Oxidation of Succinate and the Control of the Citric Acid Cycle in the Mitochondria of Guinea-Pig Liver, Mammary Gland and Kidney

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Under physiological conditions the control of various steps of the citric acid cycle is governed by the supply and demand of its intermediates. It is likely that the steady state of the cycle *in vivo* is at least partly controlled by the supply of acetyl-CoA. This compound interacts with oxaloacetate and oxalosuccinate to give citrate or malonyl-CoA. The rate of removal of oxaloacetate is an important factor in the control of the oxidation of succinate and of malate, as well as in determining the supply of aspartate and of α -oxoglutarate from glutamate via transamination. In isolated mitochondria, when the supply of substrates is largely controlled by selected additions, one can study the interactions between individual pairs or larger groups of reactions as well as the steady-state control. The present study is a continuation of our investigations into the properties of mitochondrial enzymes in the isolated form and in intact mitochondria, and we were especially interested in the interactions between the oxidation of succinate and other mitochondrial enzyme systems.

Studies of the interaction of glutamate dehydrogenase (Jones & Gutfreund, 1962) and of glutamate transaminase (Gutfreund, Ebner & Mendiola, 1961) with other steps of the citric acid cycle revealed relations with the oxidation of succinate. The synthesis of glutamate is considerably increased by the oxidation of succinate, and the synthesis of aspartate removes oxaloacetate, a powerful inhibitor of the oxidation of succinate. It appeared to be worth while to continue and extend studies of the control of the oxidation of succinate and of other reactions by the succinate-oxidase system. This subject has received considerable attention during the last few years (Tyler, 1955; Krebs, 1961; Chance & Hollinger, 1961; Slater & Hülsmann, 1961; Chappell, 1961a, 1962). Of particular relevance to our studies is Chappell's (1961a) discussion of the importance of the order of addition and of 'compartmentation' in the control of the oxidation of succinate and his finding (Chappell, 1962) that the oxidation of succinate can be much more rapid in uncoupled systems than during oxidative phosphorylation. Our studies also throw further light on Tyler's (1955) and Slater & Hülsmann's (1961) finding that there is an energy

requirement for the removal of inhibitory oxaloacetate during the oxidation of succinate.

The main features of the structure and functions of mitochondria are very similar in a wide range of species and organs. There are, however, several important qualitative and quantitative differences in the enzymic composition of mitochondria, reflecting special needs of particular tissues [see references cited by Jones & Gutfreund (1961) and by Gutfreund *et al.* (1961)]. It is of considerable value to study such kinetic features as appear important to the control of metabolism in mitochondria from several tissues and species so as to be able to assess whether a phenomenon is of specific importance to a particular tissue or whether it is of wider significance. We have made a number of comparisons between the properties of the mitochondria of guinea-pig liver, used for most of our studies, with those of guinea-pig mammary gland and kidney. The mitochondria from these three tissues proved to have sufficient significant differences in behaviour to be of value for comparisons of control phenomena.

EXPERIMENTAL

Mitochondria were prepared as described by Jones & Gutfreund (1961). Kidney mitochondria were prepared from the whole organ. Rates of oxygen uptake were measured either with a Clark oxygen electrode and recording equipment as described by Jones & Gutfreund (1962) or by the conventional Warburg method. The curvilinear recorder used for the oxygen electrode gives traces that are apparently faster at the top of the paper than at the bottom. The rates given were obtained with a more detailed curvilinear grid than that reproduced in the figures. The initial rapid decrease in oxygen concentration on the addition of mitochondria is largely due to the volume of anaerobic solution added. All studies were carried out at 25°. For measurements with the oxygen electrode the total volume of the reaction mixture was 3 ml., of which 2.5 ml. was the following medium: triethanolamine hydrochloride (0.1M); KCl (50 mM); MgCl (20 mM); KH_2PO_4 (20 mM); adjusted to pH 7.4 with KOH. Comparative experiments with the oxygen electrode indicated that tris and triethanolamine could be interchanged without any effect on the respiration. The basic medium used in all Warburg experiments was: tris (20 mM); KH_2PO_4 (16 mM); MgSO_4 (4 mM); sucrose (20 mM); KCl (30 mM);

adjusted to pH 7.4 with KOH. The total volume of the reaction mixture used was 2.5 ml. The phosphorylating medium contained in addition: glucose (8 mM); ATP (0.4 mM); hexokinase (type III; Sigma Chemical Co., St Louis, Mo., U.S.A.) (1 mg.).

