Antimicrobial properties of steroid compounds have been recorded in the literature from time to time. Although Faulkner (1943) was not able to demonstrate definite antimicrobial effects of several natural estrogenic compounds, diethylstilbestrol did exhibit some degree of bactericidal action on gram-positive bacteria. Reiss (1947a, b) observed inhibitory effects of methyl testosterone and deoxycorticosterone on the growth of *Trichophyton purpureum* and *Trichophyton gypseum* in cultures and a certain curative effect on experimental infection in castrated rabbits. The antymycotic properties of androgenic and estrogenic compounds having the position 3 blocked with methyl groups, was pointed out by Rebell and Lamb (1953). Kull, Castellano, and Mayer (1953) reported on the inhibitory effect of water-soluble amino steroids on the growth of mycobacteria and Nocardiae. Fox, Carroll, and Glacy (1957) observed that none of the naturally occurring estrogens had fungistatic action; ethynyl estradiol showed some inhibitory effect on *Nocardia asteroides* at very high levels. Recently the antimicrobial properties of deoxycorticosterone on *Neurospora crassa* and other microorganisms have been pointed out (Lester, Stone, and Hechter, 1958; Kurosawa, 1958; Lester and Hechter, 1958, 1959; Chattaway, Townsley, and Barlow, 1959) and the inhibition of *Mycobacterium tuberculosis* by corticosterone and hydrocortisone was also indicated (Hennes et al., 1959). The inhibition of growth of *Tetrahymena pyriformis* by several C-21 steroids was furthermore observed by Conner (1959).

During our studies on microbial transformations of steroids we have observed several effects of steroid compounds on growth, spore germination, germ tube growth, or activities of some microorganisms. On the basis of these cursory observations, a detailed investigation was undertaken to ascertain the effects of various progesterone analogues on the growth and activities of a selected group of bacteria, actinomycetes, and fungi, including animal and plant pathogens.

**MATERIALS AND METHODS**

*Organisms.* The microorganisms used for these experiments were originally obtained from the American Type Culture Collection, from other institutions, or isolated from natural sources.

*Inhibition of radial growth.* Sabouraud-glucose-agar or potato-glucose-agar plates were prepared containing graded amounts of the steroid compounds (1 to 100 μg per ml); the compounds were incorporated into the melted agar in ethanol solution keeping the solvent concentration below the inhibitory levels. Discs of 4 mm in diameter of 1-week-old mycelial growth of the test fungus were used as inoculum. When testing dermatophytes, a standard blended mycelium obtained from submerged culture was utilized as inoculum in a procedure similar to that described by Hok et al. (1956). Depending on the organism, the plates were incubated to 28 or 37 C during periods of time ranging from 7 to 14 days. Measurements of the radial growth were recorded and the results given in terms of percentage of inhibition, taking the control culture as 100 per cent growth.

*Inhibition of growth in shaken cultures.* The test organisms were grown in liquid media containing graded amounts of the steroid (1 to 100 μg). In the case of fungi and actinomycetes, 25 ml of a medium containing 2 per cent peptone and 1 per cent glucose in distilled water dispensed in 125-ml Erlenmeyer flasks were used. After inoculation with 0.1 ml of spore (2 to 3 million per ml) or mycelial (5 mg dry weight per flask) suspensions, the cultures were incubated with agitation using rotary shakers. After 24 to 72 hr the mycelium was separated, dried to 80 C for

1 Presented at the 59th Annual Meeting of the Society of American Bacteriologists, St. Louis, Missouri, May 1959.
24 hr, and weighed. In a few instances growth was estimated as volume of wet cellular material.

**Inhibition of spore germination.** For this test, the slide method recommended by the American Phytopathological Society (1943) was used.

**Minimal inhibitory concentration.** The agar-streak dilution method (Waksman and Reilly, 1945) was used to determine the minimal inhibitory concentration of the steroids on bacteria, actinomycetes, and fungi, using in each case the appropriate agar medium: Sabouraud-glucose or potato-glucose-agar for testing fungi, nutrient agar in the case of bacteria, and Emerson's medium for actinomycetes. A dilution method using Proskauer and Beck's medium (Youmans, Doub, and Youmans, 1953; Bojalil and Medina, 1959) was used to test the inhibitory effects of the steroid on pathogenic mycobacteria and Nocardiae. Readings were recorded at the appropriate times, as a rule 24 hr for bacteria and 48 hr to 1 week for fungi; with the pathogenic mycobacteria the incubation period was extended for 4 weeks.

