Klingmuller, W. and H. G. Truper. Determination of hexokinose ond other enzymes which possibly phosphorylate fructose in Neurospora crassa.

In an effort to characterize mutants resistant to sorbose-toxicity, we have checked the first steps of fructose utilization for differences between wild type and mutants. Utilization is initiated by phosphorylation, which in the case of fructose could be achieved by three different enzymes: hexokinase (ATP: D-hexose 6-phosphotransferase, E.C.No. 2.7. 1. 1), fructokinase (ATP: D-fructose 6-phosphotransferase, E.C.No. 2.7.1.4) and ketohexokinase (ATP: D-fructose 1-phosphotransferase, E.C.No. 2.7. 1 .3).

A method for measurement of kinases is that described by Sherman (1962 Analyt. Biochem. 5: 548) which takes advantage of the adsoption characteristics of phosphorylated and unphosphorylated sugars on ion exchange paper. Since this method does not permit differentiation-between the three different kinases mentioned, it was discontinued after some preliminary investigation. A useful technique permitting such differentiation, at least to a certain extent, is the application of combined optical enzyme tests (B. D. Sanwal and H. G. Schlegel, personal communications). The use of these enzyme techniques for the above measurements of kinases in Neurospora crassa are described in this communication.

Reparation of crude extracts: Mycelia were grown in Fries-minimal solution with 1% filter-sterilized fructose (3 x 10^4 conidio of wild type 74-OR23-1A per 100 ml solution in 200 ml Erlenmeyer flask. on a shaking machine at 25°C). Mycelia were harvested at 3-6 days, washed twice with distilled water, pressed slightly to remove excess water, and taken up in 0.05 M triethanolamine HCl/NaOH buffer of pH 7.6 (ca. 40 mg dry weight/ml buffer).

Good enzyme activities were obtained by grinding the mycelia with quartz sand in an ice both, but minute pieces of sand and mortar debris, produced during the grinding process, remained in the crude extract after low speed centrifugation and interfered with subsequent optical measurements. High speed centrifugation is not feasible, since kinases have been reported in soluble and particle-bound forms (Medina and Sols 1956 Biochim. Biophys. Acta 19: 378). This complication was avoided, and good consistent results were obtained by disrupting mycelia by means of a Hughes press (Hughes 1951 Brit. J. Exp. Pathol. 32: 97) at -40°C. In this case low speed centrifugation (0°C, 4,000 x g, 30 min.) of the broken mycelial moss gave a clear, slightly opalescent supernatant , containing IO-20 mg protein/ml, depending on the buffer volume used.

Protein was measured by the biuret method, comparing the readings with a calibration curve obtained for bovine serum albumin. Since several shortcuts of the biuret method are in use, it should be mentioned that the protein of the samples to be measured has to be precipitated with 3.0 M trichloroacetic acid. Triethanolamine HCl/NaOH buffer, recommended for kinases and applied here, produces a strong blue color itself with the reagent and interferes with the measurements. If such care is taken, protein contents from 5-25 mg/ml con be measured with reasonable precision.

Enzyme measurements:

Phosphorylation of fructose to fructose-6-phosphate was measured according to the Baehringer instructions for hexokinase (Gottschalk 1964 Arch. Mikrobiol. 49: 96) in the following coupled enzyme test:

fructose + ATP $\xrightarrow{\text{kinase}}$ fructose-6-phos$_p$hate + ADP.

fructose-6-phosphate $\xrightarrow{\text{phosphoglucoisomerase}}$ glucose-6-phosphate.

glucose-6-phosphate + NADP $\xrightarrow{\text{glucose-6-phosphate dehydrogenase}}$ 6-phosphogluconate + $NADPH_2$.

The Eppendorf photometer connected to an automatic recorder was found useful for tracing the increase in adsorption of $NADPH_2$ at 366 mμ against time. By use of a cell holder with temperature control adjustment, connected to an ultrathermostat, it could be demonstrated that the reaction rate at 25°C is higher than that at 20°C, but equals that at 30°C. Crude extracts may be stored at 4°C for several days without loss of activity. Also storage of mycelia at -20°C does not influence the activity of crude extracts.

Three and six day old wild type mycelia exhibited activities of 114.0 ± 2.6 μ moles/min/g protein and 144.0 ± 8.8 μmoles/min/g protein, respectively. Two sorbose-resistant mutants (sor^r A1 and sor^r B57), mapping in separate linkage groups, gave similar results. Thus their resistance cannot be explained by an alteration of their hexokinose and/or fructokinase activities.

Reaction rates in crude extracts with fructose and glucose as substrates were equal. At first sight this seems to indicate that the hexokinase produced during growth of mycelia in fructose media has equal affinity for both hexoses, which is in contrast to other reports on hexokinase activity in glucose-grown mycelia (Medina and Nicholas 1957 Biochem. J. 66: 573; Sols et al. 1960 Biochem. Biophys. Res. Commun. 2: 126). It should be kept in mind, however, that in our case besides hexokinase a specific fructokinase could be at work as demonstrated in other organisms. The latter enzyme, together with a hexokinase of high glucose affinity and low fructose affinity, could well effect results as indicated. Only purification (and separation) of the respective enzyme(s) would permit a decision in favor of one or the other possibility. This task was not in the scope of our present research.

silky sheen is usually observed when a suspension of the crystals is agitated; the silky appearance is usually not present on initial crystallization.

It is apparent that an enormous number of variations is possible in carrying out the described procedure. It is therefore of interest that each of the twelve systems with which we hove tried this method has allowed crystallization without recourse to changer in pH, temperature or other conditions except for the inclusion of a mercaptan where warranted. Our experience at this time includes dehydrogenases, decarboxylases, transferases and protein hormones and involves proteins usually sensitive to room temperature, proteins with high ond low polysaccharide content and complexes of more than one protein. The molecular weight range has been between 30,000 and 3,500,000. The only requirement for crystallization appears to be that the preparation is at a stage of purity where the enzyme comprises more than 30% of the total protein. - - - National Instituter of Health, Bethesda, Maryland.