METHODS

Chromatographic separation of labelled intermediates. Reactions were carried out in the Warburg apparatus and stopped by the addition of 0.5 ml. of N-sulphuric acid. Each mixture was then centrifuged, and a known volume of supernatant was continuously extracted with diethyl ether for 12 hr. The ether was allowed to evaporate at room temperature and the residue dissolved in 1 ml. of ethanol (95%, v/v). A known volume of ethanolic solution was spotted on Whatman no. 1 chromatography paper and the descending chromatogram developed overnight with one of the solvents of Ranson, Walker & Clarke (1960), 2-methylbutan-2-ol-water-formic acid (3:3:1, by vol.), the organic phase containing bromophenol blue. The chromatograms were dried in still air, the yellow spots were identified by means of known markers, and the strips containing them were cut out and scanned for radioactivity.

Electrophoretic separation of labelled intermediates. Paper electrophoresis was carried out as described by Jones & Gutfreund (1962) for the separation of glutamate and aspartate from other ninhydrin-positive material. Trial runs with ^{14}C -labelled succinate, fumarate and malate showed that these compounds ran as a single, rather broad, band ahead of the dicarboxylic amino acids. The paper strips were scanned for radioactivity in the same way as the chromatograms.

Scanning of radioactive chromatograms. The scanning apparatus consisted of a drum moving the strip at 6 in./hr. past a $\frac{1}{8}$ in. slit in front of a Geiger-Müller tube (type EW/3H, 20th Century Electronics). The output of the Ekco Ratemeter type N522C was passed through a Pye Integrating Amplifier (cat. no. 12360) connected to a 1500 Ω , 1 ma recorder (Record Electrical Co. Ltd.) moving at 6 in./hr. An adjustable offset potential was applied to the Ratemeter output to enable the signal due to background radiation to be balanced out. The resultant trace consisted of a horizontal line with steps marking the radioactive spots, the height of the step being proportional to the total number of counts obtained from the spot. The strip was scanned successively on both sides and the mean value taken. The maximum difference between the two sides was 10%.

In control runs in which known amounts of $[2,3-^{14}\text{C}_2]$ -fumarate were chromatographed and scanned, a linear relationship was obtained up to an addition of 64 μmC between the activity added and the counts obtained. The counting efficiency in terms of the activity added was 2.5%. This efficiency was not affected when different amounts of unlabelled fumarate were run with the labelled acid on the chromatogram. Similar results were obtained with $[2,3-^{14}\text{C}_2]$ succinate. In a series of six recovery experiments, in which 0.6 μC of $[2,3-^{14}\text{C}_2]$ succinate was added to mitochondria and the complete extraction and chromatography procedure carried out, the final counts/sec. obtained from the chromatograms were: 39, 37, 39, 36, 37, 39 (expected value, 40 counts/sec.).

In experiments where recycling of labelled compounds

was prevented by inhibition, or which were of short duration, the specific activity of fumarate and malate formed can be assumed to be equal to that of the added $[2,3-^{14}\text{C}_2]$ -succinate or $[2,3-^{14}\text{C}_2]$ fumarate, and the absolute amount of product can be calculated from the total counts and the known added amount of substrate.

Determination of malate. The method of Hummel (1949) was used, with the omission of the initial precipitation. In direct determinations on trichloroacetic acid-precipitated reaction media an internal standard was included with each assay mixture to allow for the interfering colour produced by carbohydrates.

Determination of oxaloacetate. The change in extinction at 340 μm in the presence of NADH and malate dehydrogenase was used.

Other analytical procedures. Proteins were determined by the micro-Kjeldahl technique, citrate by the method of McArdle (1955) and α -oxoglutarate by the method of Friedemann & Haugen (1943).

RESULTS

Records of oxygen uptake measured with the Clark electrode. Fig. 1 shows the relative rates of oxygen uptake by the mitochondria of guinea-pig liver, in the presence of an excess of substrate and phosphate acceptor and when respiration was limited by phosphate acceptor ('state 3' and 'state 4' respectively of Chance & Williams, 1956), as well as uncoupled by 2,4-dinitrophenol. These measurements with the oxygen electrode were made with succinate as substrate. Table 1 summarizes the results obtained by this method with a number of substrates and inhibitors under conditions of oxidative phosphorylation, as well as the results

Fig. 1. Record of the rate of oxygen uptake by mitochondria from guinea-pig liver (3 mg. of protein/ml.) in the presence of succinate (3.3 mM) on the addition of ADP (0.33 mM) and 2,4-dinitrophenol (75 μM). The additions were made at the times indicated as follows: A, succinate; B, mitochondria; C, ADP; D, 2,4-dinitrophenol. The rates of oxygen uptake at X and Y were 0.055 and 0.11 $\mu\text{g. atom of O/min./mg. of protein}$ respectively.

