Effect of Chloroneb on the Growth
and Metabolism of Ustilago maydis

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ABSTRACT

Chloroneb (8 μg/ml) prevented multiplication of sporidia of *Ustilago maydis*, but had little effect on increase in absorbance at 450 nm or dry weight until after 3 hr. Subsequent increases in absorbance and dry weight were strongly curtailed. Colony-forming ability was not affected when sporidia were incubated in phosphate buffer with chloroneb for 12 hr, but was reduced 80% when sporidia were incubated 12 hr in nutrient medium with chloroneb. The greatest uptake of chloroneb by sporidia in nutrient medium or phosphate buffer occurred within the first 15 min of incubation. Additional uptake of the toxicant was proportional to the increase in sporidial dry weight. No detectable metabolism of chloroneb occurred during a 24-hr incubation period. Concentrations of chloroneb (8 or 12 μg/ml) which prevented sporidial multiplication had no effect on the oxidation of glucose or acetate. Protein, RNA, and DNA of treated and untreated cultures increased at approximately the same rate until failure of sporidial division in the treated cultures. Subsequent increase of protein, RNA, and DNA in treated samples was strongly inhibited. Synchronous sporidia exposed to chloroneb did not form buds, but underwent mitosis and formed binucleated sporidia with a cross wall between the two nuclei. Thus, inhibition of protein, RNA, and DNA syntheses appears to result from the failure of sporidia to form buds and divide normally, rather than from a direct action of the toxicant on these syntheses.

A mutant of *U. maydis* resistant to chloroneb was isolated from medium containing 8 μg/ml of the toxicant. There was no appreciable difference in the uptake of chloroneb by the mutant or wild type sporidia. Neither mutant nor wild type metabolized chloroneb. The chloroneb-resistant mutant was also resistant to 2,6-dichloro-4-nitroaniline, diphenyl, hexachlorobenzene, naphthalene, *p*-dichlorobenzene, pentachloronitrobenzene, and sodium-*o*-phenylphenate. Tolerance to chloroneb in progeny from a cross of the resistant mutant to a sensitive wild type indicated single gene control of the resistance.

Additional key words: mechanism of fungicidial action, budding.

Chloroneb is a recently developed systemic fungicide effective against certain species of *Rhizoctonia* (8), *Pythium* (19), and *Ustilago* (21). It is taken up by the roots and accumulated in the roots, lower stem, and cotyledons when applied as either a soil drench or a seed overcoat (8). Hock & Sisler investigated the metabolism (12) and mode of antifungal action (13) of chloroneb in *Rhizoctonia solani* and found that the fungicide did not inhibit oxygen uptake but moderately inhibited uptake of inorganic orthophosphates and incorporation of phenylalanine into protein and uridine into RNA. Incorporation of thymidine into the DNA fraction of *R. solani* mycelium was inhibited 85-90%, but it was not determined whether this inhibition resulted from a direct or indirect action of the toxicant on the DNA synthetic process.

Interference of chloroneb with mitosis or cytokinesis could indirectly bring about inhibition of DNA synthesis. Several aromatic hydrocarbon compounds with properties similar to chloroneb are known to cause mitotic aberrations in fungi (18) and higher plants (16, 31). The present investigation was an attempt to further clarify the relationship of chloroneb toxicity to effects on DNA synthesis, nuclear division, and other cellular processes.

MATERIALS AND METHODS.—*Culture methods.*—The test organism, *Ustilago maydis* (DC.) Corda, American Type Culture Collection 14826, was grown in liquid nutrient medium (5) supplemented with yeast extract (2 g/liter) at 30 C on a water bath shaker for 16 to 20 hr. Sporidia were collected by centrifugation, washed 3 times in 0.02 M phosphate buffer (pH 6.6), and adjusted to a standard concentration of 0.7 mg dry wt/ml unless otherwise stated.

All tests were made in the nutrient medium of Coursen & Sisler (5), modified by a doubling of the concentration of phosphate buffer. Incubations were carried out in a water bath shaker at 30 C unless otherwise stated. Chloroneb was added in methanol. The final concentration of methanol in the treated and untreated cultures never exceeded 1%.

