A RAPID CELL VOLUME ASSAY FOR FUNGITOXICITY USING FUNGUS SPORES

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In investigations of the physiology of spores of the cellulolytic fungus *Myrothecium verrucaria*, it was observed that rapid swelling and germination occur in the presence of sugar and yeast extract. This swelling is not a purely osmotic phenomenon but is rather a typical growth process since more or less parallel increases in dry weight occur. Increases in cell volume of the order of 4 to 6 fold occur in about three hours and can be measured easily by centrifugation. Since the rate of swelling is markedly affected by toxic substances, it was reasoned that an extremely rapid bioassay for fungitoxic compounds could be based on this inhibition of swelling. The time for screening or evaluating such compounds can be reduced from the day or week required for conventional tests currently in use to a period of two or three hours. Aside from the relative rapidity of the method, a distinct advantage is that aseptic precautions need not be observed because of the short time intervals involved. The procedure is used most easily for determinations of fungistatic activity. Fungicidal properties can be measured with equal ease although the duration of the test must be greater to allow time for the compound to act upon the spores and to allow for washing the compound from the spores. The assay has been developed using spores of *M. verrucaria* which are particularly suitable because of their high rate of metabolism and because of the manner of germination under the conditions employed. A number of other organisms have been tested in preliminary manner to determine their possible application to the method.

METHODS

Spores of *M. verrucaria* (strain QM 460) were obtained from cultures growing at 30 C on filter paper placed on agar in 250 ml Erlenmeyer flasks as described previously (Mandels, 1951). By inoculating with 1 ml of a suspension of spores, sporulation is well advanced by 3 to 4 days and is essentially complete by about 6 days. The spores are removed from the cultures easily by simply shaking gently with distilled water. After washing twice they are suspended in distilled water or buffer. No appreciable contamination with fragments of mycelium occurs. Quantitative removal of the spores is effected by this method, and the yield of spores from each culture is essentially constant for practical purposes. A culture normally yields approximately 6×10^9 spores having a centrifuged cell volume of 280 μl and a dry weight of about 90 mg. This is adequate for 15 to 25 determinations.

To test the *fungistatic* properties of a compound 15 ml of a spore suspension in 0.05 M KH_2PO_4—K_2HPO_4 buffer pH 6.2 was added to 20 ml of sucrose and yeast extract (Difco) in buffer containing the test chemical in the desired concentration in 125 ml Erlenmeyer flasks. Final concentration of sucrose and yeast extract was 1 per cent each, final spore density 1 to 2 μl per ml. After incubating on a reciprocal shaker at 30 C for 3 hr, cell volumes were determined on three 10 ml aliquots from each culture.

Fungicidal tests were carried out by combining 15 ml of suspensions of spores with 15 ml of the compound. After incubating overnight on the shaker at 30 C, the suspensions were washed 2 times and resuspended in the sugar-yeast extract solution. Cell volumes were determined then after three hours' incubation on the shaker.

So-called *fungistatic-fungicidal* tests were similar to the fungicidal tests except that the spores were not washed after incubation overnight, the sugar-yeast extract solution being added directly to the suspensions which still contained the chemical under test.

Disinfectant tests were made by incubation with the test substance for 30 minutes at 30 C, washing, resuspending in buffer, adding sugar and yeast extract, and determining cell volumes after 3 hours' incubation.

When viability determinations were to be

made, samples were removed from the fungicidal tests prior to washing and diluted to give about 50 spores per ml. Petri dishes containing sucrose-yeast extract agar were inoculated with 1 ml of the diluted suspensions. Colony counts were made after about two days' incubation. Since the dilution required was about 1:500,000, washing the spores was considered unnecessary. In all cases where viability determinations were made, manipulations up to the time of removing aliquots for plating out were aseptic. The test

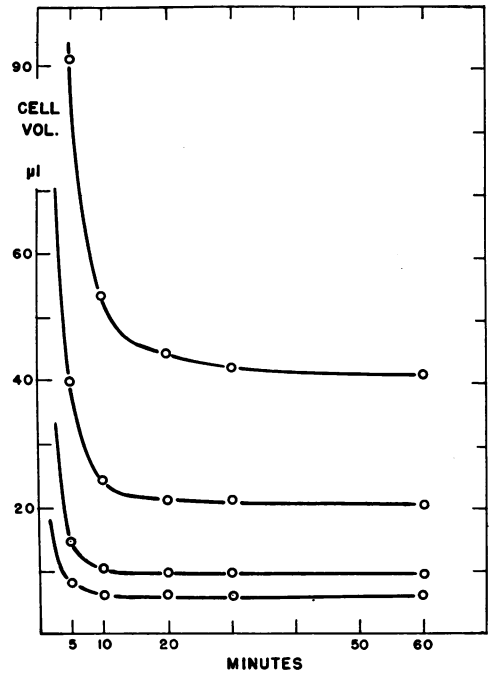

Figure 1. Curves of fungus spore suspensions of different densities. 1,000 × G.

compounds were never autoclaved; sterile solutions were obtained by making up strong stock solutions and allowing them to stand a short while before diluting them aseptically.

