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Location of Aryl Sulfatase in Conidia and Young Mycelia of *Neurospora crassa*

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Aryl sulfatase (arylsulfate sulfohydrolase, EC 3.1.6.1) was found to have multiple locations in *Neurospora* conidia. Some enzyme activity remained in the supernatant when a spore suspension was centrifuged or filtered. Part of the cell-bound activity could be detected by adding the assay ingredients to a suspension of intact spores (patent enzyme), and additional activity was only detectable when the spores were first treated to destroy their permeability barriers (cryptic enzyme). Such treatments include: disruption with an X-press, brief rinsing with chloroform or acetone, incubation at 60 C for 5 min, and incubation with phenethyl alcohol, nystatin, or ascosin. Part of the patent aryl sulfatase was inactivated by briefly acid treating the intact spores (no loss of conidial viability). This enzyme was considered to have a cell surface location. Some enzyme was acid-resistant in intact spores, but all of the enzyme was acid-sensitive in spores whose permeability barriers had been disrupted. The *p*H dependence, kinetic properties, and *p*-nitrophenyl sulfate uptake were investigated in acid-treated conidia. No aryl sulfatase was detected in ascospores. Young mycelia contained more aryl sulfatase than did conidia, but little, if any, was secreted into the growth medium. Cryptic activity was demonstrated in young mycelia by brief chloroform treatment or by rinsing the cells with 0.1 M acetate buffer. Enzyme activity in young mycelia was completely labile to acid treatment, as was cell viability.

Aryl sulfatase (arylsulfate sulfohydrolase, EC 3.1.6.1) is usually located in lysosomal structures in higher plants and animals, although the mammalian type C sulfatase is predominantly microsomal (37). Little is known about lysosomes in fungi. The appearance of acid hydrolase activities in lysosome-like organelles has been documented in *Saccharomyces cerevisiae* (10, 26), *Candida albicans* (33), and in a number of phytopathogenic fungi (36, 42). Only in the plant pathogens has the association of aryl sulfatase activity with such structures been demonstrated.

Zalokar (43), using cytochemical techniques, observed acid phosphatase and β-galactosidase activity in the mitochondrial region of centrifuged hyphal segments of *Neurospora crassa*—a result to be expected if those enzymes had a lysosomal location. He did not report an attempt to locate aryl sulfatase.

Some enzymes which are lysosomal in higher plants and animals are found outside of the plasma membrane in bacteria (11). Likewise, in fungi, some typically lysosomal activities, including acid phosphatase (12, 35, 40), ribonuclease (39), β-galactosidase (15), and acid protease (20, 25), have been shown to be located either extracellularly or in the periplasmic space.

If at least part of an enzyme activity is at or near the cell surface, useful information may be obtained by determining enzyme activity in whole-cell suspensions before and after subjecting the cells to treatments which will inactivate surface enzymes or release them into the suspension fluid. By using this approach, it has been possible to show that aryl sulfatase in *Neurospora* has a complicated locational distribution, particularly in conidia. Some enzyme activity is present as exoenzyme, and some is clearly located at the cell surface, but a significant portion of the activity is cryptic and is contained within a permeability barrier.

MATERIALS AND METHODS

Strains. All of the mutant strains of *Neurospora* have been described elsewhere (29). They include: *eth-1*^{r}, an ethionine-resistant mutant which cannot be repressed for aryl sulfatase synthesis by growth on methionine; *cys-11* (allele 85518), a mutant lacking adenosine triphosphate (ATP) sulfurylase (24) and, consequently, not repressed for aryl sulfatase synthesis when inorganic sulfate is included in the

growth medium; *fl*, a morphological mutant which does not produce conidia; and *ars* (*101*), a prototrophic strain which cannot make aryl sulfatase under any growth conditions, apparently due to a structural gene mutation. Except where otherwise indicated, the double mutant, *eth-1*^{r}, *cys-11-A*, was used.

Media and growth conditions. Conidia were produced on petri dishes containing 1.5% sucrose and 1.5% agar (Difco) or 1.0% Oxoid Ionagar (Colab Laboratories, Inc.) supplemented with Fries salts (1) and 5 mM L-methionine. They were harvested after 5 to 7 days at 24 C.

After the conidia were harvested (27), they were suspended in 0.1 M sodium acetate-acetic acid buffer (acetate buffer), *p*H 5.0. In experiments in which the conidial suspension is referred to as "washed and acid-treated," the following procedure was followed (all steps at 0 to 4 C). A conidial suspension in acetate buffer was filtered twice through glass wool. Conidia passed through, but bits of mycelia remained in the glass wool filter. Conidia were then collected from the filtered suspension by centrifuging 10 min at 700 × *g*. The pellet was resuspended and washed twice in acetate buffer by centrifuging and resuspending. HCl was added to give a *p*H of 1.4 ± 0.1, and the suspension was stirred for 15 min. A predetermined amount of NaOH was then added to bring the *p*H between 4.6 and 5.2. The suspension was again centrifuged, and the conidia were resuspended in fresh acetate buffer.

Young mycelia were prepared by inoculating conidia at a concentration of approximately 10^6/ml [estimated from the turbidity of the conidial suspension (16)] into 1.5% sucrose supplemented with Fries salts and 5 mM L-methionine. Cultures (in siliconized flasks) were shaken rapidly for 12 hr at 25 C. Young mycelia from such cultures were sampled by using a large-bore 0.5-ml pippette. The reproducibility of the sampling was only about ±10% due to a tendency for cells of this age to clump.

To determine the number of colony-forming units in suspensions of conidia or young mycelia, appropriately diluted samples were plated onto medium P (28). Colonies were counted after 2 to 3 days.

