(5–10 min)[15]. After washing in 0.1 *M* cacodylate buffer, the cells were resuspended in a stabilizing protoplast medium (18 ml 0.55 *M* *D*-mannitol; 1.5 ml McIlvain's citrate phosphate buffer pH 5.6; 0.75 ml $MgSO_4$ $7H_2O$)[3]. Purified β-(1→3) glucanase (80–100 units) was added to 1 ml of cells suspended in the protoplast medium and protoplast formation was followed by phase contrast microscopy.

A polar mode of protoplast formation, suggesting a possible concentration of the β-(1→3) glucan can be observed in the first sequence of a budding cell of *W. fluorescens* (Figure 1). The cell wall has been broken open at the opposite pole to that at which bud formation takes place. As no septum has yet been formed between mother cell and bud, the cytoplasm of both mother cell and bud are incorporated into the forming protoplast. The different stages of protoplast formation can be clearly seen (Figure 1, a–h). It is interesting to note that the cell wall is almost intact at the end of protoplast formation, suggesting that degradation proceeds slowly in the protoplast medium. The fully released spherical protoplasts were obtained after 5–10 min (Figure 1, h).

In the second sequence (Figure 2), which shows a budding cell of *S. cerevisiae*, the protoplast is formed from the cytoplasm of the bud, as septum formation between bud and mother cell is already complete. In this case protoplast formation is nonpolar, which might indicate a random distribution of β-(1→3) glucan in the cell wall. The different stages of protoplast formation are shown in Figure 2, a–h. The fully released protoplast (h) is again spherical and the bud retained its original shape throughout the sequence of protoplast formation, which lasted for three minutes.

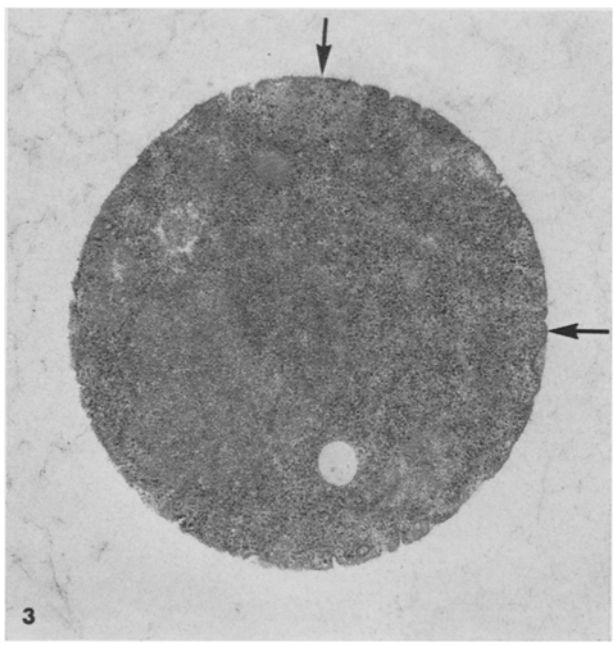

Fig. 3. Electron micrograph of a true protoplast of *Saccharomyces cerevisiae*. No adhering cell wall material is present. The arrows indicate regions where the unit membrane structure of the plasmalemma is clearly revealed. ×24,500.

The protoplasts released using β-(1→3) glucanase were examined by electron microscopy to check whether any wall material still adhered to them. Figure 3 shows a true *S. cerevisiae* protoplast obtained as previously described. The arrows indicate particularly well revealed sections of the cytoplasmic membrane, the whole of which is shown to be entirely free of adhering cell wall fragments. The β-(1→3) glucanase is thus capable of producing true protoplasts from yeast cells, even after they have been prefixed in glutaraldehyde.

