Bioenergetics (1975), 7, 1-16

Correlation of the Effects of Citric Acid Cycle Metabolites on Succinate Oxidation by Rat Liver Mitochondria and Submitochondrial Particles

M. Hillar, V. Lott, and B. Lennox

Department of Biology, Texas Southern University, Houston, Texas 77004

Received 29 March 1974

Abstract

1. Succinate dehydrogenase is inhibited by citrate and β-hydroxybutyrate in a complex manner, both in mitochondria and submitochondrial particles. Kinetics of inhibition in the particles points to a competitive component in the mechanism involved.
2. Pyruvate, α-ketoglutarate, malate, and glutamate stimulate oxidation of succinate by mitochondria.
3. Stimulation by α-ketoglutarate and glutamate is not influenced by the presence of rotenone.
4. Stimulation by pyruvate is higher in the absence of rotenone and increases significantly in the presence of K^+ and valinomycin. Pyruvate supplies in mitochondria reducing equivalents for malate dehydrogenase operating in the reverse direction—reduction of oxaloacetate to malate.
5. Stimulation by malate is higher in the presence of rotenone.

Introduction

It is known that substrates of various respiratory chain-linked flavoproteins inhibit the oxidation of each other by mitochondria or particulate preparations. Succinate inhibits DPNH, choline, α-glycerophosphate oxidase activity and vice versa [1, 2, 3, 4, 5,6]. As turnover of succinate dehydrogenase (EC 1.3.99.1), 18,000/min., is much higher than the activity of the respiratory chain it feeds [1, 7] the uncontrolled succinate oxidation would restrict the oxidation of DPNH.

The regulatory mechanism for SDH in heart muscle was postulated by Singer *et al.* [1, 8, 9, 10, 11, 12, 13, 14] in which $CoQH_2$, succinyl-CoA and ATP are the regulatory factors. SDH activity depends on the metabolic state of the mitochondria and declines in the state $4 \rightarrow 3$ transition and on adding uncouplers [9, 15, 16]. Deactivation of SDH in state 3 is associated with the change in $CoQ/CoQH_2$ ratio which can be as high as tenfold in transition from the controlled to the active state and almost complete oxidation of $CoQH_2$ occurs in the presence of uncouplers [17]. In addition the ATP generated, due to its activating action on SDH in mitochondria [1, 9, 18], may also play a role in determining what fraction of the enzyme is in the activated state at any given moment. Thus competition between substrates involves electron transport from the dehydrogenase to the respiratory chain, presumably the CoQ pool. Ideal conditions for fully active SDH in mitochondria are also those for reversed electron transport.

This mechanism, however, takes into account only one aspect of the multiple types of control operating in mitochondria for SDH. The enzyme seems to be controlled by a variety of substrates and inorganic phosphate. Fumarate and malate are competitive inhibitors with less affinity for the enzyme than succinate (K_D respectively 2.6, 4.9 and 0.1 mM) [19, 20, 21]. Inorganic phosphate is weakly inhibitory (K_D 20 mM) and binds at the active site [22]. Oxaloacetate is highly competitive to succinate (K_I 4.5 μM) and also binds to the enzyme in a pseudo-irreversible manner at a 1 : 1 molar ratio [23, 24, 25]. The activity of SDH in soluble or membrane preparations can also be increased several-fold if the enzyme is preincubated with succinate, inorganic phosphate, fumarate or malonate [8, 26, 27, 28, 29]; the enzyme returns to the deactivated (unactivated) state upon removal of the activator [30].

Overall regulatory mechanisms for succinate oxidation in intact mitochondria by SDH are, thus far, not clearly understood [24, 25, 31]. The enzyme still seems to be affected mostly by oxaloacetate [25]. Investigations correlating effects of several metabolic substrates on succinate oxidation in mitochondria and submitochondrial particles are presented in this report. It was found that SDH is inhibited only by citrate and β-hydroxybutyrate in a complex manner, both in mitochondria and particles. Pyruvate, α-ketoglutarate, malate and glutamate stimulate oxidation of succinate by mitochondria. Stimulation by α-ketoglutarate and L-glutamate is not influenced by the presence of rotenone. Stimulation by pyruvate is higher in the absence of rotenone and that of malate is higher in the presence of rotenone. Results indicate that citrate and β-hydroxybutyrate inhibit oxidation of succinate affecting enzyme. Mechanism of action of other substrates is discussed in the light of data available in the literature: α-ketoglutarate and malate remove oxaloacetate by exchange translocation on dicarboxylate carrier;

glutamate removes oxaloacetate by transanimation; pyruvate remains in equilibrium with malate dehydrogenase system, supplying reducing equivalents for oxaloacetate reduction.

Methods and Materials

Rat liver mitochondria were isolated in a solution of 0.25 M sucrose, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA-Tris, pH 7.2, as a fraction sedimented between 800-8000 x g at 0° C for 10 min. and washed three times in sucrose–tris solution. Only mitochondria showing respiratory control index of 5 were used for experiments.

Submitochondrial particles were prepared by sonification of isolated mitochondria in a Heat Systems sonifier at 100 watts for 2 min. with a 5 min. interval at 0° C (cooled with NH_4Cl–ice mixture); this operation was repeated. Particles were sedimented as a fraction between 25,000-144,000 x g and suspended in sucrose–tris solution [32].

Succinate oxidation by mitochondria was measured with the Clark oxygen electrode at 25° C in a YSI Biological Oxygen Monitor, Model 53. The basic incubation medium contained in 3 ml final volume:sucrose 0.25 M; Tris-HCl buffer, pH 7.2, 10 mM; EDTA–Tris, pH 7.2, 1 mM; substrates were added as Tris salts in concentrations as indicated in the graphs.

Succinate oxidation by submitochondrial particles was followed spectrophotometrically by measuring reduction of 1 mM potassium ferricyanide at 420 nm in DW-2 Aminco spectrophotometer. The incubation medium used was the same as for mitochondria.

