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Mellein and 4-Hydroxymellein Production by *Aspergillus ochraceus* Wilhelm[1]

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Mellein and 4-hydroxymellein are isocoumarin compounds produced by *Aspergillus ochraceus* Wilhelm. They are structurally similar to the dihydroisocoumarin moiety of ochratoxin A, a toxic metabolite of the same fungus, and they possibly have similar biological properties. Production of mellein and 4-hydroxymellein on synthetic media and natural solid substrates was determined. Several carbon and nitrogen sources supported production of these metabolites in stationary culture. Additional zinc and molybdenum increased production of both metabolites in stationary culture, but were not required for maximum production in shaken culture. Mellein and 4-hydroxymellein were produced on yellow corn, but neither was produced on wheat, peanuts, or soybeans.

Aspergillus ochraceus has been isolated from a variety of agricultural products (1, 6, 12), and Scott (6) found that three of five isolates were toxic to mice, rats, and ducklings. The toxic chemical was later identified and named ochratoxin A (7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methyl-isocoumarin linked by an amide bond to L-β-phenylalanine) (12) (Fig. 1).

Recently, the isocoumarin compounds, mellein (3-methyl-8-hydroxy-3,4-dihydroisocoumarin) and 4-hydroxymellein (3-methyl-4,8-dihydroxy-3,4-dihydroisocoumarin) (Fig. 1) were isolated from the culture media of *A oniki* and *A. ochraceus* (2, 5). They are also produced by an ochratoxin-producing strain of *A. ochraceus* (2) and are structurally similar to the dihydroisocoumarin moiety of ochratoxin A (13). Sasaki et al. (5) reported the LD_{50} of mellein and 4-hydroxymellein in intraperitoneally injected mice as 250 to 500 and 1,000 to 1,500 mg/kg, respectively. The objective of this research was to develop a medium and establish cultural conditions suitable for production of mellein and 4-hydroxymellein.

MATERIALS AND METHODS

Organism. *Aspergillus ochraceus* Wilh., NRRL 3174, was used throughout this investigation. Cultures were maintained at 25 C on Czapek solution agar with 20% sucrose (11) supplemented with 0.7% Difco yeast extract (3) and transferred to slants 7 to 14 days prior to use. Medium for these slants consisted of the following in grams per liter: dextrose, 20; KH_2PO_4, 0.5; $MgSO_4$, 0.5; yeast extract, 7; and agar, 25.

Culture medium. The basal synthetic medium was that of Ferreira (4) modified to the following composition on a gram-per-liter basis: sucrose, 30; L-glutamic acid, 3; KH_2PO_4, 1; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$ (Fe), 0.024, $ZnSO_4 \cdot 7H_2O$ (Zn), 0.001; $MnSO_4 \cdot H_2O$ (Mn), 0.011; $CuSO_4$ (Cu), 0.004; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (Mo), 0.0025. Glass-distilled water was used to prepare all media. Erlenmeyer flasks (125 ml) containing 25 or 50 ml of liquid medium or 50 g of natural solid substrate (corn, wheat, peanuts, soybeans) were stoppered with foam plugs and autoclaved at 121 C for 15 min. Media containing heat-labile components were filter sterilized. Flasks were inoculated with conidia from 7- to 14-day-old cultures of *A. ochraceus*. All experiments were replicated three times.

Metabolite extraction. Cultures were filtered through weighed 33-cm Whatman no. 2 filter papers that had been dried at 70 C for 12 hr. Mycelial mats were washed with demineralized water, and weights were determined after drying for 12 hr at 70 C and cooling in a desiccator for 1 hr. Filtrates were adjusted to their original volume with demineralized water and their *p*H values were measured with a Corning model 12 *p*H meter. Filtrates were adjusted to *p*H 4 with either 40 to 50% KOH or 25% HCl. Mellein and 4-hydroxymellein were extracted from 25 ml of each filtrate with two 25-ml portions of chloroform in a 500-ml separatory funnel. Fifty

