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Biochemical Studies of Phenoloxidase and Utilization of Catecholamines in *Cryptococcus neoformans*

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Protoplasts of *Cryptococcus neoformans* contain phenoloxidase as a membrane-bound enzyme. The enzyme appeared to be attached on the inner side of cytoplasmic membranes. Synthesis of the enzyme was derepressed by low levels of glucose but was not affected by the level of ammonium. Copper chelators which inhibited the phenoloxidase of other organisms did not affect cryptococcal enzymes. However, cyanide- or iron-chelating agents such as hydroximide derivates or 8-hydroxyquinoline were effective inhibitors, suggesting that cryptococcal phenoloxidase is an iron-containing enzyme. Phenoloxidase of *C. neoformans* catalyzed the oxidation of various diphenols via dopachrome and labile intermediates to melanin polymers. The kinetic constants (K_m) of the phenoloxidase and the permease for dopamine and norepinephrine were low. The correlation between phenoloxidase and the preferential growth of *C. neoformans* in the host brain is discussed.

Cryptococcus neoformans is a pathogenic fungus which grows preferentially in the brains of humans and experimental animals (10). The factors which enhance the growth of this fungus in the brain remain unknown. *C. neoformans* is unique among the members of the genus in that it is the only true pathogen and produces melanin when grown on media containing diphenolic compounds (34).

Melanogenesis, in various biological systems, starts with the oxidation of tyrosine to dihydroxyphenylalanine (dopa). The dopa is then oxidized through a series of extremely labile intermediates to 5,6-indoquinone, which is polymerized to form melanin (18, 20, 21). Tyrosinase, the enzyme responsible for these steps in mammalian cells, is highly specific for tyrosine (18, 20). In other organisms, melanin can be formed from dopa or catecholamines by the enzyme phenoloxidase (20). In this process, an enzyme is needed only for the oxidation of tyrosine and dopa. The other reactions in melanogenesis are extremely fast and probably nonenzymatic (22). The reactions leading to various intermediates are controlled by a number of chemical conditions; as a result, the melanin polymers show a varying structure (21, 36). There is no known enzyme which can hydrolyze melanin, nor is there an antibody which can recognize the polymers. This may be due to the fact that the melanin polymers are highly diverse in their structure and complexity. In some fungi, melanin on the cell wall has been implicated as a protective shield against microbial and enzymatic degradation (2, 16, 27).

Since Staib's discovery of melanin formation by *C. neoformans* (34), extensive work has been published on the subject. These studies, however, were limited mostly to the identification of suitable substrates and growth conditions for melanin production (5, 6, 23, 35). In spite of its importance, only one paper has been published specifically on the phenoloxidase. Shaw and Kapica (32) tested a range of substrates and the optimal assay conditions for the enzyme.

In this paper, we report the result of our studies on the biochemical aspects of melanin formation with emphasis on the properties of phenoloxidase in *C. neoformans*. This is the first of a series of papers which show correlation between virulence and the presence of phenoloxidase in *C. neoformans*.

MATERIALS AND METHODS

Strain, media, and growth conditions. *C. neoformans* B-3501 (serotype D) was grown on yeast nitrogen base medium (Difco Laboratories, Detroit, Mich.) supplemented with 1 g of glucose per liter. The pH of the medium was adjusted to 6.5 with 1 M KH_2PO_4. Conditions of inoculation and incubation were as described previously (26) except that the cells were grown in 2-liter flasks containing 800 ml of medium and harvested at the end of the exponential growth (absorbancy at 600 nm [A_{600}] = 1.0 to 1.2; Gilford spectrophotometer 250).

To investigate the effect of carbon starvation, cul-

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tures were grown for 18 h (A_{600} = up to 0.6) in yeast nitrogen base medium containing 3 g of glucose per liter. The cultures were then divided into three equal portions and centrifuged (12,000 × g; 15 min at 25°C). One part was washed with the same medium, whereas the other two parts were washed with the same medium lacking glucose (glucose-starved cells). After the washing, each part was resuspended in 800 ml of the respective medium and was shaken for 5 h at 25°C. Cycloheximide (50 μg/ml; The Upjohn Co., Kalamazoo, Mich.) was added to one of the glucose-free media.

Asparagine broth medium used in some experiments contained 1 g of asparagine, 1 g of glucose, 0.5 g of $MgSO_4$, 3 g of KH_2PO_4, and 50 μl of thiamine vitamin solution (Abbott Laboratories, Chicago, Ill.) per liter. The pH of the asparagine broth was adjusted to 6.5 with 1 M NaOH.

