who detected circannual patterns of mitosis in lens epithelium of 2 species of frog. The possible factors that may modulate the onset of mitosis in the spring are numerous, e.g., nutritional levels, day length, temperature, and various hormones. Previous studies have shown that temperature can regulate the kinetics of the cell cycle in the frog lens[21,22]. In addition, growth hormone can stimulate and hypophysectomy can curtail the level of mitosis in the normal frog lens[23]. Moreover, the hypophysectomized frog failed to show a mitotic response when challenged by either mechanical or chemical insult[24]. Thyroxin and triiodothyronine alter the locus of proliferation in the frog lens[25], extending it into the polar epithelium, resulting in patterns of DNA synthesis and mitosis comparable to those noted here in spring-summer toadfish. With respect to other species, perch reportedly do not grow in the winter when the pituitary contains little growth hormone (GH)[26]. Whether growth in the toadfish lens is under constraints similar to those noted in the frog and perch remains to be documented. More importantly however, the current study provides a base of information which permits such questions to be considered.

Summary. The toadfish lens epithelium is essentially amitotic from January to May. From June to August mitotic activity is noted. The onset of proliferation approximately corresponds to the time of year when the fish enter the shallow water to spawn. The epithelial mitotic response to needle injury is not propagated in a wave-like manner from the site of insult.

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High-Frequency Fusion of Fungal Protoplasts

In a previous communication[1], we described a method for obtaining an increased fusion frequency (2.5×10^{-3}) of *Aspergillus nidulans* protoplasts, based on spontaneous aggregation of protoplasts in inorganic osmotic stabilizers. This method was later further improved by using KCl as stabilizer, Ca ions in the concentration range of 10 to 100 m*M* and high pH. However, the frequency of protoplast fusion, measured via the complementation of nutritionally deficient *Aspergillus nidulans* mutants, remained of the same order of magnitude.

In a search for highly efficient procedures of protoplast fusion, with the aim of bringing about biochemical complementation of genetically distant fungal species, we found that higher molecular weight polyethylene glycols (PEG), known agents for stabilizing bacterial protoplasts[2] and aggregating and fusing higher plant protoplasts[3–8], can satisfy the requirements.

We describe here a simple technique which results in high-frequency intraspecific protoplast fusion and complementation of auxotrophic mutants of *Aspergillus* (*A.*) *flavus*, *A. nidulans*, *A. niger*, *Penicillium* (*P.*) *frequentans* and *P. ramigena*. The method presented may also be suitable as a means of interspecific genetic transfer.

Materials and methods. Large numbers of nutritionally-deficient stable UV-mutants of the strains *A. flavus* (SzMC 0552), *A. nidulans*[9,10] (pabal, y, ts6) *A. niger* (SzMC 0145), *P. frequentans* (SzMC 0531) and *P. ramigena* (SzMC 0519) were produced, and the mutant pairs requiring lysine (Lys) and methionine (Met) were used throughout.

Maintenance and cultivation of the mutants, and the protoplast formation were carried out as previously described[1], with the exception that protoplast formation was performed in 0.6 *M* KCl for *Aspergilli* and in 0.8 *M* KCl for *Penicillia*, at pH 6, in both cases unbuffered. After collection of protoplasts and removal of the enzymes by repeated centrifugation with isoosmotic KCl solution, protoplast suspensions (about 1 million/ml) of the Lys mutants were mixed with those of the corresponding Met mutants. The mixed suspensions were again centrifuged, the supernatant was removed by suction.

One ml solutions of PEG (m. w. 4000) of different concentrations, containing 10 m*M* $CaCl_2$, were added to the protoplasts to find the optimum concentration of the fusion agent. The suspensions were stirred vigorously for a few sec. No additional osmotic stabilizer was used in the experiments reported in this paper. Samples were taken at 15 min intervals, mixed with osmotically-stabilized minimal medium, plated for regeneration, and then checked for segregetion as described earlier[1]. Osmotically-stabilized yeast-extract medium served as control to determine both the stabilizing effect of PEG at various concentrations and the ratio of complemented protoplasts. In the stabilized yeast-extract medium, practically 100% protoplast regeneration could be attained.

In other experiments, the PEG concentration was kept on the level found to be optimum and the Ca concentration was varied.

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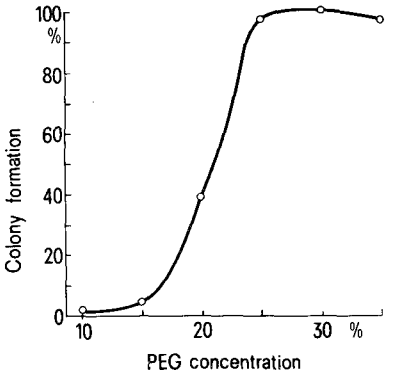

Fig. 1. Relationship between PEG concentration and osmotic stabilization of protoplasts as expressed in colony-forming units after a 1-hour PEG treatment. The average result obtained at the optimum PEG concentration (30%) is taken as 100%.

