The Influence of Exogenous Steroids on the Growth of *Aspergillus niger* and *Torula utilis*

W. E. JEFFERSON, JR., and GLADYS SISCO

From the Division of Chemistry, University of Tennessee Medical Units, Memphis

ABSTRACT The yield of *Aspergillus niger* mycelium from a synthetic medium can be increased by the addition of microgram quantities of cholesterol, ergosterol, cholestanol, 7-dehydrocholesterol, stigmasterol, sitosterol, pregnenolone, and the vitamins D. The stimulation is not due to degradation to the acetate level. It is obtained only in highly aerated cultures. The rate of growth of *Torula utilis* was not increased.

Both organisms were inhibited by desoxycorticosterone, testosterone, androstenedione, cortisone acetate, progesterone, and diethylstilbestrol. *T. utilis* was also inhibited by estradiol.

A small decrease in progesterone inhibition of *T. utilis* was obtained by adding ergosterol, cholesterol, or pregnenolone.

Of the compounds which have been adequately tested the order of stimulatory activity for *A. niger* is: ergosterol > cholesterol > stigmasterol > 7-dehydrocholesterol > cholestanol > pregnenolone. Progesterone was inhibitory at low concentrations but stimulatory at higher ones, while 17-hydroxyprogesterone was neither inhibitory nor stimulatory. Desoxycorticosterone and testosterone were inhibitory at all concentrations. Complete inhibition of the growth of the fungus was not obtained with any of the steroids.

It is concluded that *A. niger* has a metabolic requirement for a steroid with a hydroxy group on carbon 3, a double bond in the 5-6 position, and a side chain similar to that in ergosterol or cholesterol and that this material is growth-limiting in the early stages of the cultures described.

The occurrence in fungi of sterols and of enzymes which utilize sterols as substrates is well known (1), but relatively little is known about the role of these compounds in fungal metabolism. Several reports exist, however, which suggest a vital physiological role for the sterols in fungi as well as in many other organisms. Vishniac (2) found an absolute requirement in the marine fungus, *Labyrinthula vitellina*, for a 27, 28, or 29-carbon steroid with either a 3β-hydroxy or a 3-keto configuration, and Andreason (3) found that ergosterol was

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required for anaerobic but not for aerobic growth by a strain of *Saccharomyces cerevisiae*. In addition to these two reports dealing with fungi, protozoa (4), pleuropneumonia organisms (5), insect larvae (4), and Mycobacteria (6) have been shown to require sterols or to be stimulated by sterols. In general the steroid requirements of all the above organisms are quite similar (4).

There are numerous examples of inhibition of the growth and metabolism of fungi by steroids (7–10). The effect of desoxycorticosterone on *Neurospora crassa* has been extensively studied (8, 9) and appears to be quite specific. This steroid interferes with the uptake of a variety of materials by *Neurospora* mycelium including sugars and amino acids. Its effect on the intracellular accumulation of rubidium and potassium has aroused considerable interest (11, 12).

These varied reports suggested that fungi might be a fertile field for the study of steroid function. An initial attempt to approach the problem by the isolation of steroid-requiring mutants proved unsuccessful. However, in the course of this attempt several effects of added steroids on the growth and metabolism of *A. niger* were observed and investigated. This paper reports findings demonstrating that steroids can have a considerable inhibitory or stimulatory effect on the growth of fungi.

MATERIALS AND METHODS

Cultures *Aspergillus niger* NRRL-3 and *Torula utilis* NRRL-Y-900 were used throughout.

Medium A completely synthetic medium of the following composition was used for both organisms:

Sucrose		50 gm
KH_2PO_4		1 gm
NH_4NO_3		2.5 gm
$MgSO_4 \cdot 7H_2O$		0.25 gm
Fe	as $FeCl_3 \cdot 6H_2O$	1.3 mg
Zn	as $ZnCl_2$	0.25 mg
Ca	as $CaCl_2 \cdot 2H_2O$	1.3 mg
B	as H_3BO_3	0.05 mg
Mo	as $Na_2MoO_4 \cdot 2H_2O$	0.05 mg
Co	as $CoCl_2 \cdot 6H_2O$	0.05 mg
Ni	as $NiCl_2 \cdot 6H_2O$	0.05 mg
Ga	as $GaCl_3$	0.05 mg
Cu	as $CuCl_2 \cdot 2H_2O$	0.06 mg
Mn	as $MnSO_4 \cdot H_2O$	0.10 mg
V	as $NaVO_3 \cdot 4H_2O$	0.05 mg
Se	as $Na_2SeO_4 \cdot 10H_2O$	0.05 mg
H_2O	to make	1.0 liter

The pH was adjusted to 3.3 with glass-distilled HCl.

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Trace element contamination of the medium was reduced by purifying various of the ingredients as follows:—

H_2O was distilled and then treated in a Barnstead demineralizer.

Sucrose was prepared from ordinary commercial sucrose by passing a 50 per cent (w/v) solution in demineralized water through a Dowex-50 (H-cycle) column.

NH_4NO_3 was prepared as a 50 per cent (w/v) solution by combining HNO_3 and NH_3, both of which had been distilled in an all glass still.

