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The microestimation of succinate and the extinction coefficient of cytochrome *c*

Phenazine methosulphate has been shown to be a very good electron acceptor in the oxidation of succinate by succinic dehydrogenase[1], whereas cytochrome *c* is an extremely poor acceptor in this system. However, cytochrome *c* is reduced rapidly in a non-enzymic reaction with reduced phenazine methosulphate[2]. Hence the oxidation of succinate by succinic dehydrogenase can be coupled to the reduction of cytochrome *c* in the presence of trace amounts of phenazine methosulphate to give an extremely sensitive method for the estimation of succinate.

$$\text{succinate} + \text{phenazine methosulphate} \xleftarrow[\text{dehydrogenase}]{\text{succinic}} \text{fumarate} + \text{reduced phenazine methosulphate} \qquad (1)$$

$$\text{reduced phenazine methosulphate} + 2\ \text{cytochrome } c \longrightarrow \text{phenazine methosulphate} + 2\ \text{reduced cytochrome } c \qquad (2)$$

The optimal conditions for this estimation have not been explored fully, but the following conditions have been found satisfactory. Spectrophotometer cells are filled with the following reagents to a total volume of 2.95 ml; phosphate, pH 7.6, 150 μmoles; cytochrome *c*, 0.125 μmole; succinate, 0–0.05 μmole; succinic dehydrogenase prepared according to the method of SINGER *et al.*[1], 0.2 mg. The cells are incubated at 25° and the reaction begun by the addition of 0.05 ml of 0.01 % phenazine methosulphate (freshly diluted from a 1 % stock solution).

A blank without any succinate but containing all the other reagents must be run, since a red colour develops on the incubation of phenazine methosulphate with most proteins, due to a complex with sulphydryl groups[2]. The absorbance at 550 mμ is recorded until the difference between sample and blank becomes constant. Fig. 1 shows the results of a number of estimations carried out in this way. It will be noted that the amount of cytochrome *c* reduced is the same in the presence or absence of oxygen. As reduced phenazine methosulphate is very auto-oxidizable (in fact its reaction with O_2 is the basis of the usual assay for succinic dehydrogenase[1]) it is evident that the rate of reaction of reduced phenazine methosulphate with cytochrome *c* must be very much greater than that of its re-oxidation with O_2. As cyto-

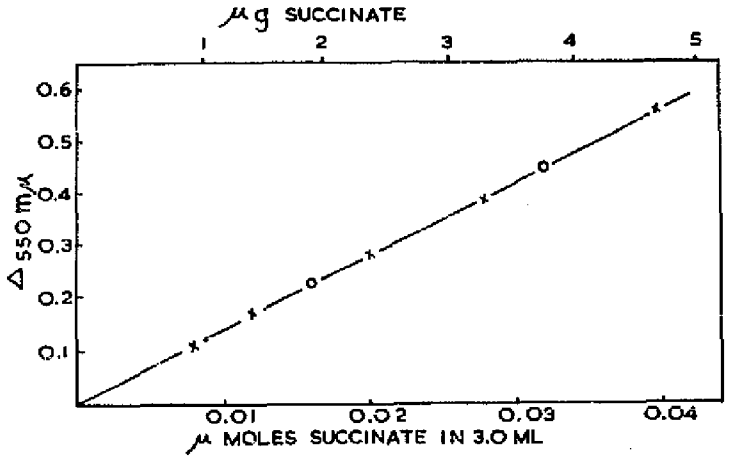

Fig. 1. The increase in absorbance at 550 mμ due to reduction of cytochrome *c* by succinate. The absorbancies plotted are after blank correction. Conditions, as in text. Enzyme used, 0.5 mg 40% pure succinic dehydrogenase. Succinate, neutralized Hopkins & Williams succinic acid, analytical grade. X aerobic, O anaerobic.

chrome *c* is a one-electron acceptor, and as phenazine compounds are reduced in two distinct one-electron stages[3,4], it is not unreasonable to assume that the rapid reaction with cytochrome *c* is with the semiquinoid form of phenazine methosulphate, which in turn is probably formed from the semiquinoid flavin form of succinic dehydrogenase.

This coupled reaction of succinate oxidation and cytochrome *c* reduction may also be used to calculate the extinction coefficients of cytochrome *c*. There is considerable variation in the published values for the oxidized and reduced forms of cytochrome *c*, which have been based on calculations from dry weight or iron content. The reaction with succinate offers standardization against a stable compound of known purity and concentration, and therefore should be considerably more accurate. From the slope of Fig. 1 it can be calculated that ε_{550} (reduced-oxidized) for cytochrome *c* is $2.10 \cdot 10^4$ cm²/mmole. The highest value previously reported[5] is $1.89 \cdot 10^4$. The cytochrome *c* used in this work was prepared according to the method of KEILIN AND HARTREE[6] and further purified by fractionation on a column of calcium phosphate gel suspended on cellulose. Brown impurities were eluted with 0.1 *M* phosphate, pH 7.6 and the cytochrome *c* eluted with the same buffer + 5% (w/v) $(NH_4)_2SO_4$, leaving behind considerable amounts of greenish impurities. The ratio of absorbance at 550 mμ (reduced with $Na_2S_2O_4$) to that at 280 mμ (oxidized with $K_3Fe(CN)_6$) was 1.30. The molar extinction coefficient of the oxidized form at 550 mμ was found to be $0.89 \cdot 10^4$ cm²/mmole and of the reduced form, $2.99 \cdot 10^4$ cm²/mmole.

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