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Chemical and Cytotoxicity Survey on the Production of Ochratoxins and Penicillic Acid by *Aspergillus ochraceus* Wilhelm[1]

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Thirty-three strains of *Aspergillus ochraceus* isolated from foods in Japan were examined for the bioproduction of toxic metabolites, and the formation of ochratoxins by two strains and of penicillic acid by twenty-eight strains was proved. The two toxins and other metabolites were isolated and identified. Toxicities shown by the metabolites were examined by HeLa cells and the relation with some metabolites was discussed from the similarities of morphological pattern by a group of strains.

The fungus, *Aspergillus ochraceus* Wilhelm, and its allied species are widely distributed in agricultural commodities and have been reported as constituents of microflora characteristic of fermented fish preparations. Members of the *A. ochraceus* group are quite active biochemically. There have been several reports on the metabolites of the fungi belonging to this group, *i.e.* ochracin (mellein)[3] (I), penicillic acid[4] (II), hydroxyaspergillic acid[5,6] (III), neohydroxyaspergillic acid[7] (IV), 3-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one[8] (V), and aspochracin[9] (VI), but the fungus had not been assumed as toxigenic till the isolation of ochratoxins from the strain K-804 originated from sorghum grain in South Africa.[10] The chemical structures (VII—IX) of ochratoxins A, B, and C were thereafter suggested[11] and confirmed by synthesis.[12] Recently the minor metabolites (X—XII) related to VII—IX,[5] the biosynthesis of VII,[13] and the production of VII by the strains of *A. melleus* Yukawa and *A. sulphureus* (Fres.) Thom et Church of the same group[14] and by *Penicillium viridicatum* Westling[15] were also reported. Although it has not yet been known whether ochratoxins

1) A part of this work was presented at the Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969.
2) Location: *a*) *Kamiyoga-1-chome, Setagaya-ku, Tokyo*; *b*) *Shirokanedai, Minato-ku, Tokyo.*
3) T. Yabuta and T. Sumiki, *Nippon Nogeikagaku Kaishi*, **9**, 1264 (1933); *idem*, **10**, 703 (1934); H. Nishikawa, *ibid.*, **9**, 772, 1059 (1933); M. Matsui, K. Mori, and S. Arasaki, *Agric. Biol. Chem.*, **28**, 896 (1964).
4) E.O. Karow, H.B. Woodruff, and J.F. Foster, *Arch. Biochem.*, **5**, 279 (1944); J.H. Birkinshow, A.E. Oxford, and H. Raistrick, *Biochem. J.*, **30**, 394 (1936); J.H. Ford, A.R. Johnson, and J.W. Hinman, *J. Am. Chem. Soc.*, **72**, 4529 (1950).
5) P.S. Steyn and C.W. Holzapfel, *J. South African Chem. Inst.*, **20**, 186 (1967).
6) J.D. Dutcher, *J. Biol. Chem.*, **232**, 785 (1958).
7) R.G. Micetich and J.J. MacDonald, *J. Chem. Soc.*, **1964**, 1507.
8) S.D. Mills and W.B. Turner, *J. Chem. Soc.* (*C*), **1967**, 2242.
9) R. Myokei, A. Sakurai, C.-F. Chang, N. Takahashi, Y. Kodaira, and S. Tamura, *Tetrahedron Letters*, **1969**, 695; R. Myokei, A. Sakurai, C.F. Chang, Y. Kodaira, N. Takahashi, and S. Tamura, *Agric. Biol. Chem.*, **33**, 1491 (1969); C.-F. Chang, R. Myokei, A. Sakurai, N. Takahashi, and S. Tamura, *ibid.*, **33**, 1501 (1969).
10) K.J. van der Merve, P.S. Steyn, L. Fourie, De B. Scott, and J.J. Theron, *Nature*, **205**, 1112 (1965).
11) K.J. van der Merve, P.S. Steyn, and L. Fourie, *J. Chem. Soc.*, **1965**, 7083.
12) P.S. Steyn and C.W. Holzapfel, *Tetrahedron*, **23**, 4449 (1967).
13) J.W. Searcy, N.D. Davis, and U.L. Diener, *Applied Microbiol.*, **18**, 622 (1969).
14) M. Lai, G. Semeniuk, and C.W. Hesseltine, *Phytopathol.*, **58**, 1056 (1968).
15) W. van Walbeek, P.M. Scott, J. Harwig, and J.W. Lawrence, *Can. J. Microbiol.*, **15**, 1281 (1969).

