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## RAPID ASSAY FOR GROWTH: DETERMINATION OF MICROBIOLOGICAL SUSCEPTIBILITY AND FUNGISTATIC ACTIVITY

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Present methods for evaluating the susceptibility of different materials to microbiological degradation require from two to six weeks. There is a need for more rapid methods. From the analysis of existing procedures it was concluded that modifications such as using more potent organisms or more nearly optimum conditions could lead only to slight improvements. This paper presents a method that is applicable to this type of assay as well as to the testing of germicides. Results can be obtained within one or two days. The method is a manometric one in which growth of the test organism is measured by oxygen absorption.

Analysis of some data on the growth of the fungus *Myrothecium verrucaria* on cotton cloth showed that, while no measurable changes in tensile strength were found during a period of 48 hours, significant growth had occurred. Development of an assay based on a 48-hour period of incubation then hinged on two points: (1) a reliable method for measuring small amounts of growth, (2) verification of the hypothesis that one can predict the susceptibility of a material to fungus attack on the basis of the amount of growth in the first two days.

In the search for a suitable criterion of growth it was concluded that changes in fresh weight or dry weight of the sample plus mycelium are small and too variable to be reliable. The impossibility of separating fungus from material precludes any direct measurement of the amount of mycelium. Measurement of a particular cellular constituent did not offer much promise. For example, the changes in nitrogen were small and the presence of this element in some fungicides or finishing materials would limit the value of such a method. Furthermore the large amount of organic material in the sample renders oxidation difficult. Calculation of the volumes of CO<sub>2</sub> evolved and of O<sub>2</sub> absorbed indicated a promising lead. Thus 1 mg of cellulose oxidized completely would result in the absorption of about 0.9 ml of O<sub>2</sub> or evolution of an equal volume of CO<sub>2</sub>. The use of a manometric determination of O<sub>2</sub> absorption seemed to offer more advantages than chemical or physical measurement of CO<sub>2</sub> evolution from the standpoint of simplicity of apparatus, ease of manipulation by untrained technicians, and the like. Conductometric or volumetric determination of CO<sub>2</sub> may be employed advantageously in certain instances.

The applicability of standard microrespirometric techniques adapted to a macro scale was next evaluated. Use of Warburg type manometers was not feasible because of the large thermobarometer corrections occasioned by changes in barometric pressure over a 48-hour period and by the more precise tempera-

ture control required. Use of differential manometers employing a closed system was found to be satisfactory. The effects of temperature fluctuations encountered in the standard incubators are largely, but not entirely, eliminated by the similar effects on the temperature of the control and experimental flasks. The principal modifications from the standard Barcroft-Haldane differential manometers are the use of 250-ml flasks as vessels and of flexible tubing to connect the manometers with the flasks.

This paper presents experimental data on the effectiveness of the method for determining fungistatic activity as well as susceptibility to degradation by microorganisms. The success of this respirometric technique as applied to the original problem suggests the possibility of more extensive applications. Potentially, any assay in which the factor under test limits or determines growth of an organism may be adapted. Application to biological assays for vitamins, amino acids, etc., may be feasible.

#### METHODS

Construction of the manometers used in this study has been illustrated in a preliminary report (Siu and Mandels, 1950). The manometer proper is constructed of capillary tubing of 2-mm bore. The length of the capillary from the U-bend to the bulbs should be about 200 to 300 mm. The bulbs should have a capacity of at least twice the volume of manometric fluid to prevent the fluid from being sucked back. The manometers are filled with Brodie's solution (Dixon, 1943) and are mounted vertically in an incubator maintained at 30 C. The manometers are connected to 250-ml Erlenmeyer flasks having 24/40 standard taper joints by means of flexible plastic tubing attached to the stopcock of the connectors. To absorb CO<sub>2</sub> a small cup containing 1.5 ml 10 per cent KOH and a filter paper wick is suspended from the hook inside the connector to the experimental flask by means of a nichrome wire. An improved design illustrated in figure 1 increases the ease of manipulation considerably and permits the manometers to be placed outside the incubator if desired. The stopcock at the bottom of the manometers is for filling them with fluid. The dimensions of the apparatus described here are such that the simplified expression (Dixon, 1943) commonly used for calculating the vessel constants can be employed. The vessel constant is of the order of 25 for apparatus of the design shown in figure 1. Thus absorption of 1,000  $\mu$ l of oxygen during growth in such a flask should produce a pressure change of about 40 mm of the manometer fluid.

