FLUOROMETRIC DETERMINATION OF THE SUCCINATE DEHYDROGENASE CONTENT OF RESPIRATORY CHAIN PREPARATIONS*

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The solution of a number of problems of considerable current interest hinges on the availability of a reliable method for the determination of the succinate dehydrogenase content of enzyme samples. Thus values for the turnover number of this enzyme in the literature cover a wide range. One reason for this has been the unavailability of a proven method for expressing the concentration of the dehydrogenase in multienzyme preparations. Another instance is the chemical basis and physiological significance of the reactivation of the succinate oxidase system in alkali-treated heart muscle preparations on the addition of soluble succinate dehydrogenase (Keilin and King, 1958), which is discussed in the next paper (Singer et al., 1962).

The fortunate property of mammalian succinate dehydrogenase that its flavin is peptide-bonded (Kearney and Singer, 1955; Kearney, 1960) provides a basis for the determination of the concentration of the enzyme independently of its activity. Although analysis for flavin peptides liberated by proteolysis has been widely used for the determination of the succinate dehydrogenase content of purified preparations, there have been several difficulties in applying the method to crude samples, such as mitochondria. Thus proteolytic digestion of samples containing hemoproteins leads to the contamination of the digest with heme-peptides which absorb light in the spectral range where flavins are determined and are reduced by hydrosulfite. Fluorometric determination, based on the unusual pH-fluorescence properties of flavin peptides, circumvents this difficulty but the pH-fluorescence relations have been previously determined for only

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one flavin hexapeptide derived from highly purified heart succinate dehydrogenase (Kearney, 1960) and could not be assumed to be identical for the mixture of flavin peptides found in proteolytic digests of this enzyme. It is also uncertain whether succinate dehydrogenase is the only flavoenzyme present in animal tissues whose prosthetic group becomes acid-soluble only after proteolytic digestion. In fact, there is an indication in the literature that liver mitochondria may contain other flavoproteins endowed with this property (Frisell and Mackenzie, 1962).

One purpose of this note is to present conditions for the determination of peptide-bonded flavin in heart muscle preparations, which have been found to be reliable in extensive use in this laboratory, and to point out that the method may be used to calculate the succinate dehydrogenase content of such samples. An enzyme sample of known protein content is treated with 10 volumes of cold acetone and 0.08 volume of 6 N HCl in the cold for 5 minutes. This and subsequent manipulations are carried out in the dark. The residue collected by centrifugation is resuspended in the original volume of 0.1% TCA (trichloroacetic acid) and treated with 20 volumes of cold acetone. After centrifugation the residue is extracted with 2.5 x the original volume of 5.5% TCA; the resulting centrifuged residue is washed with the same volume of 0.1% TCA and then with water. The acid acetone step serves to remove the type of cytochrome which is apt to interfere with spectrophotometric determinations and the TCA extraction is needed to remove all acid-soluble flavin.

The residue obtained above is resuspended in a small convenient volume of 0.1 M Tris base and the pH is adjusted to 8.0. For each mg. protein originally present 0.05 mg. each of crystalline trypsin and chymotrypsin are added and the sample is incubated for 4 hours at 38° with occasional agitation, while the pH is maintained around 8. The sample is then cooled to 0°; 0.1 volume of 55% TCA are added; insoluble protein is removed by centrifugation, and the supernatant is incubated overnight at 38° in order to hydrolyze the flavin peptides to the mononucleotide level. The pH during hydrolysis is 0.3 to 0.4. Aliquots of the hydrolyzate are used for the fluorometric determination of the flavin content in glycine buffer, pH 3.2, as previously described (Kearney, 1960). After suitable correction for internal quenching with internal riboflavin standards the bound flavin content is calculated with reference to the fluorescence of riboflavin at pH 7.0.

The choice of 3.2 as the pH of the fluorescence determination is based on the observation that the total bound flavin in proteolytic digests of Keilin-Hartree preparations shows the same pH of maximum

fluorescence at the mononucleotide level as does the pure hexapeptide isolated by Kearney (1960) (Fig. 1). It should be noted that if the hydrolysis were omitted, a different pH-fluorescence curve would result.

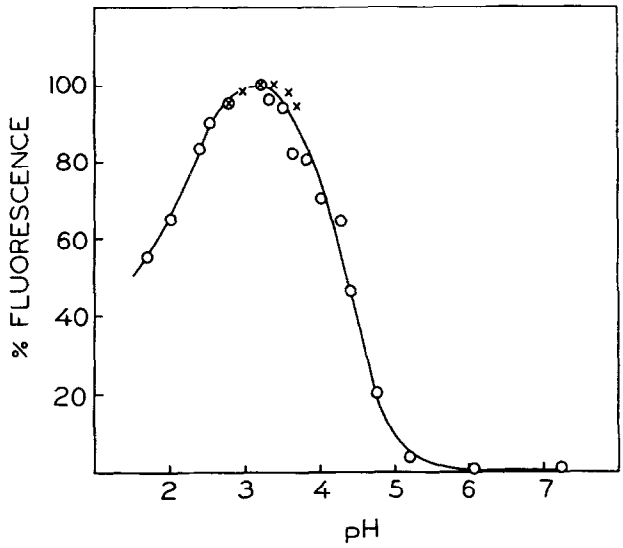

Fig. 1: Comparison of pH-fluorescence curves of a pure flavin hexapeptide derived from pure succinate dehydrogenase and of the mixture of flavin peptides present in proteolytic digests of beef heart Keilin-Hartree preparations. Solid line and open circles: data of Kearney (1960) for the hexapeptide; crosses, Keilin-Hartree preparation. The latter sample was prepared as described above except that the flavin mononucleotides were first adsorbed on a Florisil column and total flavin was eluted with 5% pyridine.

Comparison of the fluorometric and spectrophotometric (ΔD_{450}, hydrosulfite) determinations of bound flavin in several samples of beef heart mitochondria and Keilin-Hartree preparations gave, on the whole, satisfactory agreement, but the former appears to be more reliable and requires far less sample (0.03 mμmole or less against 10 mμmole for a single sample in the spectrophotometric method with conventional equipment).

The constancy of the turnover number of succinate dehydrogenase in different types of preparations from heart muscle, as illustrated in the next paper (Singer et al., 1962) and experience in this laboratory in the fractionation of mitochondria which indicates that bound flavin always follows succinate dehydrogenase activity, suggest that at least in mammalian heart, although not necessarily in other tissues, succinate dehydrogenase content may be calculated from bound flavin analysis.

As regards the application of the method, it may be pointed out that it presents the determination of an enzyme in samples approaching the physiologically intact state without the necessity of purification and possible attendant inactivation or reliance on activity determinations.

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