Mitochondrial protein was determined by the biuret method of Layne [33].

Substrates and other chemicals were purchased from Sigma Chemical Co.

Results and Discussion

Oxidation of succinate by rat liver mitochondria is presented in Fig. 1 in the medium without rotenone, and in Fig. 2–with rotenone (1 μg/3 ml volume). It can be observed that citrate and β-hydroxybutyrate inhibit oxidation of succinate in both experiments; α-ketoglutarate and L-glutamate were stimulatory in both experiments. L-malate stimulates slightly oxygen uptake in the absence of rotenone and pyruvate has a marked stimulatory effect in the absence of rotenone. In the presence of KCl and valinomycin potassium pyruvate increases oxygen uptake about two times (Fig. 3).

In Fig. 4 succinate oxidation by sonicated submitochondrial particles

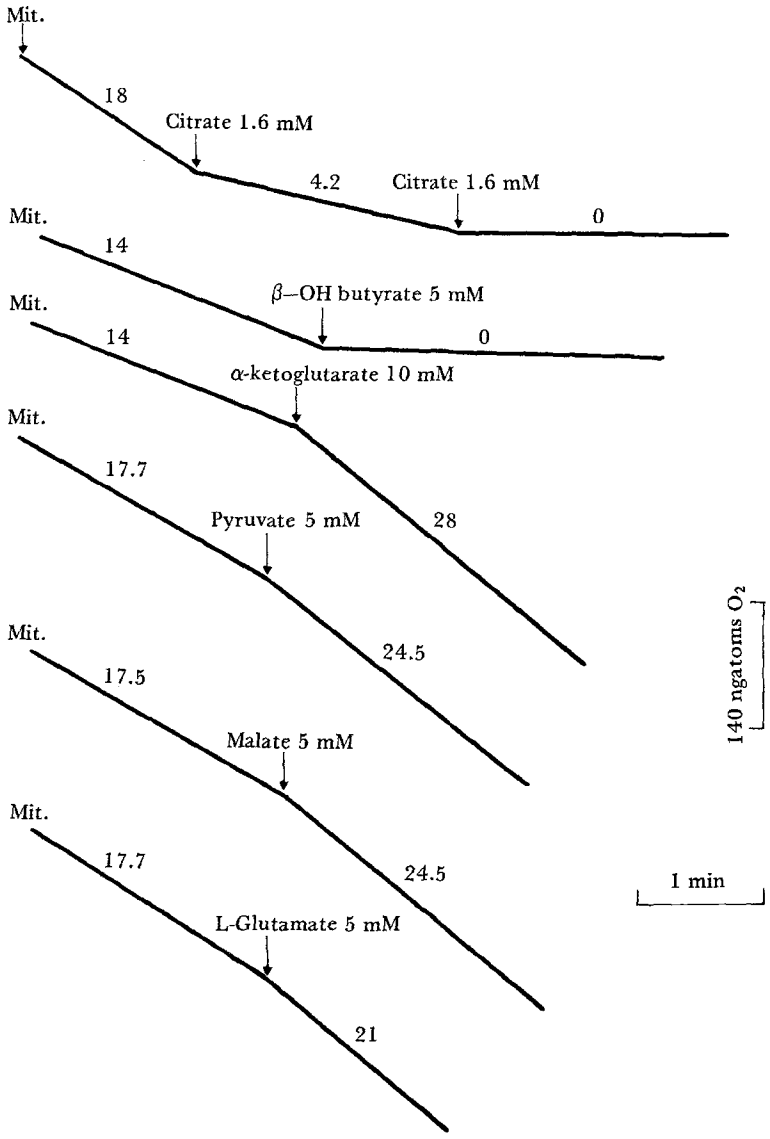

Figure 1. Oxygen uptake by mitochondria oxidizing succinate in the medium without rotenone. Medium (see Methods) contained in 3 ml final volume phosphate-tris buffer 5 mM, succinate-tris 16.6 mM, mitochondria—10 mg protein. Figures represent nanogramatoms O_2/min/mg protein.