$MgSO_4 \cdot 7H_2O$ was recrystallized from demineralized water containing 0.1 gm of ethylenediaminetetraacetic acid per 100 ml.

KH_2PO_4 was recrystallized by the same procedure as was used for the magnesium sulfate.

$GaCl_3$ and *$ZnCl_2$* were prepared by dissolving the metals.

The remaining ingredients of the culture medium were "reagent" grade chemicals.

Steroids and related compounds were obtained from Mann Research Laboratories except for cholesterol and diethylstilbestrol which were Merck and Co. products.

Steroids and other lipid-soluble substances were dissolved in ether and added to the medium prior to sterilization. The same amount of ether was added to all flasks in the same experiment.

Inoculation was accomplished by pipetting a suspension of *A. niger* conidia in demineralized water or of *T. utilis* cells in culture medium.

Incubation was carried out at 26°C on a reciprocating shaker until adequate growth was obtained. This required 24 to 48 hours for *T. utilis* and 48 to 72 hours for *A. niger*. Unless otherwise stated, each culture consisted of 30 ml of medium in a 300 ml Erlenmeyer flask.

The *amount of growth* was determined as dry weight of mycelium in the case of *A. niger* and by turbidity measurement in the *T. utilis* experiments. Dry weights were determined by collecting the mycelium on weighed filter papers in a Büchner funnel and drying the pads to constant weight in a vacuum oven at 50°C. A preliminary drying in an air stream at 50°C was used in some experiments.

Statistical Procedures The probability values in Table I were calculated for a two-sided test of the difference in two means without assuming equal variances (13). The 95 per cent confidence intervals given in Tables II to VIII were calculated by the method of Dunnett (14) for comparing several treatments with a control. The values are for a one-sided test.

The Scheffé test (15) was used in Table IX to calculate 95 per cent confidence limits for the difference between means which permit comparisons between all means. (The standard error of the mean is also included to give a more familiar estimate of the reliability of the data.)

The ranges indicated in the figures are standard errors of the mean.

Occasional values which differed widely from their replicates were discarded if the rejection quotient (16) indicated a 90 per cent probability that they were not drawn from the same population.

EXPERIMENTAL

The effects of a variety of steroids and related materials on the dry weight of *A. niger* mycelium obtained after a 72 hour incubation are shown in Table I.

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Considerable variation between replicate dry weight determinations was encountered. Statistical validity was, therefore, possible only for compounds which caused a large change in the amount of growth. A stimulatory group (ergosterol, cholesterol, 7-dehydrocholesterol, vitamin D_2, and vitamin D_3) and an inhibitory group (progesterone, desoxycorticosterone, androstenedione, cortisone acetate, testosterone, and diethylstilbestrol) are clearly

TABLE I

THE EFFECT OF VARIOUS STEROIDS ON THE RATE OF GROWTH OF ASPERGILLUS NIGER

	Dry weight of mycelium					
	Experiment I			Experiment II		
Steroid or related material*	*mg*	Treatment minus control	*p*‡ less than	*mg*	Treatment minus control	*p*‡ less than
None	101.4	—	—	278.0	—	—
Ergosterol	210.3	108.9	0.01	368.3	89.4	0.01
Cholesterol	191.7	90.3	0.01	368.5	89.6	0.01
7-dehydrocholesterol	173.4	71.9	0.2	323.6	53.7	0.01
Vitamin D_2	151.1	49.7	0.01	—	—	—
Vitamin D_3	177.0	75.6	0.2	331.1	52.2	0.01
Pregnenolone	122.0	20.6	0.2	287.5	8.6	0.9
Estrone	104.5	3.1	0.8	266.9	−12.1	0.7
Estradiol	116.6	15.2	0.2	270.6	−8.4	0.3
Diethylstilbestrol	65.8	−35.6	0.02	103.1	−175.8	0.01
Progesterone	81.1	−20.3	0.2	170.9	−108.0	0.01
Testosterone	75.0	−26.4	0.05	195.0	−83.9	0.1
Androstenedione	83.0	−18.4	0.1	194.5	−84.5	0.01
Desoxycorticosterone	89.5	−11.9	0.3	190.5	−88.4	0.01
Cortisone acetate	82.0	−19.4	0.1	180.6	−98.3	0.01
Squalene	110.4	9.0	0.3	237.4	−41.5	0.02
Phytol	73.6	−27.8	0.2	263.5	−15.5	0.2

* All substances tested at a concentration of 10 μg/ml.

‡ Calculated for the difference between each treatment and the control without assuming equal variances.

evident in spite of this limitation. Subsequent experiments also showed cholestanol, stigmasterol, pregnenolone and sitosterol to be stimulatory.

Two of the compounds in Table I, estradiol and estrone, produced no effect in this experiment but had appeared to be as stimulatory as ergosterol in preliminary experiments. This seeming anomaly was resolved when it was realized that the preliminary experiments were conducted in smaller flasks. That this was indeed an important factor is shown in Table II. The effects of estrone and ergosterol on the rate of growth of *A. niger* when the same quantity of medium is incubated in various size flasks are shown. The effect of estrone was slightly greater in the small flasks while that of ergosterol was much

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greater in the larger ones. The response to these two steroids is, therefore, dependent on the degree of aeration.

