Distribution of Steroid 1-Dehydrogenation and Side-Chain Degradation Enzymes in the Spores of \textit{Fusarium solani}: Causes of Metabolic Lag and Carbohydrate Independence

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The spores of a strain of \textit{Fusarium solani} 1-dehydrogenate ring A and cleave the 17β-acetyl side chain of 17α-hydroxyprogren-4-ene-3,20-dione (17α-hydroxyprogesterone) to give 17α-hydroxyprogren-1,4-diene-3,20-dione (the 1-dehydro analogue) and little androsta-1,4-diene-3,17-dione (androstadienedione). A 4-h lag period is observed in the course of metabolism, and there are no requirements for external additives. Exoenzymes or surface enzymes bound to the cell outside the plasma membrane, either in the periplasmic space or bound to the cell wall, cannot be detected. The spore activity is not destroyed by treatment with aqueous HCl (pH 1.50), indicating that the 1-dehydrogenation and side-chain degradation enzymes are located away from the surface of the spores. Phenethyl alcohol destroys the spore permeability barriers, and it is also likely that it exposes its enzymes to acid inactivation. The action of phenethyl alcohol is reversible at low concentrations and irreversible at high concentrations. This investigation shows that: (i) the spore 1-dehydrogenating and side-chain-degrading enzymes appear to be bound to, or imbedded in, the plasma membrane; (ii) the lag period observed in the course of metabolism of the steroid by the spores might be required for enzyme activation or diffusion of the substrate through the cell wall; and (iii) the internal metabolites of the spores, that might be required for the conversion process, appear to be present in a nondiffusible form or bound to intrasporal macromolecules.

The chemical activities of fungal spores were first discovered in 1958 by Gehrig and Knight (3). In recent years a number of investigations (R. Plourde, H. Hafez-Zedan, and J. P. Lemoine, Abst. 39 Congr. Assoc. Can. Franc. Avanc. Sci., Sherbrooke, Québec, p. 95, 1971; references 1, 6, 17, 20, 24, 25, 28, 29) established that nongerminating spores of fungi and actinomycetes can accomplish a wide range of conversions of steroid molecules. The spores of certain microorganisms showed more selective enzymatic activities as compared to corresponding growing cultures. For instance, although spores and mycelium of \textit{Aspergillus ochraceus} (28) are both highly active steroid 11α-hydroxylators, the spores exhibit very little \(6\beta\)-hydroxylation activity as compared to the mycelium. In a previous communication (17), we reported that the growing mycelium of a strain of \textit{Fusarium solani} 1-dehydrogenated ring A and degraded the side chain of 17α-hydroxyprogesterone; the spores of the same culture converted the same steroid into its 1-dehydro analogue (Fig. 1) without side-chain cleavage. Alternative explanations have been given for the cleavage of the side chain by the mycelium and not by the spores. (i) The spores may contain only 1-dehydrogenase and lack the side-chain-degrading enzymes. (ii) The main reaction in both mycelium and spore processes may be 1-dehydrogenation; further incubation may induce de novo protein synthesis of the side-chain-cleaving enzymes in the mycelium. This induction could not take place in spores due to absence of adequate nitrogen supplement. (iii) Both mycelium and spores contain both 1-dehydrogenase and side-chain-degrading enzymes. In spore-mediated reactions, the side-chain-degrading enzymes may be inactive due to a lack of certain cofactors which may be
present in the mycelium-mediated reactions. In addition, it could be assumed that certain spore enzymes are located to the exterior of the spore permeability barriers, whereas others could be within the permeability barriers. As for vegetative cells, it is possible that all enzymes may be present in one compartment. Consequently, the observed differences in enzymatic activity of spores and vegetative cells of a single microorganism on a given steroid substrate may reflect the influence of the transport systems on substances of different polarity or structure, especially when it is known that steroid conversion involves more than one enzymatic reaction; one enzyme utilizes the product(s) of the other as substrate(s).

Two other noteworthy phenomena have been observed in spormediated steroid bioconversions. (i) A lag period has always been observed before any conversion products could be detected (17, 28, 29). This lag in metabolism was not eliminated by repeated use of one batch of spores in successive fermentations; no explanation could be given for this phenomenon. (ii) Enzymatic reactions carried out by washed, nongerminating spores may or may not require external additives. For example, exogenous sugars or nitrogen sources were not essential for steroid 1-dehydrogenation or side-chain degradation by spores of S. affinis (24), nor were they essential for 15α-hydroxylation by spores of Fusarium moniliforme (1), whereas steroid hydroxylation by spores of A. ochraceus (28) and Mucor griseo-cyanus (25) were carbohydrate dependent.