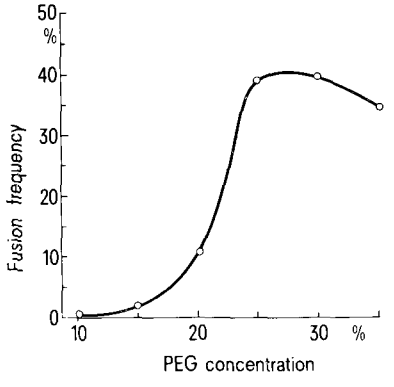

Fig. 2. Relationship between PEG concentration and frequency of protoplast fusion, based on complementation and colony formation of *A. nidulans* (Lys and Met) protoplasts in minimal medium, compared to colony formation in nutritionally-sufficient medium.

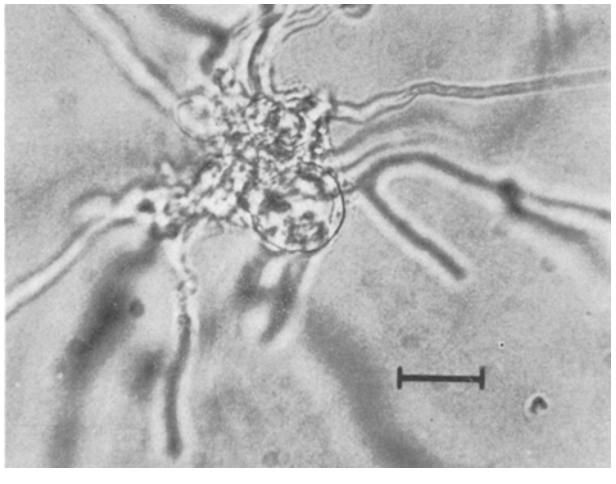

Fig. 3. Regenerating aggregate of nutritionally-complemented protoplasts of *Aspergillus nidulans* auxotrophic mutants. Marker represents 20 μm.

All procedures were carried out under strictly aseptic conditions.

Results and discussion. Complementation of Lys- and Met-requiring mutant pairs, followed by nutritional segregation of conidia or hyphae in the heterokaryotic colonies, was accepted as evidence of protoplast fusion. In the following, as an example, data concerning protoplast fusion of auxotrophic mutants of *A. nidulans* will be presented.

PEG is a good stabilizing compound, preventing bursting of protoplasts at 25% or higher concentrations. Results regarding relationship between PEG concentrations and stabilizing activity are shown in Figure 1.

PEG brought about intensive aggregation of protoplasts from 25% upwards. Calculations indicated that in the case of *A. nidulans* an average aggregation consisted of about 30 protoplasts. It may be mentioned that in the cases of *A. flavus*, *A. niger* and *Penicillia*, the aggregates contained a lower number of protoplasts, presumably because these are larger. Results concerning PEG concentration and fusion frequency of *A. nidulans* are given in Figure 2.

For osmotic stabilization and high-frequency protoplast fusion, a PEG concentration of 25% was needed. At 25 and 30% of PEG, the complementation data were similarly high and reached a level of 40% if postaggregation colony-forming units served as the basis of calculation, and 2,6% if the total number of protoplast pairs involved was used to calculate fusion frequencies. The low-frequency complementation at 20% or somewhat less PEG was due more to the hypotonic solutions and protoplast bursting than to an infrequency of aggregation. When an additional osmotic stabilizer was added to avoid protoplast bursting, a higher fusion frequency was attained. Results concerning experiments with PEG mixed with other osmotic stabilizers will be published elsewhere.

Samples taken after different intervals exhibited no significant differences. The high-frequency fusion obtained in the first 15 min was very similar to that in the second hour of sampling.

The effect of Ca ions on fusion frequency was significant. Without Ca ions, despite the good aggregation of protoplasts at higher PEG concentrations, the complementation frequency was negligible. On the other hand, as little as 1 m*M* $CaCl_2$ was effective in stimulating the fusion process. Interestingly, with from 10 m*M* to 100 m*M* $CaCl_2$, the stimulating effect proved similar.

After protoplast fusion and complementation, hyphae appeared within 10 h of incubation in osmotically-stabilized minimal medium (Figure 3); this was followed by rapid and morphologically characteristic development of a heterokaryotic colony.

In our cases, all kinds of conidial segregation pattern were found. However, in most colonies growing on minimal medium, many more Lys than Met conidia were obtained. Mycelia remained in the heterokaryotic state on minimal medium, and could be maintained indefinitely. On the other hand, colonies grown on minimal medium supplemented with lysine gave very rapidly only Lys conidia and hyphae. In rare cases the same held true in respect to minimal medium supplemented with methionine, which gave rise to only Met conidia and hyphae.

The frequency of protoplast complementation of *A. niger* was significantly lower, while that of *A. flavus* and *Penicillium* mutants was rather similar to that of *A. nidulans* auxotrophs.