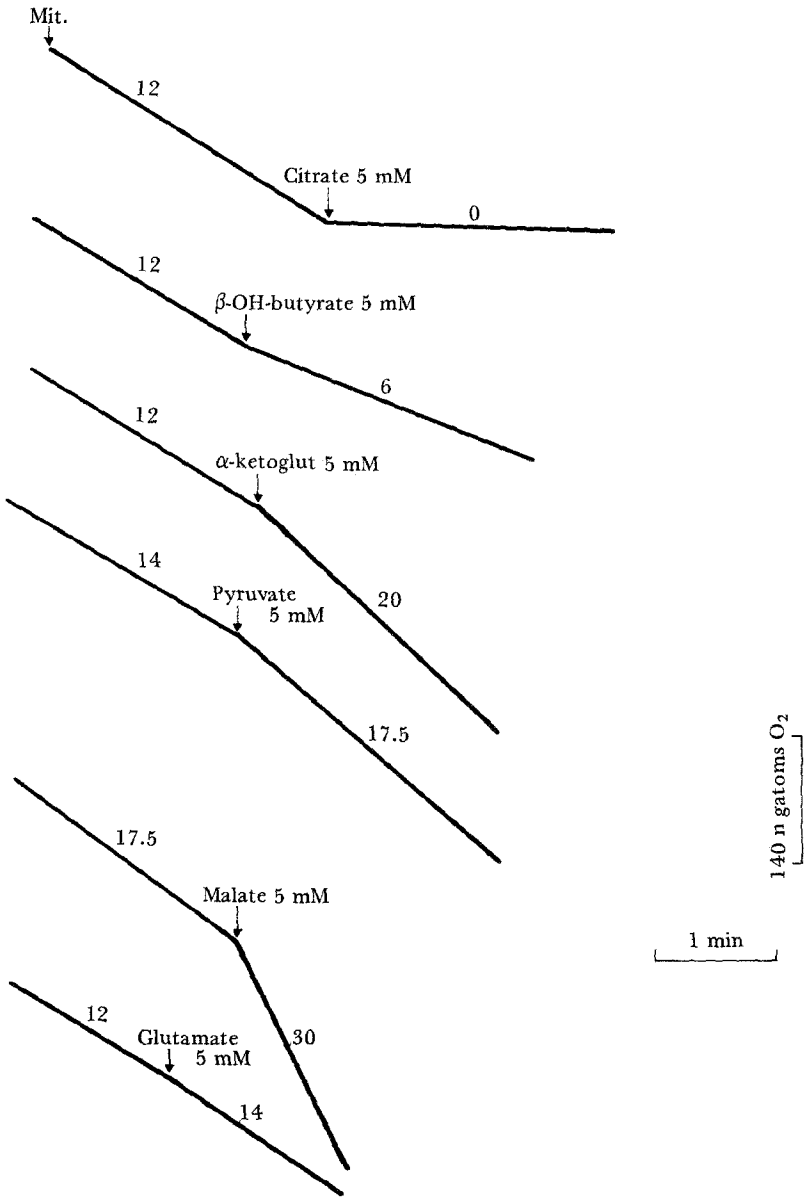

Figure 2. Oxygen uptake by mitochondria oxidizing succinate in the medium with rotenone (1 μg). Other experimental conditions as in Fig. 1. Mitochondria—7 mg protein.

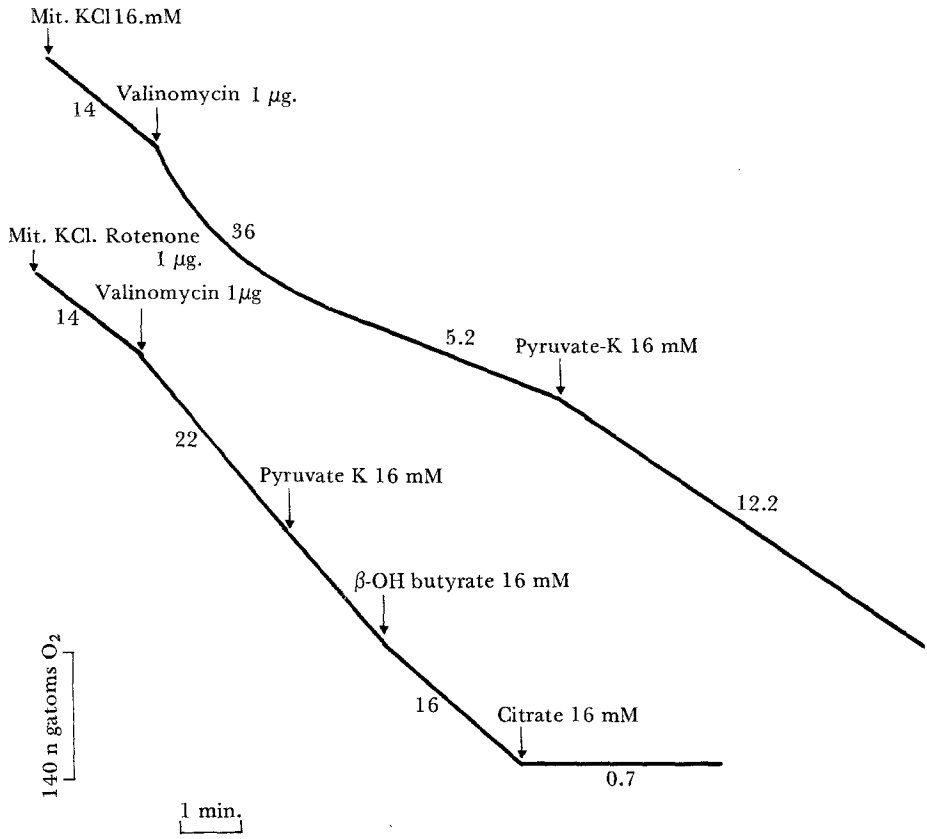

Figure 3. Effect of valinomycin, KCl and pyruvate on oxygen uptake by mitochondria oxidizing succinate in the presence or absence of rotenone. Experimental conditions as in Fig. 1. Mitochondria—8 mg protein.

is inhibited by citrate and β-hydroxybutyrate. This inhibition can be reversed by phosphate-tris. The other substrates—α-ketoglutarate, malate, L-glutamate, and pyruvate—do not exhibit an influence on succinate oxidation in this system. Moreover, phosphate alone only slightly enhances succinate oxidation. The experiments were performed in the presence of n-heptylquinoline N-oxide (HQNO) in order to prevent the flow of electrons to oxygen and increase in this way the efficiency of the reduction of potassium ferricyanide.

The inhibitory effects of citrate and β-hydroxybutyrate in submitochondrial particles depend on the concentration of inhibitor (Fig. 5, 6, 7) and are dependent on the concentration of succinate used. Half maximum inhibition is reached with about 4.5 mM β-hydroxybutyrate