Toxicity studies.—Toxicity of chloroneb to a standard suspension of *U. maydis* was evaluated by measurement of the effect of the toxicant on increase in absorbance and sporidial number, and colony-forming ability. At various times, 1 ml of suspension was removed and diluted with 9 ml of 0.02 M phosphate buffer, and the absorbance was measured at 450 nm. Increase in sporidial number/ml

was determined microscopically using a bright line hemacytometer. Colony-forming ability of cells incubated with the inhibitor for various time intervals was determined according to the method of Clemons & Sisler (4).

Uptake and metabolism of chloroneb-^{14}C.—Sporidia (1.7 mg dry wt/ml) were incubated in nutrient medium or 0.02 M phosphate buffer (pH 6.6) containing 8 μg/ml of either methyl-labeled chloroneb-^{14}C (specific activity, 1.8 mc/mmole) or ring-labeled chloroneb-^{14}C (specific activity, 0.48 mc/mmole). After various intervals, the suspension was centrifuged and 50-μliters of supernatant were collected and mixed with 10 ml of toluene-methanol scintillation fluid (13). Radioactivity in this and subsequent experiments was determined with a Packard Tri-carb Model 500B liquid scintillation counter. Extraction of sporidia treated with radiolabeled chloroneb and the chromatographic analysis of these extracts and the culture filtrate followed the procedures of Hock & Sisler (12).

Respiration.—The effect of chloroneb on the oxidation of glucose or acetate (0.25% w/v) was determined with a Gilson recording oxygen electrode according to the method of Clemons & Sisler (4).

Phenylalanine and uridine incorporation.—Radiolabeled phenylalanine and uridine were utilized to study effects of chloroneb on protein and nucleic acid syntheses. A standard suspension of sporidia was incubated at 30 C in nutrient medium for 1 hr prior to the addition of 8 μg of chloroneb/ml and L-phenylalanine-^{14}C (uniformly labeled; specific activity, 2 mc/mmole; final concentration, 1.0×10^{-4} M) or uridine-2-^{14}C (specific activity, 4.5 mc/mmole; final concentration, 1.1×10^{-4} M). Controls received only L-phenylalanine-^{14}C or uridine-2-^{14}C. At 20- or 30-min intervals, 1-ml samples of the sporidia were processed, and radioactivity in protein, DNA, and RNA fractions was determined according to the procedures of Clemons & Sisler (4).

RNA and DNA analysis.—The ribose-orcinol method (23) was used to determine the RNA content of chloroneb-treated and untreated sporidia. Every 2 hr, 5-ml samples of sporidial suspension were centrifuged and the packed sporidia were extracted with 1-ml volumes of 95% ethanol and ether-ethanol (3:1, v/v). Sporidia were chilled to 0 C and washed with 1 ml of 0.2 M perchloric acid. We extracted RNA by suspending the sporidia in 1 ml of 1 M perchloric acid overnight at 4 C. Sporidia were centrifuged, resuspended in fresh 1 M perchloric acid, and incubated 4 hr at 4 C. Perchloric acid extracts were combined, and 1 ml was added to 10 ml of orcinol reagent. The color reaction was developed in stoppered test tubes for 8 min at 100 C. The solution was cooled, and we estimated the RNA content by comparing the absorbance at 640 nm with values of a standard curve prepared using purified yeast RNA. The DNA content of sporidia was determined by the diphenylamine method (3) following the procedure outlined by Clemons & Sisler (4).

High energy nucleotide phosphate.—A standard suspension of sporidia was incubated for 2 hr in nutrient medium prior to the addition of 8 μg of chloroneb/ml. The sporidia were incubated another hour, and the free nucleotides were then extracted with cold 4% perchloric acid (v/v). The nucleotides were purified over Norit (28), and the acid-labile phosphorous was released by treatment with 1 N HCl at 100 C for 7 min (29). Inorganic phosphate released was determined by the method of Fiske & Subbarow (9).

