the presence in this fraction of a substantial amount of pituicytes. Nearly 60% of the total non-specific acetylcholinesterase activity is also sedimented in fraction I, strongly suggesting an association between this enzyme and the pituicytes. However, since the neural lobe also contains endothelium and blood cells, it cannot be excluded that part of the enzyme activities demonstrated in fraction I originates in these structures.

Vasopressin and $Mg^{2+}+Na^{+}+K^{+}$-ATPase may be used as markers for the nerve endings of the neural lobe[24]. The distribution by differential centrifugation of these substances indicates that the neurosecretory nerve terminals mainly accumulate in fraction II, but also to some extent in fraction I. Electron microscopic examination of fraction II demonstrates the presence in this fraction of large quantities of broken off nerve endings identified by their contents of neurosecretory granules (Figure 2). The concentration in fraction II of non-specific acetylcholinesterase, however, is much lower than that of fraction I and it is reasonable to conclude that the nerve endings only contain small amounts of that enzyme if any at all. The non-specific esterase activity observed in fraction II could be due to the presence in that fraction of a small number of pituicytes.

The specific acetylcholinesterase is found in relatively high concentrations in both fraction I and II and cannot be specifically associated with either the pituicytes or the nerve endings, but it may be a constituent of both particles. A much larger part of this enzyme was found in a soluble form (fraction VI) than was the case for the non-specific acetylcholinesterase.

In conclusion, this investigation indicates that the non-specific acetylcholinesterase may be primarily located in the pituicytes of the neural lobe. In this respect the pituicytes resemble the glial cells of brain tissue. The nerve endings display specific acetylcholinesterase activity and very little non-specific activity.

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Factors Affecting High-Frequency Fungal Protoplast Fusion

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Summary. Factors influencing the fusion frequency of protoplasts have been examined with auxotrophic mutants of *Aspergillus nidulans*. The optimum conditions were a total of 5 to 15 million protoplasts per ml, and 25% polyethylene glycol (PEG) 4000 or 6000 as fusogenic agent in 10 to 100 m*M* $CaCl_2$ solution.

Formerly we had developed methods for fusion of fungal protoplasts[1–3], including a high-frequency procedure carried out with polyethylene glycol (PEG) and Ca ions[4]. Details of PEG-induced protoplast fusion were given elsewhere[5], and independently by ANNÉ and PEBERDY[6–8].

Here we present results concerning 1. the concentration of the protoplasts involved in the aggregation and fusion by PEG, 2. the stabilizing, aggregating and fusogenic capacities of PEG preparations of different molecular weights at different concentrations, and 3. different inorganic materials as osmotic stabilizers and promoters or inhibitors of protoplast fusion. Appropriate control of these factors renders the fusion method more reliable for 'genetic transfusion', i.e. for transfer of genetic material via protoplast fusion.

Materials and methods. Aspergillus nidulans[9] mutants requiring lysine (SzMC 0443) and methionine (SzMC 0442) were used. The methods of protoplast formation, fusion, nutritional complementation and heterokaryon formation were similar to those described previously[2,4], except that no buffer was included in the stabilizing 0.6 *M* KCl solution; prior to PEG treatment the protoplasts were suspended in 0.05 ml osmotic stabilizer and then PEG solution was added to give a total of 1 ml; after PEG treatment for 15 min the samples were diluted with 0.6 *M* KCl and plated. The fusion frequency is expressed as the number of colonies developing on minimal medium as a percentage of those growing on nutritionally-sufficient medium, in both cases after PEG treatment.

PEG preparations (Fluka and Hoechst) with molecular weights of 400, 1540, 4000, 6000 and 20,000 were used for comparison at different concentrations (%, w/v).

