A NEW PATHOGENIC SPECIES OF ASPERGILLUS

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SUMMARY

A new species, *Aspergillus bisporus,* isolated from soil collected at Clarksburg, Maryland, and Bainbridge, Georgia, is described. On standard media the species is characterized by conidial heads with uniseriate phialides bearing short chains of large, globose, black, coarsely dentate conidia and more specifically by the production of two conidial forms on high-sugar medium. The second type of conidial structure developed on M4OY agar bears long, light-olivish conidial chains consisting of smooth to slightly rough, globose to elliptical conidia. Incubation at 37–45 C enhances the development of the second conidial form.

Conidia of *A. bisporus* survive for several months in brain, lung, liver and spleen of mice without any evidence of multiplication. However, extensive growth causing chronic nephritis occurs in the kidney.

In May, 1959, Dr. Chester W. Emmons, then at the Medical Mycology Section, National Institute of Allergy and Infectious Diseases, collected soil samples from four different sites in Clarksburg, Maryland. The soil sample from each site was divided in two equal portions, one portion was sterilized and the other was untreated. Both portions were then inoculated with a heavy suspension of mycelium and conidia of *Histoplasma capsulatum* Darling and stored at 30 C. In April, 1969, one of the authors (K-C) inoculated mice with suspensions of these soils as described by Emmons (**1958**), in an attempt to recover the culture of *H. capsulatum.* While no *H. capsulatum* survived, three strains of an interesting *Aspergillus* were isolated from one of the four untreated samples. A fourth strain had been received by one of the writers (F) in February, 1967, from Dr. Charles S. Hodges, Jr. of the Forestry Sciences Laboratory, Research Triangle Park, North Carolina.

These isolates differ sufficiently from all previously known aspergilli to warrant their description as a new species.

The purpose of this paper is to describe the fungus and to present the information obtained to date on its pathogenicity in mice.

MATERIALS AND METHODS

All cultures were grown upon a variety of agar substrata at different temperatures of incubation. The following agar media were utilized: Czapek's solution, high-sugar Czapek's, malt extract, M4OY (Raper and Fennell, **1965**), and oatmeal-tomato (Weitzman et al., **1967**). Incubation was at 25, 37, and 45 C. The species description is based primarily upon cultural characteristics and details of morphology exhibited on Czapek's solution agar at room temperature (25 C). Additional observations from cultures on other substrates at different temperatures are also incorporated. Color references are based upon plates in Ridgway's "Color Standards and Color Nomenclature" (**1912**).

Pathogenicity of the organism was tested as follows: Fifty mice were injected intravenously with about 1.7×10^5 conidia and sacrificed at designated time intervals. Four mice were sacrificed at each interval and the organs of the animals were used to recover the organism and to prepare the tissue sections as described elsewhere (Kwon-Chung, **1968**).

