The Physiological Basis of Carboxin Sensitivity and Tolerance in Ustilago hordei

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ABSTRACT

The effect of carboxin (5,6-dihydro-2-methyl-1, 4-oxathiin-3-carboxanilide) on six strains of *Ustilago hordei* was studied, using two carboxin-sensitive parent strains (1 and 2), two tolerant mutants derived from them (*car*-1 and *car*-2, respectively), and two progenies obtained from the cross between the tolerant mutants (*car*-3 and *car*-4). These six strains were tolerant to 0.2, 0.2, 25, 50, 50, and 150 μg/ml of carboxin, respectively. No leakage of cell metabolites from 1 and *car*-1 was detected in the presence of growth-inhibiting carboxin concentrations. No active uptake of carboxin-U-^{3}H by any of the four strains tested (1, *car*-1, *car*-3 and *car*-4) as well as by *Saccharomyces cerevisiae* could be detected. In both sensitive and tolerant strains, minimal growth-inhibiting concentrations of carboxin totally inhibited succinate oxidation and ^{32}P uptake, whereas glucose oxidation was inhibited only by 42-75%. Activity of succinate-DCIP reductase in cell-free extracts and in mitochondrial fractions of these strains was less affected than that of ^{32}P uptake or succinate oxidation, whereas succinate-PMS-DCIP reductase activity was only slightly inhibited by the fungicide. Lineweaver-Burk plot of succinate-DCIP reductase activity of strain no. 1 in presence of carboxin revealed that the inhibition was both competitive and noncompetitive. Growth-inhibiting concentrations of carboxin decreased the ratio of C_6/C_1 of $^{14}CO_2$ produced from glucose-6-^{14}C and gluclose-1-^{14}C, respectively, in four out of the six strains. Development of tolerance to carboxin in *U. hordei* was accompanied by an increase in carboxin tolerance of succinate-DCIP reductase activity, a decrease in its level, and with an increased isocitrate lyase and malate synthetase activities which were not affected by carboxin. It is concluded that the sensitivity of *U. hordei* to carboxin is due to inhibition in succinic dehydrogenase activity, as well as to uptake through membranes. Carboxin tolerance in *U. hordei* mainly depends on the increased activity of the glyoxylate pathway, a tolerant system of phosphate uptake, and only partially on the development of tolerant succinic dehydrogenase.

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Additional key words: succinic dehydrogenase, isocitrate lyase, malate synthetase.

In their early studies on the mode of action of carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) on the fungus *Ustilago maydis,* Mathre (13) and Ragsdale and Sisler (16) reported that carboxin inhibited oxidation of acetate, pyruvate, and succinate. Mathre (13) also observed an accumulation of ^{14}C-labeled succinate, and a decrease in ^{14}C-labeled fumarate, malate, and citrate in carboxin-treated *U. nuda* teliospores metabolizing glucose-U-^{14}C. Further investigations with a mitochondrial fraction of *U. maydis* (23) showed that the carboxin action-site was either succinic dehydrogenase or its adjacent electron acceptor; Ulrich and Mathre (19) suggested that it was between succinate and coenzyme Q. Georgopoulos et al. (6) in studies on the genetics of sensitive and tolerant strains of *U. maydis,* confirmed that the site of action was the succinic dehydrogenase system. The latter also suggested that carboxin tolerance development in *U. maydis* was associated with an increased tolerance of the succinic dehydrogenase system to the fungicide. On the other hand, Mathre (12) suggested that increased carboxin tolerance in *Saccharomyces cerevisiae* was associated with lower permeability to the fungicide. No information is available concerning the kinetics of carboxin uptake by fungal cells. This study deals with carboxin uptake and its effect on the physiology of six strains of *U. hordei* which differ in carboxin tolerance.

MATERIALS AND METHODS. — *Strains.* — *Ustilago hordei* strains I_4^+ and *arg*$^-$ used (which will be referred to as strain no. 1 and strain no. 2, respectively) were tolerant only to 0.2 μg/ml carboxin. A strain tolerant to 25 μg/ml carboxin (*car*-1) and a strain tolerant to 50 μg/ml carboxin (*car*-2) were obtained by irradiating sporidia of strains no. 1 and 2, respectively, with ultraviolet irradiation (2). Two other strains, *car*-3 and *car*-4 (tolerant to 50 μg/ml and 150 μg/ml, respectively) were obtained from progenies of a cross between *car*-1 and *car*-2 [Ben-Yephet et al. (3)].

Sensitivity to carboxin was expressed as the minimal inhibitory concentration (MIC) which completely inhibited macroscopically visible growth during 7-10 days of incubation in a complete medium.