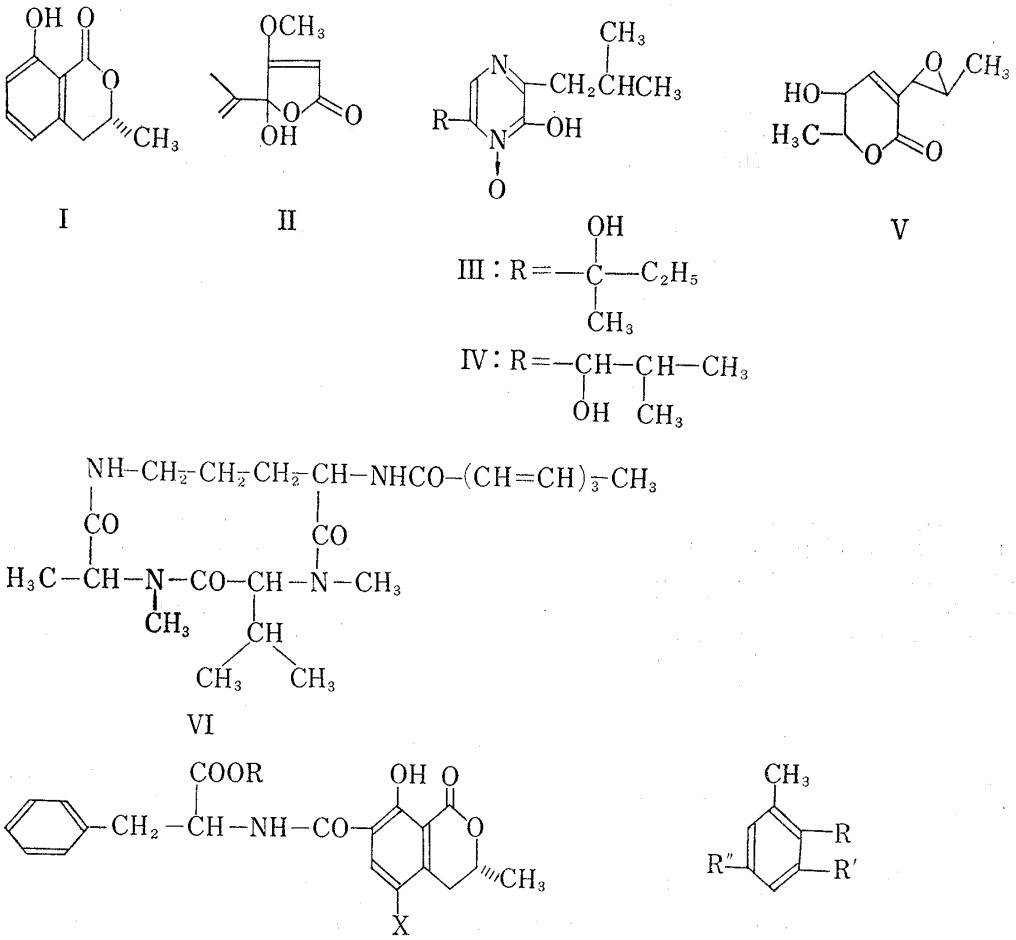

Chart 1

are carcinogenic, acute toxicity[10,16] shown by the toxins, especially by VII, is remarkable. Furthermore, penicillic acid (II) has now been reported as carcinogenic.[17]

In the course of our studies on the detection and isolation of mycotoxin-producing fungi from foodstuffs collected in this country along with epidemiological investigation,[18,19] several strains of *A. ochraceus* have been often encountered growing upon a wide variety of foods, especially cereals and dried sardines collected at Minamikushiyama Village, Nagasaki Prefecture, where the mortality rate by liver diseases including liver cirrhosis has been recorded high.

16) J.J. Theron, K.J. van der Merve, N. Liebenberg, H.J.B. Joubert, and W. Nel, *J. Pathol. Bacteriol.*, **91**, 521 (1966).
17) F. Dickens, "Carcinogenesis; A broad critique," The Williams and Wilkins Co., Baltimore, 1967, pp. 447—470.
18) S. Udagawa, M. Ichinoe, and H. Kurata, in M. Herzberg (ed), Proc. First U.S.-Japan Conf. on Toxic Microorganisms, Unnumb. Pub. (In Press), U.S. Dept. of Interior and UJNR Panels on Toxic Microorganisms, Washington, D.C., 1970.
19) M. Saito, M. Umeda, K. Ohtsubo, H. Kurata, S. Udagawa, and S. Natori, Proc. Japan. Cancer Assoc., 27th Ann. Meeting, Tokyo, Oct. 1968, p. 59; M. Saito, M. Enomoto, M. Umeda, K. Ohtsubo, H. Kurata, S. Udagawa, S. Natori, S. Yamamoto, and H. Toyokawa, Proc. Japan. Cancer Assoc., 28th Ann. Meeting, Kanazawa, Oct. 1969, p. 255.

These isolations have led to the question of possible toxigenic potentialities for human health as non-intentional carcinogenic contaminants. Unfortunately in Japan, no information is available on the field survey of ochratoxin-producing fungi.

Under such circumstances this paper describes the chemical detection by thin-layer chromatography (TLC), the isolation, and toxicity bioassay using HeLa cells of the metabolites produced by certain cultures of *A. ochraceus*, including those recently isolated from various kinds of foods in the National Institute of Hygienic Sciences.

Methods

Source of Isolates——Thirty-three strains of *A. ochraceus* were studied. A list of sources and localities is given in Table I. All strains were routinely maintained on slants of potato-dextrose agar in the cold.