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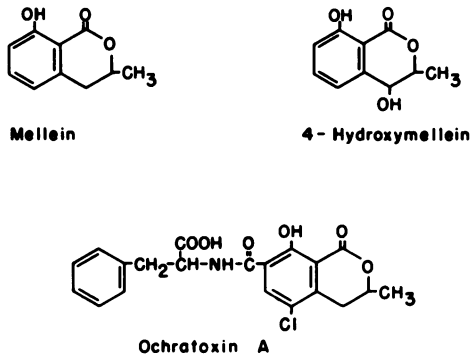

FIG. 1. *Chemical structures of mellein, 4-hydroxymellein, and ochratoxin A.*

grams of solid substrate was extracted by blending with 200 ml of chloroform for 1 min in a Waring blendor. The slurry was filtered and the residue was washed with another 100 ml of chloroform. The chloroform was evaporated to dryness on a boiling-water bath, and the residue of each flask was redissolved in 0.5 ml of chloroform. A 5-μliter sample from each flask was spotted on a thin-layer chromatography (TLC) plate (20 by 20-cm glass plate coated with a 500-μm layer of Brinkman Instruments MN Silica Gel-G-HR). Reference samples of authentic mellein and 4-hydroxymellein were spotted on each plate. Plates were developed in lined chromatography tanks containing a 2-cm layer of toluene-ethyl acetate-90% formic acid (5:4:1, v/v/v). When the solvent had ascended to within 3 cm of the top, the plates were air dried and examined for mellein and 4-hydroxymellein under shortwave (254 nm) UV light.

Identification. Identity of the extracted metabolites was confirmed by TLC co-chromatography with authentic mellein and 4-hydroxymellein in four solvent systems: chloroform-acetone (93:7, v/v) (2); methanol-glacial acetic acid-benzene (5:5:90, v/v/v) (7); glacial acetic acid-benzene-water (10:90:1, v/v/v) (10); and toluene-ethyl acetate-90% formic acid (5:4:1, v/v/v) (2, 3). UV and low-resolution mass spectral analyses were performed on authentic and extracted mellein and 4-hydroxymellein by using a Beckman DK-2A ratio-recording spectrophotometer and a Dupont Instruments 21-490 mass spectrometer, respectively. Results of these analyses agreed with those reported by Cole et al. (2). Other properties of the mellein and 4-hydroxymellein investigated were solubility in sodium bicarbonate and response to ammonium hydroxide fumes after separation by TLC.

Quantitation. A weighed sample of authentic mellein or 4-hydroxymellein was placed in a known volume of chloroform. Dilutions were prepared and optical density (OD) values were determined with a Perkin-Elmer (Coleman 139) spectrophotometer at wavelengths of 314 and 315 nm (2) for mellein and 4-hydroxymellein, respectively. Light path width was 1.8 mm. OD values and concentrations were plotted on linear graph paper, and these curves were used to quantitate production.

The recovery of authentic mellein and 4-hydroxymellein from TLC was determined. A weighed sample of each was placed in a volume of chloroform to provide a concentration of 1 μg/μliter. Samples of 5, 10, 15, and 20 μg were spotted 3 cm from the bottom of a silica gel (100-μm layer) TLC sheet (Eastman 6061 chromagram). Chromatograms were developed in lined chromatography tanks containing a 2-cm layer of chloroform-acetone (93:7, v/v). Solvent was allowed to ascend to within 3 cm of the top, and spots of mellein and 4-hydroxymellein were located under shortwave UV light. Equal-size portions (2 by 3 cm) of the chromatogram containing each fluorescing spot were marked with a pencil and transparent plastic template. Spots were cut from the chromatogram sheet and the chemical was eluted with 4.1 ml of chloroform in a 125-ml Erlenmeyer flask. A reference solvent blank was cut from a portion of the developed chromatogram sheet at the same R_F, but contained no mellein or 4-hydroxymellein.

To quantitate the metabolites, a 5-μliter sample of each crude chloroform extract was spotted on a chromatogram sheet. Authentic mellein and 4-hydroxymellein were included on each TLC sheet as internal identification standards. Also, a known amount of each chemical was spotted on one sheet of each experiment as a check on elution efficiency. Sheets were developed, spots located, and chemicals eluted and quantitated as described above.

Data analysis. The data were analyzed by analysis of variance procedures, and differences between means were determined by applying Duncan's new multiple-range test (8).