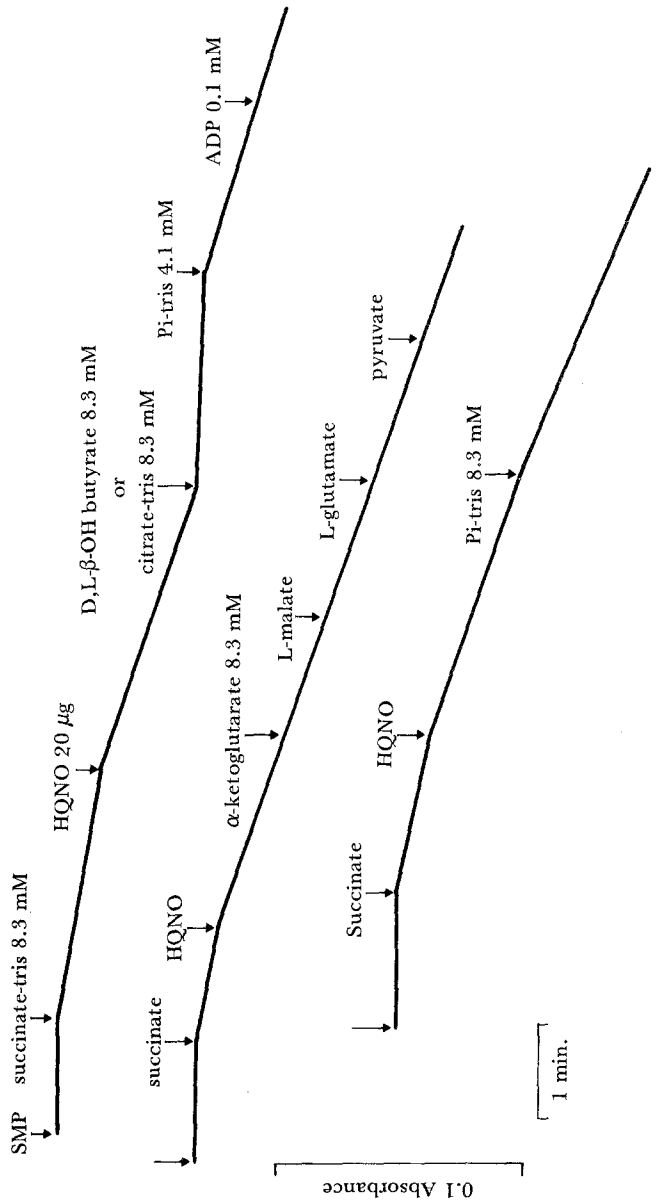

Figure 4. Reduction of potassium ferricyanide by submitochondrial particles oxidizing succinate. Incubation medium (see Methods) contained in 3 ml final volume 1 mM potassium ferricyanide, submitochondrial particles 0.58 mg protein (SMP). HQNO—n-heptyl hydroxyquinoline N-oxide.

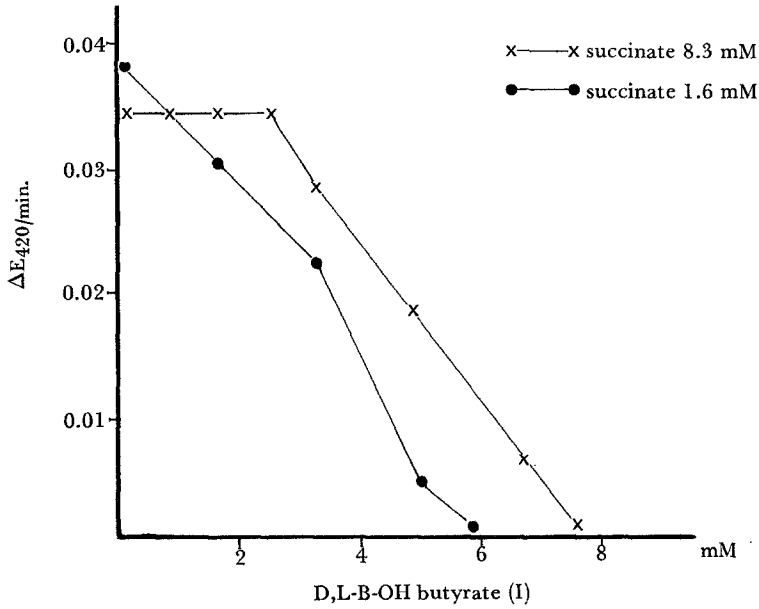

Figure 5. Inhibition by β-hydroxybutyrate of succinate oxidation by submitochondrial particles. Incubation medium as in Fig. 4. SMP–1.16 mg protein; HQNO–25 μg.

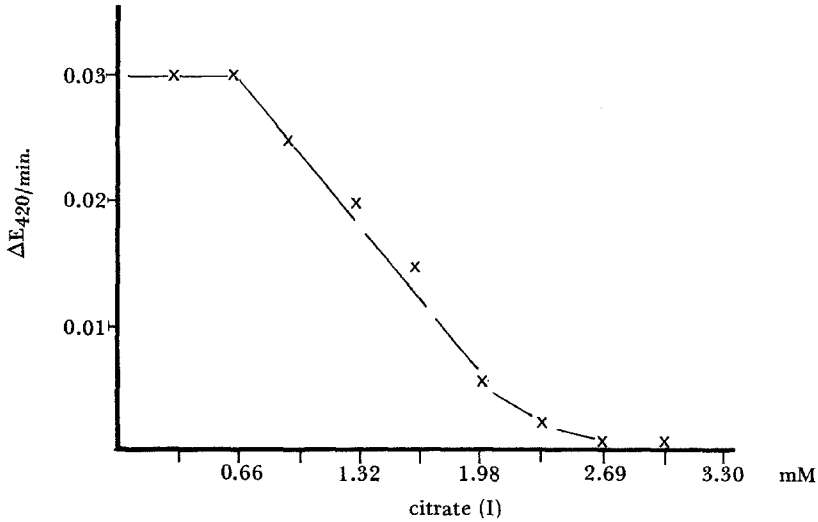

Figure 6. Inhibition by citrate of succinate oxidation by submitochondrial particles. Incubation medium and conditions of the reaction as in Fig. 4. Succinate used at 8.3 mM, submitochondrial particles–1.16 mg protein.

