"Spore Plate Method" for Transformation of Steroids by Fungal Spores Entrapped in Silica Gel G

HAMDALLAH HAFEZ-ZEDAN AND ROSAIRE PLOURDE
Faculty of Pharmacy, University of Montreal, Montreal, Quebec, Canada

Received for publication 12 January 1971

A new technique for investigating steroid biotransformations involving the use of glucose-treated Silica Gel G thin-layer chromatography plates spotted with fungal spores and steroid substrates is described. The conversion is followed by the detection and identification of steroid metabolites and is carried out on single plates by using the spores of different fungi. During the entire process, the spores remain on the original spots and microscopical examination revealed no germination. The method was successfully applied to as little as 30 μg of substrates, and a single plate could be used to detect the steroid metabolizing activity of spores of as many as 15 different cultures.

The chemical activities of fungal spores were first discovered by Gehrig and Knight (1) who reported the formation of 2-heptanone from caprylic acid by spores of Penicillium roqueforti. Subsequent reports (2, 5, 6, 9) established that spores of fungi and actinomycetes can effect a wide range of conversions, especially on steroid molecules. Some of these fungal transformations with spores (7) proved to be commercially feasible. Spore-mediated reactions offered many advantages over the transformation carried out in shaken flasks, which is tedious, time-consuming, and expensive. An agar plate method was described by Vézina et al. (10) for detecting growing microorganisms which produce equilin and other estrogens from various steroids.

The work presented here describes a simple, economic, and comparatively rapid screening method for the transformation of steroids by fungal spores entrapped in Silica Gel G thin-layer chromatography (TLC) plates.

MATERIALS AND METHODS

Sporulation. Stock cultures were maintained on slants of nutrient agar dextrose (Difco) supplemented with 1% yeast extract (Difco). Cultures of Cylindrocarpon radicicola (ATCC 11011), Fusarium solani, Aspergillus niger (Faculty of Pharmacy, University of Cairo), Neurospora sitophila, and a Fusarium sp. (Faculty of Pharmacy, University of Montreal) were sporulated on Sabouraud dextrose agar (Difco). Spores of A. terreus, P. chrysogenum, an Aspergillus sp., and a Penicillium sp. (Faculty of Pharmacy, University of Montreal) were produced on Czapek-Dox agar (Difco). For Streptomyces roseo-chromogenus (ATCC 13400) and Sporotrichum epigaeum (ATCC 7145), nutrient agar (Difco) was used, and for Septomyxa affinis (ATCC 6737) the sporulation medium consisted of 1% glucose, 0.5% yeast extract, 3% malt extract (Difco) and 2.5% agar (Difco). In all cases, 100 μg of progesterone per ml of medium was added as an inducer. Abundant sporulation was attained after incubation for 5 to 7 days at 26 to 28 C. Spores were harvested in 1% phosphate buffer, pH 6.0. Spore suspensions were filtered through sterile cheesecloth. Spores were washed five times by centrifugation, resuspended in the buffer solution, and stored at 4 C.

Preparation of spore plates. Glass plates (20 by 20 cm) were coated with a 0.25-mm layer of Silica Gel G (Merck); the slurry was made with 0.1% glucose solution instead of water and spread with the DESAGA applicator. The plates were allowed to set, but not to dry, for 7 to 10 min in air at room temperature and were immediately used. Spots, 0.5 cm in diameter (2 cm away from the edge of the plate), were applied 1 cm apart in 5.0-μliter portions, each containing 30 μg of substrate dissolved in N,N-dimethylformamide. To each spot, 10 μlitters of spore suspension was added; the spore concentration was adjusted to approximately 2 × 10^8 spores per spot. On the same plate, authentic reference steroids and spore suspensions in duplicate were spotted separately as controls.

Steroid conversion. A thick pad of filter paper soaked with an aqueous 0.1% glucose solution was placed at the bottom of an air-tight rectangular chromatographic jar with internal walls lined with filter paper moistened with water. The prepared “spore plates” were transferred to the jar and incubated at 26 to 28 C for 70 hr.

Test for germination. At the end of the incubation period, the plates were removed from the jar. From each plate, one of the duplicate control spore spots was scraped off and transferred to a small test tube containing 1 ml of water. The mixture was shaken and the Silica Gel G particles were allowed to settle. The
supernatant spore suspension was pipetted and examined microscopically for germination.

