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METABOLISM AND ENZYMOLOGY OF FLUOROSUCCINIC ACIDS

I. INTERACTIONS WITH THE SUCCINATE OXIDASE SYSTEM

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SUMMARY

1. Both monofluorosuccinate and 2,2-difluorosuccinate are metabolized by Keilin–Hartree particles containing succinate oxidase and fumarate hydratase to give oxaloacetate as a final product.

2. DL-Monofluorosuccinate is a substrate oxidized by the succinate oxidase system, with a v_{max} from 35 to 40% that of succinate and a K_m of about 1.0 mM, slightly larger than that for succinate. The product is monofluorofumarate.

3. 2,2-Difluorosuccinate inhibits the oxidation of succinate by succinate oxidase in a competitive manner with a K_i of approx. 0.5 mM. It also slowly eliminates HF in the presence of the submitochondrial particles to give monofluorofumarate.

4. Monofluorofumarate reacts in the presence of fumarate hydratase to give an unstable 2-fluoromalate which eliminates HF to give oxaloacetate. The production of HF was monitored by complex formation with yeast peroxidase; the production of oxaloacetate by inhibition of succinate oxidase activity and by its spectrum in the ultraviolet region.

5. Oxaloacetate inhibition could be treated as if oxaloacetate were a competitive inhibitor of the particulate succinate oxidase with an apparent association rate constant of $6 \cdot 10^3$ $M^{-1} \cdot sec^{-1}$, a dissociation constant of $2 \cdot 10^{-3}$ sec^{-1} and an effective equilibrium constant of 0.3 μM at pH 7.4 and 25°. No evidence for any more complex binding process was obtained, although the occurrence of other steps during the reaction cannot be eliminated.

6. The metabolic pathway followed by the fluorosuccinates explains their relative lack of toxicity compared with fluoroacetate, and the corresponding marked differences in toxicity between compounds (such as ω-fluoro odd-carbon fatty acids) which give fluorinated 3-carbon fragments and those (such as ω-fluoro even-carbon fatty acids) which give fluorinated 2-carbon fragments.

7. The significance of the inhibitor and substrate relationships for various succinate analogs reacting with succinate dehydrogenase is discussed.

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INTRODUCTION

Fluoroacetate is a deadly poison. ELLIOTT AND KALNITSKY[1] showed that this was due to the synthesis of fluorocitrate from oxaloacetate and fluoroacetyl-CoA, and the subsequent inhibition of aconitate hydratase by the citrate analog so formed. PETERS[2] recognized the phenomenon as an example of "lethal synthesis" by the enzymes of the Krebs cycle, one enzyme acting on a substrate to produce an inhibitor of a following enzyme, thus blocking the entire cycle. Monofluorosuccinate, however, is a very poor metabolic poison. Nevertheless, by analogy with other monosubstituted succinic acids[3], at least the L-isomer may be expected to act as a substrate for succinate dehydrogenase. The resulting product, monofluorofumarate, has recently been shown to act as a substrate for fumarate hydratase[4]. Monofluorosuccinate should thus enter the Krebs cycle and be metabolized.

The anticipated behavior of monofluorosuccinate may be contrasted with that of other fluorosubstituted analogs of succinate. Difluorosuccinate (2,2-substituted), trifluorosuccinate and perfluorosuccinate are all incapable of donating reducing equivalents to the respiratory system. Their effects upon succinate dehydrogenase, however, may be expected to throw light on the curious specificity shown by this enzyme. Thus both oxaloacetate (a naturally occurring member of the Krebs cycle) and malonate are powerful inhibitors of succinate dehydrogenase, the former binding with an affinity 500 times greater than the apparent affinity for succinate, the latter with an affinity some 50 times greater (comparing K_i and K_m). The reaction with oxaloacetate differs in some kinetic respects from that with other inhibitors, and it has even been suggested that it can behave as a noncompetitive rather than a competitive inhibitor[6].

We have therefore studied the response of the succinate oxidase system to these fluorosubstituted succinate analogs and compared the effects to those produced by the naturally occurring inhibitor oxaloacetate. A later paper[7] will describe the extension of these studies to the soluble dehydrogenase. Spectrophotometric and ^{19}F nuclear magnetic resonance experiments with the isolated enzyme may be expected to facilitate a more detailed study of the enzyme–substrate interaction. The present paper outlines the general "metabolic" features of such systems. A preliminary account of some of this work has appeared previously[8].

MATERIALS AND METHODS

Keilin–Hartree beef heart submitochondrial particles were prepared essentially according to the method of KING[9], with the substitution of 1-min homogenization at high speed in a large stainless steel Waring Blendor for the traditional sand grinding in a mechanical mortar. Fumarate hydratase and malate dehydrogenase were commercial enzyme preparations (Calbiochem). Cytochrome *c* peroxidase (employed as an assay system for F^- release) was prepared by the method of YONETANI AND RAY[10] and was the gift of Dr. E. Mochan of this institution. Cytochrome *c* was Sigma Type III.

DL-Monofluorosuccinic acid was synthesized by the method of DEAN AND PATTISON[11], and recrystallized from ethyl acetate when necessary. Preparations used had a melting point of from 143 to 145° (see below for a discussion of fumarate contamination). Difluorosuccinic acid was synthesized by the procedure of RAASCH AND

CASTLE[12], and recrystallized from nitromethane when necessary (see below for a discussion of fluorofumarate contamination). Monofluorofumaric and trifluorosuccinic acids were kindly provided by Dr. M. S. Raasch (Central Research Department, Experimental Station, E. I. DuPont Co., Wilmington, Del., U.S.A.). Oxaloacetic acid (Calbiochem) was prepared in 0.1 M phosphate buffer and adjusted to a pH of 7.8. Other reagents used were of analytical grade wherever possible.