The method of high-frequency intraspecific protoplast fusion might also lead to successful interspecific fusion

and genetic transfer. Among others, '*Aspercillium*' or '*Penigillus*' strains, the results of *Aspergillus-Penicillium* protoplast fusion, would be especially interesting from both theoretical and practical points of view. Results of attempts at interspecific complementation will be published elsewhere.

Zusammenfassung. Es gelang, die intraspezifische Protoplastfusion von *Aspergillus flavus*, *A. nidulans*, *A. niger*, *Penicillium frequentans* und *P. ramigena* mit grosser Häufigkeit zu verwirklichen. Die Fusionshäufigkeit wurde durch Komplementation von Mangelmutanten festgestellt.

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Electron-Microscopic Investigation on Peroxisomes in the Epithelia of Mice Gall-Bladder

The term 'peroxisome' or 'microbody' is understood as a special type of cytoplasmic organelle characterized biochemically by the association of one or more oxidases producing hydrogen peroxide with catalase, which destroys the hydrogen peroxide[1–3]. The name 'microbody' was first proposed by Rhodin in 1954[4] to describe roughly spherical singl-membrane limited particles in the proximal convoluted tubular cells of the mouse kidney 0.3–0.5 nm in diameter containing a fine-grained moderately electron dense matrix. Subsequently, analogous organelles were detected by Rouiller et al.[5,6] in hepatic parenchymal cells of the rat. The microbodies of the liver cells have an inner electron density or nucleoid. These initial studies have been confirmed and extended. Further studies indicated that microbodies or peroxysomes were constituent organelles of animal cell[7–14]. In the course of electron-cytochemical investigation, peroxisomes were found in the epithelial cells of mice gall-bladder.

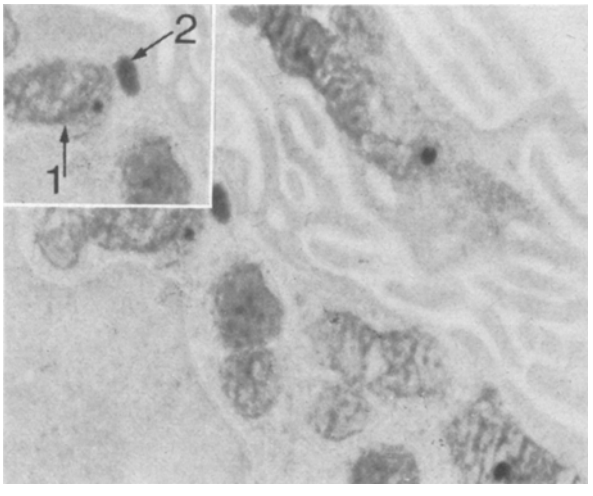

Fig. 1. Fragment of epithelial cell of mice gall-bladder. Mitochondria (1). Peroxisome (2). ×23,000.

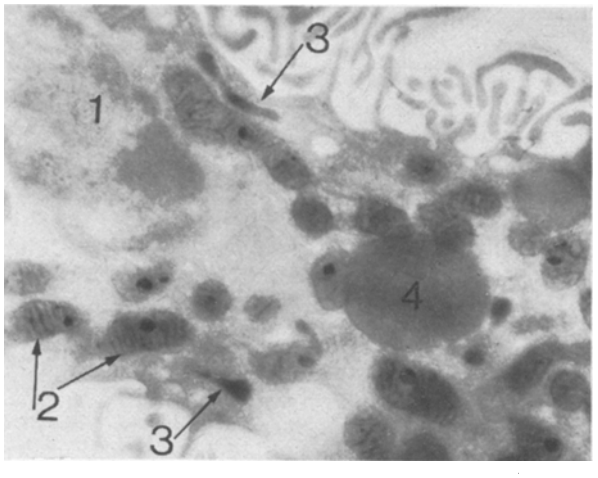

Fig. 2. Epithelial cell of mice gall-bladder. Nucleus (1). Mitochondria (2). Peroxisomes (3). Lipid drop (4). ×12,000.

Materials and methods. Normal male albino mice (18–20 g) were anesthetized with ether and small pieces of the gall-bladder were removed, fixed in ice-cold 4% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.2 for 6 h at 0 °C. The specimens were preincubated for 5 min in *Tris*-HCl, pH 9.5 containing 3.5% sucrose (v/v) and incubated for cytochemical demonstration of the peroxidatic activity of catalase in a modified DAB-medium[15] pH 9.5 at 37 °C for 3 h[16,10]. The final medium contained 20 mg DAB (3,3′-diaminobenzidine tetrahydrochloride, Chemapol, Praha), 0.2 ml 1% H_2O_2, 9.8 ml *Tris*-HCl, pH 9.5 and 3.5% sucrose.

The following control experiments were carried out: a) omission DAB, b) omission H_2O_2, c) heating at 100 °C for 10 min. After the incubation, the specimens were washed in 7% sucrose solution and postfixed in unbuffered 4% O_sO_4 for 15 h at 0 °C. They were dehydrated through graded concentrations of acetone and embedded in Epon

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