In most of the work the cellulolytic fungus *M. verrucaria* USDA 1334.2 was used. It was grown in 250-ml flasks containing 50 ml of nutrient agar (3.0 g NH<sub>4</sub>NO<sub>3</sub>, 2.22 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.59 g KH<sub>2</sub>PO<sub>4</sub>, 2.21 g K<sub>2</sub>HPO<sub>4</sub>, 1,000 ml distilled H<sub>2</sub>O, 20 g agar). The carbon source was a 7-cm disk of filter paper placed on the surface of the cooled agar. These stock cultures were inoculated with 1 ml of a spore suspension. To prepare the inoculum for experimental use 100 ml of sterile distilled water are added to a culture. After being shaken for a few minutes all the spores will have been washed from the surface of the culture. This suspension is diluted 1:10 for use as an inoculum.

Cultures for use with the manometers are prepared by adding 50 ml of the nutrient agar to the 250 ml flasks. Circular disks (7 cm in diameter) of the material to be tested are placed in the experimental flasks; no substrate is added to the control flasks. The experimental flask is inoculated with 1 ml of the standard inoculum. Both flasks are then attached to the manometers in the 30 C incubator. After the flasks attain temperature equilibrium—a few hours—the stopcocks are closed. At appropriate intervals the pressure difference between the two flasks is measured in mm.

Although it is not essential that aseptic precautions be observed, it is desirable to do so when possible. Occasionally the presence of foreign microorganisms has been found to inhibit growth of the test organism. This difficulty may be

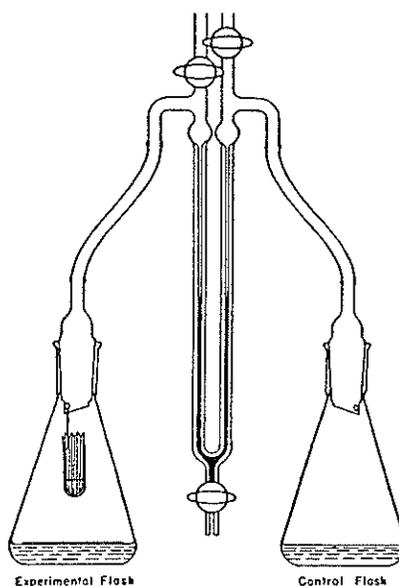


Figure 1. Design of differential manometers.

encountered in any technique in which pure culture conditions are not practiced.

Breaking strength determinations were made with a motor-driven Scott tester. The strips were conditioned prior to breaking.

#### RESULTS

The shape of the growth curve of *M. verrucaria* as measured by determination of the oxygen absorbed during growth on filter paper in the manometer flasks is shown in a semilog plot (figure 2). Oxygen absorption is seen to follow an essentially autocatalytic course typical of a normal growth curve. The data are taken from an experiment in which 21 manometers were set up to determine the variability of the method. Data from the maximum and minimum as well as the average values for all manometers are presented in the curves. The data are

summarized for groups of three manometers selected at random in table 1. With only seven sets the coefficient of variability was less than 10 per cent after 24 hours. The variation is considered to be due more to differences in growth than