Table 1. *Rates of oxygen uptake during different reactions of mitochondria from guinea-pig liver*

The rates (at 25°), as measured with the oxygen electrode (see the Experimental section for details), are in $\mu\text{g. atoms of O/min./mg. of protein}$ and present the range of values obtained for the numbers of experiments given in parentheses. The initial concentrations were: substrate, 6.6 mM; ADP, 0.3 mM; 2,4-dinitrophenol, 75 μM ; malonate, 6.6 mM; arsenite, 1 mM.

Substrate	Additions	Mitochondrial preparation	Rate
Succinate	ADP	Intact mitochondria	0.045–0.060 (8)
Succinate	Dinitrophenol	Intact mitochondria	0.080–0.110 (8)
Succinate	—	Frozen mitochondria	0.10–0.12 (6)
NADH	—	Frozen mitochondria	0.03–0.04 (4)
NADH	ADP	Intact mitochondria	<0.005 (2)
Citrate	ADP	Intact mitochondria	0.030–0.035 (3)
Citrate	ADP, arsenite	Intact mitochondria	0.016 (2)
α -Oxoglutarate	ADP	Intact mitochondria	0.020–0.030 (5)
α -Oxoglutarate	ADP, malonate	Intact mitochondria	0.010–0.015 (3)

for the oxidation of succinate by mitochondria uncoupled by 2,4-dinitrophenol and by disrupted mitochondria.

The large increase in the rate of oxygen uptake with succinate on the uncoupling of oxidative phosphorylation suggested that different rate-determining steps are in control of coupled and uncoupled oxidation. We attempted to determine the apparent K_m for succinate under different conditions in terms of the succinate concentration at which half the optimum rate of oxygen uptake is observed. The affinity for succinate is high and small changes in oxygen concentration had to be recorded. The method used allowed only an estimate of the apparent K_m values from experiments over a range of initial succinate concentration 0.1–3.3 mM: for phosphorylating mitochondria, K_m was less than 0.1 mM; for 2,4-dinitrophenol-treated and frozen mitochondria, K_m was 0.3 mM. At succinate concentrations of about 0.3 mM the rate of the oxidation of succinate was about the same for coupled and uncoupled mitochondria.

The experiments with mammary-gland mitochondria illustrated in Figs. 2 (a) and 2 (b) show a somewhat different behaviour in the following respects: the ADP-stimulated oxidation of succinate is slower than the oxidation of α -oxoglutarate. The 2,4-dinitrophenol-stimulated oxidation of succinate is slower than the ADP-stimulated oxidation of succinate, and the 2,4-dinitrophenol-stimulated oxidation of α -oxoglutarate is slower than the ADP-stimulated oxidation of α -oxoglutarate, the latter rate being 35 $\mu\text{m-moles of substrate/min./mg. of mitochondrial protein}$. The rate of oxygen uptake in the presence of α -oxoglutarate plus succinate is the same as that found in the presence of α -oxoglutarate alone.

The mitochondria of guinea-pig kidney contain approximately 2.5 times as much cytochrome and NAD/g. of protein as do liver mitochondria (Estabrook & Holowsinsky, 1961; Chance & Hollinger, 1961). The kidney mitochondria oxid-

Fig. 2. Record of the rate of oxygen uptake by mitochondria from guinea-pig mammary gland (2.8 mg. of protein/ml.) in the presence of succinate (3.3 mM) and α -oxoglutarate (3.3 mM) on the addition of either (a) ADP (3 mM) or (b) 2,4-dinitrophenol (50 μM). The additions were made at the times indicated as follows: A, succinate; B, mitochondria; C, either (a) ADP or (b) 2,4-dinitrophenol; D, α -oxoglutarate. The rates of oxygen uptake at (a) X and Y and (b) Z were 0.017, 0.035 and 0.021 $\mu\text{g. atom of O/min./mg. of protein}$ respectively.

ized succinate in the presence of 70 μM -2,4-dinitrophenol only slightly faster (Fig. 3) than under conditions of coupled oxidative phosphorylation.