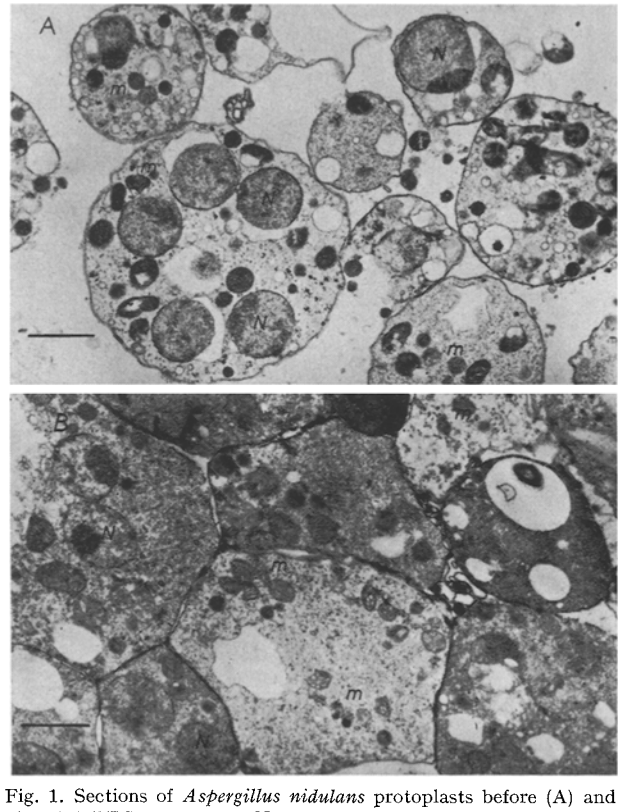

Fig. 1. Sections of *Aspergillus nidulans* protoplasts before (A) and after (B) PEG treatment. N, nucleus; m, mitochondria. Markers represent 2 µm.

For electron microscopy, the protoplasts were collected by centrifugation, fixed for 2 h in KARNOVSKY's solution[10], washed twice in Millonig's buffer (pH 7.2), and postfixed for 1 h in 1% osmium tetroxide in the same buffer. The protoplasts were rinsed twice in Millonig's buffer (pH 7.2) for 5 min each, dehydrated through ethanol series, treated with propylene oxide twice for 10 min each, then put in Durcupan I mixture for 1 night at room temperature, and finally embedded in Durcupan II mixture and polymerized at 56 °C for 48 h. Ultrathin sections were double-stained with uranyl acetate and lead citrate[11].

Results and discussion. Various amounts of the protoplasts of the 2 mutants in a 1:1 mixture were aggregated with 25% PEG 4000 in the presence of 100 m*M* $CaCl_2$. The best fusion frequencies were found at a total of 5 to 15 million protoplasts of the 2 mutants per ml.

Before and after PEG treatment, samples were taken for electron microscopy. Typical preparations are shown in Figure 1. The treated protoplasts displayed very strong adherence of the cells.

Mixtures of the protoplasts of the mutants were treated with various concentrations of PEG of different mole-

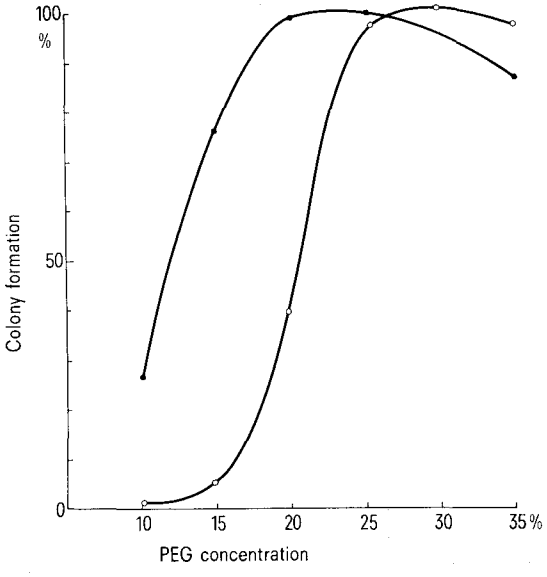

Fig. 2. Relationship between PEG 4000 concentration and osmotic stabilization of protoplasts as expressed in colony-forming units. The result obtained at a 25% PEG concentration in 100 m*M* $CaCl_2$ solution is taken as 100%. ○, PEG 4000 in 10 mM $CaCl_2$; ●, PEG 4000 in 100 m*M* $CaCl_2$.

