(*Tokyo*) *31*, 831), has appeared. Asperyellone is apparently identical with asperenone. These authors suggest the structure 7-methyl-13-phenyl-3-oxotrideca-4,6,8,-10,12-pentaene for asperyellone.

References

Bycroft, B. W., Dobson, T. A., and Roberts, J. C. (1962), *J. Chem. Soc.*, 40.
Gansser, Ch., and Zechmeister, L. (1957), *J. Am. Chem. Soc.* *79*, 3854.
Gripenberg, J. (1952), *Acta Chem. Scand.* *6*, 580.
Jefferson, W. E., Jr. (1967), *Biochemistry* *6*, 3484 (this issue; following paper).
Lund, E., Budzikiewicz, H., Wilson, J. M., and Djerassi, C. (1963), *J. Am. Chem. Soc.* *85*, 1528.
Peifer, J. J. (1962), *Microchim. Acta* 529.
Reid, W. W. (1950), *Nature* *165*, 190.
Zajic, J. E., and Kuehn, H. H. (1962), *Mycopathol. Mycol. Appl.* *17*, 149.

Steroids and Other Factors Influencing the Accumulation of Asperenone and Fermentation Acids by *Aspergillus niger* in Replacement Cultures*

W. E. Jefferson, Jr.

ABSTRACT: Asperenone and unknown B accumulate together in nitrogen-free replacement cultures of *Aspergillus niger* with no evidence for a precursor–product relationship. There is an inverse relationship between the quantities of asperenone and the citric and oxalic acids produced.

The amount of asperenone accumulated is increased by some steroids, especially estradiol and estrone, while other steroids, especially testosterone and androstenedione, cause increases or decreases depending on the mineral content of the growth or replacement medium. Copper deficiency prevented the accumulation of asperenone and the effect of estradiol on the production of asperenone and citric and oxalic acids. Asperenone accumulation is decreased by cycloheximide and puromycin.

During an investigation of the effects of steroids on fungi, accumulations of an intramycelial yellow pigment accompanied by decreased accumulations of citric and oxalic acids in some estrogen-containing replacement cultures of *Aspergillus niger* were noted. The major yellow pigment was subsequently isolated and characterized as a methyl derivative of 13-phenyltrideca-4,6,8,10,12-pentaen-3-one and given the trivial name, asperenone (Jefferson, 1967). It is accompanied by smaller amounts of two other substances with similar absorption spectra and solubilities. These two uncharacterized materials have been designated as unknowns A and B. This paper reports the results of efforts to determine the factors responsible for the accumulation of asperenone.

Materials and Methods

Organism. A. niger NRRL-3 was employed in these experiments.

Cultural Conditions. The original observations were made using the cultural methods of Shu and Johnson (1948) for the production of citric acid. Except where it is stated otherwise in the text, subsequent cultures were grown as described by Jefferson and Sisco (1961).

Replacement cultures were prepared by pipetting ethyl ether solutions of the steroids, etc., into empty 300-ml erlenmeyer flasks. The ether was evaporated in an air stream prior to the addition of 50 ml of replacement medium which was 5% sucrose unless it is stated otherwise. Approximately 0.75-g portions of washed mycelium obtained by filtration in a Büchner funnel were weighed into each flask. The cultures were incubated at 26° on a reciprocating shaker for 48 hr.

Assays. The exact weight of mycelium in each flask was determined after filtration and drying at the end of the incubation. In most of the experiments the relative amount of asperenone was determined by extracting the dry mycelium with 95% ethanol at 60° for 1 hr.

* From the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee. *Received April 14, 1967.* This investigation was supported in part by Research Grant No. Nonr(G)-00057 from the Office of Naval Research and in part by the U. S. Public Health Service, Institute of General Medical Sciences Grant GM-12645.

The resulting extracts were diluted to a convenient volume (usually 15 ml) in the same solvent and their absorbancy at 410 mμ was determined. In a few cases the extraction procedure of Folch *et al.* (1951) was utilized. The chloroform layer was dried with Na_2SO_4, filtered, and evaporated. Absorption spectra were determined on the residue after it was dissolved in hexane.

Citrate was determined by the method of Saffran and Denstedt (1948) modified by the omission of the protein precipitation step. Oxalate was determined by $KMnO_4$ titration of the calcium salt.

