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THE USE OF RADIOISOTOPES IN STUDYING THE AFFINITY OF VARIOUS TOXICANTS FOR FUNGUS SPORES

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DISCLAIMER

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In testing the toxicity of chemicals to biological structures such as fungus spores it has been customary to express the effectiveness on the basis of the concentration of the applied solution (1, 2). This has been necessary because of the small quantities involved and the lack in practically all instances of a suitable method for quantitative determination of the toxicants taken up by the spores. In investigations of the effectiveness of different materials, comparisons on this basis are subject to serious error if there are large differences in the doses received by the spores from applied solutions of comparable concentrations.

With the ready availability of radioactive isotopes, quantitative toxicity studies on a spore weight basis can be carried out quite easily. In connection with a program of investigations undertaken in cooperation with the United States Atomic Energy Commission (Contract AT(30-1)-788) innate toxicity on a weight basis has been determined for a number of toxicants and species of fungus spores. The results have shown that the concentration of the applied solution is indeed a poor measure of the amount of chemical received by the spores. Concentrations associated with the spores may reach values 10,000 or more times that of applied solutions.

With the use of isotopes it has also been possible to study the relationship of some toxicants to each other in competition for receptor sites and to study displacement of one material for another in fungus spores. It has also been found that spores of various species may act markedly different in their affinity for various toxicants.

MATERIALS AND METHODS

Toxicants used in these studies have included ^{35}S, ^{110}Ag, ^{144}Ce, ^{203}Hg, ferric dimethyldithiocarbamate labeled with ^{35}S, and 2-heptadecyl-2-imidazoline and 2,3-dichloro-1,4-naphthoquinone labeled with ^{14}C. The isotopes and labeled

compounds have been obtained from the Oak Ridge National Laboratories, Oak Ridge, Tenn., U. S. A., and from commercial sources. All of the materials were available at sufficiently high specific activity to be suitable for use at the concentrations required for their toxic effects.

Conidia of the following species of fungi have been included in these studies: Neurospora sitophila (Mont.) Shear & Dodge, N. crassa Shear & Dodge, Monilinia fructicola (Wint.) Honey, Alternaria oleracea Milbraith, Glomerella cingulata (St.) Sp. & von S., Aspergillus niger van Tiegh, Rhizopus nigricans Ehr., Cephalosporium acremonium Corda, Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr., Stemphylium sarcinaeforme (Cav.) Wilts., Venturia inaequalis (Cke.) Wint., and V. pyrina (Aderh.). Cells of Saccharomyces cerevisiae Hansen were also used in some of the tests. Methods employed for culturing the fungi, harvesting the spores, determining the spore weights, and the nutrients added for germination tests are given in previous papers (3, 4).

For studies on the uptake of toxicants by spores, known weights of spores were suspended in aqueous solutions of the toxicants for various periods of time and the loss in radioactivity of the ambient solution after centrifugation determined. Values obtained by this procedure were also checked by determining the radioactivity of the spores after removal from the solutions of toxicant. Specific activities of the radioactive toxicants were sufficiently high so that radioactivity determinations could be made directly without correction for self-absorption. Similarly, because of their small size, samples of fungus spores could also be taken in small enough quantities for direct counting without loss from self-absorption even with the ^{14}C labeled compounds.

The radioactivity was determined by the use of conventional scaling equipment and end window type Geiger tubes with thin windows. When mixtures of isotopes were used in the tests, the determination of the relative quantities of the various isotopes present was carried out with the aid of absorbers. Automatic counting equipment was available and the samples were counted in the absence and presence of

suitable absorbers. From a knowledge of the effectiveness of the different absorbers used in reducing the counts obtained with the various isotopes under the conditions employed, it was possible to set up simultaneous equations and determine the relative quantities present.

With elemental sulfur the results were obtained largely by conventional chemical methods. In contrast to the other toxicants studied, sulfur is not accumulated in spores to any extent but is rapidly reduced to hydrogen sulfide which is then released. The quantity of hydrogen sulfide given off before germination was reduced to 50 per cent was considered the ED50 value (3).