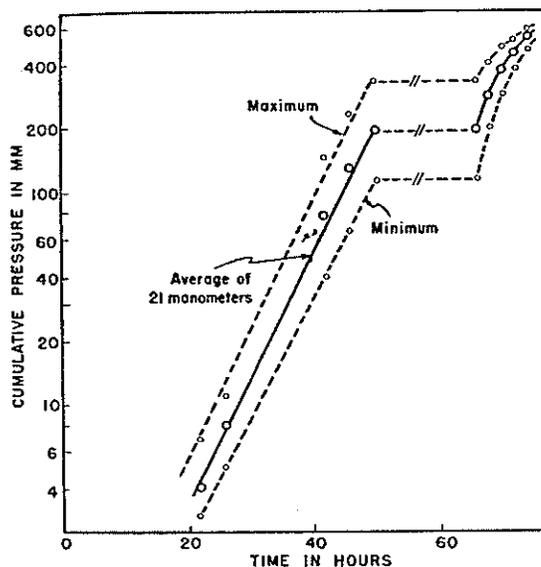


Figure 2. Course of respiration curve of *M. verrucaria* growing on filter paper.

TABLE 1

Average cumulative pressure changes of sets of three manometers containing filter paper and inoculated with *M. verrucaria*

EXPT. NO.	22 HR	26 HR	42 HR	46 HR	50 HR	68 HR	70 HR	72 HR	74 HR
1	5	8	74	125	188	275	365	459	543
2	3	7	70	118	181	271	364	454	546
3	5	8	66	110	169	244	343	438	524
4	4	8	84	140	214	309	400	479	545
5	4	8	84	137	208	290	371	453	529
6	3	8	85	138	201	281	364	444	519
7	5	9	79	129	193	282	373	457	530
Grand avg.....	4.1	8.0	77.4	128.1	193.4	278.9	368.6	454.9	533.7
Standard deviation....	0.65	0.54	7.0	10.4	14.5	18.2	15.7	11.9	10.3
Coefficient of variability.....	15.9	6.8	9.0	8.1	7.5	6.5	4.3	2.6	1.9

to inherent variability in the manometric apparatus. Thus, first growth and sporulation were visible in those manometers showing the most rapid respiration.

Data showing the changes in breaking strength of 8.25-ounce duck as related to dry weight loss of the system cloth, plus fungus and oxygen absorption of

*M. verrucaria* growing on the cloth, are illustrated in figure 3. In this experiment disks of the cloth 7 cm in diameter were used for the dry weight determinations and in the manometers. In harvesting the disks for dry weight determinations the mycelium was not washed off. Strips of the cloth raveled to 3-by-1 inch were used as substrate in the cultures used to determine breaking strength changes. The initial weight of disks was about 1.02 g. The curves show that 48 hours after inoculation there was an easily measurable change of about 190 mm in pressure in the manometers. In this period the breaking strength had not changed significantly, and the dry weight loss amounted to only 1 to 2 per cent of the original dry weight. The latter measurements are of such a low magnitude as to be of no practical use.

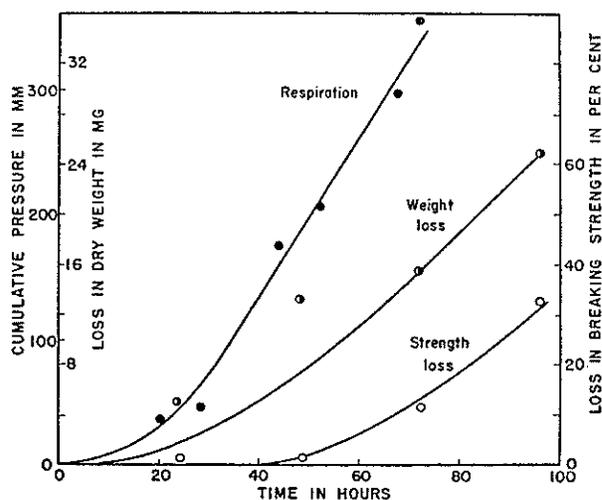


Figure 3. Respiration of *M. verrucaria* growing on cotton and dry weight and breaking strength losses.

