THE FAILURE OF MALATE TO AFFECT RADICAL CONCENTRATION AT ROOM TEMPERATURE IN SYSTEMS CONTAINING SUCCINIC DEHYDROGENASE*

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Summary. Malate does not appear to inhibit or potentiate the formation of radicals with the succinic dehydrogenase preparations employed in any discernible manner at room temperature. Malate, therefore, appears to have no part in the mechanism of radical formation.

Experimental. The appearance of electron spin resonance (ESR) signals at room temperature in systems containing heart muscle succinic dehydrogenase (SDH) depends on the presence of succinate and fumarate (Commoner and Hollocher, 1960; Hollocher and Commoner, 1960, 1961). The presence of fumarase as a minor but persistent contaminant in the preparations of SDH raises an ambiguity in the sense that any system initially prepared to contain succinate and fumarate, if given sufficient time, will contain L-malate in equilibrium with fumarate. Until recently there was no reason to believe that malate might effect or seriously affect radical formation with SDH at room temperature; however, Dervartanian et al. (1963, 1965) have reported that preparations of SDH possess what appears to be a low level of D- and L-malate dehydrogenase activity and that SDH is directly or indirectly reduced by malate. These findings prompted us to examine the possible role of malate in our systems.

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Two approaches are being followed in this regard: 1) the removal of the last traces of fumarase from preparations of SDH, and 2) the use of D-malate and analogues of fumarate which are not affected by fumarase. This report concerns the latter approach, and Table I summarizes a few results of these experiments. SDH was prepared from beef heart by a modification of the method of Singer et al. (1956), and was taken to a purity of between 30 and 50%. The preparations were found to be free of heme proteins and acid-labile flavins. Estimates of SDH purity and concentration were made by means of spectra (Singer et al., 1956), spectrophotometric assay (Arrigoni and Singer, 1962), and analysis for acid-resistant flavin (Kearney, 1960, and Singer et al., 1962). Details of the preparative method will be presented in a later paper (Griffin and Hollocher, paper in preparation). The ESR method and instrumentation employed have been described briefly (Hollocher et al., 1963). Sample temperature in the cavity was only slightly above room temperature at the low RF levels employed.

In Table I, systems 1 through 6 confirm and extend previous observations to the effect that succinate, fumarate and enzyme are required for the detection of ESR signals. Since methyl fumarate is unaffected by fumarase, systems 8 through 10 indicate that L-malate is not required for the appearance of ESR signals. The signals observed with systems containing the substrate analogues indicated are the same as those seen with systems 7, 15, and 16, insofar as we can determine. Systems 11 through 16 show that systems containing malate exhibit no ESR signal except where succinate and fumarate are present simultaneously, and that where signals appear, they are neither diminished nor enhanced by malate. According to Dervartanian and Veeger (1964, 1965), the product of the oxidation of L- and D-malate in preparations of succinic dehydrogenase is oxaloacetate, which is a potent inhibitor of succinic dehydrogenase. System 17 shows that low concentrations of oxaloacetate

Table I

Substrate requirements for the appearance of ESR signals with succinic dehydrogenase at room temperature

All systems contained potassium phosphate buffer, pH 7.4-7.6, at a concentration of 0.1 M and succinic dehydrogenase preparation to give a succinic dehydrogenase concentration estimated to be 1 to 3 x 10^{-5} M. Concentrations of the constituents indicated below were all 10^{-2} M initially, except for oxaloacetate, which was 10^{-4} M. The enclosure of L-malate and fumarate in parentheses indicates that these materials were not added initially, but were known to be formed through the action of fumarase. No detectable ESR signal (-); a small but detectable signal, (+); the substantial signal normally observed, (+++).

System	Constituents in addition to succinic dehydrogenase preparation	ESR signal
1	-	-
2	succinate	+*
3	L- or D,L-chlorosuccinate	-
4	D,L-methylsuccinate	-
5	fumarate (L-malate)	-
6	methylfumarate	-
7	succinate + fumarate (L-malate)	+++
8	succinate + methylfumarate	+++
9	D,L-methylsuccinate + methylfumarate	+++
10	L- or D,L-chlorosuccinate + methylfumarate	+++
11	D-malate	-
12	L- or D,L-malate (fumarate)	-
13	succinate + D-malate	+*
14	fumarate + D-malate (L-malate)	-
15	succinate + fumarate + D-malate (L-malate)	+++
16	succinate + fumarate + L-malate	+++**

Table I, cont.

17	succinate + fumarate+oxaloacetate (L-malate)	+
18	D,L-methylsuccinate + methylfumarate+ D-malate	+++
19	D-malate, 1.5 hrs. later D,L-methylsuccinate + methylfumarate	+++

*The succinate used (Calbiochem disodium succinic acid hexahydrate, A grade) was found to contain fumarate as a minor contaminant.

**Fumarate and L-malate were added at the mole ratio estimated to provide the equilibrium ratio attainable in the reaction of fumarase.

decrease ESR signal amplitudes. Extensions of this experiment indicate that oxaloacetate decreases radical concentration in the same way as does malonate (Hollocher and Commoner, 1961), but with greater effectiveness by an order of magnitude of at least 100. In fact, the concentration of oxaloacetate required to provide a discernible reduction in signal amplitude is so low (about 3×10^{-5} M) that wherever D- or L-malate (of order 10^{-2} M) occurs in our systems, we can deduce that less than 1% of the malate could have been oxidized by any process yielding oxaloacetate during several hours at room temperature. We have not been able to detect as yet the formation of oxaloacetate by ESR at room temperature. Potential oxidants of malate exist, since the concentration of SDH is always 1 to 3×10^{-5} M and the systems, while sealed, contain dissolved oxygen from air at concentrations of the order 10^{-4} M. Systems 18 and 19, in which fumarase can have no effect, illustrate this point. Even without succinate as a competing reductant, the formation of oxaloacetate is not detected.

With our preparations of SDH, at least, D- and L-malate appear to be

indifferent constituents and do not seem to contribute to or interfere with the process of radical formation at room temperature. Contaminating levels of fumarase in the preparations of SDH have only small effects on radical concentration, and these can be attributed to the change in the concentration of fumarate caused by formation of L-malate (Griffin and Hollocher, paper in preparation). Studies with other substrate analogues are in progress.

To a certain but unknown extent the above negative results may be attributable to differences in enzyme preparations. For example, Dervartanian and Veeger (1964, 1965) observe spectrophotometric changes with their SDH preparations, in response to succinate or malate, which they attribute to the reduction of a significant fraction of the flavin of SDH. Under similar circumstances we observe less extensive spectrophotometric changes. While the changes we observe are qualitatively similar to those cited above, we cannot attribute them unambiguously to a reduction of the flavin of SDH. In addition, Dervartanian and Veeger (1965) can detect the oxidation of malate in an assay system with their preparations, whereas we have been unsuccessful in this regard with ours.

On the other hand, we confirm with our preparations the finding of Dervartanian et al. (1963) that both succinate and malate induce the formation of very similar ESR signals in systems observed at 77° K. This confirmation is important in itself, since it provides additional evidence that the process whereby paramagnetic species appear at room temperature and at 77° K with SDH must be quite different.

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