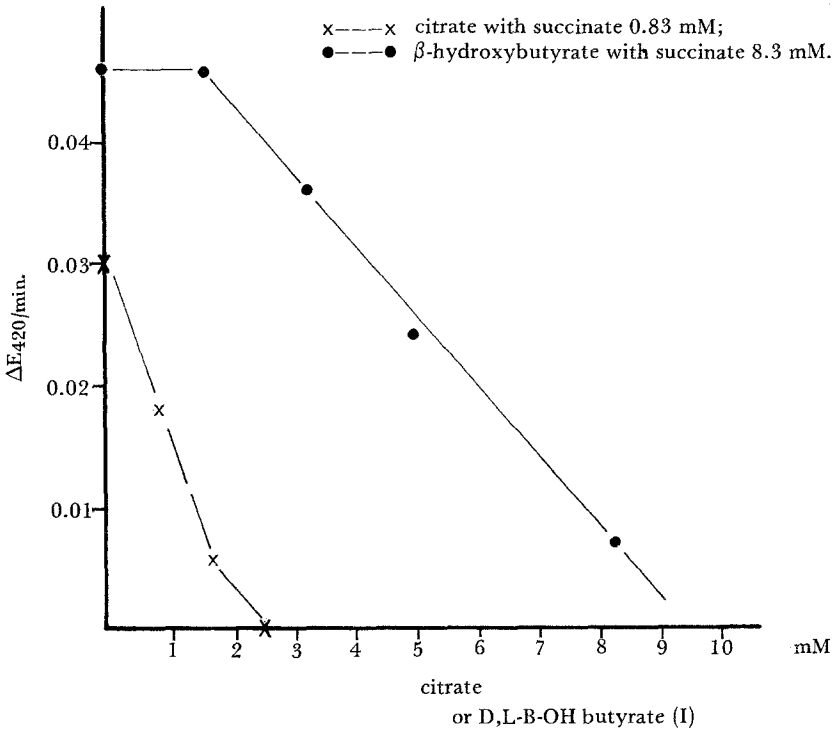

Figure 7. Inhibition by citrate and β-hydroxybutyrate of succinate oxidation by submitochondrial particles. Incubation medium and conditions as in Fig. 4. SMP—1.16 mg protein.

and 1.4 mM citrate. The mechanism of inhibition is complex as Dixon plots show sigmoidal character (Fig. 8, 9). Similar shape of Dixon plot was found for the inhibition of SDH by HCO_3^- and the mechanism may involve competition with succinate for active center [34]. However, it is interesting to speculate on the involvement of metal ions in the postulated mechanism [35, 36]. Reversal of the inhibition by phosphate depends on the phosphate concentration (Fig. 10) being maximum at about 5 mM concentration. This reversal mechanism does not seem to operate in intact mitochondria (Fig. 1).

It was reported previously by Quagliariello and Palmieri [37] in experiments under somewhat different conditions (in the presence of KCl and gramicidin) that citrate, isocitrate and α-ketoglutarate (at high concentrations—20 mM) inhibited succinate (1 mM) oxidation by mitochondria in the presence of rotenone in a competitive manner. At the same time it was found also that the uptake of succinate was strongly inhibited by citrate, isocitrate, α-ketoglutarate, malate, and malonate but

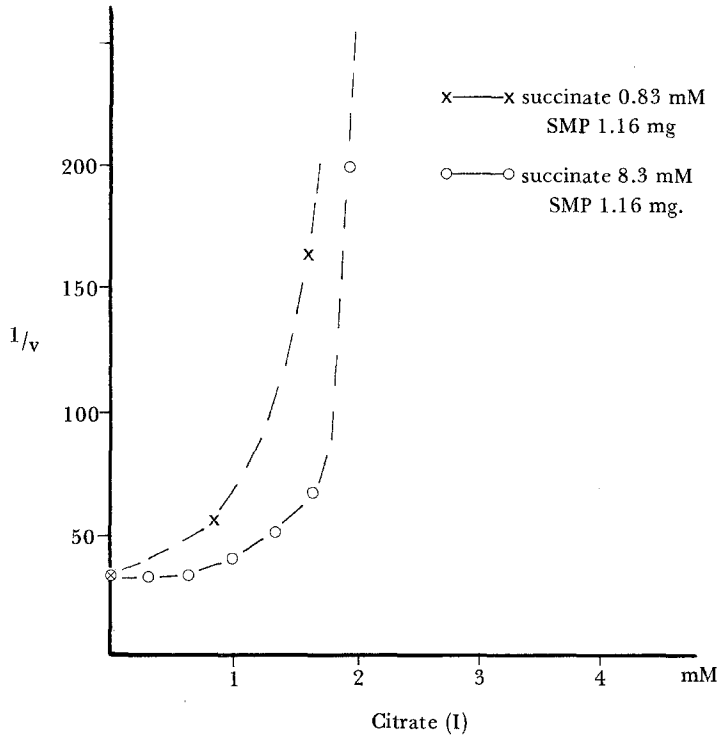

Figure 8. Dixon plot for citrate inhibition of succinate oxidation. Calculated from data in Fig. 6, 7.

not by pyruvate, glutamate and β-hydroxybutyrate. Citrate decreased internal succinate concentration, succinate–enhanced internal concentration of citrate. The authors suggested that the group of anions that inhibit succinate uptake can exchange with succinate, thus decreasing the intramitochondrial concentration of succinate. Van Dam and Tsou [38] reported, however, that also anions, which do not inhibit the oxidation of succinate, inhibit succinate uptake. Corresponding K_I values (mM) were as follows: malate–1.1; α-ketoglutarate–2.2; citrate–1.5; β-hydroxybutyrate–3.5; glutamate–3.5; isocitrate–1.0.