For melanin production on agar media, the procedure of Chaskes and Tyndall (6) was used, except that the concentration of glucose was 1 g/liter and that of various diphenols was 0.5 mM.

Breakage and fractionation of the yeast cells. The yeast cells were harvested by centrifugation at 12,000 × g for 15 min in the cold and washed once with 0.05 M phosphate buffer, pH 6.5. The cells were then resuspended in the same buffer to give a final volume of 2 ml (1 g/ml, wet weight). The same volume of glass beads (0.45 to 0.50 mm) was added in a 17 by 100-mm plastic tube with cap (Falcon Plastics, Oxnard, Calif.), and the cells were broken by rapid mixing on a Vortex mixer as previously described (26). After the breakage, supernatant was decanted, the glass beads were washed three times with the breakage buffer, and the combined supernatant was centrifuged at 105,000 × g for 45 min in the cold. The membrane cell wall fraction was washed once more and resuspended in 2 ml of the same buffer. The suspension was divided into 0.5-ml portions and stored at −18°C. In one experiment, the cell-free supernatant was also stored at −18°C.

Protoplast formation and solubilization of the phenoloxidase. The yeast cells were grown on asparagine broth medium containing 20 g of glucose per liter and harvested during the logarithmic phase (A_{600} = 0.4 to 0.6). The cells were collected by centrifugation and washed once with 0.05 M citrate-phosphate buffer, pH 5.9. The cells were then treated with mercaptoethanol and converted into protoplasts by using the procedure described by Cabib (4) with the following modifications: 0.05 M citrate-phosphate buffer, pH 5.9, containing 0.6 M KCl and 1 mM EDTA was used as osmotic stabilizer. The cells, after the mercaptoethanol treatment, were washed once with twofold-concentrated buffer. Each 1 g (wet weight) of the cells was resuspended in 3 ml of concentrated buffer, and then an equal amount of snail enzymes was added. The suspension was incubated at 30°C with slight shaking. The progress of protoplast formation was checked every 30 min. It required at least a 3-h treatment to obtain the protoplasts, and the cell debris was discarded by centrifugation at 100 × g for 15 min in the cold.

The protoplasts were washed three times for 10 min at 750 × g in the cold. The supernatant was separated from the pellet and saved for future use of the glusulase. The protoplast pellet was resuspended in 1 ml (per 1 g [wet weight] of cells) in the same buffer-stabilizer containing aureomycin (0.05 mg/ml) as a preservative. The protoplasts were stored overnight at 4°C.

Protoplasts were lysed according to the procedure described by Duran et al. (8). The pellet obtained after lysis was washed three times with 0.05 M Tris-hydrochloride buffer, pH 7.5, containing 2 mM $MgSO_4$.

The solubilization of the phenoloxidase from the protoplast membranes with various detergents was carried out according to the method of Duran and Cabib (9) except that the cholate treatment was omitted. The extracts were diluted 10-fold and concentrated at 4°C in an Amicon (Danvers, Mass.) pressure cell equipped with YM-10 membrane. After 10-fold concentration, the solution was brought up to the original volume, and the concentration step was repeated once more to eliminate most of the detergent. The final volume was adjusted to about 0.5 ml per g of cells used.

Assays of phenol oxidase. *o*-Diphenoloxidase activity was assayed either by measuring the accumulation of chromophoric intermediates during the oxidation of the diphenol substrates or by determining the radioactivity of melanin formed by the enzyme from radiolabeled substrate.

In the spectrophotometric method, the absorbance maxima of dopachrome and 5,6-dihydroxyindole were measured at 480 and 300 nm, respectively. The reaction mixture contained 0.05 M phosphate buffer (pH 6.5), 1 mM substrates, and 50 μl of enzyme in a total volume of 1 ml.

After incubation in a shaking water bath for 30 min at 25°C, the reaction was stopped by adding 10 μl of 1 M NaCN. The reaction mixtures were then centrifuged for 10 min at 22,000 × g at 20°C. The absorbancy of the supernatant was measured at 300 and 480 nm in a Gilford 250 spectrophotometer. In all experiments, L-dopa and L-norepinephrine (Sigma Chemical Co., St. Louis, Mo.) were used as substrates; in some additional experiments, other diphenolic compounds were also used as described in Results. For highly active enzyme preparations, the incubation time was decreased to 5 min. In such cases, appropriate controls of shorter and longer incubation times were also checked to ensure that the activity was in a linear range. When the enzyme preparation had extremely low activity, the incubation time was extended up to 5 h. All readings were corrected by using controls containing 10 mM NaCN. One unit of enzyme was defined as the amount which caused a change in absorption at 480 or 300 nm of 0.001 at 25°C, pH 6.5, under the specified conditions.