Experiment 1. Primary Survey of Toxin Production——Two-week-old culture of each strain of *A. ochraceus* grown on potato-dextrose agar was used to inoculate three Sakaguchi's flasks, each containing 100 ml of the following medium: sucrose, 30 g; glutamic acid, 10 g; KH_2PO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3 \cdot 6H_2O$, 24 mg; $ZnSO_4 \cdot 7H_2O$, 22 mg; $MnSO_4 \cdot 5H_2O$, 11 mg; $CuSO_4 \cdot 5H_2O$, 4 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.5 mg; distilled water, 1 liter, pH 6.0.[20] Cultures were incubated at 25° for 7 days on a reciprocal shaker. At the end of the incubation period, fresh mycelium from each strain was separated from liquid medium by filtration and soon homogenized with chloroform (60 ml per each flask) in a Waring Blender. The chloroform layer was evaporated to obtain mycelial extract. The cultural filtrate (final pH 2.4 to 8.2) was acidified (to pH *ca.* 2.0) with HCl and extracted also with chloroform.

For the detection of metabolites, both chloroform extracts were dissolved in a definite amount (3 ml) of $CHCl_3$, a definite amount of which (20 μl) was applied on thin-layer plates. For toxicity bioassay, they were dissolved in dimethyl sulfoxide and administered to HeLa cells as described later.

Experiment 2. Isolation of Ochratoxin A——According to a primary survey, only two strains NHL 5093 and 5112 were determined as ochratoxin-producers. Culture method for this experiment was the same as described in experiment 1, except that the scale was rather larger. The mycelium was dried up at 45°, resulting 29—61 g from three liter of the medium. Extraction of the filtrate (pH 6.4—8.6) with $CHCl_3$ was performed after acidification (pH 3.0).

Experiment 3. Isolation of Penicillic Acid——*A. ochraceus* NHL 5111 was grown in a stationary cultures on the modified glucose-Czapek's medium containing 0.5% malt extract and 0.2% yeast extract. From the cultural filtrate (final pH 6.1 to 7.0), penicillic acid was extracted with chloroform after the saturation with ammonium sulfate. This strain was further surveyed by extracting 25 g of the dried mycelium (yield: 7—13 g per liter of the medium) in a Soxhlet apparatus with hexane, ether, chloroform and methanol, successively.

Analytical Methods (TLC of the Metabolites of *A. ochraceus* and the Related Compounds)——(a) Samples: The metabolites of *A. ochraceus*; ochracin (I), penicillic acid (II), hydroxyaspergillic acid (III), ochratoxin A, B, and C (VII—IX). The suggested precursors of penicillic acid[21] and the related compounds; orsellinic acid (XIII), everninic acid (XIV), isoeverninic acid (XV), orcinol (XVI), orcinol monomethyl ether (XVII), and 6-methylsalicylic acid (XVIII).

(b) Plate: Silica gel G and Silica gel G treated with 3% oxalic acid solution; 20×20 cm; thickness, 250 μ.

(c) Developer: i) Benzene–MeOH–EtOAc (12:2:1), ii) Benzene–MeOH–EtOAc (15:2:1), iii) Benzene–acetone (3:2), iv) EtOAc–isopropanol–water (5:2:1), v) Benzene–$CHCl_3$–EtOAc (8:3:2), and vi) Benzene–EtOAc (4:1).

(d) Detection: Fluorescence under a ultraviolet (UV) lamp (365 mμ) for I (blue), VII, VIII, and IX (blue green) and spraying $KMnO_4$ solution for other compounds. Detection limit, I, VII—IX, *ca.* 0.02 μg; II, 0.2 μg.

(e) *Rf* Values: The reported methods for the detection of VII—IX[22,23] were comparatively examined and the best separation for all compounds was obtained by the solvent (ii) on the acid-treated Silica gel G plate; thus the system was used throughout this work. *Rf* values are as follows: I, 0.96, II, 0.45, III, 0.27, VII, 0.60, VIII, 0.30, IX, 0.95, XIII, 0.27, XIV, 0.58, XV, 0.19, XVI, 0.25, XVII, 0.50, XVIII, 0.97. As for the simplified method for the determination of II, the linearity between the amount of II (0.3—20 μg)

20) N.P. Ferreira, *J. Microbiol. Serol.*, **34**, 433 (1968); N.P. Ferreira, "Biochemistry of some Foodborne Microbial Toxins," ed. R.I. Mateles and G.W. Worgan, M.I.T. Press, Cambridge, 1967, p. 157.

21) A.J. Birch, G.B. Blance, and H. Smith, *J. Chem. Soc.*, **1958**, 4582; K. Mosbach, *Acta Chem. Scand.*, **14**, 457 (1960); R. Bentley and J.G. Keil, *Proc. Chem. Soc.*, **1961**, 111; R. Bentley and J.G. Keil, *J. Biol. Chem.*, **237**, 867 (1962); J.H. Birkinshow and A. Goulland, *Biochem. J.*, **84**, 342 (1962).

22) P.S. Steyn and K.J. van der Merve, *Nature*, **211**, 418 (1966).

23) P.M. Scott and T.B. Hand, *J. Assoc. Official Agric. Chemists*, **50**, 366 (1967).

and the diameter of the spot was confirmed and applied for further work. For the confirmation of the presence of VII—IX the solvent (iv) in Silica gel G was also employed. The *Rf* values are VII, 0.42, VIII, 0.25, and IX, 0.95.