**Steroid detection and characterization.** For detection, the duplicate plates were allowed to dry in air, activated at 110°C for 20 min in an oven, and cooled to room temperature. Activated plates were developed for 35 to 45 min in a mixture of benzene-isopropanol (6:1), air dried, and examined under ultraviolet light. One of the duplicate plates was sprayed with an aqueous iodine reagent (0.3% solution in 0.5% potassium iodide) and the other was sprayed with methanolic isonicotinic acid hydradize (INH) solution (4 g of INH in 1,000 ml of absolute methanol and 5 ml of concentrated hydrochloric acid) of Smith and Foell (8). Steroid spots appeared dark against a fluorescent background under ultraviolet light; they were brown or blue on a faint brownish yellow background when sprayed with the iodine reagent. With the INH reagent, they appeared yellow on a white background in visible light and exhibited yellow fluorescence under ultraviolet light. In certain cases, spots required 1 to 2 hr to produce a color with the INH reagent.

For identification, heavy reliance was placed on comparative mobility values ($R_f$), on color reactions of the unknowns established with authentic steroids, and on published data obtained from fermentation of a large number of steroids with various microorganisms.

**RESULTS AND DISCUSSION**

Steroid conversions with the spore plate method with fungal spores entrapped in Silica Gel G TLC plates are shown in Table 1. Many of the reported steroid transformations with spores in shaken flasks were also accomplished on plates: hydroxylation in $11\alpha$- and $16\alpha$-positions; reduction of the 20-keto group; oxidation of the $17\beta$-hydroxy group; epoxide cleavage; side-chain degradation with formation of a $17\beta$-ketone or a $17\beta$-hydroxyl; 1-dehydrogenation; ring A aromatization of 19-norsteroids; and lactonization of ring D.

Silica Gel G showed a good capacity to absorb water and to entrap the spores. The TLC plates made with 0.1% aqueous glucose entrapped the spores and kept them in contact with the substrate at a sufficient humidity level throughout the incubation period. Glucose solution was used to prepare the plates because spore activity requires the presence of a suitable carbohydrate (2, 3, 9; H. H. Zedan, M. S. Thesis, Univ. of Cairo, Cairo, U.A.R.) possibly for active transport of substrate, regeneration of reduced enzymatic systems involved in the reaction(s), or both.

The fungal spores were retained within the silica gel G at the original starting spot during the incubation and development with benzene-isopropanol mixture; this phenomenon may be attributed to physical and chemical entrapment as described by Johnson and Ciegler (4).

An increase in the spore concentration to more than $2 \times 10^6$ spores per spot resulted in scaling of the spots and falling from the plates on activation. Scaling was also observed by heating the plates at a temperature higher than 110°C or at 110°C for more than 20 min. When the steroid concentration was more than 75 μg per spot, inadequate separation of the conversion products on development of the plates was noticed.

Although sulfuric acid is widely used for the detection and identification of low concentrations of steroids on TLC plates, it was not suitable in this process. The iodine and INH reagents were used for detection, since glucose will char upon heating at 110 to 120°C in the presence of sulfuric acid and make the spot detection impossible.

Detection by the iodine reagent became difficult when the substrate concentration was lower than 20 μg per spot, whereas INH reagent was used with lower concentrations in a few cases. This seems to depend upon the steroid concentration per spot, the activity of spores, as well as the sensitivity of the reagent. However, INH reagent offered another advantage with spores capable of 1-dehydrogenating the $\Delta^4$-3-ketosteroids since it was used for differential detection of $\Delta^4$-3-ketosteroids and $\Delta^1$-4-3-ketosteroids on paper chromatograms (8). On the plates, $\Delta^4$-3-ketosteroids produced yellow spots within 5 to 10 min, whereas $\Delta^1$-4-3-ketosteroids exhibited colored spots only after 1 to 2 hr.

With steroid conversion systems that require no glucose, as that described by Singh et al. (6) for S. affinis, possibly because of the presence of adequate reserve carbohydrate material in the spores, sulfuric acid can be used as a spot color reagent. In addition, other in situ color reagents used for the detection of steroids on paper chromatograms may be applied.