The stimulatory response obtained suggests an important metabolic role for some sterol or sterols related to cholesterol or ergosterol in the metabolism of *A. niger*. The possibility exists, however, that some degradative fragment formed from these compounds is the material which is actually stimulatory. The possibility that we were measuring "acetate replacing" activity was disproven by testing the effects of ergosterol and of sodium acetate on similar cultures. The results of this test are shown in Table III. Sodium acetate did

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TABLE II

THE EFFECT OF AERATION ON THE RESPONSE OF ASPERGILLUS NIGER TO ESTRONE AND ERGOSTEROL

	Dry weight					
	50 ml flasks		125 ml flasks		300 ml flasks	
Steroid	*mg*	Treatment minus control*	*mg*	Treatment minus control*	*mg*	Treatment minus control*
Experiment one						
None	49.4	—	112.9	—	115.0	—
Estrone	57.1	7.7±16.1	135.1	22.4±25.0	152.2	37.2±18.6
Ergosterol	60.0	10.9±16.1	126.3	13.4±25.0	212.2	97.2±18.6
Experiment two						
None	12.2	—	40.0	—	56.2	—
Estrone	25.6	13.4±6.4	58.3	18.3±12.6	66.3	10.1±14.8
Ergosterol	21.4	9.2±6.4	56.5	16.5±12.6	127.0	70.8±14.8
Experiment three						
None	14.6	—	66.4	—	58.4	—
Estrone	16.7	2.1±9.7	84.7	18.3±23.0	44.2	−14.2±22.4
Ergosterol	21.7	7.1±9.7	99.2	32.8±21.8	119.5	61.1±22.4

All flasks contained 30 ml of sucrose-mineral medium. Steroids were at a concentration of 10 μg/ml.

* Plus or minus 95 per cent confidence interval for the difference in two means (Dunnett test).

stimulate the cultures but the maximum rate of growth in the presence of sodium acetate was less than that obtained when ergosterol was present. Experiment 2 shows that the effect of ergosterol could also be superimposed on that of sodium acetate. Thus, even though the possibility of some degradation has not been eliminated, the stimulation is not due to complete fragmentation of the steroid molecule.

The group of sterols which consistently produced the greatest stimulation were all thought to be purified natural products and might, therefore, contain traces of non-steroid substances which could account for their activity. Such a possibility has not been completely eliminated but it has been shown that the stimulation obtained is not due to contamination with any of the vitamins which are usually added to fungal media. Table IV shows that the

steroid response was at least as great in a medium supplemented with a vitamin mixture or with biotin alone as in the control medium. D,L-Thioctic acid was tested separately with no effect.

The possibility that the effects seen were due to chemical conversions of the steroids which were induced by autoclaving was eliminated for ergosterol, progesterone, and estrone. This was done by adding filter-sterilized solutions of these steroids to sterile flasks. The solvent was then removed *in vacuo* at room temperature after which sterile medium was added and the response

TABLE III

THE EFFECT OF ERGOSTEROL COMPARED TO THAT OF ACETATE ON THE RATE OF GROWTH OF ASPERGILLUS NIGER

Compound added	Concentration	Mycelium weight	Treatment minus control*
	μg/ml	*mg*	
Experiment one			
None	—	47.1	—
Ergosterol	10.0	103.0	55.0±18.4
Ergosterol	20.0	124.0	76.9±19.1
Sodium acetate	10.0	45.4	−1.7±18.4
Sodium acetate	20.0	60.3	13.2±18.4
Sodium acetate	40.0	62.1	15.0±18.4
Sodium acetate	80.0	67.3	20.2±18.4
Experiment two			
None	—	51.1	—
Ergosterol	10.0	108.7	57.1±19.2
Acetic acid	100.0	73.1	22.0±19.2
Ergosterol	10.0		
plus acetic acid	100.0	130.6	56.9±19.2‡

* Plus or minus 95 per cent confidence interval for the difference in two means (Dunnett test).
‡ Compared to acetic acid alone.

determined as previously described. Cultures prepared in this way responded in a manner essentially identical to those in which the steroids were added prior to autoclaving.

Results essentially similar to those already described were obtained when either glucose, fructose, or glycerol was substituted for the sucrose in the culture medium.

The effect of incubation time on the stimulation of *A. niger* by ergosterol was also investigated. It proved to be non-critical in that stimulation was present at the earliest time that provided a weighable quantity of mycelium (33 hours) and continued until very heavy growth was obtained (59 hours).