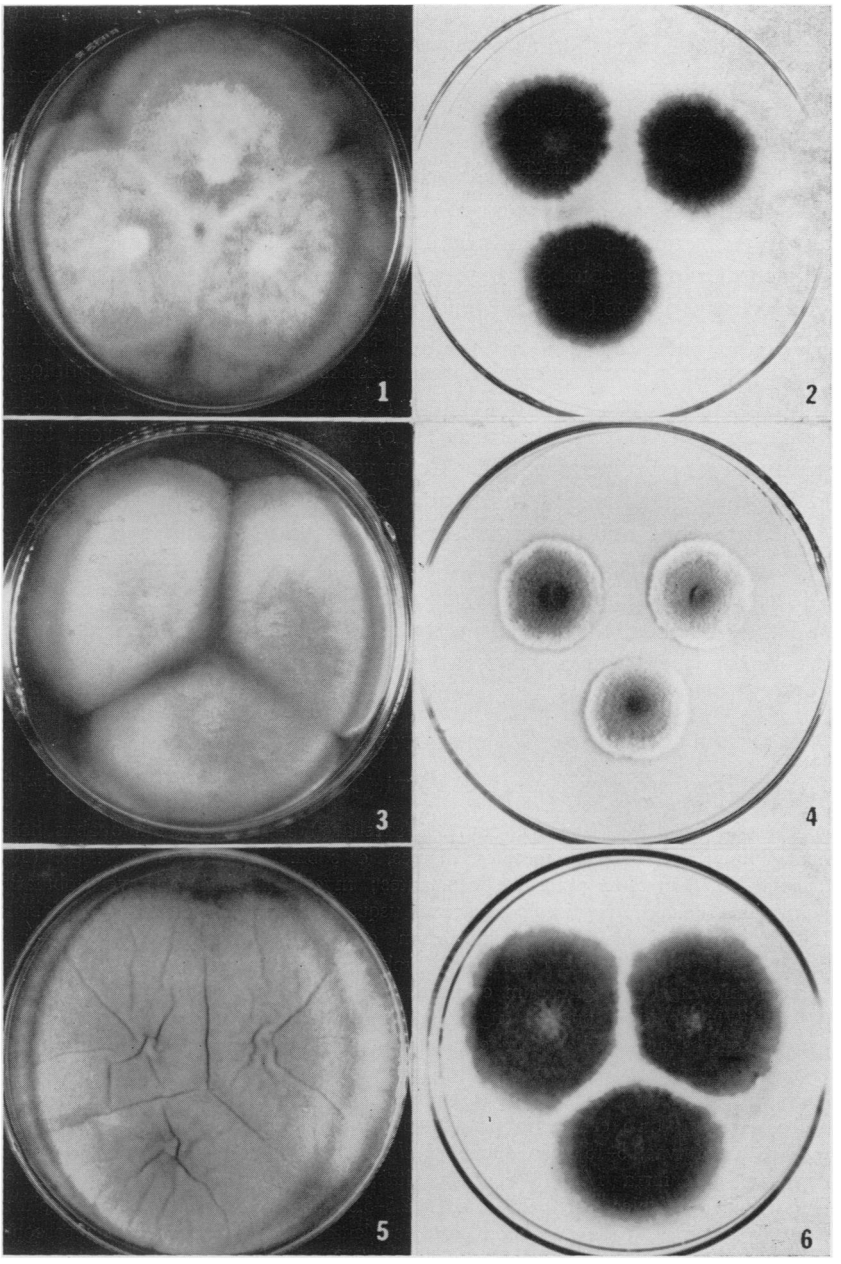
DESCRIPTION

Aspergillus bisporus Fennell & Kwon-Chung, sp. nov. FIGS. 1–14

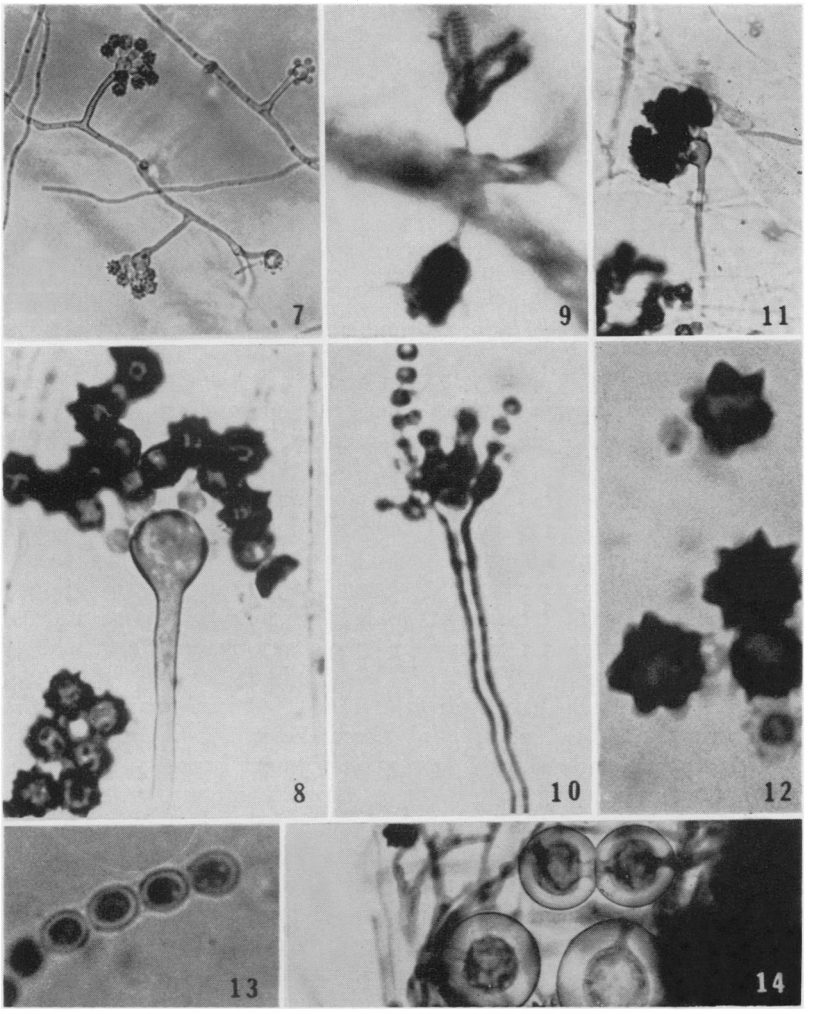
Coloniae in agaro Czapekii (cum 3% saccharo) 1–4 cm in diam duabus hebdomadibus 25 C lente crescentes, tenues, e submerso, intense olivaceo (R., Tabula XL), mycelio praecipue constantes; mycelium, fimbriatis vel lobulatis marginibus, deinde atrogriseolo-olivaceum usque chaetura-avellaneum (R., Tabula XLVI) propter abundorum conidicorum capitulorum formationem, limitatam subalbarum vel subflavarum aeriarum hypharum tramam aliquando gerens; reversum fere nigrum, pigmentum effusum.