The maximal concentration of carboxin which still allowed any macroscopically visible growth after 7-10 days of incubation in a complete medium was defined as MNIC (maximal noninhibitory concentration).

Chemicals.—Carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) formulated as dust with 75% active ingredient (a.i.) was used (UniRoyal Chemical, Division of UniRoyal Inc., Naugatuck, Conn., USA). Stock solutions were prepared in methanol [final concentration of methanol never exceeding 1% (v/v) in treated and control samples]. Carboxin -U-^{3}H 100% active ingredient (a.i.) specific activity (s.a.) 196.56 mCi/mmole and ^{32}P-phosphate (s.a.) 124.3 mCi/mmole were supplied by Nuclear Research Centre-Negev, Beer Sheva-Israel. D-glucose-1-^{14}C (s.a.) 261 μCi/mmole, and D-glucose-6-^{14}C (s.a.) 3 mCi/mmole were supplied by Radiochemical Centre, Amersham, England. Radioactivity was measured by a Model 3003 Packard liquid scintillation spectrometer.

Culturing.—Sporidia were grown on either liquid or solid (solidified by adding 2% Bacto-agar) Vogel's complete medium (CM) (21) at 20 C. In experiments with a liquid medium, Erlenmeyer flasks, containing 10% of

their volume of liquid CM, were incubated in a shaker bath. Growth was followed by measuring turbidity with a Coleman Junior spectrophotometer at 450 nm, and by counting the cells in a hemacytometer. In experiments involving oxygen uptake, the sporidia were grown on a solid CM and washed in a 0.1 M phosphate buffer pH 6.0. All four tolerant strains (*car*-1, *car*-2, *car*-3, and *car*-4) were grown on a fungicide (1 μg/ml) supplemented medium, whereas the sensitive strains 1 and 2, were grown on a fungicide-free one. Since the stability of tolerance was shown by maintaining it through 50 transfers on fungicide-free medium, there was no chance of adaptation by the above procedure.

Carboxin-U-^3H uptake.—Sporidia from liquid or solid CM were suspended in a medium composed of 0.1 M (pH 6.0) phosphate buffer 0.05 M glucose and 0.084 μCi or 0.84 μCi/ml of carboxin-U-^{3}H. Carboxin-U-^{3}H was determined in two different ways: (i) the incubated sporidia (3×10^7 in 1.5 ml) were filtered on a Millipore membrane filter (0.45-μ HA 25 mm in diameter), dried, and their radioactivity measured. (ii) Aliquots (0.1 ml) of one sample containing 3.2 to 6×10^8 sporidia, and of a second sample of the supernatant of the suspension, centrifuged at 5,000 *g* for 5 minutes were placed into scintillation vials and their radioactivity measured. Carboxin-U-^{3}H uptake was estimated by subtracting the radioactivity reading of the supernatant from that of the incubated sporidial suspensions.

^{32}P uptake.—Sporidia were grown on a modified liquid CM, containing 4 g $NaNO_3$ and 1 g $MgSO_4 \cdot 7H_2O$ per liter instead of Vogel's basic mineral solution which contains a high phosphate concentration. The sporidial concentration was adjusted to 2.10^7/ml, with fresh modified CM. Labeled phosphate (0.5 μCi of ^{32}P/ml) was added and samples of 1.5 ml were filtered at intervals on a 0.45-μ Millipore filter, washed three times with unlabeled 0.05 M phosphate buffer pH 6.0, dried, placed in scintillation vials, and their radioactivity measured.

Cell metabolite leakage.—For leakage experiments, the sporidia grown in a liquid medium were harvested by centrifugation, washed three times with sterile distilled water, and suspended in citrate buffer 0.02 M (pH 6.0) at a final concentration of 1.5×10^8 cells per ml. Amino acids were determined by the ninhydrin technique (22). Inorganic phosphate was determined according to Fiske and Subbarow (5), potassium cations with an EEL flame photometer, and calcium cations with a Model 303 Perkin-Elmer atomic absorption spectrophotometer. Sugars were determined with paper chromatography using Whatman 3MM paper with benzene:butanol:pyridine:water (3:3:5:1, v/v) as the running solution and 1% $AgNO_3$ in acetone and 2% NaOH in ethyl alcohol as developing solutions, further clearing being done in 10% $Na_2S_2O_3$ (4, 18).

Oxygen uptake.—Oxygen uptake and CO_2 output were measured with a Warburg apparatus (B. Braun, Melsungen) using 10-ml flasks, according to Umbreit et al. (20).