**Metabolic studies.** The inhibitory effect of 21,21-dimethoxy progesterone on the conversion of Reichstein's compound S to hydrocortisone by *Curvularia lunata* was studied in liquid shaken cultures. Peptone-glucose medium, 25 ml in 125-ml Erlenmeyer flasks, was sterilized and inoculated with spores or mycelial suspensions. In a set of cultures, amounts of steroid ranging from 1 to 100 μg per ml were added to the culture medium along with the inoculum and 10 mg of the steroid to be converted (compound S); in another set the organism was first grown for 48 hr and then graded concentrations of 21,21-dimethoxy progesterone and 10 mg of compound S were added. After 24 hr of oxidation, the cultures were extracted with methyl dichloride and the extracts analyzed by paper chromatography (Zaffaroni, 1953). The conversion product and the remaining starting material were determined by ultraviolet spectroscopy (240 m) after elution of the spots with methanol.

**RESULTS AND DISCUSSION**

From a group of 30 progesterone analogues tested for inhibitory action on radial growth of *C. lunata* and *Trichophyton mentagrophytes*, 21,21-dimethoxy progesterone (DMP) and 21,21-diethoxy progesterone showed the highest fungistic effect, the first organism being the most sensitive (Table 1). The fungistatic action of dimethoxy progesterone decreased with the complexity of modifications at C-21, as shown in the case of diisopropyl and benzyl derivatives. It is interesting to note the reversal of the comparative sensitivity of *C. lunata* and the dermatologic effects of the steroids tested on *Trichophyton mentagrophytes*.
Dione and progesterone, giving the activity (pregnane compounds) caused elimination of the activity; ATCC 10031, Pseudomonas aeruginosa ATCC 10145, Pseudomonas fluorescens ATCC 11251, Pseudomonas oleovorans ATCC 8062, Pseudomonas testosteroni ATCC 11996, Erwinia ananass E-B, ENCB, Erwinia ananass E-C, ENCB, Agrobacterium radiobacter ATCC 10311, Rhizobium phaseoli ATCC 10521, Rhizobium trifolii ATCC 10528.

Test organisms were originally received from the following institutions: RU, Rutgers University; ATCC, American Type Culture Collection; HI, Hospital Infantil, Mexico, D. F. through Dr. J. Olarte; ENCB, Escuela Nacional de Ciencias Biologicas, Mexico, D. F.; NRRL, Northern Utilization Research and Development Division, U. S. Department of Agriculture.

C. lunata. The introduction of chlorine at C-4 decreased the activity of 21,21-dimethoxy progesterone in particular on C. lunata and the introduction of 11-α-hydroxy, 17-α-hydroxy, 6-β-nitro, or 6-α-methyl groups in the progesterone molecule had a similar effect, decreasing considerably the fungistatic properties of the steroid at least on one of the test organisms. The combined introduction of 17-α-hydroxy...
and 6-α-methyl groups or 6-α-fluoro and 17-acetoxy groups in the progesterone molecule deprived the steroid of fungistatic properties on C. lunata. On the other hand, the fungistatic effect on T. mentagrophytes was influenced to a lesser degree by specific modifications of the dimethoxy progesterone molecule, but complexity of groups attached to C-21 and reduction of Δ^3-3-keto group, decreased the fungistatic action to some extent. Progesterone, 19-norprogesterone, and deoxycorticosterone were highly active on T. mentagrophytes, the fungistasis being affected by further modifications in the steroid molecule as hydroxylations in position 11 or 17, singly or in combination; this can be illustrated comparing the effect of 19-norprogesterone and the 11-α-

hydroxy derivative or compound S and hydrocortisone. The introduction of chlorine or fluorine nullify the fungistatic activity exhibited by the parent steroid. In a recent study, Lester et al. (1958) demonstrated that the inhibitory effect of deoxycorticosterone on the growth of N. crassa was almost lost by esterification at C-21, unsaturation of ring A, reduction of Δ^3-3-keto function, or introduction of hydroxyl groups. Conner (1959), studying the effect of steroids on the growth of T. piriformis, observed that the presence of the side chain was necessary for inhibitory action; the introduction of oxygen at C-21 or C-11 enhanced the effect, but hydroxylation at C-17, unsaturation of ring A, and substitution at C-9 lowered the inhibitory properties. It is clear from these observations that antimicrobial properties are not only dependent on the steroid structure but also on the particular test organism. In this respect, a number of species of Curvularia showed different degrees of sensitivity to dimethoxy progesterone (Fig. 1).
TABLE 5
Inhibition of pathogenic fungi by 21,21-dimethoxy progesterone