The radiometric assays for the diphenoloxidase were carried out as previously described (11, 12). The assays were run in duplicate at 37°C for 1 h. The radiolabeled substrates used were L-[*U*-C^{14}]tyrosine (specific activity, 745 mCi/mmol; New England Nuclear Corp., Boston, Mass.), DL-[7-^{14}C]norepinephrine (specific activity, 30 to 60 mCi/mmol; New England Nuclear), L-[3-^{14}C]dihydroxyphenylalanine (specific activity, 5 to 20 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), DL-[3-^{14}C]dopa (40 to 60 mCi/mmol; Research Production International Corp., Mt. Prospect, Ill.), and [7-^{14}C]dopamine (30 to 50 Ci/mmol; Amersham). One unit was defined as the amount of enzyme oxidizing 1 mmol of substrate to melanin per h at 37°C under the assay conditions. Protein was measured according to the method of Bramhall et al. (3).

Testing of inhibitors against phenoloxidase. Several reducing agents, substrate analogs, and metal-chelating agents were tested for their inhibitory effect on the phenoloxidase. Soluble enzyme was used throughout the tests to achieve a better contact between the enzyme and the inhibitor. Parallel experiments were carried out by using bound enzyme. Enzyme (30 μg/ml) was preincubated with 1 mM inhibitors for 30 min at 25°C before L-dopa or norepinephrine was added. For the studies on iron-chelating agents, 1 and 3.5 mM concentrations were used; the enzyme concentration was 74 μg of protein/ml.

Uptake measurement. Cellular uptake of the following compounds was measured: tyrosine, DL-dopa, dopamine, and norepinephrine. In different experiments, the cells were grown in 400 ml of asparagine broth medium and were harvested at A_{600} = 0.8. After washing once with the same medium lacking asparagine, the cells were suspended in 50 ml of the washing medium. Thirty-five milliliters of the cell suspensions was washed three times in cold distilled water and centrifuged, and then the pellet was dried in a 75°C oven to measure the dry weight. To 5 ml of the cells, 0.02% (vol/vol, final concentration) sodium azide was added as a preservative. These cells were used as a control for the uptake experiments. To different portions of the remaining 10 ml of cells and to the azide control, a 1-μCi/ml concentration of the following radiolabeled compounds was added: DL-3(3,4-dihydroxyphenyl)[3-^{14}C] alanine, tartrate (58 mCi/mmol; Research Production International); DL-(*Methylene*-^{14}C)noradrenaline DL-bitartrate (55 mCi/mmol; Amersham); [7-^{14}C]dopamine hydrochloride (60 mCi/mmol; Amersham); and L-[μ-^{14}C]tyrosine (450 mCi/mmol; New England Nuclear). In some experiments, 0.05 mM (final concentration) unlabeled compound (purchased from Sigma) was added as the carrier. The uptake experiments were carried out at 25°C in a shaking water bath. Reactions were started by the addition of the labeled compound. Samples of 0.5 ml were removed periodically and filtered through Gelman A/E glass fibers. The filters were washed three times with 5 ml of the cold glucose-free asparagine medium containing 5 mM nonradioactive substance. The filters were dried, and the radioactivity on the filters was determined in a Beckman LS8100 scintillation spectrophotometer. The specific activity of each reaction vessel was determined by directly measuring the radioactivity of two separate samples of 20 μl. The counts were corrected for the appropriate background and nonspecific adherence. Similar uptake experiments were carried out in yeast nitrogen base medium containing 20 g of glucose per liter.

RESULTS

Distribution of phenoloxidase in cryptococcal cells. After breakage of cryptococcal cells, all of the phenoloxidase activity was found in the particulate fraction (specific activity, 158.2 U/mg; Table 1), whereas no detectable activity was found in either the 105,000 × *g* supernatant or the culture medium. Protoplasts of the yeast cells were prepared as shown in Table 1. Most of the phenoloxidase activity in the particulate fraction was attached to the protoplast membranes.