Bioassay Using HeLa Cells——HeLa cells grown in Eagle's minimum essential medium supplemented with 10% calf serum and antibiotics were used throughout. A modification of plastic panel technique by Toplin[24] was employed for toxicity bioassay, using cover glasses.[25] The test samples were dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml and diluted with the medium. Final concentrations tested for each sample were 100, 32, 10 and 3.2 μg/ml. All assays were examined after 3 days' incubation for evidence of cytotoxicity of the cells on cover-glasses which were fixed with Carnoy's fixative and stained with hematoxylin and eosin. The degree of toxicity was estimated on a scale ranging 0 (no cellular damage) through 4 (complete cytolysis). An arbitrary criterion for the estimation of cytotoxicity was also employed, consisting of ± (producing cytotoxicity over 3 at the concentration of 100 μg/ml) through ## (producing cytotoxicity over 3 at the concentration of 3.2 μg/ml).[25] Cytomorphological changes were examined under microscopic observation on the stained preparations.

Table I. *A. ochraceus* Cultures studied

NHL[a] strain No.	Isolate No.	Source	Locality
5087	67-MI-81	kidney bean	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5088	67-MI-82	kidney bean	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5089	67-MI-83	polished rice	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5090	67-MI-84	polished rice	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5091	67-MI-85	wheat grain	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5092	67-MI-86	wheat product	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5093	67-MI-87	adzuki bean	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5094	67-MI-88	wheat flour	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5095	67-MI-89	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5096	67-MI-90	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5097	67-MI-91	wheat product	Minamikushiyama Villega, Nagasaki Pref., collected in Aug. 1967
5098	67-MI-92	polished rice	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5099	67-MI-93	polished rice	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5100	67-MI-94	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5101	67-MI-95	adzuki bean	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5102	67-MI-96	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5103	67-MI-97	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5104	67-MI-98	wheat flour	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5105	67-MI-99	adzuki bean	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5106	67-MI-100	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967

24) I. Toplin, *Cancer Res.*, **19**, 959 (1959).
25) M. Umeda, A. Saito, and M. Saito, *Jap. J. Exp. Med.*, in print.

5107	67-MI-101	polished rice	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5108	67-MI-102	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5109	67-MI-103	dried sardines	Minamikushiyama Village, Nagasaki Pref., collected in Aug. 1967
5110	SF-1-3	soybean flour (market product)	Setagaya-ku, Tokyo City, collected in Jan. 1965
5111	SF-13-1	soybean flour (market product)	Setagaya-ku, Tokyo City, collected in March 1965
5112	TO-4	pepper flour (market product)	Setagaya-ku, Tokyo City, collected in Feb. 1965
5113	WF-18-6	wheat flour (market product)	Setagaya-ku, Tokyo City, collected in March 1965
5114	RF-9-5	rice flour (market product)	Setagaya-ku, Tokyo City, collected in Feb. 1965
5115	68-MR-1	polished rice	imported from Republic of China, March 1968
5116	68-MR-2	polished rice	importrd from Republic of China, March 1968
5117	68-MR-3	polished rice	imported from Republic of China, March 1968
5118	68-MR-4	polished rice	imported from Republic of China, March 1968
5119	68-MR-5	polished rice	imported from Republic of China, March 1968

a) NHL, National Institute of Hygienic Sciences, Tokyo

Result

Toxicities of Ochratoxin A, Penicillic Acid, and Ochracin (Mellein)

Ochratoxin A (VII) and penicillic acid (II) are known to be the major mycotoxins of *A. ochraceus* and ochracin (mellein) (I) is structurally related to ochratoxins (VII—XII). As a preliminary examination of bioassay with HeLa cells, these representative toxins were comparatively examined for their toxic concentrations and morphological changes. As shown in Fig. 1, II was most toxic and the toxicity estimate was evaluated as ++. VII was less toxic, producing + toxicity. I showed only slight toxicity. An approximate ratio of the toxicities on HeLa cells among II:VII:I was calculated as 320:100:1.

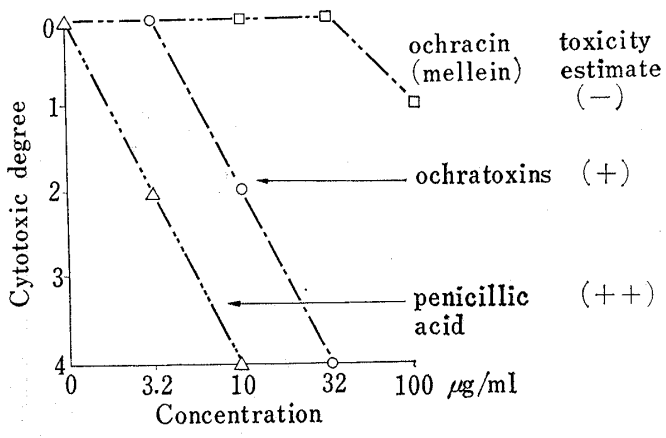

Fig. 1. Toxicity Bioassay of Ochratoxins, Mellein and Penicillic Acid on HeLa Cells by Plastic Panel Method

Morphologically, only atrophic cells remained at 32 μg/ml of ochratoxin A. At 10 μg/ml, there observed increase of mitotic cells. Shortened chromosomes in such cells were apt to arrange dispersed. Interphasic cells showed relatively unaffected (Photo 2). At 3.2 μg/ml, there observed little changes.