Linear growth experiments were performed in 10 cm petri dishes containing 35 ml of agar + sucrose + yeast extract. Periodic measurements were made of two diameters at right angles on each of three replicate plates. Inhibitors were added to the melted agar just before pouring the plates.

Respiration experiments were performed using conventional Warburg techniques. Assimilation was measured by determinations of the increase in dry weight using sintered glass crucibles of fine porosity to filter and wash aliquots of the suspensions.

Cell volumes were determined in triplicate for each treatment using standard "constable" hematocrit tubes of 10 ml total capacity with the capillary part graduated to 0.10 ml in 0.005 ml intervals. Preliminary calibration showed that most tubes were not appreciably different. Those having significant errors were discarded. Up to 32 determinations could be made at one time using eight 4-place trunnions and 15 ml Cornell tubes in an International Centrifuge, size 2, with an IEC no. 240 head. With a size 3 centrifuge and a 16-place head 64 determinations can be made simultaneously. Standard centrifuging conditions were established from the relations shown in figure 1 as 15 minutes at 1,000 × G (*ca* 2,000 rpm).

A variety of fungicides and inhibitors was used in the development of the method. The work reported was done with sodium azide, phenol, merthiolate (sodium ethylmercurithiosalicylate), G-4 (2,2′ dihydroxy-5,5′ dichlorodiphenylmethane), Copper-8 (copper-8-quinolinolate).

For testing substances relatively insoluble in water (such as G-4) they were dissolved in absolute ethanol at 350 × the desired concentration, and 0.1 ml was added to 35 ml of the sugar-yeast extract solution with ABA automatic pipettes. In such cases the same quantity of ethanol was added to the controls since even at 0.3 per cent alcohol is slightly inhibitory to the swelling. Attempts to use cellosolve as a solvent as employed by Shirk and Byrne (1951) showed that it was more toxic than ethanol. To add Copper-8 which is insoluble in any of these solvents, the compound was synthesized in the cultures by combining $CuSO_4$ and 8-hydroxyquinoline at appropriate concentrations in stoichiometric quantities—i.e., two moles of 8-hydroxyquinoline per mole of $CuSO_4$.

Results are expressed in terms of changes (i.e., in cell volume, etc.) as percentage of control. Thus for cell volume determinations:—

$$\frac{\text{final volume in presence of inhibitor} - \text{initial volume}}{\text{final volume without inhibitor} - \text{initial volume}} \times 100$$

RESULTS

Course of swelling in relation to growth and germination. The changes in cell volume and in dry weight occurring under the standard conditions employed are shown in figure 2. After a lag

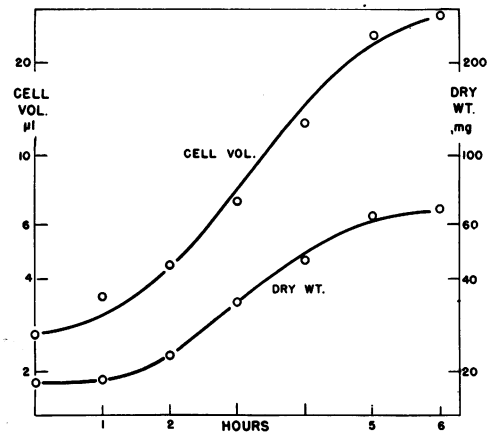

Figure 2. Changes in dry weight during swelling and early growth of fungus spores. Cell volume = µl/ml; dry weight = mg/20 ml.

of about an hour the dry weight increases logarithmically. The cell volume changes do not follow such a well defined pattern—the rate of change in volume is not constant but increases gradually. This is ascribed to the less dense packing of the cells, under given conditions of centrifugation, with increased cell volume and to entangling of the germ tubes decreasing the rate and extent of packing. The growth curve as measured by this cell volume procedure follows an empirical course due to these factors. Plotting the data as the cube root of the cell volume as proposed by Emerson (1950) does not result in linear curves. The morphological changes occurring during swelling and germination are seen in figure 3. Spores swell considerably before the protrusion of a germ tube at about two hours. As the germ tube grows, there is a significant shrinkage of the original spore. It is clear from these data that there is no sharp change in rate of increase in dry matter or in cell volume with germination (protrusion of the germ tube). The entire process of germination here can be considered as a phenomenon of growth.

Factors influencing the rate of swelling. As a phenomenon of growth, spore swelling is influenced markedly by a number of environmental conditions. The optimum temperature for swelling is between 30 and 35 C (figure 4). Whether growth occurs at 40 C is problematical since the thermoregulator failed during this experiment and the temperature fell. In citrate or phosphate buffers, pH has little effect on the rate of swelling from pH 5.5 to 8.5 (figure 5). Below pH 5.5, the rate decreases rapidly. Similar results were obtained with phthalate buffers, while acetate is inhibitory at pH 5.5 due to toxicity of the undissociated acetic acid molecules (*unpublished data*).