RESULTS

RATE OF UPTAKE OF VARIOUS TOXICANTS

2-Heptadecyl-2-imidazoline

An unexpected result obtained in these studies was that the spores took up large quantities of toxicant very rapidly from dilute solutions. Rate of uptake and final concentration reached on a spore weight basis was considerably influenced by the species of spore used. With 2-heptadecyl-2-imidazoline, by far the major portion of toxicant was taken up in 15 seconds or less (4). Only when the amount of toxicant present was more than 10,000 p.p.m. on a spore weight basis with Neurospora sitophila (with a concentration of 2.16 p.p.m. in the ambient solution) was the rate of uptake decreased so that less than 50 to 70 per cent was taken up in 0.5 to 2.0 minutes. Even when the possible dose was 34,000 p.p.m., 40 per cent was taken up in 30 seconds and a further 23 per cent in 1.5 more minutes (4). On the other hand, spores of Aspergillus niger took up relatively small quantities of 2-heptadecyl-2-imidazoline, and when these spores were mixed with those of N. sitophila, they interfered with the uptake of the toxicant by the latter. This effect remains unexplained.

Silver and Cerium

Silver and cerium are also taken up rapidly (4) as shown by the data summarized in Tables I and II.

TABLE I

Uptake of Silver by Fungus Spores When Exposed to 1.04 P.P.M. in 10 Ml. of Solution

Species	Maximum dose possible	Cumulative uptake of silver as p.p.m. of spore wt. after various intervals in minutes		
		0.5	1.5	6.5
Neurospora sitophila	2080	1085	1475	1715
	1040	585	740	700
Aspergillus niger	1040	480	534	675
	520	310	330	385
Alternaria oleracea	1040	745	955	—
	520	445	490	—
Monilinia fructicola	4160	2125	2580*	—
	2080	1530	1310*	—

* These values for 120 minutes.

TABLE II

Uptake of Cerium by Fungus Spores When Exposed to 10 P.P.M. in 10 Ml. of Solution

Species	Maximum dose possible	Cumulative uptake of cerium as p.p.m. of spore wt. after various intervals in minutes		
		0.5	1.5	6.5
Neurospora sitophila	10,000	630	590	910
Aspergillus niger	10,000	4500	5220	5400
Alternaria oleracea	8,620	2255	3820	5990
Monilinia fructicola	10,000	1400	1450	—

It is apparent that, with silver, spores of all species included in the tests, covering possible doses of from 520 to 4160 p.p.m. of spore weight, removed 50 to 90 per cent of the available silver from the solutions in the first 30 seconds or less. With cerium also the greater part of the toxicant taken up by the spores

was removed in 30 seconds except for spores of Alternaria oleracea. It will be noted that spores of Aspergillus niger take up much more cerium than those of Neurospora sitophila and most of the other species, while with the other toxicants studied such as sulfur, 2-heptadecyl-2-imidazoline, and silver, Aspergillus niger was found to be among the least reactive.

Mercury

Recent tests with mercury and 2,3-dichloro-1,4-naphthoquinone have shown that these toxicants are also taken up in relatively large amounts and that large doses are required to reduce germination capacity as will be shown below. Mercury differed from the toxicants previously considered, i.e., 2-heptadecyl-2-imidazoline, silver and cerium in that uptake took place more slowly. This is shown in Table III for Monilinia fructicola and Table IV for Neurospora sitophila. It is obvious that 16 and more minutes are required for maximum uptake in contrast to 30 seconds or less for some of the other toxicants previously considered.

TABLE III

Uptake of Mercury by Spores of Monilinia fructicola

from Solutions Containing 10 P.P.M. in 10 Ml.

Maximum dose possible, p.p.m. of spore wt.	Cumulative uptake of toxicant in p.p.m. of spore wt. after various intervals in minutes			
	1	6	16	86
20,000	4760	6625	6695	11320
10,000	3255	4120	4775	6235
5,000	2990	3650	3885	4250
2,500	1930	2210	2260	2320
1,250	1150	1190	1190	1200

TABLE IV

Uptake of Mercury by Spores of Neurospora sitophila from Solutions Containing 10 P.P.M. in 10 Ml.

