THE QUANTITATIVE ESTIMATION OF CYTOCHROME b IN SUB-MITOCHONDRIAL PARTICLES FROM BEEF HEART

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Quantitative estimation of the cytochrome b content of sub-mitochondrial particles by direct spectrophotometric analysis has previously been impractical because of uncertainties in absorbancy of the particle bound cytochrome. Although the absorbancy index for the isolated cytochrome has been determined (Goldberger, et al., 1961) the exact value for this cytochrome in sub units of the mitochondrial system has remained to be evaluated. Previous determinations of the amount of cytochrome b in particles have been based on an assumed absorbancy index (Chance, 1958). Hatefi, et al. (1962) obtained widely different results when comparing a direct spectrophotometric analysis with the differential heme analysis technique for cytochrome b estimation in QH_2-cytochrome c reductase.

In this communication a procedure is described for the spectrophotometric determination of cytochrome b in QH_2 - cytochrome c reductase by a technique which was also found applicable to preparations of the elementary particle (EP)** and the electron transport particle (ETP). It is likely that this method can be applied to other particles in which both cytochrome b and c_1 are present in a combined form, since it is evident that once cytochrome b is separated from cytochrome c_1 there is a decrease in its absorbancy.

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** A submitochondrial fraction containing all components of the electron transport chain except cytochrome c.

In a previous communication from this laboratory (Rieske and Zaugg, 1962) mention was made of a procedure used to cleave the QH_2 - cytochrome c reductase complex into its individual cytochrome components by exposure to bile salts and ammonium sulfate. A modification of this method resulted in a relatively rapid cleavage (to about 95%) with essentially complete recovery of the cytochromes. EP and ETP were likewise cleaved into 2 fractions by this method with cytochrome b being recovered in the insoluble precipitate and cytochromes a, c_1 and c (in ETP) in the supernatant liquid.

Cleavage was accomplished by exposure of the QH_2 - cytochrome c reductase (final concentration 7 - 30 mg protein/ml) at 0° first to sodium taurocholate (1 - 2 mg/mg protein) with subsequent addition of acetate buffer, pH 5.8 (final concentration 0.14 M), followed by the slow addition of saturated ammonium sulfate with constant stirring to a final concentration of 25 - 26%. The solution became slightly turbid with the addition of the final portions of ammonium sulfate. The mixture was then warmed to 30° and incubated for 40 minutes after which the precipitated cytochrome b was removed by centrifugation and washed with water. The washed precipitate was suspended in H_2O then dissolved by making the suspension 0.25 M in KOH and 3% cholate. The amount of cytochrome b in this solution was determined at 0° as the pyridine hemochromogen, using recrystallized hemin ($C_{34}H_{32}O_4N_4FeCl$) as a standard.

The supernatant liquid, containing cytochrome c_1 and a small amount of uncleaved cytochrome b, was cooled to 0° and the contents completely oxidized by addition of a small amount of ferricyanide. A few grains of solid potassium ascorbate were added to a sample of the oxidized supernatant solution and the cytochrome c_1 content determined by a difference spectrum using the value of 17.1 as a millimolar absorbancy index (reduced - oxidized at 554 mμ). This value was established by Green, et al. (1959) and can also be applied to the particle bound cytochrome. The remaining cytochrome b (generally about 5%) was determined by initially reducing the cytochrome c_1 in the supernatant liquid with ascorbate and subsequently obtaining a difference spectrum of a portion which had been reduced by dithionite. At the pH (5.8) and temperature (0°) used in these determinations ascorbate will not reduce a significant amount of cytochrome b

although cytochrome $\underline{c}_1$ becomes fully reduced in a very short time. A millimolar absorbancy index of 23.0 (reduced - oxidized) at the α peak was applied to the absorbancy value obtained for cytochrome $\underline{b}$. This value was established in the following manner:

Samples were withdrawn at varying time intervals during the 40 minute incubation period from a mixture of QH_2 - cytochrome $\underline{c}$ reductase undergoing cleavage (T = 1, 5, 10 and 15 min.). These samples were immediately diluted with water in order to stop the splitting reaction and then centrifuged. The cytochrome $\underline{b}$ content of the precipitates was determined as mentioned earlier. Absorbancy values of the cytochrome $\underline{b}$ remaining in the supernatant liquid were obtained by difference spectra for the dithionite reduced samples as outlined previously. Comparing the increases of cytochrome $\underline{b}$ content in the precipitates between T = 1 min. and T = 5, 10 and 15 min. with the corresponding decreases in absorbancy values of the supernatant liquid the value of 23.0 was obtained as a millimolar absorbancy index (reduced - oxidized at the α peak) for cytochrome $\underline{b}$ in the soluble particle under conditions of splitting.