RESULTS

Metabolite extraction and characterization. Mellein, 4-hydroxymellein, and ochratoxin A were produced on some natural substrates. Properties useful in identifying these metabolites in crude extracts are summarized in Table 1. Separation was accomplished by extracting the crude chloroform extract with 0.5 M sodium bicarbonate. Mellein and 4-hydroxymellein remained in the organic phase, and bicarbonate soluble ochratoxin A passed into the aqueous phase (3, 9). Fluorescing spots in chloroform extracts of the culture filtrate had the same mobility and fluorescent color as authentic mellein or 4-hydroxymellein in all solvent systems (Table 1).

Quantitation. Standard OD regression curves were determined for authentic mellein and 4-hydroxymellein in chloroform. Concentrations of 1 to 10 μg/ml gave a linear response. Simple regression equations derived from these data are: $Y = 0.5 + 34.852X$ and $Y = 0.05 + 44.095X$ for mellein and 4-hydroxy-

TABLE 1. *Characteristics useful in identifying mellein, 4-hydroxymellein, and ochratoxin A in crude extracts*

Solvent system[a]	Chromatography tank	Silica gel thickness (μm)	R_F			Original fluorescent color (with 254 nm of UV)			Fluorescent color after exposure to ammonia fumes		
			Mellein	4-Hydroxymellein	Ochratoxin A	Mellein	4-Hydroxymellein	Ochratoxin A	Mellein	4-Hydroxymellein	Ochratoxin A
1	Lined	500[b]	0.79	0.36	0.0	Yellow-green	Yellow-green	Green	Blue	Blue	Dark blue
1	Lined	100[c]	0.54	0.34	0.0	Yellow-green	Light blue	Green	Blue	Blue	Dark blue
2	Lined	500[b]	0.50	0.18	0.22	Yellow-green	Yellow-green	Green			
2	Unlined	100[c]	0.90	0.55	0.54	Yellow-green	Yellow-green	Green	Blue	Blue	Dark blue
3	Lined	500[b]	0.57	0.25	0.32	Yellow-green	Yellow-green	Green			
4	Lined	500[b]	0.71	0.54	0.64	Yellow-green	Yellow-green	Green	Blue	Blue	Dark blue

[a] 1, Chloroform-acetone (93:7, v/v); 2, methanol-glacial acetic acid-benzene (5:5:90, v/v); 3, glacial acetic acid-benzene-water (10:90:1, v/v); 4, toluene-ethyl acetate-90% formic acid (5:4:1, v/v).

[b] Glass plate (20 by 20 cm).

[c] Eastman chromogram (6061).

mellein, respectively.

Recovery efficiency of known amounts of mellein and 4-hydroxymellein from developed TLC sheets was determined. Amounts of 5, 10, 15, and 20 μg were eluted with 4.1 ml chloroform from equal size portions of the chromatogram. Recovered amounts were not significantly different at the 5% level from the original amounts spotted.

Sucrose concentration. Mellein production increased from 129 μg/25 ml at 2% to 1,430 μg/25 ml at 32% sucrose, but there was no significant difference in the production efficiency (amount produced per mg of mycelium) at sucrose concentrations from 4 to 16%. 4-Hydroxymellein production increased from 153 μg/25 ml at 2% sucrose to 354 μg at 4%. Production efficiency of 4-hydroxymellein was similar at sucrose concentrations of 2, 4, 8, and 16%; production was significantly lower at 32%.

Carbon source. Effects of various carbon sources, at equimolar concentrations, on production of mellein and 4-hydroxymellein are summarized in Table 2. Production of mellein based on equimolar concentrations of the various carbon sources converted to micrograms per 25 ml per mole of C indicated that gluconolactone was the best carbon source, supporting a yield of 16,010 μg/mole of C. Dextrose and sucrose supported production of 11,901, and 8,240 μg/mole of C, respectively. Production of 4-hydroxymellein from gluconolactone was highest of all sources evaluated, with a yield of 1,930 μg/mole of C, but this was not significantly greater than the 1,810 μg/mole of C produced with glycerol.

