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Mode of Action of the Antibiotic Siccanin on Intact Cells and Mitochondria of *Trichophyton mentagrophytes*

KEINOSUKE NOSE[1] AND AKIRA ENDO

Fermentation Research Laboratories, Sankyo Co., Ltd. Hiromachi, Shinagawa-ku, Tokyo, Japan

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Siccanin at 3 μg/ml completely inhibited the growth of *Trichophyton mentagrophytes*. The primary site of action of siccanin on *T. mentagrophytes* is succinate dehydrogenase in the terminal electron transport system. At a concentration of siccanin giving 50% inhibition of growth (0.3 μg/ml), respiration of intact cells was inhibited more strongly than any other cellular functions tested, including the syntheses of cellular ribonucleic acid, deoxyribonucleic acid, phospholipid, protein, and cell wall fractions. In addition, at the same concentration siccanin did not cause any detectable damage in the permeability of the cells. Furthermore, the oxidation of succinate in mitochondrial preparation is more sensitive to the antibiotic than respiration in intact cells. Oxidation of other substrates tested was less sensitive to siccanin than that of succinate. The antibiotic inhibited both phosphorylation and oxidation, without causing changes in the P:O ratio. Siccanin at 0.03 μg/ml, which caused 50% inhibition of succinate oxidation in mitochondria, had effect neither on the exchange reaction between inorganic phosphate (P_i) and adenosine triphosphate (ATP) nor on that between adenosine diphosphate and ATP. An ATP phosphohydrolase activity was also insensitive to the antibiotic. At very high concentrations, however, the antibiotic slightly inhibited the P_i-ATP exchange reaction. From those results, it was concluded that siccanin inhibits fungal growth by inhibiting the respiratory electron transport system.

The antibiotic siccanin was first isolated from culture filtrates of *Helminthosporium siccans* (10) and its chemical structure has been established (9). The antibiotic inhibits the growth of various pathogenic fungi, especially *Trichophyton*, *Epidermophyton*, and *Microsporum* (1, 10). In earlier studies, it was reported that both nucleic acid synthesis and respiration of *Trichophyton mentagrophytes* were sensitive to the antibiotic (11) and that the energy-transferring reaction in oxidative phosphorylation of rat liver mitochondrial system was likely inhibited (8). From those studies, however, the primary sites of siccanin action are poorly understood. In this work, we have further studied the mode of action of siccanin on *T. mentagrophytes* and have shown that respiration is the primary site to be inhibited by the antibiotic and further that succinate dehydrogenase is the most sensitive site of the mitochondrial respiration.

[1] Present address: Institute for Enzyme Research, The University of Wisconsin, Madison, Wis. 53706

MATERIALS AND METHODS

Materials. Crystalline siccanin, which was prepared in our laboratory, was dissolved in ethanol at an appropriate concentration and used throughout the experiments. Labeled chemicals were obtained from the Radio Chemical Centre, Amersham, England. Rutamycin was kindly supplied by L. Delcambe of International Center of Information on Antibiotics, University Liege, Belgium. *T. mentagrophytes* F63-6, which used in the experiments, was obtained from the collection of our laboratory. The strain was maintained at 10 C on the agar slants containing 2% glucose and 1% Difco neopeptone. Before cultivation in liquid medium, subcultures were made on the same solid medium and incubated at 27 C for about 2 weeks. A dense spore suspension was obtained by washing the subcultures with sterilized water.

Growth inhibition curve. Spore suspension was inoculated to 10 ml of the medium consisting of 2% glucose and 1% Difco neo-peptone (*p*H 6.5), contained in 50-ml L-flasks, and the flasks were shaken on a reciprocal shaker at 27 C. Where indicated, 0.1 ml of siccanin solution was added to the flasks. After growing for ap-

propriate periods of time, the mycelia were harvested by filtration, dried at 100 C for 1 hr, and then weighed.

