Manometric Method for the Evaluation of Preservatives

By JOHN L. WAILES

A manometric method using the Warburg apparatus has been investigated as a possible screening technique for the evaluation of agents used as preservatives in pharmaceutical preparations. This procedure also was utilized to determine the relative effectiveness of different preservatives, and preservative-surfactant combinations. The respiratory effects on baker's yeast were determined with methylparaben, propylparaben, and sorbic acid, separately, and each in the presence of polysorbate 80. Each of the three preservatives caused a decrease in oxygen consumption by baker's yeast. In general, the order of effectiveness was propylparaben > sorbic acid > methylparaben. Polysorbate 80 decreased the effectiveness of each of the three preservatives. Sorbic acid was the least affected, except at a preservative concentration of 0.025%. These manometric results are rapidly obtained, informative, and in general agreement with results found by more time-consuming procedures.

VARIOUS METHODS such as the agar-cup-plate, agar streak, agar-disk-plate, and direct inoculation, with and without complex nutrients, have been used to test preservatives against a variety of organisms. These methods, though informative, are time consuming. Also, some question may arise as to the interaction of preservative with the complex nutrients (1, 2) and the different rates of diffusion of preservatives in these nutrients.

This investigation was undertaken to determine the feasibility of using a Warburg manometric procedure as a screening technique for the evaluation of agents used as preservatives in pharmaceutical preparations. The method was devised to reduce the time element and to employ a simple medium, thus obviating complex nutrients and the varying rates of preservative diffusion in a medium. Also, it was of interest to determine whether or not the method would reveal the relative effectiveness of different preservatives and preservative-surfactant combinations.

Sabalitschka and Boehm (3) were among the first investigators to use the *p*-hydroxybenzoate esters as antibacterial and antifungal agents. Since that time these esters and many other substances have been investigated, compared, and recommended for their preservative activity (4–12). The evidence in the literature is abundant concerning the interaction between methyl- and propylparaben with nonionic surfactants (13–20).

The use of sorbic acid as a pharmaceutical preservative is relatively new compared with the parabens. Cosgrove, *et al.* (21), and later deNavarre (22) were among the first investigators to employ sorbic acid as a preservative in pharmaceuticals. In addition, deNavarre (19, 23), Barr and Tice (18), Charles and Carter (24), and recently Blaug and Ahsan (25) have investigated the effectiveness of sorbic acid in the presence of nonionic surfactants.

EXPERIMENTAL

The direct method of Warburg (26) was used to measure the amount of oxygen consumed by the yeast cells. The respiratory effects on baker's yeast were determined with methylparaben, propylparaben, and sorbic acid. Each preservative was tested separately with five different w/v concentrations: 0.025, 0.05, 0.1, 0.2, and 0.4%. In addition, each concentration of preservative was combined with five separate w/v concentrations of polysorbate 80:[1] 0.25, 0.5, 1.0, 2.0, and 5.0%. The oxygen uptake by the yeast cells in the presence of the preservative and the preservative-surfactant combination was compared with the control over a period of 90 minutes.

Each Warburg reaction flask contained a final volume of 3.0 ml. The center well of each flask contained 0.2 ml. of 20% potassium hydroxide in which a small, pleated, filter paper was inserted. The side arm contained 0.2 ml. of yeast, equivalent to 1.25 mg. of dried baker's yeast, suspended in sterile 0.05 *M* phosphate buffer (27) pH 6.0. The yeast suspension was prepared by adding 100 ml. of the sterile phosphate buffer to 625 mg. of dried yeast. The suspension was stirred for 30 minutes prior to pipetting to ensure an even dispersion of the yeast cells.

The main compartment of the flask contained 2.6 ml. of the preservative dissolved in an alcohol-phosphate buffer-dextrose solution. As an example, in order to prepare a 0.1% solution of the preservative, 107.7 mg. of the preservative was dissolved in 3.4 ml. of 95% ethyl alcohol and diluted to 100 ml. with sterile 0.05 *M* phosphate buffer, pH 6.0,

[1] Tween 80, Atlas Powder Co., Wilmington, Del.