Results

Asperenone is not produced exclusively in response to steroids. Variable but usually small amounts of the pigment are detectable in untreated replacement cultures and quite large amounts are formed in cultures which are either grown or replaced in media containing glycerol as the sole source of carbon. The addition of glycerol in amounts up to 100 μg/ml to sucrose replacement medium did not increase the asperenone content, however. The results of experiments dealing with the effects of various steroids and related materials on the accumulation of asperenone showed that estradiol, estrone, progesterone, and dethylstilbestrol at a concentration of 10 μg/ml caused an increase, the effect of adding other compounds was variable but small by comparison. Little or no effect was found with cortisone acetate, cholesterol, or tristearin when these were tested in our usual medium. The effect of adding testosterone, androstenedione, or desoxycorticosterone was generally small and sometimes negative.

The amount of asperenone found and the extent of the steroid-induced changes was consistent within experiments but varied between experiments to such an extent as to suggest some uncontrolled factor in the growth or replacement medium. Since contamination with additional minerals appeared to be the most likely uncontrolled variable, an experiment to test this possibility was conducted by modifying the mineral content of the growth medium in various ways as indicated in Table I.

TABLE I: The Influence of the Mineral Content of the Growth Medium on the Response of Replacement Cultures of *A. niger* NRRL-3 to Estradiol and Diethylstilbestrol.[a]

Medium	Steroid	*A*/g
A	None	0.78–0.91
A	Estradiol	3.22–3.46
A	Diethylstilbestrol	1.85–1.85
B	None	1.52–1.74
B	Estradiol	5.00–5.08
B	Diethylstilbestrol	4.05–5.25
C	None	0.87–1.04
C	Estradiol	1.64–1.69
C	Diethylstilbestrol	0.43–0.59
D	None	5.81
D	Estradiol	13.05
D	Diethylstilbestrol	0.75
E	None	0.86–0.89
E	Estradiol	0.88–1.07
E	Diethylstilbestrol	0.28–0.32
F	None	0.45–0.56
F	Estradiol	2.08–3.00
G	None	0.51–0.73
G	Estradiol	1.09

[a] Shu and Johnson (1948) sucrose–mineral medium modified as follows:

Medium	Modification
A	None
B	Tap water was substituted for distilled water.
C	A solution of the phosphate and sucrose in distilled water was passed through a permutit column prior to the addition of NH_4NO_3 and $FeSO_4$. No other minerals were added.
D	Medium C plus 60 μg/l. of Cu^{2+} as $CuCl_2$.
E	Medium C plus 250 μg/l. of Zn^{2+} as $ZnCl_2$.
F	Medium C plus 100 μg/l. of Mn^{2+} as $MnSO_4$.
G	Medium C plus a complete mineral supplement.

Supplements were added to a concentration of 10 μg/ml. Tabulated values are the absorbance of 15-ml ethanolic extracts at 410 mμ divided by the weight of dry mycelium extracted. Ranges are the range of duplicate cultures.

It is apparent that these modifications of the medium did have a considerable influence on the accumulation of asperenone and on the response of the fungus to estradiol and to diethylstilbestrol. The most obvious effect was due to the addition of cupric ion but this can not be the only factor influencing the response since medium G contained the same amount of copper as did medium D but gave a smaller yield of asperenone.

The effect of copper was tested in a more complete medium as shown in Figures 1–3. Figure 1 shows the effect of simultaneous variation in the copper content of the growth medium and the estradiol content of the replacement medium on the accumulation of materials absorbing at 410 mμ in ethanol. Figure 2 depicts the accumulation of citric acid in the same cultures. This inverse relationship between citrate and asperenone accumulation has been noted consistently. The similarity between the two sets of curves (except for the direction of the response) appears to indicate a relationship between these two effects of estradiol. Figure 3 shows that the oxalic acid content of the same culture filtrates parallels the citrate concentration.

Further exploration of the effects of variation of the mineral composition of the medium failed to implicate any additional individual ions but permitted the observation that variations in the medium can change the response to some of the steroids. That is, some steroids