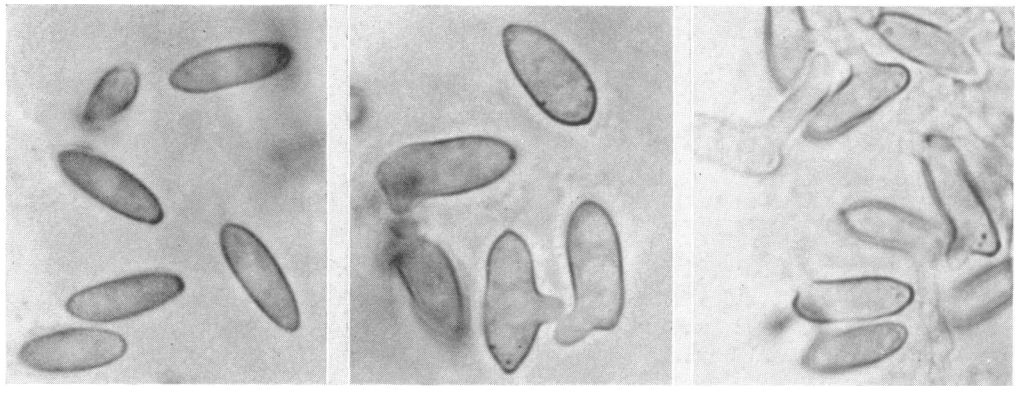

Figure 3. Appearance of fungus spores during swelling and germination: (a) normal unswelled spores, (b) appearance after 2 and, (c) 3 hours in sucrose + yeast extract.

Very slow swelling occurs in the presence of sugars alone. Various substrates were tested for their ability to stimulate swelling or germination in the presence of glucose or sucrose. These included various amino acids, organic acids, vitamins, and mixtures of these either as synthetic or natural mixtures including malt extract,

yeast extract, and casamino acids. While most compounds tested had a slight to moderate effect in stimulating swelling, only yeast extract had a marked effect.

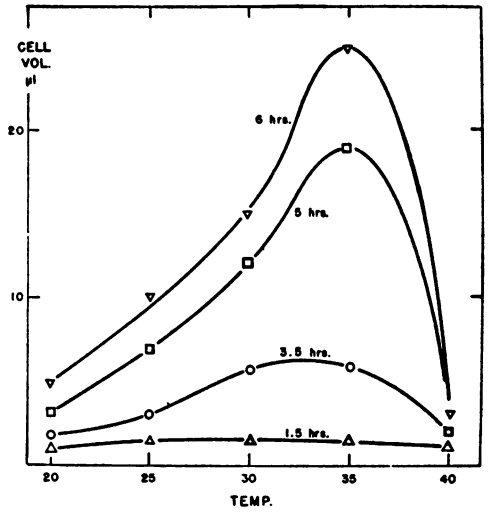

Figure 4. Effect of temperature on swelling rate (µl/ml) of fungus spores.

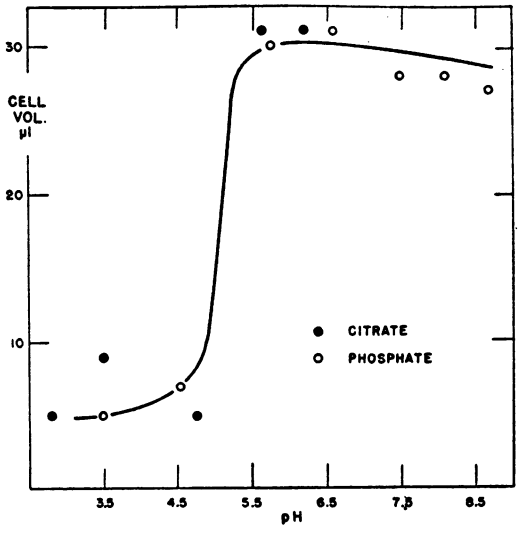

Figure 5. Effect of pH on swelling of fungus spores. Five hours in 0.5 per cent sucrose + 0.5 per cent yeast extract at 26 C.

Yeast extract is required in relatively high concentration for maximum rate of swelling (figure 6). Below 0.25 per cent the rate decreases rapidly. The yeast extract does not act as a trigger to break dormancy and set the germinative processes going but must be kept available if rapid metabolism is desired.

The process of germination in spores of *M. verrucaria* is highly aerobic (table 1). No growth or germination occurs under the conditions employed here unless the suspensions are shaken or aerated. Aeration tends to cause more deposition of spores on the walls of the containers and is less convenient to use than a shaker.