L-Glutamic acid concentration. At 6 g of glutamic acid/liter, mellein production was 2,050 μg/25 ml. Total production was not significantly different at glutamic acid concentrations of 3, 6, or 12 g/liter, but the production efficiency was greatest at 6 g/liter. A yield of 3.96 μg/mg of mycelium was obtained, and this was significantly greater than for any other concentration. Production of 4-hydroxymellein increased with increasing glutamic acid concentration. Yields of 130, 540, and 600 μg/25 ml were obtained from 3, 6, and 12 g of glutamic acid/liter, respectively, but as with mellein, production efficiency was highest at 6 g/liter. At this concentration, 1.04 μg/mg of mycelium was obtained.

Organic nitrogen. Mellein production (Table 3) was 1,810 μg/25 ml with urea as the nitrogen source. L-Glutamic acid and glycine supported yields of 1,680 μg of mellein per 25 ml. L-Methionine and phenylalanine supported some growth and production of small amounts of mellein, but no production occurred on tryptophan or anthranilic acid. Production of 4-hydroxymellein on yeast extract and peptone was 674 and 500 μg/ml, respectively. Peptone was the most efficient nitrogen source, supporting a yield of 2.10 μg/mg of mycelium.

Inorganic nitrogen. Production of mellein and 4-hydroxymellein on equimolar concentrations of nitrogen from various inorganic sources is summarized in Table 4. Mellein production (2,610 μg/25 ml) in the glutamic acid control was highest. Yields of 1,890 and 1,850 μg/25 ml were produced on potassium nitrate and calcium nitrate, respectively. Highest production efficiency occurred with

TABLE 2. *Production of mycelium, mellein, and 4-hydroxymellein by A. ochraceus in stationary culture on equimolar concentrations of various carbon sources*[a-g]

Carbon source	Concn (g/liter)	Final pH[h]	Mycelial dry wt (mg/25 ml)	Mellein			4-Hydroxymellein		
				μg/25 ml	μg/mg of mycelium	μg/25 ml/mole of C	μg/25 ml	μg/mg of mycelium	μg/25 ml/mole of C
None		8.42	77[f]	T[d]	T[f]	T[g]	T[d]	T[e]	T[e]
Sucrose	80.0	6.77	442[a]	2,320[a]	5.25[bc]	8,240[c]	309[ab]	0.69[abcd]	1,400[bcd]
Maltose[i]	80.0	6.96	399[ab]	1,371[bc]	3.43[cd]	4,880[cde]	276[abc]	0.69[abcd]	980[bcd]
Cellobiose[i]	80.0	6.92	332[bc]	555[d]	1.67[def]	1,980[e/g]	73[d]	0.22[de]	480[cde]
Trehalose	80.0	8.37	271[cd]	0[d]	0[f]	T[g]	25[d]	0.10[de]	T[e]
Soluble starch[j]	80.0	7.36	402[ab]	1,846[ab]	4.59[bc]	6,570[cd]	376[a]	0.94[ab]	1,320[bc]
Glucono-δ-lactone[i]	41.7	7.38	310[bcd]	2,249[a]	7.25[a]	16,010[a]	271[abc]	0.87[abc]	1,930[a]
Dextrose[i]	42.2	6.29	282[cd]	1,674[ab]	5.94[ab]	11,910[b]	145[bcd]	0.51[bcd]	1,030[bcd]
Fructose[i]	42.2	6.56	262[cde]	721[cd]	2.75[cde]	5,120[cde]	134[cd]	0.51[bcd]	960[bcde]
Sorbitol	42.7	7.44	330[bc]	573[d]	1.74[def]	4,080[def]	102[cd]	0.31[cde]	730[cde]
Galactose[i]	42.2	7.01	264[cde]	429[d]	1.63[def]	3,060[def/g]	90[d]	0.34[cde]	640[cde]
Citric acid	45.0	7.12	227[de]	81[d]	0.36[f]	860[f/g]	T[d]	T[e]	T[de]
Xylose[i]	35.2	6.74	252[cde]	765[cd]	3.04[cde]	6,520[cd]	104[cd]	0.41[cde]	890[bcde]
Fumaric acid	27.2	9.10	213[de]	0[d]	0[f]	0[g]	0[d]	0[e]	0[e]
Glycerol	22.6	7.07	169[e]	200[d]	1.18[ef]	2,720[ef/g]	133[cd]	0.79[abc]	1,810[ab]
Sodium acetate	19.2	5.11	11[f]	0[d]	0[f]	0[g]	0[d]	0[e]	0[e]

[a-g] Mean values within any one column followed by the same letter are not different ($P < 0.05$). T = trace.
[h] Initial pH was 5.0.
[i] Sterilized by filtration.
[j] Moles of carbon estimated.

