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.

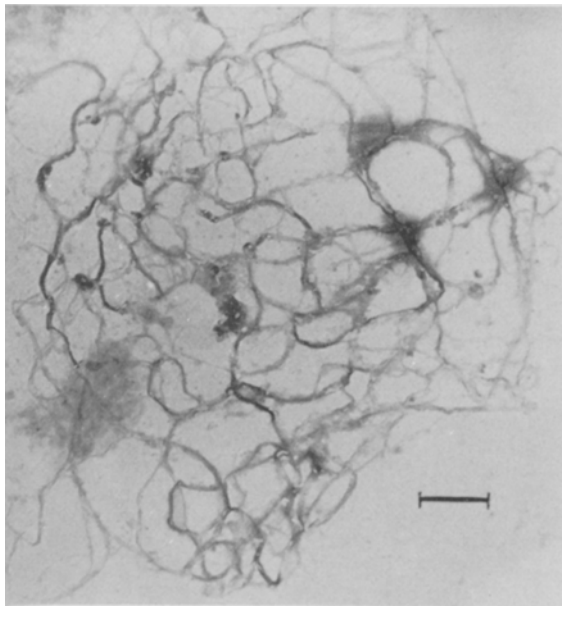

Fig. 2. Fibrillar network of the new cell wall of a regenerating protoplast after a 1-h incubation in osmotically-stabilized liquid culture medium. The protoplasts were burst, washed, treated with 0.1% trypsin for 2 h, washed again, applied to grids and carbon coated. Bar represents 1 μm.

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

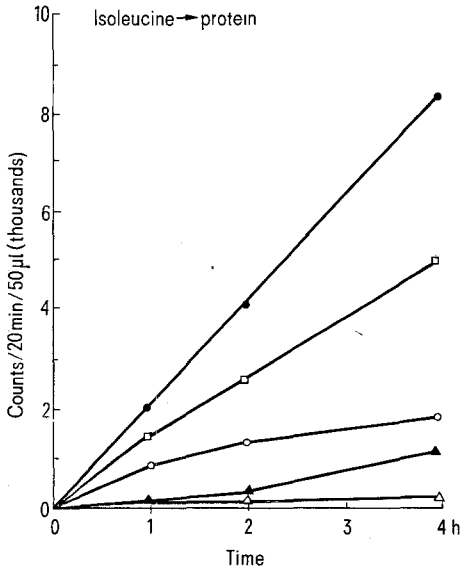


Fig. 1. Effect of protease inhibitors on incorporation of isoleucine into protein. △, normal red cells; ●, infected red cells; □, infected + 50 µg/ml chymostatin; ○, infected + 50 µg/ml pepstatin; ▲, infected + 2 m*M* PMSF.

similar studies, performed on *P. berghei*-infected mouse red cells. In this case, however, the incorporation of labeled precursors into protein or nucleic acids were used as indicators of growth. Our data show that parasite protein and nucleic acid synthesis were inhibited by several protease inhibitors.

Figure 1 shows the effects of 3 protease inhibitors on the incorporation of isoleucine into protein of infected blood cells. Pepstatin, an inhibitor of acid proteases[8] inhibits the partially purified plasmodial protease by 50% at a concentration of 0.25 µg/ml ($4 \times 10^{-10}$ *M*)[1]. Chymostatin, which inhibits cathepsin B[9] is almost equally effective, whereas phenylmethane sulfonyl fluoride (PMSF) an inhibitor of serine proteases, completely inhibits the enzyme at about 1 m*M*[1]. Pepstatin and PMSF inhibit incorporation of label into protein by about 80 and 90%, respectively, at the concentrations tested. Chymostatin was less effective. Leupeptin, another inhibitor of the parasite protease[1,2] also inhibited incorporation of isoleucine into protein, but concentrations of 1 mg/ml or more were required for 50% inhibition (not shown).

