Table II. Comparison between testicular growth induced by orange radioluminous paint and continuous light

Treatment	Combined testicular weight (mg) after the following number of days of photostimulation			
	0	7	12	18
Short days	14.9 ± 1.3 (16)	16.9 ± 2.5 (5) [b]	24.9 ± 3.1 (23) [b]	77.4 ± 23.6 (6)
Continuous light	14.9 ± 1.3 (16)	208 ± 33 (8)	635 ± 36 (11)	2153 ± 319 (8)
25 mg Orange RLP [a]	14.9 ± 1.3 (16)	91 ± 20 (5)	516 ± 91 (10)	2290 (2)

[a] RLP is an abbreviation for radioluminous paint. [b] Mean ± SEM. Number of animals in parentheses.

Green radioluminous paint was without effect on gonadotrophin secretion. This is perhaps not too surprising as red/orange light is much more effective than blue/green light in inducing gonadal growth[4,16,21]. Whether this reflects the spectral responsivity of the photoreceptor or merely the greater tissue penetrance of the longer wavelength red light is unknown. The present results say nothing as to the site of the extra-retinal receptor. Other evidence, however, argues against it being the pineal gland[12,15,16,18,22] although this organ might play a minor role in quail[23]. This does not of course exclude a function for the pineal in other species[24]. A final caution must be entered as HOMMA et al.[13] have recently reported some experiments in quail where they claim that the eyes do have a role in the photoperiodic control mechanism. They might be necessary for birds to distinguish short days under some circumstances for enucleation does not always lead to gonadal regression when birds are transferred from 18L/6D to 8L/16D.

Résumé. Pour stimuler le photorécepteur extrarétinal de cailles japonaises (*Coturnix coturnix japonica*), des disques enduit de couleurs radiolumineuses ont été introduite sous la peau de leur crâne et soumis à un éclairement de courte durée en lumière naturelle. A une dose de 25 mg, une teinte maintenue dans la région de l'orange de spectre provoque un notable accroissement testiculaire. Le taux de cette augmentation ne différa pas de celui qui s'observe chez la caille soumise à un éclairement continu. Des enduits émettant la couleur verte furent sans effet. Ces données laissent supposer que la caille n'utilise pas son œil comme photorécepteur stimulant le développement périodique de ses gonades.

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Increased Fusion Frequency of *Aspergillus nidulans* Protoplasts

We earlier reported that protoplast fusion and heterokaryon formation could be achieved with auxotrophic mutants of *Geotrichum candidum*[1]. Nutritional complementation occurred in low frequencies, depending on the conditions. It was further mentioned that protoplast fusion could also be attained with nutritionally-deficient *Aspergillus nidulans* mutants. We describe here the aggregation and an increased fusion frequency of *Aspergillus nidulans* protoplasts.

Materials and methods. Nutritionally-deficient stable UV-mutants of the strain *Aspergillus nidulans* paba1, y, ts6[2-3] were produced, and the mutants requiring lysine (lys) and methionine (met) were used in these experiments. The mutants were cultivated and maintained on culture medium containing 0.5% yeast-extract, 1% glucose and 2% agar, at pH 6–6.2.

If not otherwise stated, the medium for protoplast formation consisted of 0.6 *M* KCl in McIlvain citrate-phosphate buffer (pH 6.0), containing 1% freeze-dried digestive juice of the snail *Helix pomatia*. This enzyme solution was filtered through a membrane sheet (pore size 0.22 µm) to exclude particles deleterious to aggregation and to fusion of protoplasts. For protoplast fusion the same solution was used, without snail enzyme. For regeneration of protoplasts use was made of a minimal medium ($(NH_4)_2SO_4$, 5 g; KH_2PO_4, 1g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; glucose, 10 g in 1000 ml distilled water) supplemented with vitamins[4], KCl (0.6 *M*) and agar (2%). The same medium with added L-lysine and L-methionine (each 50 µg/ml) was employed to determine the number of colony-forming units and the frequency of protoplast fusion.

In order to cultivate the auxotrophic mutants under optimum conditions for growth and protoplast production, a modification of the previously-described cellophane-

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sheet technique[5,6] was applied. It may be mentioned that with these mutants liquid medium can also be used for cultivation; however, in many other species of filamentous fungi, the cultivation in liquid is very unfavourable for protoplast formation. Sheets of cellophane, placed on the surface of the culture medium in Petri-dishes 10 cm in diameter, were inoculated with small drops of dense conidial suspensions. The distance between the drops was about 1 cm. After a 30 h-incubation at 30 °C, the cellophane sheets with the fast-growing colonies were lifted off and placed into protoplast-formation medium. For protoplast production, it is practical to use Petri-dishes of the same diameter as in the case of cultivation. 2 ml of enzyme solution is sufficient per sheet and 2 cellophane sheets can be applied in each dish. Gentle shaking at 30 °C led to rapid protoplast formation, and within 90 min a fair number of protoplasts were released.