Comparative determinations of the susceptibility of treated fabrics to mildew as measured by a conventional loss in breaking strength technique and by the manometric technique are illustrated in figures 4 and 5. Cotton khaki twill (8.2 ounces) was treated with varying concentrations of a solution of copper naphthenate "nuocide copper 8" in toluene. The material was given two dips and two nips at 30 pounds pressure in a Butterworth padder and dried 10 minutes at 200 F. The copper content of the fabric was determined by polarographic analysis and expressed as percentage of the dry weight of the cloth. For use the cloth samples were wetted in 0.05 per cent gardinol, rinsed in distilled water, placed in the culture flasks containing 50 ml of inorganic nutrient agar and inoculated with a suspension of *M. verrucaria* spores. Autoclaved cloth was used in the manometers but not in the cultures used to determine tensile strength loss. The curves showing oxygen absorption during growth (figure 4) demonstrate very clearly the effect of the different concentrations of fungicide. At 0.02 per cent Cu there is slight protection indicated, but at 0.58 per cent Cu very little growth

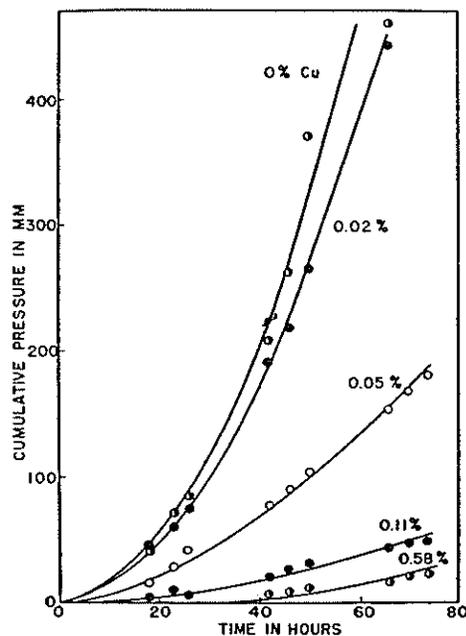


Figure 4. Respiration of *M. verrucaria* growing on cotton khaki twill containing different concentrations of copper naphthenate.

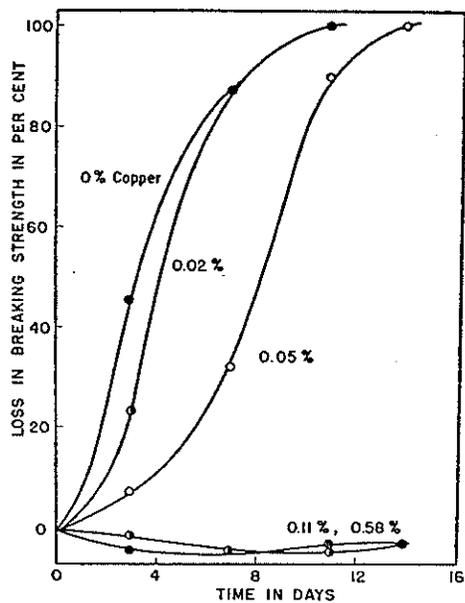


Figure 5. Breaking strength losses of cotton khaki twill containing different concentrations of copper naphthenate.

occurred. Qualitatively the picture obtained using breaking strength loss as a measure of susceptibility differs in that complete protection is indicated at 0.11

per cent and 0.58 per cent Cu. For comparative purposes the respiration at 30 hours on the various samples expressed as percentage of the control is shown as a function of the copper concentration along with similar plots of the relative breaking strengths (figure 6).

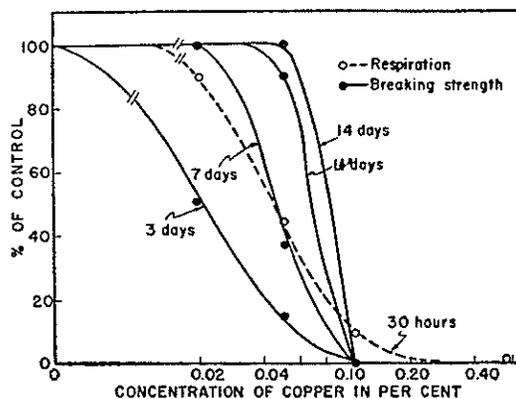


Figure 6. Relative respiration and tensile strength loss of treated twill during incubation with *M. verrucaria*.