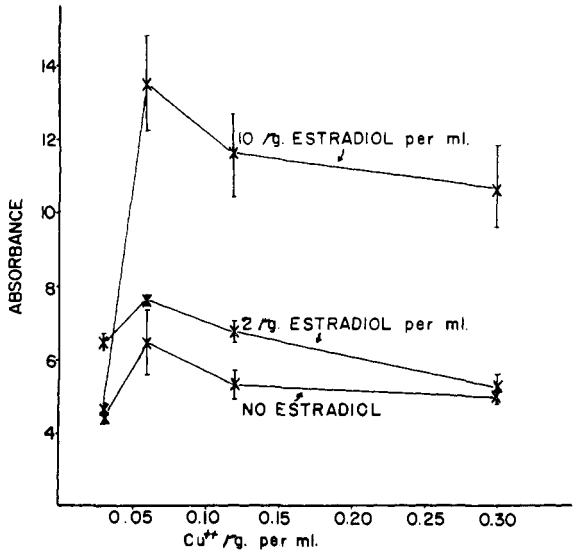

FIGURE 1: The influence of estradiol and copper on the production of asperenone by *A. niger* in replacement culture. A solution prepared by mixing the water, phosphate, and sucrose of the Shu and Johnson (1948) medium was treated with permutit to remove contaminating ions. Except for copper the remaining ingredients of that medium were added and the medium was further supplemented with $CaCl_2 \cdot 2H_2O$ (4.8 μg/ml), $Na_2MoO_4 \cdot 2H_2O$ (0.13 μg/ml), H_3BO_4 (0.26 μg/ml), and $CoCl_2 \cdot 6H_2O$ (0.2 μg/ml). The medium was divided into several portions and cupric ion in the form of $CuCl_2$ was added in the amounts indicated. Replacement was in 5% sucrose. Ranges are the range of duplicate cultures.

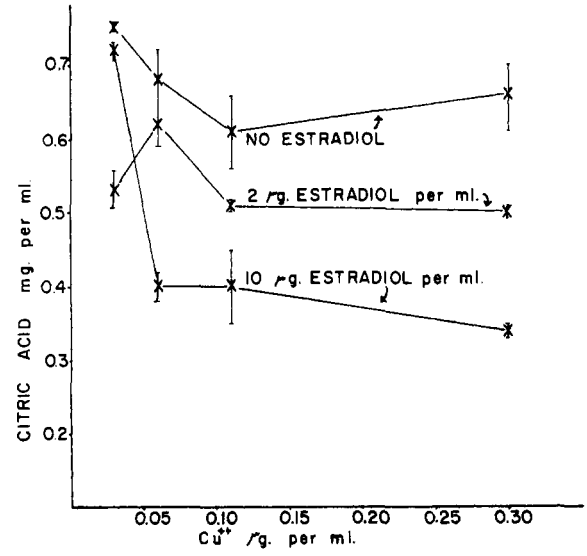

FIGURE 2: The influence of estradiol and copper on the production of citric acid by *A. niger* in replacement culture. Determined on the cultures described in Figure 1.

can cause a decrease in asperenone content in one medium and an increase in another. These effects were observed by varying the quantity of the trace element supplement consisting of all of the mineral components of the growth medium other than NH_4NO_3, KH_2PO_4, $FeSO_4$, and $ZnCl_2$. When the quantity of supplement was increased by a factor of four, the effect of adding estrone or estradiol was unchanged while the addition of testosterone, androstenedione, or progesterone also resulted in an increased asperenone production.

Similar but larger effects were seen when the replacement medium was supplemented with a mineral mixture. An example of such an experiment is shown in Table II. In it mycelium from several flasks was harvested, pooled, washed, and then divided into three portions and replaced on three different media as described in Table II. In each case the concentration of the individual minerals was the same as that used in the growth medium. The response was similar to that described above but more pronounced. The addition of the mineral constituents of the growth medium to the replacement cultures caused a change in the direction of the response to testosterone, androstenedione, and desoxycorticosterone. There was also a considerable quantitative change in the amount of asperenone in the untreated cultures and in the response to estradiol and progesterone. The highest concentrations of asperenone were seen in a medium which was complete except for a nitrogen source. Inclusion of ammonium or sodium nitrate in this medium inhibited asperenone accumulation.

The time course of asperenone accumulation in the presence and absence of estradiol is shown in Figure 4. In this experiment the initial extraction was by the Folch technique. Absorbancies were determined in hexane to bring out the fine structure of the spectrum to permit estimation of the contribution of unknown B to the absorption. Simultaneous equations based on the spectra of the purified materials were used to esti-

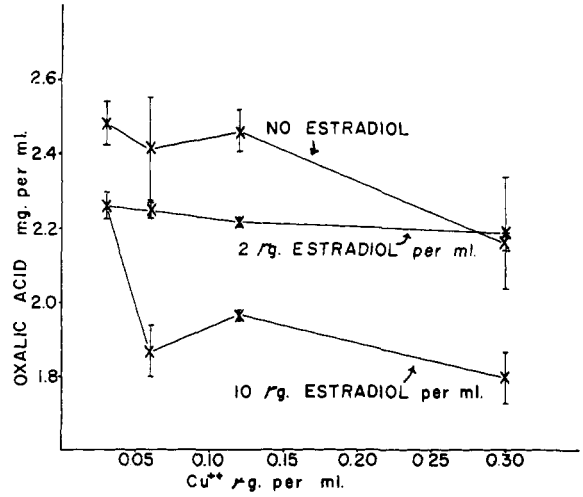

FIGURE 3: The influence of estradiol and copper on the production of oxalic acid by *A. niger* in replacement culture. Determined on the cultures described in Figure 1.

