S.P. Colowick and N.O. Kaplan (edS).
Methods of Enzymology 60, Academic Press, N.Y. 1955

[17] Enzymatic Degradation of Cytochrome C

$$\text{Cytochrome c } (Fe^{+++}) + H_2O \rightarrow \text{Hematin peptide } (Fe^{+++}) + \text{Amino acids} \quad (1)$$

$$\text{Succinate} + \text{Hematin peptide } (Fe^{+++}) \rightarrow \text{Fumarate} + \text{Hematin peptide } (Fe^{++}) \quad (2)$$

$$O_2 + \text{Hematin peptide } (Fe^{++}) \rightarrow \text{Verdohemochrome peptide} \quad (3)$$

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Assay Method

Principle. The over-all reaction produces a visible color change from red to green.[1] The green compound has absorption bands at 655 mμ, 530 mμ, and 410 mμ. The course of the primary reaction which hydrolyzes about 80% of the cytochrome peptide bonds can be observed by means of quantitative ninhydrin analyses.[2] The second, oxidative step, which requires reduction of the iron (in this case by means of succinic dehydrogenase and succinate) can be followed spectrophotometrically, fluorometrically, or manometrically. The first method, based on the large decrease of the optical density at 410 mμ during the course of the reaction, is described below.

Reagents

Buffers. 0.05 *M* phosphate, pH 6.9, containing 75 g./l. of mannitol. 0.1 *M* Tris-HCl, pH 8.7.

Sodium succinate, 2.5×10^{-2} *M* in buffer.

Cytochrome c (Sigma), 5 mg./ml. in buffer (2.5×10^{-4} *M*).

Enzyme. Mitochondria from young cultures of the *poky* strain of *Neurospora* as described below.

Procedure. Flask A containing 0.8 ml. each of cytochrome, succinate, and enzyme plus 2.0 ml. of pH 6.9 buffer, and flask B containing 0.8 ml. each of cytochrome and enzyme plus 2.8 ml. of pH 6.9 buffer are incubated with shaking at 35°. At the time of mixing and at 10-minute intervals, 0.5 ml. samples are removed and pipetted into a mixture of 1.0 ml. of pH 6.9 buffer and 1.5 ml. of pH 8.7 buffer. The pH of the resulting mixture is 8.3. The optical density at 410 mμ of the A series is measured in a

[1] F. A. Haskins, A. Tissieres, H. K. Mitchell, and M. B. Mitchell, *J. Biol. Chem.* **200**, 819 (1953).

[2] W. Troll and R. K. Cannan, *J. Biol. Chem.* **200**, 803 (1953).

Beckman DU spectrophotometer using the B series as blanks for zeroing the instrument.

Purification Procedure

The cytochrome-destroying system described here is found in the mitochondria of the *poky* strain of *Neurospora* but not in those from the wild-type strain. The mitochondria are prepared by centrifugation in essentially the same way as described for animal tissues by Hogeboom *et al.*[3]

Growth of the Mold. Inoculate 7 l. of Fries minimal medium[4] contained in a 9 l. bottle equipped with a tube and cotton filter for aeration with sterile air with the conidia from a 6 to 12 day old, 25 ml. agar-minimal culture of the *poky* strain of *Neurospora*. Aerate at 25° for 2 to 3 days to give about 15 g. wet weight of mold after filtering through paper or cheesecloth. Wash the mold twice with 50 parts of distilled water. Grind the mold in an ice-cold mortar with 0.5 part of sand and 3 parts of cold phosphate-mannitol buffer, pH 6.9. Centrifuge at 2000 × *g* for 3 minutes, and repeat the grinding and centrifugation with the residue. Centrifuge the combined supernatant solutions in a refrigerated centrifuge for 20 minutes at 30,000 × *g*. Resuspend, and wash the red pellet twice with 100 vol. of buffer. Suspend the particles in a volume of buffer equal to half the weight of the original moist mold.

Aging the Particles and Storage of the Preparation. With freshly prepared particles there is a lag of 2 to 4 hours in the cytochrome destruction reactions, and this is reduced to 5 to 30 minutes by storing the particle suspension at 8° for 2 to 4 days. The suspension is then frozen quickly in dry ice and Cellosolve and stored at −20° for use over a period of at least 30 days. Thawed particles can be used directly or after centrifugation and resuspension in fresh buffer.

Properties

Specificity. Aged mitochondria from *poky* cause the release of amino acids from cytochrome c (beef or *Neurospora*) and from protamine or casein but not appreciably from egg albumin or hemoglobin. The aging process itself involves an extensive self-proteolysis which does not occur with mitochondria from wild-type *Neurospora*. It is not known whether more than one particle-attached proteolytic enzyme is involved, but the action on cytochrome c does produce a definite residual fragment which retains the absorption spectrum of the undigested cytochrome. At neutral

[3] G. H. Hogeboom, W. C. Schneider, and G. E. Palade, *J. Biol. Chem.* **172**, 619 (1948); see also Vol. I [3].

[4] G. W. Beadle and E. L. Tatum, *Am. J. Botany* **32**, 678 (1945).

pH values this residual fragment is either insoluble or is adsorbed by the particles.

The second stage of the reaction, the oxidation of the hematin peptide after reduction of the iron through succinic dehydrogenase, does not occur with hematin, hemoglobin, or catalase, although indirect evidence suggests that it does occur with cytochromes a and b. The isolated hematin peptide decomposes spontaneously in the presence of hydrosulfite and oxygen, and it is quite possible that its breakdown in the presence of succinate, succinic dehydrogenase, and oxygen is also spontaneous after reduction, analogous to the reactions described for pyridine hemochromogen.[5] However, ascorbate or glutathione do not serve as satisfactory reducing agents.

Inhibitors. The proteolytic action on cytochrome c is inhibited by an unknown water-soluble substance(s) present in extracts of *Neurospora*. It is also inhibited by protamine (0.05 mg./ml.). The oxidative step of the reaction is inhibited by malonate and fumarate when succinate is used for reduction, and by cyanide.

Effect of pH. The over-all reaction has a pH optimum between pH 6.8 and 7.0, and it does not occur appreciably below pH 6 or above pH 7.3.

[5] R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," p. 499, Interscience Publishers, New York, 1949.