Preparation of active cell-free extracts from spores that can transform steroids could give useful information about the locational distribution of the steroid-metabolizing enzymes in the spores, the lag commonly observed in the metabolism, and the requirement for exogenous carbohydrate supply in some spore-catalyzed reactions. Truly intracellular steroid-metabolizing enzymes (D. M. El-Tayeb, Ph.D. thesis, Univ. of Wisconsin, Madison, 1965; references 7, 18, 19) have been isolated from the soluble fraction of cell homogenates of various microorganisms (vegetative growth). On the other hand, because of unknown causes, it was not possible to obtain active cell-free extracts from the spores (29), preventing further factual investigations of these phenomena.

In the present work, the ability of phenethyl alcohol (PEA) to disrupt the permeability barriers of the spores of a strain of F. solani and expose its steroid 1-dehydrogenation and side-chain degradation enzymes to acid inactivation is examined. Finally, an investigation was undertaken to determine: (i) the location of these enzymes in the spores; (ii) the possible causes of the lag period usually encountered in spore-mediated steroid conversions; and (iii) the reasons for the nonrequirement of the spores of certain microorganisms for external additives.

MATERIALS AND METHODS

Organism. The spores of a large number of fungi were screened for their ability to 1-dehydrogenate or cleave the 17β-acetyl side chain of progesterone or related steroids, or both. Of these, a strain of F. solani (from the stock culture collection of the Faculty of Pharmacy, University of Montreal) was selected for the present investigation as the spores were found to carry out these transformations and could be incubated for long periods in hydrochloric acid (pH 1.50) without significant loss in viability.

The culture was maintained on nutrient dextrose agar (Difco) supplemented with 1% yeast extract (Difco) stored at 4 C, and subcultured monthly.

Chemicals. Chromatographically pure steroids were obtained from K & K Laboratories, Inc., Plainview, N.Y. Cycloheximide was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Nystatin was the product of Sigma Chemical Co., Inc., St. Louis, Mo. L-Alanine, L-proline, and L-serine were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Other chemicals were of reagent grade.

Sporulation. The spores were produced on a medium consisting of glucose (10 g), peptone (20 g), yeast extract (10 g), agar (27 g), and water to 1 liter. Unless otherwise indicated, progesterone (100 μg per ml of medium) was added as an inducer. All subsequent processes were carried out under aseptic conditions according to the procedure described earlier (17). For convenience, spores which were not subjected to any treatment before incubation with the steroid substrate are referred to as “intact” spores.

Cell viability tests. Spore suspensions were diluted in 1% phosphate buffer (pH 6.0) so that cell counts could be obtained. Diluted suspensions were then plated in triplicates on Sabouraud dextrose agar
(Difco) as follows. Measured portions of the resulting suspensions were mixed with 5 ml of molten medium and poured onto a 15-ml solid base layer of the same medium in petri plates (100 by 15 mm). The plates were then incubated for 72 h at 26 to 28 C, and the colonies were counted. After any treatment, viability of the spores was estimated from plate counts and expressed as percentage of the total.

**Acid treatment of spores.** In experiments in which the spores are referred to as "acid-treated," the following procedure was carried out aseptically. A portion of the spore suspension was kept at 26 C and acid treated by adding 1 N hydrochloric acid to give a pH of 1.50; the spores were kept in suspension by agitation. After appropriate periods of time, samples were taken from the "acid-treated" mixture, and the effect of acid was terminated by adding a predetermined amount of 1 N NaOH to bring the pH to approximately 6.0. These samples were then centrifuged, suspended in the buffer solution to the original volume, and used for the transformation of the steroid substrate. As a control, HCl and NaOH, in quantities equivalent to those used in the test, were mixed prior to addition to the suspension, and the process was completed as above. Samples of acid-treated and control spores were diluted and plated for viable count determination.

**Phenethyl alcohol treatment of spores.** Measured volumes of the spore suspensions kept at 26 C were treated with HCl, pH of 1.50, containing various concentrations of PEA, for 30 min, or as indicated. At the end of the incubation period, the suspensions were brought to an approximate pH of 6.0 with NaOH. The spores were separated by centrifugation, washed once with 1% phosphate buffer (pH 6.0), and resuspended in the buffer to the original volume. Controls were prepared by mixing appropriate volumes of HCl and NaOH, in quantities equivalent to those used in the test, before addition to a spore suspension containing various concentrations (as those used in the test) of PEA. Viable count determinations were carried out as described.

