressed and especially that of *A. flavus* (brown); the decrease was greater with 0·5% (Fig. 2).

Methyl hydroxybenzoate 0·01% had no effect on *A. flavus* (green and brown) but the other organisms were stimulated as were all with 0·1%; the response with 0·01% was greatest with *A. niger* and *Rh. cohnii*. Propyl hydroxybenzoate 0·01% also had a slight stimulating effect and 0·05% more so, except with *A. flavus* (brown) (Fig. 3). Methyl and propyl hydroxybenzoates, 0·023 and 0·012% (equivalent to the concentrations in Solution for Eye-drops), stimulated the oxygen uptake of *Rh. arrhizus* (from 113 μl in 12 hr for the control to 213 μl) but that of *A. flavus* (brown) was relatively unchanged.

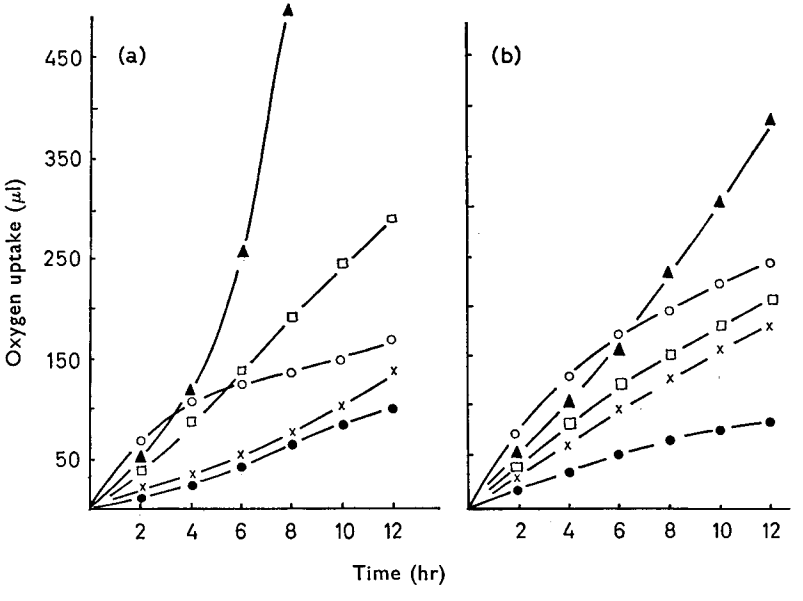

FIG. 3. The effect of (a) methyl hydroxybenzoate, 0·1% and (b) propyl hydroxybenzoate, 0·05%, on oxygen uptake. Symbols as in Fig. 1.

A. flavus (brown) and *Rh. arrhizus* gave visible growth on the culture medium within 1 and 4 days respectively but in the absence of oil there was no growth even after 14 days. Media containing benzoic acid, 0·1 and 0·2%, and phenol, 0·25 and 0·5%, inhibited the growth of both organisms. *Rh. arrhizus* was more susceptible, since its growth was also inhibited by benzoic acid 0·02%, and methyl hydroxybenzoate 0·1% (Table 1). Normal metabolism was affected by propyl hydroxybenzoate and phenol since the mycelia, where growth occurred, were more felted and sporulation was retarded.

TABLE 1. EFFECT OF ANTIFUNGAL AGENTS ON COLONY GROWTH AND ON OXYGEN UPTAKE OF SPORES

Organism	Antifungal agent	Overall concentration %	Colony diameter mm after: 7 days	Colony diameter mm after: 14 days	Oxygen uptake µl in 12 hr (or stated hr)
A. flavus (brown)	Benzoic acid	0·01	56	71·5	467 (5 hr)
		0·02	54	74	—
		0·1	0	0	23
		0·2	0	0	—
" "	Methyl hydroxy-benzoate	0·01	57	67	420 (10 hr)
		0·02	53	64	—
		0·1	39	60	506 (8 hr)
" "	Propyl hydroxy-benzoate	0·01	55	68	426 (8 hr)
		0·02	52	64	—
		0·05	38	60	389
" "	Methyl and propyl hydroxy-benzoate	0·023 and 0·012	54	68	524 (7 hr)
" "	Phenol	0·05	38	72	445
		0·1	14	27	—
		0·25	0	0	83
		0·5	0	0	21
" "	Controls: with oil	0	55	75	471 (7 hr)
	without oil	0	0	0	81
Rh. arrhizus	Benzoic acid	0·01	17	38	220
		0·02	0	0	—
		0·1	0	0	0
		0·2	0	0	—
" "	Methyl hydroxy-benzoate	0·01	16	37	149
		0·02	11	33	—
		0·1	0	0	164
" "	Propyl hydroxy-benzoate	0·01	15	38	160
		0·02	13	34	—
		0·05	5	16	245
" "	Methyl and propyl hydroxy-benzoate	0·023 and 0·012	6	27	213
" "	Phenol	0·05	7	24	112
		0·1	4	5	—
		0·25	0	0	75
		0·5	0	0	41
" "	Controls: with oil	0	17	30	111
	without oil	0	0	0	60

Colony diameters and oxygen uptake are means of results of replicate plates and reaction flasks at 30°
Agar culture medium: 2 ml arachis oil and 18 ml inorganic salts-agar, inoculated on the surface with one drop of spore suspension.
Oxygen uptake reaction mixtures: 0·15 ml arachis oil, 0·5 ml spore suspension, 1·5 ml salts solution, water to 3 ml.
Antifungal agents at overall concentrations given were included.