TABLE 1. Orientation of phenoloxidase in the membrane

Fraction	Phenoloxidase	
	U[a]	Sp act (U/mg of protein)
Particulate fraction	1,132	158
Protoplast membrane	1,101	550
Protoplast membranes treated with glutaraldehyde[b,c]	<10	<10
Protoplasts treated with glutaraldehyde[b]	812	406

[a] See Materials and Methods.

[b] Glutaraldehyde was added to a final concentration of 1% to a suspension of protoplasts. After 30 s at 20°C, 20 volumes of 0.6 M KCl in citrate-phosphate buffer, pH 5.9, was added. Protoplasts were centrifuged for 5 min at 1,000 × *g* and washed again with the same buffer.

[c] Lysates were treated with glutaraldehyde in the same way, but dilution and washing were performed with 0.05 M Tris-hydrochloride buffer containing 2 mM $MgSO_4$, and centrifugation was for 10 min at 25,000 × *g*.

Protoplasts were used to study the orientation of phenoloxidase in the membrane by using a method similar to that described by Duran et al. (8). In contrast to *S. cerevisiae*, the cell walls of *C. neoformans* were not completely disrupted by the hydrolytic enzymes. The protoplasts were released through a break in the cell walls that remained intact as ghosts. This may explain the slow protoplast formation in *C. neoformans*.

When the protoplasts were briefly treated with glutaraldehyde before lysis, only the external side of the membrane was exposed, and the subsequent manifestation of enzyme activity was not affected. When the same treatment was performed after the lysis of protoplasts, the internal side of the membrane had been exposed to glutaraldehyde, and most of the enzyme activity was irreversibly lost (Table 1). These results indicate that phenoloxidase is so oriented in the membrane that it is accessible only from the inside of the cells.

Solubilization of phenoloxidase. The enzyme was solubilized from particulate fraction of broken cells or membranes isolated from protoplasts. Many detergents, such as sodium dodecyl sulfate, cetylmethylammonium bromide, cholate, deoxycholate, Zwittergent (3-08 3-16; Calbiochem), Triton X-100, Tween 80, and digitonin, were tested (see Materials and Methods) in four different concentrations (0.01, 0.1, 0.5, and 2.0%). Digitonin proved to be effective in releasing the bound enzyme at a concentration

of 0.5% (data not presented). Even with digitonin, the solubilization was efficient only with the protoplast membrane fractions and not with the pellets of broken cells. The solubilization efficiency varied between 50 and 70%. The soluble enzyme was unstable and lost its activity within 2 weeks at −70°C. Efforts to retain the enzyme activity by changing the storage conditions and adding exogenous proteins or phosphatidylserine failed.

Effect of glucose and ammonia on the synthesis of phenoloxidase in *C. neoformans*. Phenoloxidase in *C. neoformans* is a constitutive enzyme. It is synthesized by the cells in minimal medium in the absence of its substrates. When exponentially growing cells were suspended in a medium lacking glucose and incubated for 5 h, a striking increase in phenoloxidase activity was seen (Table 2). The same derepression effect was obtained by growing the cells in a complete medium to stationary phase when most of the glucose was exhausted. The addition of cycloheximide to a culture in glucose-free medium prevented the rise in enzyme activity, indicating that protein synthesis was necessary for the increase. The repression was specific for glucose, since the enzyme activity in cells growing exponentially on xylose was equal to that of glucose-starved cells (data not shown).

No ammonia repression of phenoloxidase has been observed in *C. neoformans*. As shown in Table 2, cells pregrown in a medium containing ammonium sulfate and starved for glucose in the presence of ammonium sulfate had higher enzyme activity than cells grown and starved in the presence of asparagine as a nitrogen source. Differences in enzyme level, however, were found only by using the colorimetric assay.

Enzymatic properties of phenoloxidase. Cryptococcal phenoloxidase had a broad optimal pH around 6.5 (data not shown). The optimal temperature was 37°C, with the maximal ranging between 25 and 40°C. Since autoxidation of the substrates is very fast at 37°C, the enzyme assay was run at 25°C, where only slight autoxidation occurred during the experiments.