Synthesis of cellular constituents. Young filamentous (not pellet forming) mycelia, which were used in the experiments described, were obtained as follows. One liter of the medium consisting of 2% glucose, 2% sodium lactate, 1% Difco neo-peptone, 0.3% KH_2PO_4, 0.2% $MgSO_4 \cdot 7H_2O$, and 0.1% Fries' trace elements solution (2) contained in a 5-liter Fernbach flask, was inoculated with a heavy spore suspension of *T. mentagrophytes* and incubated at 30 C for approximately 40 hr with shaking. The young mycelia were harvested by filtration and then suspended (4.6 mg, dry weight, per ml) in the medium containing 50 mM tris(hydroxymethyl)aminomethane(Tris)-maleate (*p*H 6.0), 10 mM glucose, 10 mM sodium lactate, and 4 mM KH_2PO_4. In the incorporation experiments of labeled compounds into cellular constituents, 3.1 μCi of ^{32}P-H_3PO_4, 2.0 μCi of uniformly labeled ^{14}C-glutamate, or 2.0 μCi of glucosamine-*1*-^{14}C hydrochloride, respectively, was added to 10 ml of the mycelial suspension contained in 50-ml flasks at zero time. At the same time, 0.1 ml of siccanin solution was added to the flasks. The flasks were incubated at 27 C with shaking for the indicated periods. To terminate the incubation, mycelia was quickly separated from medium by filtration in a Büchner funnel and washed thoroughly with distilled water. Fractionation of the cell constituents was carried out by the method of Schneider (20) with a slight modification. The cells were suspended in 5 ml of ice-cold 5% trichloroacetic acid and disrupted in a Potter-Elvehjem homogenizer. The suspension was centrifuged at 24,000 $\times$ *g* for 30 min, and the supernatant solution was referred to as the acid-soluble fraction. The acid-insoluble residue was washed twice with ice-cold 5% trichloroacetic acid, once with 50% (v/v) ethanol, and finally with 99% (v/v) ethanol, and then extracted with 5 ml of ethanol-ether (3:1) mixture by stirring for 10 min at room temperature. The extract (phospholipid fraction) was obtained by centrifugation. The residue after lipid extraction was washed twice with ethanol-ether (3:1) and once with 5% trichloroacetic acid, and then was incubated at 37 C for 16 hr after being suspended in 5 ml of 1 N KOH solution. After neutralization with HCl, the suspension was centrifuged, and the supernatant solution obtained was referred to as ribonucleic acid (RNA) fraction. The residue was washed with ice-cold 5% trichloroacetic acid and incubated in 5 ml of 5% acid at 90 C for 20 min. After centrifugation of the incubation mixture, the supernatant solution was obtained and referred to as deoxyribonucleic acid (DNA) fraction. In the experiment with ^{14}C-glutamate incorporation into protein, the residue after DNA extraction was washed twice with 5% trichloroacetic acid and once with 50% ethanol, suspended in 5 ml of 1 N KOH, and then heated at 100 C for 10 min. The suspension was centrifuged, and the supernatant solution obtained was referred to as protein fraction. In the incorporation of ^{14}C-glucosamine into cell wall fraction, phospholipid and nucleic acid extractions were omitted. The residue after the extraction of protein was washed once with 1 N KOH and twice with distilled water and referred to as cell wall fraction.

The incorporation of ^{32}P-orthophosphate into cell constituents was measured with a thin-window counter. Radioactivity of ^{14}C-containing fractions was determined with a liquid scintillation spectrophotometer in dioxane scintillation fluid: 2,5-diphenyloxazole, 8 g; 1,4-bis-2-(5-phenyloxazolyl)-benzene, 0.2 g; dioxane, 800 ml; toluene, 200 ml.

Respiration of intact cells. Respiration was measured polarographically in a semiclosed reaction vessel of an oxygen meter of Ohkura Electric Co., Japan, originally designed by Hagihara (7). The reaction mixture in the vessel contained 150 μmoles of phosphate buffer (*p*H 6.8), 300 μmoles of sodium lactate, and mycelial cells equivalent to 1 to 2.5 mg (dry weight) in a total volume of 1.5 ml. The incubation was started by the addition of cell suspension and carried out at 30 C with stirring. Oxidation reaction was followed for several minutes on a recorder. Per cent inhibition was calculated from the slopes on a chart before and after the addition of siccanin.

^{14}C-glutamate uptake and leakage. Young mycelia of *T. mentagrophytes* (4.2 mg, dry weight) was suspended in 10 ml of the medium containing 500 μmoles of Tris-maleate (*p*H 6.0), 200 μmoles each of glucose and sodium lactate, 40 μmoles of KH_2PO_4, 20 μmoles of $MgCl_2$, and 0.01 μmoles of nonlabeled glutamate. The mixture was transferred to a 50-ml flask and incubated at 27 C with shaking. ^{14}C-glutamate was added to the mixture at zero time. Incubation was stopped by separation of the cells from the medium by filtration. The cells were thoroughly washed with the above medium, homogenized in dioxane scintillation fluid supplemented with 3% Cab-O-Sil (Packard Instrument Co.), and then submitted to the determination of radioactivity.