Capitula conidica pusilla, nigra, asterinea, e curtis divaricatis conidiorum catenis composita; *conidiophorae* e substrato praecipue crescentes, crassitunicatae, levigatae aut pigmenti grana gerentes, rectae vel leniter sinuosae, brunneae, sine conspicuis cauliculis, $5–50 \times 2–6$ μ; *vesiculae* globosae usque inaequaliter subglobosae vel spatulae, erectae in summis conidiophoris aut verticali axe transversim emergentes, fulvae, 5–15 μ in diam; *phialides* uniseriatae, valde divaricatae, non densatae, in summa 1/2–2/3 vesicula sitae, ampulliformes, subfusco pigmento, magnitudine variables, 5–6 usque $8–10 \times 2.5–3.0$ usque 4–5 μ; *conidia* raro plura quam quatuor in catena, globosa, crasse dentata, atrobrunnea in microscopii preparationibus, nigra oculo nudo, 4–6 usque 7–9 μ.

Secundaria capitula in marginalibus coloniarum in agaro M4OY 37–45 C areis, vel in vetustis coloniis 24 C crescentibus minus picta (olivaceo- lutea, R.,



FIGS. 1–6. Two-week-old colonies of *Aspergillus bisporus,* NRRL 3693, on various media. 1–2. On Czapek's solution agar (3% sucrose) at 37 C and 25 C, respectively. 3–4. On malt extract agar at 37 C and 25 C, respectively. 5–6. On M4OY at 37 C and 25 C, respectively.



FIGS. 7–14. Conidial structures and hülle cells of *A. bisporus,* strain NRRL 3693. 7–8. Details of the primary conidial structure, × 300 and × 1,000, respectively. 9. Columnar to loosely columnar secondary conidial heads produced on a bundle of mycelium on M4OY at 45 C, as seen with transmitted light, × 200. 10. Details of the secondary conidial structure, × 1,000. 11. A vesicle produced at an angle to the main axis of the conidiophore, × 350. 12. Large, dark, coarsely dentate, globose primary conidia, × 1,250. 13. Oval, smooth conidia from the secondary conidial head, × 1,250. 14. Globose hülle cells infrequently produced among tufts of mycelium on the surface of old colonies on oatmeal-tomato agar, × 485.

Tabula XL, usque griseolo-olivacea, R., Tabula XLVI), laxe columnaria, 20–30 × 60–95 μ, et glabra usque leniter rugulosa, ovata usque elliptica, conidia 4–7 × 3.0–3.5 μ producentia.

Hülle cellulae aliquando productae sed non copiosae in avenae agaro 25 C, globosae usque ovatae vel instar duarum conjunctarum sphaerularum formatae, 15–22 × 20–35 μ.

Typica cultura, NRRL 3693, in anno 1969 a K. J. Kwon-Chung per artem inoculati muris isolata, e solo in anno 1959 in Clarksburg, Maryland, a C. W. Emmons collecto.

Etymology; *bi-*(prefix, two) [types of], *spora, ae* (spore).

HOLOTYPE: Dried agar cultures of NRRL 3693 have been deposited in the Herbarium of the National Fungus Collection, USDA, Beltsville, Md.