Substrate specificity and cofactors. The cryptococcal enzyme oxidized a variety of phenolic compounds. It seemed from the data shown in Table 3 that the only requirement was for a phenyl compound which contained two hydroxyl groups in an *ortho*-(3,4) position. However, when the hydroxyl group in position 3 or 4 was replaced by an amino group (aminotyrosine, 2-amino-3-hydroxybenzoic acid, or 3-hydroxyanthranilic acid) or when an additional hydroxyl group was added in position 3, a compound different from the regular dopachrome was formed. This compound had a yellow color instead of red, with peaks of absorbancy at 210, 260, and 480 nm. The same compound was produced when chlorogenic acid was the substrate. However, no activity was observed when a methoxy group or oxygen replaced one of the hydroxyl groups (normetanephrine) or when a monophenol compound like tyrosine served as a

TABLE 2. Effect of glucose and ammonia on phenoloxidase synthesis

Treatment	Phenoloxidase sp act (U/mg of protein)[a]					
	Colorimetric assay				Radiometric assay	
	300 nm		480 nm			
	L-Dopa	L-Norepinephrine	L-Dopa	L-Norepinephrine	L-Dopa	L-Norepinephrine
Exponentially growing cells[b]	2,070	2,930	597	950	52	54
Cells starved for glucose[c]	23,600	42,000	6,470	13,200	462	466
Cells starved for glucose in presence of cycloheximide[d]	1,360	1,950	411	502	62	67
Cells grown on ammonium sulfate and starved for glucose[e]	12,500	25,400	3,490	9,160	482	556
Cells grown on asparagine and starved for glucose[f]	5,650	11,100	1,690	3,890	497	688

[a] Particulate enzyme was used in all experiments; substrate concentration was 1 mM.

[b] Cells were grown in yeast nitrogen base (YNB) medium containing 3 g of glucose per liter and were harvested at A_{600} = 0.6.

[c] As in *b*, but the cells were transferred into glucose-free medium for 5 h.

[d] Cycloheximide concentration was 50 mg/liter.

[e] Cells were grown in YNB medium containing 5 g of $(NH_4)_2SO_4$ per liter as the nitrogen source and then starved for glucose 5 h before harvest.

[f] Cells were grown in asparagine broth medium containing 1 g of asparagine per liter as the nitrogen source and then starved for glucose 5 h before harvest.

TABLE 3. Substrate specificity of phenoloxidase

Substrate[a]	Sp act (U/mg of protein)	
	300 nm	480 nm
Catechol	5,980	809
L-Dopa	5,430	1,090
D-Dopa	5,480	1,150
Gallic acid	10,820	2,160
Caffeic acid	1,320	1,210
Chlorogenic acid	409	423
Epinephrine	11,950	3,700
Dopamine	5,210	910
Norepinephrine	7,570	2,890
Methyldopa	4,170	1,190
3-Aminotyrosine	<10	<10
Hydroquinone	<10	<10
Hydroxy anthranilic acid[b]	NT[c]	1,225
Mimosine	<10	<10
Tyrosine	<10	<10
Normetanephrine	<10	<10
Hydroxybenzoic acid	<10	<10

[a] Particulate enzyme at a concentration of 0.22 mg/ml was used. Incubation time was 30 min, and the substrate concentration was 1 mM. All of the products except for those of 3-aminotyrosine, hydroxyanthranilic acid, and hydroxybenzoic acid have A = 300 and 480 nm.

[b] The substrate absorbs strongly at 300 nm, and therefore the product at 300 nm was not measured.

[c] Not teted.

substrate. All of the oxidized products of the different substrates were quinone compounds of the dopachrome type. Each of these had peaks of absorbancy at 300 and 480 nm. The quinone compound was oxidized further into a dark brown melanin. The K_m values of phenoloxidase for dopa, dopamine, norepinephrine, and epinephrine were 5.9×10^{-4}, 1.0×10^{-4}, 3.0×10^{-4}, and 6.0×10^{-5} M, respectively.

The experiment described in Table 4 confirmed that the enzyme involved in the catecholamine metabolism was truly an oxidase-type enzyme and not a peroxidase or superoxide dismutase enzyme. Peroxidase oxidized dopa only in the presence of peroxide. Catalase and superoxide dismutase had no activity toward dopa.

Cations such as Mg^{2+}, Cu^{2+}, Fe^{3+}, and Ca^{2+} were tested for their ability to improve the phenol oxidase activity. Except for Cu^{2+} and Fe^{3+}, the cations tested had no effect on the phenol oxidase activity (data not shown). Cu^{2+} or Fe^{3+} stimulated the oxidation reaction, but the autoxidation was accelerated simultaneously.