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Minimal Inhibitory Conc</th>
<th>µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colletotrichum caffeanum CF-2</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>ENCB</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Colletotrichum gloesporioides CBS</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Curvularia lunata 190 Sy</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Fusarium moniliforme ATCC 9851</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Fusarium solani CBS</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Microsporum canis ATCC 9865</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Rhizoctonia solani ATCC 10145</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes ISET</td>
<td></td>
<td>25-50</td>
</tr>
<tr>
<td>Trichophyton mentagrophytes ATCC</td>
<td></td>
<td>25-50</td>
</tr>
<tr>
<td>9129</td>
<td></td>
<td>25-50</td>
</tr>
</tbody>
</table>

Test organisms received from the institutions indicated in previous tables; CBS, Centraalbureau voor Schimmelcultures; Sy, Syntex Collection.

In view of these results, a more extensive study was carried out to determine the antimicrobial spectrum of the compound using dilution methods; the highest amount of steroid utilized was 100 µg per ml. The results revealed that the steroid was inactive on gram-negative bacteria (Table 2); however, some gram-positive bacteria were inhibited at concentrations ranging from 1 to 100 µg per ml (Table 3). It was observed that mycobacteria were the most sensitive organisms, particularly photochromogens and scotochromogens. The photochromogen, strain P-18, is resistant to 50 µg per ml of isoniazid or streptomycin. A particular strain of M. tuberculosis, resistant to 50 µg per ml of isoniazid, showed sensitivity to 25 µg per ml of the steroid. These results are similar to those previously reported by Kull et al. (1953) who observed that Mycobacterium smegmatis strain 607 and M. tuberculosis strain H37Rv were inhibited in vitro by several amino steroids at concentrations between 30 and 130 µg per ml. Furthermore, Lester and Hechter (1958) indicated the inhibitory effect of deoxy corticosterone on Mycobacterium ranae at concentrations from 150 to 250 µg per ml and Hennes et al. (1959) found that hydrocortisone and cortisone inhibited the growth of M. tuberculosis at concentrations of 100 µg per ml, although 20 µg per ml gave slight inhibition. No differences were observed by these authors with isoniazid sensitive or resistant strains. 21, 21-Dimethoxy progesterone was active on M. tuberculosis strain H37Rv at 50 µg per ml, however, some differences in sensitivity can be observed with different isolates.

A number of species of Nocardia, both saprophytic and pathogenic, were inhibited by dimethoxy progesterone at concentrations from 5 to 50 µg per ml, N. asteroides and Nocardia brasiliensis being the most sensitive (Table 4). In this connection it is interesting to note that several workers indicated the inhibitory effect of some steroids on Nocardiae. Kull et al. (1953) reported the inhibition of growth of N. asteroides by 3-keto-21-(1-piperidyl)-4, 17-pregnadiene mono-hydrobromide at concentrations between 10 to 20 µg per ml, and Rebell and Lamb (1953) indicated a similar effect of 3-β-methoxy-17-β-hydroxy-Δ4-androstene-17-α-acetate at a concentration of 10 µg per ml. Data in Fig. 3 show the growth of N. asteroides strain 8694 in shaken cultures was inhibited by amounts of dimethoxy progesterone ranging from 1 to 25 µg per ml; apparently the inhibition rate was higher than observed in the case of C. lunata and T. mentagrophytes.