We next investigated the rate of oxygen uptake by the mitochondria of guinea-pig liver during the oxidation of succinate on the addition of oxalo-

acetate both during oxidative phosphorylation and in the presence of 2,4-dinitrophenol. The addition of oxaloacetate to give a final concentration of 1.7 mM (half that of succinate) did not affect the rate of oxygen uptake by mitochondria actively phosphorylating ADP. On the addition of 2,4-dinitrophenol, however, the presence of such a concentration of oxaloacetate is inhibitory. Fig. 4 shows that the addition of oxaloacetate to succinate-treated mitochondria in 'state 4' causes a small stimulation of respiration, that the addition of

2,4-dinitrophenol briefly stimulates oxygen uptake to the level found in the absence of oxaloacetate but that complete inhibition soon occurs. When mitochondria were preincubated with 2,4-dinitrophenol and oxaloacetate for 1 min. the addition of succinate did not result in any significant increase of respiration.

Two further observations made during the experiments on the oxidation of succinate in the presence of 2,4-dinitrophenol may have some bearing on the mechanism of inhibition by oxaloacetate. These are the inhibition of the oxidation of succinate at high 2,4-dinitrophenol concentrations (Chappell, 1961b) and the time-dependence of such inhibition at moderately high 2,4-dinitrophenol concentrations. The first phenomenon is illustrated with the mitochondria of guinea-pig kidney in Fig. 3, which shows the rate of respiration in the presence of succinate on the addition of ADP, 2,4-dinitrophenol (70 μ M) and more 2,4-dinitrophenol (175 μ M). Further, it was discovered during experiments with liver mitochondria that, if measurements were carried out with more dilute mitochondrial suspensions than were used for those recorded in Fig. 1, then, during the longer time-course before anaerobiosis, the rate of oxygen uptake decreased progressively.

Oxidation of [2,3- 14 C]₂succinate. Using rat-liver mitochondria, Greengard, Minnaert, Slater & Betel (1959) showed that with 6.6 mM-succinate a proportion of malate from succinate was further oxidized. To determine the amount of oxidation of

Fig. 3. Record of the rate of oxygen uptake by mitochondria from guinea-pig kidney (1.5 mg. of protein/ml.) in the presence of succinate (3.3 mM) on the addition of ADP and 2,4-dinitrophenol. The additions were made at the times indicated as follows: A, succinate; B, mitochondria; C, ADP (final concn. 0.2 mM); D, 2,4-dinitrophenol (final concn. 70 μ M); E, 2,4-dinitrophenol (final concn. 175 μ M). The rates of oxygen uptake at X and Y were 0.12 and 0.137 μ g. atom of O/min./mg. of protein respectively.

Fig. 4. Record of the rate of oxygen uptake by mitochondria from guinea-pig liver (3.5 mg. of protein/ml.) in the presence of succinate (3.3 mM) on the addition of oxaloacetate (1.7 mM) and 2,4-dinitrophenol (75 μ M). The additions were made at the times indicated as follows: A, succinate; B, mitochondria; C, oxaloacetate; D, 2,4-dinitrophenol. The rates of oxygen uptake at X and Y were 0.024 and 0.10 μ g. atom of O/min./mg. of protein respectively.

Fig. 5. Fate of 14 C-labelled succinate oxidized by mitochondria from guinea-pig liver. The final concentration of added [2,3- 14 C]₂ succinate (0.6 μ C) was 4 mM. The phosphorylating reaction medium, conditions of reaction and method of separation of labelled compounds are described in the Experimental and Methods sections. The counts/sec. given are the average of counts from both sides of the chromatogram. ●, Total radioactivity; ▲, succinate radioactivity; ○, malate radioactivity; △, fumarate radioactivity.

Table 2. *Effect of succinate on the oxidation of citrate and α -oxoglutarate by mitochondria from guinea-pig liver*

The final concentration of added substrates was 4 mM. The composition of the phosphorylating medium, conditions of the reaction, and determinations of citrate and α -oxoglutarate are described in the Experimental and Methods sections. The reaction time was 10 min. Approx. 8 mg. of mitochondrial protein was present in each reception vessel.