Ascospores were obtained as follows. The two opposite mating types of an appropriate strain were inoculated simultaneously onto plates of crossing medium (41) modified by replacing magnesium sulfate with magnesium chloride. Cysteic acid (1 mM) was added to provide a sulfur source which would not repress aryl sulfatase synthesis. To provide a solid substrate, 1.2% agarose (L'Industrie Biologique Francaise, S.A.) was added, since it has been found (29) that this is the only commercial source of agar in which there are not enough sulfur-containing compounds to repress aryl sulfatase synthesis in wild-type *Neurospora*. After 7 weeks, a black layer of ascospores had accumulated on the underside of the petri dish lid. The ascospores were scraped off with a spatula and suspended in 0.1 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer, *p*H 8.1.

Disruption of conidia and ascospores. Conidia and ascospores were disrupted with a Biotech X-press (6). A frozen suspension of spores was placed under high pressure and passed through a small orifice. One pass was sufficient to break most of the conidia in a suspension (determined by observing the suspension with a light microscope). Ascospores were more resistant to the disruption procedure. Some ascospores were still intact after three passes through the orifice, but very few remained intact after seven passes.

Uptake of p-nitrophenyl sulfate into conidia. Conidia were suspended at a concentration of 10^8 cells/ml in 0.28 M Tris-hydrochloride, *p*H 8.1. To this was added 5 mM ^{35}S-labeled *p*-nitrophenyl sulfate (41,000 counts/μmole). The suspension was incubated at 37 C; at various times, 2.0-ml samples were collected on discs of Whatman no. 1 filter paper by vacuum filtration and then washed with 10 ml of cold 0.25 M Tris-hydrochloride, *p*H 8.1. The samples and the filter paper were placed in glass counting vials and each sample was oxidized to a colorless residue, by using Jeffay's modification of Pirie's wet-ashing method (14). The residues were dissolved in Bray's solution (2) and counted with a Packard Tri-Carb liquid scintillation counter.

Assays for aryl sulfatase. Aryl sulfatase was assayed by the technique of Metzenberg and Parson (31). Cycloheximide (final concentration, 0.1 mM) was routinely included in the assay mixture. One enzyme unit produces 1 pmole of *p*-nitrophenol/min. Activity is usually expressed as units per 10^6 conidia, with the number of conidia estimated from the turbidity of the conidial suspension by the method of Kappy and Metzenberg (16).

For convenience in expressing the results in this paper, the enzyme unit defined here is 1/1,000 times the unit used in previous publications from this laboratory (31).

In many cases, aryl sulfatase assays were performed on samples of conidia or young mycelia which were collected by vacuum filtration on a disc of Whatman no. 1 filter paper. When this was the case, samples were washed (by vacuum filtration) with 5 ml of ice-cold acetate buffer, and the sample and the filter paper was placed in a round-bottomed test tube (18 by 150 mm) containing a glass bead. Then the assay incubation mixture was added (total volume, 1.0 ml). The sample of conidia or young mycelia was suspended in this assay incubation mixture and shaken continuously at 37 C during the assay for aryl sulfatase activity. It was shown that the filter paper had no effect on the enzyme assay.

Chemicals. *p*-Nitrophenyl sulfate and Tris was obtained from Sigma Chemical Co. ^{35}S-labeled *p*-nitrophenyl sulfate was prepared by the method of Metzenberg and Ahlgren (30). Cycloheximide was obtained from Calbiochem. Nystatin was a gift from Richard Donovick of the Squibb Institute for Medical Research, New Brunswick, N.J. Ascosin was donated by William F. Phillips of Commercial Solvents Corp., Terre Haute, Ind. Stock solutions of these polyene antibiotics were prepared in redistilled dimethylformamide.

RESULTS

Partial removal of aryl sulfatase from whole conidia by washing. Part of the aryl sulfatase activity in a suspension of conidia is readily separated from the spores by centrifuging (Table

1). Successive washes or a long continuous wash did not remove additional enzyme activity. The fraction of enzyme which was recovered in the supernatant solution varied widely between different lots of conidia in the range of 30 to 75% of the activity in the original conidial suspension. A soluble fraction of enzyme activity in this range was also found if conidia were suspended and washed in 0.1 M Tris-hydrochloride buffer, *p*H 8.1.

Enzyme activity (either soluble or cell-bound) did not appear or disappear during the assay. This was confirmed by showing that, with both fractions, the formation of nitrophenol was linear for at least 120 min. By comparing the concentration of colony-forming units in an unwashed conidial suspension (in water) with a suspension of conidia washed in sterile acetate buffer, it was shown that washing with acetate buffer did not cause loss of viability.

Brief treatments with a variety of reagents (including 1 M KCl, 1 M sucrose, 5 mM disodium ethylenediaminetetraacetic acid, 0.1 M Na_2HPO_4, 0.1 M Na_2SO_4, and 5 mM *p*-nitrophenyl sulfate) did not elute any cell-bound activity.

TABLE 1. *Partial removal of aryl sulfatase from conidia by washing with acetate buffer*[a]

Sample[b]	Aryl sulfatase activity
	units/ml
Original conidial suspension	8.4×10^3
Supernatant from original suspension	3.9×10^3
Supernatant from first wash	0.5×10^3
Supernatant from second wash	0.2×10^3
Resuspended pellet after second wash	5.1×10^3
Conidia maintained 3.5 hr in dilute suspension, collected, and resuspended[c]	4.8×10^3

[a] Conidia were harvested and filtered through glass wool. They were not further washed or treated. A sample of the original suspension was assayed for aryl sulfatase activity. A 1.0-ml portion of the suspension was centrifuged (10 min at 700 × *g*), and a sample of the supernatant solution from this centrifugation was assayed. The pellet from this centrifugation was resuspended in 1.0 ml of acetate buffer. The suspension was centrifuged as before, and a sample from the supernatant solution (designated first wash) was assayed for aryl sulfatase. The pellet from this centrifugation was resuspended again, and the procedure was repeated to give a second wash and a final pellet. The final pellet was suspended in 1.0 ml of acetate buffer, and a sample of the suspension was assayed.

[b] Each sample corresponds to a volume of the original suspension which contained 5.4×10^8 conidia.