Succinate and monofluorosuccinate oxidation were measured either manometrically[13], spectrophotometrically by cytochrome *c* reduction at 550 mμ (ref. 14), or by the appearance of unsaturated bonds at 230 mμ (ref. 15). Manometric studies were made by means of the Gilson differential respirometer with semi-micro flasks at 30°. Spectrophotometry was carried out with a Zeiss PMQ II instrument with Sargent linear-log recorder, the cell compartment at 25°. Fumarate and fluorofumarate were estimated by the fumarate hydratase-sensitive absorption between 260 and 220 mμ (ε_{mM} fumarate at 230 mμ = 4.7), oxaloacetate by the absorption between 300 and 240 mμ (ε_{mM} oxaloacetate at 270 mμ = 1.0). By coincidence, the absorption of oxaloacetate at 230 mμ is closely similar to that of an equilibrium fumarate–malate mixture of equivalent total concentration; this temporarily obscured the significance of the results with monofluorosuccinate (below).

RESULTS

Oxidation of monofluorosuccinate by succinate oxidase

As reported previously[8], monofluorosuccinate proved to be a substrate for succinate oxidase by all methods employed. A maximum rate between 35 and 40% of that found with succinate itself was obtained. The fact that four methods (phenazine methosulfate reduction, cytochrome *c* reduction, oxygen uptake, and product appearance) gave the same initial rate indicates that the only differences from the succinate reaction lie at the level of the dehydrogenase itself.

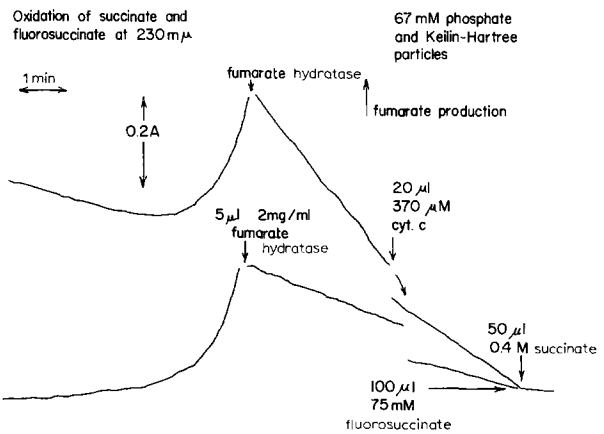

Fig. 1. Oxidation of succinate and fluorosuccinate measured by fumarate and fluorofumarate appearance at 230 mμ. 6.7 mM succinate or 2.5 mM fluorosuccinate in 3 ml phosphate buffer (pH 7.4) with 0.2 mg·ml^{-1} (protein) Keilin–Hartree particles in 1-cm cells at 25°. Cytochrome *c* (2.1 μM) and fumarate hydratase additions as indicated. Note: direction of time is from right to left.

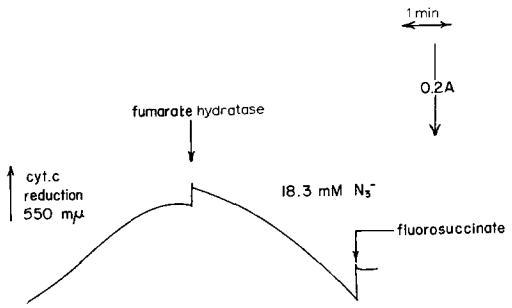

Fig. 2. Reduction of cytochrome *c* by fluorosuccinate and reoxidation following fumarate hydratase addition. 2.5 mM fluorosuccinate added to 37 μM cytochrome *c* solution (pH 7.4 phosphate buffer, 67 mM) in presence of 0.48 mg·ml^{-1} (protein) Keilin–Hartree particles. 18 mM N_3 added to increase steady-state reduction measured at 550 mμ. 1-cm cells, 3 ml final volume at 25°. Note: direction of time is from right to left.

Fig. 1 shows the oxidation of both succinate and fluorosuccinate measured spectrophotometrically at 230 mμ. Following an addition of fumarase to the system oxidizing succinate (upper curve) there is a rapid disappearance of the fumarate absorption at this wavelength, followed by a slower rise at approx. one fourth of the initial rate, corresponding to the equilibrium constant of 4 for the malate–fumarate system under these conditions. The lower curve shows that monofluorosuccinate also gives rise to a product with absorption at 230 mμ. In this case, however, addition of fumarate hydratase results in a total cessation of the reaction.

Fig. 2 shows that when monofluorosuccinate is added to particles in the presence of a high azide concentration (to inhibit cytochrome oxidase activity by some 99%), the reduction of exogenous cytochrome *c* can be followed in the same way as with succinate[14], until a steady state is reached. In this case the addition of fumarate hydratase causes the cytochrome *c* to be reoxidized after a brief lag period. This suggests that fumarate hydratase has induced the formation of an inhibitor of the

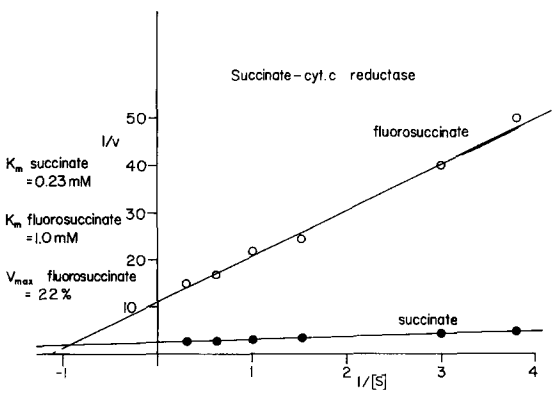

Fig. 3. Lineweaver–Burk plot for oxidation of succinate and fluorosuccinate assayed by cytochrome *c* reductase. Reduction of 38 μM cytochrome *c* by 0.5 mg·ml^{-1} particles in 67 mM phosphate (pH 7.4) in presence of 0.33 mM KCN. Velocity in $\Delta A \cdot \text{min}^{-1}$ at 550 mμ. Succinate or fluorosuccinate concentration in mM. 1-cm cells, 3 ml final volume at 23°.