Mitochondrial preparations.—Sporidia grown on liquid CM, were washed three times with sterile distilled water, suspended (at a final concentration of 2.5×10^9/ml) in a medium prepared according to White (23) and composed of 0.24 M sucrose, 0.005 M EDTA and 0.15% bovine albumin (BSA, fraction V), and homogenized for 10 minutes with a Model MSK cell homogenizer. The homogenized cell suspension was centrifuged at 3,000 *g* for 10 minutes, and the supernatant centrifuged again at 3,500 *g* for 10 minutes. The supernatant was centrifuged at 10,000 *g* for 20 minutes and the precipitated pellet was suspended in White's medium and centrifuged at 20,000 *g* for 10 minutes. The resulting pellet was washed gently with a BSA-less White's medium, and resuspended in the same fresh medium. For the preparation of crude extracts, sporidia were homogenized in a BSA-less White's medium. The remaining unbroken cells and cell debris were precipitated by successive centrifugations at 3,000 *g* and 3,500 *g* for 10 minutes. All operations were performed aseptically at 5 C.

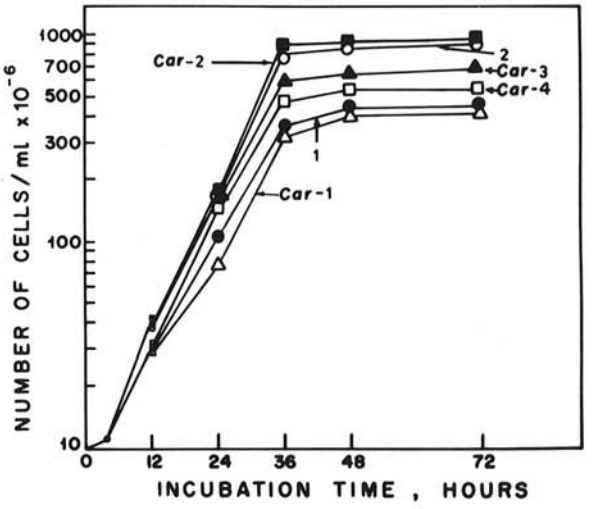

Fig. 1. Growth curves of 1, 2, *car*-1, *car*-2, *car*-3, and *car*-4 *Ustilago hordei* strains on carboxin-free complete liquid medium.

TABLE 1. Carboxin-U-^{3}H uptake by the sensitive strain no. 1, and the tolerant (*car*-1, *car*-3, and *car*-4) strains of *Ustilago hordei* and by *Saccharomyces cerevisiae*

Strain[a]	Carboxin-U^3-H uptake (%) 0.1 μg/ml	1 μg/ml	Cell volume in reaction mixture[c] (%)	No. of cells/ml ($\times 10^9$)
1	11	9.2	33	3.7
car-1	11	11.7	36	4.4
car-3	10	10	37.5	4.8
car-4	11	11	50	6.0
Saccharomyces cerevisiae	5	7	40	3.2
No cells	0	0	0	0

[a] *Car*-1, *car*-2, and *car*-4 are carboxin-tolerant, whereas strain 1 is carboxin-sensitive. *Saccharomyces cerevisiae* is tolerant to carboxin.

[b] Radioactivity: 0.084 and 0.84 μCi/ml in 0.1 μg/ml and 1 μg/ml, respectively. Carboxin uptake calculated as percentage of cpm lost from the supernatant. Incubation time: 20 minutes; incubation temperature: 20 C.

[c] Cells were suspended in phosphate buffer 0.1 M (pH 6.0).

TABLE 2. Effect of carboxin on oxygen uptake by sensitive (1 and 2) and tolerant (*car*-1, *car*-2, *car*-3, and *car*-4) strains of *Ustilago hordei*

Strain[a]	Carboxin concentrations		Oxygen uptake, μliters per mg dry cells per hour[b]					
			Succinate (0.05 M)			Glucose (0.05 M)		
	MIC[c] (μg/ml)	MNIC[d] (μg/ml)	Control	0.5 MNIC	MIC	Control	0.5 MNIC	
1	0.5	0.2	2.04	0.18	0	5.9	4.2	1.41
2	0.5	0.2	2	0.21	0	6.2	3.8	1.6
car-1	30	25	1.6	0.26	0.15	6.6	2.62	2.07
car-2	60	50	1.92	0.28	0	5.8	5	2.84
car-3	60	50	1.5	0.2	0.1	6.1	4.3	2.44
car-4	200	150	0.9	0	0	4.6	3.6	2.65

[a]Strains 1 and 2 are carboxin-sensitive, *car*-1, and *car*-2 are the two respective tolerant mutants derived from them and *car*-3 and *car*-4 are two progenies obtained from the cross between the tolerant mutants.