[c] A 1.0-ml portion from the original conidial suspension was diluted into 200 ml of acetate buffer and stirred continuously to keep the spores in suspension. At the end of 3.5 hr, the spores were collected on a paper filter and suspended in a total volume of 1.0 ml (acetate buffer). Aryl sulfatase activity was determined on a portion of the resuspended conidia.

Inactivation of conidial aryl sulfatase by acid treatment. Acid treatment quickly inactivates a fraction of the aryl sulfatase that is detectable in whole washed conidia (Fig. 1). In other similar experiments, all of the acid-sensitive portion of the enzyme activity was inactivated if exposed for only 1 min at the low *p*H. The remaining, acid-resistant (but patent) enzyme activity was not inactivated after extended acid treatment.

The acid inactivation of aryl sulfatase was not accompanied by loss of conidial viability—92% of the colony-forming units survive after 15 min at *p*H 1.5. The fraction of enzyme resistant to acid was determined in a number of experiments

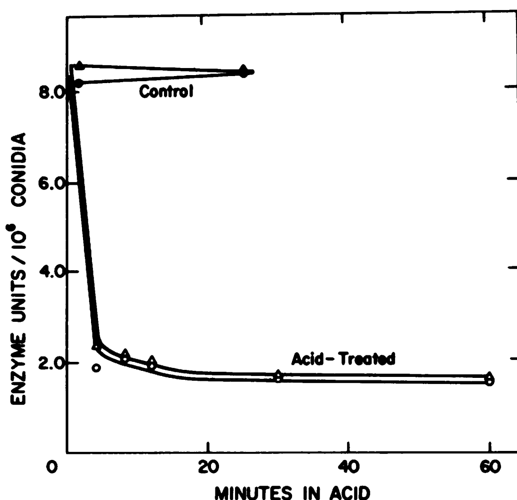

FIG. 1. *Inactivation of conidial aryl sulfatase by acid treatment. Conidia were grown, suspended in acetate buffer, and filtered twice through glass wool. The filtered suspension was washed twice with acetate buffer by centrifuging and resuspending. A portion of the washed suspension was acid-treated by adding HCl to give a pH of 1.3. After various periods of time, samples were taken from the acid-treatment mixture, and the pH of each sample was readjusted to 5.1 by adding a predetermined amount of NaOH. These samples were then assayed for aryl sulfatase activity. Controls were run by diluting a portion of the washed conidial suspension with an appropriate concentration of NaCl. Symbols:* ○, ●, *Cycloheximide (0.05 mM) present during the enzyme assay;* △, ▲, *no cycloheximide during the assay.*

and fell in a range of 20 to 37% of the activity in washed conidia before acid treatment. The results were substantially the same in conidia which were prewashed with 0.1 M Tris-hydrochloride (*p*H 8.1), 1 M KCl, or 5 mM disodium ethylenediaminetetraacetic acid. In one experiment, acid treatment was carried out by adding 2 M glycerol at the same time that the *p*H was decreased. Precisely the same fraction of enzyme remained after acid treatment as in a control, acid-treated without glycerol. This result indicates that conditions which plasmolyze the cell do not affect the fraction of aryl sulfatase that is acid-resistant.

It should be pointed out that there is no acid-resistant component of enzyme activity in conidia which have been mechanically disrupted or the permeability barriers of which have been destroyed by any of a variety of techniques (*see* below).

The following simple explanations for the existence of a fraction of patent aryl sulfatase, resistant to acid-inactivation, were considered. (i) Conidial proteins and polysaccharides might buffer out the added hydrogen ions. (ii) Protein synthesis might occur during the aryl sulfatase assay (which usually lasted 2 hr), and "acid-resistant" enzyme could appear during this time. (iii) Aryl sulfatase might exist as an acid-resistant proenzyme (which could be converted into an active form during the enzyme assay), or it could be in an internal compartment, inaccessible to acid (from which it might be transported during the enzyme assay). (iv) Acid treatment might generate an enzyme inhibitor, rather than inactivating enzyme molecules.

Successive acid treatments on the same sample of conidia do not inactivate additional enzyme. Furthermore, variation of the concentration of conidia does not change the fraction of enzyme which is resistant to acid inactivation. These results eliminate the first possibility.

The fraction of aryl sulfatase which is acid-resistant is not influenced by the presence of cycloheximide during the enzyme assay (Fig. 1). This result argues against the second possibility.

If either hypothesis ii or iii were true, the assay for aryl sulfatase in acid-treated conidia should not be linear with time. Figure 2 shows the time course of the aryl sulfatase-catalyzed reaction in acid-treated conidia. It can be seen that, if a conversion process occurs which causes aryl sulfatase activity to appear during the enzyme assay, it must be complete within the first 40 min of the assay. The assay is linear after that time. If such a process were occurring, preincubation of conidia at 37 C should make the activity acid-sensitive. In fact, when acid-treated conidia were incubated for 3 hr in 0.1 M Tris-hydrochloride at 37 C (conditions comparable to the aryl sulfatase assay), only one-third of the aryl sulfatase activity was lost on subsequent acid treatment. These results make it very unlikely that the apparently acid-resistant enzyme activity is actually due to acid-sensitive enzyme which has appeared during this assay.

The fourth alternative listed above was directly tested by looking for an inhibitor in cell-free extract. Disrupted, acid-treated conidia were tested for their ability to inhibit the reaction catalyzed by purified aryl sulfatase. The results are shown in Table 2. No inhibitor of the kind postulated was detected. It is, of course, conceivable that such an inhibitor could occur at high concentration in close proximity to the enzyme molecules in conidia with intact structure but could be too dilute in the cell-free extract to be effective. No evidence is available concerning this possibility.

The results outlined above appear to eliminate a number of trivial explanations for the existence of an acid-resistant, patent aryl sulfatase activity. This unique enzyme fraction will be considered further below.