Colonies on Czapek's solution agar (3% sucrose) growing slowly, diameter varying with the strain and ranging from 1–4 cm in 2 weeks at 25 C, thin, consisting primarily of Dark Olive (R. Pl. XL) submerged mycelium (FIG. 2), margins fimbriate to irregularly lobed, becoming Dark Grayish Olive to Chaetura Drab (R., Pl. XLVI), with the production of abundant small and inconspicuous conidial heads, some strains showing a localized surface growth of gray-white to yellowish short aerial hyphae; reverse unwrinkled, nearly black, with pigment diffusible and eventually staining the whole substratum Sepia (R, Pl. XXIX) to Fuscous (R., Pl. XLVI). Conidial heads (FIGS. 7, 8) diminutive, black, radiate with divergent short conidial chains; conidiophores (FIGS. 8, 10) arising mainly from the substrate, heavy-walled, smooth or occasionally showing granular pigment deposits, straight or slightly sinuous, shading to brown, occasionally septate, without well-defined foot cells, variable in length from 5–50 μ, and in diam from 2–6 μ; vesicles globose to irregularly subglobose or spathulate, erect on the conidiophores or borne at an angle to the vertical axis (FIGS. 8, 11) infrequently sessile 5–15 μ in diam, yellow-brown; phialides uniseriate (FIG. 8) divergent, widely spaced over the upper half or two-thirds of the vesicular surface, ampulliform, slightly brownish, occasionally borne singly on the hyphae, variable in size, (5–)6–8(–10) × (2.5–)3–4(–5) μ; conidia rarely more than four per chain, globose, coarsely dentate (FIG. 12), dark brown in mounts but black to the naked eye, (4–)6–7(–9) μ.

Colonies on malt extract agar at 25 C (FIG. 4) growing slowly, slightly smaller but with somewhat more abundant aerial mycelium than on Czapek's solution agar, submerged mycelium extending 2–3 mm beyond the edge of the surface growth, otherwise plane, velvety, heavily sporing, near Light Grayish Olive (R., Pl. XLVI) to Dark Olive Gray (R., Pl. LI); reverse grayish yellow. Conidial structures generally as on Czapek's agar but with conidiophores frequently longer, ranging

from 7–100 μ. Yellowish tufts of short aerial hyphae appear on the surface of some colonies.

Colonies on oatmeal-tomato agar grow and sporulate luxuriantly, 2.5–6 cm in diam in 2 weeks at 25 C, plane and black, margins irregularly lobed, conidial heads as described above. Limited numbers of globose to ovate or dumbbell-shaped hülle cells, 15–22 × 20–35 μ, infrequently produced by some strains in localized surface tufts of white hyphae (FIG. 14).