Expt. no.	Substrates		Inhibition by succinate of the utilization of citrate (%)	Substrates		Inhibition by succinate of the utilization of α -oxoglutarate (%)
	Citrate	Citrate + succinate		α -Oxo- glutarate	α -Oxo- glutarate + succinate	
Utilization of citrate (μ moles)		Utilization of α -oxoglutarate (μ moles)				
1	2.3	1.6	30	2.5	1.6	20
2	3.1	2.2	30	—	—	—
3	—	—	—	2.8	2.4	14
4	2.7	1.8	33	3.7	2.8	24

malate taking place with 4 mM-succinate, the concentration used in most of our subsequent experiments, mitochondria from guinea-pig liver were allowed to oxidize $[2,3-^{14}\text{C}_2]$ succinate, and the changes in the radioactivities of substrate and of product were followed as described above. The results are shown in Fig. 5.

The fall in the radioactivity of succinate is linear for 12 min., indicating that recycling of active material is not important during this period. At 12 min. the radioactivity of fumarate plus malate accounts for 70 % of the loss of the radioactivity of succinate, so malate dehydrogenase is not completely inhibited. If it is assumed that the fall in total radioactivity represents the oxidation of malate to undetected products, the rate of oxidation of malate can be calculated to be $14.5 \mu\text{moles of malate/min./mg. of protein}$. The rate of oxidation of succinate during the first 12 min. is $58 \mu\text{moles/min./mg. of protein}$ (see also Table 1). Detectable radioactivity did not appear in the citrate or α -oxoglutarate spots or at any other place on the chromatogram. The accumulation of oxaloacetate measured by direct analysis was below the limits of the analytical method ($0.1 \mu\text{mole}$).

Effect of the oxidation of succinate on other citric acid-cycle reactions. To study the competition between the oxidation of succinate and other citric acid-cycle oxidations, experiments were carried out in which the rates of oxidation of citrate and of α -oxoglutarate were studied in the presence and absence of succinate. The results are shown in Table 2.

The utilization of citrate was decreased by about 30 % and that of α -oxoglutarate by about 20 % in the presence of succinate. When ^{14}C -labelled succinate was used the appearance of radioactivity in citrate or α -oxoglutarate could not be detected, suggesting that competition between added and

Table 3. *Effect of glutamate on the oxidation of malate by mitochondria from guinea-pig liver*

The final concentrations of the added $[2,3-^{14}\text{C}_2]$ fumarate ($0.6 \mu\text{C}$) and glutamate were 4 mM. 2 mM-Malonate was present in all cases. The composition of the phosphorylating medium, conditions of reaction and determinations of labelled compounds are described in the Experimental and Methods sections. The rate of oxidation of malate was determined from the utilization of fumarate plus malate, which rapidly form an equilibrium mixture.

Expt. no.	Reaction time (min.)	Substrates	
		Fumarate	Fumarate + glutamate
		Utilization of fumarate plus malate ($\mu\text{moles/min./mg. of protein}$)	
1	10	13.5	34.0
2	15	12.3	24.0
3	20	7.7	28.0

internally generated citrate and α -oxoglutarate was not an important factor.

Oxidation of $[2,3-^{14}\text{C}_2]$ fumarate. Fumarate (or malate) is oxidized slowly by the mitochondria of guinea-pig liver, but the rate of oxygen uptake is greatly increased by the addition of glutamate plus malonate (to prevent oxygen uptake due to succinate produced from glutamate). This reaction was followed by using ^{14}C -labelled fumarate to determine how much of the increased oxygen uptake was actually due to increased utilization of fumarate. The results of three experiments are shown in Table 3 where the rate of utilization of fumarate plus malate in the presence of malonate is given in the presence and absence of glutamate.

In all three experiments the rate of utilization of fumarate plus malate was stimulated two- to four-fold by the addition of glutamate. Pyruvate was

less effective than glutamate in stimulating the oxidation of malate.

The rate of oxidation of malate in Expts. 1 and 2 agrees reasonably well with the rate calculated from the data given in Fig. 5, which was $14.5 \mu\text{moles/min./mg.}$ of protein, in the presence of added succinate. Thus it appears that the accumulation of malate during the oxidation of succinate is not due to respiratory-chain competition but to inhibition of malate dehydrogenase by local formation of oxaloacetate.

Effect of oxaloacetate on the oxidation of succinate. The presence of 4 mM-oxaloacetate in the phosphorylating medium only slightly inhibited oxygen uptake with succinate as substrate when measured in the Warburg over a 5 min. period, after an initial 5 min. equilibration period. The rates of oxygen uptake (means of three experiments) were $0.055 \mu\text{g. atom of oxygen/min./mg.}$ of protein with succinate and $0.051 \mu\text{g. atom of oxygen/min./mg.}$ of protein with succinate plus oxaloacetate. Oxygen consumption in the presence of oxaloacetate alone (4 mM) was too low to be detected but the rate of

utilization of oxaloacetate was relatively constant in a variety of conditions. Table 4 shows the utilization of oxaloacetate in the presence and absence of succinate and in the presence and absence of phosphate acceptor and 2,4-dinitrophenol.