Demonstration of a cryptic compartment of aryl sulfatase activity in conidia. X-press disruption of the conidial structure resulted in a marked increase in the aryl sulfatase activity detectable in a conidial suspension (Table 3). In addition, all of the enzyme activity became acid-sensitive as a result of the disruption.

Cryptic enzyme activity can also be demonstrated by treating conidia briefly with chloroform or acetone (Table 4). The technique illustrated in this table was previously described by Eberhart and Tatum (5). The aryl sulfatase activity became completely acid-sensitive as a result of chloroform treatment, although the acid inactivation was not instantaneous as it was for the acid-sensitive fraction of enzyme in conidia with an intact permeability barrier (Table 4). The time dependence of the aryl sulfatase assay of chloroform-treated conidia is presented in Fig. 2. The rate of appearance of product decreased at later times in the enzyme assay, perhaps due to endogenous protease activity. For this reason, a fixed assay time period was adopted for all samples in any given experiment.

Brief heat treatment will also reveal the cryptic compartment of aryl sulfatase (Fig. 3). A similar phenomenon was observed for catalase activity in distiller's yeast (7). Aryl sulfatase is quite stable at elevated temperatures; however, after the first 5 min of heat treatment, aryl sulfatase activity decreased at a rate parallel to the loss of activity in conidia whose permeability barrier was previously destroyed with chloroform. This slow de-

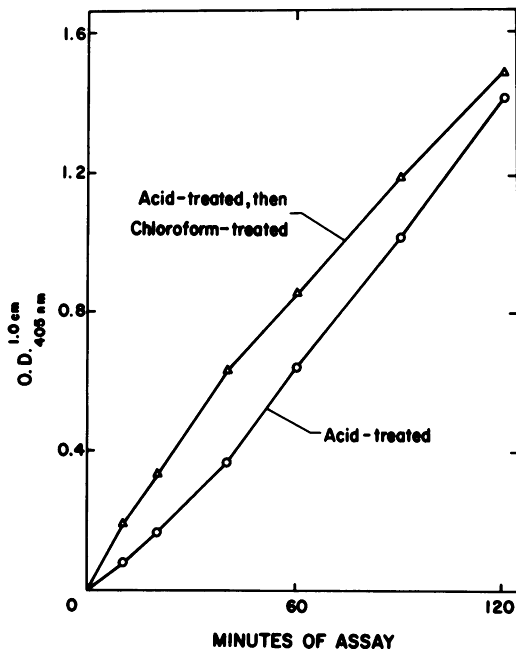

FIG. 2. *Time dependence of the aryl sulfatase-catalyzed reaction in acid-treated conidia with and without chloroform treatment. All assays were run in the presence of 0.1 mM cycloheximide. Optical density readings at 405 nm (1-cm light path) were corrected for background. Both time curves were run on the same conidial suspension; 0.35-ml samples (1.7 × 10⁸ conidia) were used for assays of acid-treated conidia; 0.15-ml samples (7.1 × 10⁷ conidia) were used for assays of conidia further treated with chloroform.*

crease in activity was probably due to heat inactivation of the enzyme. Within the first 5 min of heat treatment, there was a marked increase in the aryl sulfatase activity detectable in a suspension of previously intact conidia. This corresponded to exposure of the cryptic compartment of enzyme. Within this same time interval, most of the enzyme activity became acid-sensitive.

The cryptic aryl sulfatase was exposed by preincubation with polyene antibiotics at 0 C (Table 5), and the enzyme also became susceptible to acid inactivation (Fig. 4). Acid inactivation was not as rapid with conidia which were treated with nystatin as with chloroform-treated conidia. Ascosin also exposed cryptic enzyme activity but allowed acid inactivation of aryl sulfatase to proceed even more slowly.

Incubation of conidia with phenethyl alcohol (30 min at 37 C) was also effective in exposing the cryptic enzyme compartment and in making all of the aryl sulfatase acid-sensitive. Details of these experiments have been reported elsewhere (Scott and Metzenberg, Neurospora Newsletter, vol. 15, p. 8, 1969). As with the polyene antibiotics, accessibility of substrate to enzyme in a standard assay was achieved by milder conditions than were required to make the enzyme activity susceptible to rapid acid inactivation. Intermediate concentrations of phenethyl alcohol (0.25 and 0.5%) revealed the cryptic compartment almost completely, but left a large portion of the enzyme protected from brief acid treatment (*p*H 1.3, 0 C, 2 min). After incubation with 0.75 or 1.0% phenethyl alcohol, however, all of the cryptic compartment was accessible to enzyme substrate, and all of the enzyme activity had become susceptible to acid inactivation.

Several general properties of the cryptic enzyme activity have been demonstrated.

(i) The size of the cryptic compartment of enzyme is independent of the treatment used to reveal it (Table 6). The size does vary between different lots of conidia harvested on different days. The cryptic compartment of enzyme activity

TABLE 2. *Effect of mixing disrupted, acid-treated conidia with purified aryl sulfatase on the aryl sulfatase determination*

Sample[a]	Aryl sulfatase activity
	units
Purified aryl sulfatase, 0.10 ml....	4.7×10^3
Disrupted, acid-treated conidia, 0.15 ml[b]........	0.9×10^3 [c]
Mixture of the above........	5.6×10^3 [c]

[a] The final assay volume was made up to 1.0 ml by adding 0.1 M Tris-hydrochloride (*p*H 8.1). The same buffer was used for diluting the purified enzyme and for suspending and washing the conidia.

[b] Washed and acid-treated conidia were disrupted by passing the suspension through an X-press once.

[c] Assays contained 8.1×10^7 disrupted, acid-treated conidia.

TABLE 3. *Aryl sulfatase activity and aryl sulfatase acid-sensitivity after passing washed-and acid-treated conidia through the orifice in an X-press*

No. of passes through the X-press	Aryl sulfatase activity[a]	
	Direct assay	Assay after acid-treatment
0	4.3	4.2
1	8.1	0.4
2	7.7	<0.2
3	7.2	<0.2

[a] Expressed as enzyme units per 10^6 conidia.

is more striking if the conidia were previously acid-treated; however, the amount of enzyme activity that is exposed by these techniques was the same regardless of whether the conidia were previously acid-treated or not.