Results observed in various laboratories are probably an expression of multiple actions of anions (substrates) on the SDH [26], on the transport systems across mitochondrial inner membrane [39, 40, 41, 42] and on enzyme equilibria in the mitochondria [43, 44, 45, 46]. It was also found that pH shift [47] affects the enzyme activity. SDH is reversibly converted in the submitochondrial particles from inactive to

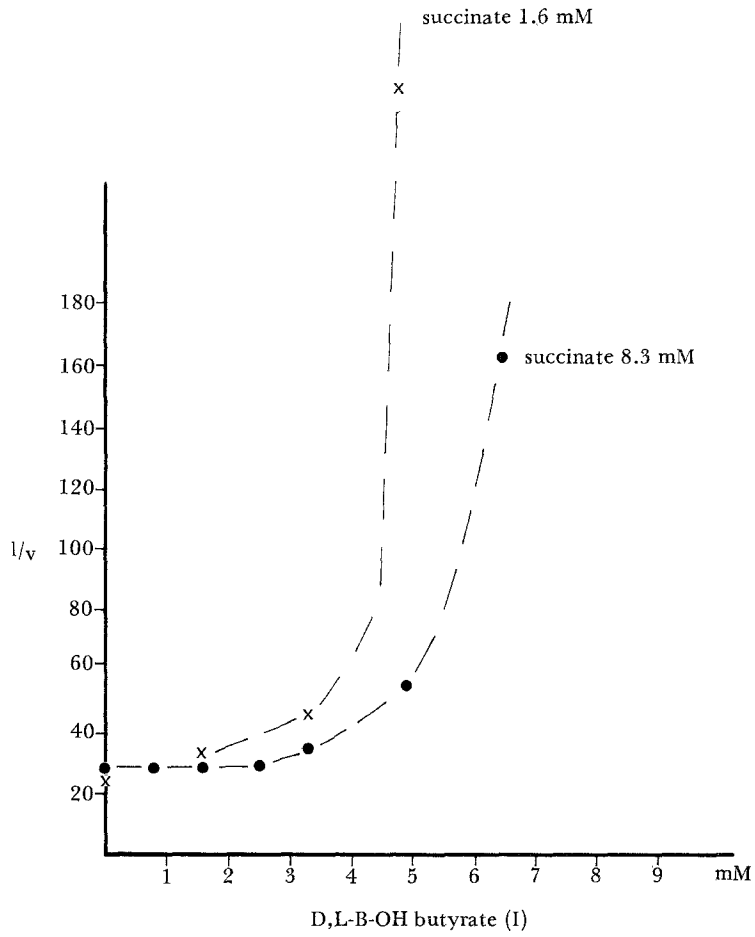

Figure 9. Dixon plot for β-hydroxybutyrate inhibition of succinate oxidation. Calculated from data in Fig. 5.

active form at a pH of about 6. Whether the pH shift associated with the state 4 → 3 transition has any effect on SDH remains to be established.

Strong inhibitory effects of citrate and β-hydroxybutyrate seem to be the result of the inhibition of the enzyme itself (as it can be seen from experiments with submitochondrial particles) (Fig. 4-10). We do not think that the other substrates except oxaloacetate may be inhibitory for SDH under physiological conditions. The enzyme in the membrane of submitochondrial particles is in the active state which is the closest to that in intact mitochondria.

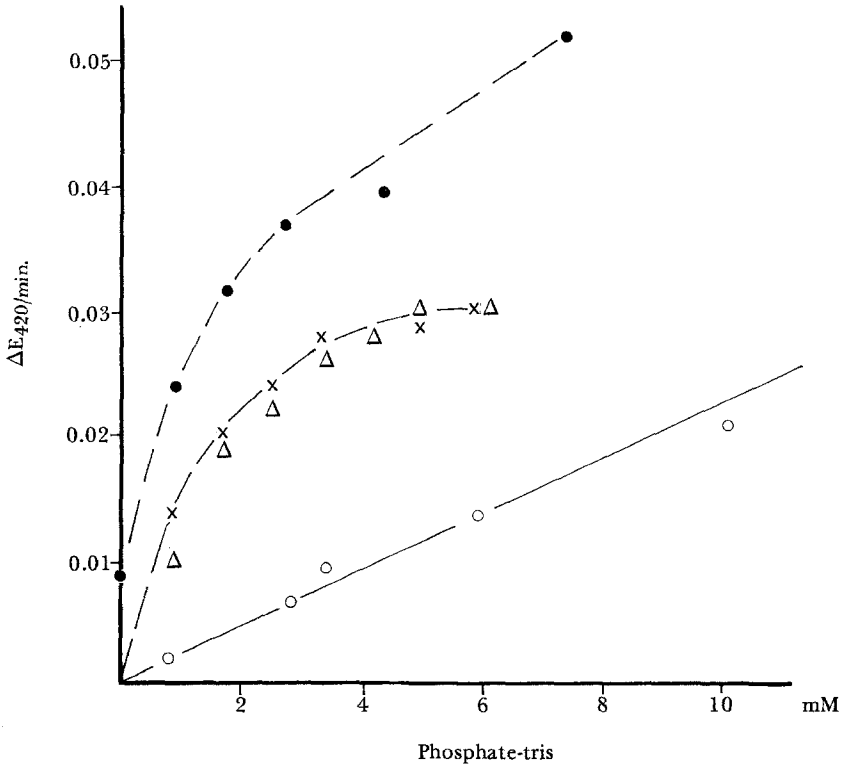

Figure 10. Effect of Pi on inhibition of succinate oxidation in SMP produced by citrate and β-hydroxybutyrate. Reaction medium as in Fig. 4. SMP 1.16 mg protein; HQNO–25 μg.

●——● β-hydroxybutyrate 8.3 mM and succinate 8.3 mM;
x——x β-hydroxybutyrate 7.5 mM and succinate 8.3 mM;
△——△ β-hydroxybutyrate 5.8 mM and succinate 1.6 mM;
○——○ citrate 2.5 mM and succinate 0.83 mM.

The inhibition of succinate uptake observed by Van Dam and Tsou [38] is related to the interaction of various metabolites with three carriers present in the inner mitochondrial membrane [41]. Dicarboxylate carrier catalyzes the exchange of a dicarboxylate for either Pi or a dicarboxylate as well as exchange of Pi for Pi. The tricarboxylate carrier can catalyze the exchange of a tricarboxylate for either tricarboxylate or dicarboxylate, as well as a dicarboxylate–dicarboxylate exchange. Finally, the ketoglutarate carrier can catalyze the exchange of ketoglutarate for dicarboxylate and a dicarboxylate–dicarboxylate exchange [48].