[b]Each Warburg flask contained 3 ml of reaction mixture in the main compartment, and a piece of Whatman filter paper soaked in 0.2 ml of 30% NaOH. The reaction mixture contained a substrate (0.05 M) solution in 0.1 M phosphate buffer pH 6.0, with or without carboxin. Incubation temperature was 20 C. No. of strokes of the Warburg apparatus/min = 84; observation period = 2.5 hours; equilibration time = 10 minutes.

[c]MIC = Minimal inhibitory concentration of carboxin in complete medium. Composition of complete medium: 20 ml Vogel's solution; 50 mg tryptophan; 5 g casein hydrolyzate (acid) vitamin-free; 5 g Bacto yeast extract (Difco); 10 g glucose; 10 ml vitamin solution; 250 mg tetracycline hydrochloride; 20 g Bacto agar (Difco); 1,000 ml distilled water.

[d]MNIC = Maximal noninhibitory concentration of carboxin in complete medium.

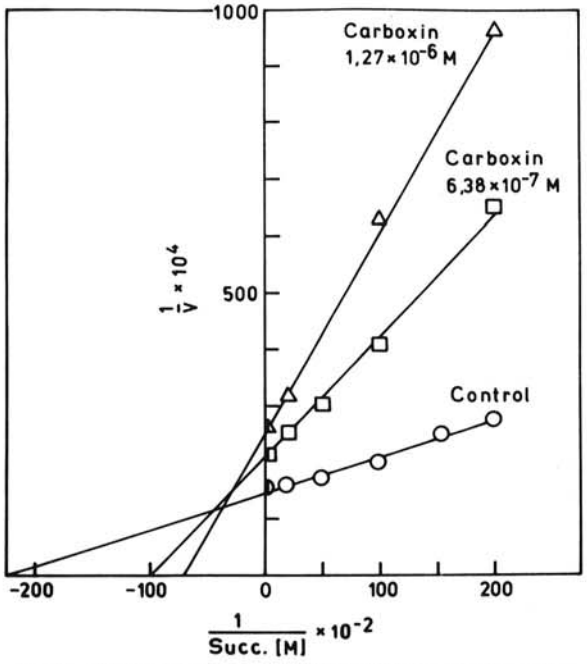

Fig. 2. Lineweaver-Burk plot of the inhibition by carboxin of succinate oxidation by the mitochondrial fraction of *Ustilago hordei* (strain no. 1). The reaction mixture contained KH_2PO_4-K_2HPO_4 50 mM pH 7.4; KCN 1.5 mM; dichlorophenolindophenol (DCIP) 0.05 mM; carboxin and succinate concentrations as shown. Incubation temperature 30 C. Endogenous rates of DCIP reduction were subtracted. (V) is expressed as moles of succinate oxidized per minute per mg protein, calculated using an extinction coefficient of 21 × 47.6 × △ 600 (7).

Protein content and enzymatic activity assay.—Protein content was measured according to Lowry et al. (8), using bovine serum albumin for the preparation of a reference curve. The activity of succinic dehydrogenase (SD) was measured as succinate dichlorophenolindophenol (DCIP) reductase by following the reduction of DCIP for 15 minutes, and as succinate phenazine methasulphate (PMS)-DCIP reductase, by following DCIP reduction for 2 minutes in the presence of PMS. These reactions were done according to Arrigoni and Singer (1) as modified by Ulrich and Mathre (19). Isocitrate lyase and malate synthetase activities were examined according to Maxwell and Bateman (15) and McGilvray and Morris (11), respectively.

RESULTS.—*Growth rate of U. hordei strains and the effect of carboxin on growth and morphology.*—Growth rate of *U. hordei* strains grown in a carboxin-free medium expressed as number of cells per milliliter is shown in Fig. 1. The data shows that in some strains growth of the tolerant mutant is faster and in others it is slower than in the sensitive original strains. Values for mg dry weight per milliliter after 48 hours of incubation were 9.8, 10.9, 9.74, 10.4, 10.2, 8.6, for 1, 2, *car*-1, *car*-2, *car*-3, and *car*-4, respectively. Carboxin in the growth medium slowed down the budding of sporidia. At 48 hours of incubation, budding of the original sensitive strain was slightly inhibited already by a carboxin concentration of 0.1 μg/ml, and was completely arrested at a concentration of 0.5 μg/ml. The tolerant strains *car*-1, *car*-3, and *car*-4 were slightly inhibited at 5, 20, and 75 μg/ml, respectively. They stopped budding at 30, 60, and 200 μg/ml, respectively. Addition of carboxin to the growth medium [i.e., below minimal inhibitory concentrations (MIC)] resulted in a morphological change of the sporidia from an ellipsoidal to a circular shape.