Penicillic acid at 10 μg/ml produced increase of mitotic cells and enlargement of interphasic cells. Enlarged interphasic cells contained large nuclei with dotty chromatin and irregular and larger nucleoli. Pleomorphism was relatively marked (Photo 3).

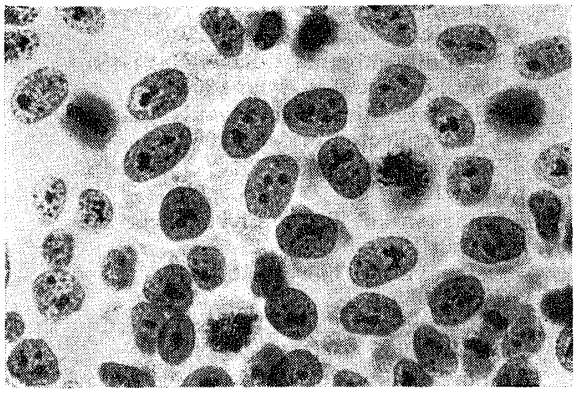

Photo 1. Control Culture of HeLa Cells. 40×10

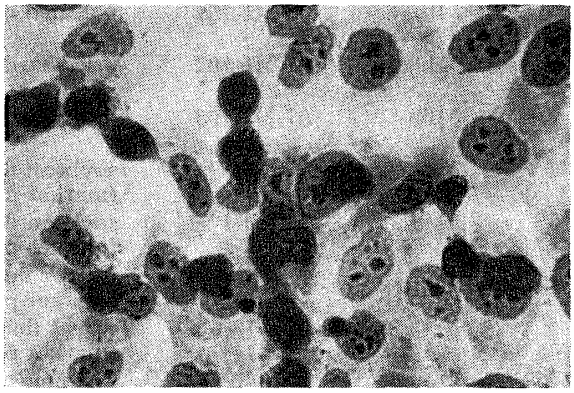

Photo 2. Culture treated with 10 μg/ml of Ochratoxin A for 3 days. 40×10. Irregularity of the Size of Interphasic Cells was slight. There is an Increase of Metaphasic Cells (Round Black Spots in the Photo)

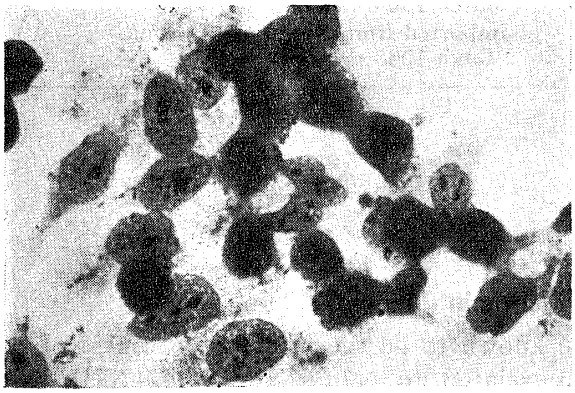

Photo 3. Culture treated with 5.6 μg/ml of Penicillic Acid for 2 Days. 40×10. The Nuclei and the Nucleoli of Interphasic Cells became enlarged. Metaphasic Cells are increased

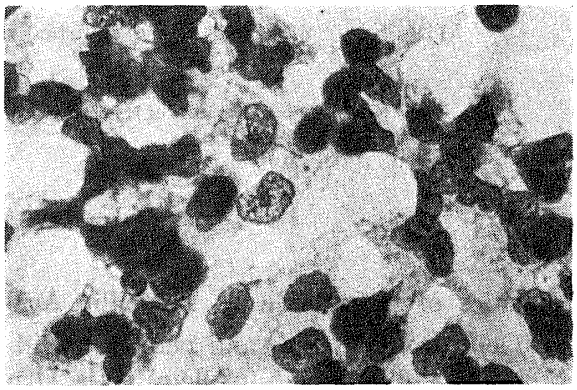

Photo 4. Culture treated with 10 μg/ml of the Chloroform Extract from the Cultural Filtrate of NHL 5105 for 3 Days. 40×10. Cytoplasmic Vacuolation, and Increase of Mitotic Cells are prominent (Pattern 1)

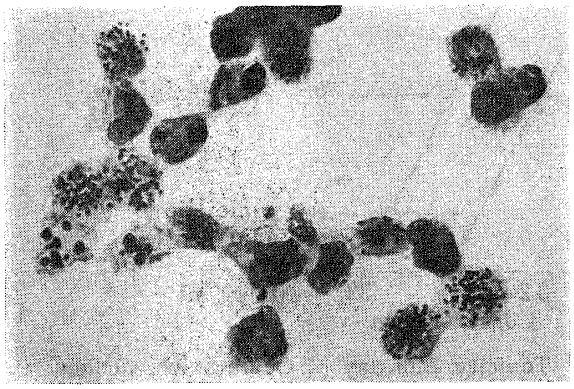

Photo 5. Culture treated with 3.2 μg/ml of the Chloroform Extract from the Mycelium of NHL 5105 for 3 Days. 40×10. Atrophic Cells and Mitotic Cells with shortened dispersed Chromosomes are prominently observed. There are Several Cells with Karyorrhexis (Pattern 2)