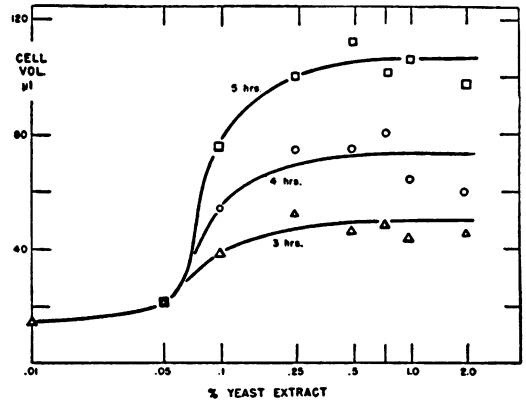

Figure 6. Effect of concentration of yeast extract on swelling of fungus spores in 1 per cent sucrose.

TABLE 1

Effect of aeration on swelling of fungus spores

TREATMENT	CELL VOLUME AT 3.5 HR
	µl
Standing	11*
Intermittent shaking	13
Continuous shaking	54
Aerated	61

* No significant increase over original volume.

Age has a very marked effect on the physiology of the spores. Under the conditions used here for propagating spores, sporulation starts after about 3 or 4 days and is completed by about the 5th day. Spores taken from cultures up to about 16 days old will swell rapidly. If older cultures are used, however, the rate of swelling becomes progressively slower than with younger spores.

Effect of time of incubation on degree of inhibition. In the data reported here on the influence of various chemicals on swelling, cell volumes were measured after three hours' incubation with substrate and inhibitor. This time was selected

more or less for convenience. Data obtained from several experiments with different inhibitors or fungicides show (table 2) that the degree of inhibition increases with time of incubation up to three hours in all the cases studied. In other words, the proportionate difference between the control growth curves and those in the presence of inhibitor increases rather markedly up to about three hours. The change after this is much slower and appears to reach a more or less constant value.

Effect of spore concentration. Measurements of swelling of spores were made, using suspensions having 10 to 20 μl spores per 10 ml of suspension.

TABLE 2

Effect of time of incubation on degree of inhibition

TREATMENT	CELL VOLUMES AS PER CENT OF CONTROL				
	1 hr	2 hr	3 hr	4 hr	5 hr
Azide 10^{-5} M	100	93	92	95	
Azide 10^{-4}	58	30	14	17	
Azide 10^{-3}	0	0	0	0	
Merthiolate 1 ppm	50	58	35	29	
G-4 *0.6 ppm		78	91	89	94
1.5		83	79	78	54
4		72	54	51	28
10		44	23	11	11
15		0	0	0	0

* G-4 (2,2′ dihydroxy-5,5′ dichlorodiphenylmethane).

If less dense suspensions are used, the initial cell volume cannot be measured accurately because of a relatively large error in the first calibration mark in the hematocrit tubes. On the other hand, if denser suspensions are used the increase in volume may be so great as to exceed the graduated limits of the capillary in the centrifuge tube. The possibility that the toxicity of a low concentration of inhibitor is a function of spore density must be considered. This was checked only for Copper-8. For initial spore volumes of 9 and 17 μl per 10 ml no significant effect was noted.

Use of stored suspensions for toxicity determinations. A distinct advantage to laboratories making daily tests of toxicity would be gained if it were possible to make up stock suspensions and store them for periods of a week or more. To test this possibility a suspension of spores in buffer was prepared. Samples were removed at this time to measure the rate of swelling. The remainder of the suspension was stored in a refrigerator at about 4 C. At intervals up to 9 days samples were withdrawn for test. Since the resistance of the spores to toxic agents might change during such storage, a check of this was made by making simultaneous measurements of swelling in the presence of merthiolate at 1 ppm. Data for measurements at three hours are summarized in table 3. No significant differences are found during 9 days' storage. It is not known how long such suspensions can be kept without deterioration. A suspension kept refrigerated for eight months, however, did not swell at all when sucrose and yeast extract were added.

TABLE 3

Effect of storing suspensions on swelling and on susceptibility to merthiolate

TIME OF STORAGE	CELL VOLUME—3 HR			INCREASE AS PER CENT OF CONTROL
	Control	1 ppm merthiolate	Substrate	
days	μl	μl	μl	
0	38	19	9	35
1	37	20	—	41
2	34	20	8	46
3	42	19	8	32
9	34	18	8	38

Another useful procedure would be to lyophilize aliquots of a stock suspension. Preliminary tests show that lyophilization decreases the amount of swelling at three hours after resuspending the spores and adding sugar and yeast extract. This is offset by the advantages of having large stocks of readily usable spores on hand which should not deteriorate as rapidly as suspensions. The spores were lyophilized by centrifuging aliquots in 50 ml centrifuge tubes, pouring off the supernatant, freezing rapidly in dry ice, and drying under high vacuum. The dried pellets were suspended easily by adding a small quantity of water, stirring to form a paste, and adding buffer.