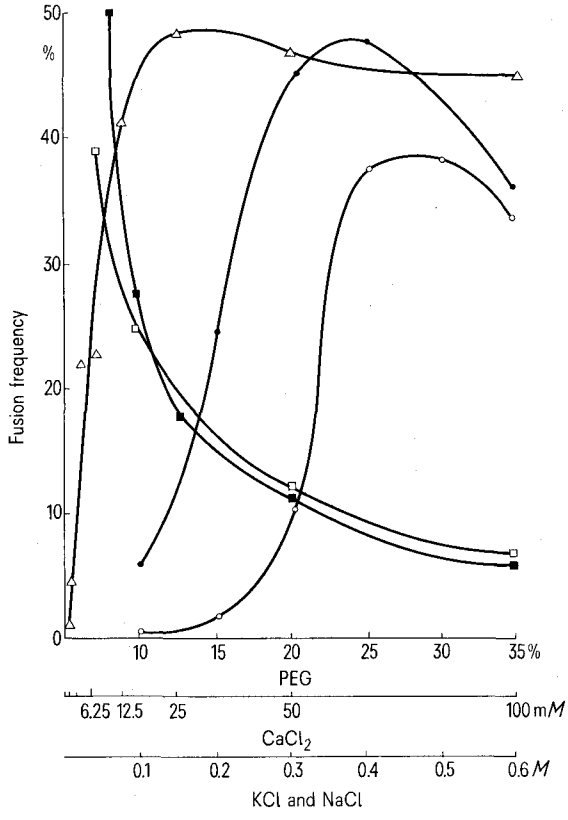

Fig. 3. Relationship between PEG 4000, $CaCl_2$, KCl and NaCl concentrations, and frequency of protoplast fusion, based on complementation and colony formation of protoplasts of auxotrophic mutants in minimal medium, compared to colony formation in nutritionally-sufficient medium. O, PEG 4000 in 10 m*M* $CaCl_2$; ●, PEG 4000 in 100 mM $CaCl_2$; △, $CaCl_2$; □, KCl; ■, NaCl.

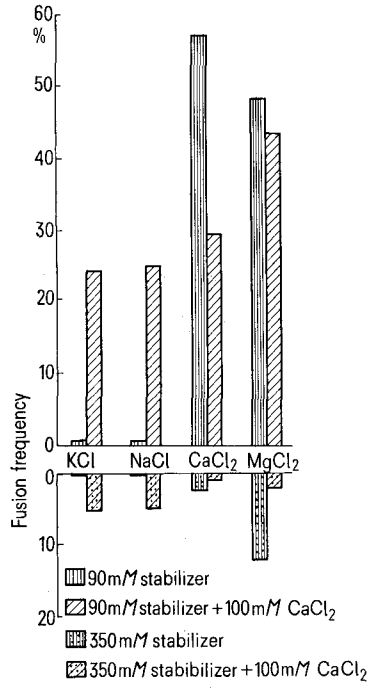

Fig. 4. Relationship between different osmotic stabilizers and frequency of protoplast fusion in 25% PEG in the presence and absence of Ca.

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cular weights. The $CaCl_2$ concentrations was kept at 100 m*M*. All varieties of PEG were able to stabilize and aggregate protoplasts and induce protoplast fusion at appropriate concentrations. PEG 4000 and 6000 proved to be the most effective.

The protoplast-stabilizing effects of PEG 4000 in the presence of 10 m*M* or 100 m*M* $CaCl_2$ are shown in Figure 2. Bursting of protoplasts was prevented at and above 25% PEG 4000 in 10 m*M* $CaCl_2$ solution, and at and above 20% PEG 4000 in the case of 100 m*M* $CaCl_2$.

Intensive aggregation and fusion of protoplasts was brought about in both 20% and 25% PEG 4000, depending on the Ca concentration (Figure 3). The further experiments were carried out with 25% PEG 4000.

The $CaCl_2$ concentration of the PEG 4000 solution added to the protoplasts basically influenced the frequency of protoplast fusion. The data are also presented in Figure 3. The KCl or NaCl concentration in the fusion solution containing 100 m*M* $CaCl_2$ was also important (Figure 3).

Not only the concentrations of the monovalent cations and Ca were critical, but also the means of administering them: a fusion frequency of more than 10% could be attained in a solution containing 25% PEG 4000 in 0.1 *M* $CaCl_2$ and 0.3 *M* NaCl or KCl, but the frequency was negligible if the protoplasts were first suspended in 0.5 ml 0.6 *M* NaCl or KCl, and then 0.5 ml PEG 4000 in 0.2 *M* $CaCl_2$ was subsequently added to give the previous final concentrations. In this case no fusion at all occurred without Ca, although the intensity of aggregation was high. The plated protoplasts of the 2 auxotrophs remained viable for as long as 1 week.