Blood from normal and *Plasmodium berghei*-infected mice was collected using EDTA as anticoagulant. Samples were mixed with Trigg's medium (TM)[10] with the isoleucine concentration reduced by 50%, and centrifuged for 5 min at 250 × *g*. The supernatant fluid and buffy layer were removed, cells were suspended in TM, and layered over a solution of 10% (w/v) Dextran (MW 500,000) in saline. Cells were then centrifuged for 7 min at 400 × *g*, resuspended in TM to which inhibitors were added and recentrifuged. They were finally suspended in TM which contained the appropriate inhibitor. Duplicate aliquots of 0.4 ml were transferred to test tubes and were incubated with 0.05 µCi of isoleucine-$^{14}$C (70 mCi/mmole). At the times indicated, 50 µl samples were removed and pipetted onto filter paper discs, which were processed for scintillation counting[11]. The points shown are the average


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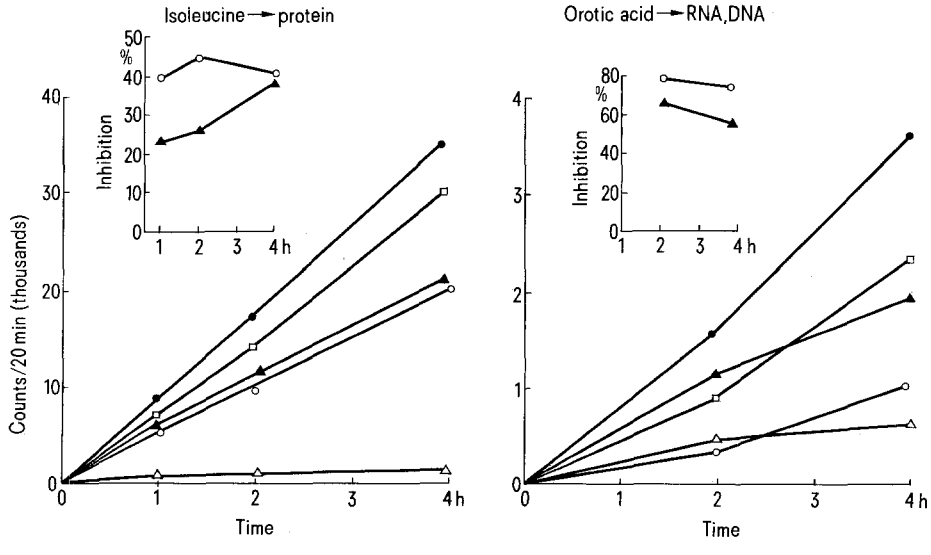


Fig. 2. Effects of pepstatin on incorporation of precursors into protein and nucleic acids. △, normal red cells; ●, infected red cells; □, infected + 12.5 µg/ml pepstatin; ▲, infected + 25 µg/ml pepstatin; ○, infected + 50 µg/ml pepstatin. The insert shows the inhibition when a mixture of 15 amino acids was added to the cells. Isoleucine and methionine are already present in the medium. ○, infected cells + 50 µg/ml pepstatin; ▲, same, but supplemented with amino acids.

of duplicate tubes. The parasitemia was about 4%, and the reticulocyte count was less than 0.5%. The washing procedure removes over 90% of the leukocytes.

Figure 2 shows the effects of pepstatin on the incorporation of precursors into protein and nucleic acids. In the latter case less than 10% of the incorporation is into DNA, as shown by selective removal of RNA, from the filter paper discs[12]. A dose dependent response was obtained. Protein synthesis was inhibited by about 40% and nucleic acid synthesis by 80% at the highest pepstatin concentration tested. Some variability in the sensitivity to the inhibitors was encountered in different experiments. We have not yet been able to account for this.

Cells were prepared as described for Figure 1. However, the adenosine content of the medium was reduced by 50% (to 5 μg/ml), the parasitemia was 11%, the volume of the incubation medium was reduced to 0.225 ml, and 0.075 μCi of isoleucine-$^{14}$C were added. For incorporation into nucleic acids, cells were incubated with 0.125 μCi of orotic acid-6-$^{14}$C (specific activity 25.2 mCi/mmole). In this experiment, counts in infected cells have been corrected for counts into an equivalent number of red cells.