Under the experimental conditions used, succinate uptake is not the

rate limiting factor for its oxidation. Some anions (α-ketoglutarate, malate, glutamate) in spite of inhibitory effect on succinate transport, still stimulate its oxidation. Stimulation of succinate oxidation by α-ketoglutarate and malate is associated with the removal of oxaloacetate from mitochondria in the exchange translocation of oxaloacetate for malate and α-ketoglutarate [41, 46]. Moreover, stimulation of succinate oxidation by malate is stronger in the presence of rotenone, i.e. under conditions when the formation of oxaloacetate is prevented. Also, in the presence of glutamate, oxaloacetate is transaminated to aspartate which in turn can be exchanged for glutamate. Klingenberg and Schollmeyer [49, 50] showed decreased level of oxaloacetate upon incubation of mitochondria with glutamate or pyruvate.

The observed stimulatory effect of pyruvate on succinate oxidation is related to the shift in malate dehydrogenase equilibrium toward the reduction of oxaloacetate. From the studies of Papa *et al.* [51] it was suggested that the translocation of pyruvate across the inner mitochondrial membrane is directly coupled to hydroxyl ion counterflux or to proton symport (the same mechanism as for phosphate translocation) [52, 53]. Phosphate decreases pyruvate uptake by competition for OH^-. Valinomycin in the presence of K^+ increases uptake of pyruvate. There is no direct exchange of pyruvate with inorganic phosphate, malate, citrate or α-ketoglutarate. Also pyruvate–pyruvate exchange is not inhibited either by mersalyl or by butylmalonate. This indicates that a pyruvate translocator is different from these mediating the transport of inorganic phosphate and citric acid cycle intermediates. It was shown also that succinate uptake which is normally potentiated by Pi [39] shows little requirement for Pi in the presence of rotenone. This has been explained as being due to the production of fumarate from malate, which is unable to leave the mitochondria; however, fumarate leaves the mitochondria and now promotes succinate uptake [39].

Pyruvate, which does not interfere with the translocation of the substrates may supply reducing equivalents for the reduction of oxaloacetate, thus enhancing its removal and activating SDH at the same time. In addition, resulting increased level of acetyl-CoA might activate malate dehydrogenase in the reverse direction or SDH.

Our findings and interpretation are in agreement with observations from other laboratories. Haslam and Krebs [44], Haslam and Griffiths [45] and Gimpel *et al.* [46] found that intramitochondrial NAD(P)H is oxidized by added oxaloacetate with the formation of malate. Lynis and Tapscott [54] using ferricyanide as an electron acceptor in coupled mitochondria found that succinate oxidation in rat liver mitochondria becomes inhibited after 1-3 minutes of incubation. This inhibition could be prevented by rotenone or glutamate. The authors suggest that higher level of oxaloacetate in the ferricyanide reaction is due to an increased rate of synthesis of this intermediate. They postulate a control

mechanism which blocks normally oxidation of malate, which is lost in the presence of ferricyanide.

Recently, Garber and Salganiceff [43], in their studies on the control of malate oxidation, demonstrated an intramitochondrial accumulation of oxaloacetate during malate oxidation which was inhibitory toward further malate oxidation (with K_I for oxaloacetate toward malate dehydrogenase of 0.25 mM) [55]. The addition of glutamate or pyruvate reduced the level of oxaloacetate. These studies showed that malate dehydrogenase functions in an equilibrium state in intact mitochondria and that other metabolic pathways play an important role in influencing the steady state concentration of oxaloacetate.

Acknowledgment

Research supported by N.I.H. grant #PR 0806-02.

References

1. M. Gutman, E. B. Kearney and T. P. Singer, *Biochemistry*, 10 (1971) 4763.
2. R. L. Ringler and T. P. Singer, *J. Biol. Chem.*, 234 (1959) 2211.
3. C. Y. Wu and C. L. Tsou, *Sci. Sinica*, 4 (1955) 137.
4. T. Kimura and T. P. Singer, *Nature*, 184 (1959) 791.
5. T. Kimura, T. P. Singer and C. J. Lusty, *Biochim. Biophys. Acta*, 44 (1960) 284.
6. R. L. Ringler and T. P. Singer, *Arch. Biochem. Biophys.*, 77 (1958) 229.
7. T. P. Singer, in: *Biological Oxidations*, ed. T. P. Singer (1968), p. 339, John Wiley-Interscience, New York.
8. M. Gutman, E. B. Kearney and T. P. Singer, *Biochemistry*, 10 (1971) 2726.
9. M. Gutman, E. B. Kearney and T. P. Singer, *Biochem. Biophys. Res. Comm.*, 44 (1971) 526.
10. E. B. Kearney, M. Mayr and T. P. Singer, *Biochem. Biophys. Res. Comm.*, 46 (1972) 531.
11. T. P. Singer, E. B. Kearney and M. Gutman, in: *Biochemical Regulatory Mechanisms in Eukaryotic Cells*, eds E. Kun and S. Grisolia (1972), p. 271, Wiley-Interscience.
12. T. P. Singer, E. B. Kearney and W. C. Kenney, *Advan. Enzymol.*, 37 (1973) 189.
13. P. Cerletti and A. Manzocchi, *Acta Vitamin. Enzymol.*, 1-4 (1973) 5.
14. M. Gutman, E. B. Kearney and T. P. Singer, *Biochem. Biophys. Res. Comm.*, 42 (1971) 1016.
15. T. P. Singer, M. Gutman and E. B. Kearney, *FEBS Lett.*, 17 (1971) 11.
16. P. A. Srere, in: *Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria*, eds M. A. Mehlman and R. W. Hanson (1972), p. 79, Academic Press, New York.
17. M. Klingenberg, in: *Biological Oxidations*, ed. T. P. Singer (1968), p. 3, John Wiley-Interscience, New York.
18. T. P. Singer, E. B. Kearney and M. Gutman, in: *Biochemistry and Biophysics of Mitochondrial Membranes*, eds G. F. Azzone, E. Carafoli, A. L. Lehninger, E. Quagliariello and N. Siliprandi (1972), p. 41, Academic Press, New York.
19. W. P. Zeylemaker, D. V. DerVartanian, C. Veeger and E. C. Slater, *Biochim. Biophys. Acta*, 178 (1969) 213.
20. D. V. DerVartanian and C. Veeger, *Biochim. Biophys. Acta*, 92 (1964) 233.
21. D. V. DerVartanian and C. Veeger, *Biochim. Biophys. Acta*, 105 (1965) 424.
22. E. C. Slater and W. D. Bonner, *Biochem. J.*, 52 (1952) 185.

