Toxic Fungi Isolated from Fermented Foodstuffs

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ABSTRACT: A lot of fungal strains which were isolated from popular fermented foodstuffs consumed daily in quantity in Japan, were found to produce toxic culture filtrates through chemical checks, biological tests and short-term animal experiments in our previous study. The long-term animal experiments using several strains of fungi which were selected for further chronic toxicity tests from the results of the above study and other additional strains of fungi which were isolated from Japanese, American and Korean fermented foodstuffs such as "miso", "shoyu", "koji" and cheese were carried out for the determination of chronic toxic effects on animals and carcinogenesity of the fungus metabolites. Out of 25 fungal culture filtrates, 16 filtrates showed development of chronic pathological changes in mice. Moreover, several strains of fungi were found to produce liver cell tumors and hemangioma of the liver. Tubular dilatation and degeneration of the kidney and atrophy of the testis were also observed. In the additional experiments using four fractionated materials of the fungal culture filtrate of M−3 *Alternaria tenuis*, different results were obtained by each fraction.

Nowadays, toxic metabolites of fungi in foodstuffs show epidemiological significance in causing several diseases such as stomach cancer, liver cancer and other diseases (Wogan, 1964; Alpert *et al.*, 1969; Enomoto *et al.*, 1972).

In 1968, we reported mycotoxins in Japanese fermented foods (Kinosita *et al.*, 1968). Those foodstuffs were represented by "miso" or fermented soy bean paste, "katsuobushi" or fermented dry bonito and "tane-koji" or a starter used for preparation of

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fermented foodstuffs in their fermentation process. In that investigation we isolated many strains of fungi from those foodstuffs and carried out chemical checks of kojic acid, β-nitropropionic acid and fluorescent metabolites. Biological tests, carried out with the culture preparations, revealed significant biological activities in bacteriocidal effects, induction of bacteriophage and influence on propagation of hamster cells in vitro. Moreover, short-term animal experiments showed development of miscellaneous pathological changes. From the results of the above study, several strains of fungi were selected for further long-term experiments. The present study was carried out to detect chronic toxic effects and carcinogenesity of metabolites of fungi isolated from mainly Japanese (partially American and Korean) foods and was undertaken to contribute to the etiological analysis of cirrhosis of the liver and hepatoma especially high in incidence among Japanese people as shown by biometric statistics in Japan.

MATERIALS AND METHODS

1. Preparation of culture filtrates.

Twenty-five strains of fungi, which were isolated from Japanese, American and Korean foodstuffs and found to have acute toxicities on mice as reported in the previous paper (Kinosita *et al.*, 1968), were cultured at 25°C for two weeks without shaking on the surface of 150 ml of glucose ammonium nitrate medium (B mebium) in 500-ml Erlenmyer flasks to obtain culture filtrates. The culture filtrates were adjusted to pH 5.0–5.5. The B medium was composed of 50 g of glucose, 2.4 g of NH_4NO_3, 10 g of KH_2PO_4, 2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $ZnSO_4 \cdot 7H_2O$, 0.002 g of $CuSO_4 \cdot 5H_2O$, 0.001 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.06 g of $CaCl_2$ and 1,000 ml of distilled water.