Preparation of T. mentagrophytes mitochondria. Young mycelial cells were ground lightly in a mortar with two volumes of silica sands and then suspended in the preparation medium consisting of 500 mM sucrose, 10 mM sodium ethylenediaminetetraacetate (EDTA), 0.15% bovine serum albumin, 6 mM potassium citrate, and 10 mM Tris adjusted to *p*H 6.8. The suspension was first centrifuged at 1,000 $\times$ *g* for 10 min, and the pellet was discarded. The supernatant solution was then centrifuged at 10,000 $\times$ *g* for 10 min, and the resultant pellet was homogenously suspended in the medium mentioned above. The suspension was recentrifuged at 1,000 $\times$ *g* for 10 min, and the supernatant suspension, which had a final concentration of 10 to 30 mg of protein per ml, was referred to as mitocondrial fraction. Experiments with the mitochondria were conducted on the day they were prepared since respiratory control index (RCI) of the fungal mitochondria had been found to decrease in several hours after their preparation even at 0 C. All operations were carried out between 0 to 4 C. Protein was determined by the method of Lowry et al. (16).

Oxidation and phosphorylation in mitochondria. Oxygen uptake was measured in the oxygen meter at 30 C. A 1.5-ml amount of the basal medium (*p*H 6.9, adjusted with KOH), containing 125 mM sucrose, 10 mM KH_2PO_4, 10 mM $MgCl_2$, 1.5 mM EDTA, and 4.5 mg of bovine serum albumin, was first transferred to the vessel. Incubation was started by addition of 20 to 100 μliters of mitochondrial suspension (1 to 2 mg of pro-

tein). Substrate (20 μliters), adenosine diphosphate (ADP; 10 μliters), and inhibitors (15 μliters) were also added to the incubation mixture. RCI was calculated from the rates of oxygen uptake before and after the addition of ADP. Effect of inhibitors was followed in the presence of both substrate and ADP, and per cent inhibition was calculated as described above. For assay of oxidative phosphorylation, about 5 μCi of orthophosphate, 0.5 mg of hexokinase (Sigma Chemical Co., type 4), and 150 μmoles of glucose were added to the reaction mixture. At predetermined intervals 100-μliter samples of the incubation mixture were taken out for the assay of adenosine triphosphate (ATP), which was carried out by the method of Nielsen et al. (18). Free orthophosphate was determined by Takahashi's method (21).

Partial reactions of phosphorylation in mitochondria. ^{32}P-inorganic phosphate (P_i)-ATP exchange reaction was performed by the method of Conover et al. (5). ^{14}C-ADP-ATP exchange reaction was done by the method of Wadkins et al. (22) except that ATP was isolated from the reaction mixture by paper chromatography. In those experiments reaction was stopped by adding 20 mg of charcoal (norit A) and by cooling rapidly. The charcoal was washed with water, and ATP adsorbed was then eluted with 5 ml of 50% ethanol. The extracts were concentrated to a minimal amount under vacuum and submitted to paper chromatography on Whatman no. 3MM paper with a solvent system of isobutyric acid-28% ammonium hydroxide-water (66:1:33). Radioactive spots corresponding to ATP were cut out, eluted with 2 ml of distilled water, and then submitted to the determination of absorption at 260 nm and of radioactivity in dioxane scintillation fluid, from which specific radioactivity of ATP fractions was calculated.

ATP phosphohydrolase (EC 3.6.1.3; ATPase) reaction was carried out by the method of Pullman et al. (19). The free P_i formed was determined by Takahashi's method, the volumes being scaled down by one-half.

Assay of succinate dehydrogenase in mitochondria. Succinate dehydrogenase (EC 1.3.99.1) was assayed by measuring succinate-phenazine methosulfate reductase by using phenazine methosulfate and 2,6-dichlorophenolindophenol as sequential electron acceptors (6).

RESULTS

Effect of siccanin on the growth of T. mentagrophytes. The growth of *T. mentagrophytes* from spores was inhibited approximately 50% by siccanin at a concentration of 0.1 μg/ml (Fig. 1A). Almost the same extent of the growth inhibition was observed when siccanin was added to the cultures at 70 hr, by which time considerable mycelial mass had developed (Fig. 1B). Those results indicate that inhibition by siccanin is not specific for germination of the fungus in its life cycle. To study the primary attacking sites of siccanin in the cells, experiments were carried out to examine changes in some cellular activities of the fungus in the presence of various amounts of the antibiotic to inhibit its growth moderately.

Effect of siccanin on the synthesis of cellular materials, on respiration, and on a cell membrane function. Incorporation of ^{32}P-P_i into RNA, DNA, phospholipid, and acid-soluble fractions, of ^{14}C-glutamate into protein, and of ^{14}C-glucosamine into cell wall fraction of *T. mentagrophytes* were first followed as shown in Fig. 2 and 3. The synthesis of RNA, DNA, and phospholipid was slightly inhibited by 0.3 μg of siccanin per ml. Inhibition rates of these three syntheses at 30 to 50 min after the addition of siccanin were not more than 30%. There was obviously no fraction in which synthesis was inhibited specifically and remarkably. The syntheses of protein and cell wall were not affected under the same conditions.