**Treatment of spores with organic solvents.** Measured samples from the spore suspension, each 15 ml, were collected on 60-ml Buchner funnels (fritted, fine porosity). The spores on the disk of each funnel were treated with a 3-ml portion of cold chloroform or acetone by adding the solvent while constantly applying vacuum. The spores were then washed with 30 ml of cold buffer, and suspended in the buffer to the original volume. A portion of the spores was acid-treated, whereas another portion served as a control. Samples from test and control suspensions were diluted and plated for viable count determinations.

**Nystatin treatment of the spores.** Appropriate volumes of a 3,000 U per ml solution of nystatin dissolved in N,N-dimethylformamide (DMF) were added to samples of the spore suspension to give a concentration of 50 U per ml of medium; sterile glucose solution was also added to a concentration of 0.5 mg per ml. The flasks were incubated at 26 C on the shaker for various periods of time and chilled rapidly, and the spores were removed by centrifugation. The collected spores were suspended in the buffer solution to the original volume and divided into two portions; one portion was acid-treated, and the other served as a control. Viable count determinations were carried out.

**Measurement of nystatin uptake by spores.** Spores were incubated for various periods of time with known concentrations of nystatin. The addition of nystatin was considered as the zero time. Nystatin was estimated in the supernatant fluids by measuring its absorption at 321 nm as described by Lampen et al. (8). Control solutions were prepared by mixing appropriate volumes of DMF with the spore suspensions to give dilutions of the solvent corresponding to its concentration in the nystatin incubation mixtures. The process was completed as under nystatin-treated spores, and the supernatant fluids were used as controls. By using such a control, the possibility of leakage of cell materials which might contribute to the absorption at 321 nm was eliminated. It was therefore, assumed, that the nystatin disappearing from the supernatant fluid was absorbed by the spores.

**Treatment of the spores with various reagents.** Measured portions of the spore suspension were centrifuged, and spores were resuspended to the original volume in one of the following solutions: 1 M KCl, 1 M sucrose, 0.1 M Na2HPO4, 0.1 M Na2SO4, or 5 mM diisodimethylamine tetraacetate. In another set of experiments, the spores were suspended in 0.05 M citric acid-0.1 M Na2HPO4 (pH 3.0 to 5.0) or 0.033 M KH2PO4-K2HPO4 (pH 6.0 to 8.0) buffers. After 30 min of shaking at 26 C, spores were separated by centrifugation and suspended in distilled water. Control samples were prepared by shaking in distilled water under the same conditions.

**Steroid conversion by spores.** Transformations were carried out in 50-ml Erlenmeyer flasks, each containing 10 ml of the spore suspension (2 × 10⁴ spores per ml). Unless otherwise specified, sterile glucose solution was added to each flask to give a final concentration of 0.5 mg per ml, and a 50 mg per ml solution of the steroid substrate dissolved in DMF was added to give a concentration of 0.5 mg per ml of medium. The flasks were incubated on a reciprocating shaker (4-cm stroke, 150 cycles per min) at 26 to 28 C for the required period.

**Steroid determination.** Qualitative and quantitative estimation of the transformation products and residual substrate during the course of transformation was carried out by using the same procedures as described (17).

**RESULTS**

**Transformation of 17α-hydroxyprogesterone by intact spores.** Spores of *F. solani* converted 17α-hydroxyprogesterone to 17α-hydroxyprogren-5a,14-diene-3,20-dione, as the main product, (45%), and androstadienedione (4%). The transformation products were isolated and identified by comparison with authentic samples (melting point, mixed melting point, infrared spectra, and chromatographic
behavior) according to the procedure described (17).

The time course of conversion of 17α-hydroxyprogesterone by the spores is illustrated in Fig. 2. No reaction takes place before 4 h of incubation. On prolonged incubation, the first product to appear was 17α-hydroxy-pregna-1,4-diene-3,20-dione. As the substrate continued to disappear gradually and its 1-dehydro analogue to accumulate, a second product, androstadienedione, began to appear in the medium after 10 h of incubation. Both products exhibited an increase in their concentrations and reached a plateau within 24 h. Addition of glucose (0.5 mg per ml) to the reacting mixture did not enhance the 1-dehydrogenation or the side-chain degradation of the substrate by the spores, indicating that these reactions do not require external energy-yielding materials.