Synchronous growth and analysis of sporidia.—Sporidia were synchronized according to the methods of Holliday (15). After synchronization, the sporidia were incubated in nutrient medium with or without 8 μg of chloroneb/ml. At various times, 20-ml samples were removed, chilled, and extracted with 2-ml volumes of absolute ethanol, ice-cold 10% trichloroacetic acid (w/w), 50% ethanol (v/v), and ether-ethanol (3:1, v/v). The RNA fraction was removed (4) and the cellular residue was resuspended in 1 ml of 0.5% trichloroacetic acid (w/w) and hydrolyzed at 90 C for 15 min. After a cooling period, the sporidia were centrifuged and washed twice with 0.5 ml of 0.5% trichloroacetic acid. The trichloroacetic acid hydrolyzate and washes were combined and the DNA content was determined by the diphenylamine method (3). The cellular residue was resuspended in 3 ml of 0.1 N NaOH and then washed twice with 0.5 ml of 0.1 N NaOH. The protein content of the combined NaOH extracts was determined by the method of Lowry et al. (20).

Nuclear stain.—Sporidia were fixed with 95% ethanol-glacial acetic acid (3:1, v/v) for 30 min at room temperature. We removed the excess fixative before hydrolysis by washing the sporidia with distilled water. We performed hydrolysis at room temperature using 5 N HCl for 5 to 7 min. The cells were immediately washed with distilled water (approximately 10 times the volume of HCl used) and incubated in de Thomasi Schiff's reagent (2) in the dark for 30 to 40 min. The unbound stain was removed by rinsing in tap water for 10 min.