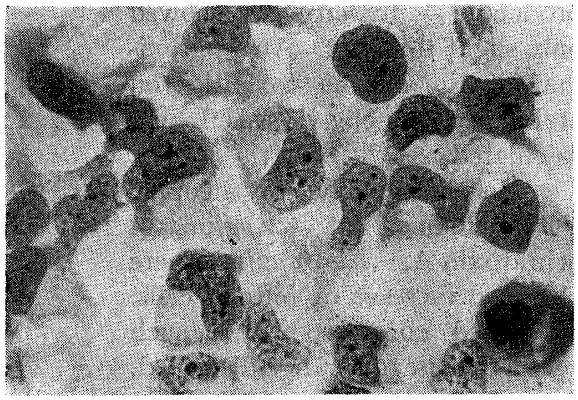

Photo 6. Culture treated with 32 μg/ml of the Chloroform Extract from the Mycelium of NHL 5098 for 3 Days. 40×10. Enlarged Cells with Enlarged Nuclei are marked. There are Clear Zones (Golgi's apparatus?) at the Juxta-nuclear Position (Pattern 3)

Detection of the Metabolites from Strains of *A. ochraceus* by TLC and Bioassay by HeLa Cells

A survey of 33 strains using TLC in Expt. 1 showed more than twenty kinds of spots as a whole on the plates. Their *Rf* values by the solvent (ii) and fluorescence under a UV light were recorded as follows (from the top): 1 (0.96, blue), I, IX, 2 (0.95, orange), 3 (0.95, –), 4 (0.94, –), 5 (0.92, yellow-green), 6 (0.90, –), 7 (0.88, orange), 8 (0.85, blue-green), 9 (0.78, –), 10 (0.78, yellow-green), VII, 11 (0.59, blue), 12 (0.59, blue-green), 13 (0.54, orange), 14 (0.53, –), II, VIII, 15 (0.30, violet), 16 (0.30, blue), 17 (0.26, pink), 18 (0.24, blue-green), 19 (0.20, –), 20 (0, –). Since penicillic acid (II) was proved to be produced by almost all strains, the amount was estimated from the area on the plate. The results are shown in Table II.

TABLE II. Metabolites and Toxicities of the Strains of *A. ochraceus*

Strain	Filtrate				Mycelium			
	Total amount of extract (mg/100 ml)	No. of spot detected[a]	Penicillic acid (mg/100 ml)	Toxicity on HeLa cells	Total amount of extract (mg/100 ml)	No. of spot detected[a]	Penicillic acid (mg/100 ml)	Toxicity on HeLa cells
5087	48	5, 16	—	—	36	4, 5, 6, 16	—	±
5088	44	4, 5, 9, 14, 16	36	++	41	4, 5, 9, 14, 16	—	±
5089	131	4, 5, 8, 9, 12, 14, 18, 19	48	+	36	4, 5, 8, 9, 14, 18, 19	—	—
5090	140	ochracin, 2, 4, 8, 9, 10, 14	33	—	48	1, 4, 5, 9, 10, 14	—	—
5091	9	2, 5, 9, 14, 19	2	±	54	4, 5, 14, 18, 19	4	—
5092	246	1, 11, 14, 19	28	—	56	4, 5, 14, 19	—	—
5093	94	ochratoxins, 17, 20	7	+	49	4, 5, 6, 9, 20	—	±
5094	138	2, 10, 15, 17, 19, 20	24	±	47	4, 5, 10, 13, 15, 19, 20	—	±
5095	118	1, 11, 16, 17, 20	72	+	56	3, 20	6	±
5096	186	9, 10, 19	42	+	53	2, 4, 5, 9, 10, 13, 14, 19	4	—
5097	226	2, 15	—	—	80	4, 5	—	±
5098	179	9, 15, 19	63	+	13	5, 9, 13, 15, 19	—	+
5099	181	7, 9, 15, 19, 20	61	±	7	5, 9, 10, 15, 19	—	+
5100	172	3, 15, 20	26	±	12	3, 5, 10, 15, 20	—	+
5101	146	5, 19, 20	11	±	73	3, 4, 5, 15, 19, 20	—	—
5102	110	3, 15, 20	11	±	75	3, 5, 15, 19	—	—
5103	122	3, 15, 18, 20	22	+	87	3, 4, 5, 8, 19, 20	—	—
5104	178	4, 5, 19, 20	1	±	35	4, 5, 19, 20	0.5	—
5105	19	7, 9, 12, 19, 20	11	++	55	2, 3, 4, 5, 7, 14, 19, 20	—	+++
5106	240	2, 7, 12, 15, 20	29	+	16	2, 4, 5, 10, 19, 20	—	++
5107	113	2, 7, 12, 19, 20	34	+	56	4, 5, 9, 12, 19, 20	—	+
5108	—[b]	2, 4, 7, 8, 12, 19, 20	8	+	16	2, 4, 5, 14, 20	0.8	++
5109	—[b]	12, 15, 18, 20	29	+	30	4, 5, 12, 19, 20	—	+
5110	—[b]	1, 4, 7, 10, 16	11	+	32	1, 8, 9, 10, 14, 15, 20	1	±
5111	25	7, 11, 14, 19, 20	11	++	24	4, 5, 20	—	+
5112	60	ochratoxins, 5, 19	—	±	40	ochratoxins, 2, 5, 9, 11, 14	—	±
5113	104	2, 3, 7, 12, 14, 15	17	±	14	2, 5, 7, 12, 14, 19, 20	3	+
5114	148	2, 5, 12, 19, 20	—	++	46	2, 3, 5, 7, 12, 19, 20	—	++
5115	119	3, 19, 20	11	±	31	3, 4, 5, 19, 20	—	±
5116	87	3, 20	1	±	24	4, 5, 20	—	±
5117	172	5, 15, 19, 20	17	±	11	5, 20	—	—
5118	—[b]	3, 15, 20	26	—	36	5, 15, 20	—	—
5119	—[b]	5, 14, 19	—	—	32	5	—	—

