APPLICATION OF BOUND FLAVIN ANALYSIS TO THE STUDY OF THE TURNOVER NUMBER OF SUCCINATE DEHYDROGENASE AND OF THE RECONSTITUTION OF THE SUCCINOXIDASE SYSTEM*

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The availability of a sensitive method for the determination of the succinate dehydrogenase content of heart muscle preparations, based on bound flavin content and therefore independent of the activity of the enzyme (Singer *et al.*, 1962) opened the way for the investigation of several problems concerning this enzyme. One of these is the question of the turnover number of the enzyme in respiratory chain preparations and in soluble purified ones. Values in the literature for the soluble enzyme cover a wide range; CoQ is said to be a better acceptor than phenazine methosulfate at least in one type of particle (Ziegler and Doeg, 1959); and the dehydrogenase has been reported to have a higher turnover number in particulate than in certain soluble preparations (Ziegler and Doeg, 1962).

Table I is a compilation of available turnover numbers for heart succinate dehydrogenase from literature data and unpublished studies in this laboratory, with activities expressed per mole of bound flavin. With certain preparations only data based on the manometric phenazine assay are available. Since it has been recently shown that in this test the reoxidation of the dye may become the limiting factor under certain conditions (Arrigoni and Singer, 1962) where turnover numbers based on the spectrophotometric phenazine assay are unavailable, they are regarded as provisional. It may be seen in this Table that when turnover numbers are based on bound flavin content and care is taken to eliminate presently known limitations of the assay procedures, a reasonably constant turnover number of 11,500 ± 1,000 is obtained for all particulate preparations examined.

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TABLE I

TURNOVER NUMBERS OF SUCCINATE DEHYDROGENASE FROM HEART

Preparation	Assay method	Turnover number (Moles succinate / min./mole bound flavin)	Reference
Beef heart mitochondria	Phenazine, spectrophot., V_{max} dye	11,000 to 13,000	c
Keilin-Hartree, beef heart	Phenazine, spectrophot., V_{max} dye	7,000 to 11,300	c
" " " "	Oxidase (+ cytochrome c)	5,100 to 7,500	c
ETP, beef heart	Phenazine, manometric, V_{max} dye and succinate	9,700	d
" " "	CoQ_{10}, fixed dye[a]	10,000	d
Succ.-CoQ reductase, beef heart	Phenazine, manometric, V_{max} dye and succinate	11,300	d
" " " " "	CoQ_{10}, fixed dye[a]	12,600	d
" " " " "	Phenazine, menometric, V_{max} dye	11,400	c
" " " " "	CoQ_{10}, fixed dye (at 30°)	11,300	c
" " " " "	Phenazine, spectrophot., V_{max} dye	11,900	c
Soluble enzyme, pig heart, Wang et al. method[e]	Phenazine, manometric, fixed dye[b]	4,600	e
Soluble enzyme, beef heart, Wang et al. method	Phenazine, spectrophot., V_{max} dye	4,400	c
Soluble enzyme, beef heart Singer et al. method[f]	Phenazine, manometric, V_{max} dye and succinate	3,600 to 4,100	f,d
Soluble enzyme, beef heart Bernath and Singer method[g]	Phenazine, manometric, V_{max} dye	6,240	g
Soluble enzyme, beef heart, Bernath and Singer method[g]	Phenazine, spectrophot., V_{max} dye	6,200 to 10,700	c

Except as noted all assays refer to 38° and fixed (0.02 M) succinate concentration and pH 7.6. Values obtained in this laboratory (references c,f,g) were preactivated samples. Correction from 0.02 M succinate to V_{max} succinate gives of the order of 10 to 15% increase. [a]Assay at pH 7.0, 0.01 M succinate. [b]Assay at pH 7.8, 0.033 M succinate. [c]Unpublished results from this laboratory. [d]Ziegler and Doeg (1962). [e]Wang et al. (1956). [f]Singer et al. (1956). [g]Bernath and Singer (1962).