Carboxin-U-^3H uptake.—Identical amounts of carboxin-U-^{3}H were found to be associated with cells of all the *U. hordei* strains and *S. cerevisiae* at zero time and after 60 minutes of incubation, indicating a passive penetration or adsorption of fungicide into the cells. In another experiment, 3.2 to 6.0 × 10^9 cells/ml were incubated with carboxin-U-^{3}H for 20 minutes, centrifuged, and the radioactivity of the supernatant compared with that of the noncentrifuged *U. hordei* strains and *S. cerevisiae* cell suspension. Only a 10%

TABLE 3. Relative levels of succinate-DCIP reductase, succinate-PMS-DCIP reductase in mitochondrial fractions and crude extracts of sensitive and tolerant *Ustilago hordei* strains and their inhibition by carboxin

Strain[a]	Activity of succinate-DCIP-reductase (%)				Activity of succinate-PMS-DCIP reductase (%)			
	Control		Carboxin[b]		Control		Carboxin[b]	
	Mitochondria	Crude extract	Mitochondria	Crude extract	Mitochondria	Crude extract	Mitochondria	Crude extract
1	100[c]	100[d]	34	35	100[e]	100[f]	64	62
2	100	100	24	28	125	104	62.5	63
car-1	10.2	30	4.0	27	42	66	32	51.5
car-2	10	16	8	10.7	52	62	42	41.5
car-3	4	11	2.4	11	16.6	67	13.3	60
car-4	1.6	4.5	1.0	3.9	17.8	63	14.8	60

[a]Strains 1 and 2 are carboxin-sensitive, *car*-1, and *car*-2 are the two respective tolerant mutants derived from them and *car*-3 and *car*-4 are the progenies obtained from the cross between the tolerant mutants.
[b]Carboxin concentrations: 0.5, 0.5, 30, 60, 60, and 200 μg/ml for 1, 2, *car*-1, *car*-2, *car*-3, and *car*-4, respectively.
[c, d, e, f]Succinate oxidized μmoles per mg protein per minute: c, 48; d, 18; e, 394; f, 198.

reduction in the supernatants radioactivity as compared to the noncentrifuged cell suspension was observed (Table 1).

Effect of carboxin on ^{32}P uptake.—The effect of carboxin on ^{32}P uptake in 4 *U. hordei* strains is shown in Fig. 3. The decrease in uptake is gradual with the increase of carboxin concentrations, reaching a 95% inhibition level for each strain at the MIC concentration. The inhibition is observed immediately upon the addition of carboxin and it is maintained at least for 50 minutes.

Metabolite leakage from cells.—*U. hordei* 1 and *car*-1 strains were suspended in 0.02 M citrate buffer pH 6.0 for 8 hours in the absence and presence of 1 μg/ml and 100 μg/ml of carboxin, respectively. Aliquots (8 ml) were taken at intervals, centrifuged at 5,000 *g* for 5 minutes, and the supernatant tested for the presence of amino acids, sugars, phosphate, potassium, calcium ions and substances absorbing light at 260 nm. No leakage of those substances from the treated cells was observed compared to control cells.

Effect of carboxin on oxidation of glucose and succinate.—Minimal growth inhibiting concentrations of carboxin inhibited glucose oxidation in sensitive (strains 1 and 2) and tolerant (*car*-1, *car*-2, *car*-3, and *car*-4) strains of *U. hordei* by 76, 76, 69, 51, 60, 42 percent, respectively. Half MNIC concentration inhibited glucose oxidation by less than 50%. With succinate as a substrate, oxygen uptake in sensitive and tolerant strains was almost totally arrested already at half of the MNIC (Table 2).

Analysis of the inhibition type of succinate-DCIP reductase by carboxin.—Succinate-DCIP reductase activity of the carboxin-sensitive strain no. 1 was tested in mitochondrial fraction and in the supernatant, obtained after precipitation of the mitochondria, at various substrate concentrations. The results plotted according to Lineweaver-Burk (Fig. 2) show that the inhibition of succinate oxidation in mitochondrial fraction is both of a competitive and noncompetitive type. Similar results were also obtained with the supernatant.