No activity was noticed against yeasts (Table 2) but several filamentous fungi were inhibited at concentrations between 25 and 100 µg per ml (Table 5). Particular attention was given to the study of the inhibitory effect of dimethoxy progesterone on C. lunata, an organism utilized to carry out the 11-β-hydroxylation of some steroid compounds (Shull, Kita, and Davison, 1953). Both radial and mycelial growth in shaken cultures were used to ascertain the antifungal effect. Concentrations of dimethoxy progesterone between 6.25 and 100 µg per ml limited the radial growth significantly (Fig. 2). Concentrations of dimethoxy progesterone from 1 to 10 µg per ml were inhibitory for mycelial growth in shaken cultures (Fig. 3). As indicated in the results corresponding to 72 hr growth, the fungistatic effect on C. lunata was definitely transitory. In this regard, further experiments demonstrated that the fungus is able to metabolize the dimethoxy progesterone molecule converting it into more polar unidentified compounds having diminished fungistatic properties; when isolated and tested for inhibition of radial growth on C. lunata, these compounds showed 30 to 60 per cent less activity than the original one. The growth rate recovery of C. lunata runs parallel with the modification of the dimethoxy progesterone molecule. This behavior resembles...
that previously indicated in studies with *N. crassa* (Lester et al., 1958) and *Rhizopus nigricans* (Capek, Pavlu, and Hanc, 1958) postulating a detoxifying mechanism during the microbial transformation of steroids. Complementary experiments demonstrated definite fungistatic effects by using spores or mycelial inoculum. Spore germination was inhibited by concentrations of steroid as low as 2.5 μg per ml giving 25 per cent inhibition in 6 hr; 50 μg per ml gave 80 per cent inhibition under the same conditions (Fig. 4). The effect of steroid on *C. lunata* was compared with the activity on several plant and human pathogenic fungi (Fig. 5). Most of the inhibitory effect of dimethoxy progesterone on *C. lunata* and *Helminthosporium sativum* was attained at levels between 1 and 10 μg per ml and above this point the inhibition proceeded at a very low rate. In contrast, *T. mentagrophytes* was inhibited at a higher rate between 10 and 100 μg per ml. Moderate inhibition was obtained with the other test organisms.

It was observed that several steroids and precursors (cortisone, hydrocortisone, ergosterol, squalene) did not affect the growth of *C. lunata*. When tested in mixtures with dimethoxy progesterone, these compounds did not modify the
inhibition of radial growth (Table 6). In shaken cultures, however, a reversible action was observed with squalene and deoxycorticosterone at concentrations between 1 and 20 μg per ml. No clear explanation can be given for this behavior but experimental evidence obtained so far indicates that under submerged conditions the presence on either squalene or deoxycorticosterone increases the conversion rate of dimethoxy progesterone to less toxic compounds. Ergosterol was stimulatory and hydrocortisone did not modify the inhibitory effect (Table 7).

The results obtained in the study of the effect of dimethoxy progesterone on the course of the transformation of Reichstein's compound S to hydrocortisone by a particular strain of C. lunata are shown in Fig. 6. When dimethoxy progesterone was added to the culture medium

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**Fig. 4.** Effect of 21,21-dimethoxy progesterone (DMP) on spore germination in *Curvularia lunata*

**Fig. 5.** Comparative effect of 21,21-dimethoxy progesterone (DMP) on pathogenic fungi
TABLE 6
Effect of 21,21-dimethoxy progesterone on radial growth of Curvularia lunata in the presence of other steroids

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Concentration (ppm)</th>
<th>Inhibition Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>21,21-Dimethoxy progesterone</td>
<td>100</td>
<td>75-85</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>50</td>
<td>14-20</td>
</tr>
<tr>
<td>Cortisone</td>
<td>50</td>
<td>0-11</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>17-β-Estradiol</td>
<td>50</td>
<td>33</td>
</tr>
<tr>
<td>Estrone</td>
<td>50</td>
<td>17-18</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Squalene</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>DMP + deoxycorticosterone</td>
<td>100 + 50</td>
<td>76-78</td>
</tr>
<tr>
<td>DMP + cortisone</td>
<td>100 + 50</td>
<td>75-78</td>
</tr>
<tr>
<td>DMP + hydrocortisone</td>
<td>100 + 50</td>
<td>73-77</td>
</tr>
<tr>
<td>DMP + estradiol</td>
<td>100 + 50</td>
<td>65-76</td>
</tr>
<tr>
<td>DMP + ergosterol</td>
<td>100 + 50</td>
<td>65-75</td>
</tr>
<tr>
<td>DMP + squalene</td>
<td>100 + 50</td>
<td>65-75</td>
</tr>
</tbody>
</table>

along with the inoculum and the steroid to be converted (compound S), a striking decrease in the 11-β-hydroxylating reaction was observed (curve A); concentrations ranging from 1 to 10 μg per ml inhibited the conversion to a considerable extent (30 to 86 per cent). Increased amounts of the inhibitory steroid gave the same effect; this behavior suggests that some limiting mechanisms, such as steroid solubility or binding on the mycelial surface, were in operation. When the compound was added after a 48-hr growing period along with the compound S, the inhibitory effect on the 11-β-hydroxylation was lessened, requiring higher concentrations of dimethoxy progesterone to attain the same degree of inhibition (curve B). This result indicates that dimethoxy progesterone interferes with the formation and activity of the 11-β-hydroxylating mechanisms in C. lunata. However, experiments utilizing a particular strain of Cunninghamella bainieri, which converts compound S into a mixture of epimeric compounds (hydrocortisone and epi-compound F), and with adrenal gland homogenates, which convert compound S into hydrocortisone, demonstrated that the steroid has no appreciable effect on the enzymatic action (Fig. 6). This indicates that a specific mechanism is acting in the case of C. lunata.