The 4-h lag period in the metabolism of the substrate suggests the presence of an "inducible" system. Lawrence (9, 10) reported that the ability of spores of Penicillium roqueforti to oxidize fatty acids and triglycerides was markedly stimulated by adding small amounts of certain amino acids including l-alanine, l-proline, and l-serine. However, addition of 20 μmol of any of these amino acids per ml of medium could not enhance the bioconversion of 17α-hydroxyprogesterone by F. solani spores. Furthermore, the addition of 0.05 mg of either peptone, yeast extract, or ammonium sulfate per ml of fermentation medium as well as the reuse of the spores in two successive fermentations did not eliminate the lag period or enhance the yield. The same reaction course was observed when the transformation was conducted in the presence of tetracycline-hydrochloride (100 μg per ml), chloramphenicol (100 μg per ml), or cycloheximide (50 μg per ml), antibiotics which inhibit protein synthesis.

**Resistance of spore enzymes to successive and continuous washing.** Successive and continuous washes of F. solani spores with 1% phosphate buffer (pH 6.0) did not reduce its 1-dehydrogenation or side-chain degradation activity on 17α-hydroxyprogesterone.

Spores washed successively for five 1-h intervals or continuously for 5 h both transformed the substrate into its 1-dehydro analogue and androstadienedione to the same extent as unwashed spores. The presence of cycloheximide (50 μg per ml) in the reaction mixture did not affect the process.

**Resistance of spore enzymes to brief washing with various reagents and buffers.** Spores of F. solani were exposed to brief treatment with aqueous 1 M KCl, 1 M sucrose, 0.1 M Na₂HPO₄, 0.1 M Na₂SO₄, 5 mM ethylenediaminetetraacetate, citrate-phosphate buffers (pH 3.0 to 5.0), and phosphate buffers (pH 6.0 to 8.0). However, these washes did not remove any enzymes from the spores; no significant decrease in spore activity was observed, and the presence of cycloheximide did not affect the course of transformation.

**Resistance of spore enzymes to acid treatment.** In one experiment (see below) "intact" spores of F. solani were tested for conversion of 17α-hydroxyprogesterone at different pH values. No significant difference in activity was observed when transformation was carried out at a pH ranging from 3.0 to 8.0. For this reason the present study was undertaken to examine the effect of acid treatment on spore activity.

Acid treatment of spores for 1 h did not inactivate or reduce spore 1-dehydrogenation or side-chain degradation activity. After such treatment, spores could transform 17α-hydroxyprogesterone as did untreated spores. Successive acid treatments on the same sample of spores, for as much as 4 times, as well as variation of spore concentration, from 5 × 10⁷ to 2 × 10⁸ spores per ml, could not induce any reduction in activity. It is noteworthy that spores suspended in aqueous HCl, pH 1.50, were as active on the steroid substrate as spores suspended in the buffer solution. No appreciable loss in spore viability occurred during these treatments.

**Effect of PEA on spore activity.** Spores of F. solani, suspended in 1% phosphate buffer (pH 6.0) containing increasing concentrations of PEA (0.1 to 1%), were sampled at 30-min intervals during a 90-min period. Spores were separated by centrifugation, and viability was determined as described before. Plate counts indicated that no loss of viability occurred.

PEA-treated spores converted 17α-hydroxy-
progesterone into its 1-dehydro analogue and androstadienedione, even in the presence of cycloheximide (50 \( \mu \)g per ml), as did untreated spores. A 4-h lag period was observed in the course of transformation, and the process did not require the presence of carbohydrates.

Spores shaken for different periods of time in HCl (pH 1.50) containing high concentrations of PEA could not transform 17\( \alpha \)-hydroxyprogesterone even in the presence of an energy source, although there was no appreciable loss in viability. Substantial reduction in spore activity was readily seen at PEA concentrations as low as 0.2%, and maximal effect was obtained by using 0.5% PEA (Fig. 3). Acid treatment for 30 min in the presence of 0.5% PEA (Fig. 4) inhibited the steroid-metabolizing activity of the spores; however, repetition of this treatment with spores collected from different batches showed variation in the concentration of PEA required to render spore activity sensitive to acid treatment at a fixed period of time. However, with spores from one batch, this variation was insignificant.

In one experiment, spores were shaken for 30 min in 0.5% PEA in the buffer solution, washed once with water, and then suspended in the buffer to original volume. The resulting suspension was divided into portions. One portion was immediately acid-treated; likewise, the other portions were kept for various periods of time at 26 C before acid treatment. Immediate acid treatment on the first portion completely inactivated the spores. When PEA-treated spores were allowed a 1-h or more “recovery” period before hydrochloric acid treatment, 1-dehydrogenation and side-chain degradation activity were not affected. However, when the same experiment was repeated with 1% PEA, a 1-h “recovery” period could not protect the spores from acid inactivation.