6-Methyl-5,6,7,8-tetrahydropterine, 2-amine-4-hydroxy 6,7-dimethyl-5,6,7,8-tetrahydropterine, and tetrahydrofolic acid are cofactors essential for adrenal tyrosine hydroxylase activity

TABLE 4. Oxidation of dopa by catalase, peroxidase, superoxide dismutase, and cryptococcal phenoloxidase[a]

Enzyme	Sp act (U/mg protein)	
	300 nm	480 nm
Catalase	<1	<1
Catalase + H_2O_2	<1	<1
Peroxidase	<1	<1
Peroxidase + H_2O_2	6,370	2,500
Peroxidase + catalase	110	<1
Peroxidase + catalase + H_2O_2	238	1
Superoxide dismutase	<1	<1
Superoxide dismutase + H_2O_2	<1	<1
Cryptococcal enzyme	5,430	1,090
Cryptococcal enzyme + H_2O_2	336	<1
Cryptococcal enzyme + H_2O_2 + catalase	4,660	930

[a] H_2O_2 concentration, 5 mM; dopa concentration, 1 mM; peroxide, 65 purpuro gallin units (19); catalase, 175 Sigma units; superoxide dismutase, 30 U. Assay conditions: see Materials and Methods for the colorimetric assay.

toward tyrosine (14, 22, 33). We tested these compounds with tyrosine as a substrate, but observed no activity (data not shown).

Uptake of radiolabeled catecholamines. The kinetic constants of the transport system for dopa, dopamine, norepinephrine, and epinephrine were the same. There was no incorporation of tyrosine into *C. neoformans* cells (data not shown). Azide eliminated the incorporation of each of the catecholamines tested into the cells, indicating that active transport was involved. The K_m values for uptake of the four catecholamines shown in Table 5 were in the same order of magnitude. However, there were differences in the V_{max} values. The highest V_{max} value was for dopamine (3.5 nmol/min per mg of cells), and

TABLE 5. Kinetic constants of the catecholamines transport system[a]

Substrate	K_m (μM)	V_{max} (nmol/min per ml of cells)
Dopa	450	0.15
Norepinephrine	320	0.23
Dopamine	610	2.33
Epinepherine	120	0.03

[a] Catecholamine concentration in the medium ranged from 0.015 to 4.0 mM. Radioactivity varied in the range of 1 to 5 μCi/ml. Kinetic parameters of each system were separately determined, using $[S]/V \times [S]$ and $1/V \times 1/[S]$ plots. Incubation time was 10 min.

the lowest was seen for epinephrine (0.05 nmol/min per mg of cells).

Effect of inhibitors on phenoloxidase. Table 6 shows the effects of 15 different compounds used in these experiments. Reducing agents such as mercaptoethanol, tetrahydrofolic acid, and phenylthiourea were extremely effective and inhibited over 90% of the enzyme activity. Substrate analogs like 2-methyldopa and 3-methoxytyrosine were not effective, whereas mimosine drastically suppressed the observed phenoloxidase activity. To our surprise, copper chelators such as penicillamine, diethylthiocarbamic acid, and 4',4'-diaminophenyl sulfone were not effective. This is an important observation, since phenoloxidase from other biological sources is a copper-containing enzyme and is very sensitive to these agents. Cyanide was the most powerful inhibitor. The K_i of NaCN was 50 μM, one order of magnitude lower than the K_i of the other effective inhibitors (data not shown). Since sodium cyanide is an effective iron-chelating agent, eight more iron-chelating agents such as 8-hydroxyquinoline and hydroxamic acid derivatives were tested (Table 7). Six of eight agents inhibited nearly 25 to 60% of the enzyme activity.

TABLE 6. Effect of inhibitors on phenoloxidase of *C. neoformans*[a]

Inhibitor[b]	% Activity[c]
D-Glucose	88
Thio-D-glucose	99
O-Fluoro-DL-phenylalanine	101
1-Phenyl-2-thiourea	2[d]
6-Mercaptopurine	85
2-Thiouracil	41
Diethylthiocarbamic acid	92
Pencillamine	87
4',4'-Diaminodiphenylsulfone	90
Mimosine [3-hydroxy-4-oxo-1(4H)-pyridinealanine]	8[d]
Methyldopa	86
3-Methoxytyrosine	85
2-Mercaptoethanol	3[d]
Tetrahydrofolic acid	<1[d]
Sodium cyanide	<0[d]

[a] L-Norepinephrine was used as a substrate. Similar results were obtained when dopa or norepinephrine was used as a substrate. The test with soluble enzyme gave results similar to those obtained with the membrane-bound enzyme.