Respiration was more sensitive to siccanin; it was inhibited approximately 60% by the antibiotic at a concentration of 0.1 μg/ml (Fig. 4). The amount of siccanin per milligram (dry weight) of cells used in those experiments was about one-half of the amounts shown in Fig. 2 and 3. Notwithstanding this difference, it is concluded that oxidative respiration is more sensitive to siccanin than other macromolecular syntheses. For the measurement of oxidation activity by the oxygen meter we had to use the cells of *T. mentagrophytes* with high endogenous oxidation activity, since the cells were found to lose their oxidation activity from starvation during incubation. It was also observed that several minutes is required for the full inhibitory effect of siccanin on respiration to appear. This time lag was not observed in the mitochondrial system as mentioned later; thus, it is likely that this lag is the time required for the penetration of siccanin to its target. Figure 5 shows a typical experiment on the relationship between inhibition of oxidation and siccanin concentration; siccanin could not inhibit the respiration more than about 80%, even at high concentrations. The measurement of oxidation activity of the *T. mentagrophytes* cells was done as soon as possible after preparation of cells, since the inhibition rate was apt to decrease with aging of the cells. Antimycin A (10^{-5} M), azide (3.3×10^{-2} M), and rutamycin (3.3 μg/ml) inhibited the oxygen uptake of these cells by 70, 80, and 60% respectively. At various concentrations tested, 2,4-dinitrophenol (DNP) did not stimulate oxidation activity of the intact cell system.

The possibility that siccanin causes damage directly to the cell membrane was tested, as has been reported with polymyxins (17), nystatin (3), and in other antibiotics. Siccanin (0.1 and 10 μg/ml) was added to the medium in which the fungal cells were being incubated with ^{14}C-gluta-

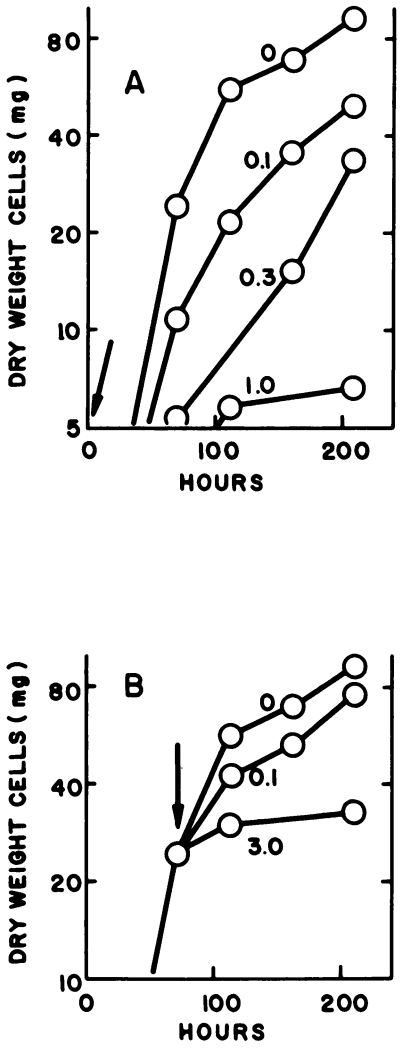

FIG. 1. *Effect of siccanin on germination and mycelial growth of T. mentagrophytes. Siccanin was added to spore (A) or to growing mycelia (B) at the time indicated by the arrow. Concentrations (μg/ml) are shown by each curve. Results are the average of two estimations.*