(ii) The K_m of aryl sulfatase for *p*-nitrophenylsulfate in permeabilized conidia is no different from the K_m of purified aryl sulfatase acting on the same substrate. This observation was confirmed with chloroform-treated conidia and with nystatin-treated conidia. In both cases, as with the purified enzyme, the K_m was 2.0 mM.

(iii) In all cases in which the cryptic compartment of aryl sulfatase was exposed to substrate by some treatment, the enzyme also became acid-sensitive. Acid inactivation of such treated conidia was not instantaneous, as it was with purified aryl sulfatase or with the acid-sensitive compartment in untreated conidia. Not only did the cryptic enzyme activity become acid-sensitive in the course of these experiments, but also the enzyme that was previously patent and acid-resistant.

(iv) Although these treatments make the cryptic aryl sulfatase activity accessible to enzyme

TABLE 4. *Aryl sulfatase activity and acid sensitivity after treatment of conidia with chloroform or acetone*

Treatment[a]	Aryl sulfatase activity[b]
No treatment	5.2
Washed once with 5 ml of cold chloroform	14.1
Washed twice with 5 ml of cold chloroform	14.1
Washed four times with 5 ml of cold chloroform	13.8
Washed once with 5 ml of cold acetone	14.1
Washed once with 5 ml of chloroform at 37 C	13.7
Washed once with 5 ml of cold chloroform and then with 5 ml of cold acetate buffer	14.0
Acid treatment after chloroform treatment[c]	
1 min	1.8
5 min	0.6
15 min	<0.4

[a] Samples of a washed and acid-treated conidial suspension were collected on filter paper discs and further washed with 5 ml cold acetate buffer. The samples were then treated with chloroform or acetone by adding the solvent as indicated while constantly applying vacuum.

[b] Expressed as enzyme units per 10^6 conidia.

[c] An aliquot from the conidial suspension was collected on a filter paper disc and treated with 10 ml of cold chloroform. The treated conidia were then resuspended in acetate buffer to the original volume of the aliquot. This suspension was acid-treated for the time periods indicated. Samples were then collected on filter paper discs and assayed for aryl sulfatase activity. They were not further washed with buffer.

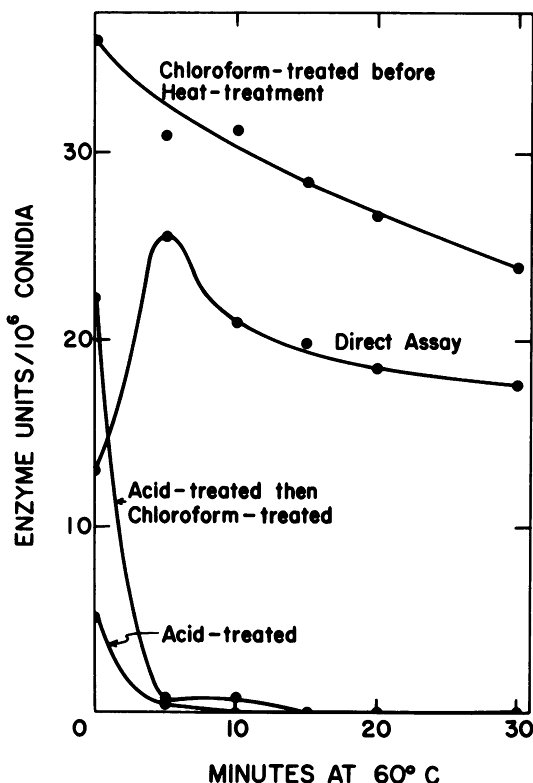

FIG. 3. *Effect of heat treatment on aryl sulfatase activity and acid sensitivity in conidia. A conidial suspension was filtered through glass wool and washed (but not acid-treated), and part of the washed suspension was incubated at 60 C. At intervals, portions were pipetted from the incubation tube into assay tubes, and aryl sulfatase was measured by adding the assay components and incubating at 37 C (indicated "direct assay"). An additional portion was taken at each time point, subjected to acid treatment (pH 1.4, 0 C, 15 min), and then readjusted to pH 4.6 with NaOH. Samples from these acid-treated samples were collected and washed on filter paper discs; one sample from each time point was further washed with cold chloroform to find any aryl sulfatase remaining cryptic. Enzyme assays were run on these samples as described in the text. Part of the original conidial suspension was treated with chloroform on a filter paper disc, resuspended in acetate buffer, incubated at 60 C, and sampled at various time intervals for direct assay as described above. The results of these assays are designated "chloroform-treated before heat treatment."*

TABLE 5. *Effect of polyene antibiotics on aryl sulfatase in acid-treated conidia*

Treatment[a]	Aryl sulfatase activity[b]	
	After incubation	After incubation and $HCCl_3$ treatment
Control[c]	1.5	6.0
Nystatin (100 μg/ml, 30 min)	6.3	5.8
Ascosin		
100 μg/ml, 30 min	6.1	5.6
100 μg/ml, 55 min	5.9	5.9

[a] Portions of a conidial suspension (washed and acid-treated) were mixed with equal volumes of 200 μg of nystatin or ascosin per ml in acetate buffer. After the indicated time at 0 C, samples were collected, washed on filter paper discs, and assayed for aryl sulfatase activity. Additional samples from each incubation mixture were treated with chloroform to look for any enzyme activity that remained cryptic after treatment with polyene antibiotics.

[b] Expressed as enzyme units per 10^6 conidia.

[c] Control contained about 3.6% (v/v) redistilled dimethylformamide which corresponded to the dilution of this solvent in the nystatin incubation mixture.

substrate, only in the case of the conidial suspension that was disrupted by the X-press was the enzyme activity made soluble. With X-press-disrupted conidia, only a small portion of the enzyme activity will sediment in 20 min at 30,000 × *g*, and only about 10 to 15% of the activity is retained on a filter paper disc when a sample is collected and washed by vacuum filtration. With all of the other treatments described above, the enzyme activity was quantitatively retained on a filter paper disc and rapidly sedimented at low centrifugal forces.