Soluble purified samples of the flavoprotein show lower values, which vary with the method of isolation. This lower value may be in part due to inactivation during isolation, in part to the differential loss of one of the two reaction sites for phenazine methosulfate thought to be present in all bound forms of the heart enzyme but not in soluble ones (Giuditta and Singer, 1958). The loss of this presumed second site would occur in the alkaline extraction step common to all procedures for the isolation of the enzyme, since it is at this point that the turnover number abruptly changes in the phenazine assay. That the inactivation - or loss of reaction site - does not invariably occur in the isolation of the soluble enzyme is indicated by the fact that in occasional preparations, as isolated by the method of Bernath and Singer (1962), the turnover number is the same as in mitochondria (Table I). Of the three soluble preparations available this also yields the highest turnover number in the average preparation (Table I).

It may be noted that in suitable respiratory chain preparations the CoQ and phenazine assays yield about the same turnover number. This is the only indication that these methods may be measuring the full rate of the substrate-flavoprotein interaction. Oxidation via the respiratory chain in Keilin-Hartree preparations measures a much lower activity. Hence in this preparation the activity of the dehydrogenase outstrips that of the overall system.

The ready inactivation of the succinate-phenazine reaction without loss of activity in the fumarate-$FMNH_2$ assay on storage and other manipulations of soluble preparations (Singer et al, 1957) and on incubation of particulate ones with CN^- (Giuditta and Singer, 1958) suggested that $FMNH_2$ might provide a more reliable measure of the basal activity of the dehydrogenase than does phenazine methosulfate. When the turnover numbers in the $FMNH_2$ assay per mole of bound flavin were examined, however, the surprising fact emerged that this value is not constant and, in fact, may increase abruptly on purification of the enzyme. It is variable even in multi-enzyme preparations. The following values were obtained for the beef heart enzyme at pH 7.6, 38° (V_{max} with respect to $FMNH_2$): mitochondria, 230 to 290; Keilin-Hartree preparation, 290; succinate C Q reductase complex, 475 to 586; Wang et al. (1956) soluble enzyme, 4; Singer et al. (1956) soluble enzyme, 400 to 475; Bernath and inger (1962) soluble enzyme, 690 to 700. It appears that the reaction of $FMNH_2$ with the bound flavin is inhibited by steric factors and that detachment from the respiratory chain and preparative modification may remove this barrier. In line with this reasoning an increase in turn-

over number occurs during the alkaline extraction step and of the three soluble preparations examined the one extracted at the lowest pH (Singer et al., 1956) shows the smallest increase. Thus an increase in the turnover number in the $FMNH_2$ assay might be diagnostic of structural modification in the vicinity of the flavin and even particulate preparations retaining full activity with CoQ (e.g., the reductase) may be somewhat modified.

Another problem open to solution with the availability of the bound flavin method is the mechanism of the reactivation ("reconstitution") of succinoxidase on the addition of soluble succinate dehydrogenase to alkali-inactivated preparations (Keilin and King, 1958). In this report the mechanism of the alkali-inactivation of succinoxidase was suggested to be some type of "displacement" of the dehydrogenase, but later King (1962) claimed that an actual dissociation of the dehydrogenase from the respiratory chain occurred and that the alkali-treated sample was devoid of the dehydrogenase. From this the hypothesis was elaborated that the dehydrogenase is held by simple ionic bonds to the respiratory chain. These speculations were based on catalytic activity, not on the measurement of dehydrogenase content. It was clearly desirable to follow the content of bound flavin through the cycle of inactivation and reactivation.

Numerous experiments of the type shown in Table II have led to the following conclusions. (1) Under the experimental conditions used by Keilin and King (1958) alkali inactivates succinate dehydrogenase but does not dissociate it. Even a modest dissociation (solubilization) of the dehydrogenase without prior treatment with organic solvents requires considerably more alkaline pH values. This is in accord with previous findings in this (Singer et al., 1957) and other laboratories (Basford et al., 1957; Wang et al., 1956). (2) Contrary to King's (1962) report, it makes no difference in our experiments whether the alkali-treated particles are centrifuged before or after neutralization (cf. expts. 2 and 3). Slight differences appear only if the inactivation is carried out at a much more alkaline pH than is required for complete inactivation of the dehydrogenase and oxidase in a relatively short time. Thus the results cannot be explained as a re-association of dissociated dehydrogenase on neutralization. (3) On treatment with soluble succinate dehydrogenase partial, sometimes complete, reactivation of succinoxidase occurs, as reported by Keilin and King (1958). This is indeed accompanied by an increase in bound flavin content, which suggests some type of association with the alkali-treated particles. Thus far, however,