Maximum dose possible, p.p.m. of spore wt.	Cumulative uptake of toxicant in p.p.m. of spore wt. after various intervals in minutes	
	10	20
40,000	6780	20400
20,000	6500	13100
10,000	4500	7400
5,000	3350	4800
2,500	2200	2300

2,3-Dichloro-1,4-naphthoquinone

Results of similar studies carried out with 2,3-dichloro-1,4-naphthoquinone are given in Table V. In general this material was taken up rapidly in conformity with various toxicants previously investigated except mercury. As will be shown, this toxicant was more active on a spore weight basis than any previously considered except silver.

TABLE V

Uptake of 2,3-Dichloro-1,4-naphthoquinone by Fungus Spores When Exposed to 2.8 P.P.M. in 10 Ml.

Species	Maximum dose possible	Cumulative uptake of toxicant in p.p.m. of spore wt. after various intervals in minutes		
		5	15	75
Neurospora sitophila	2800	830	755	800
	933	550	535	505
	311	250	240	225
Monilinia fructicola	2800	1120	1320	1445*
	933	700	775	810*
	311	290	295	295*
Myrothecium verrucaria	2800	1360	—	—
	933	725	—	—
	467	410		

* Values after 216 minutes.

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Some Effects of Mixtures of Toxicants When Used Simultaneously or Consecutively

In recent experiments spores of a number of species of fungi were exposed to various combinations of 2-heptadecyl-2-imidazoline, silver, and cerium both in simultaneous and consecutive treatments. The results have shown (5) that the spores take up these toxicants at about the same rate and at about the same degree whether they are presented to them singly, simultaneously or consecutively. Toxicants are not released on resuspension in water or on further treatment with a second toxicant. The results suggest that receptor sites for the three toxicants are not similar. It would be expected, of course, that, if toxicants more closely related chemically were studied, certain interferences would be noted.

With the availability of both mercury and silver as radioactive isotopes, it seemed of special interest to study these two chemicals in their relationship since they would be expected to have some receptor sites in common. The results have shown, somewhat unexpectedly, that spores previously treated with silver took up mercury much more rapidly than spores not pretreated. Results of such an experiment with spores of Neurospora sitophila are shown in Table VI. In these tests spores were treated with silver followed by mercury, mercury followed by silver and with silver and mercury simultaneously. Exposures were for 10-minute periods. In the simultaneous treatments two consecutive 10-minute treatment periods were used. Determinations of both isotopes were made so that release of toxicants previously taken up was taken into account. Silver was applied as 1.00 p.p.m. in 10 ml. and mercury at 10 p.p.m. This difference in concentration was used because of the greater toxicity of silver compared to mercury. The maximum concentrations that could be attained in the 10-mg. lots of spores were 1000 and 10,000 p.p.m. for silver and mercury respectively and correspondingly less for the larger lots of spores. It is apparent that more mercury is taken up during the time periods under study if the spores have been treated with silver previously or if silver is present simultaneously. For example, with the 10-mg. lots, spores not previously treated took up 4450 p.p.m. while those previously exposed to silver

as a result of which they took up 760 p.p.m. of silver, took up 7800 p.p.m.of mercury in ten minutes. When silver and mercury were present simultaneously, 6700 p.p.m. of mercury were taken up in the first ten minutes. Another lot of spores, data for which are not shown in the table, but which was treated simultaneously with those in this experiment took up 4800 p.p.m. of mercury in ten minutes. When the amount of mercury available is relatively low as in the 40 and 50 milligram lots the mercury is taken up equally readily whether or not pretreatment with silver is involved. Further experiments have shown that this effect of silver on the uptake of mercury is primarily an influence on the rate. When longer time periods are observed, spores not pretreated with silver take up about as much mercury as those pretreated.