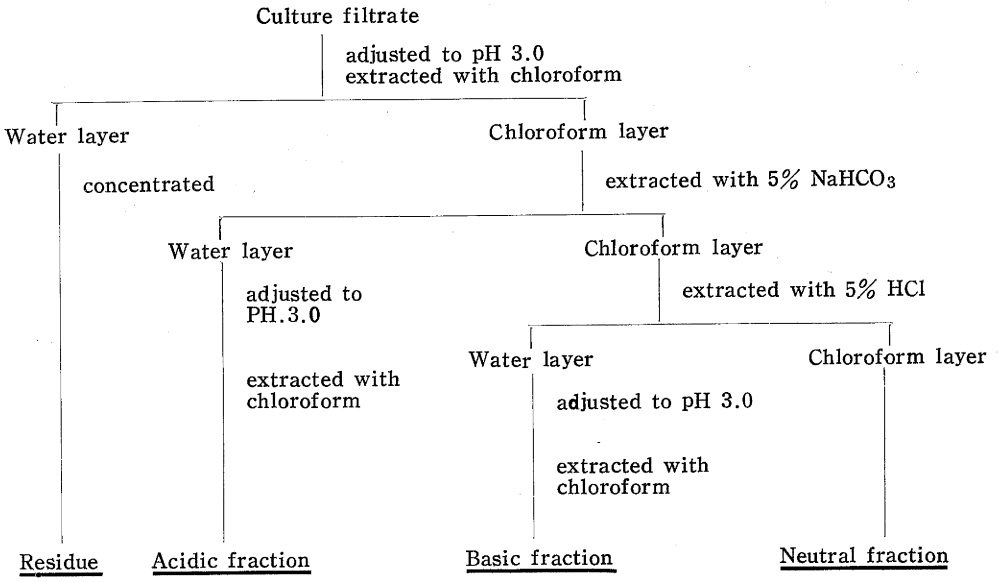
The strains of fungi examined, sources (places) and sampled foodstuffs from which the fungi were obtained are as follows: M–1 *Aspergillus* (*Asp.*) *oryzae*, Wakayama, Kinzanzi-miso; M–3 *Alternaria tenuis*, Wakayama, miso; M–8 *Asp. flavus*, Wakayama, nukamiso; M–9 *Mucor* species, Wakayama, nukamiso; M–10 *Asp. flavus*, Amami Ooshima, cycad miso; M–11 *Penicillium* (*Pen.*) *cyclopium*, Korea, miso; M–12 *Scopulariopsis brevicaulis*, Korea, miso; M–14 *Pestalotia* species, Korea, miso; M–16 *Asp. oryzae*, Wakayama, miso starter; M–19 *Asp. oryzae*, Nagoya, miso starter 1; M–20 *Asp. flavus*, Nagoya, shoyu starter 1; M–23 *Asp. flavus*, Nagoya, shoyu starter 3; M–24 *Asp. oryzae*, Nagoya, shoyu starter 4; M–25 *Asp. oryzae*, Nara, miso koji 1; M–28 *Asp. candidus*, Yamagata, miso koji; M–30 *Asp. oryzae*, Nara, miso koji 2; C–1 *Pen. roqueforti*, Los Angeles, blue cheese; C–3 *Pen. miczynskii*, Los Angeles, blue cheese; C–4 *Penicillium* species, Los Angeles, blue cheese; S–1 *Pen. implicatum*, Ooshima, cycad; S–3 *Aspergillus* species, Ooshima, cycad; S–4 *Asp. flavus*, Ooshima, cycad; K–4 *Aspergillus* species, Kochi, katsuobushi No.1.

2. Fractionation of fungal culture filtrates.

For further study on the toxicity of fractionated materials of fungal culture filtrates, a strain of M–3 *Alternaria tenuis* Nees isolated from Wakayama miso was selected as a

test fungus. The fungus was cultured at 25°C for two weeks on B medium. The obtained culture filtrate was fractionated by the method of Chart 1. Each fraction was adjusted to pH 5.0−55.

Chart 1. Fractionation of culture filtrate of M−3 *Alternaria tenuis* Nees



3. Animal experiments.

Experiment I

As a method for administration, 0.5 ml of the culture filtrate of each fungus was daily intraperitoneally injected to C3H/HeJ male mice, 4−5 weeks old, (usually 4−35 mice in each group for each sample; 319 mice in total, including 10 control mice treated with B medium only and 20 control mice with no treatment), at a total 10 times in all in the beginning of this experiment. Thereafter, no treatment was performed. On 300−400 days after the first injection, mice were sacrificed to observe pathological changes in several organs. The mice were fed with low protein diet (Nutritional Biochemicals Corp.) containing 8% protein during the experiment.

Experiment II

To examine the toxicities of the fractionated materials such as residue, acidic, basic and neutral fractions of fungal culture filtrates of M−3 *Alternaria tenuis* Nees mentioned above, 0.5 ml of each fraction was injected to C3H/HeJ male mice, 4−5 weeks old, in the same way as in Experiment I. In this experiment, 30 mice were used for each fraction. On approximately 350 days after the first injection, mice were sacrificed for pathological study. The mice were fed with low protein diet throughout the experiment.