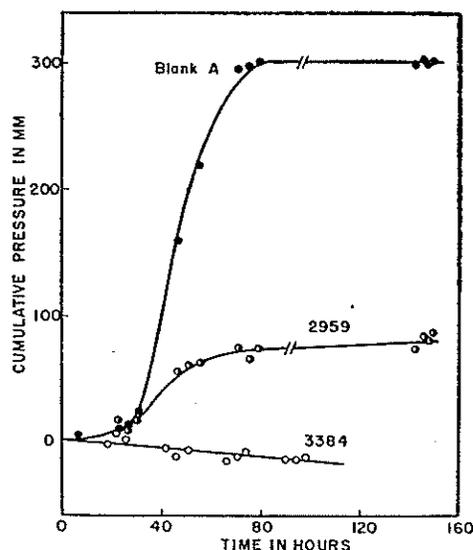


Figure 7. Respiration of *A. versicolor* growing on plastic films.

The relative susceptibility determined by breaking strength measurements changes radically with time. At 3 days 50 per cent protection would be indicated by 0.2 per cent Cu and about 85 per cent by 0.05 per cent Cu. The 7-day harvest indicates no protection at 0.02 per cent and about 60 per cent by 0.05 per cent, whereas at 14 days no protection is indicated by either of these concentrations of fungicide. In marked contrast to this, the respiration data show no significant change in relative susceptibility within the incubation time of 20 to

60 hours. The most striking feature of these data is the demonstration that observations taken after only 1 or 2 days on the manometers give a good picture of relative susceptibility. Interpretation of the low respiration at the two highest concentrations of fungicide is not certain. It is considered that the respiration at 0.58 per cent copper represents growth on impurities in the agar. It has not

TABLE 2  
Composition of plastic films

COMPONENT	BLANK A	FILM NO. 2959	NO. 3384
Vynlite resin.....	70	70	70
Methyl acetyl ricinoleate.....	15	15	—
Diethyl phthalate.....	15	15	30
Plumbo-sil-B.....	1	1	1
BVS.....	1	1	1
Milmer I.....	—	0.6	—

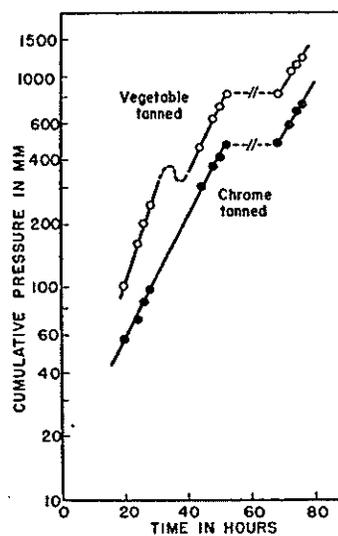


Figure 8. Respiration of *A. niger* growing on leather.

been determined whether or not the oxygen uptake at 0.11 per cent copper represents very slow degradation.

Measurement of the susceptibility of vinylite resin plastic films to microbiological degradation is shown in figure 7. Three films of different susceptibilities were inoculated with *Aspergillus versicolor* QM-4g. The composition of these films is shown in table 2. The plumbo-sil-B is a stabilizer and the BVS a lubricant. Rapid growth occurred on the "Blank A" film. This film contains a ricinoleate plasticizer that is available as substrate for the fungus. Inclusion of 0.6 per cent "Milmer I" (a fungicide) markedly retarded, but did not stop, growth of the fungus (film no. 2959). Film 3384 containing only diethyl phthalate as a plas-

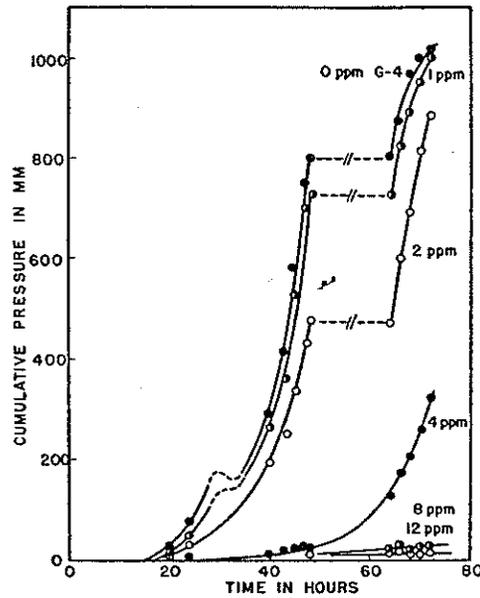


Figure 9. Respiration of *T. mentagrophytes* growing on peptone sucrose agar containing different concentrations of G-4.