TABLE VI

Silver and Mercury Contents and Germination of Spores of Neurospora sitophila Treated with Silver Followed by Mercury, Mercury Followed by Silver, and with Silver and Mercury Simultaneously in Two 10 Minute Treatment Periods

Treatment	Spore wt., mg.	Ion content in p.p.m. of spore weight and germination in per cent				
		First period		Second period		
		Ag	Germination	Ag	Hg	Germination
Silver followed by mercury	10	760	0	615	7800	0
	20	405	15	460	4600	7
	30	290	25	320	3010	25
	40	210	37	240	2300	27
	50	185	46	200	1650	36
		Hg		Hg	Ag	
Mercury followed by silver	10	4450	64	5120	780	0
	20	3430	77	4070	480	0
	30	2300	86	2360	260	20
	40	1780	102	1700	235	32
	50	1650	96	1880	190	41
		Ag / Hg		Ag	Hg	
Silver and mercury together	10	425 / 6700	2	700	8190	0
	20	360 / 4360	22	275	4410	28
	30	300 / 2925	37	320	2990	36
	40	235 / 2070	43	240	2200	48

INNATE TOXICITY OF THE FUNGICIDES

With these techniques the effect on germination based on the actual dose received by the spores can be readily determined. When plotted on logarithmic-probability paper a straight line is obtained as usually happens also when the dose is expressed on a concentration basis. Such a curve is illustrated in Figure 1 in which the effect of mercury on the germination of spores of Neurospora sitophila was determined. Values obtained in two different experiments, as indicated by the different kinds of circles, were plotted. The ED50 value was found to be 5030 p.p.m. of spore weight.