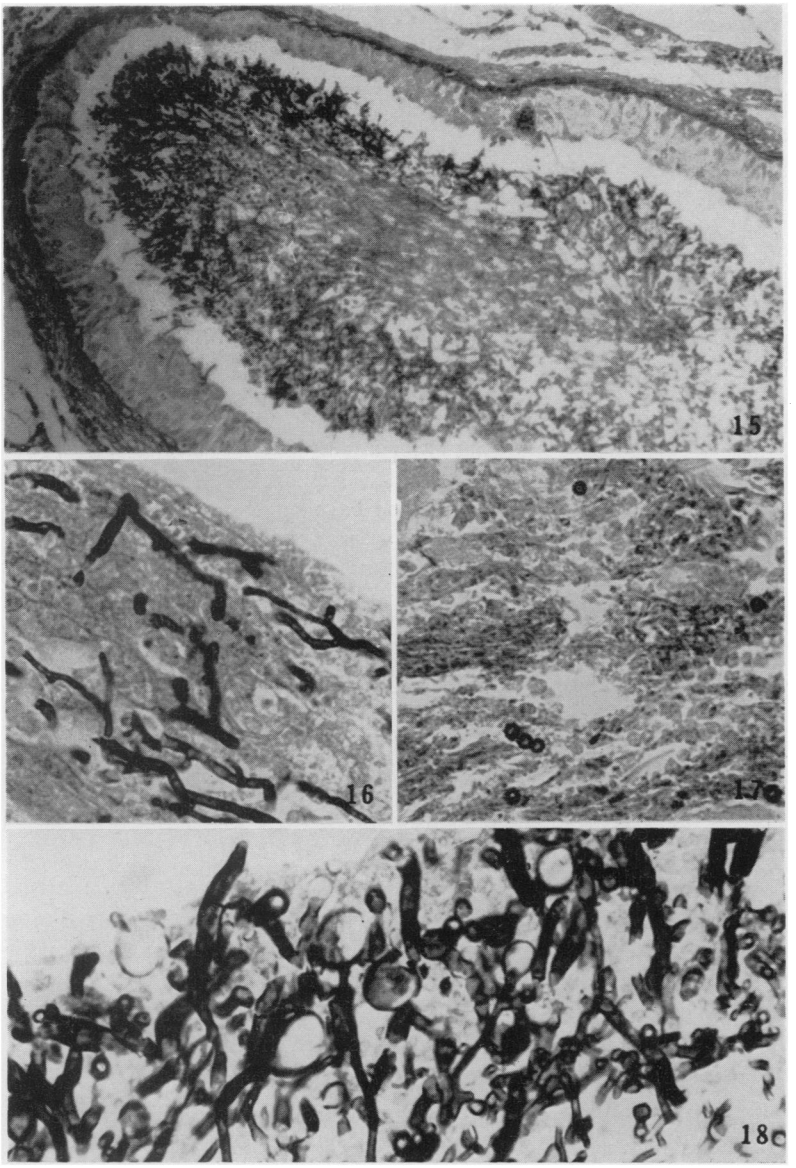
At 37 C on these three media conidial structures are less crowded, but the rate of growth is much increased (FIGS. 1, 3, 5). Colonies generally appear lighter in color than those at 25 C owing to the enhanced development of aerial hyphae.

Colonies on M4OY grow rapidly, reaching diameters up to 6 cm in 2 weeks at 25 C, with margins fimbriate or lobed (FIG. 6), Deep Grayish Olive to Dark Grayish Olive (R., Pl. XLVI) from the abundant conidial structures produced from the basal mycelium and from thin to ropy aerial mycelium. The conidial heads first produced are as described above but bear coarsely echinulate rather than dentate conidia. A second type of conidial head is produced in marginal areas of aging colonies of some strains incubated at 25 C and in all strains incubated at 37–45 C. These secondary heads are more lightly pigmented (Olive Buff, R., Pl. XL, to Light Grayish Olive, R., Pl. XLVI), loosely columnar (FIG. 9), 20–30 × 60–95 μ, and produce smooth to slightly roughened ovate to elliptical conidia (FIG. 13) 4.0–7.0 × 3.0–5.0 μ. The two types of conidial heads have been observed to arise from the same hypha and colonies derived from single conidia of either type are identical on all media.

The type culture, NRRL[1] 3693, is one of three strains isolated by Kwon-Chung in 1969 by the mouse-passage technique from a soil sample collected by C. W. Emmons at Clarksburg, Maryland, in 1959 and since stored at 30 C. Other strains representative of the species include NRRL 3691 and 3692 from the same soil as the type, and NRRL 3690 isolated by C. S. Hodges, Jr., from soil collected in an 18-year-old plantation of *Pinus elliottii* located near Bainbridge, Georgia. Of the four strains, NRRL 3690 produces the secondary spore form most abundantly. NRRL 3691, while producing conidial structures characteristic of the species, is an extremely slow-growing strain.

The species epithet *bisporus* was selected to denote the two conidial forms produced.

[1] Northern Regional Research Laboratory, Peoria, Illinois.



FIGS. 15–18. Tissue sections of kidney or liver from mice infected with *A. bisporus,* strain NRRL 3693. (GMS stain). 15. A portion of renal pelvis showing an extensive hyphal growth, × 125. 16. Hyphal growth seen in the tip of papilla, × 485. 17. Conidia observed in the liver at 140 days post-inoculation, × 300. 18. Hyphae swollen at one end or at intercalary position growing in the renal pelvis, × 485.

Aspergillus bisporus does not fit satisfactorily into any of the groups of *Aspergillus* species delineated by Raper and Fennell (**1965**). However, relationships to several of these groups are readily apparent. Conidia of two kinds are produced in a number of species. Of these, only *A. funiculosus* in *A. sparsus* group has phialides in a single series. In *A. ornatus* group, which is characterized by uniseriate phialides, *A. raperi* with globose to subglobose hülle cells and *A. brunneo-uniseriatus* with large coarsely roughened dark conidia show affinities with the new species but are easily separable on the bases of other characters.