TABLE II: The Effect of Steroids on Pigment Accumulation in Replacement Cultures of *A. niger* on Three Different Replacement Media.[a]

Steroid	Steroid Concn (μg/ml)	Medium	A/mg	Difference	p Less Than
None		A	0.40		
Estradiol	10	A	0.81	+0.41	0.01
Estrone	10	A	0.91	+0.51	0.01
Testosterone	10	A	0.10	−0.30	0.01
Androstenedione	10	A	0.09	−0.31	0.01
Desoxycorticosterone	10	A	0.16	−0.23	0.01
Progesterone	10	A	0.47	+0.08	0.10
None		B	0.46		
Estradiol	10	B	0.58	+0.12	0.01
Estrone	10	B	0.69	+0.24	0.01
Testosterone	10	B	0.39	−0.07	0.01
Androstenedione	10	B	0.61	+0.15	0.01
Deoxycorticosterone	10	B	0.68	+0.22	0.01
Progesterone	10	B	0.85	+0.39	0.01
None		C	0.65		
Estradiol	10	C	0.82	+0.17	0.01
Estrone	10	C	1.20	+0.55	0.01
Testosterone	10	C	0.72	+0.07	0.05
Androstenedione	10	C	0.91	+0.26	0.01
Desoxycorticosterone	10	C	0.79	+0.14	0.01
Progesterone	5	C	0.67	+0.02	0.5
Progesterone	10	C	0.91	+0.26	0.01
Progesterone	20	C	0.94	+0.29	0.01

[a] Tabulated values are the absorbance of 15-ml ethanolic extracts divided by the dry weight of the mycelium extracted. Replacement medium A was 5% sucrose, B was 5% sucrose plus KH_2PO_4, $MgSO_4$, and $CuCl_2$ at concentrations equal to those in the growth medium, and C was growth medium minus NH_4NO_3. N equals three in all cases.

mate the absorbancy due to each of the two components as follows. The absorbancy at 392 mμ due to asperenone = $(1.297(A_{392}) - A_{415})/0.465$ and also = $(0.988(A_{392}) - (A_{438}))/0.808$. The absorbancy at 392 mμ, due to unknown B, is equal to the absorbancy at 392 minus this value. Figure 4 was constructed from the average of both values for asperenone. Figure 5 was constructed similarly. Attempts to apply the same methods to unknown A were unsuccessful because of the small quantities present and the presence of interfering substances at the lower wavelengths.

The accumulation of asperenone and unknown B follows approximately the same time course suggesting that they are metabolically related but there is no indication that one is a precursor of the other. Figures 4 and 5 also show that estradiol has no effect on the time of formation of asperenone and unknown B although it strongly increases the quantity formed. In this experiment the maximum accumulation of asperenone was 0.43 mg/g of dry mycelium in the presence of estradiol and 0.11 mg/g of dry mycelium in the controls. The quantity of unknown B could not be determined because of a lack of reliable estimate of its extinction coefficient.

Other work in this laboratory (L. B. Foster and W. E. Jefferson, unpublished data) has shown that testosterone can induce an increased synthesis of some of the enzymes of carbohydrate metabolism. Because of this, the effect of puromycin and of cycloheximide on asperenone accumulation was investigated. The results are shown in Table III. Both protein synthesis inhibitors did reduce the accumulation of asperenone.

Discussion

The accumulation of asperenone and its companions is associated with a nongrowing condition, a certain balance of mineral elements, the presence of certain steroids in μg quantities, and the presence of glycerol as a carbon source. Its formation is inhibited by protein synthesis inhibitors and is associated with reduced yields of citric and oxalic acids. The picture is further complicated by an interrelationship between the mineral composition of the medium and the structure of the applied steroid.