The effect of azide on various processes. The effect of sodium azide (as a representative water soluble toxic compound) on various physiological processes of the spores was studied in relation to its effects upon spore swelling. Data summarizing results from several experiments are presented

in figure 7. It should be pointed out that azide, as well as several other poisons *data (unpublished)*, markedly stimulates the endogenous respiration of *M. verrucaria* spores. Consequently, the respiration data were corrected for this effect by subtracting the endogenous values at each concentration of inhibitor from the oxygen uptake in the presence of substrate at the same inhibitor concentration. Somewhat higher concentrations of azide are required to inhibit respiration than assimilation or swelling. This is in accord with the commonly demonstrated greater resistance of respiration than growth to toxic chemicals. Linear growth is most sensitive. The close relation of the fungicidal and viability curves to the fungistatic curve must be considered a coincidence since the time of exposure to the azide would certainly affect the position of the curves on the concentration axis. The arbitrary selection of an overnight period of incubation prior to washing off the azide placed the curve in its pictured position. If longer or shorter periods of exposure were used, the curve would have been shifted to the left or right, respectively. The shape of the curve would perhaps also change.

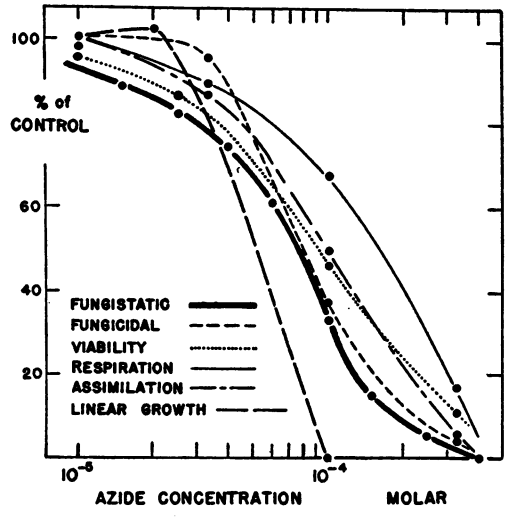

Figure 7. Effect of sodium azide on various processes.

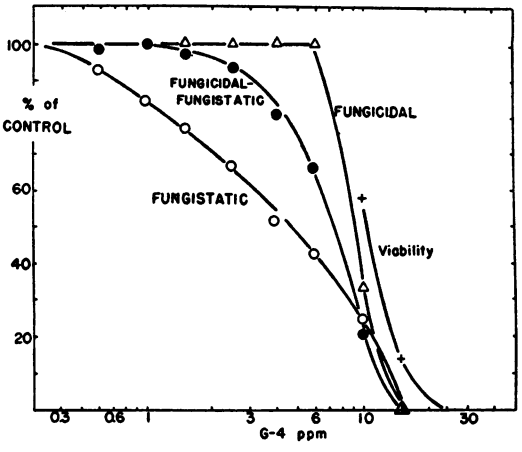

Figure 8. Effect of G-4 (2,2′ dihydroxy-5,5′ dichlorodiphenylmethane) on swelling and viability.

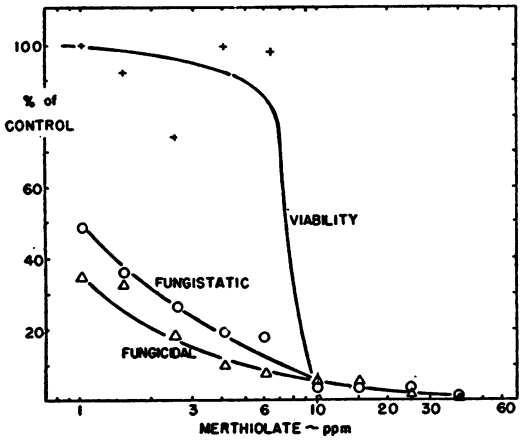

Figure 9. Effect of merthiolate on swelling and viability.

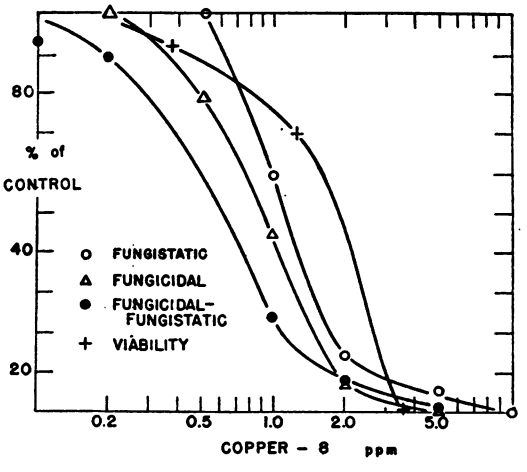

Figure 10. Effect of Copper-8 (copper-8-quinolinolate) on swelling and viability.

Toxicity measurements of various fungitoxicants. The effect of the fungicide G-4 on swelling and viability is shown in figure 8. The clear distinction between the fungistatic and fungicidal curves indicates that the inhibition below about 10 ppm is reversible, i.e., removing the G-4 from solution results in more or less complete restoration of the capacity for growth. Above 10 ppm the effect is not reversible. The shape of the fungicidal curve can be accounted for only by assuming death of varying numbers of spores. Presumably the failure of the viability and fungicidal curves to coincide is due to slow recovery of some spores. In the fungistatic-fungicidal curves presumably some adaptation or detoxification of G-4 occurs at sublethal concentrations since the curve is displaced to the right of the fungistatic curve.