a) except penicillic acid b) no record

Two strains, NHL 5093 and 5112, showed the same fluorescence spots as the authentic sample of the mixtures of ochratoxins (VII—IX) both in the solvent (ii) on acid-treated Silica gel G and in (iv) on Silica gel G. Penicillic acid (II) was detected from 28 strains and the highest yield was *ca.* 70 mg from 100 ml medium. One strain (NHL 5090) showed the spot identical with ochracin (I) and the identity was confirmed by the two other solvent systems (hexane–benzene, benzene). All of the twenty spots other than these were different from those of the known metabolites (I—IV, VII—IX) and the proposed precursors of penicillic acid (XIII—XVIII), and have not yet been identified.

Toxicity bioassay shown in Table II revealed twenty-seven extracts from the filtrate and 21 extracts from mycelium exhibited over ± toxicity. Nineteen strains produced toxic substances both in culture filtrate and mycelium. Only four strains produced little or weak toxic substances in the present study.

Toxicity by the filtrates of NHL 5093 and 5112 could be attributed to the presence of ochratoxin A. When toxic concentration of each extract producing penicillic acid was calculated from the amount of penicillic acid in the extract, almost all strains exhibited corresponding toxicity at the expected concentration. Morphological changes caused by penicillic acid such as increase of mitotic cells and enlargement of interphasic cells were similarly observed in the cultures administered with the filtrates of NHL 5088, 5091, 5095, 5099, 5100, 5102, 5106, 5107, 5108, 5109 and 5115. Although mitotic cells did not increase, the filtrates of NHL 5089, 5094 and 5101 produced enlargement of interphasic cells similar to those produced by the administration of penicillic acid.

Morphological examination revealed another patterns of changes caused by several samples. The filtrates of NHL 5105 and 5111 produced a similar pattern of morphology which consisted of atrophic cells with vacuoles in the cytoplasm, shranken nuclei with irregular karyoplasm and increased mitotic cells with clumped chromosomes (pattern 1) (Photo 4). The changes induced by the administration of the filtrate of NHL 5114 and the mycelial extracts of NHL 5105, 5106, 5108, 5113 and 5114 were similar to but a little different from pattern 1; that is, appearance of polynuclear cells and increase of mitotic cells with peculiarly dispersed chromosomes as well as shrinkage of the nuclei with irregular karyoplasm and atrophic cells with vacuoles (pattern 2) (Photo 5). Another morphological changes were enlargement of the cytoplasm with faintly eosinophilic masses at juxta-nuclear position (pattern 3) (Photo 6). The samples producing such effect were the mycelium extracts of NHL 5088, 5095, 5098, 5100, 5107, 5110 and 5113. There were still several samples which could not be applied to the above-described patterns and produced toxic effects.

The Isolation of Ochratoxin A and Other Metabolites from the Strains NHL 5093 and 5112

Chloroform extracts of the filtrates in Expt. 2 (150—170 mg from 3 liter) were chromatographed through a column of acid-treated silica gel using benzene–EtOAc (3:1) as the solvent. The fraction showing fluorescence was applied on preparative TLC under the conditions described before. The combination of the two chromatographic methods finally afforded ochratoxin A (2 mg), which showed mp 159° after recrystallization from benzene–hexane; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1720, 1674, 1613, 1521; UV $\lambda_{max}^{CHCl_3}$ $m\mu$: 215, 333. TLC by two solvents (ii and iv) and the comparison of infrared (IR) spectra indicated the identity with ochratoxin A (VII).

The dried mycelium (53 g) of strain NHL 5112 was successively extracted with hexane, benzene, chloroform, and MeOH. The pressence of a minute amount of ochratoxin A and C was proved by TLC. The methanol extract afforded D-mannitol, mp 164—170° (from MeOH, 0.2 g).

Toxicities of Chromatographic Fractions of the Filtrate of Strain NHL 5112

The chloroform extract of the filtrate of strain NHL 5112 showed ± toxicity and production of ochratoxins. Three hundred mg of the extract obtained from 6 liter of the medium

was further applied on preparative TLC and by monitoring under a UV lamp the plate was separated into as many as 22 fractions, which were extracted with MeOH. The recovery is more than 90% of the original extract. Each fraction was separately examined for toxicity bioassay on HeLa cells and the fractions 4, 8, 10, 11, 12, and 22 from the top showed ± to + toxicities. The fractions 4 and 8 corresponded to those of ochratoxin C and A, respectively. The fractions 10 to 12 corresponded to that of penicillic acid from the *Rf* value but the absence of the acid in this strain was confirmed by further examinations. Morphological examination showed the changes produced by the fractions were different from those by penicillic acid. Thus the toxicities shown by the fractions 10, 11, 12, and 22 remained obscure.