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containing 5% dextrose. When 2.6 ml. of this solution was diluted with 0.2 ml. of the yeast suspension contained in the side arm, the resulting solution consisted of 0.1% of the preservative and 3.0% ethyl alcohol. The control flasks contained 2.6 ml. of the alcohol-phosphate buffer-dextrose solution in the main compartment in addition to the 0.2 ml. yeast suspension in the side arm, and the potassium hydroxide in the center well.

The flasks were placed in the constant temperature water bath. All tests were conducted at 37 ± 0.01°. The Warburg was adjusted to 120 cycles per minute with a 3-inch stroke. After a 10 minute equilibration period, the contents of the side arm were tipped into the main compartment of the reaction flasks. Manometer readings were recorded every 10 minutes for a 90-minute period.

At the end of 90 minutes, one standard loopful was transferred from the main compartment of each flask to 20 ml. of sterile Sabouraud maltose broth. These tubes were incubated at 37° for 18 hours, and then observed to determine either the absence or presence of growth. Those tubes that showed no growth were incubated for a total of 72 hours, and again observed.

All solutions and the yeast suspension were prepared immediately prior to use. Each concentration of preservative and preservative-surfactant combination was run in triplicate and each experiment repeated three times.

RESULTS AND DISCUSSION

It was considered necessary to determine the respiratory effects of each component of the substrate. Table I reveals that baker's yeast in phosphate buffer consumed very little oxygen over the 90-minute time period, thus showing little endogenous respiration. However, approximately a ninefold increase in oxygen consumption was observed with the addition of 5% w/v dextrose. Oxygen consumption was further increased by the addition of alcohol. The increase was observed with the subsequent addition of 1, 3, and 5% (v/v) alcohol. Therefore, alcohol at any one of these concentrations could be used as a solvent for the preservatives without any inhibition of respiration caused by the presence of alcohol. Three per cent alcohol was arbitrarily chosen as the solvent in this investigation.

It was observed that a 10% concentration of alcohol caused a decrease in oxygen consumption. Therefore, a 10% concentration is in itself inhibiting to yeast respiration. This inhibition was more pronounced at 15%, and complete inhibition was noted with 20% alcohol.

When polysorbate 80 was added to the phosphate buffer-5% dextrose-3% alcohol substrate, no significant change in oxygen consumption was observed during the 90-minute period. Barr and Tice (18), although using different organisms, concluded that microorganisms produce esterases that split the fatty acid from the surfactant. They also stated that the possibility of the microorganisms utilizing the surfactants for nutritive purposes was remote. The insignificant change in oxygen consumption by yeast cells in the presence of polysorbate 80 tends to confirm their work.

Table II reveals that propylparaben is superior to methylparaben in inhibiting the oxygen consumption of baker's yeast. These manometric results are in accordance with those obtained by Sabalitschka (5). Also, it may be seen that sorbic acid has a greater inhibitory effect than methylparaben, but not as great as propylparaben in the dextrose-alcohol substrate buffered at a pH of 6.0. These results are in agreement with those reported by Osman and El-Mariah (28). Methylparaben is superior to sorbic acid at the lowest preservative concentration of 0.025%. This exception was also observed when the preservatives were tested in the presence of polysorbate 80 (Tables III–V).

A comparison of the results found in Tables III–V shows that an increase in the percentage of polysorbate 80 causes a decrease in the effectiveness of methylparaben, propylparaben, and sorbic acid in inhibiting the respiration of the yeast cells.

Although sorbic acid is the least affected by the surfactant, propylparaben is superior in the presence of small quantities (0.25 and 0.5%) of polysorbate 80. Thus, in pharmaceutical preparations containing less than 1% polysorbate 80, propylparaben would be the best preservative for inhibiting the growth of yeast. In instances where the percentage of surfactant is greater than 1%, sorbic acid would be more effective. These results support the findings of Charles and Carter (24) in that sorbic acid in the presence of nonionic surfactants provided protection of cosmetic formulations against contamination by various organisms.

Experiments also were undertaken in an attempt to correlate either the killing or the inhibiting action with the per cent of respiratory inhibition. At the end of the 90-minute period, one standard loopful from the main compartment of each flask was transferred to a tube containing 20 ml. of sterile Sabouraud dextrose broth. The tubes were incubated at 37° for a period of 18 hours and then observed for growth. If no growth was apparent, the tubes were incubated for a total of 72 hours, and observed again for growth.