We noted that spore activity was higher in shaken flasks than on plates. This increased activity can be attributed to different factors associated with agitation. For instance, the random distribution of the spores through agitation will produce an intimate contact between the spores and the substrate; furthermore, it provides adequate and continuous aeration to the system which is important in the transformation reactions. In the case of the plate method, these conditions cannot be fully satisfied.

Variations in substrate concentration and number of spores per spot, or thickness of the Silica Gel G layer (or both) may improve the overall conversion yield and prevent spot scaling and falling.

For screening purposes, the process appears to be as successful as transformation in shaken
Table 1. Transformation of various steroids with spores of various microorganisms by the “spore plate method”

| Microorganism          | Steroid substrate                  | Product(s)                                  |
|------------------------|------------------------------------|---------------------------------------------|
| *Cylindrocarpon radicicola, Fusarium solani, Septomyxa affinis* | Progesterone<sup>b</sup>           | Androstadienedione                          |
|                        | Progesterone<sup>b</sup>           | 1-Dehydrotestololactone                     |
|                        | Androstadienedione                 | Androstadienedione                          |
|                        | Androstenedione                    | 1-Dehydrotestololactone                     |
|                        | Estradiol                          | Estrone                                     |
|                        | 19-Nortestosterone<sup>c</sup>     | Estradiol                                   |
|                        | 16α,17α-Epoxyprogesterone<sup>e</sup> | Unidentified product                       |
|                        | Testosterone                       | 20α-Hydroxy derivative                      |
|                        |                                    | 20α-Hydroxy-1-dehydro derivative           |
|                        |                                    | 16α-Hydroxy-1-dehydrotestosterone         |
|                        |                                    | 16α-Hydroxy-1-dehydrotestololactone        |
|                        |                                    | Androstadienedione                          |
|                        |                                    | 1-Dehydrotestololactone                     |
| *Aspergillus niger*    | Progesterone<sup>b</sup>           | 11α-Hydroxy derivative                      |
| *Aspergillus* sp., *A. niger, A. terreus* | Estradiol                           | None                                        |
|                        | 19-Nortestosterone                 | None                                        |
|                        | Testosterone                       | 2 Unidentified products                     |
|                        | Androstadienedione                 | None                                        |
|                        | 16α,17α-Epoxyprogesterone          | 3 Unidentified products                     |
|                        | Androstenedione                    | 2 Unidentified products                     |
| *Aspergillus* sp., *A. terreus* | Progesterone<sup>d</sup>           | 11α-Hydroxy derivative                      |
|                        | Estradiol                          | Unidentified product                        |
| *Streptomyces roseochromogenus* | Progesterone               | 16α-Hydroxy derivative                      |
|                        | Androstadienedione                 | 16α-Hydroxy derivative                      |
|                        | Androstenedione                    | 16α-Hydroxy derivative                      |
|                        | Testosterone                       | None                                        |
|                        | Estradiol                          | None                                        |
|                        | 19-Nortestosterone                 | None                                        |
|                        | 16α,17α-Epoxyprogesterone          | None                                        |
| *Sporotrichum epigaeum* | Testosterone                       | Androstadienedione                          |
|                        | Progesterone                       | None                                        |
|                        | Androstadienedione                 | None                                        |
|                        | Androstenedione                    | None                                        |
|                        | 19-Nortestosterone                 | None                                        |
|                        | Estradiol                          | None                                        |
|                        | 16α,17α-Epoxyprogesterone          | None                                        |

<sup>a</sup> Spores: 2 × 10⁴/spot; steroid: 30 μg/spot; incubation: 70 hr; silica gel G layer thickness: 0.25 mm. The following common names are used: progesterone: pregn-4-ene-3,20-dione; testosterone: androst-4-en-17β-ol-3-one; androstenedione: androst-4-ene-3,17-dione; androstadienedione: androsta-1,4-diene-3,17-dione; 19-nortestosterone: estr-4-en-17β-ol-3-one; estradiol: estradiol: estra-1,3,5(10)-triene-3,17β-diol; 16α,17α-epoxyprogesterone: 16α,17α-oxido-pregn-4-ene-3,20-dione; 1-dehydrotestololactone: 17α-oxa-androsta-1,4-diene-3,17-dione; estrone: estra-1,3,5(10)-tri-en-3-ol-17-one.