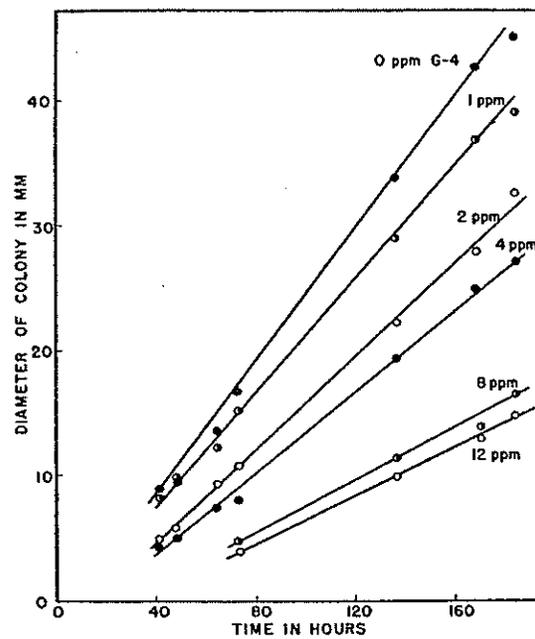


Figure 10. Linear growth of colonies of *T. mentagrophytes* growing on peptone sucrose agar containing different concentrations of G-4.

ticizer, which is not available as a substrate for the fungus, supported no growth as indicated by oxygen absorption. These results conform with those obtained

with stiffening tests (Harvey, 1949*a,b*), in which it was shown that the susceptibility of these films to fungi depends on the nature of the plasticizer used.

Manometric growth curves with leather as substrate are shown in figure 8. Two types of leather were used: (1) straight vegetable-tanned upper and (2) straight chrome-tanned upper. The samples were not washed to remove soluble materials added to the leather in the tanning process nor were they sterilized. The inoculum was *A. niger* TC 215-4247. A significant difference is noted between the two types of leather, growth on the chrome-tanned being somewhat slower than on the vegetable-tanned. Similar differences were found between a straight vegetable-tanned sole leather and a vegetable-retanned chrome upper leather.

Several experiments were set up using *M. verrucaria* or *Trichophyton mentagrophytes* QM-252 to determine the applicability of the manometric method to the evaluation of fungicides. In these experiments the fungicide G-4 (2,2'-dihydroxy-5-5'-dichlorodiphenyl methane) was dissolved in ethanol. Appropriate

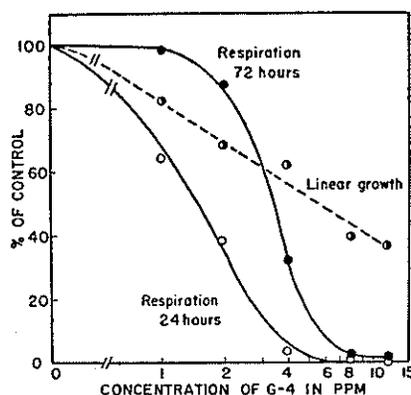


Figure 11. Relative respiration and linear growth rates of *T. mentagrophytes* at different concentrations of G-4.

