to undertake purification on a large scale. I would appreciate hearing from other Neurosporologists if they have any suggestions in this regard.

Preliminary kinetic experiments conducted with partially purified preparations of PFK indicate that the enzyme is inhibited both by fructose-6-phosphate and ATP at higher concentrations. At least in some respects the Neurospora PFK appears to be similar to that of other microorganisms. = = = Department of Biology, University of Calgary, Calgary, Alberta, Canada.

Kapoor, M. and D. Bray. Purification procedure for glutamine synthetase. Neurospora cress strain pe Y8743m (FGSC#37) was used as a source of glutamine synthetase. Lyophilized mycelial powders were prepared as described for phosphofructokinase above. Extraction and purification were carried out at 3°C in a cold room. Twenty-five grams of the powder was extracted with 50 ml of 0.05 M phosphate buffer (5 x 10^-4 M in EDTA and 10^-4 M in β-mercaptoethanol), pH 7.5 for 30 min. The mixture was strained through four layers of cheese-cloth and the supernatant was centrifuged at 15,000 rpm for 15 min in a Sorvall RC-2 refrigerated centrifuge. The supernatant from this step was treated with a saturated solution of ammonium sulfate and the precipitate formed was dissolved in the original buffer and further fractionated by adsorption on alumina Cy gel, as described in a recent publication (Kapoor and Bray 1968 Biochemistry 7:3583). Elution from the gel was carried out according to our published procedure with the modification that the elution buffer was made 3 x 10^-3 M with regard to MgCl2. The addition of Mg++ leads to a much more efficient elution and in addition to stabilization of the enzyme. The gel eluate can be stored in the form of lyophilized powder for several weeks without any loss of activity. The powder is dissolved in a small quantity of water and passed through a column of Sephadex G25 equilibrated against the original buffer containing Mg++. The enzyme obtained at this stage shows an increase of 20-fold in specific activity over that of the crude extracts.

In the next step, the enzyme preparation is purified by ion exchange chromatography on a DEAE-sephadex column (2.5 x 31 cm) equilibrated with 0.02 M phosphate buffer with β-mercaptoethanol 5 x 10^-4 M, EDTA 5 x 10^-4 M, ribitol 0.1 M, MgCl2 10^-3 M (pH 7.7). Approximately 60 mg of protein is applied on the column and washed down with buffer. Elution is then carried out by means of a linear gradient of 0-0.6 M NaCl prepared in the above-mentioned buffer. Five ml fractions are collected with an effluent flow rate of 1.0 ml per minute. The enzyme appears towards the end of the gradient, preceded by a major non-enzymatic protein peak. Without ribitol and Mg++ in the eluting buffer, all enzyme activity is lost within a few hours of collection of the fractions, but ribitol stabilizes the enzyme considerably. The fractions containing enzyme activity are pooled and concentrated by ultrafiltration, using Amicon Diaflo Ultrafilter with XM-50 membrane. This gives an enzyme preparation with an increase in specific activity of ca. 100 to 150-fold over that of the crude extract. The peak fractions show a much higher specific activity but, again, a loss occurs during concentration of the enzyme preparation. The addition of sorbitol has proved very useful in this case also. = = = Department of Biology, University of Calgary, Calgary, Alberta, Canada.


