Cassady, W. E. and R. P. Wagner. An assay method for kynurenine-3-hydroxylase: enzyme marker for the outer membrane of mitochondria.

Recently Cassady and Wagner (1968 Genetics 60: 168) and Cassady (1960 Ph. D. Thesis, University of Texas at Austin) hove shown that the enzyme L-kynurenine-3-hydroxylase (KH) (EC 1. 14. 1.2) is localized on the outer membrane of Neurospora mitochondria. It was also found that KH is onlv present in the mitochondria. KH had previously been shown by Okamato et al. (1967 Biochem. Biophyr. Res. Comm. 26:309), Schnaitman and Greenawalt (1968 J. Cell Biol. 38: 158) and Beattie (1968 Biochem. Biophyr. Res. Commun. 31:901) to be localized on the outer membrane of rat liver mitochondria. Okamato et al. (1967) also observed 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 added in order: potassium cyanide 10 µ moles, phosphate buffer pH 7.5 200 µmoles, potassium chloride 20 µmoles, glucore-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 too total volume of 2 ml.

Following addition of protein and water, reaction mixtures were incubated one hour at 30°C in a Warner-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 .O ml 0. 1 N HCl, shaken and the optical density at 400 mµ 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 mµ determined again. Sodium nitrite at acid pH reacts with the 3-hydroxykynurenine farming a pale yellow diazo-oxide which absorbs at 400 mµ. The increased absorbance at 400 mµ following addition of sodium nitrite is a measure of the 3-hydroxykynurenine produced. The difference in absorbance at 400 mµ 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:

$$\frac{(\text{µg 3-hydroxykynurenine produced/hour})\ (\text{dilution factor})}{(\text{mg protein in assay})\ (0.224)} = \text{mµmoles 3-hydroxykynurenine produced/hour/mg protein.}$$

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 mµ immediately following the addition of sodium nitrite because the diozo-oxide formed is not stable. Determinations of optical density were done at 25°C using cuvettes with a 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 Eley Rwd, Eglin AFB, Florida 32542).