Torula To determine whether or not the results obtained with *A. niger* were applicable to a wider group of organisms, some of the experiments

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TABLE IV

THE EFFECT OF STEROIDS ON THE GROWTH OF ASPERGILLUS NIGER IN THE PRESENCE OF A VITAMIN MIXTURE AND BIOTIN ALONE

Vitamins	Steroid	Concentration	Mycelium	Treatment minus control*
		μg/ml	*mg*	
None	None	—	126.1	—
None	Ergosterol	10	175.9	49.8±49.6
None	Estrone	10	169.1	43.0±55.7
None	Progesterone	12	98.2	−27.9±49.6
None	Testosterone	10	104.0	−22.1±49.6
Mixture	None	—	182.2	—
Mixture	Ergosterol	10	238.3	56.1±57.2
Mixture	Estrone	10	240.3	58.1±57.2
Mixture	Progesterone	12	98.8	−83.4±57.2
Mixture	Testosterone	10	118.9	−53.3±57.2
Biotin	None	—	134.1	—
Biotin	Ergosterol	10	208.4	74.3±50.0
Biotin	Estrone	10	188.1	54.0±54.5
Biotin	Progesterone	12	64.1	−70.0±50.0
Biotin	Testosterone	10	92.1	−42.1±50.0

The components of the vitamin mixture and the amount of each contained in 1 liter of medium were: biotin, 2 μg, Ca pantothenate, 400 μg, folic acid, 2 μg, inositol, 2 mg, niacin, 400 μg, *p*-aminobenzoic acid, 200 μg, pyridoxine hydrochloride, 400 μg, riboflavin, 200 μg, and thiamine hydrochloride, 400 μg.

* Plus or minus 95 per cent confidence interval for the difference in two means (Dunnett test).

TABLE V

THE EFFECT OF VARIOUS STEROIDS ON THE GROWTH OF TORULA UTILIS

Steroid	Turbidity	
	Klett units	Treatment minus control*
None	190	—
Ergosterol	213	23.0±39.4
Cholesterol	192	2.7±39.4
Estrone	153	−37.0±39.4
Progesterone	15	−174.7±39.4
Estradiol	114	−75.4±39.4
Testosterone	87	−103.0±39.4
Androstenedione	32	−157.7±39.4
Desoxycorticosterone	40	−150.3±39.4

All steroids were tested at a concentration of 10 μg/ml.

* Plus or minus 95 per cent confidence interval for the difference in two means (Dunnett test).

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TABLE VI

THE PARTIAL REVERSAL OF PROGESTERONE INHIBITION OF TORULA UTILIS BY ERGOSTEROL AND CHOLESTEROL

Steroid	Turbidity	
	Klett units	Treatment minus control*
None	190	—
Progesterone	15	−174.7±39.4
Ergosterol	213	23.0±39.4
Cholesterol	192	2.7±39.4
Progesterone plus ergosterol	32	16.7±15.6‡
Progesterone plus cholesterol	52	36.7±15.6‡

All steroids at 10 μg/ml.

* Plus or minus 95 per cent confidence limit for the difference in two means (Dunnett test).

‡ Compared to progesterone alone.

described above were repeated with *T. utilis*. The effects of several steroids on the rate of growth of this organism are shown in Table V. It can be seen that the inhibitions obtained were more pronounced than those obtained when the same compounds were tested against *A. niger*, but there was no evidence for stimulation by any of the compounds tested. With the exception of estradiol, which was slightly inhibitory to *T. utilis* but not to *A. niger*, it can be said that those materials which inhibited *A. niger* also inhibited *T. utilis* and that those which stimulated *A. niger* had no effect on *T. utilis*.

The existence of stimulation and inhibition of the same organisms by structurally related compounds suggested that some kind of competitive relation-

TABLE VII

THE PARTIAL REVERSAL OF PROGESTERONE INHIBITION OF TORULA UTILIS BY PREGNENOLONE

Progesterone	Pregnenolone	Turbidity Klett units	Treatment minus controls*
μg/ml	*μg/ml*		
—	—	165.8	—
2	—	121.2	−44.6±7.8
4	—	96.2	−69.6±7.8
6	—	83.4	−82.4±7.8
—	8	165.8	0.0±7.8
—	16	159.8	−6.0±7.8
2	8	134.8	13.4±9.5‡
2	16	129.3	8.1±16.0‡
4	8	120.6	22.4±10.2‡
4	16	105.6	9.4±10.2‡
6	8	92.6	9.2±6.8‡
6	16	94.2	10.8±6.8‡

* Plus or minus 95 per cent confidence limit for the difference in two means (Dunnett test).

‡ Compared to same concentration of progesterone alone.

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ship might exist; *i.e.*, that ergosterol or a related sterol played an essential role in these fungi and that the inhibitory steroids were interfering with its function or synthesis.

A preliminary test of this idea was made using *T. utilis* as the test organism. First we attempted to reverse progesterone inhibition with cholesterol or ergosterol. The results are shown in Table VI. Both compounds appeared to cause a slight decrease in inhibition but it was far from complete, and was not increased by further increases in the concentration of the sterols. Pregnenolone was tested similarly with the results shown in Table VII. Again there was some

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TABLE VIII

A COMPARISON OF THE RATE OF GROWTH OF PROGESTERONE-INHIBITED AND NORMAL TORULA UTILIS

Time of incubation	Turbidity Klett units		
	Control	10 μg/ml progesterone	20 μg/ml progesterone
hrs.			
9.6	13, 13	9, 11	10, 9
11.4	19, 20	16, 15	19, 15
13.8	55, 51	40, 38	37, 32
16.0	117, 127	76, 79	93, 93
18.9	299, 273	158, 160	155, 187
23.0	610, 600	395, 375	425, 350
33.5*	1370, 1370	1290, 1270	1270, 1240
35.2*	1380, 1400	1300, 1280	1280, 1270

Each figure represents an individual culture.
* Diluted for reading.

decrease in inhibition but no evidence for a competitive relation between the two steroids. Some specificity is suggested, however, since pregnenolone had no effect on the inhibition of *T. utilis* by desoxycorticosterone.