Cell wall analysis.—The hexose and hexosamine content of the cell walls was determined by the methods of DeTerra & Tatum (6). A standard suspension of sporidia was incubated for 6 hr in nutrient medium with or without 8 μg of chloroneb/ml. The sporidia were centrifuged, washed, and lyophilized. The dried sporidia were ground in a mortar and extracted with 1% sodium deoxycholate for 24 hr at 4 C. The wall fragments were then washed 10 times with distilled water, passed through a graded ethanol series, washed 5 times with absolute ethanol, and dried overnight at 37 C. Microscopic examination of the wall preparation revealed no intact sporidia or contamination of the wall fragments. Ten-mg samples of the wall material were hydrolyzed in 2 ml of 3 N HCl for 3 hr in a boiling water bath. After a cooling, the residue was centrifuged and the excess HCl was removed by vacuum evaporation. The samples were brought to equal volumes and the hexose and hexosamine
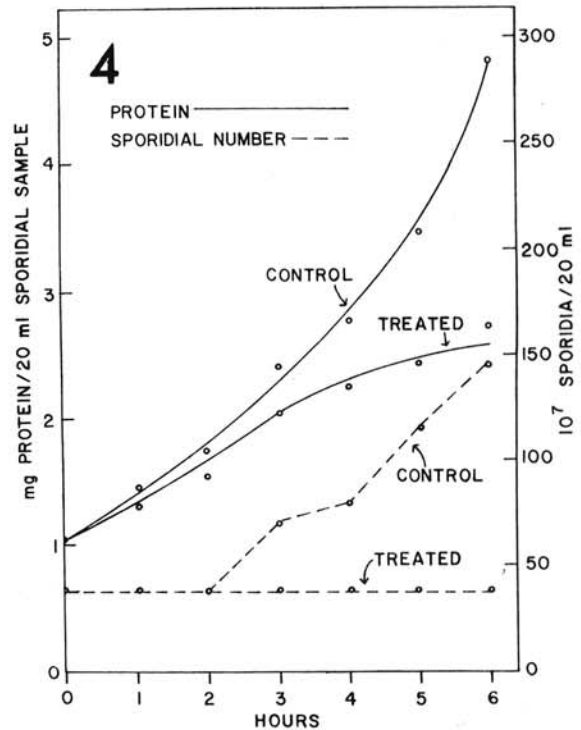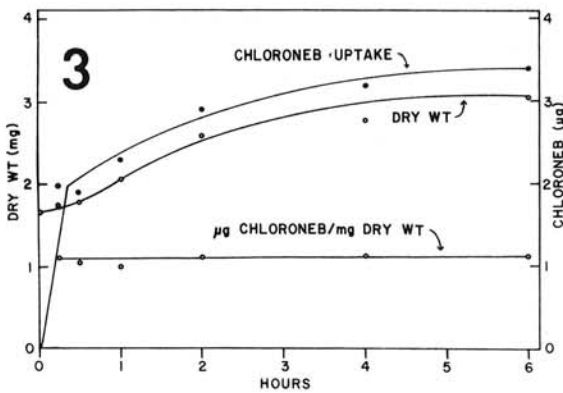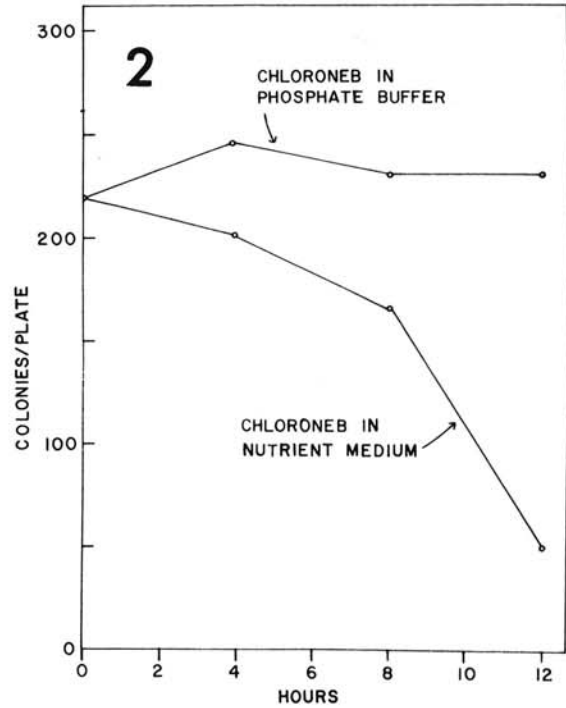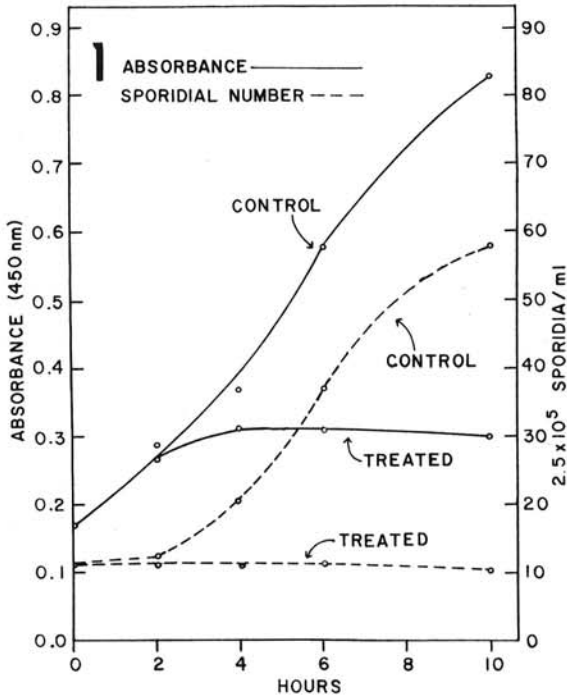
Fig. 1-4. 1) Effect of 8 μg of chloroneb/ml on the increase in absorbance and sporidial number of *Ustilago maydis* sporidia incubated in nutrient medium. Absorbance and sporidial number values plotted are for a 10-fold dilution of the standard suspension used. **2)** Effect of exposure time on survival (ability to form colonies) of *U. maydis* sporidia in a medium containing 8 μg of chloroneb/ml. **3)** Increase in sporidial dry weight and uptake of chloroneb by *U. maydis* sporidia incubated in a nutrient medium containing 8 μg of chloroneb/ml. **4)** Effect of 8 μg of chloroneb/ml on increase in protein and sporidial number of synchronous cultures of *U. maydis* sporidia incubated in a nutrient medium.

concentrations were determined by the diphenylamine (7) and N, N-dimethyl-*p*-amino-benzaldehyde (26) methods, respectively.