Results and Discussion

Summarized results of Experiment I and Experiment II are given in Table 1 and Table 2, respectively. In Experiment I, 16 strains out of 25 strains of fungi showed positive chronic toxic effects on mice. In the following discussion, the results of chemical checks, biological tests and short-term animal experiments refer to our previous study (Kinosita *et al.*, 1968).

M−3 *Alternaria tenuis* Nees (Exp. 2 and Exp. 31) caused slight tubular dilatation of the kidney and liver cell tumors in two cases out of four (Exp. 2). This strain showed positive results of bacteriocidal effects against *E. coli*. M−12 *Scopulariopsis brevicaulis* (Exp. 7) caused moderate pleomorphism of liver cell nuclei and liver cell tumor (one case out of four). This strain also showed positive results of bacteriocidal effects against *E. coli*. M−14 *Pestalotia* species (Exp. 8) caused degeneration of proximal tubules of the kidney in the previous short-term (5 days) experiment. In this long-term experiment, it caused liver cell tumor (one case out of four), slight atrophy of the liver and slight tubular dilatation of the kidney. M−19 *Asp. oryzae* (Exp. 12 and Exp. 37) caused slight atrophy of the liver, moderate pleomorphism of liver cell nuclei and liver cell tumor in one case out of four (Exp. 12). Moreover, nervous symptoms such as mild tremor, tumble, gait disturbance and moving in only right hand circles were observed. This strain produced 0.2 mg/ml of kojic acid in B medium. M−20 *Asp. flavus* (Exp. 13, Exp. 14, and Exp. 32) caused swelling of the liver. Microscopically, marked pleomorphism of liver cell nuclei, cirrhotic change in the liver, liver cell tumors (three cases out of four, Exp. 13) and hemangioma of the liver (one case out of two, Exp. 14) were observed. This strain produced 0.5 mg/ml of kojic acid and 0.2 mg/ml of β-nitropropionic acid in B medium. M−23 *Asp. flavus* (Exp. 15) caused marked pleomorphism of liver cell nuclei, slight vacuolar degeneration of periportal regions and liver cell tumor (one case out of four). This strain produced 0.2 mg/ml of kojic acid in B medium and 0.1 mg/ml of β-nitropropionic acid in the same medium. M−25 *Asp. oryzae* (Exp. 17) caused moderate pleomorphism of liver cell nuclei, irregular arrangement of liver cell cords and liver cell tumor (one case out of four). In the previous short-term experiment this strain caused necrosis of proximal tubules of the kidney and degeneration of islets of the pancreas and produced 0.1 mg/ml of kojic acid in B medium. M−28 *Asp. candidus* (Exp. 19 anb Exp. 38) caused slight atrophy of the liver and liver cell tumor (one case out of two) and tubular dilatation of the kidney. All mice in this group showed a decrease in their movement. In the previous study, M−28 *Asp. candidus* produced a large amount of β-nitropropionic acid (1.1 mg/ml) and only a small amount of kojic acid (0.1 mg/ml) in B medium and the B preparation given intraperitoneally caused lesion of the kidney and hemorrhage of the pancreas and the glandular stomach. C−1 *Pen. roqueforti* (Exp. 22 and Exp. 36) caused slight atrophy of the liver. Microscopically, moderate periportal degeneration of the liver, irregular arrangements of liver cell cords and swelling of proximal tubular epithelia of the kidney were observed (Exp. 36). Liver

cell tumors were also observed (two cases out of three, Exp. 22). C−3 *Pen. miczynskii* (Exp. 24) caused marked pleomorphism of liver cell nuclei and liver cell tumor (one case out of one). S−3 *Aspergillus* species (Exp. 27) caused moderate pleomorphism of liver cell nuclei and irregular arrangement of liver cell cords. S−4 *Asp. flavus* (Exp. 28 and Exp. 35) caused liver cell tumors (two cases out of three), slight cirrhotic change and cholangiosis of the liver (Exp. 28). Moderate periportal degeneration, irregular cell cord arrangements of the liver and degeneration of proximal tubular epithelia of the kidney were also observed (Exp. 35). K−4 *Aspergillus* species (Exp. 29) caused slight atrophy of the liver. This strain showed positive bacteriocidal effects against *E. coli*. M−10 *Asp. flavus* (Exp. 44) caused marked pleomorphism of liver cell nuclei, irregular liver cell cords and liver cell tumor (one case out of 13 cases). Marked degeneration of proximal tubules and dilatation of collecting tubules of the kidney were also observed microscopically. One case of subcutaneous fibrosarcoma out of thirteen examined cases was obtained. Control animals, which were treated with B medium only, showed slight pleomorphism of liver cell nuclei and slight periportal vacuolar changes of the liver. Control animals with no treatment showed almost normal.