An experiment was also conducted to determine the stage of the growth of *T. utilis* which was sensitive to inhibition by progesterone. A large number of identical cultures were prepared, 10 μg/ml of progesterone were added to one-third, 20 μg/ml to another one-third while the remainder were kept as controls. They were all inoculated with *T. utilis* and incubated simultaneously. At the intervals indicated in Table VIII two flasks from each set were removed and the turbidity of the contents determined. The values obtained are shown in Table VIII. Inhibition was present at ~10 hours, the earliest time tested, and apparently ceased at about 19 hours when the increase in turbidity in all three sets became linear and approximately equal. There was essentially no difference in the response to progesterone at 10 and 20 μg/ml.

The effect of varying the concentration of the steroids on the rate of growth of *A. niger* has been determined for ergosterol, progesterone, testosterone,

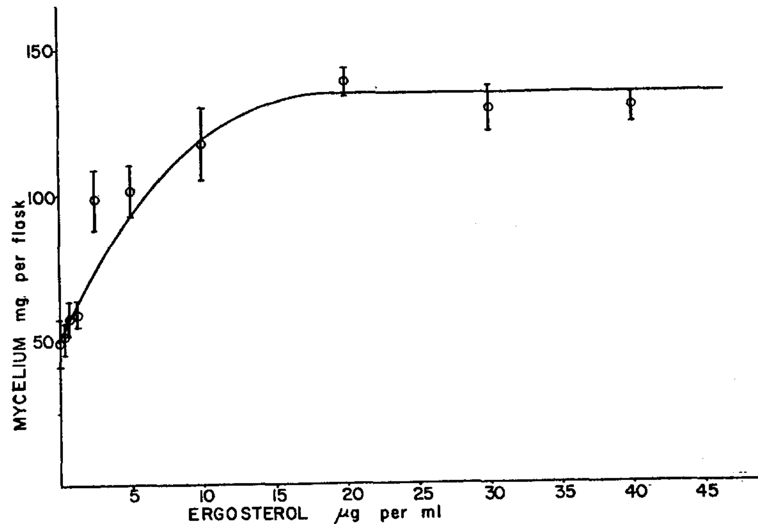

FIGURE 1. Dose response curve of *Aspergillus niger* to ergosterol. The ordinate gives the dry weight of mycelium collected after incubation of a sucrose-mineral medium containing the indicated amount of ergosterol and inoculated with a conidial suspension.

cholestanol, stigmasterol, pregnenolone, desoxycorticosterone, and 17α-hydroxyprogesterone. The first five of these are shown in Figs. 1 through 5 respectively. For ease of comparison, the responses to the pregnene derivatives: pregnenolone, progesterone, desoxycorticosterone, and 17α-hydroxyprogesterone are shown together in Fig. 6.

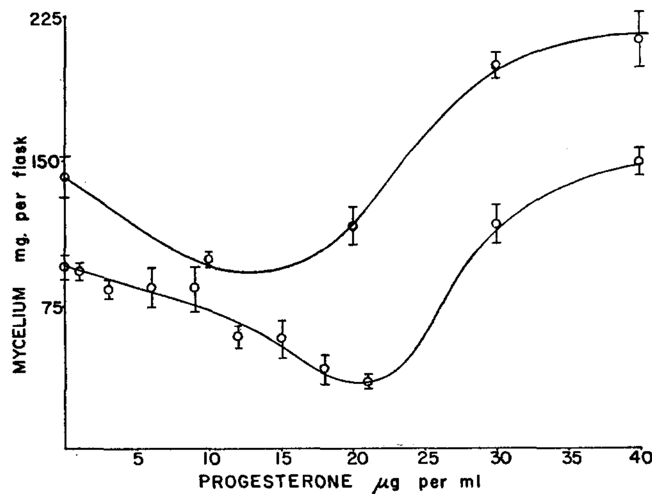

FIGURE 2. Dose response curve of *Aspergillus niger* to progesterone. The two curves are for separate experiments. The ordinate gives the dry weight of mycelium collected after incubation of a sucrose-mineral medium containing the indicated amount ot progesterone and inoculated with a conidial suspension.