The neutralized acid hydrolyzate, glucose standard, and glucosamine standard were analyzed by paper chromatography with an ethyl acetate-pyridine-water (10:4:3, v/v) solvent system. Reducing sugars were detected by the silver nitrate-sodium hydroxide method (17).

RESULTS.—*Toxicity studies.*—Chloroneb prevented sporidial multiplication, but had little effect on increase in absorbance of sporidial suspension during the first 3 hr of incubation. Subsequent increase in absorbance (or of dry weight), however, was sharply curtailed by the toxicant (Fig. 1).

Lethal effects of the toxicant were evident only when conditions supporting growth prevailed. About 80% of the sporidia incubated for 12 hr in nutrient medium containing toxicant (8 μg/ml) failed to form colonies when plated on agar medium (Fig. 2). There was no loss of colony-forming ability in sporidia incubated with chloroneb for 12 hr in 0.02 M phosphate buffer.

Uptake and metabolism of chloroneb-^{14}*C.*—Sporidia in nutrient medium accumulated chloroneb rapidly during the first 15 min of incubation (Fig. 3). Toxicant accumulated thereafter was proportional to the increase in sporidial dry weight, and the quantity per mg of fungal dry weight remained constant even though the concentration in the medium declined. This is in contrast to the findings of Hock & Sisler (12) with *R. solani* that the quantity of toxicant in both hyphae and medium declined as the fungal dry weight increased. Sporidia incubated in 0.02 M phosphate buffer absorbed the same amount of toxicant initially as those incubated in nutrient medium, but dry weight did not increase and no additional chloroneb was taken up. The toxicant absorbed was not irreversibly bound even after 3 hr, because 99% of the radiolabel could be removed from the sporidia with 0.02 M phosphate buffer. The sporidia did not metabolize the toxicant during a 24-hr incubation period. All radiolabel chloroneb initially added to the cultures could be recovered from the medium or sporidia as unaltered chloroneb.

Energy generation.—Chloroneb apparently does not interfere with energy generation in *U. maydis.* Concentrations (8 or 12 μg/ml) which prevented sporidial multiplication had no effect on the oxidation of glucose or acetate during a 6-hr exposure period. After 1 hr of incubation, free nucleotide levels, measured by absorbance at 260 nm, were similar in chloroneb-treated (8 μg/ml) and untreated samples, as were the levels of high energy phosphate released from the free nucleotides by 7 min of acid hydrolysis.

Protein synthesis.—Incorporation of L-phenylalanine-^{14}C into the protein fraction of sporidia was inhibited less than 10% by 8 μg of chloroneb/ml during a 3-hr period. The effect on protein synthesis in synchronized cultures

TABLE 1. The effect of chloroneb (8 μg/ml) on the incorporation of uridine-2-^{14}C into the RNA and DNA fractions of *Ustilago maydis* sporidia

	Min	Count per min per mg dry wt		% Inhibition
		Control	Treated	
RNA				
	20	14,880	7,360	50
	40	30,560	17,920	40
	60	48,080	34,640	28
	80	63,680	49,280	23
DNA				
	20	101	63	38
	40	369	166	55
	60	760	345	55
	80	1,638	813	51

determined by the method of Lowry et al. (20) is shown in Fig. 4. After 3 hr, the protein content of the treated cultures was 85% of the untreated cultures. Subsequent increase in protein was largely prevented by the fungicide, and by 6 hr, the content of the treated cultures was only 58% of the control. Onset of the marked decline in the rate of protein synthesis at 3 hr in treated cultures coincided with the doubling of sporidial number in the untreated cultures.

Uridine incorporation.—The effect of chloroneb on the incorporation of uridine-2-^{14}C into RNA and DNA fractions of sporidia is shown in Table 1. After 20-min incubation, chloroneb inhibited uridine incorporation into RNA 50%; however, inhibition gradually decreased and after 80 min it was only 23%. Incorporation of uridine-2-^{14}C into DNA was inhibited about 40% after 20-min incubation, and after 40-min inhibition increased to 55% where it remained constant through 80 min. Radiolabel in the ethanol extracts of treated sporidia was reduced 20 to 50% of the control, indicating chloroneb may have interfered with the uptake of uridine-2-^{14}C.