dilutions were made and aliquots of the solution of fungicide were added to the agar media. An equal volume of ethanol equivalent to 1 per cent final concentration in the medium was added to the controls. The media were autoclaved after addition of fungicide. In the experiments with *M. verrucaria* the substrate was 2 per cent sucrose, but with *T. mentagrophytes* 2 per cent peptone was also added. Parallel cultures were set up in the manometers and in petri dishes. In the latter case growth was determined by periodically measuring the diameter of the colonies. Cumulative pressure changes with *T. mentagrophytes* are shown in figure 9 and the linear growth in figure 10. Significant differences are seen within 24 hours after inoculation in the manometers. At this time the colonies in the petri dishes were too small to be measured accurately without the use of some means of magnification. Comparison of the curves shows that the same picture does not obtain in both methods. This is brought out more clearly in figure 11, in which the rate of linear growth and the total respiration at various times are plotted as percentage of the controls without fungicide as a function

of the log of the fungicide concentration. At higher concentrations of G-4 there was growth on the petri dishes, but the growth in the manometers was proportionately much less. Results of a similar experiment with *M. verrucaria* are shown in figure 12. Here it is seen that there is a much closer relation between the results of both methods. In the manometers, however, there was a significant stimulatory effect at low concentrations of G-4 (1 ppm). In the petri dishes this effect was not so great and was not observed in this particular experiment.

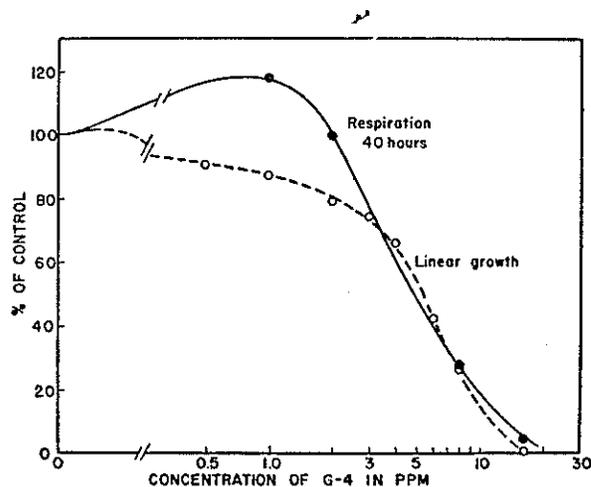


Figure 12. Relative respiration and linear growth rates of *M. verrucaria* at different concentrations of G-4 in sucrose agar.

#### DISCUSSION

The application of manometric measurements of respiratory activity to the evaluation of germicides is not new. No such techniques, however, appear to have met the qualifications for practical application or for the determination of susceptibility to microbiological attack. The reasons for this are probably either the unsuitability of the technique for routine determinations by semiskilled personnel or an inadequacy of the principle upon which the method is based.

The methods that have been proposed fall into two fundamentally distinct groups. First are those which depend upon a measurement of the effect of a particular substance upon the respiration of a given amount of organism (Bronfenbrenner *et al.*, 1939; Ely, 1939; Nickerson, 1946). Theoretically this approach does not rest on too solid a foundation. It presupposes a direct relation between the effects of the poison on the respiration of a given cell under conditions in which growth is not occurring and the potential effect of the poison on growth. It has been demonstrated repeatedly (see reviews of Goddard, 1948; Needham, 1942; Thimann, 1948) that growth may be inhibited completely by exposure to physical or chemical treatments that may result in either no effect on respiration or only partial inhibition. Obviously one might overlook valuable substances by placing too much faith in such a method. This would seem particularly true in a

search for a substance of not too drastic action that might be expected to display more or less specific toxicity to an organism or group of organisms. Neither fungicidal or fungistatic properties can be determined very reliably by this method. Techniques based on this principle would seem also to be of little value in determining the susceptibility of relatively inert materials such as cellulose and plastics to microbiological degradation.

The second type, which has been proposed by Greig and Hoogerheide (1941*a,b*), depends upon the effect of a germicide upon the growth of bacteria as measured by the respiration during growth in the presence of the chemical. The method developed in the present paper has the same theoretical basis and will be evaluated below.

Methodologically the technique proposed here differs from earlier developments by replacing the standard microrespirometric techniques with a macro-modification. There is a resultant simplification in manipulation. The use of differential manometers obviates the requirement of a thermostated water bath; the temperature fluctuations ( $\pm 1$  F) encountered in a standard air incubator can be tolerated. A differential system is also immune to the large fluctuations in barometric pressure which may occur during the course of an experiment or test.