TABLE I

KIDNEY INFECTIONS FOUND IN 50 MICE INJECTED (I.V.) WITH 1.7×10^5 CONIDIA OF A. BISPORUS

	No. of days from injection to autopsy								
	1	7	14	20	28	35	50	120	140
No of Mice sacrificed	4	4	4	4	4	4	4	10	12
No of mice with diseased kidney	0	0	0	0	2	1	2	5	4
Appearance of kidney	N[a]	N	N	N	1-mouse, atrophy of both kidneys	1-mouse, both kidneys discolored and enlarged	1-mouse, one kidney enlarged	3-mice, one each kidney discolored and enlarged	3-mice, atrophy of one kidney
					1-mouse, one kidney partially discolored	3-mice, normal	1-mouse, atrophy of one kidney	1-mouse, atrophy of one kidney, the other enlarged	1-mouse, one kidney enlarged
					2-mice, normal		2-mice, normal	1-mouse, atrophy of one kidney, 5-mice, normal	8-mice, normal

[a] Normal.

Diminutive conidial heads with vesicles frequently borne at an angle to the vertical axes of very short conidiophores are known in several species of the *A. fumigatus* and *A. cervinus* groups. Of these species, only those from the *A. fumigatus* group show an increase in growth rate at 37 C similar to that of *A. bisporus*. These same species grow well on high-sugar Czapek's and on M4OY. The fact that strains of *A. bisporus* produce both types of conidial heads on the latter medium and grow well on malt-yeast extract agar containing 700 gr/l sucrose suggests relationship to the osmophilic species of the *A. restrictus* group. Additionally, most of these osmophilic species produce colum-

nar heads somewhat similar to the secondary heads produced by strains of the new species.

PATHOGENICITY

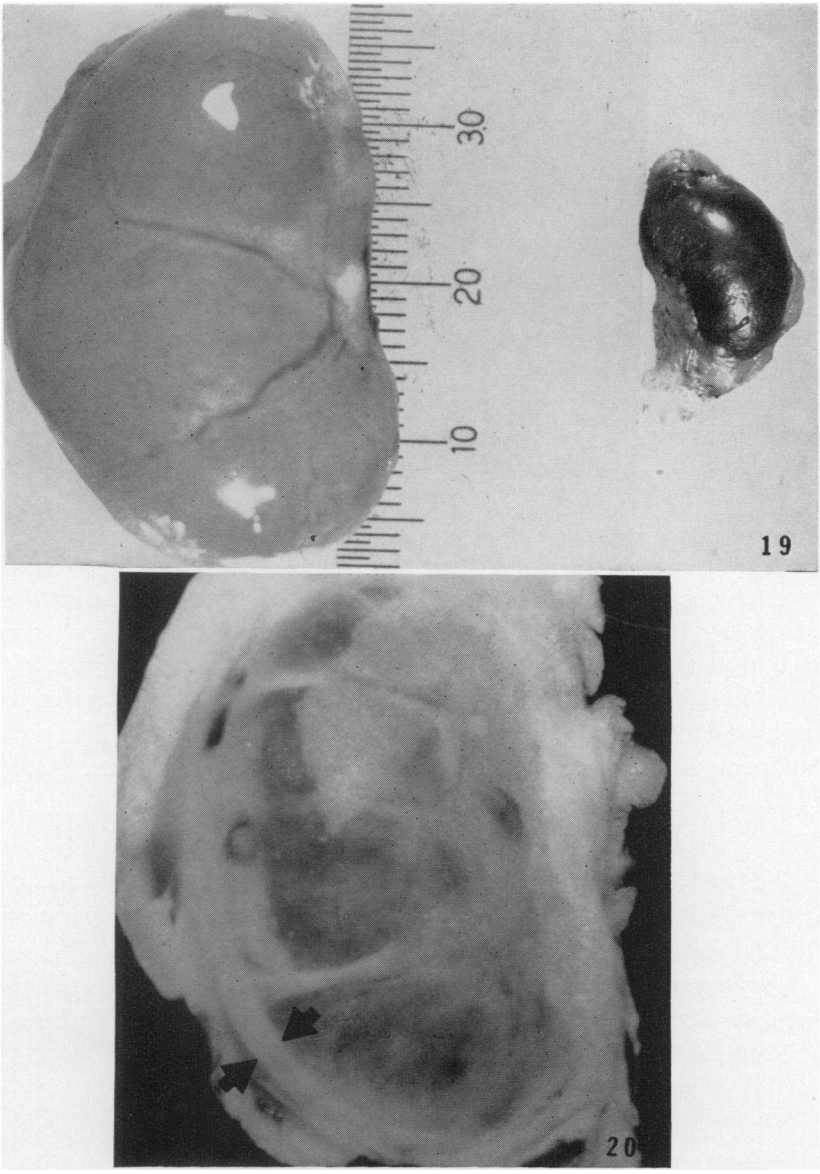
The fungus was isolated from liver, spleen, lung and brain for 140 days after injection. Gross lesions were not observed in these organs. Sections of the tissues stained both with Gomori methenamine-silver stain (GMS) and periodic acid-Schiff stain (PAS) showed intact conidia but with no evidence of germination (FIG. 17). In some mice, when autopsied at 120 to 140 days post inoculation, cultures were recovered only from liver and kidney but not from lung, brain or spleen.