In Experiment II, in which mice were treated with four fractionated materials of fungal culture filtrate of M−3 *Alternaria tenuis* Nees, different results were obtained by each fraction. Residue of the fractionation caused marked pleomorphism of cell nuclei and periportal degeneration of the liver as well as moderate swelling and dilatation of proximal tubules of the kidney. In some cases (four cases out of thirteen) minute abscesses were observed in the liver. One case was suspected to be leukemia. Acidic fraction caused slight atrophy of the liver. Mice in this group were all weakened. The basic fraction and neutral fraction showed no remarkable change in any organ macroscopically, but moderate pleomorphism of liver cell nuclei and periportal degeneration microscopically.

As for tumorous change, liver cell tumors (adenoma and carcinoma) occur spontaneously in a low incidence in a number of inbred strains, but are common in older males of strains C3H and CBA (Burns *et al.*, 1940; Andervont, 1950). Heston found an incidence of 85% in C3H males, 72% in C3Hf males and 78% jn C3He males at 14 months of age (Heston *et al.*, 1960). The incidence of sponteneous hepatomas is influenced by diet (Tannenbaum *et al.*, 1949), castration (Andervont, 1950), chemical carcinogens and radioactive compounds (Heston *et al.*, 1960). Although this experiment was designed to confirm the acceleration of the so-called spontaneous hepatoma development in males of the C3H/He strain due to mycotoxins, the incidence of liver cell tumor (including adenoma and liver cell carcinoma) in this study was not so higher than expected. The mice were fed with low protein diet to make experimental animals more sensitive in response to pathogenic agents in the beginning of this experiment as in the previous study (Kinosita *et al.*, 1968). However, the low protein diet might have less influence on the occurrence of hepatoma. It is doubtful that subcutaneous fibrosarcoma in this study were caused by mycotoxins only, because the incidence of this lesion is too low. Further studies are needed to reveal the relationship between such tumorous changes and mycotoxins.

Table 1. Summarized results of the long-term animal experiments (Experiment I)

Experimental number	Strain of fungus (Source (place) and foodstuff)	Pathological findings[a),b)]
2.	M−3 *Alternaria tenuis* (Wakayama, miso)	Liver: pleomorphism (′), liver cell tumor (2/4) Kidney: p.t. dilatation (′) Testis:hyposperm. (′)
31.	M−3 *Alternaria tenuis*	Liver:pleomorphism (′′′) Kidney: p.t. dilatation (′)
7.	M−12 *Scopulariopsis brevicaulis* (Korea, miso)	Liver: pleomorphism (′′), liver cell tumor (1/4) Testis: hyposperm. (′)
8.	M−14 *Pestalotia* species (Korea, miso)	Liver: atrophy (′), pleomorphism (′′), liver cell tumor (1/4) Kidney: p.t. dilatation (′) Testis: hyposperm. (′)
11.	M−18 *Asp. oryzae* (Wakayama, miso starter)	Liver: pleomorphism (′′), irregular liver cell cords Testis: hyposperm. (′)
12.	M−19 *Asp. oryzae* (Nagoya, miso starter 1)	Liver: atrophy (′), pleomorphism (′′), liver cell tumor (1/4) Testis: hyposperm. (′)
37.	M−19 *Asp. oryzae*	Liver: pleomorphism (′′) Thymus: atrophy (′) Nervous symptom:tremor, tumble or gait disturbance, moving in only right hand circles
13.	M−20 *Asp. flavus* (Nagoya, shoyu starter 1)	Liver: pleomorphism (′′), cirrhotic change (′), liver cell tumor (3/4)
14.	M−20 *Asp. flavus*	Liver: swelling (′), pleomorphism (′′′), hemangioma of liver (1/2)
32.	M−20 *Asp. flavus*	Liver: swelling (′), pleomorphism (′′′) Kidney: p.t. swelling (′), degeneration (′)
15.	M−23 *Asp. flavus* (Nagoya, shoyu starter 3)	Liver: periportal vacuolar degeneration (′), pleomorphism (′′′), liver cell tumor (1/4) Testis: hyposperm. (′)
17.	M−25 *Asp.oryzae* (Nara, miso koji 1)	Liver: pleomorphism (′′), irregular liver cell cords, liver cell tumor (1/4) Testis: hyposperm. (′)
19.	M−28 *Asp.candidus* (Yamagata, miso koji)	Liver:atrophy(′), pleomorphism (′′), fatty degeneration (′), liver cell tumor (1/2) Testis: hyposperm. (′) Kidney: p.t. dilatation (′)
38.	M−28 *Asp.candidus*	Liver: pleomorphism (′′) Heart: Endocardial swelling Decrease in thier movement
20.	M−30 *Asp. oryzae* (Nara, miso koji 2)	Liver: pleomorphism (′)
22.	C−1 *Pen. roqueforti* (Los Angeles, blue cheese)	Liver: liver cell tumor (2/3)