TABLE 7
Effect of 21,21-dimethoxy progesterone on submerged growth of Curvularia lunata in the presence of other steroids

<table>
<thead>
<tr>
<th>Steroids</th>
<th>Concentration (μg/ml)</th>
<th>Dry Weight per Flask (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No steroid</td>
<td>88.0</td>
<td></td>
</tr>
<tr>
<td>Ergosterol</td>
<td>102.7</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>98.0</td>
<td></td>
</tr>
<tr>
<td>Ergosterol</td>
<td>81.4</td>
<td></td>
</tr>
<tr>
<td>Squalene</td>
<td>84.0</td>
<td></td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>92.7</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>85.3</td>
<td></td>
</tr>
<tr>
<td>21,21-Dimethoxy progesterone</td>
<td>95.1</td>
<td></td>
</tr>
<tr>
<td>Squalene + DMP</td>
<td>92.0</td>
<td></td>
</tr>
<tr>
<td>Ergosterol + DMP</td>
<td>89.8</td>
<td></td>
</tr>
<tr>
<td>Deoxycorticosterone + DMP</td>
<td>84.0</td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone + DMP</td>
<td>54.0</td>
<td></td>
</tr>
</tbody>
</table>

The fungistatic action was parallel to the inhibitory effect on the conversion of compound S to hydrocortisone, which indicates interference with enzyme synthesis, although interference with cell permeability is not excluded. When the steroid was added to the system after 48 hr growth, at which point the 11-β-hydroxylating mechanisms were in operation, a definite inhibition of enzyme action was also observed.
Thirty progesterone analogues were tested for inhibitory activity on radial growth of Curvularia lunata and Trichophyton mentagrophytes. 21, 21-Dimethoxy progesterone and 21, 21-diethoxy progesterone were the most active compounds on C. lunata and dimethoxy progesterone, progesterone, 19-norprogesterone, and deoxycorticosterone were most inhibitory on T. mentagrophytes. Dimethoxy progesterone also inhibited Colletotrichum coffeicinum, Fusarium moniliforme, Fusarium sativum, and Rhizoctonia solani. Gram-positive bacteria, particularly saprophytic and pathogenic mycobacteria were inhibited by concentrations of dimethoxy progesterone ranging from 1 to 100 µg per ml. Nocardia asteroides and Nocardia brasiliensis showed sensitivity to the steroid at concentrations from 5 to 50 µg per ml. No inhibitory effect was observed on gram-negative bacteria and yeasts. Dimethoxy progesterone was essentially bacteriostatic or fungistatic and its effect on radial growth of C. lunata was not modified by inactive steroids (cortisone, hydrocortisone) or precursors (squalene); however, the inhibition of mycelial synthesis in submerged cultures was reversed by squalene or deoxy cortisolosterone. Under the same growth conditions, ergosterol was stimulatory and hydrocortisone inactive. The complexity of groups attached to C-21, the suppression of the double bond between C-4 and C-5, reduction of A4-3 keto function, introduction of a double bond at C-1, and halogenations were modifications which brought about marked decrease in the fungistatic action of dimethoxy progesterone on C. lunata. The inhibition of T. mentagrophytes was influenced to a lesser degree by modifications of the steroid molecule. Further modifications of progesterone molecule resulted in an almost complete loss of activity on both test organisms. Metabolic studies in connection with the 11-β-hydroxylation of Reichstein's compound S by C. lunata demonstrated that dimethoxy progesterone at the concentrations from 1 to 10 µg per ml significantly inhibited the formation of hydrocortisone. Results indicate that the inhibitory steroid prevents both cellular synthesis and enzyme action in C. lunata. In contrast, dimethoxy progesterone did not affect the steroid hydroxylating abilities of Cunninghamella bainieri or adrenal gland homogenates.

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