Succinate-DCIP and -PMS-DCIP reductases, isocitrate lyase and malate synthetase activities in sensitive and tolerant strains of U. hordei.—In this experiment, the relative levels and sensitivity to carboxin of succinate-DCIP and -PMS-DCIP reductases were tested in crude extracts and mitochondrial fractions of six strains of *U. hordei*. Activity of succinate-PMS-DCIP reductase was considerably higher than that of succinate-DCIP reductase (Table 3). Mitochondrial fractions and crude extracts prepared from all strains showed a decrease in the activity of succinate-DCIP reductase with increased carboxin tolerance. Some decrease, although much less pronounced, was also observed with succinate-PMS-DCIP reductase activity. Succinate-DCIP reductase activity in crude extracts of the tolerant strains, was less affected by carboxin than that of their corresponding mitochondrial fraction.

TABLE 4. Isocitrate lyase and malate synthetase activities in *Ustilago hordei* strains

Strain[a]	Isocitrate lyase[b]	Malate synthetase[c]
1	2.2	286
2	0.76	124
car-1	8.6	680
car-2	1.76	265
car-3	2.2	372
car-4	14.0	894

[a]Strains 1 and 2 are carboxin-sensitive, *car*-1 and *car*-2 are the two respective tolerant mutants derived from them and *car*-3 and *car*-4 are two progenies obtain from the cross between the tolerant mutants.
[b]Enzymatic activity expressed as μg glyoxylate/mg protein/min.
[c]Enzymatic activity expressed as (Δ_{412} per mg protein per minute) × 10,000.

Isocitrate lyase and malate synthetase were tested in crude extracts of six strains. The results (Table 4) showed that the activities of those enzymes were considerably higher in tolerant strains as compared to the respective parent sensitive strains.

Comparative effect of carboxin on succinate-PMS-DCIP reductase, succinate-DCIP reductase, isocitrate lyase, malate synthetase and ^{32}P uptake.—Succinate-DCIP reductase was more sensitive to carboxin than succinate-PMS-DCIP reductase. Isocitrate lyase and malate synthetase activities in cell-free crude extracts were not affected by carboxin. In all *U. hordei* strains ^{32}P uptake by intact cells was inhibited by lower carboxin

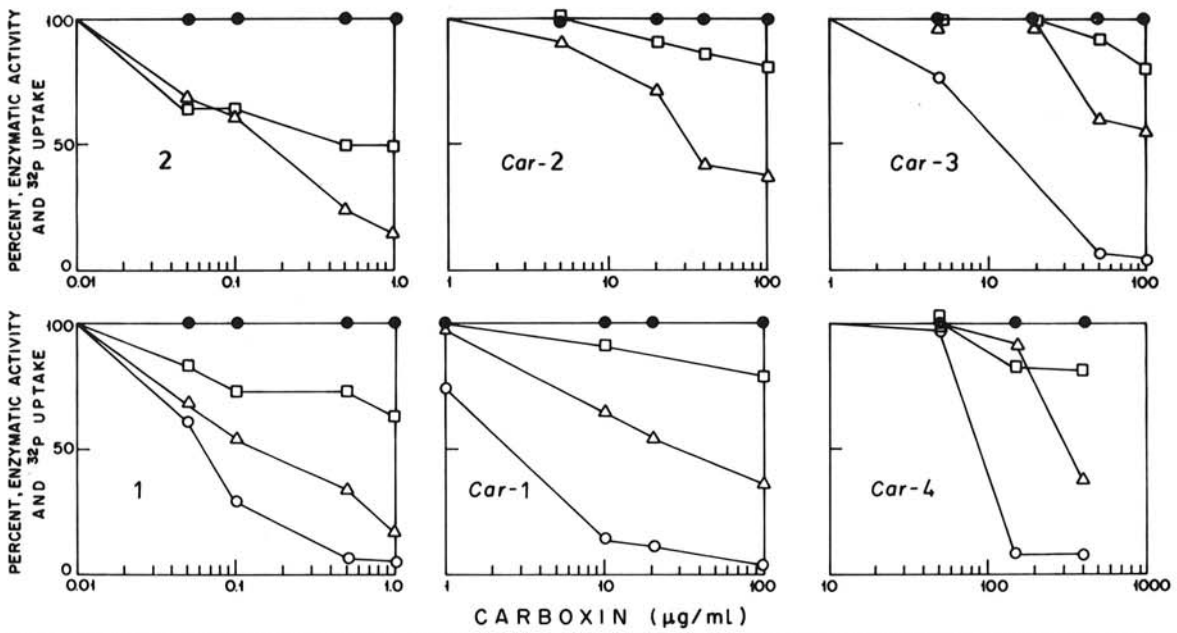

Fig. 3. Effect of carboxin on succinate-phenazine methasulphate (PMS)-dichlorophenolindophenol (DCIP) reductase, succinate-DCIP reductase, isocitrate lyase, malate synthetase, and ^{32}P uptake in 1, 2, *car*-1, *car*-2, *car*-3, and *car*-4 *Ustilago hordei* strains. Symbols: (●—●) isocitrate lyase and malate synthetase; (□—□) succinate-PMS-DCIP reductase; (△—△) succinate-DCIP reductase; (°—°) ^{32}P uptake.