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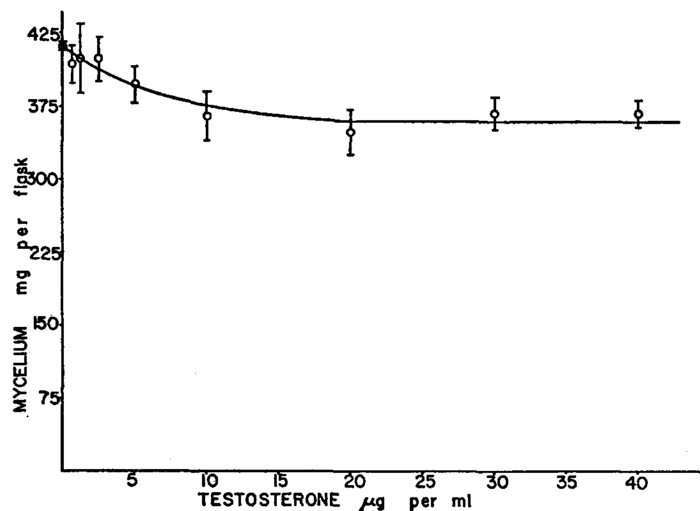

FIGURE 3. Dose response curve of *Aspergillus niger* to testosterone. The ordinate gives the dry weight of mycelium collected after incubation of a sucrose-mineral medium containing the indicated amount of testosterone and inoculated with a conidial suspension.

A variety of responses is evident, varying from considerable stimulation with the sterols through slight stimulation with pregnenolone to inhibition with testosterone and desoxycorticosterone. Progesterone surprisingly caused an inhibition at low concentrations and a stimulation at higher ones. This unusual response was repeated with a larger number of replicates in order to

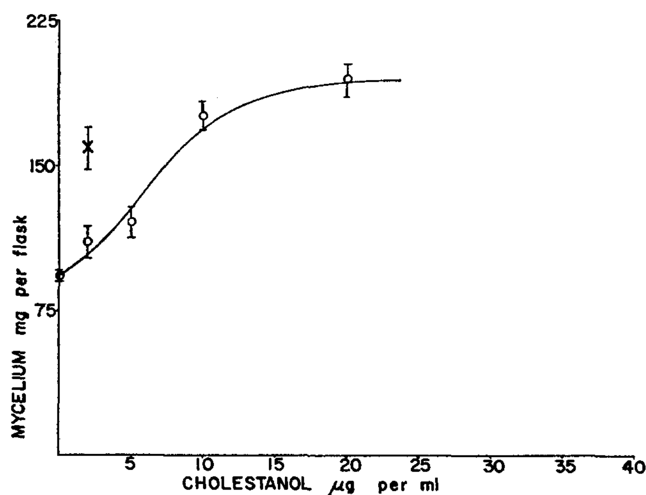

FIGURE 4. Dose response curve of *Aspergillus niger* to cholestanol. The ordinate gives the dry weight of mycelium collected after incubation of sucrose-mineral medium containing the indicated amount of cholestanol and inoculated with a conidial suspension. X, cholesterol determined simultaneously.

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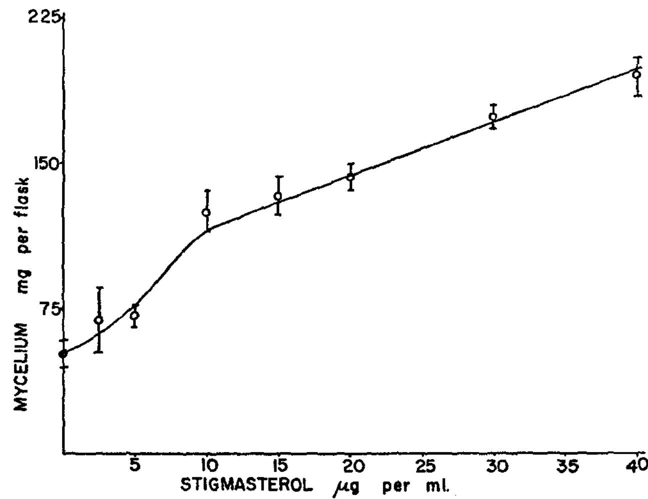

FIGURE 5. Dose response curve of *Aspergillus niger* to stigmasterol. The ordinate gives the dry weight of mycelium collected after incubation of a sucrose-mineral medium containing the indicated amount of stigmasterol and inoculated with a conidial suspension.

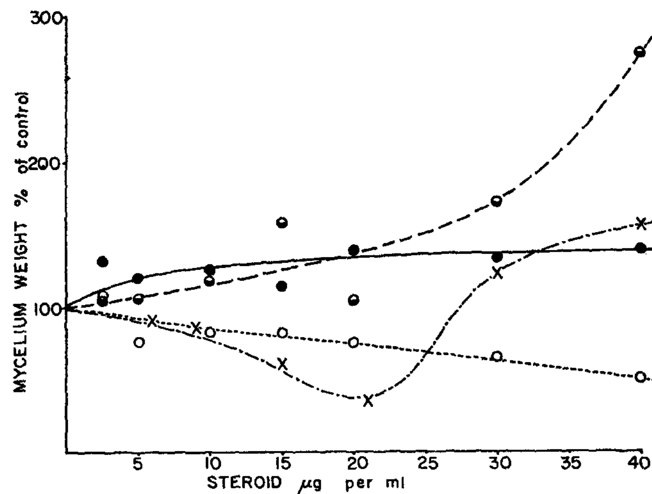

FIGURE 6. Dose response curve of *Aspergillus niger* to several pregnene derivatives. The data have been adjusted (average dry weight for each treatment/average dry weight of control × 100) for ease of comparison. The ordinate gives the adjusted dry weight of mycelium collected after incubation of a sucrose-mineral medium containing the indicated amount of steroid and inoculated with a conidial suspension.