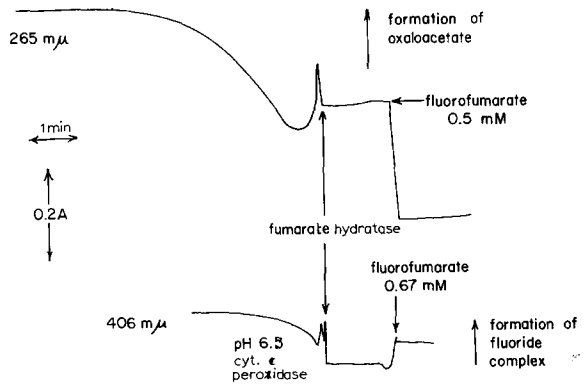

Fig. 4. Formation of fluoromalate and oxaloacetate, and release of F^- in reaction between fluorofumarate and fumarate hydratase. The upper trace shows the hydration of 0.5 mM fluorofumarate by 25 μl 2 mg·ml^{-1} fumarate hydratase in 60 mM phosphate (pH 7.4) to form fluoromalate; measured at 265 mμ in 1-cm cell, 3 ml volume at 25°. The lower trace shows the effect of the hydration of 0.67 mM fluorofumarate in 40 mM phosphate (pH 6.5) upon 11 μM cytochrome *c* peroxidase, which is converted to its F^- complex upon the addition of 25 μl 2 mg·ml^{-1} fumarate hydratase to the system (measured at 406 mμ). Note: direction of time is from right to left.

succinate–cytochrome *c* reductase reaction. The reoxidation, by that part of the oxidase that is not bound by azide, reflects the decreased ratio of reductase to oxidase activity in the particles.

Fig. 3 summarizes the results of a series of experiments of the type shown in Fig. 2 at different monofluorosuccinate concentrations. A Lineweaver–Burk plot of the reciprocal of the initial velocity of cytochrome *c* reduction in the absence of added fumarate hydratase is given against the reciprocal of substrate concentration for both succinate and monofluorosuccinate. The K_m for monofluorosuccinate was found to be 1.0 mM (4 times greater than that for succinate) with a v_{max} varying from 20 to 40% of that with succinate.

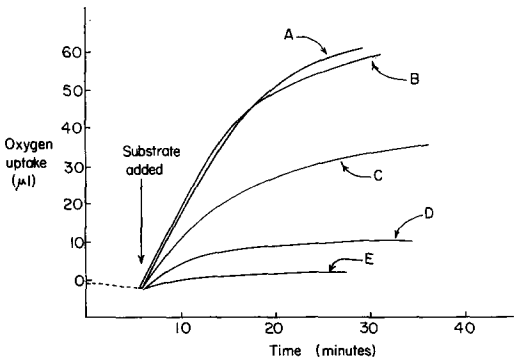

Fig. 5. Time course of monofluorosuccinate oxidation measured manometrically. Manometric experiments carried out at 30° in 67 mM phosphate (pH 7.4) containing 5.5 μM cytochrome *c* and 2 mg particles in 1.5 ml final volume. A, 13 mM succinate alone (control); B, 13 mM succinate + 11 mM monofluorosuccinate; C, 13 mM succinate + 11 mM monofluorosuccinate + 20 μg fumarate hydratase; D, 11 mM monofluorosuccinate alone; E, 11 mM monofluorosuccinate + 20 μg fumarate hydratase.

The expected product of fluorosuccinate oxidation, monofluorofumarate, is known to be a substrate for fumarate hydratase[4,16]. Fig. 4 is a composite summary of two experiments upon the reaction of monofluorofumarate with fumarate hydratase. At 230 mμ, monofluorofumarate reacts in a closely similar fashion to fumarate itself (*cf.* Fig. 1). However, at 265 mμ (top trace) a biphasic response is seen on the addition of enzyme. An initial product is formed with little absorption at this wavelength, followed by the secondary production of a strongly absorbing material with the spectrum of oxaloacetate. The lower curve shows that the appearance of oxaloacetate is accompanied by a release of F^-. The method employed is made possible by the unusually high affinity of yeast peroxidase (cytochrome *c* peroxidase) for F^-. At pH 6.5, the dissociation constant for cytochrome *c* peroxidase and F^- at 25° is about 75 μM (refs. 17, 18). The generation of 660 μM F^- from fluorofumarate therefore results in almost complete formation of the cytochrome *c* peroxidase–F^- complex from 5 μM cytochrome *c* peroxidase as measured at 406 mμ, the Soret band (Fig. 4). Experiments at pH 7.5, where the dissociation constant is 10 times larger[18], correspondingly showed only partial formation of the complex under these conditions. The time-course for cytochrome *c* peroxidase–F^- formation does not show the lag phase expected from the delay in the fluoromalate to oxaloacetate reaction shown in the upper curve. This, however, is understandable if it is remembered that 50% complex formation in Fig. 4 corresponds to only 12% breakdown of fluoromalate. The effect of the peroxidase assay is to "compress" the lag phase.