In the kidney, however, the conidia germinated and produced a hyphal mass causing chronic nephritis. TABLE I lists the kidney lesions found in mice sacrificed at each interval. Macroscopic lesions were not observed in mice sacrificed at 1, 7, 14 and 20 days. Grossly abnormal kidneys were seen in 14 of 34 mice autopsied between 28–140 days after injection. The most common abnormalities were those of discoloration with enlargement of one kidney or less frequently of both kidneys. Some abnormal kidneys appeared as a balloon (FIG. 19) due to the severe hydronephrosis. They were saturated with fluid and contained fungal balls measuring up to 5 mm in diam. The cortex of such kidneys remained as a thin layer (FIG. 20, see arrows). Unilateral atrophy was frequently observed while the other kidney appeared normal.

In the early stages of infection, sections of the kidneys showed extensive hyphal growth only in the renal pelvis (FIG. 15) and in the tip of the papilla (FIG. 16). Erosion of the epithelium lining the pelvis was seen in several areas contiguous to the mass of highly branching hyphae. Swelling of hyphae at the intercalary position or at the tip stimulating vesicles was commonly seen in the sections (FIG. 18).

DISCUSSION

The light colored small conidia are produced only on media with high sugar concentration such as M4OY and Czapek's solution with 40% sucrose or dextrose, preferably at 37–45 C. This phenomenon leads one to believe that the conidia of *A. bisporus* that survived in the soil sample for 10 years at 30 C might have been the black, large, coarsely dentate form. The thick, black wall of the conidia may have protected them from deterioration by metabolites of other microorganisms in the soil as well as from extreme dehydration. These conidia also showed prolonged survival without germination in the various



FIGS. 19–20. Unilateral enlargement of kidney found in mice infected with *A. bisporus,* strain NRRL 3693. 19. Severe case of hydronephrosis in the right kidney while the left kidney appears normal, × 2.1. 20. Longitudinal section of a hydronephrotic kidney showing a thin layer of cortex (see arrows), × 2.1.

tissues of mice. The number of colonies developing from lung, liver and spleen of infected mice decreased very slowly. It would be of interest to investigate the behavior of the light colored small conidia in animals.

Although over 40% of the mice infected with 1.7×10^5 conidia developed abnormal kidneys at 28–140 days post inoculation, no mice receiving 1×10^4 conidia showed such abnormalities during six months of observation. These results indicate that a minimal dose is necessary to induce progressive infection in the kidney. The reason for confinement of germination of the conidia to pelvis and papilla of the kidney is not clear. It may be that the fungus prefers the hypertonic condition in vivo as was found in vitro. It is well established that the medulla of the kidney is much more susceptible to infection than the cortex. The susceptibility of the medulla to infection was found to be determined at least in part by its habitual hypertonicity (Andriole and Epstein, **1965**).

Frequent observation of unilateral kidney enlargement or atrophy is unique and most puzzling. The right kidney was about three times more frequently involved than the left. Since the conidia reached the kidneys by hematogenous spread, it is unlikely that the right one received more spores than the left. The only difference between the left and right kidney in mice is that the right one is slightly heavier in weight and higher in position (Dunn, **1967**). Whether these differences play a role in the frequent unilateral involvement is not known.

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