[b] All of the compounds were purchased from Sigma, and the concentrations used were 1 and 3.5 mM. The substrate concentration was 1 mM. Soluble enzyme was used for this experiment in a concentration of 30 μg/ml. The enzyme was preincubated with the inhibitors for 30 min at 25°C.

[c] The percentages of activity obtained by colorimetric assay were almost the same as those obtained by the radiometric method. One hundred percent values were 636 and 595 nmol/h per mg of protein for norepinephrine and dopa, respectively.

[d] Concentration of chelating agents was 1 mM.

DISCUSSION

The phenoloxidase in *C. neoformans* is a membrane-bound enzyme. It is found only in the particulate fraction of a broken cell preparation after centrifugation at a high speed (105,000 × *g*). Shaw and Kapika (32) found the enzyme activity in the supernatant; however, no data about the particulate fraction were reported. In their experiment, Shaw and Kapika used a low-speed centrifugation (20,000 × *g*), and as a result, some membrane debris may have been in the supernatant. When we used low-speed centrifugation, some enzyme activity was found in supernatant but in much lesser amounts than were found in the pellet.

The study using protoplast confirmed that the phenoloxidase is attached to the membrane. Since the protoplasts are liberated through a localized opening rather than by complete digestion of the cell wall (25), they are free from cell wall debris. This finding eliminates the possibility that the enzyme is bound to the cell walls. The procedure used in our study gave 60 to 70% of protoplast formation within 3 h compared with 24 h as reported by Peterson et al. (25).

For enzymatic assay, both the radiometric method and the colorimetric method were essential. The former method was more sensitive, and the assay was based on the insoluble final product of melanogenesis. The colorimetric method was less sensitive, but was based on the accumulation of two intermediates, dopachrome and 5,6-dihydroxyindole. These two compounds were the only stable intermediates found during melanogenesis in *C. neoformans*.

The enzyme activity is determined more directly by measuring the accumulation of the intermediates rather than of the melanin. This may explain the discrepancies between colorimetric and radiometric assay results for some cases in Table 2. The intermediates are synthesized at early steps of the reaction pathway close to the enzymatic oxidation of diphenols. The two intermediates were isolated and characterized by using high-performance liquid chromatography (data will be published elsewhere).

The phenoloxidase of *C. neoformans* is different from those of plant and mammalian sources. The mammalian tyrosinase from brain (18), adrenal glands (13, 22), or melanoma (11, 12) has externally restricted substrate specificity, primarily for tyrosine or dopa.

Like the phenoloxidase from plant and mushroom sources (7), the enzyme of *C. neoformans* can oxidize a variety of phenolic compounds.

TABLE 7. Effect of chelating agents on phenoloxidase activity

Ferric chelator	Phenoloxidase (% activity)	
	Dopa	Norepinephrine
8-Hydroxyquinoline	56	53
DL-2-Alanine hydroxamate	54	59[a]
L-Arginine hydroxamate	37	61
L-Glutamic acid monohydroxamate	69	63[a]
DL-Methionine hydroxamate nitrilotriacetic acid	62	72
Nitrilotriacetic acid	76	61[a]
DL-Phenylalanine hydroxamate	88[b]	81[b]
8-Hydroxyquinoline-β-D-glucopyranoside	81[b]	82[b]

[a] The results are for 3.5 mM chelating agents; 1 mM gave around 80% of the activity. Higher concentration did not increase the inhibition. The substrate concentration was 1 mM.

[b] The results are for 3.5 mM; higher concentration did not increase the inhibition.

Whether only one or more enzymes are involved for the oxidation of many diphenols cannot be proven until a study is made with purified enzyme. However, a strong evidence for the involvement of one enzyme can be found in the spontaneous single-gene mutants deficient only in phenoloxidase (J. C. Rhodes et al., unpublished data) and their revertants. The mutants were unable to oxidize any substrate, whereas the revertants gained the ability to oxidize many diphenols. This observation is consistent with the theory that the substrate specificity of tyrosinase becomes more restricted as the organism rises in the evolutionary scale (20). Unlike the plant and mushroom enzyme, however, cryptococcal phenoloxidase has an absolute requirement for two hydroxyl groups in the 3,4 position on the phenyl compound. Tyrosine, therefore, cannot serve as a substrate for the enzyme (Table 3).