Is the acid-resistant, patent enzyme activity really cryptic enzyme detected under suboptimal assay conditions? The demonstration that considerable aryl sulfatase is present in a cryptic state in conidia suggests additional possible explanations for the acid-resistant, patent enzyme activity described above. A compartment of enzyme which is entirely intracellular may show patent activity if the enzyme substrate can enter the spores to a limited extent, presenting the intracellular enzyme with a subsaturating substrate concentration. Any treatment which would destroy the permeability barriers of the conidia to *p*-nitrophenyl sulfate would increase the intracellular concentration of this compound and could account for the observed crypticity of aryl sulfatase.

On the basis of this hypothesis, it would be predicted that the K_m for the patent enzyme should be high. To account for the observed increase in the enzyme-catalyzed reaction when the conidial permeability barriers are disrupted, the apparent K_m for acid-resistant, patent aryl sulfatase should fall in the range of 9 to 30 mM. Four determinations of this parameter, however, gave a range of 0.9 to 2.7 mM—a result which does not support this simple explanation.

Another possible explanation is that the cryptic enzyme is readily accessible to substrate in intact cells, but operates at a suboptimal *p*H. When the permeability barriers are disrupted, the buffer added with the standard enzyme assay mixture would have access to the cryptic enzyme molecules, and the activity detected in the assay would increase.

To evaluate this possibility, the *p*H dependence of the patent, acid-resistant enzyme was deter-

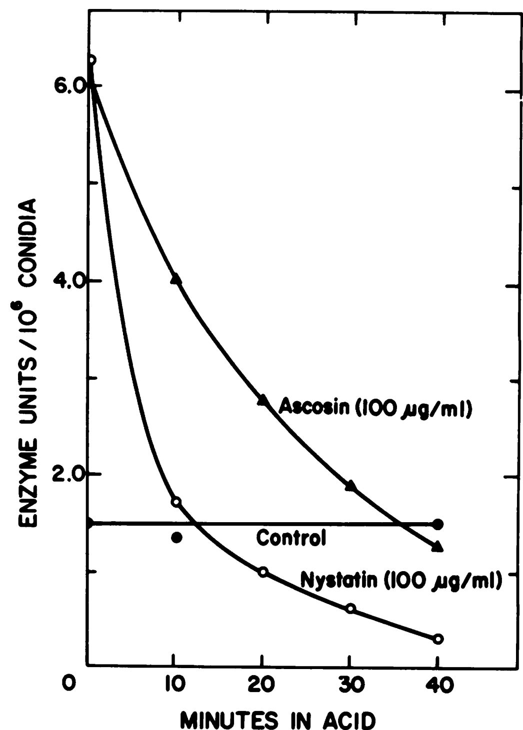

FIG. 4. *Effect of acid treatment on aryl sulfatase activity in polyene antibiotic-treated conidia. The conidial suspensions are those described in Table 5. The antibiotic incubation was continued for 50 min. At that time, a portion from each incubation mixture was taken to pH 1.4 with HCl, and samples were collected at 10-min intervals. These samples were first adjusted to pH 4.9 with NaOH, collected on filter paper discs, washed, and assayed for aryl sulfatase activity.*

mined by using Tris-dimethylglutarate buffers over the *p*H range of 4 to 9. If this hypothesis were correct, the patent aryl sulfatase activity in acid-treated spores should be relatively insensitive to a change in the *p*H of the assay incubation mixture, and the enzyme activity in spores with disrupted permeability barriers should be more sensitive to the extracellular *p*H.

This was not found to be the case. The acid-resistant, patent aryl sulfatase showed a sharp optimum activity at *p*H 7.6 to 7.8. Aryl sulfatase determined in washed and acid-treated conidia which had been further treated with chloroform showed a similar *p*H dependence, as did activity measurements on purified aryl sulfatase.

The simplest interpretation of this result is that patent, acid-resistant enzyme activity is completely accessible to hydrogen ions, but somehow protected from acid denaturation. In cells with disrupted permeability barriers, as pointed out above, this enzyme activity is no longer resistant to acid denaturation.

A third possibility is that *Neurospora* conidia contain an acid-resistant uptake system which transports *p*-nitrophenyl sulfate into the cells under the conditions of the standard aryl sulfatase assay and which is rate-limiting for the enzyme-catalyzed reaction in intact cells. Such a system could have the *p*H optimum and K_m observed for the acid-resistant, patent component of conidial aryl sulfatase (described above).

To investigate this possibility, uptake of *p*-nitrophenyl sulfate was measured directly (Fig. 5). A small, but unequivocal, uptake of labeled substrate was detected. One experiment was done under the exact conditions used in the standard aryl sulfatase assay. An additional experiment was done in the presence of 50 mM Na_2SO_4, which should prevent possible uptake of traces of labeled inorganic sulfate known to be contaminating the labeled substrate (30). Under both of these conditions, radioactivity entered the conidia. In the presence of inorganic sulfate, the uptake was nearly linear over a 2-hr period.

From the amount of cryptic enzyme in *ars*+ conidia and the measured uptake of *p*-nitrophenyl sulfate, it can be shown that all of the substrate that was taken up would be cleaved as rapidly as it entered the cell. If the uptake in the first hour in the presence of inorganic sulfate is taken as the initial rate of transport of *p*-nitrophenyl sulfate, the total *p*-nitrophenol formed in 120 min would be 4.3 nmoles/ml of the conidial suspension in the uptake incubation mixture. This corresponds to only 7 to 20% of the *p*-nitrophenol produced by acid-treated, *eth-1*^{r},*cys-11-A* (*ars*+) conidia with intact permeability barriers in a normal enzyme assay.