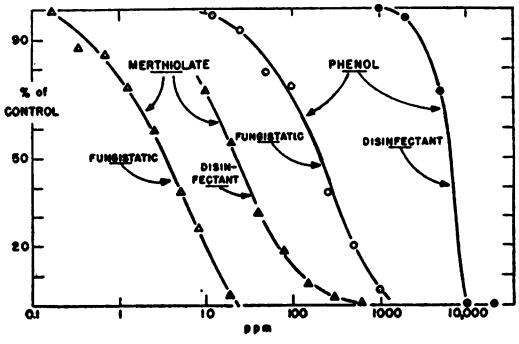

Figure 11. Inhibition by merthiolate and phenol when employed as fungistatic or disinfectant compounds.

The action of merthiolate (figure 9) is quite different from that of G-4 in that its toxicity changes much more slowly with changes in concentration. It differs also in that the fungicidal curve is to the left of the fungistatic one. Furthermore, the viability curve is radically different from the fungicidal curve. This is ascribed to slow reversal of toxicity at sublethal concentration. In other words, the inhibitory effects of merthiolate decrease slowly after washing the spores, whereas with G-4 or azide the effect is more immediate. This hypothesis is supported by the slightly smaller size of the colonies from treated spores in the viability tests. This difference is of a magnitude, as calculated from the linear growth rate, to indicate a lag of somewhat more than 4 hours in germination.

Data obtained with Copper-8 (figure 10) are more comparable to the relations found with merthiolate.

Measurement of disinfectant properties by spore swelling. The feasibility of using the swelling of spores as a measure of disinfectant properties following treatment with disinfectant concentrations was tested with merthiolate and with phenol. We are using disinfectant here as in the sense of Rahn (1945), i.e., high concentrations for a short period of time. For comparative purposes the data are plotted along with fungistatic measurements (figure 11).

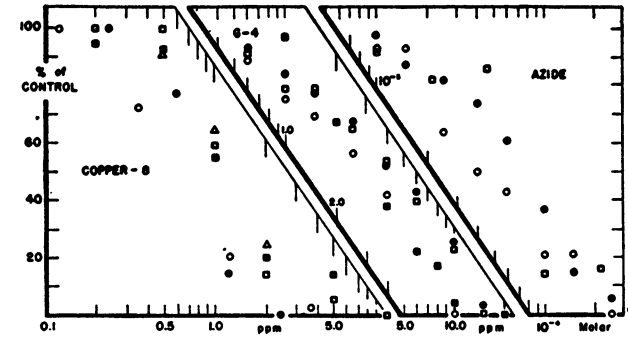

Figure 12. Reproducibility of inhibition by Copper-8, (copper-8-quinolinolate) G-4 (2,2′ dihydroxy-5,5′ dichlorodiphenylmethane), and azide. Data are from comparable experiments.

Reproducibility of the method. Reproducibility of the method is illustrated in figure 12, where data from separate experiments for several inhibitors are plotted together. These experiments were performed over a period of about six months. In each case the similar points represent an individual experiment; different suspensions were used in each experiment.

Adaptability to other organisms. The applicability of the method to other fungi and several bacteria was investigated. Of the large number originally considered, many were eliminated be-

cause of such factors as pathogenicity, poor spore production, poor wetting-out characteristics, and difficulties in handling spores.

Organisms showing some promise as test organisms are listed in table 4. Qualitative observations with *Trichoderma viride* (strain QM 6a), and *Alternaria tenuis* indicate these organisms to have some possibilities.

Organisms which in actual test were found not to swell significantly under the conditions used for *M. verrucaria* were *Aspergillus terreus* (strain QM 82j), *A. niger* (strain QM 458), *Cladosporium herbarum* (strain QM 489), *Helminthosporium* sp (strain QM 763), *Stemphylium* (*Macrosporium*) *sarcinaeforme*,[1] *Memnoniella echinata* (strain QM 1c), *Cephalothecium roseum* (strain QM 599), *Chaetomium globosum* (strain QM 459), *Pestalotia bicolor* (strain QM 664), *Sclerotinia fructicola*,[1] *Botrytis* sp (strain QM 1059).

TABLE 4

Swelling of various organisms after 3 hours in nutrient media

ORGANISM	QM NO.	VOLUME	
		0 hr	3 hr
		μl	μl
Curvularia brachyspora	639	35	79
Penicillium expansum	921	19	31
Aspergillus fumigatus	6b	25	41
Pullularia pullulans	279c	11	50
Bacillus cereus	476	6	13
B. subtilis	942	10.5	100
B. megaterium	603	6.5	17.7
Escherichia coli	1465	1.0	7.5*

* 2 hr data.