TABLE 3. *Production of mycelium, mellein, and 4-hydroxymellein by A. ochraceus in stationary culture on equimolar nitrogen from organic sources*[a-e]

Source	Concn (g/liter)	Final pH[f]	Mycelial dry wt (mg/25 ml)	Mellein		4-Hydroxymellein	
				μg/25 ml	μg/mg of mycelium	μg/25 ml	μg/mg of mycelium
None		5.03	4[d]	0[e]	0[e]	0[e]	0[d]
Yeast extract[g]	5.68	5.34	378[a]	1,340[bcd]	3.54[d]	674[a]	1.78[ab]
Urea	2.7	5.54	191[bc]	1,810[a]	9.48[a]	140[de]	0.73[bcd]
L-Glutamic acid	6.0	6.69	315[ab]	1,680[ab]	5.33[bc]	350[bc]	1.11[b]
Glycine	3.08	4.23	245[bc]	1,680[ab]	6.86[b]	250[cd]	1.02[bc]
Alanine	3.65	4.51	204[bc]	1,430[bc]	7.00[b]	270[cb]	1.32[ab]
L-Glutamine	2.30	4.87	315[ab]	1,290[cd]	4.09[cd]	260[cd]	0.83[bc]
Asparagine	3.08	4.63	254[ab]	1,240[cd]	4.88[cd]	400[bc]	1.57[ab]
L-Aspartic acid	5.46	6.75	296[ab]	1,160[cd]	3.92[cd]	390[bc]	1.32[ab]
Peptone	3.38	4.53	238[bc]	970[d]	4.08[cd]	500[ab]	2.10[a]
L-Methionine	6.12	3.13	214[bc]	120[e]	0.56[e]	40[e]	0.19[d]
Phenylalanine	6.77	3.66	116[cd]	T[e]	T[e]	T[e]	T[d]
Tryptophan	8.37	3.95	119[cd]	0[e]	0[e]	0[e]	0[d]
Anthranilic acid	5.62	5.13	11[d]	0[e]	0[e]	0[e]	0[d]

[a-e] Mean values within any one column followed by the same letter are not different ($P < 0.05$). T = trace.
[f] Initial pH was 5.0.
[g] Some ochratoxin produced, but not quantitated.

potassium nitrate (7.33 μg/mg of mycelium). The next most efficient nitrogen sources were, in decreasing order, glutamic acid (control), calcium nitrate, ammonium chloride, and ammonium nitrate. Only a trace of mellein was produced with ammonium sulfate. Production of 4-hydroxymellein was highest with glutamic acid (control) and calcium nitrate (230 and 220 μg/25 ml, respectively). Production per milligram of mycelium was not significantly different with any source except ammonium chloride and ammonium sulfate,

TABLE 4. *Production of mycelium, mellein, and 4-hydroxymellein by A. ochraceus in stationary culture on equimolar concentrations of nitrogen from inorganic sources*[a-d]

Source	Concn (g/liter)	Final pH[e]	Mycelial dry wt (mg/25 ml)	Mellein		4-Hydroxymellein	
				μg/25 ml	μg/mg of mycelium	μg/25 ml	μg/mg of mycelium
None		5.0	2[d]	0[c]	0[c]	0[b]	0[b]
L-Glutamic acid (control)	6.0	6.51	391[a]	2,610[a]	6.68[ab]	230[a]	0.59[a]
Calcium nitrate	3.37	6.23	373[ab]	1,850[b]	4.96[b]	220[a]	0.59[a]
Potassium nitrate	4.56	6.22	258[bc]	1,890[b]	7.33[a]	70[b]	0.27[ab]
Ammonium nitrate	3.28	3.46	299[abc]	470[c]	1.57[c]	73[b]	0.24[ab]
Ammonium chloride	2.39	2.40	172[c]	300[c]	1.74[c]	T[b]	T[b]
Ammonium sulfate	2.71	2.41	216[c]	T[c]	T[c]	T[b]	T[b]

[a-d] Mean values within any one column followed by the same letter are not different ($P < 0.05$). T = trace.
[e] Initial pH was 5.0.

which produced only trace amounts.