No growth appeared at the end of 72 hours in the tubes containing inoculum from the flasks that showed an inhibition of 95% or greater. Those preservatives that produced this degree of inhibition evidently killed the yeast cells within this 90-

TABLE I.—MICROLITERS OF OXYGEN CONSUMED BY 1.25 MG. OF BAKER'S YEAST IN VARIOUS SUBSTRATES

Substrate	Av. μl. O_2 Consumed in 90 min.
Phosphate buffer pH 6.0	16
5% Dextrose[a] in phosphate buffer pH 6.0	143
5% Dextrose + 1% alcohol[b] in phosphate buffer pH 6.0	162
5% Dextrose + 3% alcohol in phosphate buffer pH 6.0	186
5% Dextrose + 5% alcohol in phosphate buffer pH 6.0	217
5% Dextrose + 10% alcohol in phosphate buffer pH 6.0	140
5% Dextrose + 15% alcohol in phosphate buffer pH 6.0	56
5% Dextrose + 20% alcohol in phosphate buffer pH 6.0	0

[a] w/v Solution. [b] v/v Solution.

TABLE II.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY METHYLPARABEN, PROPYLPARABEN, AND SORBIC ACID IN 90 MINUTES

Concentration of Preservative, % w/v	Per Cent Inhibition		
	Methylparaben	Propylparaben	Sorbic Acid
0.4	92 ± 0.632[a]	100 ± 0.000	96 ± 3.346
0.2	43 ± 1.549	99 ± 1.000	83 ± 0.812
0.1	33 ± 2.024	99 ± 1.000	65 ± 1.483
0.05	26 ± 1.048	90 ± 1.000	30 ± 1.500
0.025	18 ± 1.483	58 ± 0.707	6 ± 0.812

[a] Standard deviation.

TABLE III.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY METHYLPARABEN AND METHYLPARABEN-POLYSORBATE 80 COMBINATION IN 90 MINUTES

Concentration of Methylparaben, % w/v	Concentration of Polysorbate 80, % w/v					
	0.0	0.25	0.5	1.0	2.0	5.0
0.4	92 ± 0.632[a]	81 ± 1.897	75 ± 2.121	63 ± 1.581	48 ± 1.897	30 ± 2.121
0.2	43 ± 1.549	43 ± 2.121	39 ± 1.000	32 ± 1.581	26 ± 0.000	19 ± 1.581
0.1	33 ± 2.024	25 ± 1.732	22 ± 1.224	21 ± 2.121	16 ± 1.000	9 ± 1.000
0.05	26 ± 1.048	19 ± 1.000	18 ± 1.000	15 ± 0.707	7 ± 1.000	0 ± 0.000
0.025	18 ± 1.483	8 ± 0.707	7 ± 1.000	5 ± 0.000	0 ± 0.000	0 ± 0.000

[a] Standard deviation.

TABLE IV.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY PROPYLPARABEN AND PROPYLPARABEN-POLYSORBATE 80 COMBINATION IN 90 MINUTES

Concentration of Propylparaben, % w/v	Concentration of Polysorbate 80, % w/v					
	0.0	0.25	0.5	1.0	2.0	5.0
0.4	100 ± 0.000[a]	99 ± 1.000	98 ± 1.000	98 ± 1.000	82 ± 0.707	55 ± 0.707
0.2	99 ± 1.000	98 ± 0.000	96 ± 1.224	75 ± 1.000	61 ± 2.073	38 ± 0.707
0.1	99 ± 1.000	88 ± 0.707	64 ± 1.581	58 ± 1.788	47 ± 1.581	20 ± 2.144
0.05	90 ± 1.000	58 ± 1.224	55 ± 0.707	42 ± 1.732	24 ± 1.000	5 ± 0.000
0.025	58 ± 0.707	46 ± 1.000	36 ± 1.732	24 ± 2.236	12 ± 1.303	0 ± 0.000

[a] Standard deviation.