In these experiments the utilization of oxaloacetate was lower in the presence of succinate, but this effect was not consistently observed and was always small. A control experiment showed that the spontaneous decomposition of oxaloacetate was negligible under the conditions used.

The reactions taking place when succinate and oxaloacetate were both present were studied in more detail with $[2,3-^{14}\text{C}_2]$ succinate to differentiate between the malate synthesized from succinate and that synthesized from oxaloacetate. To analyse the changes in the concentrations of malate and of oxaloacetate parallel incubations were carried out with unlabelled succinate and the reaction was terminated with trichloroacetic acid instead of sulphuric acid. The results of two such experiments are recorded in Table 5.

The total malate synthesized minus the ^{14}C -labelled malate must represent the malate synthesized from the added oxaloacetate. Table 5 shows that, though oxaloacetate does not inhibit oxygen uptake, the utilization of succinate is decreased by about a third and the accumulation of ^{14}C -labelled malate is decreased still more. However, the total accumulation of malate is increased, owing to the malate synthesized from oxaloacetate. In Expt. 1 the synthesis of malate from oxaloacetate was stimulated fourfold by succinate, and though in Expt. 2 the stimulation was smaller it was still significant. Despite the increased conversion of oxaloacetate into malate the overall utilization of oxaloacetate is unaffected.

The fate of the oxaloacetate not converted into malate was not determined. Analysis for phosphoenolpyruvate gave a negative result and the

Table 4. *Utilization of oxaloacetate by mitochondria from guinea-pig liver*

The final concentrations of the added oxaloacetate and succinate were 4 mM. The reaction media referred to are described in the Experimental section. Incubation in Warburg flasks was for 10 min. at 25° . Approx. 8 mg. of mitochondrial protein was present in each reaction vessel.

Substrates	Utilization of oxaloacetate (μmoles)		
	In phosphorylating medium	In basic medium	In basic medium containing dinitrophenol ($160 \mu\text{M}$)
Oxaloacetate	6.7	6.1	6.2
Oxaloacetate + succinate	5.9	5.0	5.9

Table 5. *Metabolism of succinate and oxaloacetate by mitochondria from guinea-pig liver*

The final concentrations of the added $[2,3-^{14}\text{C}_2]$ succinate ($0.6 \mu\text{C}$) and oxaloacetate were 4 mM. The composition of the phosphorylating medium, conditions of reaction, determinations of labelled products, and determinations of total malate and oxaloacetate are described in the Experimental and Methods sections. The reaction times were 10 min. Approx. 8 mg. of protein was present in each reaction vessel.

Expt. no.	Substrates	Oxygen uptake ($\mu\text{g. atoms}$)	Utilization of $[^{14}\text{C}]$ succinate (μmoles)	Formation of $[^{14}\text{C}]$ malate (μmoles)	Total formation of malate (μmoles)	Calc. formation of $[^{14}\text{C}]$ malate (μmoles)	Utilization of oxaloacetate (μmoles)
1	Succinate	6.4	7.2	4.9	4.7	—	—
	Oxaloacetate	0	—	—	0.8	0.8	5.3
	Succinate + oxaloacetate	6.5	4.4	2.2	5.4	3.2	5.3
2	Succinate	5.6	6.1	3.9	3.6	—	—
	Oxaloacetate	0	—	—	1.9	1.9	5.8
	Succinate + oxaloacetate	5.8	4.1	1.6	4.3	2.7	6.1

accumulation of pyruvate accounted for less than 20 % of the utilized oxaloacetate.

Effects of 2,4-dinitrophenol on the oxidation of succinate. As shown in short-term experiments with the oxygen electrode, the oxidation of succinate stimulated by 2,4-dinitrophenol (70 μ M) was about twice as fast as that in the presence of ADP (greater than 100 μ M). This effect was not shown in Warburg experiments which involve a preliminary 5 min. equilibration period before recording begins. The rate of oxygen uptake by liver mitochondria in the presence of 12 mM-succinate and 160 μ M-2,4-dinitrophenol was 0.033 μ g.atom/min./mg. of protein and in the phosphorylating medium 0.055 μ g.atom/min./mg. of protein. Oxaloacetate (4 mM) completely inhibited oxygen uptake in the presence of 2,4-dinitrophenol, and by using [2,3- 14 C]₂succinate it was confirmed that succinate metabolism was completely suppressed in this system.