Isolation of Penicillic Acid and Other Metabolites from Strain NHL 5111

The chloroform extract from the culture filtrate of strain NHL 5111 in Expt. 3 was concentrated and the deposited crystalline substance (0.28 g from 1 liter) was recrystallized from hexane–benzene or xylene to colorless needles of mp 85—86°; IR ν_{max}^{Nujol} cm^{-1}: 3260, 1748, 1643, 1343, 1051, 1036, 800; NMR ($CDCl_3$) τ: 4.60 (s, 1H), 4.88 (m, 1H), 4.96 (m, 1H), 6.16 (s, 3H), 8.27 (br. s, 3H). A mixed fusion and the comparison of the spectra with the authentic sample of penicillic acid (II) showed the identity. The acetate showed mp 70° (lit.[4] mp 72°).

The hexane extract from the dried mycelium in Expt. 3 left a small amount of oily residue. From the ether extract colorless crystals (0.2 g) of mp 119° (from EtOH) was obtained and identified with meso-erythritol. The chloroform extract afforded a minute amount of colorless crystals of mp >265°. The methanol extract gave colorless crystals of mp 166° (from MeOH, 0.7 g) and identified with D-mannitol. All the extracts showed the presence of penicillic acid (II) by TLC.

From the mycelium (12.0 g) in Expt. 3, the benzene extract was applied for column chromatography on acid-treated silica gel using benzene–$CHCl_3$ (4:1) as the developer. After the elution of fatty material showing yellow-green fluorescence, a fraction showing yellow fluorescence was obtained. After recrystallization from EtOH pale yellow needles of mp 243° (8 mg) was obtained; M^+ 380.087 *m/e* (Calcd. for $C_{21}H_{16}O_7$, 380.090). *Anal.* Calcd. for $C_{21}H_{16}O_7$: C, 66.31; H, 4.24. Found: C, 66.50; H, 3.96. IR ν_{max}^{Nujol} cm^{-1}: 1750, 1637, 1623, 1558, 1520, 1234, 1080, 1039, 768. UV $\lambda_{max}^{CHCl_3}$ mμ: 240, 253 (inf.), 285, 314, 384. The compound showed no toxicity on HeLa cells.

Discussion

The bioproduction of ochratoxins by *A. ochraceus* isolated from foodstuffs in this country has now been proved for the first time. The frequency of ochratoxin-producing strains within the *A. ochraceus* group has been reproted to be low: Nesheim[26] reported 4 out of 14 and Lai, *et al.*[14] found 4 out of 34. In our case (Expt. 1) 2 out of 33 strains were proved to produce the toxin under the conditions employed. Since the toxin is easily detectable by its strong fluorescence under the UV light (*ca.* 0.01 μg), the low rate of frequency, along with the low yield from the filtrate (2 mg from 3 liter in the highest), suggested that the production of the toxin, especially in the high amount, is assumed to be restricted to some strains in the group under some specified nutritional conditions.[27]

On the contrary the production of penicillic acid has been proved for almost all of the strains and the yields were quite high under the condition employed (*ca.* 70 mg from 100 ml in the highest). Toxicity bioassay on HeLa cells and simultaneous morphological examination supported that toxic effects by most of the strains producing penicillic acid were caused by penicillic acid itself. Penicillic acid is one of the ubiquitous mold metabolites and has

26) S. Nesheim, *J. Assoc. Offic. Anal. Chem.*, **1967**, 370.
27) N.D. Davis, J.W. Searcy, and U.L. Diener, *Appl. Microbiol.*, **17**, 742 (1969).

also been isolated from several species of *Penicillium*. As the first description on the toxic character, Alsberg and Black,[28] studying the possible relation between pellagra and moldy maize, isolated this acid from *P. puberulum* Bain. Recently the acid has been also reported to be carcinogenic.[17]

The suggested precursors[21] (XIII—XVIII) of penicillic acid (II) could not been detected in the strains tested in the present study probably due to their formation in a early stage of cultivation and the rapid turn-over to (II).

Thus, the toxicities shown by most strains were due to the formation of penicillic acid and of ochratoxins in some cases. There were, however, several strains which exhibited high toxicity and morphological changes different from those by penicillic acid or ochratoxins. Among these, a certain pattern of morphological changes was produced by several strains and others by several other strains. When the spots produced by the strains exhibiting interst-ing morphological changes of pattern 2 were precisely compared, spot 2 became emerged to be the ubiqitous spot and thus could be supposed to be a toxic principle of these strains. Spot 2 was produced by other strains but those were all producers of penicillic acid. Similar trials can be done for strains to produce similar morphological changes. Tissue culture method is, thus, proved to be a useful tool for investigating the relationships between the metabolites and biological activities.

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28) C.L. Alsberg and O.F. Black, *Proc. Soc. Exper. Biol. and Med.*, **9**, 6 (1911—1912); C.L. Alsberg and O.F. Black, U.S. Dept. Agr., Bur. Plant Indust., Bul. 270, 1913, pp. 1—47.