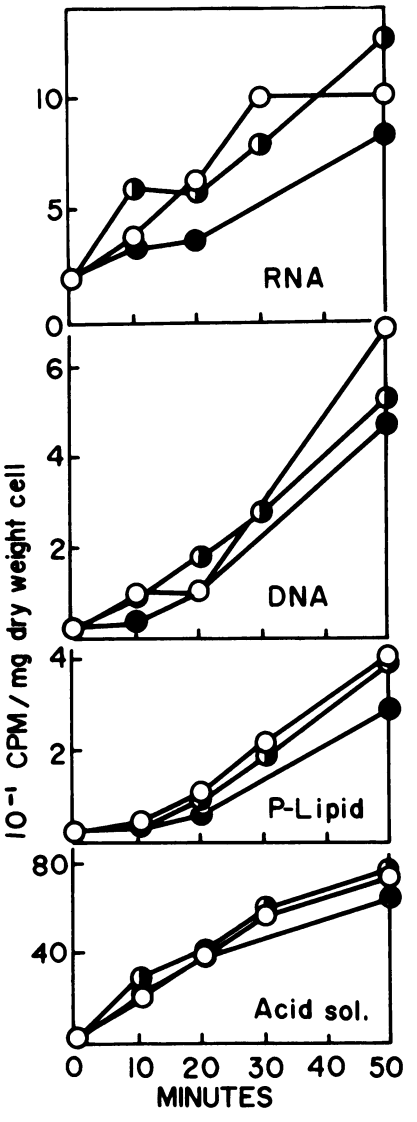

FIG. 2. *Influence of siccanin on the incorporation of ^{32}P-orthophosphate into RNA, DNA, phospholipid, and acid-soluble fractions. Concentration of siccanin (μg/ml): 0 (○), 0.1 (◑), and 0.3 (●).*

mate, and the amounts of isotope in the cells were measured periodically (Fig. 6). The cells continued taking in ^{14}C-glutamate at the same rate as control for at least 30 min after the addition of 0.1 μg of siccanin per ml. It was also observed that ^{14}C-glutamate already taken into the cells did not leak, even at a concentration of 10 μg of siccanin per ml. The results are in good agreement with those showed in Fig. 2, which essentially indicated no inhibitory effect of siccanin on the incorporation of ^{32}P-orthophosphate into the acid-soluble fraction. Thus, it is concluded that siccanin does not primarily cause damage to the cell membrane.

Effect of siccanin on the oxidation and phosphorylation in mitochondria. The experiments described above, which were carried out with intact cells, suggested that siccanin primarily inhibits respiration of *T. mentagrophytes* cells. To study those effects of siccanin on the fungal respiration in detail, experiments were performed with the mitochondrial system of the fungus. Table 1 shows the oxidation activity of the fungal mitochondria, together with the effect of siccanin

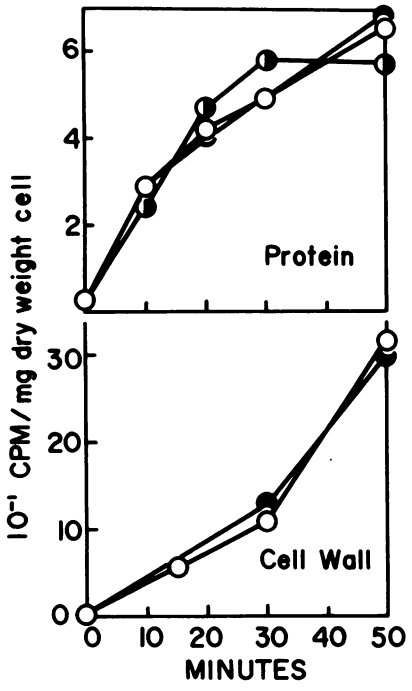

FIG. 3. *Influence of siccanin on the incorporation of ^{14}C-glutamate into protein and of ^{14}C-glucosamine into cell wall fraction. Symbols:* (○) *no siccanin added,* (◑) *0.1 μg/ml,* (●) *0.3 μg/ml.*

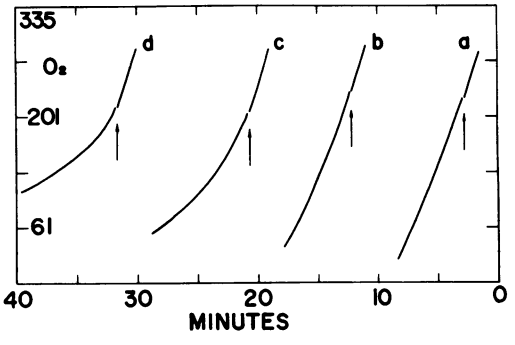

FIG. 4. *Time course of oxygen consumption by T. mentagrophytes cells in the presence or absence of siccanin. Mycelial cells (2.5 mg, dry weight, per assay) used were the same lot as in the experiments in Fig. 2 and 3. Siccanin was added to the reaction mixture at the time indicated by the arrows with the final concentration of 0 (a), 0.03 (b), 0.1 (c), and 0.3 (d) μg/ml. Per cent inhibition at those concentrations, which was calculated from the oxygen uptake rate at 5 min after addition of siccanin, is 0, 7, 60, and 70%, respectively. Dissolved oxygen is expressed as nanomoles per 1.5 ml of incubation mixture.*

on that activity. The oxidation of succinate in mitochondria was more sensitive to siccanin than it was in intact cells. The inhibitory effect on the