The protoplast suspensions were combined for each mutant separately, then filtered through a sintered glass filter (Jena G2) without suction or pressure. Protoplasts were collected by centrifugation (3,000 *g*, 10 min, 2 °C). After discarding of the supernatant, ice-cold fusion medium was added and the concentration of protoplasts in the suspensions of the 2 mutants was determined with a hemocytometer. More ice-cold fusion medium was added to give a protoplast concentration of 1 million/ml, and 1 ml protoplast suspensions of the lys mutant were mixed with 1 ml of those of the met mutant. The suspensions were made up with cold fusion medium to 10 ml and centrifuged in 1 cm diameter tubes as above. After removal of the supernatant and repetition of the centrifugation, the aggregated protoplasts were shaken in 1 ml fusion medium to yield smaller aggregates, consisting mainly of 5–20 protoplasts. Samples of 0.05 ml were mixed with 5 ml portions of a regeneration medium of 1.5% agar (42 °C) and poured over a basic regeneration plate ('pouring method'). For comparison, samples were also applied to the surface of the regeneration medium, spread carefully with a sealed capillary, dried slightly, and sprayed with regeneration medium as described earlier ('spraying method'). The plates were incubated at 30 °C and checked for complementation.

In order to determine the frequency of protoplast fusion, 1:100 and 1:1000 dilutions were made from the suspensions of the aggregated protoplasts, mixed with the regeneration medium supplemented with the required amino acids, poured, and incubated as above. Conidia from colonies grown on minimal medium were collected to prove heterokaryon formation and to determine the proportion of segregation. The conidia were spread on the surface of agar plates containing 0.5% yeast-extract and 0.02% L-sorbose. Wet velvet was used for replica plating of conidia of the resulting small colonies onto minimal medium and onto the same medium supplemented with either lysine or methionine (50 µg/ml). All procedures were carried out under strictly aseptic conditions.

Results and discussion. Protoplast fusion and heterokaryon formation could be achieved at a considerably higher frequency with *Aspergillus nidulans* than with *Geotrichum candidum*. This was mainly due to the ability of *Aspergillus nidulans* protoplasts to form firm aggregates if adpressed by centrifugation in solutions of KCl or NaCl as osmotic stabilizers. The mechanism of aggregate formation and increased protoplast fusion at high salt concentrations is not known.

The interdependence of the abilities of protoplasts to aggregate and to fuse was very clear. Without previous aggregation a similarly low (or even lower) complementation frequency could be attained as with *Geotrichum* (1.6×10^{-6} per protoplast pair). This was the case when protoplasts of the 2 mutants were mixed and immediately added to the minimal medium; when mixed protoplasts were sedimented in the presence of the snail enzyme; when a sugar was used as osmotic stabilizer; or when protoplasts were formed in inorganic stabilizer, but washed with 0.8 *M* glucose, then centrifuged and treated as described. On the other hand, good aggregation occurred and fairly good complementation was found if protoplasts were formed in a sugar solution (e.g. 0.8 *M* glucose) but washed thoroughly with 0.6 *M* KCl or NaCl.

The highest frequency of complementation was attained as described in the methodical part with the application of the 'pouring method'. The average frequency of complementation was 2.5×10^{-3} as calculated on the basis of complementation per colony-forming unit (aggregates of various size and single cells). According to further calculations, an average colony-forming unit consisted of about 10 protoplasts. KCl and NaCl yielded similar results. The 'spraying-method', which proved

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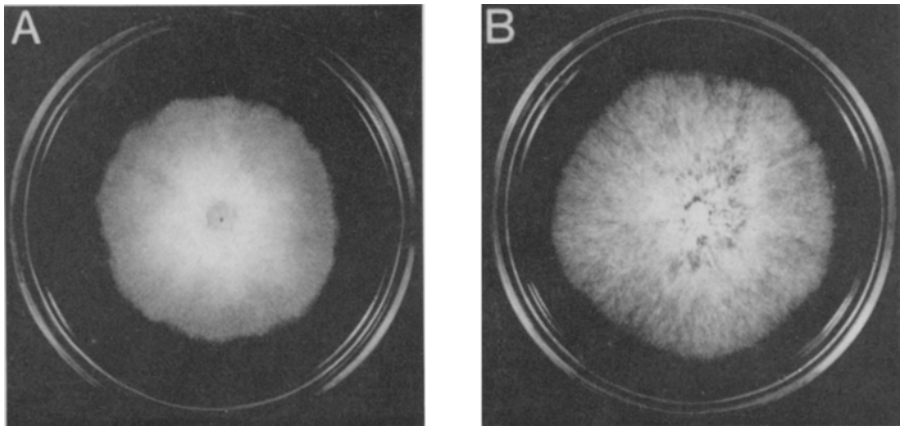

Fig. 1. 5-day old colonies of *Aspergillus nidulans* on minimal agar medium after several serial transfers. *A*) *Aspergillus nidulans* pabaI, *y*, ts6; *B*) heterokaryon colony of lys and met mutants of the same strain.

excellent with yeasts requiring high agar concentration for regeneration[7], proved less efficient in this case.