Discussion. The application of the extracellular exo-β-(1→3) glucanase from *Basidiomycete* QM 806, to the analysis of yeast cell walls and the formation of yeast protoplasts has been demonstrated. The fact that this enzyme, in a purified state, entirely dissolved yeast cell walls made it an ideal tool for analysis of the carbohydrates, as it caused changes only in its specific substrate (β-1→3 glucan). In addition it gave information on the amount of this substrate present in the walls. The endo-β-(1→3) glucanases of *Bacillus circulans*[16] and *Cytophaga johnsonii*[17] were not able to cause complete solubilization of the cell walls of *S. cerevisiae* as did the *Basidiomycete* enzyme. Nor were these endoglucanases so effective in producing protoplasts from *S. cerevisiae*.

The methods described using *Basidiomycete QM 806* β-(1→3) glucanase would be useful in a comparative study of the cell wall composition and protoplast formation in different yeasts, or in a study of the effect of growth conditions and age on cell wall composition and protoplast formation. In view of the purity of the protoplasts obtained with this enzyme, it should also prove useful for studies on the protoplasts themselves.

Résumé. L'exo-β-(1→3) glucanase, isolée du *Basidiomycete* QM 806 a été utilisée avec succès pour l'analyse des polysaccharides de la paroi cellulaire de la levure *Saccharomyces cerevisiae* et pour la formation de protoplastes à partir de *Saccharomyces cerevisiae* et *Wickerhamia fluorescens*.

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Inhibition of Mammalian Acetylcholinesterase by Phenylmethanesulfonyl Fluoride[1]

The sulfonyl fluorides are powerful irreversible inactivators of many esterases[2–6]. The mechanism of their action has been well established in the case of acetylcholinesterase (acetylcholine acetyl-hydrolase, E.C. 3.1.1.7), using methanesulfonyl fluoride as inhibitor[4]. The first step is the formation of an enzyme-inhibitor complex, followed by a nucleophilic attack on the sulfur atom by the basic group of the esteratic site. The final acceptor of

the sulfonyl group, in analogy to what occurs with chymotrypsin[7], is probably the serine hydroxyl of the active site. Among this group of compounds, phenylmethanesulfonyl fluoride (PMSF) has become prominent because it inhibits the esterase activity of trypsin and chymotrypsin at low concentrations, but has no effect, even at a concentration of 10^{-3} *M*, on the acetylcholinesterase obtained from the main electric organ of *Electrophorus electricus*[3].

In a more extensive study of the properties of rat brain acetylcholinesterase and cholinesterase (acylcholine acylhydrolase, E.C. 3.1.1.8), we have found that phenylmethanesulfonyl fluoride is a potent inhibitor of the former, but not of the latter. We now report these findings, as well as a discussion of their meaning in relation to the conformation of the active site of acetylcholinesterases obtained from different sources.

Materials and methods. Enzyme preparations. Acetylcholinesterase and cholinesterase were partially purified from whole brains of adult Sprague-Dawley rats, by the procedure of CLOUET and WAELSCH[8], up to the deoxycholate stage, but not including it.

Enzyme assays. These were based on the measurement of the acid liberated by the enzyme, using acetylbetamethylcholine or butyrylcholine as specific substrates for acetylcholinesterase and cholinesterase, respectively. The acid liberated was recorded continuously by means of an automatic recording pH-stat (Radiometer SBR 2 Titrigraph). The assay solution was placed in the reaction vessel of the pH-stat kept at 37°C, and had the following final composition, in a volume of 3 ml: NaCl 0.1 *M*, $MgCl_2$ 1 m*M*, *Tris* HCl (pH 7.5) 10^{-7} *M*, acetylbetamethylcholine or butyrylcholine, 3 m*M*. After 10 min incubation the reaction was started by adding 630 µg of enzyme protein in a volume of 0.1 ml. CO_2-free nitrogen was bubbled continuously through the solution in order to avoid atmospheric CO_2 entering the reaction vessel. A blank, from which enzyme was omitted, was run with each set of experiments. Under these conditions, reaction rates were linear for at least 40 min. When PMSF was used, it was prepared as a fresh solution in 10% isopropanol. The enzyme preparation was incubated with it at the concentrations indicated in the Figure, at 37°C for exactly 15 min, and then added to the reaction mixture, where the inhibitor was diluted 300-fold. This lowered the inhibitor concentration to a level of inactivity. Control enzyme preparations were incubated in the same way with equivalent concentrations of isopropanol.