These observations support the conclusion reached by CLARKE *et al.*[16], that the immediate product of fumarate hydratase action on monofluorofumarate is an unstable 2-fluoromalate which undergoes spontaneous hydrolysis to oxaloacetate and F^-. Indeed, TEIPEL *et al.*[4] have obtained evidence that fluorofumarate is unique in this respect among the halogenated fumarates. All the other halofumarates, which also react much more slowly with fumarate hydratase, give the corresponding halogenated malates in which the halogen is on the β-carbon atom.

The formation of oxaloacetate from fluorofumarate explains the inhibition of monofluorosuccinate oxidation shown in Figs. 1 and 2 following fumarate hydratase addition, since oxaloacetate is a potent inhibitor of the dehydrogenase[6,19].

KEILIN AND HARTREE[13] showed that the oxidation of succinate manometrically was linear with time if carried out with freshly prepared particles. Declines in the rate of succinate oxidation with time, characteristic of other particle preparations, required the presence of fumarate hydratase, malate dehydrogenase and NAD (each or all of which may be present in particles prepared by their method[9]), and could be attributed to the oxidative production of oxaloacetate. Evidently the corresponding phenomenon with monofluorosuccinate requires only the activation of particle-bound fumarate hydratase, and is independent of malate dehydrogenase and NAD. Fig. 5 compares the oxidation of succinate and monofluorofumarate by fresh and "aged" samples of particles. With both substrates, an inhibited state supervenes in the presence of "aged" particles; this inhibition occurs more rapidly and completely with monofluorofumarate. With fresh particles, fumarate hydratase is required to induce the inhibited state with monofluorofumarate, although it has no effect on the succinate reaction (the inhibition of which demands other cofactors[13]). Such inhibition of monofluorofumarate oxidation, like that of succinate oxidation[13], can be partially relieved by the presence of glutamate at relatively high concentration (0.1 M), which

presumably permits the transamination of oxaloacetate in the particles. Incubation with monofluorofumarate also inhibits the activity of the particle toward succinate almost completely, as might be expected.

Not all the peculiarities of monofluorosuccinate oxidation by submitochondrial particles can, however, be attributed to the action of fumarate hydratase upon the oxidation product, to form oxaloacetate. Firstly, even in the absence of added fumarate hydratase, and with fresh particles whose intrinsic fumarate hydratase activity is low, the manometric oxidation of monofluorosuccinate shows a decline in rate after 5–10 min (Fig. 5). This suggests that fluorofumarate itself is acting as an inhibitor of the reaction. As will be shown in the following paper[7], this is indeed the case, monofluorofumarate showing a K_i of about 0.15 mM. In the succinate–fumarate system[19], the ratio K_i (product):K_m (substrate) is 1.5:0.25 (mM) which equals 6; in the monofluorosuccinate–monofluorofumarate system, the corresponding ratio is 0.15:1.0 (mM), which equals 0.15. Thus in the latter case, product accumulation will rapidly affect reaction rate; in the former case, the reaction will be independent of product accumulation over most of its course. Secondly, samples of fluorosuccinate that have been allowed to stand in solution show a much lower activity (from 4 to 10% instead of 35 to 40% of the succinate rate) than when freshly recrystallized. Inhibitors are therefore generated directly from the substrate in the absence of enzyme. These include the products of HF elimination, fumarate and F^-, both of which can act as inhibitors of succinate dehydrogenase[15,19]. Thus spectroscopic observations on monofluorosuccinate solutions at 230 mμ, before and after fumarate hydratase addition, have identified from 0.2% fumarate (in freshly prepared solution) to 2.5% fumarate (in samples several days old) present. Only samples with very low fumarate (and presumably F^-) content give maximal oxidation rates. Whether other inhibitors such as monofluorofumarate and oxaloacetate themselves, are present in aged solutions of monofluorosuccinate is still uncertain.

Inhibition of succinate oxidase by 2,2-difluorosuccinate

2,2-Difluorosuccinate showed no hydrogen donor activity in any of the systems tested. It was incapable of supporting oxygen or cytochrome *c* and phenazine methosulfate reduction spectrophotometrically. As shown in Fig. 6, following incubation with particles under manometric conditions, it appeared to act as a largely competitive inhibitor of succinate oxidation with a rather low K_i of about 6 μM. Small deviations from strictly competitive behavior may, however, occur (Fig. 6), and spectrophotometric studies of cytochrome *c* reduction indicated a mixed type of inhibition with both competitive and noncompetitive characteristics. This was resolved into an immediate competitive inhibition, followed by a more slowly developing inhibition less rapidly reversed by succinate. Fig. 7 shows the initial inhibition observed spectrophotometrically, with a competitive K_i calculated as approx. 0.5 mM from the results at higher succinate concentrations. This appears to be the primary effect due to difluorosuccinate itself (see ref. 7).

The slow inhibition also proved to be competitive in nature, but dependent on the production of fluorofumarate from difluorosuccinate and on the presence of active fumarate hydratase in the particle preparation. It could thus account for the much lower apparent K_i found for difluorosuccinate in manometric experiments (Fig. 6). Fig. 8 shows the decline in succinate–cytochrome *c* reductase activity during incu-

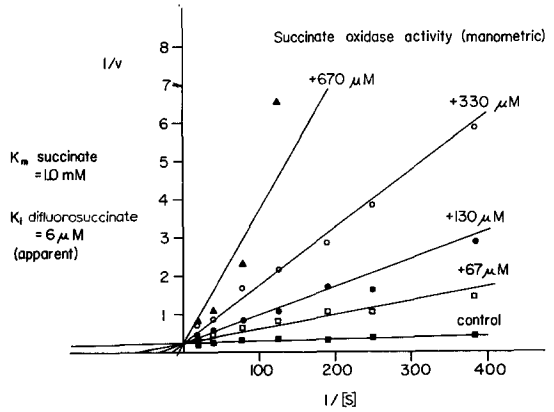

Fig. 6. Lineweaver–Burk plots for oxidation of succinate following preincubation of particles and 2.2-difluorosuccinate. Manometric experiments at 30° in 67 mM phosphate (pH 7.4). 13 μM cytochrome *c*, 0.4 mg particles in 1.5 ml final volume. 2.6–53 mM succinate (apparent K_m of 1 mM). Preincubation at 30° for 30 min with difluorosuccinate concentrations indicated. $1/[S]$ in M^{-1}, $(1/v)$ in $\mu l \cdot min^{-1}$. Maximal activity in absence of inhibitor was 4 $\mu l \cdot min^{-1}$.