TABLE V.—PER CENT INHIBITION OF RESPIRATION OF BAKER'S YEAST BY SORBIC ACID AND SORBIC ACID-POLYSORBATE 80 COMBINATION IN 90 MINUTES

Concentration of Sorbic Acid, % w/v	Concentration of Polysorbate 80, % w/v					
	0.0	0.25	0.5	1.0	2.0	5.0
0.4	96 ± 3.346[a]	98 ± 0.707	96 ± 1.581	98 ± 1.224	90 ± 0.707	87 ± 1.000
0.2	83 ± 0.812	83 ± 0.707	81 ± 1.732	81 ± 1.000	78 ± 1.581	64 ± 2.345
0.1	65 ± 1.483	64 ± 2.345	61 ± 2.121	62 ± 1.224	59 ± 1.000	49 ± 3.240
0.05	30 ± 1.500	26 ± 1.581	34 ± 0.707	32 ± 2.549	34 ± 1.224	18 ± 1.288
0.025	6 ± 0.812	0 ± 0.000	0 ± 0.000	0 ± 0.000	0 ± 0.000	0 ± 0.000

[a] Standard deviation.

minute period. This could be considered as self-sterilization.

In those experiments where the percentage of inhibition ranged from 86 to 92%, growth appeared in the tubes at the end of 72 hours, but not at 18 hours. This showed that there were some yeast cells living and able to reproduce in a favorable environment. Had the time limit been extended for more than 90 minutes, it is possible that those few remaining cells might have been killed.

All other tubes from the remaining experiments showed growth at the end of the 18 hours of incubation. Therefore, those results represent merely a degree of inhibition, but not one of self-sterilization.

CONCLUSIONS

1. A manometric method has been used to determine the relative effectiveness of preservatives, and preservative-surfactant combinations on the respiration of baker's yeast.

2. Propylparaben was superior to either sorbic acid or methylparaben in inhibiting yeast respiration in a substrate composed of 5% dextrose, 3% alcohol, and buffered at a pH of 6.0.

3. The activity of sorbic acid was the least affected in the presence of polysorbate 80.

4. In the presence of small quantities (0.25 and 0.5%) of polysorbate 80, propylparaben showed the greatest inhibitory powers. However in greater concentrations of the surfactant (1, 2, and 5%) sorbic acid was more effective.

5. Methylparaben was the least effective of the compounds tested, alone or in the presence of polysorbate 80.

6. On the basis of these manometric results using baker's yeast as the test organism, further investigation is considered justified using this technique with other organisms, preservatives, and surfactants.

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Antiradiation Compounds I

Acylated Derivatives of β-Mercaptoethylamine

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A series of S-acyl derivatives of β-mercaptoethylamine (MEA) has been prepared in an attempt to provide compounds having protective effects in animal cells against ionizing radiation and which are less toxic than MEA itself. In general, toxicity to animals was reduced and good protective ability against X-radiation was retained in some of the esters. The dithiocarbamic acid of MEA was also prepared, but attempts to acylate this compound, which showed good antiradiation effects in close to toxic dose levels in mice, gave a variety of decomposition products.

Beta-Mercaptoethylamine (cysteamine, MEA) has been reported one of the most effective compounds discovered for protecting animal cells against the deleterious effects of ionizing radiation (1). This compound is somewhat toxic for human use (2), however, so an attempt has been made to mask the toxic effects by preparing derivatives subject to hydrolysis *in vivo* which would afford a liberation of MEA over a period of time. Several acylating agents were selected for this purpose.

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The hydrochloride of MEA was utilized on the assumption that acylation of the nitrogen would be prevented. Conventional blocking agents would be difficult to remove in the presence of the thioester formed, and nonhydrolyzable substituents on the nitrogen have generally resulted in diminution or loss of radio-protective action. Attempts to prepare the S-carbobenzoxy derivative of MEA, furthermore, resulted in the formation of S-benzylmercaptoethylamine. Also, MEA hydrochloride failed to react with ethyl chloroformate. However, the use of the hydrochloride of MEA was successful in preventing formation of N-acyl derivatives.

Some difficulty was experienced in finding a suitable solvent for both MEA·HCl and the selected acyl chlorides, but the reaction was found to take place in either dimethylformamide or an