tion was measured in a Specol spectrocolorimeter at 474, 515 and 556 nm, against similarly treated reagent blanks in 10 mm glass cells. The readings were corrected for unspecific background colour by applying Ecorr. = $2E_{515}-/E_{474} + E_{556}/$[5].

The results of analysis (Table) indicated that the smallest content of estrogen-like substances occurs in unrooted dormant hyacinth bulbs, and that they are present only in leaves and inflorescence. In the non-cold-treated bulbs which were planted in soil and had grown all the time in the greenhouse, about 2-fold increase of the content of estrogen-like substances was observed in leaves and inflorescence during flowering time. However, estrogen-like substances were not found in the fleshly scales and roots.

Occurrence and distribution of estrogen-like substances in different organs of cold-treated and non-cold-treated hyacinth bulbs

Type of bulbs	Heel	Scales	Leaves	Inflore-scence	Roots
Dormant bulbs analyzed in November 1972	0	0	23	9	—
Non-cold treated bulbs analyzed at the flowering time (January 16, 1973)	0	0	42	21	0
Cold-treated bulbs analyzed at the flowering time (January 20, 1973)	0	27	62	33	21

Results are expressed in μg equivalent of estrone in 100 g of fresh weight plant material

In the cold-treated hyacinth bulbs analyzed at the flowering time, an increase of the content of estrogen-like substances in leaves and inflorescence was observed. These substances were also found in fleshly scales and roots in cold-treated bulbs.

It can be then summarized that as in the other species so far investigated the estrogen-like substances are present in hyacinth plants. After the cold treatment of hyacinth bulbs – which is necessary for normal growth of inflorescence and leaves – an increase in the level of estrogen-like substances took place. It is known from other data[6] that estrogens can replace a low temperature treatment in the flowering process of *Cychorium intybus*.

Zusammenfassung. Kälteeinwirkung auf Hyazinthenknollen, welche eine normale Entwicklung von Blütenständen verursacht, führt gleichzeitig zu einer allgemeinen Steigerung des Oestrogengehaltes in der Pflanze mit Höchstwerten in den Blättern und Blütenständen.

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Department of Ornamental Plants Research Insitute of Pomology, Skierniewice (Poland),
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Self-Inhibiting Extracellular Proteins from *Aspergillus oryzae*

Earlier we described the occurrence of both self-stimulating and self-inhibiting substances in cultures of filamentous fungi[1-3]. It was shown that the stage of action during the culture development of these organisms is of critical importance. This explains the curious response one very often gets in these cultures by different inoculum sizes. Using low concentration substrates (10 g/l carbon source) filtrates from very young cultures of *A. oryzae* when added at the stage of inoculation to fresh cultures showed inhibiting effects of growth which often had lasting effects up to later stages of growth. The implications in continuous culture operation are obvious. In fact there are great difficulties in maintaining a true equilibrium of mycelium content in homogeneous single stage continuous culture, and the range of dilution rates allowing continuous procedure, albeit with considerable variations in mycelium content, is very narrow.

With high substrate concentrations on media producing a slightly alkaline reaction we could now show that in continuous culture of *A. oryzae* self-inhibiting extracellular compounds occurred (when tested as above), although the concentration of these substances seemed out of phase with the variations of mycelium content. An example of the effect of small amounts of culture