PEA is thought to operate primarily at the level of membranes in Escherichia coli (23) and Neurospora crassa (21, 22). From our results, it appears that PEA caused a rapid and reversible breakdown in the permeability barriers of \( F. \) solani spores. Disruption of the permeability barriers was indicated by the fact that only after PEA treatment of the spores were 1-dehydrogenation and side-chain degradation enzymes inactivated on immediate or concurrent acid treatment.

At low concentrations of PEA (0.1 to 0.6% vol/vol) the action of PEA appears to be reversible. Exposure of the spores to higher concentrations of PEA (1%) resulted in permanent irreversible disruption of the permeability barriers, and a long recovery period (1 h) cannot restore barrier integrity. Similar observation has been reported by Silver and Wendt (23) using \( E. \) coli. The recovery from PEA-induced permeability (when 0.2 to 0.6% PEA was used) did not require the presence of external additives (e.g., energy sources); as reported by Silver and Wendt (23), it may be explained by the following. (i) The initial action of PEA may be a
The absorption of PEA by the membrane alters the conformation of the membrane, resulting in the breakdown of its structural integrity. When PEA is removed, the membrane returns to its original configuration. Thus, the recovery does not require energy metabolism and repair synthesis is not necessary. At high PEA concentration, membrane breakdown is so extensive that recovery becomes impossible or difficult to be accomplished. (ii) The action of PEA is a physiological or a physicochemical process, and repair synthesis is necessary. At low concentrations of PEA, energy required for such synthesis might be supplied by the spore's endogenous reserve materials. However, at high PEA concentrations, membrane breakdown will be so extensive that the spore could not accomplish the repair because of its limited internal metabolites essential for the process, or for other unknown causes, as addition of glucose was insufficient for recovery.

Effect of acetone and chloroform on spore activity. Brief treatments of F. solani spores with cold chloroform or acetone resulted in complete loss of spore activity and viability.

Effect of nystatin on spore activity. Although a significant amount of added nystatin (16.2%) was absorbed by the spores of F. solani (Fig. 5) after incubation for 30 min, consecutive treatment by HCl did not alter the enzymatic activity of the spores. Moreover, preincubation of the spores with 100 U of nystatin per ml of medium, for as long as 2 h, followed by acid treatment could not inactivate the spores; 17α-hydroxyprogesterone was transformed into its 1-dehydro analogue and androstadienedione, and no loss in spore activity or viability could be demonstrated. However, one has to bear in mind the possibility of inactivation of the antibiotic by the spores.

Effect of pH on spore activity. The effect of pH on the conversion of 17α-hydroxyprogesterone by "intact" spores and spores treated with 1% PEA is illustrated in Fig. 6. With untreated spores, no significant difference could be observed when the transformation was carried out at a pH ranging from 3.0 to 8.0 in citrate-phosphate and phosphate buffers. Moreover, acid treatment at pH 1.50 did not inactivate the spores. The activity of PEA-treated spores decreased gradually as pH was reduced from 6.0 to 3.0 and was lost completely at pH 1.50. This observed reduction in activity at low pH values could not be attributed to removal of internal metabolites necessary for the process (as a result of disruption of spore permeability barriers by PEA treatment), since addition of carbohydrate or nitrogenous compounds did not enhance the conversion process.

DISCUSSION

The nongerminating spores of this strain of F. solani are capable of 1-dehydrogenating 17α-hydroxyprogesterone with or without removal of its methyl ketone side chain to give 17α-hydroxyprogrena-1, 4-diene-3, 20-dione as the main metabolite and only a low yield of androstadienedione.

No enzyme activity was detected in the supernatant fluids after removal of the spores.
by centrifugation. This eliminates the possibility of participation of exoenzymes in spore-mediated steroid biotransformations. Thereafter, enzymes operating in these bioconversions could be: (i) surface enzymes, bound to the cell outside the plasma membrane, either in the periplasmic space or bound to the cell wall, (ii) bound to, or imbedded in the plasma membrane, or (iii) truly intracellular enzymes, within the cytoplasmic membrane.

Direct proof of the location of these enzymes in the spores is lacking because it was not possible to prepare active cell-free extracts from the spores. Nevertheless, the following reasoning would, by indirect evidence, suggest that 1-dehydrogenation and side-chain degradation enzymes of the spores of *F. solani* are located away from the spore surface.