RNA and DNA syntheses.—The effect of chloroneb on the increase of RNA and sporidial number is shown in Fig. 5. RNA levels in treated and untreated samples increased at approximately the same rate during the first 2 hr of incubation. After 2 hr, the rate of increase of RNA in treated cultures declined, and was strongly curtailed after 4 hr. RNA content of the untreated samples continued to increase rapidly; the number of sporidia/ml doubled in 3 hr and continued to increase at a rate nearly paralleling the increase of RNA.

The effect of chloroneb on the increase of DNA in *U. maydis* cultures (Fig. 6) resembled its effect on the increase of RNA. The DNA levels in both treated and untreated samples doubled in 4 hr. After this period, DNA increase in the treated samples was strongly inhibited, whereas in untreated samples it continued at an accelerated rate. The number of sporidia/ml in the untreated cultures doubled after 4 hr, and continued to increase at a rate nearly paralleling the increase in DNA, but the number of sporidia/ml in treated cultures remained constant at the initial value.

Synchronous growth and analysis of sporidia.—Holliday (14) found that shortly after mitosis in synchronized sporidia of *U. maydis*, the DNA content doubles almost concurrently with the doubling of sporidial number. Thus, newly formed single-celled sporidia are uninucleate, but contain the DNA required for the next mitotic division. Such sporidia were used to study the effect of chloroneb on morphological development, mitosis, and DNA synthesis.

Untreated sporidia formed buds, proceeded through mitosis, and doubled in number between the 2nd and 3rd hr of incubation. The DNA content of these sporidia had nearly doubled at the end of 3 hr. Treated sporidia, on the other hand, did not form buds, but became enlarged, underwent mitosis, and formed binucleate sporidia with a cross wall between the two nuclei. Although the rate of DNA synthesis was slowed, the quantity of DNA per sporidium doubled by 6 hr. There was no increase in the number of sporidia. The number of sporidia as well as the quantity of DNA doubled twice in untreated cultures during the 6-hr incubation period.

Wall analysis.—The quantity of hexose and hexosamine in acid hydrolyzates of 10 mg of wall material was 5.37 mg and 0.29 mg, respectively, in treated samples, and 5.29 mg and 0.27 mg, respectively, in untreated samples. Chromatographic analysis of sugars present in the hydrolyzates revealed no apparent differences between the treated and untreated samples.

Chloroneb-resistant mutant of U. maydis.—A mutant of *U. maydis* resistant to chloroneb was isolated from sporidia incubated 24 hr in nutrient medium containing 8 µg of chloroneb/ml. The mutant grew normally in medium containing 16 µg of chloroneb/ml; whereas growth of the wild type was prevented by 8 µg/ml. Tolerance was not related to uptake or metabolism of the toxicant because both wild type and mutant sporidia had accumulated 1.5 µg of chloroneb/mg dry weight after 30-min incubation. All radiolabel initially added to the mutant cultures was recovered after 24 hr and identified as unaltered chloroneb.

The effects of various compounds on the growth of wild type and mutant strains are shown in Table 2. In addition to chloroneb, the mutant was also more tolerant than the wild type to 2,6-dichloro-4-nitroaniline, diphenyl, hexachlorobenzene, naphthalene, *p*-dichlorobenzene, pentachloronitrobenzene, and sodium-*o*-phenylphenate. Wild type and mutant were equally sensitive to 2,4,6-trichlorophenol; 2,4,5,6-tetrachloroisophthalonitrile; *o*-benzylchlorophenol; and 1,2,4-trichloro-3,5-dinitrobenzene.