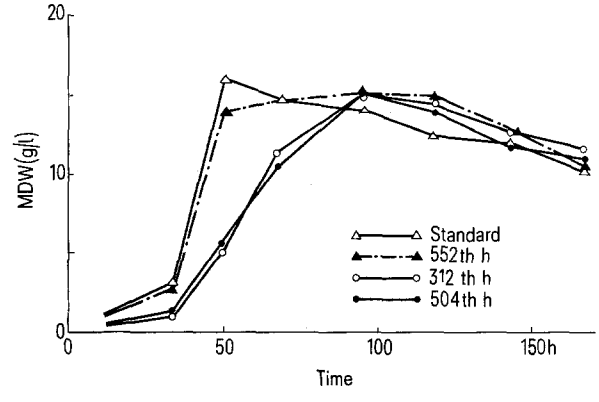

Fig. 1. Effect of culture filtrate (supernatant) from deep continuous culture of *A. oryzae* on fresh stationary cultures of the same organism. Experimental characteristics. Deep culture. Substrate: potato starch, 40 g; $(NH_4)_2SO_4$, 16 g; KH_2PO_4, 4 g; citric acid, 10 g; glacial acetic acid, 6.9 ml; $MgSO_4.7H_2O$, 0.6 g; $FeCl_3.6H_2O$, 0.1 g; $CaCl_2.6H_2O$, 10 mg; $ZnSO_4.7H_2O$, 5 mg; $CuSO_4.5H_2O$, 0.5 mg; $MnSO_4.4H_2O$, 0.5 mg; pH adjusted (NaOH) to 6.8; distilled water up to 1 l. Dilution rate, 0.04 h^{-1}; temperature 30 °C; mycelium separated by centrifuging.
Stationary culture. Substrate: glucose, 40 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0.3 g; $FeCl_3.6H_2O$, 0.003 g; Na_2HPO_4, 9.46 g; KH_2PO_4, 9.07 g; distilled water up to 1 l. 10 ml portions of sterile medium distributed in 100-ml sterile conical flasks; 1.5 ml conidial suspension in membrane-filtered supernatant (or sterile water as control) from deep culture added to each; inoculum size, 2×10^5 conidia/ml.

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filtrate taken from a deep culture of *A. oryzae* under continuous operation when added to stationary cultures of the same organism at the stage of inoculation is shown in Figure 1. It can be seen that culture filtrates derived from the submerged continuous culture at the 312th and 504th h were inhibitory to the test cultures, which showed considerably reducec growth rates in the linear phase, whereas culture filtrate at the 552nd h was devoid of any growth-inhibitory action.

Larger amounts of culture filtrates, which showed this kind of inhibitory effect were collected and purification of the desired compounds was attempted. Since the purifi-

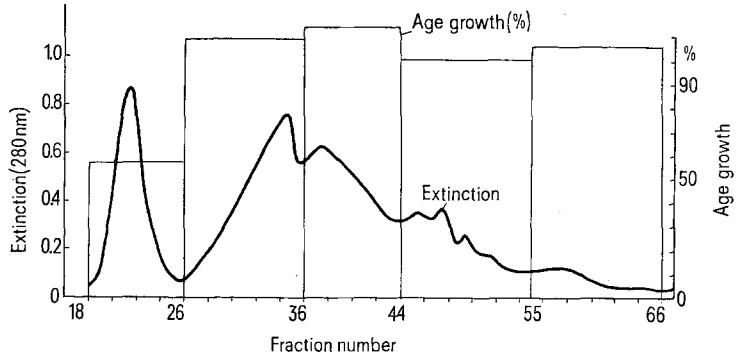


Fig. 2. UV-absorbance (280 nm) and biological activity of fractionated filtrate (G-25) from a deep culture. Experimental characteristics. Deep culture and stationary culture tests as for Figure 1. Biological activity is expressed as percentage reduction of growth in stationary cultures after 38 h incubation. Separating column: Sephadex G-25, (75 × 2.5 cm); bed volume: 370 ml; void volume: 115 ml; eluting agent: 0.05 *M* phosphate pH 6.8; fraction volume: 5 ml; 7.5 ml of an 11-fold culture filtrate were used for separation. Tests for biological activity as under Figure 1 of the pooled fractions for the individual UV-peaks after 2-fold concentration by sterile ultrafiltration on UM-05.

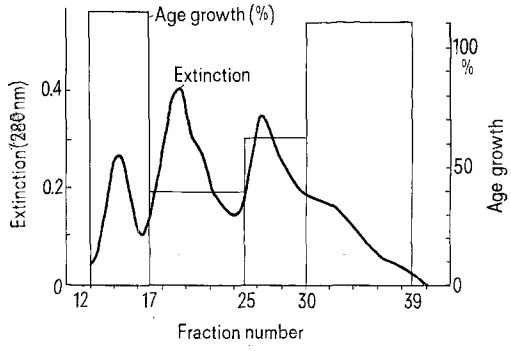


Fig. 3. UV-absorbance (280 nm) and biological activity of fractionated eluate of peak No. 1 from G-25 on G-75 Sephadex. For further details see Figure 2.