bation of particles with difluorosuccinate (a time scale similar to that involved in the manometric equilibration period). This decline may probably be attributed to the action of fumarate hydratase associated with the particles, as the addition of excess fumarate hydratase (lower curve) rapidly gave an enzyme with the same degree of inhibition.

The sample of difluorosuccinate used in the experiment of Fig. 6 was found to contain some 4% fluorofumarate by measurement at 230 mμ and conversion to oxaloacetate by fumarate hydratase (MATERIALS AND METHODS). Assuming a K_i of 0.25 μM for oxaloacetate (below), this would give an apparent K_i of $0.25 \times 100/4 =$ 6 μM (*cf.* the value given in Fig. 6). A recrystallized sample with 0.3% monofluoro-

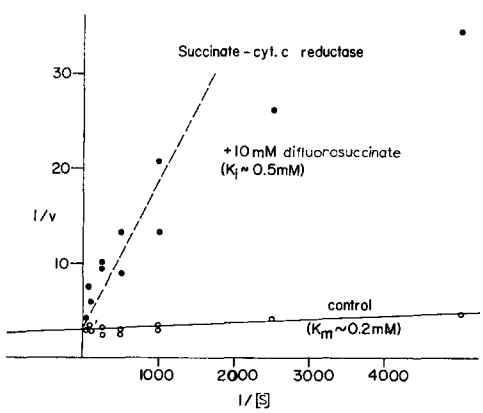

Fig. 7. Lineweaver–Burk plots for oxidation of succinate and cytochrome *c* reduction showing inhibition by 2,2-difluorosuccinate without preincubation. Reduction of 37 μM cytochrome *c* followed spectrophotometrically at 550 mμ in presence of 200 μM KCN in 60 mM phosphate (pH 7.4) using 0.1 mg·ml^{-1} particles in a final volume of 1.0 ml. $1/v$ in $(\Delta A_{550\ m\mu}/min)^{-1}$, $1/[S]$ in M^{-1}.

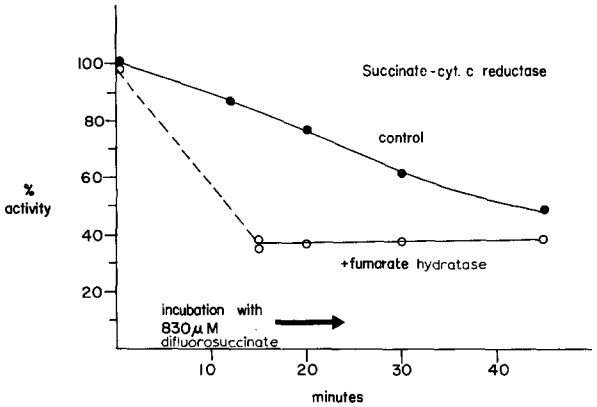

Fig. 8. Progressive inhibition of succinate cytochrome *c* reductase by difluorosuccinate in presence and absence of fumarase. 0.02 mg·ml^{-1} particles incubated in 67 mM phosphate (pH 7.4) at 25° with 830 μM difluorosuccinate in presence and absence of 3.3 μg·ml^{-1} fumarate hydratase. Cytochrome *c* reductase activity then measured with 33 μM cytochrome *c*, 6.7 mM succinate and 330 μM KCN. "100%" = 0.18 ΔA·min^{-1} ($\simeq$ 9 μM cytochrome *c*·min^{-1}).

fumarate (giving an expected apparent K_i of $0.25 \times 100/0.3 = 80$ μM) gave higher apparent K_i values of 60 μM (manometric) and 20 μM (spectrophotometric) after preincubation but the same K_i values of 1.0 mM (manometric) and 0.4 mM (spectrophotometric) without preincubation.

Fig. 9 shows that, as required thermodynamically, particles preincubated with difluorosuccinate show an initial inhibition on addition of succinate (a "pseudo-noncompetitive" phase) that then declined to the expected competitive value. Parallel experiments (described below) with oxaloacetate show that this kinetic behavior can also be matched by the behavior of oxaloacetate itself. A subsequent paper[7] will present both spectroscopic and kinetic data to support the view that the fluorofumarate

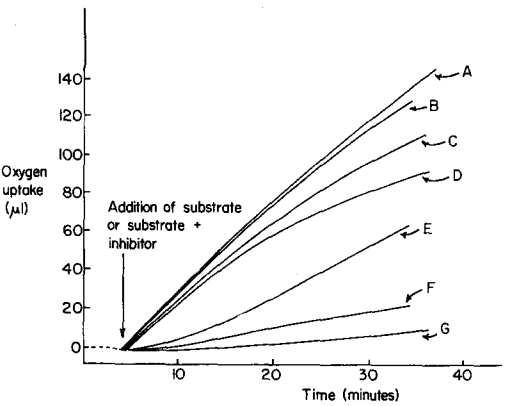

Fig. 9. Initial and final inhibitions produced by difluorosuccinate; manometric reversal of the inhibition upon addition of succinate. 0.27 mg·ml^{-1} particles oxidizing 50 mM succinate in 67 mM phosphate (pH 7.4) with 13 μM at 30°. A, no other additions (control); B, C and D, 0.13, 1.3 and 4.0 mM difluorosuccinate added simultaneously with succinate, respectively; E, F and G, preincubated for 30 min with 0.13, 1.3 and 4.0 mM difluorosuccinate, respectively.

produced (which gives rise *via* fumarate hydratase to oxaloacetate) is not only formed by HF elimination during difluorosuccinate synthesis, but is also formed by the direct action of succinate dehydrogenase on difluorosuccinate.