Materials. Acetylbetamethylcholine chloride and butyrylcholine chloride or iodide were from Sigma Chemical Co. Phenylmethanesulfonyl fluoride was a pure product from Mann Research. Its m.p. (uncorrected) was 91–92°C, the same as reported by FAHRNEY and GOLD[3].

Results and discussion. Acetylcholinesterase activity was measured after incubating the enzyme preparation with PMSF in the concentration range 0 to 9×10^{-4} *M* (Figure). 50% inhibition is obtained with 4×10^{-5} *M*, and with 9×10^{-4} *M* more than 90% of the enzyme has been inhibited. This was after allowing the enzyme-inhibitor reaction to proceed for only 15 min. Longer reaction times will lead to essentially complete inhibition. The second-order rate constant for the enzyme-inhibitor reaction, obtained by the procedure of FAHRNEY and GOLD[3], was 4.7×10^2 ($1\ mole^{-1}\ min^{-1}$). This is comparable to the reactivity of trypsin with diisopropylphosphorofluoridate and with PMSF (ca. 3×10^2 $1\ mole^{-1}\ min^{-1}$ for both of them)[3], and is at least 4 orders of magnitude higher than the rate constant for the reaction between the acetylcholinesterase of *E. electricus* and phenylmethanesulfonyl fluoride[3]. This marked difference in reactivity between the acetylcholinesterases of rat brain and *E. electricus'* electric organ indicates that the conformation of the active site is somewhat different in them, and that extrapolation from one to the other must be done with caution. FAHRNEY and GOLD[3] had already noted that the acetylcholinesterase of *E. electricus* was about one order of magnitude less reactive than the one present in crude homogenates of rat brain[2], towards methane and benzenesulfonyl fluorides. They attributed the difference to the use of a crude homogenate for the mammalian enzyme. The present results suggest that these differences were due to the enzyme per se.

Cholinesterase, on the other hand, is completely insensitive to phenylmethanesulfonyl fluoride in the concentration range studied (Figure). This is an indication that phenylmethanesulfonyl fluoride is not a non-specific sulfonylating agent that may lead to protein denaturation. A similar conclusion has been reached with other enzymes[7]. The large differences in reactivity towards this inhibitor between the two enzymes studied by us, indicate that PMSF is for them a valuable differential inhibitor.

Resumen. El fenilmetano sulfonil fluoro (FMSF) 4×10^{-5} *M* inhibe a la acetilcolinesterasa (E.C. 3.1.1.7) de cerebro de rata en más de 50%, y 9×10^{-4} *M* más de 90%. La constante de reacción inhibidor enzima es de 4.7×10^2 ($1\ mol^{-1}\ min^{-1}$). La colinesterasa (E.C. 3.1.1.8) de cerebro de rata es insensible a FMSF.

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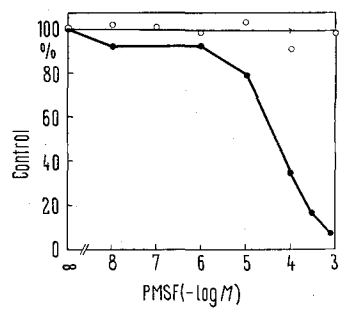

Acetylcholinesterase and cholinesterase activities at different PMSF concentrations. ●—●, acetylcholinesterase; ○—○, cholinesterase. Reaction rates were measured with acetylbetamethylcholine and butyrylcholine, respectively, in an automatic recording pH-stat as indicated under Methods. In the absence of inhibitor, the rate for acetylcholinesterase was 70 nmoles of substrate hydrolyzed per min, and for cholinesterase 12 nmoles/min. Each point represents 3 independent measurements.

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