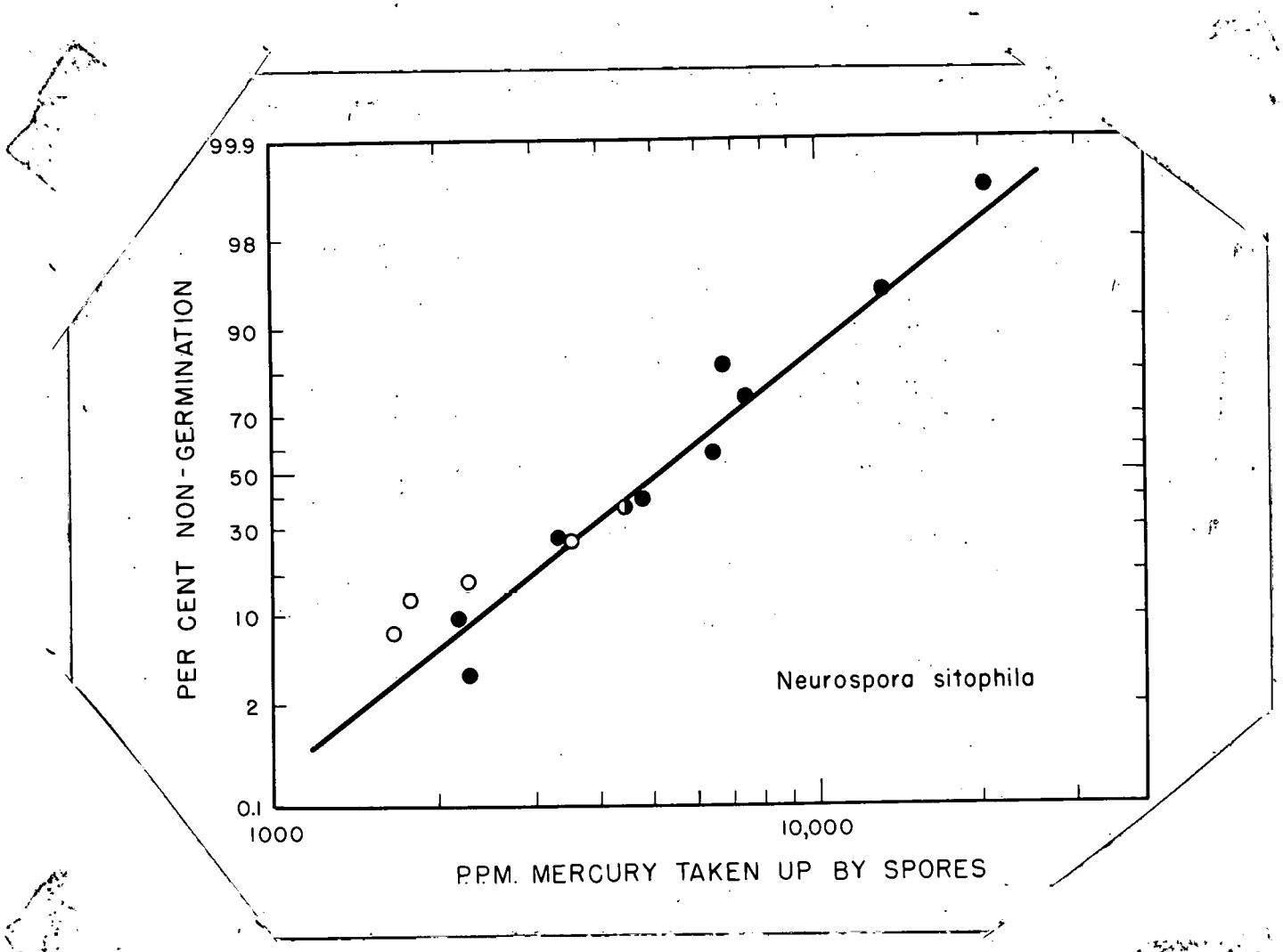

Figure 1. Dosage response curve for the effect of mercury on a spore weight basis on the germination of spores of Neurospora sitophila.

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ED50 values for a number of the fungicides under study are summarized in Table VII. Silver is the most toxic of all the materials investigated, although 2,3-dichloro-1,4-naphthoquinone approaches it closely. The quantity of 2-heptadecyl-2-imidazoline required on a spore weight basis to give a 50 per cent loss in germination capacity for spores of Venturia pyrina was found to be 9300 p.p.m. In spite of this relatively low innate toxicity this fungicide is successfully used commercially against apple scab caused by the closely related Venturia inaequalis. Cerium is the least toxic of the materials studied. It was toxic only to Monilinia fructicola. Spores of two other species were not affected by the uptake of 7000

TABLE VII

ED50 Values in P.P.M. on a Spore Weight Basis for a Number of Toxicants for Spores of Some Species of Fungi

Toxicant	Species	ED50 values
2-Heptadecyl-2-imidazoline	Neurospora sitophila	5800
	Venturia pyrina	9300
Silver	Neurospora sitophila	165
	Monilinia fructicola	216
	Alternaria oleracea	360
	Aspergillus niger	540
Cerium	Neurospora sitophila	> 970*
	Monilinia fructicola	4600
	Alternaria oleracea	>7110*
	Aspergillus niger	>8440*
Sulfur	Neurospora sitophila	11,500
	Cephalosporium acremonium	53,600 (ED72)
	Alternaria oleracea	6,800 (ED95)
Mercury	Neurospora sitophila	5030
	Monilinia fructicola	2830
2,3-Dichloro-1,4-naphthoquinone	Neurospora sitophila	560
	Monilinia fructicola	385

* No effect on germination at these concentrations.

to 8000 p.p.m. Spores of Neurospora sitophila took up only relatively small amounts.

The toxicity of all the fungicides included in these studies is relatively low compared to the effectiveness of other biological agents. Many drugs are toxic to animals at several milligrams per kilogram. Studies with ^{35}S labeled penicillin by Rowley et al. (6) have shown that only very small quantities are required per unit weight of bacteria to stop growth. Similarly many insecticides and plant growth regulators are active at low concentrations. It would therefore seem reasonable to expect that much more active antifungal substances will probably be discovered.

RECEPTOR SITES

Release and Exchange of Toxicants Taken up by Spores

When spores which have taken up various toxicants are removed from the testing solutions and resuspended in water, there is usually little release of toxicant from the spores. In general drastic measures are necessary to remove the toxicants from the spores. However, on adding a large excess of non-radioactive fungicide to the ambient solution, exchange takes place. By studying the degree and rate of exchange it is possible to get some idea as to how firmly the materials are held, and also with organic compounds, whether they have undergone chemical change in the spores. Further, by using related chemicals in the ambient solution, instead of chemicals identical with those used in the original treatment, information can be obtained as to the degree of competition for the same receptor sites. In such tests it is necessary for only one of the pair of materials to be investigated to be radioactive. This facilitates studies with organic compounds which are often difficult to obtain in radioactive form.