Inhibition was expressed as percent of control. For activity values of succinate-DCIP and -PMS-DCIP reductases, isocitrate lyase and malate synthetase see Table 4 and Table 5, respectively. Values of ^{32}P uptake in control were 1397, 941, 1023, and 916 cpm per 3×10^7 cells per minute for 1, *car*-1, *car*-3, and *car*-4, respectively.

TABLE 5. Effect of carboxin on C_6/C_1 ratio of $^{14}CO_2$ produced during respiration on glucose-6-^{14}C and -1-^{14}C by sensitive (1 and 2) and tolerant (*car*-1, *car*-2, *car*-3, and *car*-4) strains of *Ustilago hordei*

Strain[a]	Carboxin concentrations		$^{14}C_6 : {}^{14}C_1$ ratio[b]		
	MIC[c] (μg/ml)	MNIC[d] (μg/ml)	Control	0.5 MNIC	MIC
1	0.5	0.2	0.22	0.22	0.08
2	0.5	0.2	0.38	0.31	0.10
car-1	30	25	0.28	0.14	0.09
car-2	60	50	0.24	0.23	0.23
car-3	60	50	0.28	0.32	0.23
car-4	200	150	0.23	0.30	0.12

[a]Strains 1 and 2 are carboxin-sensitive, *car*-1 and *car*-2 are the two respective tolerant mutants derived from them, and *car*-3 and *car*-4 are two progenies obtained from the cross between the tolerant mutants.

[b]Each Warburg flask contained 3 ml of reaction mixture in the main compartment, and a piece of Whatman filter paper soaked in 0.2 ml of 30% NaOH. The reaction mixture contained a substrate (0.05 M) solution in 0.1 M phosphate buffer pH 6.0 with or without carboxin and 1 μCi of glucose-1-^{14}C or glucose-6-^{14}C. Incubation temperature was 20 C. Number of strokes of the Warburg apparatus per minute = 84; observation period = 2.5 hours; equilibration time = 10 minutes.

[c]MIC = Minimal inhibitory concentration of carboxin in complete medium. Composition of complete medium: 20 ml Vogel's solution; 50 mg tryptophan; 5 g casein hydrolyzate (acid) vitamin-free; 5 g Bacto yeast extract (Difco); 10 g glucose; 10 ml vitamin solution; 250 mg tetracycline hydrochloride; 20 g Bacto agar (Difco); 1,000 ml distilled water.

[d]MNIC = Maximal noninhibitory concentration of carboxin in complete medium.

concentrations than those inhibiting succinate-DCIP reductase both in cell-free extract and mitochondrial fraction (Fig. 3).

Effect of carboxin on $^{14}C_6/^{14}C_1$ ratio.—Minimal growth inhibiting concentrations of carboxin decreased C_6/C_1 ratio of $^{14}CO_2$ produced from glucose-6-^{14}C and glucose-1-^{14}C in the sensitive 1 and 2 strains and in the tolerant *car*-1 and *car*-4 but not in the tolerant *car*-2 and *car*-3 (Table 5). This means that even when respiration of these two strains is inhibited they do not shift from the TCA cycle. It is evident that 0.5 MNIC does not cause a marked shift from the TCA cycle except in the strain *car*-1.

DISCUSSION.—In the present study, the effect of carboxin on some physiological systems in *U. hordei* was examined. Two sensitive parent strains (1 and 2), two

tolerant mutants derived from them (*car*-1 and *car*-2), and two progenies (*car*-3 and *car*-4) obtained from the cross between the tolerant mutants were used. No relationship between carboxin tolerance, and growth rate could be observed in *U. hordei*. Growth rate expressed as increase in cell concentrations with time was different in the various strains but similar when expressed as mg dry weight per milliliter. This is important to point out since physiological activities are related to dry matter.

Mathre (12) reported that the difference between carboxin-sensitive and -tolerant fungi was in accordance with the ability of carboxin to penetrate into the cells. On the other hand, Lyr et al. (10) could not find any significant differences in carboxin uptake between sensitive and tolerant fungi. The results of this study indicate that *U. hordei* cells do not have an active system of carboxin uptake, as a free and passive carboxin-U-^{3}H penetration was observed in four strains, including sensitive and tolerant *U. hordei* strains as well as *S. cerevisiae*.