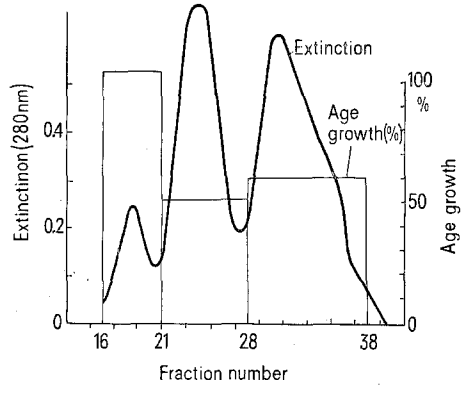


Fig. 4. A separation scheme for the self-inhibitory compounds. [a] Column length, 1 m; inner diameter, 25 mm; total bed volume: 440 ml; void volume: 165 ml; eluting agent: 0.05 *M* phoosphate buffer pH 6.8; size of fractions 10 ml. [b] Column characteristics as above, except that void volume is 160 ml. The protein-content of the pooled fractions was finally determined according to the method of Lowry using weighed amounts of serum-albumin as standards.

cation would be considerably eased if concentrated culture filtrates could be used, evaporation was carried out in vacuum at 30 °C. However, a 5-fold concentration resulted in a complete loss of the inhibitory factor. A test of the condensed liquid showed that the desired compound was non-volatile. With this concentration factor there was also considerable precipitation of amorphous matter, suggesting denaturation of proteinaceous material, possibly due in part to the high concentration of phosphates and other compounds of the medium used. A concentration by a factor of 2 was possible. however, without detectable loss of activity. All activity tests were carried out as described for Figure 1. Diffusion tests were unsuccessful; this may be due to the fact that the inhibitory effect is not an absolute one, rather is it a retardation of growth, with a possible reduction of maximum yield of mycelium, effects most difficult to detect in agar diffusion tests. The inhibitory principle was lost also upon boiling for 5 min, but maintained most of its activity after pasteurization at 70 °C for 10 min. Dialysability against both water and culture filtrate was ascertained before carrying out purification; the active factor was retained in the dialysis sac, which should prevent the passage of molecules with a molecular weight larger than 10,000 daltons. Concentration of the active compound is possible by ammonium sulphate (100% sat.) precipitation. No detectable loss of activity occurred after dissolving the precipitate in M/15 phosphate buffer at pH 6.8 and dialysis against distilled water.

For the fractionation described below, concentration was carried out by ultrafiltration. Using various grades of filters, it could be shown again that the active compound has a molecular weight larger than 10,000 and smaller than 50,000 daltons.

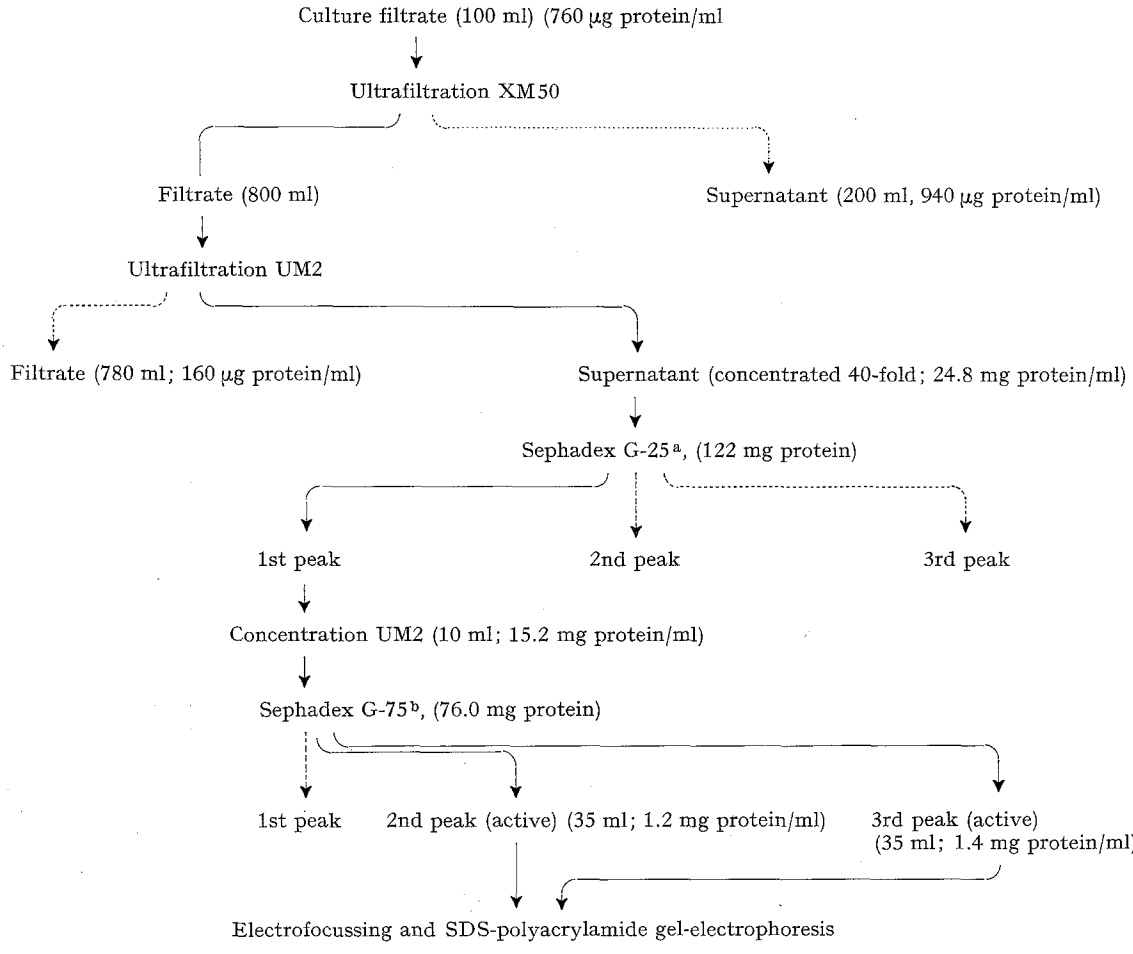
A number of fractionations were now carried out on culture filtrate concentrated by ultrafiltration.