Oxidation of succinate by mammary-gland mito-

chondria. Mitochondria from guinea-pig mammary gland oxidize succinate more slowly than they oxidize α -oxoglutarate, but the rate of oxygen uptake is greatly stimulated by pyruvate or glutamate (Jones & Gutfreund, 1961). This stimulation was postulated to be due to the removal of oxaloacetate which in mammary gland mitochondria inhibited the oxidation of both succinate and malate.

When mammary-gland mitochondria were incubated with oxaloacetate (4 mM) in the basic medium the rate of utilization of oxaloacetate was 28 μ m-moles/min./mg. of protein, i.e. about half the value for liver mitochondria. The results of a further experiment, with 14 C-labelled succinate, are given in Table 6, which shows the effects of glutamate and oxaloacetate on the utilization of 14 C-labelled succinate and on the accumulation of 14 C-labelled malate.

The utilization of 14 C-labelled succinate is stimulated by glutamate, but the stimulation was not so great in the presence of oxaloacetate. As succinate is also produced from the added glutamate the stimulation of the removal of 14 C-labelled succinate represents a minimum value for the stimulation of total oxidation of succinate. One can deduce from the results given in Table 6 that, in the presence of oxaloacetate and glutamate, the removal of malate is suppressed, as it is when no additions are made.

The results reported in Table 7 confirm that stimulation by glutamate of the oxidation of 14 C-labelled succinate involves the formation of a 14 C-labelled amino acid, presumably mainly aspartate, though our method does not separate aspartate and glutamate.

The 14 C added as succinate is partially recovered in the amino acid fraction, but the incorporation into this fraction is decreased when oxaloacetate is added. Comparison of Tables 6 and 7 suggests that this decrease is due to the inhibition of the oxidation of both succinate and malate.

Table 6. *Oxidation of succinate by mitochondria from guinea-pig mammary gland*

The final concentration of [2,3- 14 C]₂succinate (0.6 μ C) was 4 mM. The composition of the phosphorylating medium, conditions of reaction and determinations of labelled compounds are described in the Experimental and Methods sections. The reaction time was 15 min. Approx. 8 mg. of protein was present in each reaction vessel.

Additions	Utilization of succinate (μ moles)	Formation of malate (μ moles)
None	1.8	1.8
4 mM-Glutamate	4.1	2.4
4 mM-Glutamate + 10 mM-oxaloacetate	2.9	3.0

Table 7. *Formation of amino acids from succinate by mitochondria from guinea-pig mammary gland in the presence of glutamate*

The final concentrations of added [2,3- 14 C]₂succinate (0.6 μ C) and glutamate were 4 mM. The composition of the phosphorylating medium and electrophoretic separation of the dicarboxylic amino acids are described in the Experimental and Methods sections. The reaction time was 15 min. Approx. 8 mg. of protein was present in each reaction vessel.

Concn. of oxaloacetate (mM)	14 C radioactivity from paper (counts/sec.)	
	Citric acid-cycle acids	Glutamate + aspartate
At zero time	11.3	0
0	8.2	3.7
1	9.3	2.0
10	11.3	0.8

DISCUSSION

Mitochondria from guinea-pig liver oxidize succinate more rapidly than any other substrate tested (see Table 1). The comparison of the initial rate of oxidation of succinate by these mitochondria in the presence of ADP and 2,4-dinitrophenol respectively suggests that, during the oxidative phosphorylation of ADP to ATP, phosphorylation is rate-determining. When the phosphorylation is uncoupled by the addition of 2,4-dinitrophenol the rate of oxidation of succinate is nearly doubled. These results are in complete agreement with Chappell's (1962) observations with rat-liver mitochondria. There is some difficulty in ascertaining

whether the rate of mitochondrial oxidation of succinate in the presence of 2,4-dinitrophenol is limited by succinate dehydrogenase or by some other step of the succinate-oxidase system; such information should come from a detailed analysis of spectroscopic experiments and of interactions with electron acceptors at different points of the chain. The succinate-oxidase activity of mitochondria disrupted by freezing and thawing is approximately twice that of intact mitochondria coupled to phosphorylation and thus similar to that of the oxidation stimulated by 2,4-dinitrophenol. Uncertainties about the stability of the succinate-oxidase activity and of other factors affecting the yield (Thorn, 1962) do not allow one to conclude whether the oxidation stimulated by 2,4-dinitrophenol amounts to the maximum turnover of succinate oxidase from mitochondria.