Inhibition of succinate oxidase by oxaloacetate

Oxaloacetate formation has been proposed to explain: (a) the progressive inhibition of succinate oxidation in aged preparations of submitochondrial particles[13]; (b) the progressive inhibition of monofluorosuccinate oxidation in all systems (the present paper); (c) the occurrence of two types of inhibition by difluorosuccinate, immediate and slow (the present paper).

Oxaloacetate has also been widely discussed[6,20,21] as one of the major controlling substances in mitochondrial succinate oxidation, and it is recognized as the most powerful competitive inhibitor of succinate dehydrogenase[22]. It forms an unusual spectroscopic complex with the soluble enzyme[19], and its inhibition of ferri-

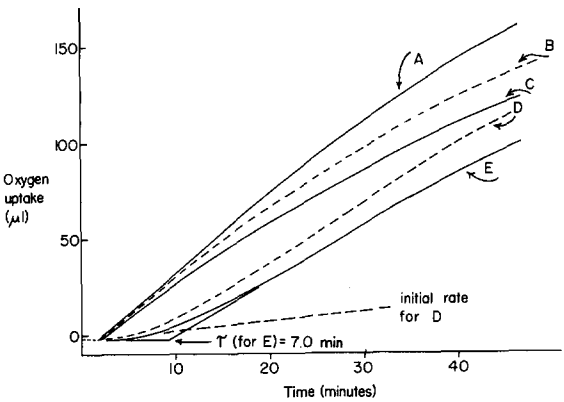

Fig. 10. Initial and final inhibitions produced by oxaloacetate, and manometric reversal of oxaloacetate inhibition upon addition of succinate. A, oxidation of 50 mM succinate (control); B and C, 2.7 and 6.7 μM oxaloacetate added simultaneously with succinate, respectively; D and E, preincubated for 30 min with 2.7 and 6.7 μM oxaloacetate, respectively. τ, lag phase for release of oxaloacetate inhibition. K_m (succinate)/K_i (oxaloacetate) $\simeq$ 4000.

cyanide reduction by the latter enzyme seems to be biphasic in character[23]. It therefore seemed worthwhile to quantitate its effect on the particulate succinate oxidase under the conditions used to study the fluorosuccinates.

Very low concentrations of oxaloacetate were required to inhibit succinate oxidase and succinate–cytochrome *c* reductase. In both systems, the initial reaction rate following succinate addition of oxaloacetate-incubated enzyme is independent of succinate concentration (*i.e.* is "noncompetitive") and indicates a K_i of 0.25 μM at 25° in pH 7.4, 67 mM phosphate buffer. Thus particles in presence of 2.5 μM oxaloacetate are initially 90% inhibited on addition of 5 mM succinate. The final reaction rate is dependent on succinate concentration, and calculation of K_i on the basis of strict competition gives the same value. That is, particles in presence of 2.5 μM oxaloacetate are 60% inhibited in steady state with 5 mM succinate. This situation can be provisionally interpreted as reflecting the slowness of oxaloacetate dissociation

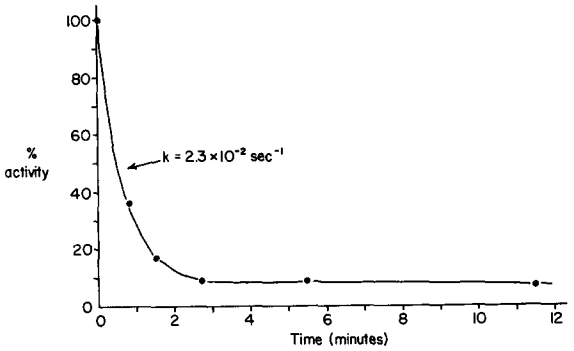

Fig. 11. Progressive inhibition of succinate cytochrome *c* reductase activity by incubation with oxaloacetate. Cytochrome *c* reductase activity measured as in Fig. 8. Inhibition of activity (monitored following addition of 6.7 mM succinate) after incubation with 4 μM oxaloacetate. Exponential decline reflects oxaloacetate binding. Measured activity is initial activity; K_i (oxaloacetate) = 0.3 μM (see text). Other conditions as in Fig. 8.

following the addition of succinate. Fig. 10 illustrates the slow onset and reversal of oxaloacetate inhibition obtained in a typical monometric experiment.

The binding of oxaloacetate to the particles can also be followed kinetically by the decline in succinate–cytochrome *c* reductase activity measured at varying intervals after oxaloacetate addition. Such a series of experiments is summarized in Fig. 11. Conversely, the dissociation of oxaloacetate can be followed by measuring the lag time before steady-state succinate oxidation is restored on addition of succinate to an oxaloacetate-inhibited system (as in Fig. 10 or comparable spectrophotometric studies). Average values obtained for these apparent kinetic constants at pH 7.4 and 25° are $6 \cdot 10^3$ $M^{-1} \cdot sec^{-1}$ for k_{on} and $2 \cdot 10^{-3}$ sec^{-1} for k_{off}. Assuming that the binding of oxaloacetate is a simple bimolecular reaction (see DISCUSSION), then the ratio k_{off}/k_{on} should equal the dissociation constant K_i. The value obtained here, 0.3 μM, may be compared with the value of 0.25 μM observed directly. Despite the unusual spectrum of the enzyme–oxaloacetate complex[19,7], and the unusual inhibitory pattern in the ferricyanide assay[23], there is thus no need to postulate more than one binding reaction for the particulate system. Calculation of K_i by three methods (i) competition with succinate, assuming $K_i = K_i (K_m + [S])/K_m$, (ii) directly, by percent inhibition before displacement by succinate, and (iii) as above, by the ratio of velocity constants, has given the same value.