With the employed mutants, heterokaryon formation could also be attained through mycelial fusion of intact growing cells under appropriate circumstances. However, in the cases presented here, heterokaryon formation of this type in the course of incubation can be considered insignificant, if it takes place at all. If protoplasts of the mutants were kept under conditions favourable for cell-wall formation, and then treated as described above for protoplasts, there was a rapid decrease of complementation to a very low, or zero frequency. The decrease was proportional to the time of development of cell-wall elements. Interestingly enough, in spite of the regenerating cell-wall, the intensity of aggregation of the cells in the first 6 h was similar as in the case of true protoplasts. Results of other experiments concerning the kinetics of heterokaryon formation and cell-wall regeneration, to be published elsewhere, also revealed that the fusion process took place before cell-wall regeneration.

The fused and complemented protoplasts could regenerate cell wall, and colonies developed. The first colonies, heterokaryotic in nature could be seen by naked eye on the 3rd day of incubation.

Colonies developed from complemented cells differed characteristically in morphology from the parent mutants.

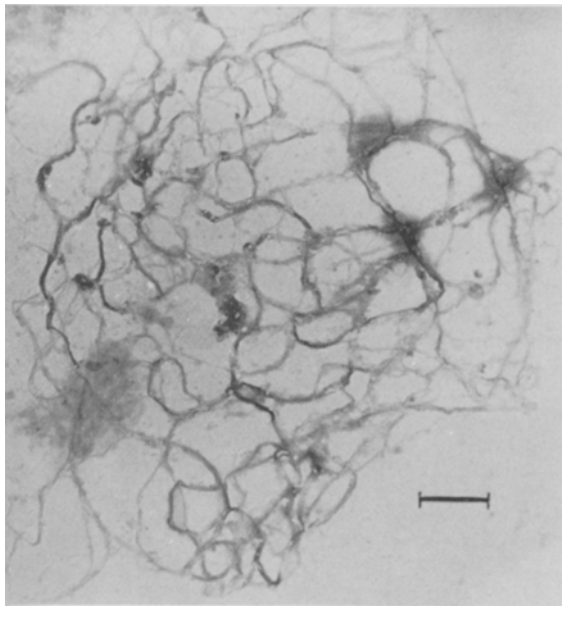

Fig. 2. Fibrillar network of the new cell wall of a regenerating protoplast after a 1-h incubation in osmotically-stabilized liquid culture medium. The protoplasts were burst, washed, treated with 0.1% trypsin for 2 h, washed again, applied to grids and carbon coated. Bar represents 1 μm.

In general, the heterokaryon colonies were irregular and heterogeneous. Figure 1 represents some differences in colony morphology between the original *Aspergillus nidulans* strain, to which the colonies of the mutants are rather similar on supplemented medium, and a typical heterokaryon colony after serial transfer on minimal medium. The morphological differences are permanent.

Aggregation experiments carried out at various pH levels (pH 3–12) and at different temperatures (0–30 °C) revealed that these factors were not decisive; mostly the size of the aggregates was affected. For two reasons, however, the use of low temperatures after protoplast formation and before plating is suggested. The aggregates were better packed at low temperatures, and the subsequent fusion frequency was somewhat higher; the development of elements of a fibrillar network was inhibited at low temperatures, but they appeared rapidly at room temperature, as seen in Figure 2. This observation is in good agreement with the earlier data obtained with *Saccharomyces cerevisiae*[8,9]. The fibrillar network can prevent protoplast fusion completely.

Serial transfer of complemented cells could be performed indefinitely on minimal medium. However, the conidia were segregated into the original mutants indicating the heterokaryotic state of the hyphae. No rule could be established as regards ratios of segregation. All types of segregation patterns existed.

The solutions of KCl or NaCl could aggregate protoplasts selectively. No aggregation occurred with *Geotrichum*, *Saccharomyces* and *Candida* species. On the other hand, intense aggregation was found not only with *Aspergillus nidulans*, but with other *Aspergilli* and *Penicillia* too, and protoplast fusion has already been achieved with *Aspergillus niger* mutants.

Zusammenfassung. Die Häufigkeit der Protoplastfusion auxotropher Mutanten von *Aspergillus nidulans* wurde durch Zugabe von 0.6 *M* KCl oder NaCl gesteigert.

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Inhibition of Macromolecular Synthesis in the Malarial Parasites by Inhibitors of Proteolytic Enzymes

Recently, we reported that acid proteases from several species of *Plasmodium* were extremely sensitive to several protease inhibitors isolated from actinomycetes cultures[1,2]. Since erythrocytic stages of the malarial parasite obtain much of their amino acids from breakdown of host cell hemoglobin[3–5], and since they also digest some of their own organelles[6], we suggested that the organism may be particularly susceptible to inhibitors of proteolytic enzymes[1,2]. This may be the case, as low concentrations of several such inhibitors inhibited growth, as measured as an increase in parasitemia of *P. knowlesi* incubated in vitro in monkey red blood cells[7]. We now report on