The simplest, but not the only possible, view connecting the above would regard asperenone as a product whose formation is induced by oxidative metabolism

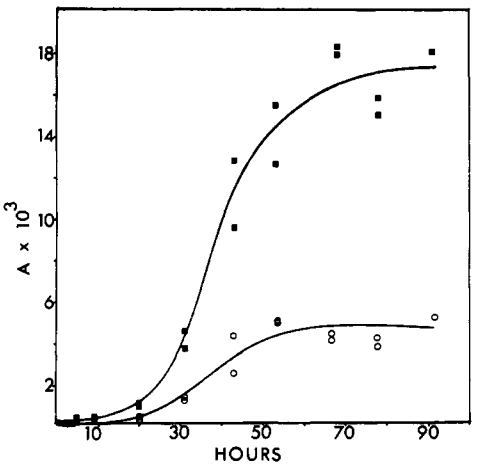

FIGURE 4: The rate of accumulation of asperenone in *A. niger* replacement cultures. Each point represents the absorption at 392 mμ due to asperenone in an extract of a single culture in 5% sucrose. Cultures represented by the upper curve contained 10 μg of estradiol/ml.

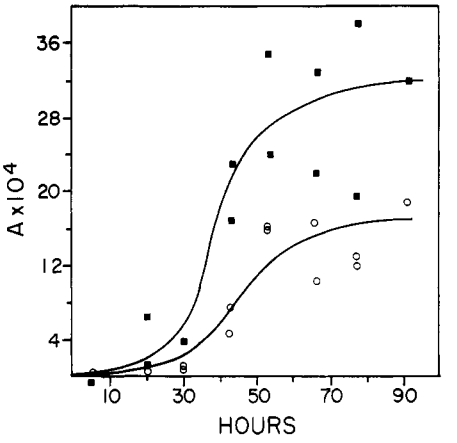

FIGURE 5: The rate of accumulation of unknown B in *A. niger* replacement cultures. Taken from the cultures described in Figure 4. Points are the absorption at 392 mμ due to unknown B.

in the absorbance of an essential nutrient (in this case nitrogen) and whose formation is very sensitive to the balance of several metallic ions. The effect of steroids on the system may be to change the effective concentration of one or more specific ions at the sensitive portion of the cell.

The accumulation of special products when microorganisms metabolize under nongrowing conditions is a common phenomenon and one that might be expected to require new protein synthesis. Marked changes in the metabolism of *A. niger* and other organisms in response to the level and balance of metallic ions in the medium is also well documented (Weinberg, 1962; Foster, 1949) and has frequently been associated with the formation of pigments. The association of some steroid hormones with specific mineral metabolism is obvious and too well known to need documenting except to mention a reported correlation between blood copper and estrogen levels (von Studnitz and Berezin, 1958). Numerous attempts to explain the physiological activity of steroid hormones on the basis of changes in the permeability of cells or organells have been made (Willmer, 1961; Kroeger, 1966) but do not appear to enjoy much current acceptance (Karlson and Sekeris, 1966).

TABLE III: The Effect of Inhibitors of Protein Synthesis on Asperenone Formation.[a]

Supplement	*A*/g, % of Control		
	Expt		
	1	2	3
Estradiol	320	201	325
Estradiol	330	197	316
Cycloheximide	73	65	28
Cycloheximide	83	49	
Puromycin	103		
Puromycin	93		
Estradiol and cycloheximide	105	173	55
Estradiol and cycloheximide	107	139	57
Estradiol and puromycin	158		
Estradiol and puromycin	190		

[a] The concentrations of supplements were: estradiol (10 μg/ml), cycloheximide (5 μg/ml), and puromycin (20 μg/ml). The concentration of asperenone was estimated on the basis of the absorbancy of extracts prepared after 48-hr incubation in sucrose solution.

References

Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N. (1951), *J. Biol. Chem. 191*, 833.

Foster, J. W. (1949), Chemical Activities of the Fungi, New York, N. Y., Academic.

Jefferson, W. E. (1967), *Biochemistry 6*, 3479 (this issue; preceding paper).

Jefferson, W. E., and Sisco, G. (1961), *J. Gen. Physiol. 44*, 1029.

Karlson, P., and Sekeris, C. E. (1966), *Acta Endocrinol. 53*, 505.

Kroeger, H. (1966), *Expl. Cell Res. 41*, 64.

Saffran, M., and Denstedt, O. F. (1948), *J. Biol. Chem. 175*, 849.

Shu, P., and Johnson, M. J. (1948), *Ind. Eng. Chem. 40*, 1202.

von Studnitz, W., and Berezin, D. (1958), *Acta Endocrinol. 27*, 245.

Weinberg, E. D. (1962), *Perspectives Biol. Med. 5*, 432.

Willmer, E. N. (1961), *Biol. Rev. 36*, 368.