Another difference between these preparations is that the plant and mushroom phenoloxidases are soluble, whereas cryptococcal phenoloxidase is a membrane-bound enzyme. The enzyme is attached to the inner side of membrane, probably to the plasma membrane. However, the possibility exists that the enzyme may be attached to membranes of cytoplasmic organelles.

Cryptococcal phenoloxidase shows many similarities to the enzyme of *Mycobacterium leprae*. The enzyme of *M. leprae* is also membrane bound, and the two enzymes utilize the same range of substrates (30, 31). It is significant to note that both organisms are neurotropic, although their site of growth is different in the mammalian host. The cells of *C. neoformans* multiply best in the central nervous system (10), and those of *M. leprae* multiply best in the peripheral nervous system. The metabolism of dopa and its derivatives is known to be important in both systems. Cryptococcal phenoloxidase, however, is distinct from the bacterial enzyme at least in two respects. Whereas the *M. leprae* enzyme oxidizes dopa only to 5,6-dihydroxyindoquinone A = 540 nm (29), the enzyme of *C. neoformans* follows the conventional pathway, yielding dopachrome A = 300 and 480 nm (18, 20). The enzyme from *C. neoformans* is completely inhibited by mimosine and cyanide, which do not affect the bacterial enzyme (28, 30). Furthermore, the substrate analog mimosine was proven to be a substrate rather than an inhibitor for *M. leprae* enzyme (28).

Three copper-binding compounds which are known to act as powerful inhibitors for the phenoloxidases of other biological origin were ineffective for cryptococcal phenoloxidase. This indicates that phenoloxidase of *C. neoformans* may not be a copper-containing enzyme. On the other hand, six of eight iron-chelating agents inhibited the cryptococcal enzyme (Table 7), suggesting that the enzyme is an iron-containing enzyme like the adrenal tyrosinase (13). A final resolution of this problem, however, cannot be made until the enzyme is purified and the atomic absorption can be measured.

Although ammonium sulfate is known to be the least desirable nitrogen source for melanogenesis in *C. neoformans* (5), ammonia repression of the enzyme was not observed (Table 2). This observation is in contrast to the conclusion of Nurudeen and Ahearn (23). They used whole cells to measure the enzyme activity, and it is not possible to interpret the results at the enzyme level.

The low K_m of the uptake mechanism for dopamine (610 μM) and norepinephrine (320 μM) demonstrates that *C. neoformans* cells possess high affinity toward the uptake of these compounds. Dopamine and norepinephrine are the major catecholamines found in the brains of many animals (1). The high maximal velocity values (2.33 and 0.23 nmol/min per mg of cells) indicate that the accumulation of these compounds takes place at a high speed. These findings, in addition to the K_m values of the phenoloxidase toward dopamine (10^{-4} M) and norepinephrine (3×10^{-4} M), suggest that *C. neoformans* can efficiently utilize catecholamines in the brain.

A medium with a high glucose concentration has been shown to be unsuitable for melanin

synthesis by *C. neoformans* (24). Our study shows that the glucose represses the synthesis of phenoloxidase (Table 2). The brain is one of the most metabolically active organs in the body, and glucose is used at the rate of 0.31 μmol/min per g of brain (31). The glucose concentration diffuses down from a plasma level of 6 to 4 mM in the cerebral spinal fluid. It enters the cerebral cells, where the glucose level ranges from 0.3 to 3 mM (31). This is the lowest steady-state concentration of glucose found in any organ in the mammalian body. Since the low glucose concentration supports the derepression of the synthesis of phenoloxidase, *C. neoformans* may utilize catecholamines more efficiently in the brain than in other organs. Consequently, melanin formation by *C. neoformans* may occur in the brain, and the brain becomes the optimal niche for its survival and growth. If this is the case, the fungus may lose virulence when it becomes deficient in phenoloxidase. In support of this view, mutants lacking phenoloxidase have been isolated by Kwon-Chung et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, F16, p. 322) and Rhodes et al. (unpublished data). These mutants were found to be avirulent for mice. The revertants which regained the enzyme simultaneously showed the recovery of a virulence level equal to that of the wild type. Furthermore, the analysis of progeny obtained by crossing a mutant with a wild type showed cosegregation of virulence and melanin-forming ability. These results clearly demonstrate that phenoloxidase activity is associated with virulence in *C. neoformans*.

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