TABLE II

VARIATIONS IN SUCCINATE DEHYDROGENASE CONTENT DURING THE REVERSIBLE ALKALI INACTIVATION

Exp.	Sample	% Initial activity		Bound flavin content mμmoles/mg. protein
		Succinate-phenazine	Succinoxidase	
1	Untreated KH	100	100	0.14
2	Alkali-treated KH	3	1	0.13
3	Same, centrifuged at pH 9.4	3	1	0.14
4	Same as 2 + 0.34 mμmoles fresh Wang enzyme/mg. KH	40	32	0.17

KH (beef heart Keilin-Hartree preparation) and succinate dehydrogenase (Wang et al. (1956) gel eluate stage) were prepared exactly as per King (1961a). A 3% suspension was slowly adjusted to pH 9.4 at 0° and then incubated at room temperature in the dark. At about 98% inactivation of succinoxidase the sample was cooled to 0°, neutralized to pH 7.4, and repeatedly washed by centrifugation (exp. 2). Another sample (exp. 3) was held at pH 9.4 the same length of time, but centrifugation was performed before neutralization. Soluble dehydrogenase was added within 15 minutes of its isolation. Following brief incubation at pH 7.4, all samples were reapeatedly washed by ultracentrifugation to remove uncombined dehydrogenase.

no correlation has been found between the degree of reactivation and the increase in bound flavin content. In all experiments, regardless of degree of reactivation, the "reconstituted" sample had a higher content of succinate dehydrogenase than the untreated one and is, hence, not identical with the original one.

Studies by Arrigoni (Arrigoni et al., 1962; Singer and Kearney, 1962) have further shown that the various catalytic activities of succinate dehydrogenase disappear at different rates on alkaline incubation and that the reactivated material differs from the original in several respects. Thus while the experimental findings of Keilin and King (1958) have been confirmed, the later interpretation that this is a reconstitution of the intact respiratory chain seems somewhat premature. It may be, perhaps, more proper to consider the phenomenon a reversible inactivation. The suggestion that this type of reincorporation is a more physiological test of succinate dehydrogenase than catalytic assays (King, 1961b) presently lacks experimental basis.

REFERENCES

Arrigoni, O., and Singer, T. P., Nature, 193, 1256 (1962).

Arrigoni, O., Hauber, J., and Singer, T. P., to be published (1962).

Basford, R. E., Tisdale, H. D., and Green, D. E., Biochim. Biophys. Acta, 24, 490 (1957).

Bernath, P., and Singer, T. P., Methods in Enzymology, V, 562 (1962).

Giuditta, A., and Singer, T. P., J. Biol. Chem., 234, 666 (1958).

Keilin, D., and King, T. E., Nature, 181, 1520 (1958).

King, T. E., J. Biol. Chem., 236, 2342 (1961a).

King, T. E., Biochim. Biophys. Acta, 47, 430 (1961b).

King, T. E., Biochim. Biophys. Acta, 58, 375 (1962).

Singer, T. P., Kearney, E. B., and Bernath, P., J. Biol. Chem., 223, 599 (1956).

Singer, T. P., Kearney, E. B., and Massey, V., Adv. Enzymol., 18, 65 (1957).

Singer, T. P., and Kearney, E. B., in The Enzymes (P. Boyer, H. A. Lardy, and K. Myrbäck, eds.) Vol. VII, Academic Press, in press.

Singer, T. P., Hauber, J., and Kearney, E. B., Biochem. Biophys. Res. Communs., 9, 146 (1962).

Wang, T. Y., Tsou, C. L., and Wang, Y. L., Scientia Sinica, 5, 73 (1956).

Ziegler, D. M., and Doeg, K. A., Biochem. Biophys. Res. Communs., 1, 344 (1959).

Ziegler, D. M., and Doeg, K. A., Arch. Biochem. Biophys., 97, 41 (1962).