If these inhibitors are indeed acting to prevent hemoglobin breakdown by the parasite, it might be possible to reverse the inhibition by supplying the cells with amino acids. Figure 2 (insert) shows the results of such an experiment. The addition of a mixture of amino acids partially reversed the inhibition due to pepstatin, at least during the first two hours. With leupeptin (not shown), a solution of either amino acids or proteose peptone partially reversed the inhibition.

Our data suggest that protease inhibitors may be considered as potential antimalarial agents. Thus, 4 compounds that inhibit the parasite protease also inhibited macromolecular synthesis in infected red cells. These findings are consistent with our working hypothesis[1,2] that agents that inhibit hemoglobin breakdown could inhibit parasite growth through deprivation of amino acids. The finding that this inhibition was partially overcome in the presence of amino acids or proteose peptone is consistent with this hypothesis. The marked inhibition of pepstatin on precursor incorporation into nucleic acid remains to be explained, as it is not clear if this is a direct effect, or if it is secondary to the inhibition of protein synthesis.

Our data are also consistent with results from experiments in which 'growth' of *P. knowlesi* was measured by following the increase in parasitemia in infected red cells over a 24 h period[7]. Pepstatin and chymostatin concentrations as low as 0.01 μg/ml markedly inhibited the increase in parasitemia. Leupeptin actually caused a decrease in the parasitemia, but much higher concentrations were required. It is not clear if the high concentrations needed for inhibition in the present study are due to a species difference or to a difference in the method for measuring 'growth'. However, the present findings indicate that it may be of value to test other inhibitors of proteolytic enzymes on growth of the malarial parasite.

*Zusammenfassung.* Nachweis, dass diverse Hemmstoffe der proteolytischen Enzyme den Einbau ihrer Vorgänger in Protein und RNS von *Plasmodium berghei* bei in vitro-Kultur in Mäuse-Erythrozyten verhindern, eine Hemmung, die teilweise durch Aminosäuren rückgängig gemacht wird. Diese Daten sprechen für einen Aminosäure-Entzug durch die Proteinase-Hemmstoffe im Parasiten, wobei der Hämoglobinabbau blockiert wird.

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## Structure Dependent Catalytic Effect of Cupric Ion on the Hydrolysis of Cephalosporins

All natural and semisynthetic penicillins are catalytically hydrolyzed by cupric ion to the corresponding microbiologically inactive penicilloic acids or a complex of penicilloyl-copper ion[1-4]. We have found that cephalosporins, the structural relatives of penicillins, have a structure-dependent sensitivity to the hydrolysing action of cupric ion.

We have examined a large number of semisynthetic cephalosporins of diverse chemical composition for their sensitivity to the hydrolysing effect of cupric ion in aqueous solution. For convenience of assay a disc-agar diffusion method with *B. subtilis* ATCC 6633 as the test organism and a fixed concentration of cephalosporin (4 μg/ml) was used. For compounds with poorer intrinsic activities a test concentration of 8 μg/ml was employed with the same general pattern of results. The activity of the compounds was determined: a) in deionized water; b) in the presence of 1 μg/ml $CuSO_4$ (the average amount in human serum); and c) in a mixture containing 1 μg/ml $CuSO_4$ together with 5 μg/ml of D-penicillamine (Cuprimine). These solutions were kept at 37°C and samples were assayed microbiologically at 0, 3, 5 and 24 h. The results obtained with several known or commercially available cephalosporins are presented in Table I. It can be seen from this table that the activity of cephalosporins containing a phenylglycine moiety (cephaloglycin and to a lesser degree cephalexin and cephradine) is progressively lost in the presence of cupric ion. This degrading effect of cupric ion can be inhibited by D-penicillamine (Cuprimine), a highly specific copper chelating agent.

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