(*continued*)

Experimental number	Strain of fungus (Source (place) and foodstuff)	Pathological findings[a),b)]
36.	C−1 *Pen.roqueforti*	Liver: atrophy (′), pleomorphism (′′′), irregular cell cords Kidney: p.t. swelling (′)
24.	C−3 *Pen. miczynskii* (Los Angeles, blue cheese)	Liver:pleomorphism (′′′), liver cell tumor (1/1)
27.	S−3 *Aspergillus* species (Ooshima, cycad)	Liver: swelling (′), pleomorphism (′′), irregular liver cell cords Testis:hyposperm. (′)
28.	S−4 *Asp. flavus* (Ooshima, cycad)	Liver: pleomorphism (′′), cirrhotic changes(′), cholangiosis (′′), liver cell tumor (2/3)
35.	S−4 *Asp.flavus*	Liver: pleomorphism (′′′), irregular cell cords Kidney: p.t.swelling (′), degeneration (′)
29.	K−4 *Aspergillus* species (Kochi, katsuobushi No.1)	Liver: atrophy (′), pleomorphipm (′′)
44.	M−10 *Asp. flavus* (Amami Ooshima, cycad miso)	Liver: pleomorphism (′′′), irregular liver cell cords, liver cell tumor (1/13) Subcutaneous fibrosarcoma (1/13) Kidney: p.t. degeneration and swelling (′′′), c.t. ditatation (′′)
33.	Control (B medium)	Liver: pleomorphism (′)
34.	Control (no treatment)	Liver: pleomorphism(′)

a) Degree of changes: (′); slight, (′′); moderate, (′′′); marked

b) Abbreviations used in findings: pleomorphism; pleomorphism of liver cell nuclei, liver cell tumor; adenoma and carcinoma of the liver (positive cases/examined cases), p.t. (of the kidney); proximal tubules, c.t. (of the kidney); collecting tubules, hyposperm.; hypospermatogenesis

Table 2. summarized results of the long-term experiments using fractionated materials of fungal culture filtrate of M−3 *Alternaria tenuis* Nees (Experiment II)

Experimental number	Fractionated material	Pathological findings[a),b),c)]
39.	Residue of chloroform extract of filtrate	Liver: pleomorphism (′′′), minute abscesses (4/13) Leukemia, suspected (1/13) Kidney: p.t. swelling (′), dilatation (′)
40.	Acidic fraction of filtrate	weakened Liver: atrophy (′), pleomorhism (′′′)
41.	Basic fraction of filtrate	Liver: pleomorphism (′′′)
42.	Neutral fraction of filtrate	Liver: pleomorphism (′′)

a) Degree of changes: (′); slight, (′′); moderate, (′′′) ;marked

b) Abbreviations used in findings: pleomorphism; pleomorphism of liver cell nuclei, p.t. (of the kidney): proximal tubules

c) Parentheses are used to indicate frequency of lesions (positive cases/examined cases)