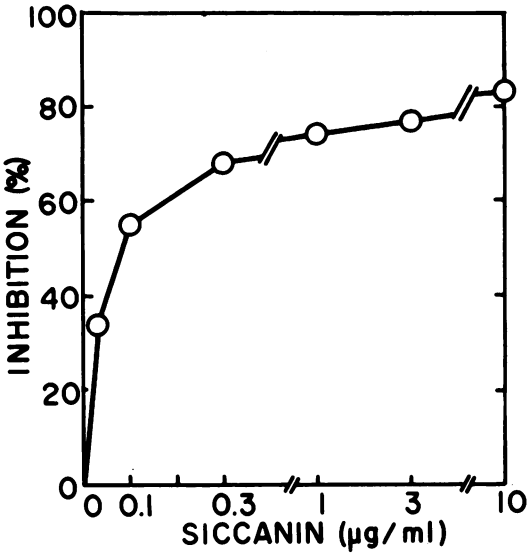

FIG. 5. *Effect of siccanin on respiration of T. mentagrophytes cells. Reaction mixtures contained 1.0 mg (dry weight) of mycelial cells. Per cent inhibition is calculated as described in the legend to Fig. 4.*

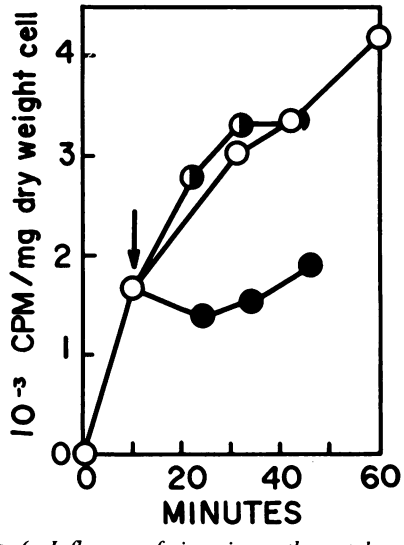

FIG. 6. *Influence of siccanin on the uptake of ^{14}C-glutamate and its leakage in T. mentagrophytes cells. Siccanin was added at 10 min as indicated by the arrow. Symbols:* (○), *no siccanin added;* (◑), *0.1;* (●), *10 μg/ml.*

mitochondria was spontaneously observed upon addition of the antibiotic; a time lag was observed with intact cells. Inhibitory effect of siccanin on the oxidation in the mitochondrial system depended on the substrates used. Among the substrates succinate was the most sensitive; its oxidation was inhibited 50% by siccanin at a concentration of 0.03 μg/ml. At this concentration

TABLE 1. *Influence of siccanin and some inhibitors on oxidation in T. mentagrophytes mitochondria*[a]

Substrate[b]	O_2 uptake without inhibitor		O_2 uptake with inhibitor		
	Amt[c]	RCI[d]	Inhibitor (μg/ml)	Amt[c]	Per cent inhibition
Expt 1					
Succinate	86	2.1	Siccanin, 0.03	43	50
α-Ketoglutarate	58	2.9	Siccanin, 0.03	47	19
l-Glutamate	47	3.0	Siccanin, 0.03	36	23
Citrate	45	2.5	Siccanin, 0.03	39	13
l-Malate	42	2.1	Siccanin, 0.03	36	14
α-Glycerophosphate	22	1.6	Siccanin, 0.03	18	18
β-Hydroxybutyrate	20	1.4	Siccanin, 0.03	18	10
NADH	19	1.1	Siccanin, 0.03	20	0
Expt 2					
Succinate	84	1.2	Siccanin, 0.3	19	77
Succinate	84	1.2	Azide, 650	23	73
Succinate	84	1.2	Rutamycin, 10	42	50
NADH	109	1.5	Siccanin, 0.3	92	16
NADH	109	1.5	Azide, 650	20	82
Ascorbate + TMPD	75	1.2	Siccanin, 0.3	60	20
Ascorbate + TMPD	75	1.2	Azide, 650	20	73

[a] Amounts of mitochondria used were 1.04 and 0.75 mg of protein for experiments 1 and 2, respectively.

[b] Ten millimoles of each substrate was applied except for NADH (1 mmole) and TMPD (0.2 mmole). NADH, reduced nicotinamide adenine dinucleotide; TMPD, *N,N,N',N'-tetramethyl-p*-phenylendiamine dihydrochloride.

[c] Nanomoles of O_2 per minute per milligram of protein.