Cellulose has been intensively studied in a wide range of organisms (Norkram 1967 Adv. Appl. Microbiol. 9: 91), and has recently been investigated in Neurospora from both an industrial (Kuroda 1968 Chem. Abs. 68: 18955d) and a theoretical standpoint (Meyers and Eberhard 1966 Biochem. Biophys. Res. Commun. 24: 782). Although there have been attempts to rationalize the cellulase assay (β-1,4-glucanase = Cx) on an absolute basis (bond broken per second = Almin and Eriksson 1968 Arch.Biochem. Biophys. 124: 129) there is a lack of standardization between published methods. For example, carboxymethyl cellulose (CMC) has been used in several different states each with various degrees of substitution (DS) and of polymerization (DP), which control the rate of the reaction. Its ionic character limits its use in viscometric assays, as the viscosity is dependent on pH, ionic strength and polyvalent cation content of the assay medium. There are limitations that are magnified in the more enzymically reactive, lower substituted CMC’s. Glycol cellulose (hydroxy ethyl cellulose = HE C) has been proposed to replace CMC in order to circumvent these difficulties (Iwasa et al. 1964 J. Biochem. (Tokyo) 55: 30) but has been rarely used, presumably because the authors noted the laborious procedure of making this non-ionic substituted cellulose. A rigorously controlled range of HE C’s is available commercially and this allows the facile development of more standardized reductometric and viscometric cellulose assays. For this purpose we have used the readily soluble Natrosol 250 M H EC (4,500-6,500 centipoises at 20°C, mean substitution 2.5, DS ca. 1.0, DP 565 = Hercules Powder Co., Wilmington, Delaware). Equivalent HE C’s are available from Farbwerke Hoechst A. G., Frankfurt, Germany (Tylose H 4000) and Union Carbide Corp., New York (Cellosolve M 4400H). Viscometric assays were carried out using a Cannon-Fenske Viscometer (No. 200: efflux time of solvent 10.2 sec.) at 25°C with a reaction mixture of 7 ml 0.44% HEC (250 M Natrosol), 1 ml 0.5 M sodium acetate buffer pH 4.8 and 2 ml enzyme. The substrate was dissolved in water by shaking overnight at room temperature. Blending to aid the rapid solution of the substrate for at least five seconds gave a much reduced viscosity level. Reductionometric analyses were performed using equivalent reaction mixture, reducing end groups being estimated by the ferricyanide method modification proposed by Pork and Johnson (1949 J. Biol. Chem. 181: 149). The colorimetric Somogyi-Nelson method could not be used as it caused precipitation of the substrate and concomitant absorption of the colored complex. Tritimetric analyses of the oligosaccharides by the Somogyi method or by direct reduction with alkaline iodine proved practical but tedious. Enzyme units are defined: one reductometric unit is that amount of enzyme which produces 1% degradation in 1 minute, under the above conditions, while a viscometric unit is that amount of enzyme which causes a change of Δf (specific fluidity) of 0.15 in 10 minutes when incubated under the above conditions. Apparent zero order kinetics were maintained up to a change of Δf of 0.10. It is important to note that substituted celluloses are degraded at a changing rate...
A comparison has been made of the levels of C\textsubscript{x} cellulose produced by Neopspora crassa STA4 (FGSC 262) and N. crassa ATCC 10355. The organisms were grown in Vogel's mineral medium N containing 0.5% glucose plus 1% "cellulase" (750 ml/2 l flask, 30°C with shaking). Celluloses used included: HEC (Natrosol 250 M), CMC (CellulosBio Inc., Ltd., England) and Avicel (Microcrystalline cellulose, FMC Corp., Marcus Hook, Pennsylvania). Relative small amounts of enzyme were produced under these conditions (Table 1). For example, a crude commercial cellulase (Trichoderma viride) has a specific activity of 20.8 (viscometric). More enzyme was excreted into the medium than retained intracellularly, but intracellular levels were measurable with these techniques. CMC proved a better inducer of cellulase than the other two substrates. Attempts to release additional bound cellulase by further incubation of the harvested mycelium or mycelial homogenates at pH 7.5 (P04) 16 hours (3°C) proved successful.

Table 1. The induction of cellulose by "celluloses" in two strains of Neopspora crassa (viscometric units).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Extracellular</th>
<th>Intracellular</th>
<th>Extracellular</th>
<th>Intracellular</th>
<th>Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µ/ml SpAct</td>
<td>µ/ml SpAct</td>
<td>µ/ml SpAct</td>
<td>µ/ml SpAct</td>
<td>µ/ml SpAct</td>
</tr>
<tr>
<td>HEC</td>
<td>0.14 0.72</td>
<td>0.30 0.21</td>
<td>0.28 2.55</td>
<td>0.12 0.06</td>
<td>0.17 1.70</td>
</tr>
<tr>
<td>CMC</td>
<td>0.68 4.86</td>
<td>0.11 0.06</td>
<td>1.20 14.20</td>
<td>0.64 0.42</td>
<td>1.46 13.2</td>
</tr>
<tr>
<td>AVICEL</td>
<td>0</td>
<td>0</td>
<td>0.10 1.25</td>
<td>0.01 0.18</td>
<td>0.42 4.2</td>
</tr>
</tbody>
</table>