Studies of this nature have shown that fungus spores do not distinguish between neodymium, lanthanum, samarium, praseodymium, and cerium. Cerium taken up by spores was found to exchange just as readily with the other elements mentioned

as with cerium itself. Some of the relationships between silver and a number of other ions are given in Table VIII. In these experiments the effect of Hg+,

TABLE VIII

Effect of Other Metal Ions on the Uptake or Release of Silver When Added Either Previous to, Simultaneous with, or Subsequent to Exposure to Silver*

Ion	Micrograms of silver for 10 mg. of spores of Neurospora sitophila				
	Uptake after pretreatment		Uptake in simultaneous treatment		Release of Ag on subsequent treatment**
	10 Min.	1150 Min.	10 Min.	932 Min.	60 Min.
None	13.9	17.7	14.1	18.2	0.9
Ag+	10.0	9.0	-	-	11.9
Hg+	6.0	12.0	0.0	4.6	5.5
Hg++	4.8	8.1	0.0	2.8	-
Cu++	6.9	15.8	2.7	13.4	1.0
Cd++	10.4	18.1	3.0	16.5	1.1
Ni++	12.7	17.4	3.9	15.6	0.9
Co++	-	-	3.2	14.3	0.9
Zn++	9.7	18.0	15.6	19.7	0.7

*Spores exposed to 10 ml. of soln. of silver containing 2×10^{-5} equivalents per liter. Other ions used at concentrations 50 times higher.

** Spores had taken up an average of 11.5 micrograms.

Hg++, Cu++, Cd++, Ni++, Co++, and Zn++ ions on the uptake and release of Ag+ when used in pretreatments, in simultaneous treatments, and subsequently for exchange, was studied. The spores were exposed to 10 ml. of a solution of radioactive silver containing 2×10^{-5} equivalents per liter and the other ions were used at concentrations 50 times higher. It is seen that Hg+ and Hg++ and Cu++ showed the greatest competition with silver for receptor sites. Interference was more pronounced in the simultaneous treatment series than when the spores were pretreated with the various ions. In the exchange studies, exchange was found to be complete upon the addition of silver and about 50 per cent with Hg+ (Hg++ was not

included in this test). The other ions did not release significant amounts of the absorbed silver.

Other tests have confirmed this finding that silver taken up by spores is readily exchanged upon the addition of more silver. This is more complete than the exchange with any of the other toxicants studied. Spores saturated with 2,3-dichloro-1,4-naphthoquinone released only a few per cent of the toxicant upon the addition of non-radioactive naphthoquinone. This is interpreted to indicate that toxicant taken up has undergone chemical change. This is being investigated further by other methods.

SUMMARY

When fungus spores are exposed to dilute solutions ~~(1 to 10 p.p.m.)~~ of toxicants such as 2-heptadecyl-2-imidazoline, 2,3-dichloro-1,4-naphthoquinone, silver, mercury, or cerium, relatively large amounts are taken up rapidly by the spores. ED50 values were found to range from 165 p.p.m. of spore weight for silver and spores of Neurospora sitophila to 9300 p.p.m. for heptadecyl-2-imidazoline and spores of Venturia pyrina. These high values show that the spores accumulate the toxicants from dilute solutions. They also show that the innate toxicity of these materials is not very high in comparison with other biologically-active agents. The results suggest that further search for new fungicides should lead to some that are more active on a spore weight basis.

The amount of toxicant taken up depends upon the toxicant and the particular species involved. Spores more active in taking up one toxicant compared to spores of other species, may not necessarily be more active in this respect with another fungicide. Specificity of fungicides may be the result of differences in the quantity taken up and in innate toxicity to the particular species involved.

When various materials not closely related chemically are used in either consecutive or simultaneous applications to fungus spores interferences are not apparent. Spores saturated with one toxicant will readily take up large amounts of a second unrelated chemical. Competition for receptor sites becomes evident,

however, when closely related materials are studied. The rare earth elements neodymium, lanthanum, samarium, praesodymium, and cerium seeem to compete for the same receptor sites.

Competition for receptor sites was found between Ag+, Hg+, Hg++, and Cu++. Spores pretreated with Ag take up mercury more rapidly than spores not pretreated. Silver taken up by spores is exchanged were readily than any of the other fungicides studied. It appears that 2,3-dichloro-1,4-naphthoquinone is rapidly changed chemically when taken up by spores, since no exchange occurs when spores apparently saturated with this fungicide are exposed to additional quantities of the toxicant.

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