Tolerance to chloroneb followed the pattern expected for single gene resistance. Progeny of a cross
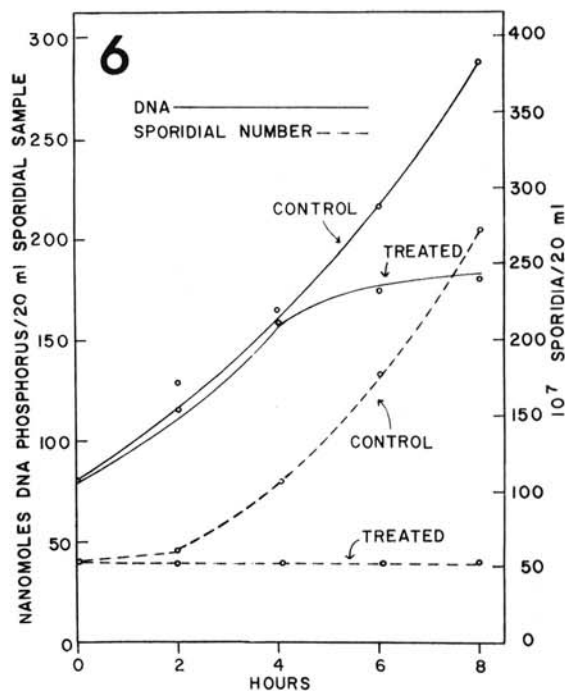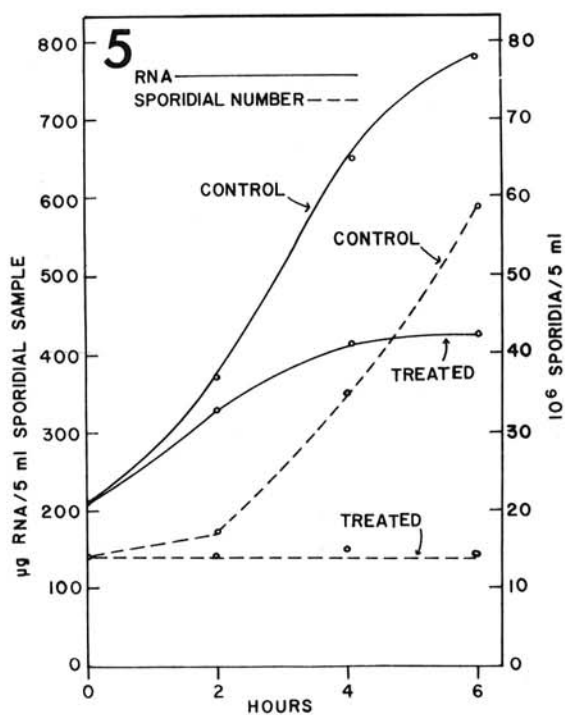

Fig. 5-6. 5) Effect of 8 µg of chloroneb/ml on the increase in RNA and sporidial number of *Ustilago maydis* sporidia incubated in a nutrient medium. **6)** Effect of 8 µg of chloroneb/ml on increase in DNA and sporidial number of *U. maydis* sporidia incubated in a nutrient medium.

TABLE 2. The effect of various compounds on growth[a] of wild
type and a chloroneb-resistant mutant of *Ustilago maydis*

Compounds	Concentration (µg/ml)	% Inhibition of growth	
		Resistant mutant	Wild type
Chloroneb	8	8	100
2,4-dichloro-4-nitroaniline	16	12	99
Diphenyl	8	15	99
Hexachlorobenzene	32	0	75
Naphthalene	16	10	100
p-dichlorobenzene	8	0	60
Pentachloronitrobenzene	16	0	100
Sodium-o-phenylphenate	16	15	100
2,4,6-trichlorophenol	4	50	50
Tetrachloroisophthalonitrile	6	100	100
	4	30	35
o-benzyl-chlorophenol	8	100	100
	4	40	53
1,2,4-trichloro-3,5-dinitrobenzene	6	100	100
	4	20	20

[a] Increase in absorbance at 450 nm of cultures between the 3rd and 6th hr of incubation. Initial sporidial concentration 0.04 mg dry wt/ml.

between the chloroneb-resistant mutant and a compatible sensitive strain were randomly isolated and analyzed according to the methods of Holliday (14). Of 92 colonies plated on medium containing 8 µg of chloroneb/ml, 47 were resistant and 45 were sensitive to chloroneb.