cassady, W. E. and R. P. Wagner. An assay method for kynurenine-3-hydroxylase: enzyme marker for the outer membrane of mitochondria. Recently Cassidy and Wagner (1968 Genetics 60: 168) and Cassidy (1960 Ph. D. Thesis, University of Texas at Austin) have shown that the enzyme L-kynurenine-3-hydroxylase (KH) (EC 1.14.1.2) is localized on the outside membrane of Neurospora mitochondria. It was also found that KH is only present in the mitochondria. KH had previously been shown by Okamoto et al. (1967 Biochem. Biophys. Res. Commun. 26: 309), Schnaitman and Greenawalt (1968 J. Cell Biol. 38: 158) and Beattie (1968 Biochem. Biophys. Res. Commun. 31: 901) to be localized on the outer membrane of rat liver mitochondria. Okamoto et al. (1967) also showed that rat liver KH was an exclusive mitochondrial enzyme. The specific localization of this enzyme makes it a valuable research tool for workers studying Neurospora mitochondria. For this reason the array method used in our laboratory is presented below. Other methods used in separating Neurospora mitochondria into outer and inner membrane fractions will be detailed elsewhere.

KH activity was assayed by determining the actual production of 3-hydroxykynurenine using the method of Ghosh and Forrest (1967 Genetics 55: 423) with minor modifications. Reaction mixtures in 25 ml Erlenmeyer flasks were composed of the following reagents in order: potassium cyanide 10 µmoles, phosphate buffer pH 7.5 200 µmoles, potassium chloride 20 µmoles, glucose-6-phosphate 100 µmoles, NADP 0.8 mg, glucose-6-phosphate dehydrogenase (Sigma type VI) from yeast) 0.2 unit, DL-kynurenine sulfate (Sigma) 2.4 mg, mitochondrial protein 1 to 4 mg, and water as needed to total volume of 2 ml.

Following addition of protein and water, reaction mixtures were incubated one hour at 30°C in a Water-Chilcot reciprocating water bath operating on setting 6. A substrate minus blank was run concurrently with each sample. Reactions were terminated by adding 0.5 ml 40% TCA. Substrate was added to the blanks following addition of TCA. Precipitated protein was removed by centrifugation at 3500 rpm for 15 min. The supernatant was carefully collected with a Pasteur pipette. A 0.5 ml sample of supernatant was transferred to a cuvette, acidified with 1.0 ml 0.1 N HCl, shaken, and the optical density at 400 µm determined with a Cary Model 14 recording spectrophotometer previously zeroed on a water blank. Next, 0.2 ml 0.25% sodium nitrite was added, the cuvette shaken, and the optical density at 400 µm determined again. Sodium nitrite at acid pH reacts with the 3-hydroxykynurenine forming a pale yellow diazo-oxide which absorbs at 400 µm. The increased absorbance at 400 µm following addition of sodium nitrite is a measure of the 3-hydroxykynurenine produced. The difference in absorbance at 400 µm between each sample and its blank is determined and the amount of 3-hydroxykynurenine produced in the reaction is read from a standard curve. Specific activity is calculated as follows:

\[
\frac{\text{µg 3-hydroxykynurenine produced/hour} \times \text{dilution factor}}{\text{mg protein in assay} \times 0.224} = \mu\text{moles 3-hydroxykynurenine produced/hour/mg protein}.
\]

The standard curve should be made using the incubation mixture, TCA and 3-hydroxykynurenine. The curve is linear between 1 and 50 µg 3-hydroxykynurenine. 3-Hydroxykynurenine can be obtained from Pierce Chemical Co. In all instances it should be verified as true 3-hydroxykynurenine by paper chromatography or other methods. It is especially important to determine the optical density at 400 µm immediately following the addition of sodium nitrite because the diazo-oxide formed is not stable. Determinations of optical density were done at 25°C using cuvettes with a 0.1 cm light path. (The assistance of Mrs. Dorothy Oliver is gratefully acknowledged. † † † Genetics Foundation, University of Texas at Austin, Austin, Texas 78712. (Present address of WEC † 306 Bly Rd, Eglin AFB, Florida 32542.)