The average of 20 determinations on the QH_2 - cytochrome $\underline{c}$ reductase complex gave the number 2.0 as a ratio of cytochrome $\underline{b}$ to cytochrome $\underline{c}_1$ with a maximum deviation of $\pm$ 5%. On this basis absorbancy indexes were calculated for cytochrome $\underline{b}$ from difference spectra of the intact QH_2 - cytochrome $\underline{c}$ reductase (Table I). The values given in the table for EP and ETP were obtained from

TABLE I

Absorbancy Indexes for Particle Bound Cytochrome $\underline{b}$ from Beef Heart

Sub-mitochondrial Preparation	Millimolar Absorbancy Index ($cm.^{-1}mM^{-1}$)
QH_2 - Cytochrome $\underline{c}$ reductase (reduced - oxidized, α peak)	23.4
QH_2 - Cytochrome $\underline{c}$ reductase (563 - 577 mμ)	28.5
EP (563 - 577 mμ)	28.5 $\pm$ .5
ETP (563 - 577 mμ)	28.5 $\pm$ .5

The values given apply to difference spectra of a dithionite reduced sample in which other cytochromes have previously been reduced with ascorbate (see Fig. 1), and were determined at 0° and pH 5.8.

experiments similar to those outlined above for QH_2 - cytochrome c reductase. Because of a shift downward in the difference spectrum with respect to the base line the value of 23.4 $cm.^{-1} mM^{-1}$ (reduced - oxidized at 563 mμ) gives estimations which are about 10% low for EP and ETP, therefore the millimolar absorbancy index of 28.5 (563 - 577 mμ) must be used. If cytochrome a is present in a particle the determination cannot be made until essentially all of the cytochrome a in the reference cell has been reduced by ascorbate, since the presence of more reduced cytochrome a in the sample cell than in the reference cell will prevent the trough at 577 mμ from reaching its minimum. A difference spectrum of cytochrome b similar to that in Fig. 1 should be obtained in order for the determined absorbancy indexes to apply.

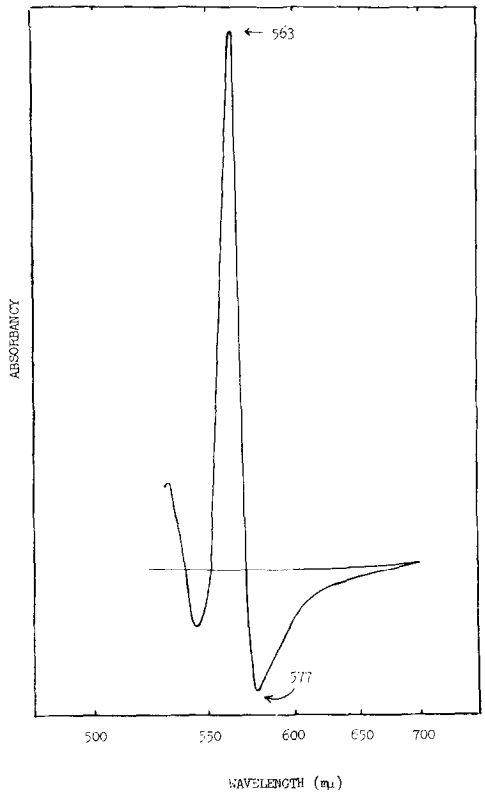

Fig. 1. A difference spectrum of cytochrome b from QH_2-cytochrome c reductase at pH 5.8 taken at 0°. The spectrum was obtained by addition of a few grains of dithionite to the sample cell containing a solution of the reductase which was previously reduced with ascorbate.

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References

Chance, B., J. Biol. Chem. 233, 1223 (1958).

Goldberger, R., Smith, A. L., Tisdale, H. D., and Bomstein, R., J. Biol. Chem., 236, 2788 (1961).

Green, D. E., Jarnefelt, J. and Tisdale, H. D., Biochim. Biophys. Acta, 31, 34 (1959).

Hatefi, Y., Haavik, A. G., and Griffiths, D. E., J. Biol. Chem., 237, 1681 (1962).

Rieske, J. S. and Zaugg, W. S., Biochem. Biophys. Res. Comm., in press (1962).