Factors which would seem to militate against a given organism for general use are: (1) the possession of a thick, rigid spore wall where no swelling occurs and germination consists of the extrusion of one or more germ tubes, (2) extreme slowness of germination, (3) pathogenicity, (4) extreme sensitivity to poisons.

With bacteria and yeast it is quite likely that cell division may occur within the time period of the test. This would have no effect on the usability of these organisms.

[1] These cultures were kindly supplied by Dr. Saul Rich of the Connecticut Agricultural Experiment Station.

DISCUSSION

To clarify discussion of the results it should be pointed out that the terms fungistatic, fungicidal, and disinfectant as used here are based on the experimental conditions used (see Methods) which in turn were established to conform with the conventional use of these terms (see McCallan and Wellman, 1942; Horsfall, 1945*a*; Rahn, 1945).

The swelling of *M. verrucaria* spores in solution containing sugar and yeast extract, as employed here, is accompanied by more or less parallel increases in dry weight which follow a typical autocatalytic growth curve. During this swelling the rate of metabolism increases rapidly as indicated by a progressive change in the initial respiration of the resting spores from a Q_{O_2} of about 1 to a value of the order of 75 within 2 hours (Mandels and Norton, 1948). Thus, under these conditions, increase in cell volume is a valid measure of growth.

The partial inhibition of growth in the presence of toxicants, as measured by cell volume, can be due to a partial inhibition in the rate of growth of each spore, to a selective action resulting from differences in sensitivity of each spore (the shape of the curve being determined by the percentage of spores inhibited), or to a combination of both processes. In other words, is the shape of the curve a manifestation of spore variability or does it represent the effect of concentration of inhibitor on an enzymic reaction or series of reactions? In the case of the fungicidal curve where the spores have been incubated with inhibitor overnight and are then washed "free" of fungicide before adding substrate, viability determinations have been made. To varying degrees the viability curve is always displaced to the right of the swelling curve, i.e., the concentration of toxicant which inhibits swelling 50 per cent kills less than 50 per cent of the spores. Furthermore, the curves are not always of the same shape. This is particularly evident with merthiolate. The shape and position on the concentration axis of the fungicidal swelling curve thus are not determined exclusively by percentage of spores killed. The implication is that the inhibitory effect at sublethal concentrations and times of exposure is reversible but not instantaneous, the reversibility taking time.

The shape of the fungistatic swelling curve

cannot be ascribed to killing spores since the inhibition at the concentration levels employed is at least partially reversible unless prolonged incubation is used—witness the differences between the fungistatic curves and the disinfectant curves. While we have no unambiguous data, it is assumed that the shape of the fungistatic curve should be ascribed to effects of concentrations of inhibitor on limiting enzyme reactions, permeability, etc.

In the fungistatic tests, where only partial inhibition is occurring, the inhibitor acts upon a rapidly metabolizing system in which growth is taking place. In the fungicidal tests the action is on a dormant spore having a very low rate of metabolism which even qualitatively must be quite dissimilar from that of the growing spore in the presence of exogenous substrates. In spite of this it is significant to note that the shapes of the two types of curves are similar, the position of the fungicidal curve on the concentration axis being a function of time of exposure (i.e., compare disinfectant curve with fungicidal). If the shape of the toxicity curve is an indication of the mechanism of action as discussed by Horsfall (1945*b*), it can be inferred that in the cases studied the inhibitor is acting in the same or similar manner upon metabolizing and dormant spores. The curves are not identical, however, but cross in some cases. The shift in the toxicity curves with time, as noted particularly in the first few hours (table 2), may indicate a difference in action upon dormant and on growing cells.

Comparison with other methods. The two principal methods for assaying fungicides in laboratory screening tests are the linear growth method using petri dishes and the spore germination test (Horsfall, 1945*a*). The former of these methods is used primarily by those interested in so-called industrial fungicides; the latter, by those concerned with agricultural fungicides.

Data obtained from measurements of the effects of toxic compounds on linear growth have been very useful yet are difficult to interpret in precise manner because of the questionable physiological meaning of increase in colony diameter as a measure of growth. In some cases of toxicity measurements by this method, clear-cut results are obtained, the rates being constant and some function of the concentration of toxicant with no significant lag evident in any of the curves. In others, however, growth occurs in the presence of toxicant only after a lag of several days or longer. In such cases the rate of growth may be slower than in the controls or it may be equal to the rate in the controls (Bateman, 1933). Presumably the lag represents adaptation to the toxicant although volatilization, decomposition, etc. must also be considered. In the procedure recommended by the Prevention of Deterioration Center (1948) for testing candidate fungicides the criterion used for effectiveness of the chemical is the decrease in growth rate as measured by the slope of the growth curve, the lag period being neglected completely. In addition to the obvious problems involved in interpretations of data of this type, there is a fundamental difficulty involved. Specifically, just what does linear growth, or its inhibition, represent? In many cases there is no consistent relation between growth as measured by spread of a fungus on agar and as measured by dry weight increase, assimilation of nitrogen, volume of mycelium, etc. This is easily demonstrated by the fact that the traces of impurities present in some types of agar are adequate to support a maximum amount of growth as measured by colony diameter, yet the amount of mycelium formed in such cases is practically nonmeasurable. In such cases addition of sugar may actually retard linear growth slightly, yet the total growth is increased greatly.