[d] Respiratory control index.

other substrates, linked to the terminal oxidation through reduced nicotinamide adenine dinucleotide (NADH), showed slight inhibition (Table 1, experiment 1). Siccanin showed essentially no effect on the oxidation of NADH or ascorbate which was mediated by *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride to the terminal oxidation (Table 1, experiment 2). DNP at various concentrations, did not release oxidation activity from the inhibition by siccanin. These results suggest that siccanin is an inhibitor of electron transport system, acting on the dehydrogenation step of succinate. And siccanin also has slight effect on the dehydrogenation step of other substrates. It must be considered, however, that mitochondrial preparations used here were not intact, as indicated by their low RCI.

The direct effect of siccanin on succinate dehydrogenation was studied as shown in Table 2. The result showed that the antibiotic actually inhibited succinate-phenazine methosulfate reductase in the mitochondria and that the inhibition was stronger than it was in succinate oxidation described above.

It has been reported that inhibitors of energy transfer and uncouplers vary in their effectiveness on mitochondrial oxidation with substrate used (4, 13, 15). Thus, effect of siccanin on phosphorylation was examined. Table 3 shows the effect of siccanin on the oxidative phosphorylation when succinate, glutamate, and citrate were substrates. Siccanin inhibited phosphorylation coupled with oxidation of succinate. The antibiotic showed little effect on phosphorylation coupled with oxidation of glutamate. The P:O ratio, however, was not changed under those conditions; i.e., the inhibition of phosphorylation was parallel to that of oxidation. In this system, both rutamycin and DNP strongly inhibited only the phosphorylation, thus reducing down the P:O ratio. When citrate was used as a substrate,

TABLE 2. *Influence of siccanin on succinate dehydrogenase in T. mentagrophytes mitochondria*

Inhibitor (μg/ml)	Succinate dehydrogenase activity[a]
Siccanin	
0	7.55
0.003	5.45 (28)[b]
0.03	2.56 (66)
0.3	1.52 (80)

[a] Expressed as diminution in absorption at 600 nm per minute per milligram of protein. Reaction mixtures contained, in 1.0 ml: 50 μmoles of potassium phosphate (*p*H 7.4), 20 μmoles of sodium succinate, 10 μmoles of potassium cyanide, 163 nmoles of phenazine methosulfate, 15.3 nmoles of 2,6-dichlorophenolindophenol, 11.9 μg of protein of mitochondria, and inhibitor in 10 μliters of ethanol.

[b] Numbers in parentheses represent per cent of inhibition.

TABLE 3. *Influence of siccanin on oxidation and phosphorylation in T. mentagrophytes mitochondria*[a]

Substrate[b]	Inhibitor (μg/ml)	O_2 uptake[c]	Phosphorylation[c]	P:O ratio
Succinate	Siccanin			
	0	68	108.5	1.6
	0.01	52	78.9	1.5
	0.03	39	66.4	1.7
	0.10	38	56.2	1.5
	0.30	26	48.4	1.9
Glutamate	Siccanin			
	0	51	94.5	1.9
	0.10	38	85.0	2.2
	0.30	34	75.0	2.2
	1.0	31	46.8	1.5
Citrate	Siccanin			
	0	38	54.6	1.4
	0.30	31	42.2	1.4
	1.0	29	42.9	1.5
Succinate	Dinitrophenol, 18.4	58	5.40	0.09
	Rutamycin, 10.4	31	1.50	0.05

[a] Incubation mixtures contained 1.52 mg of protein of mitochondria in a total volume of 1.675 ml. The respiratory control index of the mitochondrial preparation was 1.4 for succinate, 5.0 for glutamate, and 2.0 for citrate.

[b] Ten millimoles of each substrate was applied.

[c] Values expressed as nanoatoms per minute per milligram of protein.

siccanin showed essentially no inhibitory effect on either phosphorylation or oxidation.

Effect of siccanin on partial reactions of phosphorylation in mitochondria. Table 4 shows that siccanin did not affect the P_i-ATP exchange reaction. At very high concentrations, the antibiotic slightly inhibited the reaction. As compared with the effective concentration of siccanin required for growth inhibition, however, these concentrations are too high to give evidence that this inhibition is the primary action of siccanin. Rutamycin, an inhibitor of the energy transfer in rat liver mitochondria (15), also inhibited this reaction of *T. mentagrophytes* mitochondria. Siccanin did not inhibit the ADP-ATP exchange reaction (Table 5). The antibiotic showed no effect on the Mg^{2+}-stimulated ATPase reaction as its primary site (Table 6). Slight stimulation of this reaction was observed at very high concentrations of siccanin. Rutamycin also inhibited this reaction, as reported with rat liver mitochondria (15). These results indicate that siccanin neither affects the energy-transferring step in oxidative phosphorylation nor the uncoupling action as far as its primary site of action is concerned. From the results described above, it can be concluded that siccanin acts preferentially on dehydrogenation step of succinate and, with less potency, other substrates.