The information in this section does not completely resolve the question of whether the acid-resistant, patent enzyme is really a separate compartment. The results, however, seem most compatible with the existence of two separate compartments, in spite of the fact that some *p*-nitrophenyl sulfate uptake apparently occurs.

Determination of aryl sulfatase in ascospores. Crosses of *fl; eth-1*^{r}*-a* × *fl; eth-1*^{r}*-A* were made on numerous plates. The *fl* character completely prevents the formation of macroconidia, which would otherwise have been present, admixed with the ascospores. An additional advantage is that *fl* strains are extremely fertile in crosses. The strains used and the nutritional conditions chosen are those which would be most likely to give ascospores derepressed for aryl sulfatase synthesis.

Ascospores were harvested, suspended in 0.1 M Tris-hydrochloride (*p*H 8.1), and disrupted by passing seven times through the orifice in an X-press. Samples were then assayed for aryl sulfatase activity. No activity was detectable.

Aryl sulfatase activity and location in young mycelia. During germination of conidia, the aryl sulfatase activity increased dramatically. After 5 hr (when 50% of the conidia showed germination tubes), the aryl sulfatase activity increased about 12 times, whereas the protein [measured by a modification of the method of Lowry et al. (19)] increased only about 2.6 times. After 12 hours, the specific activity was 20 to 30 times that in the conidial inoculum.

Little, if any, aryl sulfatase was found in the growth medium. A sample of the medium from which mycelia (derepressed for aryl sulfatase synthesis) was removed by filtration was concen-

TABLE 6. *Comparison of aryl sulfatase activity after different treatments on the same conidial suspension*

Treatment	Aryl sulfatase activity[a]
Control[b]	3.6
Chloroform treatment[b] (one wash with 5 ml, 0 C)	10.6
Nystatin[b] (100 μg/ml, 30 min, 0 C)	10.0
Heat treatment (5 min, 60 C)[c]	11.1
X-press (one pass)[c]	10.4

[a] Expressed as enzyme units per 10^6 conidia.

[b] Assay samples were collected and washed on filter paper discs. Chloroform treatment was applied to conidia on the filter paper; nystatin treatment preceded collection of the samples.

[c] Assay samples were pipetted directly into the assay tubes. Heat treatment was carried out in the assay tubes; X-press treatment preceded collection of the samples.

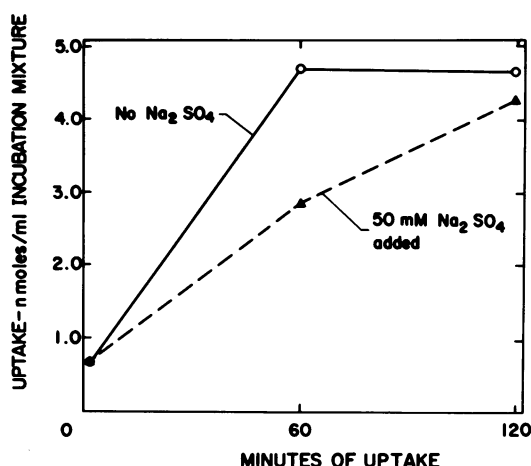

FIG. 5. *Uptake of* ^{35}*S-labeled p-nitrophenyl sulfate into washed and acid-treated conidia. The triple mutant eth-1*^{r}*, cys-11; ars(101)-A was used in this experiment to avoid cleavage of the labeled substrate. Conidia were grown, harvested, washed, and acid-treated as usual. The determination of* ^{35}*S-labeled p-nitrophenyl sulfate uptake is described in the text.*

trated by using an Amicon Diaflo Ultrafiltration apparatus (with a UM-10 filter). The activity measured in this concentrate was less than 4% of the activity found in the mycelia which was removed from the growth medium that was concentrated. An aryl sulfatase determination was run on a mixture of the concentrated medium plus purified aryl sulfatase. No evidence was obtained for the presence of an inhibitor in the medium. Medium that had not been concentrated was also not inhibitory.

Samples of young mycelia were collected on filter paper discs and assayed for aryl sulfatase activity after washing with various buffers or with chloroform (Table 7). It can be seen that the amount of enzyme activity detected by these assays depended on the buffer used for washing the cells. The viability of the young mycelia also depended on the buffer that was used for washing. Acetate buffer kills most of the cells, perhaps by altering the cell membrane (23) or by acidifying the cellular contents (34). This result is in marked contrast to the lack of effect of such a wash on ungerminated conidia.

Most of the viability was retained after washing with 0.1 M Tris-hydrochloride, *p*H 8.1. The detectable aryl sulfatase activity after such a wash was considerably lower than after chloroform or acetate washing. The activity detectable in Tris-washed cells could be increased by subsequent washing with chloroform or acetate buffer to the level achieved by washing with those reagents alone. These results suggest that, in mycelia, there is also a cryptic compartment of aryl sulfatase, but that the permeability barrier is much more fragile than in conidia. The production of *p*-nitrophenol in the standard assay conditions was not linear with time in Tris-washed mycelia. At first the rate was very low, but after 20 to 60 min it became nearly linear at a rate not greatly different from cells which were washed with acetate or chloroform. This suggests that the permeability barrier is fragile even after washing with Tris buffer, and that determination of the patent enzyme activity in young mycelia may be difficult, if any patent activity exists at all. Acid treatment (*p*H 1.5, 30 min, 0 C) of Tris-washed cells reduced the viability to less than 0.01% and also destroyed all of the aryl sulfatase activity over a period of 10 min.

DISCUSSION

The results reported above lead to the conclusion that there are at least three, and probably

TABLE 7. *Effect of the wash on detectable aryl sulfatase activity and viability of young mycelia*

Treatment[a]	Aryl sulfatase activity[b]	Viability[c]
		%
No wash	1,300	100
0.1 M Sodium acetate-acetic acid buffer, *p*H 5.0	3,500	0.5
Chloroform	3,700	
0.1 M Tris-hydrochloride buffer, *p*H 8.1	1,200	89
0.1 M Sodium succinate-succinic acid buffer, *p*H 4.9	1,300	

[a] Conidia (1.3×10^6 per ml of culture medium) were inoculated and allowed to germinate for 12 hr at 25 C; 1.0-ml portions were then collected on filter paper discs and washed, where indicated, with 5 ml of the appropriate solution (ice-cold).