Another example of this lack of correlation was encountered in an experiment where a cover slip had been placed on the surface of nutrient agar in a petri dish. A fungal contaminant which started growth at one edge of the cover slip had the same rate of linear growth under the cover slip as it did out in the open, in spite of the restricted oxygen supply. The total amount of mycelium produced, however, was immensely greater where growth was not restricted by the cover slip. On the other hand rates of linear growth are, in many cases, proportional to concentrations of accessory growth factors required by the organism as evidenced by the extensive utilization of this technique in work with *Neurospora.*

A distinct advantage of the linear growth technique is that the test extends over a sufficient period of time as to provide some opportunity for adaptation to occur. Obviously a fungitoxic compound is of limited use if adaptation occurs readily, and this cannot be detected in short

term tests. However, adaptation to DDT, sulfonamides, or certain antibiotics does not render these agents useless. For purposes of screening the significant point seems to be the initial toxicity of the compound.

The spore germination technique for assaying fungicides has been used successfully by several groups of investigators. Interpretation of the data obtained in such tests has been on a basis of inherent variability among the spores used in their resistance to the toxicant, the data following a normal distribution curve. This method is exclusively a sporostatic test. The method proposed here, as indicated earlier, cannot be interpreted on the same basis. Whereas spores are used in both types of tests, the results obtained by measuring the swelling of spores are a combination of sporostatic and mycostatic phenomena. The data do not follow the pattern expected if normal variability of spores were involved. In plots on logarithmic-probability paper (McCallan and Wellman, 1942), straight lines are not obtained. This can possibly be ascribed to a combination of sporostatic and mycostatic effects. At high concentrations of toxicant where considerable inhibition is found—say over 60 per cent—the effect may be primarily sporostatic since only partial germination has occurred in the duration of the test. On the other hand, at lower concentrations where only slight inhibition is noted, the effect may be primarily mycostatic.

Advantages and limitations of the method. The method proposed has several distinct advantages over both the spore germination test and the linear growth method. It is much more rapid—3 hours as compared with incubation overnight for the spore germination test and 5 or more days for linear growth measurements. Because of the short time of incubation necessary with the spore swelling techniques, aseptic precautions need not be observed. The linear growth procedure requires aseptic techniques. In addition to the difficulties inherent in aseptic manipulations, the problem of sterilization of the fungicide is ever present. The ease of measuring cell volumes in hematocrit tubes is a distinct advantage over tedious microscopic observations of germination.

An attractive feature of the method is the possibility of preparing standard spore suspensions for use over an extended time period. Such suspensions in buffer do not change in their potentialities for swelling over a period of 9 days when refrigerated. Lyophilization of aliquots of a standard suspension should enable storage over more prolonged periods. In addition to the time-saving features of such methods it may be that the inherent biological variability found when repeating tests over a period of time could be reduced materially.

The disadvantages of the method are based on the limitations as to organism and on the required inclusion of a complex nutrient-like yeast extract in the medium. Preliminary trials of a variety of fungi have been made. Quite possibly some of these could be adapted for the technique if there were sufficient reason for their use to justify experimental establishment of more ideal conditions. Undoubtedly bacteria and yeasts could be used; indeed, a standard method for measuring quantities of unicellular organisms is by centrifugation in hematocrit tubes.

Inclusion of a complex substrate in the nutrient solution leads to the possibility of interaction with the toxicant being tested. In none of the cases studied, however, has this factor appeared to be significant.

Applications. The simplicity and speed of the test indicate that its application may extend beyond the screening of fungitoxic compounds. The screening of chemicals for application as disinfectants, water purification, etc. is a possibility. Such application depends to a great extent upon the pattern of toxicity in the spores as compared with that in the specific organisms one is interested in. Obviously the establishment of similarity in toxicity patterns is a lengthy task and the extrapolation from one organism to another is always uncertain.

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SUMMARY

Spores of the fungus *Myrothecium verrucaria* swell rapidly and germinate in the presence of sucrose and yeast extract. This swelling is not a simple osmotic process but is accompanied by increase in dry weight and is a typical phenome-

non of growth. The cell volume, which is easily measured by centrifugation in hematocrit tubes, increases about 5 times in 3 hours at 30 C.

A rapid assay for screening compounds for fungitoxicity based on this spore swelling has been developed. The primary advantages of the assay are the speed (3 hours) and the elimination of aseptic precautions. Standard suspensions can be stored under refrigeration for at least nine days without deterioration. Lyophilization of aliquots of standard suspensions can be used to provide material over more extended periods.

In a brief survey of the spores of other fungi some were found which show promise for utilization in the technique.

The technique is particularly adapted to fungistatic testing although it can also be used for fungicidal or disinfectant testing.

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