DISCUSSION.—Chemical determination of DNA in cultures of randomly dividing *U. maydis* sporidia showed that DNA synthesis continues almost uninhibited in the presence of chloroneb until the content doubles, but doubling of DNA is not followed by a doubling of sporidial number as is normally the case in *U. maydis*. Ultimate cessation of DNA synthesis in treated sporidia, therefore, seems to result from the failure of normal cytokinesis rather than from a direct action of the toxin on DNA synthetic reactions. These data favor the assumption that inhibition of DNA synthesis by chloroneb in *R. solani* (13) is likewise based on an indirect action.

RNA and protein syntheses in chloroneb-treated sporidia follow a pattern similar to DNA synthesis. Though both processes are inhibited somewhat prior to the normal time of sporidial division, they are not strongly curtailed until after failure of division. Thus, inhibition of RNA and protein syntheses also appears to be secondary. Chloroneb inhibits the incorporation of exogenous uridine into RNA and DNA of *U. maydis* sporidia more than would be expected from analysis based on chemical determinations. Inhibition of incorporation of uridine from an exogenous source, therefore, may primarily involve interference with the transport into the cell. Diphenyl, which probably has some common sites of action with chloroneb, affects membrane permeability in *Fusarium solani* (10).

Additional evidence for a secondary action of chloroneb on DNA synthesis is shown by its effect on sporidia in synchronous cultures. Synchronous sporidia have the DNA required to complete the first

mitotic and cellular division (15). Chloroneb-treated sporidia do not bud and then divide into two single cells; but after mitosis, a crosswall develops between the two nuclei and a two-celled sporidium is formed. If DNA synthesis had been the primary site of action of chloroneb, the treated sporidia should have undergone one normal doubling before such morphological effects were evident.

The failure of chloroneb to effect respiration and high energy phosphate levels in treated sporidia agrees with the findings of Hock & Sisler (13) that inhibition of growth is not due to interference with energy production.

Although chloroneb prevents budding and normal sporidial division, nuclear division apparently proceeds normally. Macris & Georgopoulos (22) reported that pentachloronitrobenzene induced colonial type growth in *Neurospora crassa* and that the hyphal wall of treated cells contained less hexosamine than control cells. They concluded that alteration of the chemical composition of the hyphal wall was important in regard to the morphological changes observed, but it may not have been the only effect of pentachloronitrobenzene. Even though chloroneb had little effect on hexosamine and hexose content of the sporidial wall of *U. maydis*, it may have affected the budding process by other mechanisms involving the wall. The budding process in *Ustilago* is little understood; however, Nickerson (24) reported the presence of a protein disulfide reductase in yeast which induces localized areas of increased plasticity in the cell wall. These areas fail to withstand internal pressure and permit protoplast emergence from the mother cells to form bud initials. Interference of chloroneb with an . enzymatic plasticizing of the cell wall might account for the failure of the treated sporidia to bud. The toxicant might act directly on the enzyme or accumulate at the membrane-cell wall interface and prevent contact

of membrane-bound enzyme with the cell wall. The consequence of such interferences could be the cessation of growth because of the encasement of the protoplast in a rigid cell wall capsule.

The increased tolerance of the chloroneb-resistant mutant to several other hydrocarbon compounds indicates that chloroneb should be classified with the aromatic hydrocarbons discussed by Georgopoulos & Zaracovitis (11). These compounds are probably structurally nonspecific, Ferguson-type toxicants (1). The simultaneous increase in resistance to these compounds suggests they have a common mechanism of action or that a common factor regulates their activity, even though diverse chemical structures are involved. Activity in this group is probably regulated by the degree to which the compounds partition to certain regions of the cell. A mutation which makes partitioning less favorable for chloroneb may likewise be unfavorable for other members of the aromatic hydrocarbon group, and thus there would be a parallel increase in tolerance to all the compounds. Common or somewhat different mechanisms may be involved in the ultimate action of the compounds in a particular organism. Several types of toxic effects have actually been reported for aromatic hydrocarbon types of fungicides. Among these are inhibition of sporulation (16), inhibition of protein synthesis (30), alteration of membrane permeability (10), induction of abnormal growth (25, 27), and mitotic aberrations (16, 31). Some of these may be secondary effects, but aromatic hydrocarbons may inhibit different cellular processes in different organisms.

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