These results may then be used to estimate the amounts of oxaloacetate required in the present assay systems to account for progressive inhibition by mono- and difluorosuccinate. As shown above, the resulting correlations were found to be good.

DISCUSSION

A metabolic scheme for the fluorosuccinates

The fluorosuccinates whose behavior is described here represent in some ways the converse of PETERS[2], "lethal synthesis". In each of the present cases a sequence of two enzymes, succinate dehydrogenase and fumarate hydratase, catalyses the removal of the potentially inhibitory fluorine as F^- and the formation of the normal

metabolite oxaloacetate. The chemical and enzymic transformations involved are shown in Fig. 12. Succinate gives rise to fumarate and malate, but the production of oxaloacetate is dependent on the addition of malate dehydrogenase and NAD. Monofluorosuccinate forms fumarate by non-enzymic HF elimination and fluorofumarate by the action of succinate dehydrogenase. Difluorosuccinate is an inhibitor of succinate dehydrogenase but can form fluorofumarate both by non-enzymic and by enzymic[7] elimination of HF. As suggested by CLARKE *et al.*[16], in each case the fluorofumarate gives fluoromalate *via* fumarate hydratase, and the fluoromalate eliminates HF non-enzymically to give oxaloacetate. The expected "active" form of difluorosuccinate, α,β-difluorosuccinate, is not available, but the "product", difluorofumarate, can be made. This fits into the scheme of Fig. 12 by reacting with fumarate hydratase to give an unstable product, presumably 2,3-difluoromalate, which gives rise to a powerful inhibitor of succinate dehydrogenase, presumably fluorooxaloacetate[7].

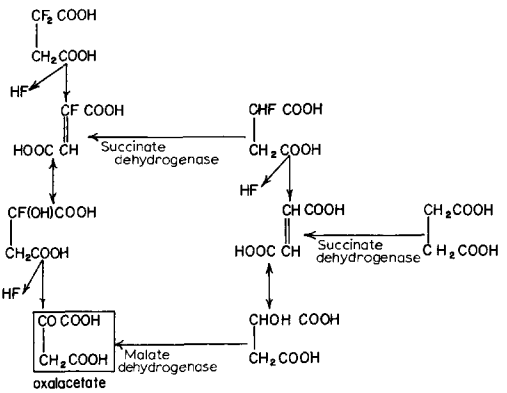

Fig. 12. Metabolic pathways for fluorosuccinates and fluorofumarate in Keilin–Hartree particles.

The "protective biosynthesis" that occurs with fluorosuccinate (Fig. 12) accounts for the remarkable difference between the toxicity of compounds giving rise to fluoro-substituted 3-carbon fragments and of compounds giving rise to fluoro-substituted 2-carbon fragments[24]. A striking example is given by PETERS[25]: a C_{12} ω-fluoro fatty acid blocks citrate metabolism by kidney mitochondria at very low concentrations, while the corresponding C_{13} ω-fluoro fatty acid has no effect at 10 times the concentration.

The behavior of mono- and difluorosuccinate toward succinate dehydrogenase (this paper), and of mono- and difluoro fumarate toward fumarate hydratase[4,7,16], also highlights the exceptional character of fluorocitrate[2] as an inhibitor. The former group of fluoro compounds bind reversibly to the enzymes concerned in a manner analogous to that found for the natural substrate and with affinities not greatly different from the affinity for the substrate. The enzymically formed fluorocitrate binds aconitate hydratase very tightly (K_i approx. $6 \cdot 10^{-9}$ M)[26] in a chemically complex process. It may be noted that the fluorine in fluorocitrate, formed by condensation of fluoroacetyl-CoA and oxaloacetate, is not expected to be on the carbon atom to which the hydroxyl group is transferred (Eqn.1)[27]. We conclude that this inhibition is the

result of some special feature of aconitate hydratase and is not a consequence of general characteristic common to all reactions involving the desaturation of a carbon–carbon bond[28].

(a) Succinate dehydrogenase

$$\begin{array}{l} \text{HOOC–CX} = \text{CH–COOH} \\ \qquad\quad \uparrow\downarrow 2\text{H} \\ \text{HOOC–CHX–CH}_2\text{–COOH} \end{array}$$

(active when X = H or F)

(b) Fumarate hydratase

$$\begin{array}{l} \text{HOOC–CX} = \text{CH–COOH} \\ \qquad\quad \uparrow\downarrow \text{H}_2\text{O} \\ \text{HOOC–CXOH–CH}_2\text{–COOH} \end{array}$$

(active when X = H or F) (1)

(c) Aconitate hydratase

$$\begin{array}{l} \text{HOOC–CH} = \text{C–COOH} \\ \qquad\qquad\quad | \\ \qquad\qquad \text{CHX–COOH} \end{array}$$

$$\begin{array}{l} \uparrow\downarrow \text{H}_2\text{O} \qquad\qquad \uparrow\downarrow \text{H}_2\text{O} \\ \text{HOOC–CHOH–CH–COOH} \qquad \text{HOOC–CH}_2\text{–C(OH)–COOH} \\ \qquad\qquad\quad | \qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad \text{CHXCOOH} \qquad\qquad\quad \text{CHXCOOH} \end{array}$$