TABLE 4. *Influence of siccanin on ^{32}Pi-ATP exchange reaction in T. mentagrophytes mitochondria*[a]

Inhibitor (μg/ml)	Specific radioactivity of ^{32}P-ATP after exchange reaction[b]
Siccanin	
0	221
0.03	214
0.30	182
3.0	161
Dinitrophenol, 18.4	105
Rutamycin, 10.0	123

[a] Reaction mixtures contained, in 1.0 ml: 20 μmoles of potassium phosphate (*p*H 7.4), 10 μmoles of $MgCl_2$, 10 μmoles of ATP, about 10 μCi of ^{32}P-orthophosphate, 1.4 mg of protein of mitochondria, and inhibitor in 10 μliters of ethanol.

[b] Values expressed as counts per minute per nanomole.

TABLE 5. *Influence of siccanin on ^{14}C-ADP-ATP exchange reaction in T. mentagrophytes mitochondria*[a]

Inhibitor (μg/ml)	Specific radioactivity of ^{14}C-ATP after exchange reaction[b]
Siccanin	
0	27.4
0.03	26.9
0.30	28.7
3.0	27.5
Rutamycin, 10.0	21.6
No mitochondria[c]	9.13

[a] Reaction mixtures contained, in 1.0 ml: 10 μmoles of Tris-sulfate (*p*H 7.0), 6 μmoles of ATP, 2 μmoles of ADP (about 10^6 counts/min), 1.4 mg of protein of mitochondria, and inhibitor in 10 μliters of ethanol.

[b] Values expressed as counts per minute per nanomole.

[c] Reaction was carried out without mitochondria but the isolation procedure of the ATP fraction was performed as for siccanin and rutamycin.

DISCUSSION

The respiration of the intact cells of *T. mentagrophytes* has been preferentially inhibited by siccanin at the concentrations which inhibited growth of the fungus. Furthermore, succinate oxidation in mitochondria was more sensitive to the antibiotic than the respiration was in intact cells. Although the possibility can not be excluded that unknown cellular functions not tested here are also sensitive to the antibiotic, the results obtained led us to the conclusion that the primary site of action of siccanin is the aerobic

TABLE 6. *Influence of siccanin on ATPase in T. mentagrophytes mitochondria*[a]

Inhibitor (μg/ml)	P_i released from ATP[b]
Siccanin	
0	14.1
0.03	14.1
0.30	14.9
3.0	17.1
Rutamycin, 10.0	4.1
No mitochondria[c]	0.79

[a] Reaction mixtures contained, in 1.0 ml; 50 μmoles of Tris-sulfate (*p*H 7.4), 6 μmoles ATP, 3 μmoles $MgCl_2$, 5 μmoles phosphoenol pyruvate (Boehringer), 0.465 mg protein of mitochondria, and inhibitor in 10 μl ethanol.

[b] Values expressed as micrograms per milligram of protein per minute.

[c] See footnote *c*, Table 5.

respiration. Inhibitors of energy transfer and uncouplers, i.e., oligomycin, rutamycin (15), usnic acid (13), nigericin (4), and aurovertin (14), were reported to inhibit mitochondrial oxidation depending on the substrates used. Lardy et al. (14) showed that NADH-linked substrates, as compared with succinate, are more sensitive to oligomycin. They explained the reason to be that the oxidation of NADH is more tightly coupled with phosphorylation. Wadkins et al. (23) showed that those antibiotics really do not affect the NADH oxidation by mitochondrial fragments. Substrate dependency of siccanin action, however, cannot be explained by this idea since the antibiotic is more inhibitory to the oxidation of succinate than to that of NADH-linked substrates, and, furthermore, succinate dehydrogenase activity was shown to be inhibited by the antibiotic. Hagihara et al. (8) suggested that siccanin is an inhibitor of energy transfer in rat liver mitochondria. In this paper, however, we further extended studies of those points in detail and indicated that siccanin at low concentrations neither reduces the P:O ratio nor inhibits partial reactions of phosphorylation in our fungi system.

With regard to preparation of mitochondria from *T. mentagrophytes* cells, several methods were tried for disrupting fungal cell wall. The treatment of the cells by snail gut enzymes according to Weiss (24) was not a good method for obtaining active mitochondria in respiration. Disruption of the cells by a homogenizer by the method of Iwasa (12) was found also ineffective to *T. mentagrophytes* cells. After the other trials, the grinding of the cells with silica sand as described in this paper was found to be an effective and simple way for *T. mentagrophytes* cells used here.

We are also studying the mode of action of siccanin on mitochondrial preparations from rat liver and beef heart; the results will be published elsewhere.

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