The theoretical validity of the method depends primarily upon justification of the premise that under a given set of conditions there is a direct relation between the amount of respiration and the amount of growth. It can be demonstrated effectively that we are measuring the respiration of the growing mycelium rather than that of the spores used for inoculum by calculating the approximate oxygen uptake of the inoculum. *M. verrucaria* spores have an endogenous  $Q_{O_2}$  of about  $4 \mu l O_2$  per mg dry weight per hour (Mandels and Norton, 1948). About 90  $\mu g$  of spores are customarily used as inoculum for each flask in the manometers. The respiration of this amount of inoculum would thus be about  $10 \mu l O_2$  per day, representing a pressure change of less than 1 mm per day. During growth on cellulose the manometers register changes of the order of 40 to 50 mm per hour during the period of maximum rate of respiration. Greig and Hoogerheide have shown, using several species of bacteria, that the rate of oxygen consumption of a culture during growth is proportional to the bacterial content. It is commonly recognized that the  $Q_{O_2}$  of an organism is more or less constant during the period of logarithmic growth. For practical purposes the applicability of the initial premise seems to depend on how widely the conditions for growth can be varied without affecting the  $Q_{O_2}$  very greatly. Yamamoto (1933) has shown that iodoacetate inhibits growth and respiration of *A. niger* to about the same degree. Data presented here have shown that the linear growth rate of *M. verrucaria* at different concentrations of the fungicide G-4 parallels the respiration. The data obtained with *T. mentagrophytes* under similar conditions show an almost complete inhibition of growth as measured by respiration at concentrations of G-4 when appreciable growth occurred in the petri dishes. No growth was observed, however, in the manometers at these concentrations. Thus the lack of correlation here must be due to some experimental factor such as the method of inoculation.

It is entirely reasonable that considerable variations in  $Q_{O_2}$  be anticipated between rapid growth on a readily available substrate and slow growth under conditions in which substrate availability or presence of an inhibitory substance is limiting growth. Even if such variations are so great as to change the  $Q_{O_2}$  by a factor of 2 or 4, or even more, this is not considered as a serious objection provided intelligent applications of the data are made.

Application of the technique to determination of the susceptibility of materials to microbiological degradation depends upon the existence of a relation between the susceptibility and the growth which occurs in the first two days. At this time very little degradation would have occurred. Data obtained by incubating cotton khaki twill containing varying concentrations of copper naphthenate after inoculation with *M. verrucaria* show a linear relation between the respiration of the cultures during the first 48 hours and the log of the copper content of the cloth. Very little respiration occurred at 0.11 per cent Cu. The breaking strength measurements of the cloth harvested after 7 days' incubation give a very similar picture. However, an earlier or a later harvest indicates quite a different picture. The correlation between respiration during 48 hours and the susceptibility of the treated fabrics as determined by breaking strength data is very good.

Undoubtedly, the same apparatus and technique can be applied to similar problems involving other materials such as plasticizers, glue, wool, wood, and paper. Other organisms, such as protozoa and insects, could also be used. The method should be useful for rapid screening. It should also reduce the requirements for diverse types of equipment currently used for testing the mildew resistance of materials. Thus cotton fabrics are conditioned at 70 F and 60 per cent relative humidity for 24 hours before the strength is determined on a Scott tensile tester. Plastic films, on the other hand, are conditioned at 73 F and 50 per cent relative humidity, and their stiffness is determined by a Clark flexibility tester. The manometric method requires neither these testers nor conditioning rooms.

#### ACKNOWLEDGMENTS

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#### SUMMARY

A rapid and reliable manometric method has been devised which is particularly useful for determining the susceptibility of treated cotton, plastics, leather, wool, and other materials to attack by microorganisms.

The method is also applicable for determinations of the fungistatic and bacteriostatic activity of compounds and, with simple modification, for determination of germicidal properties.

The method is based upon a determination of growth as measured by oxygen consumption.

The rapidity of the method is such that satisfactory measurements can be made in 24 or 48 hours.

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