(inactive and inhibited when X = F)

Inhibition by oxaloacetate and by other substrate analogs

In every case described here, final inhibition occurred because oxaloacetate was generated, no mechanism for its removal existing in the submitochondrial particles. In intact mitochondrial systems, of course, oxaloacetate can be removed by ATP and phosphoenol pyruvate carboxylase to give phosphoenol pyruvate or by acetyl-CoA and citrate synthetase to give citrate[21]. Some of the perculiarities of the oxaloacetate inhibition[6,20,23] can be attributed to the tightness of binding and slowness of release from the enzyme. The data of ZEYLEMAKER AND SLATER[23], however, suggest further complexities in oxaloacetate inhibition. In particular, the "on" and "off" constants calculated here may not be simple rate constants. But with the particulate enzyme catalyzing the reduction of oxygen and cytochrome *c*, it was not necessary to use more complicated mechanisms; a detailed analysis of the kinetics of oxaloacetate inhibition has yet to be made. The complex formed between oxaloacetate and succinate dehydrogenase also differs spectroscopically from other enzyme–inhibitor complexes[22], in giving a marked increase in absorption of the oxidized enzyme in the 500–700 mμ region. The chemical and physiological significance of all these kinetic and spectroscopic differences is unknown at present.

Fig. 13 compares the structures and affinities of six substrates or inhibitors of succinate dehydrogenase. The oxaloacetate reaction suggests the presence on the enzyme of two neighboring sites, one of which binds the terminal carboxyl group, if it is present, and the other a neighboring carbonyl group. The high affinity shown in the slow reaction with oxaloacetate is probably a metabolic control device for succinate dehydrogenase[6], and the binding of malonate may thus reflect its similarity to oxaloacetate and not any similarity to succinate itself. The slowness of oxaloacetate release, and the spectrum of the final oxaloacetate complex, also suggest a relationship between this complex and the "inactive" form of the enzyme[29]. Relief of the slow oxaloacetate inhibition and the transformation of the inactive to the active form could be chemically similar processes.

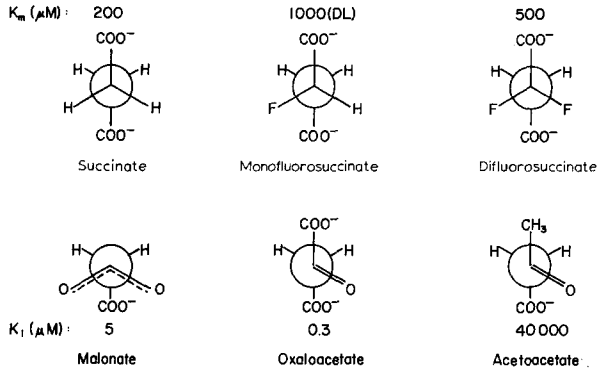

Fig. 13. Structures and affinities of certain substrates and inhibitors of succinate dehydrogenase. Note: K_m and K_i values given here and elsewhere are not directly comparable as the former are steady-state constants while the latter are equilibrium constants.

Although there are some kinetic differences between soluble and particulate succinate dehydrogenase[30], the two forms of the enzyme behave similarly towards a wide range of substrates and inhibitors. Table I compares the apparent K_m and K_i values for the fluorosuccinic acids studied here, including trifluorosuccinate, found to be a comparatively weak inhibitor[7], with the values previously reported for other substrate and inhibitor analogs.

Despite the differences in reactivity (Table I) and spectrum of the complexes produced by the analogs, it is remarkable that pairs of optical isomers, one of which is usually a substrate and the other an inhibitor (malate is an exception[22]), show no

TABLE I

AFFINITIES AND TURNOVER NUMBERS FOR COMPLEXES OF SUCCINATE DEHYDROGENASE AND SUBSTITUTED SUCCINIC ACIDS

K_m depends on nature and concentration of hydrogen acceptor system. Maximum turnover: maximal activity varies from 50 to 250 moles succinate per sec per mole flavin, at pH 7.4, 30°. This basal activity with succinate is taken as "100%". Note that the malates are an exception to the rule that only the L-configuration is active[22].

Substrates	*K_m (mM)*	*Maximum turnover*	*Ref. No.*
Succinate	0.15*–1.0**	[100]	5
DL-Fluorosuccinate	1.0**	35–40	This paper
L-Chlorosuccinate	3.3*	46	3
DL-Bromosuccinate	2.3*	?	3
L-Methylsuccinate	20.0*	7	3
Inhibitors	*K_i (mM)*		*Ref. No.*
2,2-Difluorosuccinate	0.5		This paper
Trifluorosuccinate	3.0		This paper
D-Chlorosuccinate	3.6		3
D-Methylsuccinate	18.0		3

* At low acceptor concentrations.
** At high acceptor concentration (or in succinate oxidase assay).

great difference in apparent affinity for the enzyme. Other evidence[7] suggests that monofluorosuccinate is no exception to this rule. An additional feature may be noted of the substrates other than the natural substrate, succinate. This is that unlike succinate, which can presumably bind in two ways, either of which is catalytically active[31], substrates with an L-substituent (L-fluoro, L-chloro, L-methyl) can bind in two ways, one of which is an active and the other an inactive complex. The inactive complex may be "forbidden"; in this case one would expect an effect on the apparent affinity—a doubling of the apparent K_m at low acceptor concentrations. At the other extreme, the inactive complex may be formed as well as the active complex; in this case one would expect an effect on turnover—a halving of v_{max}. The high maximal rate obtained with racemic DL-fluorosuccinate tentatively indicates the former rather than the latter possibility. These and other mechanistic questions will be treated further in a following paper[7].

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