----- O -----, desoxycorticosterone

—— ● ——, 17 α-hydroxyprogesterone

---- ⊖ ----, pregnenolone

----- × -----, progesterone

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estimate the average growth at the various progesterone levels more precisely. The results of the second experiment are shown in the upper curve in Fig. 2. Both the inhibition at 10μg/ml and the stimulation at 30 and 40 μg/ml are highly significant. The two curves do not have quite the same minima, however.

The dose response curve for testosterone is also somewhat unusual in that the inhibition reaches a maximum and is not increased by further increases in the amount of testosterone added to the culture. A similar situation was found for the inhibition of *Torula utilis* by progesterone (Fig. 7) but not for its

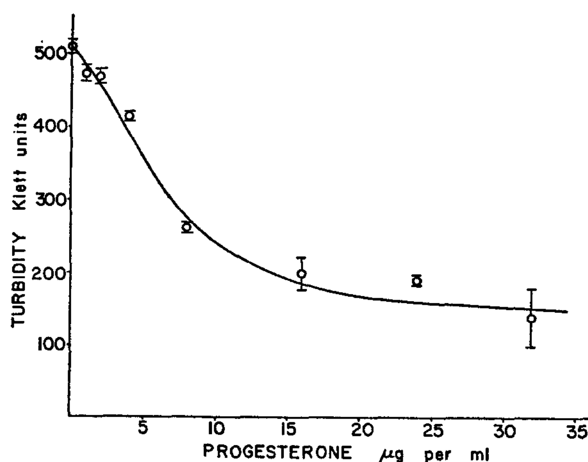

FIGURE 7. Dose response curve of *Torula utilis* to progesterone. The ordinate gives the increase in turbidity during incubation of a sucrose-mineral medium containing the indicated amount of progesterone and inoculated with a suspension of *T. utilis* cells.

inhibition by diethylstilbestrol. This latter substance gave essentially complete inhibition at 16 μg/ml.

Since it was not possible to obtain all the dose response curves simultaneously, direct quantitative comparisons between them may not be justified. An effort was made to overcome this difficulty by testing representative stimulatory compounds simultaneously at two different concentrations. The data obtained are shown in Table IX.

At the 5 μg/ml level only the cholesterol- and ergosterol-treated cultures exceeded the 95 per cent probability of being greater than the controls while at twice this concentration the list extends to include cholestanol- and stigmasterol-treated cultures also. Other comparisons which can be made at the 95 per cent confidence level are:

1. At the 5 μg/ml level, ergosterol was superior to all the other steroids except cholesterol.

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2. At the 10 μg/ml level, both stigmasterol and cholestanol were superior to 7-dehydrocholesterol and pregnenolone.
3. Cholestanol was significantly more effective at 10 μg/ml than at 5 μg/ml.

TABLE IX
A COMPARISON OF THE RELATIVE EFFECTIVENESS OF SEVERAL STEROIDS WHICH STIMULATE ASPERGILLUS NIGER

Steroid	Concentration	No. of flasks (N)	Average dry weight	Standard error of the mean
	μg/ml		mg	
None	—	10	165.5	9.3
Ergosterol	5	5	276.7	9.6
Ergosterol	10	4	283.2	5.9
Cholesterol	5	5	245.7	8.2
Cholesterol	10	4	265.4	12.5
Stigmasterol	5	5	213.2	21.8
Stigmasterol	10	4	265.7	5.5
Cholestanol	5	5	162.5	9.7
Cholestanol	10	5	277.9	8.8
7-Dehydrocholesterol	5	5	177.5	6.3
7-Dehydrocholesterol	10	4	180.6	6.2
Pregnenolone	5	5	164.6	14.7
Pregnenolone	10	5	178.6	14.6

95 per cent confidence limits (Scheffé) for the difference between any two average dry weights:

$N = 10$ *vs.* $N = 5$ .... ±66.6
$N = 10$ *vs.* $N = 4$ .... ±72.5
$N = 5$ *vs.* $N = 5$ .... ±77.2
$N = 5$ *vs.* $N = 4$ .... ±82.5
$N = 4$ *vs.* $N = 4$ .... ±86.5

DISCUSSION

The stimulation of *A. niger* by sterols under conditions permitting very rapid growth indicates that these compounds serve some essential function in the metabolism of this organism and that in the absence of a supplement their rate of synthesis can become growth-limiting. The structural similarity between the sterols which stimulate *A. niger* under these conditions and those which are required by *Labyrinthula vitellina* (2) suggests that there is an essential function of sterols which is common to all fungi. The failure to demonstrate stimulation of *T. utilis* under the same conditions probably means that the rate of growth was being limited by something other than steroids or that the requirement is more specific. The fact that the same steroids were inhibitory to both *A. niger* and *T. utilis* suggests a basic similarity in the role of steroids in their metabolism.