A first fractionation was performed by using an 11-fold concentrated (ultrafilter UM-05) culture filtrate on a Sephadex G-25 gel (for details of operation see caption to Figure 2). The eluate collected in 5 ml-portions was tested for absorbance at 280 nm; the pooled fractions

making up the individual peaks, as shown in Figure 2, were tested for their biological activity after a 2-fold concentration on sterile ultrafiltration equipment with filter UM-05.

The percentage reduction of growth, as shown in Figure 2, is taken as a measure for biological activity. It is evident that the desired compound is contained within the first fraction of a Sephadex G-25 eluate. This fraction is identical with the void volume of the Sephadex column.

Since a G-25 Sephadex is suitable for fractionation of compounds between molecular weight 1000--5000 daltons, it is understandable that the active coumpound, which by ultrafiltration indicated already a molecular weight larger than 10,000 daltons, can be detected within the void volume fraction. After a 5-fold concentration on ultrafilter UM-2 of the pooled fractions of the 1st peak from G-25, a further fractionation was carried out on a Sephadex G-75 column. Both the UV-absorbance (280 nm)



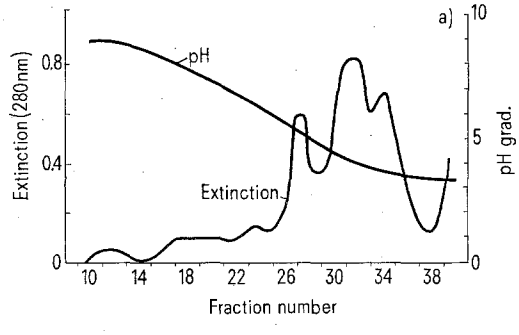


Fig. 5. UV-absorbance and biological activity of fractionated eluate of peak No. 1 from Sephadex G-25 according to the treatment scheme as shown in Figure 5. For details see Figure 2.

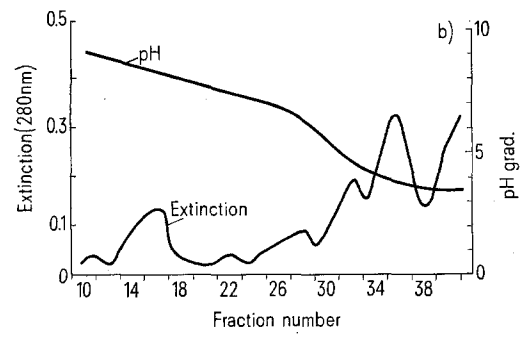


Fig. 6. Electrofocusing of eluates from G-75 as shown in Figure 4. a) peak No. 1; b) peak No. 2.

of the 5 ml-fractions and the biological activity of the pooled fractions are shown in Figure 3. It is evident that the clearly separated peaks Nos. 2 and 3 showed the expected inhibitory activity. The molecular weights, according to the K_{av}-values, are in the order of 30,000 daltons and 11,000 daltons for peak 2 and 3 respectively.