The enzymes were not released from the spores by successive or continuous washing, and the subuse of one sample of spores in two or three successive transformation processes did not reduce the activity of the spores; moreover, no enzymatic activity has been detected in the suspending medium after removing the spores by centrifugation. However, it is considered possible that the enzymes may be firmly adsorbed to the spore wall; no enzyme activity was found in any of the supernatant fluids resulting from spore washings at different pH values or with various reagents known to remove surface enzymes, and no reduction in spore activity was observed after these washes. It is also possible that these enzymes dissociate reversibly or irreversibly from the cell wall; the inactivation of any released enzymes might have occurred especially at the extremes of the pH values, and spore activity was not reduced because of a simultaneous de novo enzyme synthesis which would compensate the inactivated enzymes. As described by Mandels and Vitols (13) for induced trehalase system in spores of the fungus *Myrothecium verrucaria*, de novo protein synthesis could occur at the expense of endogenous amino acid pools of the spores. However, spores of *F. solani* which have been successively or continuously washed, briefly treated with various reagents, or suspended at different pH values transformed 17α-hydroxyprogesterone in the presence of various protein synthesis inhibitors (cycloheximide, 50 μg per ml; chloramphenicol, 100 μg per ml; or tetracycline-hydrochloride 100 μg per ml) without any reduction in activity.

Acid treatment of the spores presumably destroys or inhibits enzymes located outside the cell membrane and leaves the internal enzymes fully active. Several investigators (2, 11, 12, 16, 22, 30) have used the acid treatment to locate surface enzymes on fungal spores. By parallelism, our results suggest that the 1-dehydrogenase and side-chain degradation enzymes of the spores of *F. solani* are not located on or near the surface of the spores because they were not vulnerable to treatment with dilute HCl.

Successive acid treatments on the same sample of spores and variation in the concentration of the spores subjected to acid treatment did not inactivate the spores or even reduce their activity. Furthermore, spore activity was not affected when transformation was carried out in dilute HCl, pH 1.50. These results eliminate the possibility that sporal proteins and carbohydrates might buffer out the added hydrogen ions.

The presence of cycloheximide, chloramphenicol, or tetracycline HCl during the transformation of the substrate by acid-treated spores did not influence spore activity. This observation argues against the possibility that enzymes, presumed to be located at the surface of the spores, were inactivated by acid treatment and that de novo synthesis of acid-resistant enzymes occurred during incubation with the steroid substrate.

When the permeability barriers of the spores were disrupted with 1% PEA (see below), no cryptic activity could be revealed, and the enzymes were inactivated by acid treatment. This observation makes it unlikely that acid-resistant 1-dehydrogenase or side-chain degradation enzymes appeared during the conversion of the substrate. It also rules out the possibility that enzymes might be located in an internal compartment accessible to both hydrogen ions and substrate but are acid resistant, or somehow protected from the hydrogen ions.

Our results are in favor of the presence of the 1-dehydrogenation and side-chain degradation enzymes of the spores of *F. solani* in one compartment accessible to substrate but inaccessible to hydrogen ions. According to Scott and Metzenberg (21, 22) classification, spore enzymes might be bound to, or imbedded in, the cytoplasmic membrane.

Further evidence that these enzymes are not located at the surface of the spores can be obtained by comparison of the pH activity curves for spores with intact and disrupted permeability barriers. It was reasoned that if pH activity curves for the "intact" and 1% PEA-treated spores were similar, this would
constitute evidence that enzymes are located at, or in close proximity to, the exterior of the cytoplasmic membrane of the spores. Here, the enzymes would be subject to direct action of the solution in which the spores were suspended. On the other hand, if the enzymes are imbedded in, or located at, the interior of the cytoplasmic membrane, activity of the intact spores should be relatively insensitive to changes in the pH of the incubation mixture, since the internal pH of the spores remains essentially constant. Spores with disrupted permeability barriers should be more sensitive to the extracellular pH; addition of HCl would have access to enzymes molecules, and the activity would be destroyed.

From the pH activity curves (Fig. 6), it is evident that there is no significant change in activity of spores with intact permeability barriers when transformation of the steroid substrate was carried out at different pH values. This is consistent with the observation of Vézina et al. (29) who reported that steroid transformations by fungal spores are not strongly influenced by variations in pH within fairly wide limits. On the other hand, the curves show that activity of spores with disrupted permeability barriers was substantially reduced by lowering the pH of the suspending medium and destroyed by acid treatment.

A 4-h lag period was observed in the transformation of 17α-hydroxyprogesterone by the spores of F. solani. The presence of lag phases in the course of bioconversion of other steroids by nongerminating spores of various microorganisms has also been reported (28, 29). The lag period before transformation occurs might be an indication of the time required for: (i) synthesis of the 1-dehydrogenation and side-chain degradation enzymes; (ii) synthesis of permeases that allow or increase access of the substrate to its metabolizing enzymes; (iii) synthesis of enzyme systems that can use substrates from the endogenous pool of the spores to yield either energy or metabolic products essential for transformation, or both; (iv) activation of preformed permease(s) or enzyme systems, or both; or (v) diffusion of the substrate through the cell wall.