Discussion

When arachis oil was used as the sole carbon source, the oxygen uptake of the fungi increased in the order, *A. flavus* (green), *Rh. cohnii*, *Rh. arrhizus*, *A. niger* and *A. flavus* (brown).

Stimulation of respiration occurred with low concentrations of antifungal agents, presumably due to their metabolism, but higher concentrations were inhibitory. A similar effect has been noted with a variety of toxic substances and organisms (Owens, 1953; Simon, 1953; McCallan, Miller & Weed, 1954; Beveridge & Hugo, 1964; Smith & Shennan, 1966).

The preservative action of organic acids is due mainly to the unionised molecules (Rahn & Conn, 1944; Aalto, Firman & Rigler, 1953; Bandelin, 1958; Albert, 1965; Winsley & Walters, 1965), and the concentration of undissociated acid in the aqueous phase may be calculated by the method outlined by Martin (1960).

For benzoic acid 0·1%, the pH of the aqueous phase of the oxygen uptake reaction mixture was 3·4, $K_a = 6{\cdot}4 \times 10^{-5}$ and assuming the oil/water partition coefficient, $k = 5{\cdot}3$ (Garrett & Woods, 1953), the calculated equilibrium concentration of unionised acid in the aqueous phase, $[HA]_w = 0{\cdot}073\%$. This is a concentration which inhibited oxygen uptake, growth in agar and was fungicidal in 12 hr. That this should be so is not surprising, since unionised benzoic acid at a concentration of about 0·02% has been shown to inhibit the growth of *A. niger* (Evans & Dunbar, 1965; Winsley & Walters, 1965) and at concentrations >0·025% to inhibit the growth of *Saccharomyces ellipsoideus* (Rahn & Conn, 1944). With sodium benzoate 0·1%, the calculated value of $[HA]_w$ is 0·007%. At such low concentrations of unionised benzoic acid no growth inhibition occurs, but respiration is stimulated and presumably the unionised molecules or anions are metabolised. Thus with *A. flavus* (brown) the oxygen uptake of the control was 410 μl in 8 hr and that of a reaction mixture in which oil was replaced by sodium benzoate, 552 μl in 8 hr, but when both oil and sodium benzoate were present the oxygen uptake increased to 466 μl in 4 hr.

Species difference between the fungi was well shown by their response to phenol. *A. flavus* (brown) had the greatest oxygen uptake on arachis oil. It was also the most susceptible organism to phenol which has an arachis oil/water partition coefficient of about 5·6 (Bean & others, 1965). This greater sensitivity was evident also with the oil-soluble propyl hydroxybenzoate. The aqueous concentrations of hydroxybenzoates were, however, much less than those of 0·4% methyl and 0·1% propyl hydroxybenzoate which inhibited the respiration of yeast (Wailes, 1962) and of 0·1 and 0·03% respectively, which inhibited the growth of *A. niger* (Bandelin, 1958).

Although conditions between the manometric and culture experiments were not identical, some degree of correlation existed (Table 1). For example, benzoic acid 0·1% inhibited the growth of *Rh. arrhizus* and *A. flavus* (brown) and there was no oxygen uptake with *Rh. arrhizus* nor after about 6 hr with *A. flavus* (brown). These results, like others (Wailes, 1962; Chauhan, Rivers & Walters, 1963), indicate that oxygen uptake is much reduced when there is fungistasis; it ceases only in fungicidal conditions.

Before germination, spores swell due to water intake. Swelling is accompanied by a large increase in oxygen uptake and requires a utilisable exogenous carbon source to provide energy for the increase in plasticity of the spore wall (Ekundayo & Carlile, 1964; Ekundayo, 1966; Marchant & White, 1966). The permeability of the spore wall is also increased; thus Caltrider & Gottlieb (1963) and Chauhan & others (1963) found that spores were more sensitive to toxic agents after 4–6 hr in nutrient media

when they were swollen or producing germ-tubes. With organisms like *A. flavus* (brown) which are able to utilise oil, the effect of preservatives does not seem to be entirely dependent upon the concentration in the aqueous phase. There would appear to be an advantage in the use of a preservative with an oil/water partition coefficient sufficient to produce a suitable concentration in the oil phase. Additionally, for ionisable compounds, the pH of the aqueous phase should be such as to ensure that the biologically active form is mainly present.

Within the limitations of the need ultimately to test a preservative in the emulsion or cream in which it is to be used, simulated manometric conditions can provide activity values rapidly and with ease.

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