After a number of trials the scheme shown in Figure 4 proved to be a workable basis for the purification of the inhibitory compound. The fractionation together with the biological activity of the Sephadex G-75 is shown in Figure 5. On the basis of the determined K_{av}-values, the molecular weights of the active compounds could again be calculated as 28,000 and 11,000 daltons respectively. Electrofocusing of the 2 active peaks from G-75 filtration showed that these peaks do not represent pure compounds. The results are shown in Figure 6. When divided up into the individual peaks and percolation over G-25 (for removal of sucrose and ampholines) as obtained after electrofocussing, the biological activity was lost. No attempt at this stage was made to pool all the electrofocussed peaks and to try if biological activity could thus be restored.

Peaks Nos. 2 and 3 from Sephadex G-75 fractionation were subjected to SDS-polyacrylamide gel electrophoresis to estimate the degree of purity and possible sub-unit character of the coumponds. 2 stainable bands could be detected with apparent molecular weights of the compounds of 27,000 and 12,000 daltons respectively. The tests on differential absorbance at 260 and 280 nm of these peaks also showed that the active principles are proteins; according to this method virtually no nucleic acids could be detected. It would seem premature to present speculations on the mechanism of action of these proteins. Experiments along this line are in progress.

Zusammenfassung. Mittels Ultrafiltration und Gelelektrophorese konnten 2 extrazellulare Protein-Fraktionen, Mol.- Gew. 27000–28000 daltons und 11000–12000 daltons, aus submersen Kulturen von *Aspergillus oryzae* gewonnen werden, welche Wachstumshemmung desselben Organismus bei Verabreichung zu Beginn der Kulturentwicklung verursachen.

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[4] Technical assistance by Mrs. U. Stangl and Mr. L. Berzaczy is greatly appreciated.

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Herpes-Virus and Double-Stranded RNA

Although 14 years have passed since the discovery of interferon, the exact nature of its induction by viruses is not yet completely understood.

It appears that RNA needs a well defined secondary structure to be a good interferon inducer, true double-stranded ribonucleic acids being better inducers than single-stranded forms[1–7]. However, DNA appears to be a very poor inducer of interferon, if it works at all. The question of what the inducer nucleic acid is in DNA viruses needs to be explored further.

The report of Colby and Duesberg[8] that vaccinia virus induces a double-stranded (DS) RNA in chick embryo cells offered a possible explanation of the phenomenon. However, the necessity for DS RNA during replication of DNA viruses has not been shown.

Herpes simplex virus displays a variable pattern with regard to interferon induction; the same viral strain, in the same non-permissive cells, at a low multiplicity of infection (MOI) induces interferon production, while no viral constituents are synthesized; at a higher MOI, viral constituents are produced in a defective manner, but no detectable amounts of interferon are present[9].

If a DS RNA is a necessary step during replication of DNA viruses, one can assume that interferon appears when the virus-induced DS RNA occurs in a cell population with conserved protein biosynthesis. The present communication deals with our attempts to check such a hypothesis.

Materials. All experiments were carried out on primary chick embryo cells (CEC) and on HEp-2, MA-104 and mKS-B[10] cell lines. *Herpes simplex* virus, strain S, (a gift from Dr. G. Tarro), vesicular stomatitis virus (VSV) and vaccinia virus were replicated on Hep-2 cells. Titers: *Herpes simplex* virus, 3×10^8 PFU/ml; VSV, 5×10^6 PFU/ml; vaccinia virus, 5×10^8 $TCID_{50}$/ml on HEp-2 cells.

Results and discussion. Monolayers of barely confluent CEC were infected with *Herpes simplex* virus (MOI 10 PFU/cell), a second set of replicates was infected with vaccinia virus (MOI 10 PFU/cell), while a mock-infected group of cultures served as control. After an adsorption period of 1 h, nutrient medium (without serum) containing 5 μCi/ml of uridine-5-^{3}H was added and the cultures were incubated at 37 °C for 6 h, since it is well known that at this moment all RNA synthesis is herpes-directed[11–12].

In other series of experiments, cultures were incubated for 18 and 24 h after infection, and extraction of RNAs was performed as described by Colby and Duesberg[8].

Under identical conditions this experiment was repeated with HEp-2, mKS-B and MA-104 cell cultures with similar results. 1. LiCl precipitation of RNAs from *Herpes*-infected and uninfected cells was performed as

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