Chloramphenicol, cycloheximide, and tetracycline-hydrochloride, antibiotics known to inhibit protein synthesis, did not affect the transformation of 17α-hydroxyprogesterone by spores with intact or disrupted permeability barriers; also, the reuse of the spores in successive fermentations did not eliminate the lag period. This indicates that no adaptive enzyme synthesis had occurred after incubation with the steroid substrate and rules out the first three possibilities. Therefore, it seems more likely that the lag period may be a consequence of the activation of preformed proteins or cell wall permeability rather than of induced enzyme synthesis. However, since the lag period in the transformation of 17α-hydroxyprogesterone by the spores of F. solani was not eliminated after disruption of the permeability barriers of the spores, it is more likely that the permease(s), if any, is not dormant in the spores. Therefore, the lag period in oxidation of the substrate might be required for activation of 1-dehydrogenation and side-chain degradation enzymes or other enzymatic systems which might participate indirectly in the transformation process by generating energy or metabolic products essential for the bioconversion, or both. The reuse of the spores in successive fermentations did not eliminate the lag period, presumably due to rapid deactivation of the enzymes on removing the steroid substrate or metabolites by washing.

The possibility that the lag period observed in the conversion course of the substrate by the spores may also be attributed to cell wall permeability cannot be excluded. It is generally assumed that the permeability of a microbial cell is determined entirely by its membrane and not by the looser lattice of its wall. However, cell walls may act to bind or sieve molecules that diffuse through the aqueous channels or pores that are an integral part of their structure (4, 16, 26, 27). It has also been demonstrated that bacterial cell walls flex and change volumes in response to a changed ionic environment, possibly with dilatation and contraction of the openings in their matrix (15). However, whether the lag period observed in steroid transformations by fungal spores is related to enzyme activation, cell wall permeability, or both, or to any other causes must await the preparation of active cell-free extracts.

Vézina et al. (25, 28) reported that steroid hydroxylations by spores of A. ochraceus, M. griseo-cyanus, and other fungi are carbohydrate dependent. It appears that the internal substances essential for catalyzing these reactions by the spores could diffuse easily from the spores; therefore, little or no conversion is observed in absence of glucose when thoroughly washed or starved spores are used. For optimal hydroxylation by these spores, it is essential to maintain a minimum concentration of glucose in the medium. Our results show that the 1-dehydrogenation and side-chain degradation
of 17α-hydroxyprogesterone by washed spores of F. solani do not have a requirement for glucose or other carbohydrates. This has also been shown to be true for steroid 1-dehydrogenation with spores of Septotmyxa affinis (24) and 15α-hydroxylation with spores of F. moniliforme (1). The spores' internal reserve of substrates and cofactors appears to be adequate to sustain these reactions. Furthermore, washed spores of F. solani with disrupted permeability barriers convert the steroid substrate without any requirement for an external energy source. This indicates that spore reserve materials are present in a nondiffusible form, or are retained by binding to intra-sporal macromolecules in some manner, and that they cannot diffuse into the suspending medium.

Although the spores of this strain of F. solani can remove the side chain of 17α-hydroxyprogesterone, we found that the spores of another strain (17) cannot cleave the side chain of the same substrate. These results indicate that there is a remarkable strain specificity in the biochemical behavior of spores.