[b] Number of cells was calculated from the turbidity of the conidial suspension used for inoculation. Values indicate enzyme units per 10^6 cells.

[c] Viability was determined on different cultures than were used for the enzyme assays. Samples were washed twice with the appropriate sterile buffer by centrifuging and resuspending. One hundred per cent represents a portion diluted directly from the culture into sterile distilled water and plated to determine the number of colony-forming units. The values obtained in this manner from a 12-hr culture were not significantly different from the number of colony-forming units originally inoculated into the culture as conidia.

four, compartments of aryl sulfatase in *Neurospora* conidia. These include: (i) a true, soluble, exoenzyme; (ii) a cell-bound fraction outside the plasma membrane, either in the periplasmic space or bound to the cell wall; (iii) an intracellular fraction; and (iv), possibly, a fraction bound to or imbedded in the plasma membrane. Reservations concerning the location of this fourth category (the patent, acid-resistant enzyme) were considered in the Results section.

The location of enzyme in the second category above is based on its sensitivity to an acid treatment which does not affect conidial viability. This criterion has been dealt with extensively in connection with several carbohydrases in *Myrothecium verrucaria* and *Aspergillus luchuensis* by Mandels (22). By the same criterion, it was established that almost all of the invertase in *Neurospora* conidia is external to the plasma membrane (27).

The cryptic enzyme (category iii) is considered to be intracellular because this activity can only be detected when conidia have been disrupted (with the X-press) or when the conidial permeability barriers have been destroyed (by heat-treatment, chloroform, acetone, nystatin, ascosin, or phenethyl alcohol). Considerable information is available on the mechanism of action of the polyene antibiotics in *Neurospora* (17), and the fact that these agents reveal the cryptic aryl sulfatase can be taken as strong evidence that a membrane is responsible for the crypticity. Phenethyl alcohol is also thought to operate primarily at the level of membranes in *Neurospora* (18). A similar suggestion has been made for its primary effect in *Escherichia coli* (38). Several different treatments, physical and chemical, give the same value for the amount of enzyme that is cryptic (Table 6). This argues against the possibility that each of these treatments is acting directly on the enzyme molecules to increase their activity.

From the results presented, it cannot be determined whether these categories of enzyme activity represent sublocations in each conidium, or whether they represent subclasses of a heterogeneous population of cells. In particular, it might be asked whether the acid-sensitive, cell-bound enzyme (category ii) might be due to dead spores or to contamination with bits of mycelia. It is unlikely that dead spores account for this fraction of enzyme because a comparison of the number of spores in a conidial suspension (determined with a hemocytometer) with the number of colony-forming units showed that virtually all (greater than 92%) of the structures visually identifiable as spores formed colonies. In the same conidial suspension, 57% of the total cell-bound enzyme was acid-sensitive.

The second possibility is more difficult to test. Since mycelia contain a much higher level of enzyme activity, a small contamination of a conidial suspension with mycelial fragments could account for category ii of conidial aryl sulfatase. Microscopic examination of filtered conidial suspensions showed no appreciable contamination with mycelial fragments. Different filtration techniques were tried and all gave substantially the same fraction of acid-sensitive enzyme, so it is doubtful that all of the enzyme in category ii could be due to mycelial contamination; the point, however, is by no means proven. There are, of course, more subtle types of population heterogeneity which might account for these "compartments," but no evidence can be presented concerning these possibilities.

In many species, enzyme activities which are located in more than one cell compartment occur in structurally different isozymes corresponding to the different locations. So far, there is no reason to suspect that aryl sulfatases from different compartments have structural differences. All of the enzyme is acid-sensitive in disrupted spores and in spores lacking intact permeability barriers, all compartments are absent if conidia are produced under repressing conditions, and all are absent in *ars*⁻ mutants. Structural differences might still be found, however, especially considering the recent results of Gascón et al. (9) with yeast invertase.

Other investigations of fungal conidia have demonstrated multiple locations for single enzyme activities [invertase in *N. crassa* (27), aryl-β-glucosidase in *N. crassa* (3, 4) and in *Aspergillus oryzae* (13), laminarinase in *N. crassa* (21), and diphosphopyridine nucleotidase in *N. crassa* (44)]. None of these reports [except, possibly, that of Eberhart and Beck (4)] has shown an enzyme distribution as complicated as that found for aryl sulfatase in this study. The situation with aryl-β-glucosidase (4) is similar in many respects to that of aryl sulfatase. No patent, acid-resistant aryl-β-glucosidase and only very little cryptic activity was found in conidia; however, in conidia which were germinated 5 hr with cellibiose as a carbon source, patent, acid-resistant enzyme was detected, as well as a much increased cryptic compartment (perhaps 25% of the total activity). In yeast, multiple enzyme location has been well documented. Invertase can occur in four locations (8): free in the medium, bound to the cell but released on protoplasting, intracellular, and bound to a protoplast membrane.

Upon germination and growth under derepressing conditions, *Neurospora* synthesizes a considerable amount of aryl sulfatase; little, if any, however, is secreted into the medium or can be removed by washing. Essentially the same situation has been reported by Eberhart (3, 4) for aryl-β-glucosidase. The investigation of the location of aryl sulfatase in growing mycelia has been complicated by the sensitivity of *Neurospora* mycelia to acid treatment and to washing with 0.1 M acetate buffer. This sensitivity is surprising, since yeast cells are very resistant to acid treatment [no loss of viability was observed in 0.1 N HCl at 20 C for 18 hr (32)] and are also more resistant to acetate buffer than is *Neurospora* (34).

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