Time-temperature. Maximum mellein production of 1,400 μg/25 ml occurred at 25 C in 10 days. 4-Hydroxymellein and mellein were produced concurrently. Maximum 4-hydroxymellein production (460 μg/25 ml) occurred at 25 C in 14 days.

Trace elements. Results from 7-day stationary-culture experiments, in which the trace elements Zn, Cu, Fe, Mn, and Mo were deleted or added separately to the basic culture medium, indicated that mellein production was high in all cultures. When no trace elements were added a yield of 1,430 μg/25 ml was obtained, compared to a yield of 1,875 μg/25 ml in the presence of all five trace elements. When Mn, Cu, and Mo were added individually, reduced mycelial yields resulted, but high yields of mellein per milligram of mycelium were obtained. Production of 4-hydroxymellein was 470 μg/25 ml when all trace elements were added. Deletion of Fe, Mn, Cu, or Mo had no significant effect on production. When Zn was omitted, a yield of only 0.04 μg/mg of mycelium was obtained, compared to 0.86 μg/mg when it was the only trace element added. When either Fe, Mn, or Cu was added alone, a significant reduction in yield of 4-hydroxymellein resulted; no significant reduction in yield was observed with Mo alone.

Stationary versus shaken culture. Production of mellein and 4-hydroxymellein was compared in parallel stationary and shaken cultures containing only the trace elements Zn and Mo. Total mellein production was the same in stationary and shaken cultures; however, production efficiency was significantly greater in stationary cultures (3.75 μg/mg of mycelium compared to 1.96 μg/mg of mycelium). 4-Hydroxymellein production was greater in stationary culture (720 μg/50 ml) as opposed to shaken cultures (480 μg/50 ml); production efficiency was greater also in stationary culture. A trace of ochratoxin A was produced in shaken culture.

Natural substrates. Four natural substrates were evaluated for their ability to support production of mellein and 4-hydroxymellein. A trace of mellein was produced on yellow corn, but none was produced on wheat, peanuts, or soybeans. 4-Hydroxymellein production on corn was 5,610 μg/50 g, but none was produced on wheat, peanuts, or soybeans. Ochratoxin A was produced on all substrates. Corn yielded 6,500 μg/50 g and wheat 4,700 μg/50 g. Peanuts and soybeans supported much lower yields of 1,830 and 1,760 μg/50 g of ochratoxin A.

Medium for production of mellein and 4-hydroxymellein. As a result of this investigation, the following culture medium is suggested for mellein and 4-hydroxymellein production (per liter): sucrose, 80.0 g; L-glutamic acid, 6.0 g; KH_2PO_4, 1.0 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 1.0 mg; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.5 mg. Other carbon or nitrogen sources may be substituted, but this formulation gives high yields and is easy to prepare. Incubation may be as stationary or shaken culture at 25 to 30 C for 10 to 14 days. Initial pH of 4.0 to 6.0 is satisfactory.

DISCUSSION

The four solvent systems (Table 1) in lined chromatography tanks provided consistent R_F values and a range of separation distances on TLC plates that permitted preliminary identification of mellein, 4-hydroxymellein, and ochratoxin A. Distinct color differences were apparent when these metabolites were viewed

under shortwave UV light.

In quantitating mellein and 4-hydroxymellein by standard visualization techniques, methods used for other fungal metabolites proved to be unsatisfactory because mellein faded on developed TLC plates shortly after exposure to light. UV spectrophotometry was used to develop a method requiring minimum exposure of the metabolites to light before quantitation. OD regression curves of authentic mellein and 4-hydroxymellein revealed that concentrations of 1 to 10 μg/ml gave OD readings generally considered reliable in spectrophotometric determinations. Separation on Eastman chromagram sheets in chloroform-acetone (93:7, v/v) was satisfactory (Table 1), and the amount applied could be recovered from the TLC plate by elution with chloroform.