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LITERATURE CITED

1. Casas-Campillo, C., and M. Bautista. 1965. Microbiological aspects in the hydroxylation of estrogen by Fusarium moniliforme. Appl. Microbiol. 13:977-984.
2. Eberhardt, B. M., and R. S. Beck. 1970. Localization of the β-glucosidases in Neurospora crassa. J. Bacteriol. 101:408-417.
3. Gehrig, R. P., and S. G. Knight. 1968. Formation of ketones from fatty acids by spores of Penicillium roqueforti. Nature (London) 182:1237.
4. Gerhardt, P., and J. A. Judge. 1964. Porosity of isolated cell walls of Saccharomyces cerevisiae and Bacillus megaterium. J. Bacteriol. 87:945-951.
5. Hafez-Zedan, H., and R. Plourde. 1971. "Spore plate method" for transformation of steroids by fungal spores entrapped in silica gel. Appl. Microbiol. 21:815-819.
6. Hafez-Zedan, H., O. M. El-Tayeb, and M. Abdel-Aziz. 1970. Microbiological transformation of steroids by spores and mycelial growth of some fungi. p. 73-74. Conf. Microbiol. (2nd), Cairo.
7. Iida, M., J. D. Townsley, M. Hayano, and H. J. Brodie. 1965. Preparation and properties of a cell-free enzyme system possessing both steroid dehydrogenase and hydrogenase activities. Steroids 1:159-173.
8. Lampen, J. O., E. R. Morgan, A. Slocum, and P. Arnow. 1969. Absorption of nystatin by microorganisms. J. Biol. 79:282-289.
9. Lawrence, R. C. 1966. The oxidation of fatty acids by spores of Penicillium roqueforti. J. Gen. Microbiol. 44:393-405.
10. Lawrence, R. C. 1967. The metabolism of triglycerides by spores of Penicillium roqueforti. J. Gen. Microbiol. 46:65-76.
11. Mandels, G. R. 1953. Localization of carboxylases at the surface of fungus spores by acid treatment. Exp. Cell Res. 5:48-55.
12. Mandels, G. R. 1953. The properties and surface location of an enzyme oxidizing ascorbic acid in fungus spores. Arch. Biochem. Biophys. 42:164-173.
13. Mandels, G. R., and R. Vitos. 1967. Constitutive and induced trehalose transport mechanisms in spores of the fungus Myrothecium verrucaria. J. Bacteriol. 92:159-167.
14. Manocha, M. S., and J. R. Colvin. 1967. Structure and composition of the cell wall of Neurospora crassa. J. Bacteriol. 94:202-212.
15. Marquis, R. E. 1968. Salt-induced contraction of bacterial cell walls. J. Bacteriol. 95:156-159.
16. Metzenberg, R. L. 1963. The localization of β-fructofuranosidase in Neurospora. Biochim. Biophys. Acta 77:455-465.
17. Plourde, R., O. M. El-Tayeb, and H. Hafez-Zedan. 1972. Reduction of the 20-carbonyl group of C-21 steroids by spores of Fusarium solani and other microorganisms. I. Side-chain degradation, epoxide cleavage, and substrate specificity. Appl. Microbiol. 23:601-612.
18. Prairie, R. L., and P. Talalay. 1963. Enzymatic formation of testosterone. Biochemistry 2:203-208.
19. Rahim, M. A., and C. J. Sib. 1966. Mechanism of steroid oxidation by microorganisms. XI. Enzymatic cleavage of the pregnane side-chain. J. Biol. Chem. 241:3615-3623.
20. Schleg, M. C., and S. G. Knight. 1962. Hydroxylation of progesterone by conidia from Aspergillus ochraceus. Mycologia 54:317-319.
21. Scott, W. A., and R. L. Metzenberg. 1969. Permeabilization of Neurospora conidia with phenethyl alcohol. Neurospora Newsl. 18:8-9.
22. Scott, W. A., and R. L. Metzenberg. 1970. Location of aryl sulfatase in conidia and young mycelia of Neurospora crassa. J. Bacteriol. 104:1254-1265.
23. Silver, S., and L. Wendt. 1967. Mechanism of action of phenethyl alcohol: breakdown of the cellular permeability barrier. J. Bacteriol. 93:560-566.
24. Singh, K., S. N. Sehgal, and C. Vézina. 1965. Transformation of Reichstein's Compound S and oxidation of carbohydrates by spores of Septotmyxa affinis. Can. J. Microbiol. 11:361-364.
25. Singh, K., S. N. Sehgal, and C. Vézina. 1967. Transformation of steroids by Mucor griseo-cyanus. Can. J. Microbiol. 13:1271-1281.
26. Trevithick, J. R., and R. L. Metzenberg. 1966. Molecular sieving by Neurospora cell walls during secretion of invertase isozyme. J. Bacteriol. 92:1010-1015.
27. Trevithick, J. R., and R. L. Metzenberg. 1966. Genetic alteration of pore size and other properties of the Neurospora cell wall. J. Bacteriol. 92:1016-1020.
28. Vézina, C., S. N. Sehgal, and K. Singh. 1963. Transformation of steroids by spores of microorganisms. I. Hydroxylation of progesterone by conidia of Aspergillus ochraceus. Appl. Microbiol. 11:50-57.
29. Vézina, C., S. N. Sehgal, and K. Singh. 1968. Transformation of organic compounds by fungal spores. Advan. Appl. Microbiol. 10:221-288.
30. Zalokar, M., and V. W. Cochrane. 1956. Diaphosphopyridine nucleotidase in the life cycle of Neurospora crassa. Amer. J. Bot. 43:107-110.