Stimulation and inhibition by different members of a group of structurally related materials are frequently associated with inhibitions which are due to

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competition for sites normally occupied by either the stimulatory substance or a derivative of it. Attempts to demonstrate this by reversing the inhibitory effect of progesterone by the addition of other steroids met with very limited success. The slight reversals obtained with ergosterol and cholesterol were statistically significant but of too low a magnitude to indicate a direct competition with either of these compounds. The reversal by pregnenolone was somewhat greater but its incomplete and non-competitive nature rules out a direct competition with this compound also. The fact that these reversals were obtained and that there was some specificity involved strengthen the idea that competition between steroids exists in microorganisms, and suggest that the metabolically functional compound has not been tested for this activity.

The quantitative comparisons between the various sterols permit some conclusions concerning the molecular structure of the metabolically active sterol in *A. niger*. The materials which produced the most stimulation at low concentrations were ergosterol and cholesterol, suggesting that one of these or a closely related material is the "functional" compound. The fact that several other compounds were stimulatory but less so than the two mentioned above could indicate a relatively non-specific requirement or that the organism is capable of converting related materials to the functional sterol.

The inclusion of cholestanol in this less effective group indicates that a double bond at carbon 5 exists in the natural steroid. The data obtained with progesterone and pregnenolone indicate that the hydroxyl at carbon 3 is necessary since reduction of the 3-keto group to the 3-beta-hydroxy compound converted the toxic progesterone to the slightly stimulatory pregnenolone.

The shape of the stigmasterol curve and the comparison between it and ergosterol in Table I indicate a preference for the ergosterol or cholesterol type of side chain.

The short chain or side chainless steroids, progesterone, pregnenolone, 17α-hydroxyprogesterone, desoxycorticosterone, and testosterone, present a very complicated picture. Although all these short chain steroids are much less stimulatory than ergosterol, *A. niger* is able to utilize several of them to some extent. This is in contrast to the *Labyrinthula* strain studied by Vishniac (2) which requires a sterol of at least twenty-seven carbon atoms. The introduction of a hydroxyl at carbon 21, as in desoxycorticosterone, or substitution of a hydroxyl for the side chain, as in testosterone, prevents utilization by *A. niger* and confers toxicity, while the introduction of a hydroxyl at carbon 17 destroys both the stimulatory and the inhibitory properties of progesterone.

The data obtained with 7-dehydrocholesterol seem to indicate a deleterious effect of the double bond at carbon 7. The same bond occurs in ergosterol, however, and unless it is more than compensated for by the C-22 double bond or the 24 β-methyl group, some other explanation is needed. We are inclined

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to suspect a toxic contaminant in the 7-dehydrocholesterol even though both it and the ergosterol were recrystallized just prior to their use in the experiment shown in Table IX.

The conclusion can be drawn that *A. niger* is stimulated by and, therefore, has a metabolic requirement for, a 3-hydroxysteroid with an unsaturation in the 5-6 position. Ergosterol, since it is known to occur in a wide variety of fungi (4), is a likely candidate although the unsaturation at carbons 7 and 22 and the methyl group at carbon 24 may or may not be essential. It is equally possible that some derivative existing in the cell in much smaller quantities is the metabolically active material. The steroids which give maximum stimulation of *A. niger* are similar to those required by *Labyrinthula* (2), pleuropneumonia organisms (5), several insect larvae (4), and various protozoa (4).

The shape of the concentration *versus* growth curve obtained with *A. niger* and progesterone suggests several possible sequences of events. It is possible that the active inhibitor is a complex and that progesterone itself or a different, less easily formed complex is stimulatory. However, an extensive search for the other component of such a complex has so far proved fruitless. A second possibility is that the progesterone could have been modified by the organism with the resulting formation of a stimulatory steroid. A third possibility is that progesterone could be competitively occupying an essential site in the cell and carrying out the normal function of its competitor in a less efficient manner. It is also possible that the results seen represent the superimposition of two completely unrelated effects.

It is not possible to distinguish between these mechanisms with the data on hand. It is interesting to note, however, that steroid dose response curves of similar shape have been obtained in mammals (17).

The role of solubility of the various steroids in the culture medium is not known. For convenience we have reported the concentrations in micrograms per milliliter although in many cases these values exceeded the solubility of the steroid in water. Weight of steroid per unit of fungus would perhaps have been more desirable if it could have been determined readily. The incomplete nature of the inhibitions obtained might be referable to the limited solubility of the inhibitors but we are inclined to doubt this for the following reasons:

1. Two inhibitors which were tested, diethylstilbestrol and menadione, have solubilities which are similar to those of the steroids but gave dose response curves which terminated in complete inhibitions.
2. The response to progesterone and the sterols extends well beyond their solubility limits.

It, therefore, appears that the break in the inhibition curves encountered with several oxygenated steroids must have a physiological rather than a physiochemical explanation.

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