23. W. P. Zylemaker, A. D. H. Klasse and E. C. Slater, *Biochim. Biophys. Acta*, 191 (1969) 229.
24. E. B. Kearney, B. A. C. Ackrell and M. Mayr, *Biochem. Biophys. Res. Comm.*, 49 (1972) 1115.
25. T. P. Singer, E. B. Kearney and B. A. C. Ackrell, *9th Intern. Congress Biochemistry* (1973), Abstract Book, 4 S a 3, p. 207.
26. E. B. Kearney, *J. Biol. Chem.*, 229 (1957) 363.
27. E. B. Kearney, T. P. Singer and N. Zastrow, *Arch. Biochem. Biophys.*, 55 (1955) 579.
28. E. B. Kearney, in: *Proceedings Intern. Symp. Enzyme Chem.* (1958), Tokyo and Kyoto, p. 340, Maruzen, Tokyo.
29. T. P. Singer, E. B. Kearney and V. Massey, *Advan. Enzymol.*, 18 (1957) 65.
30. T. Kimura, J. Hauber and T. P. Singer, *J. Biol. Chem.*, 242 (1967) 4987.
31. M. Gutman and A. Gopher, *FEBS Lett.*, 35 (1973) 103.
32. C. T. Gregg, in: *Methods in Enzymology*, eds S. P. Colowick and N. O. Kaplan, vol. X (1967), p. 181, Academic Press, New York.
33. E. Layne, in: *Methods in Enzymology*, eds S. P. Colowick and N. O. Kaplan, vol. III (1957), p. 450, Academic Press, New York.
34. C. Veeger, in: *Flavins and Flavoproteins*, ed. H. Kamin (1971), p. 642, University Park Press, Baltimore, Butterworths, London.
35. W. G. Bradsley and R. E. Childs, *Biochem. J.*, 137 (1974) 55.
36. W. G. Bradsley, R. E. Childs and M. J. Crabbe, *Biochem. J.*, 137 (1974) 61.
37. E. Quagliariello and F. Palmieri, in: *The Energy Level and Metabolic Control in Mitochondria*, eds S. Papa, J. M. Tager, E. Quagliariello and E. C. Slater (1969), p. 68, Adriatica Editrice, Bari.
38. K. Van Dam and C. S. Tsou, in: *ibidem*, p. 21.
39. J. B. Chappell and K. M. Haarhoff, in: *Biochemistry of Mitochondria*, eds E. C. Slater, Z. Kaniuga and L. Wojtczak (1967), p. 75, Academic Press, New York.
40. J. B. Chappell, J. D. McGiven and M. Crompton, in: *The Molecular Basis of Biological Transport*, eds J. F. Woessner and F. Huijing (1972), p. 55, Academic Press, New York.
41. E. Quagliariello and F. Palmieri, in: *Biochemistry and Biophysics of Mitochondrial Membranes*, eds G. F. Azzone, E. Carafoli, A. L. Lehninger, E. Quagliariello and N. Siliprandi (1972), p. 659, Academic Press, New York.
42. C. L. Moore, in: *Metabolic Transport*, ed. L. E. Hokin (1972), p. 573, Academic Press, New York.
43. A. J. Garber and L. Salganicoff, *J. Biol. Chem.*, 248 (1973) 1520.
44. J. M. Haslam and H. A. Krebs, *Biochem. J.*, 107 (1968) 659.
45. J. M. Haslam and D. E. Griffiths, *Biochem. J.*, 109 (1968) 921.
46. J. A. Gimpel, E. J. DeHaan and J. M. Tager, *Biochim. Biophys. Acta*, 292 (1973) 582.
47. E. B. Kearney, M. Mayr and T. P. Singer, *Biochem. Biophys. Res. Comm.*, 46 (1972) 531.
48. W. W. Kay and H. L. Kornberg, *Eur. J. Biochem.*, 18 (1971) 274.
49. M. Klingenberg and P. Schollmeyer, *Biochem. Biophys. Res. Comm.*, 4 (1961) 38.
50. P. Schollmeyer and M. Klingenberg, *Biochem. Biophys. Res. Comm.*, 4 (1961) 43.
51. S. Papa, A. Francavilla, G. Pradies and B. Meduri, *FEBS Lett.*, 12 (1971) 285.
52. S. Papa, N. E. Lofrumento, M. Coglisci and E. Quagliariello, *Biochim. Biophys. Acta*, 189 (1969) 311.
53. S. Papa, N. E. Lofrumento, E. Quagliariello, A. J. Meijer and J. M. Tager, *J. Bioenergetics*, 1 (1970) 287.
54. G. Lynis and E. B. Tapscott, *Biochem. Biophys. Res. Comm.*, 52 (1973) 246.
55. F. C. Grim and D. G. Doherty, *J. Biol. Chem.*, 236 (1961) 1980.