Mellein production showed a linear relationship with increasing sucrose concentration over the range studied, but production efficiency was best at 4, 8, and 16%. 4-Hydroxymellein production efficiency was the same at sucrose concentrations of 2, 4, 8, and 16%, similar to ochratoxin A and B production reported by Davis et al. (3). For both mellein and 4-hydroxymellein production a sucrose concentration of 8% was optimal (based on total amount produced per flask). Evaluation of production on media, with equimolar concentrations of 15 carbon sources, revealed that compounds that are oxidized normally through both the hexose monophosphate and glycolytic pathways supported both growth and metabolite production. The organism grew poorly on the tricarboxylic acid cycle intermediates and metabolite production was poor.

L-Glutamic acid at 6 g/liter produced the maximal amounts of mellein and 4-hydroxymellein. Ferreira (4) found that 7.5 to 10 g/liter was maximal for ochratoxin A production by *A. ochraceus* in a similar medium. The 6 g/liter concentration was used as a control and as a basis for evaluating other organic and inorganic nitrogen sources.

Production of mellein and 4-hydroxymellein coincided with rapid mycelial growth at 20, 25, 30, and 35 C. Incubation for longer than 8 to 10 days resulted in decreased yields of mellein but not 4-hydroxymellein. Incubation for 10 to 14 days at 25 to 30 C was selected as optimal for production of both metabolites in the same medium.

Additional Zn and Mo were needed for maximal production of mellein and 4-hydroxymellein in stationary culture, but were not required for maximal production in shaken culture.

Possible complications, due to the production of both 4-hydroxymellein and ochratoxin A on common substrates, should be considered when production of either metabolite is being investigated because of their similar characteristics on developed TLC plates.

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LITERATURE CITED

1. Christensen, C. M., and D. R. Gordon. 1948. The mold flora of stored wheat and corn and its relation to heating of moist grain. Cereal Chem. **25**:40-51.
2. Cole, R. J., J. H. Moore, N. D. Davis, J. W. Kirksey, and U. L. Diener. 1971. 4-Hydroxymellein: a new metabolite of *Aspergillus* ochraceus. J. Agr. Food Chem. **19**:909-911.
3. Davis, N. D., J. W. Searcy, and U. L. Diener. 1969. Production of ochratoxin A by *Aspergillus ochraceus* in a semisynthetic medium. Appl. Microbiol. **17**:742-744.
4. Ferreira, N. P. 1967. Recent advances in research on ochratoxin. Part 2. Microbiological aspects, p. 168. *In* R. I. Mateles and G. N. Wogan (ed.), Biochemistry of some foodborne microbial toxins. M. I. T. Press, Cambridge, Mass.
5. Sasaki, M., Y. Kaneko, K. Oshita, H. Takamatsu, Y. Asao, and T. Yokotsuka. 1970. Studies on the compounds produced by molds. Part VII. Isolation of isocoumarin compounds. Agr. Biol. Chem. **34**:1296-1300.
6. Scott, De B. 1965. Toxigenic fungi isolated from cereal and legume products. Mycopathol. Mycol. Appl. **25**: 213-222.
7. Shotwell, O. L., C. W. Hesseltine, and M. Gouldin. 1969. Note on the natural occurrence of ochratoxin A. J. Ass. Offic. Anal. Chem. **52**:81-83.
8. Steel, R. G. D., and J. H. Torrie. 1960. Analysis of variance I: the one-way classification. *In* Principles and procedures of statistics. McGraw-Hill, New York.
9. Steyn, P. S., and K. J. Van der Merwe. 1966. Detection and estimation of ochratoxin A. Nature (London) **211**: 418.
10. Theron, J. J., K. J. Van der Merwe, N. Liebenberg, H. J. B. Joubert, and W. Nel. 1966. Acute liver injury in ducklings and rats as a results of ochratoxin poisoning. J. Pathol. Bacteriol. **91**:521-529.
11. Thom, C., and K. B. Raper. 1945. A manual of the aspergilli. The Williams & Wilkins Co., Baltimore.
12. Van der Merwe, K. J., P. S. Steyn, L. Fourie, De B. Scott, and J. J. Theron. 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. Nature (London) **205**:1112-1113.
13. Van der Merwe, K. J., P. S. Steyn, and L. Fourie. 1965. Mycotoxins. Part 2. The constitution of ochratoxins A, B, and C, metabolites of *Aspergillus ochraceus* Wilh. J. Chem. Soc. **87**:7083-7088.