<sup>b</sup> Transformation products were isolated and identified in a previous work (3) but not with *S. affinis*.

<sup>c</sup> Transformation products of these substrates were isolated and identified, and a detailed study on the kinetics of these biotransformations will be published later.

<sup>d</sup> Yields of products were too low for detection by the iodine reagent even with spores 2 × 10⁴/spot and steroid of 60 μg/spot.
flasks. Other potential uses for the method include: (i) isolation and identification of conversion products by streak application of both the spores and the substrate on preparative thin-layer chromatography plates; (ii) study of the relation between conversion of substrate and spore concentration, or substrate concentration, on single plates, as well as qualitative and quantitative estimation of the products; (iii) following the course of reaction with microscopic slides instead of large plates, one at a time, or by application of many spots on single plates and successive elution, or by using other conventional methods; (iv) adaptation of the method for other conversion systems, e.g., fatty acids and carbohydrates. (v) the method may find useful applications for continuous fermentation processes as that described by Johnson and Ciegler (4) for sucrose conversion by a fungal-spore column method. As spores can carry out steroid transformations on plates, it seems feasible to produce the same reactions in columns with the possibility of continuous fermentations. Consequently, the formation of an end product (or intermediate) could be controlled by its removal from the reaction sequence as soon as it is formed. This offers a great advantage in steroid biotransformations as the enzymes react in succession; one enzyme utilizes the product(s) of another as substrate(s). Some of these reactions are undesirable and limit the industrial use of highly active organisms, e.g., the 11α-hydroxylation is usually followed by the undesirable 6β-hydroxylation, and the 1-dehydrogenation is commonly followed by a side-chain removal or by complete degradation of the steroid molecule. By continuous fermentation, such subsidiary reactions may be by-passed. However, on account of steroid insolubility in aqueous media, one must find a method for their solubilization without inhibiting spore activity.

ACKNOWLEDGMENTS
We are indebted to Osama El-Tayeb, Faculty of Pharmacy, University of Cairo, U.A.R., for authentic samples of 1-dehydrotestolactone and its 16α-hydroxy analog. This work was supported by a research grant (CRM 3180) from The Medical Research Council of Canada.

LITERATURE CITED
1. Gehrig, R. F., and S. G. Knight. 1958. Formation of ketones from fatty acids by spores of Penicillium roqueforti. Nature (London) 182:1237.
2. Hafez-Zedan, H., O. El-Tayeb, and M. Abdel-Aziz. 1970. Microbiological transformation of steroids by spores and mycelial growth of some fungi. 2nd Conf. Microbiol., Cairo, p. 73–74.
3. Hafez-Zedan, H., O. El-Tayeb, and M. Abdel-Aziz. 1970. Transformation of progesterone with the vegetative growth and by non-germinating spores of Cylindrocarpon radicicola.
Fusarium solani, and Aspergillus niger. 2nd Conf. Microbiol., Cairo, p. 71-72.

4. Johnson, D. E., and A. Ciegler. 1969. Substrate conversion by fungal spores entrapped in solid matrices. Arch. Biochem. Biophys. 130: 384-388.

5. Schleg, M. C., and S. G. Knight. 1962. Hydroxylation of progesterone by conidia from Aspergillus ochraceus. Mycologia 54:317-319.

6. Singh, K., S. N. Sehgal, and C. Vezina. 1965. Transformation of Reichstein’s compound S and oxidation of carbohydrates by spores of Septomyxa affinis. Can. J. Microbiol. 11:351-364.

7. Singh, K., S. N. Sehgal, and C. Vezina. 1968. Large-scale transformation of steroids by fungal spores. Appl. Microbiol. 16:393-400.

8. Smith, L. L., and T. Foell. 1959. Differentiation of Δ4-3-ketosteroids and Δ4-4-3-ketosteroids with iionicotic acid hydrazide. Anal. Chem. 31:102-105.

9. Vezina, 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.

10. Vezina, C., K. Singh, and S. N. Sehgal. 1969. Agar plate method for detecting microorganisms which produce equilin and other estrogens from various steroids. Appl. Microbiol. 18:270-271.