Data concerning the effects of various osmotic stabilizers are shown in Figure 4. The protoplasts of the mutants in 0.6 *M* KCl were mixed and then washed twice by centrifugation in KCl, NaCl, $CaCl_2$ or $MgCl_2$ (0.6 *M*); the supernatant was removed and protoplasts were suspended in 0.15 ml of the osmotic stabilizers. 25% PEG 4000 solution was then added with or without $CaCl_2$, with a supplement of the stabilizing compounds (0.3 *M*) or without them. Figure 4 indicates that 1. K and Na are similar in effect and the presence of Ca is needed for high-frequency fusion, 2. a very high fusion frequency can be reached if KCl is replaced by $CaCl_2$, 3. additional $CaCl_2$ gives a lower frequency, the Ca concentration then being over the optimum, and 4. a high fusion frequency results if K is replaced by Mg. Under hyperosmotic conditions, the fusion process is diminished, to zero in some cases, independently of the osmotic stabilizer.

In other experiments it was found that most of the above conclusions can be generalized and are valid for both filamentous fungi and yeasts, and 'transfusion' of genetic material can be carried out successfully intraspecifically and interspecifically.

Das Wachstum der Zellwand in synchroner *Chlorella*

Growth of the Cell Wall in Synchronous *Chlorella*

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Summary. By pulse labelling, it is shown that the relative growth rate of cell wall substances in synchronous *Chlorella* has a sharp maximum at the time of autospore formation. The dependence on time of the rate of growth of the cell wall coincides with that of the rate of increase of the free space. Moreover, the increase of the nitrogen content of the cell wall also has a maximum roughly at the same time as the growth rate.

Kationische mehrwertige Spurenelemente treten nicht nur in das Innere von *Chlorella* ein, sondern sie werden auch durch die Zellwand stark adsorbiert[1–4]. Die Untersuchungen sollten nun auf synchrone Algen ausgedehnt werden. Dazu sollten zunächst Aussagen über die Verteilung der Zellwandsynthese über den Wachstumszyklus erhalten werden. Die Vermehrung von *Chlorella* erfolgt durch Autosporenbildung. Die reife Algenzelle teilt sich in 2–16 Tochterzellen, die nach der Bildung neuer Zellwand und teilweisem Abbau der alten Zellwand freigesetzt werden[5,6].

Die synchrone Züchtung erfolgte im Hell-Dunkel-Zyklus[7,8]. *Chlorella fusca*, Stamm 211-8b, wurde in einem Zyklus von 16 h Licht und 12 h Dunkel in einer 2,5 l Algensuspension fassenden Säule gehalten[9]. Zur Bestimmung des Zellwandzuwachses wurden die Algen jeweils 20 min in Flachgefässen, wie sie auch für Ionenaufnahmeversuche verwendet werden[10], mit $^{14}CO_2$ pulsmarkiert. Die Gefässe waren mit einer zusätzlichen, durch eine Serumkappe verschliessbaren Öffnung versehen, durch welche die Algen mittels Injektionsspritze eingebracht wurden. Nach Markierung wurden die Algen im Zellhomogenisator aufgebrochen und die Zellwände durch Zentrifugation bei 800 *g* isoliert[11,12], zweimal mit 200 ml Wasser gewaschen und bei 95°C getrocknet. Die Ausbeute an Zellwandtrockengewicht, bezogen auf Algentrockengewicht, war 8%. Nach Nassverbrennung[13] wurde die spezifische Aktivität durch Szintillationszählung bestimmt. Die isolierten Zellwände waren – je nach Wachstumsphase – mit 0–5% Stärke verunreinigt. Deren Beitrag zur Masse und Aktivität wurde nach Heisswasserextraktion und Filtration durch die Jod-Stärke-Reaktion bzw. durch Szintillationszählung nach Verbrennung bestimmt und in Rechnung gestellt.

Die spezifische Aktivität der Zellwand ist dem während des Pulses (Δt) erzielten Zuwachs (ΔZ) direkt und der Menge an bereits vorhandener Zellwand (Z) verkehrt proportional. Dabei ist $\Delta Z \ll Z$. Bei konstanter Radiokohlen-

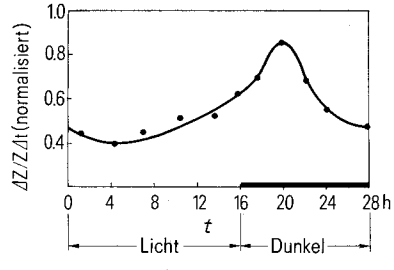

Fig. 1. Verhältnis der relativen Zuwachsgeschwindigkeiten der Zellwandmasse und der Zellmasse bei synchroner *Chlorella*.