According to Shively and Mathre (17), carboxin caused a leakage of cell metabolites in *Rhizoctonia solani*. No such effect was observed by us in either sensitive or tolerant *U. hordei* strains.

Mathre (13) and Ragsdale and Sisler (16) reported that carboxin inhibited the oxidation of pyruvate, acetate and succinate in *U. maydis* cells, but only slightly affected glucose oxidation. Lyr et al. (10) found that endogenous respiration of a carboxin-sensitive *Rhodotorula glutinis*, and oxidation of glucose and acetate in the presence of 10^{-4}M carboxin were inhibited 50%, 40%, and 54%, respectively. Furthermore, 10^{-4}M of carboxin inhibited acetate oxidation 25% in the carboxin-tolerant *Procandida albicans*. In our studies, it was found that in both sensitive and tolerant *U. hordei* strains minimal growth inhibiting concentrations of carboxin also completely inhibited respiration on succinate, whereas glucose oxidation was inhibited only by 42 to 75% and endogenous respiration was only slightly inhibited.

In contrast to Mathre (14) who demonstrated a noncompetitive inhibition of succinate oxidation in a mitochondrial fraction of *U. maydis*, inhibition by carboxin of succinate-DCIP reductase in mitochondrial fraction and in the supernatant of cell-free extract of *U. hordei* was found to be of a both competitive and a noncompetitive type. Competitive inhibition of succinate oxidation as that shown by tannic acid in rat liver mitochondria was explained by its effect on mitochondrial permeability to succinate (9).

In all *U. hordei* strains tested, succinate-DCIP reductase activity in cell-free extract and mitochondrial fraction was markedly affected by carboxin whereas succinate-PMS-DCIP reductase activity was only slightly affected. The different response of succinate reductase systems to carboxin corroborates the suggestion of White (23) and of Ulrich and Mathre (19) that the site of action of carboxin is on an intermediate electron carrier between succinate and coenzyme Q. Due to its structural relationship to salycylanilide, a known uncoupler of oxidative phosphorylation (24), carboxin could have a similar effect on sensitive fungi. However, this possibility was examined in rat liver mitochondria by Mathre (14) who concluded that carboxin inhibits mitochondrial respiration at or close to the site of succinate oxidation but exerts only a slight effect on phosphorylation reactions. The fact that ^{32}P uptake and succinate oxidation in intact cells were more affected than succinate-DCIP reductase activity in mitochondrial fractions, may point out that carboxin is affecting substrate uptake as well.

Carboxin tolerance in *U. hordei* strains was positively correlated with increase in tolerance of succinate-DCIP reductase to carboxin and with a considerable decrease in enzyme activity. Thus, the mitochondrial fraction of *car*-4 (tolerant to 150 μg/ml carboxin) was only 1.6% as compared to 100% found in the sensitive strains 1 and 2. Yet, both respiration level and growth rate were similar in all strains. Such a discrepancy between respiration rate and level of succinate-$K_3Fe(CN)$ reductase activity was also observed in sensitive and tolerant strains of *U. maydis* by Georgopoulos et al. (6) who suggested that this was due to the higher sensitivity of the enzyme prepared from the tolerant strain to adverse conditions prevailing during preparation of the cell-free extract. Our results indicate that intact cells of the more tolerant strains probably do contain less enzyme than the sensitive ones. The reduced activity of the succinate-DCIP reductase in these strains is compensated by an increase in the alternative pathway of the glyoxylate cycle as evidenced by the increase in isocitrate lyase and malate synthetase activities. It should be emphasized, however, that growth inhibiting concentrations of carboxin did not affect isocitrate lyase and malate synthetase activities and only partially affected succinate-DCIP reductase activity. Therefore, the possible existence of additional inhibition sites should not be excluded. These indications are supported by the observation that oxidation of succinate by intact cells is more inhibited than succinate-DCIP reductase at half of the maximal non-inhibiting concentrations of carboxin, and that the ratio C_6/C_1 of $^{14}CO_2$ was not changed at these concentrations, indicating no effect on citric acid cycle in the intact cell. These findings are in accordance with the fact that carboxin tolerance in *U. hordei* is controlled by a polygenic system [Ben-Yephet et al., (3)]. It seems that development of carboxin-tolerant strains from carboxin-sensitive ones in *U. hordei* depends on the development of alternative metabolic pathways such as glyoxylate cycle, or carboxin-tolerant phosphate uptake and only partially on the development of carboxin-tolerant succinate-DCIP reductase.

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