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発酵食品類より分離された毒性真菌類

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日本（及び一部韓国並びに米国）の主要な発酵食品である味噌，醤油，こうじ，わさび漬け，かつおぶし及びそれらの種こうじ，又，米国のブルーチーズなどから分離される真菌類は多く，それらの培養濾液の中に kojic acid，β-nitropropionic acid など化学物質を産生するもの，bacteriophage induction など生物活性を示すもの，Aflatoxin など螢光物質を産生するもの，更にマウスに対して急性の毒性作用を示す多くの strain を我々は得ている．本研究ではそれらの真菌類のうち 25 strain を選んで培養濾液をマウスへ腹腔内投与することにより長期の慢性毒性を観察した結果 16 strain によって明らかな慢性病変が見られた．毒性作用の標的臓器は strain によって異なるが，病理組織学的には肝細胞萎縮，肝細胞核の大小不同性，肝細胞腫瘍（肝腺腫及び肝細胞癌），腎尿細管の拡張と上皮の変性それに睾丸の萎縮などが見られた．更に上記の strain の一つである M−3 *Alternaria tenuis* の培養濾液の酸性，塩基性及び中性の各分画について慢性毒性を検索した。

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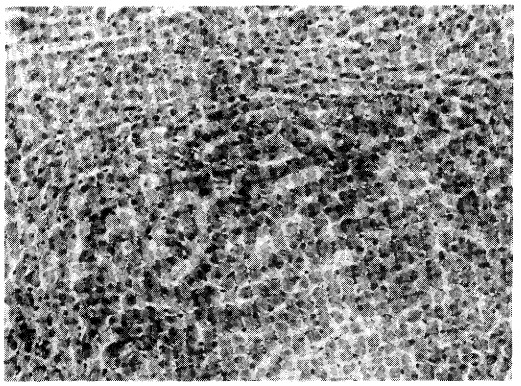

Photo. 1. Adenomatous change in the liver in Exp. 7, intraperitoneal injection of culturefiltrate of M−12 *Scopulariopsis brevicaulis*. (H. and E., ×142)

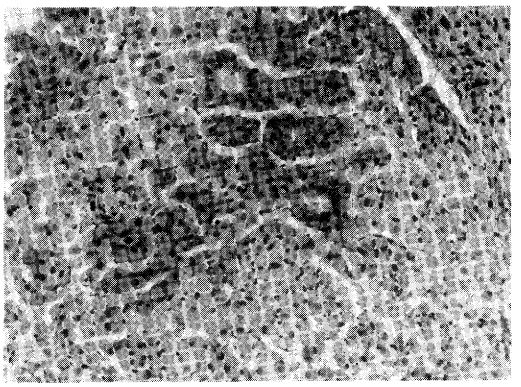

Photo. 2. Liver cell carcinoma in Exp. 2, intraperitoneal injection of culture filtrate of M−3 *Alternaria tenuis* Nees. (H. and E., ×142)

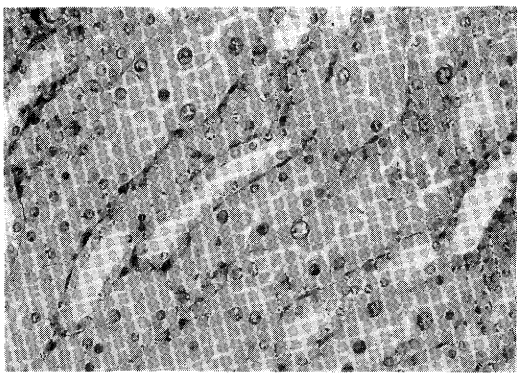

Photo. 3. Remarkable swelling and degeneration of epithelial cells of the proximal tubules of the kidney in Exp. 44, intraperitoneal injection of culture filtrate of M−10 *Asp. flavus*. (H. and E., ×350)

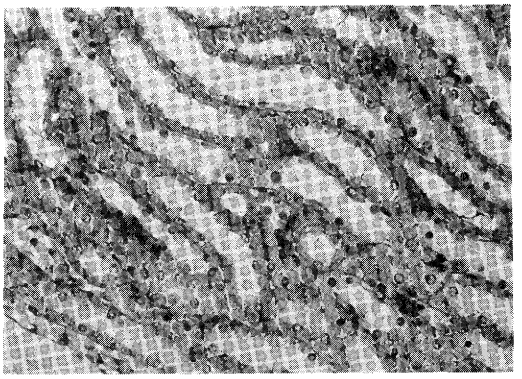

Photo. 4. Degeneration of epithelial cells of the collecting tubules in the same kidney as Photo 3. (H. and E., ×350)