The results of studies of the K_m of various mitochondrial preparations for succinate can also be interpreted in terms of different rate-determining steps. The low apparent K_m value obtained with intact phosphorylating mitochondria is likely to be due to the fact that in this system the reaction of succinate with succinate dehydrogenase is not rate-determining. The K_m values obtained with disrupted mitochondria and with mitochondria treated with 2,4-dinitrophenol are in agreement with those obtained for various succinate-oxidase preparations (e.g. Thorn, 1962). Differences between the kinetic constants of enzymes in organized systems and those of the same enzymes in their isolated form tempt one to speculate about changes in protein structure. It seems likely, however, that an apparent increase in affinity for a substrate by an enzyme in an organized system is due to a change of rate-control from one step to another. For efficient metabolic control by substrate concentration the latter should be approximately equal to the apparent K_m . Often steady-state intermediate concentrations are lower than the known K_m of the isolated enzyme concerned, but correspond to the level appropriate to the apparent K_m for this substrate in the organized multi-enzyme system.

In experiments with mitochondria from guinea-pig kidney the overall rate of oxidation of succinate, coupled to phosphorylation, is about 2.5 times that observed for the mitochondria of guinea-pig liver, but the increase in oxidation on the uncoupling of phosphorylation with 2,4-dinitrophenol is relatively small. This indicates that the phosphorylating potential of the kidney mitochondria is almost equal to the rate of oxidation of succinate. In mammary-gland mitochondria the situation is complicated by the slow removal of oxaloacetate under phosphorylating conditions (Jones & Gutfreund, 1961), and on the addition of 2,4-dinitro-

phenol the oxidation of succinate is completely inhibited.

Succinate dehydrogenase is sensitive to inhibition by oxaloacetate, the K_i being $1.5 \mu\text{M}$ (Pardee & Potter, 1948). Tyler (1955) showed that ATP (magnesium salt) prevented the inhibition by oxaloacetate of the oxidation of succinate in rat kidney homogenates. We found that, when oxaloacetate was added to mitochondria oxidizing succinate in the presence of phosphate acceptor ('state 3'), this did not produce an appreciable inhibition of oxygen uptake, and that, when added to mitochondria with no acceptor ('state 4') oxaloacetate produced an increase in oxygen uptake. The analysis of the utilization of succinate given in Table 5, however, shows that, though oxygen uptake is not affected, the utilization of succinate is decreased by about 30%. In addition, as shown in Fig. 1, the rate of oxidation of succinate is limited by the rate of formation of ATP, being stimulated twofold by the addition of 2,4-dinitrophenol. Therefore succinate dehydrogenase could be inhibited by as much as 50% without any fall in oxygen uptake. A 30% inhibition of the utilization of succinate may represent a 60–70% inhibition of succinate dehydrogenase, but even this rather high inhibition is far less than that which would be expected from the K_i of oxaloacetate when it is present at the same concentration as that of the substrate. It follows that the effective concentration of oxaloacetate in the region of the active site of succinate dehydrogenase must be far lower than the concentration in the medium.

The demonstration that the oxidation of succinate stimulated by 2,4-dinitrophenol ($50\text{--}100 \mu\text{M}$) is completely inhibited by oxaloacetate (1.7 mM) is in accordance with the theory that the oxidation of succinate is dependent on the maintenance of a 'high-energy' phosphorylated intermediate (Azzone & Ernster, 1961; Schollmeyer & Klingenberg, 1961). Slater & Kemp (1962) have questioned this theory as the stimulatory action of ATP on mitochondria depleted of phosphate by preincubation is uninhibited by oligomycin, which prevents the reversal of the terminal stages of oxidative phosphorylation. As shown in Fig. 4, the initial response of mitochondria that have been preincubated with succinate and oxaloacetate to the addition of 2,4-dinitrophenol is a rapid oxygen uptake, such as would be expected in the absence of oxaloacetate, and complete inhibition only occurs after a significant lag period. When oxaloacetate is added to mitochondria already respiring in the presence of succinate and 2,4-dinitrophenol a similar lag is observed before inhibition. The inhibition, therefore, is not due to a direct action of 2,4-dinitrophenol on a phosphorylated intermediate essential for the oxidation of succinate,

but apparently to some secondary action that allows oxaloacetate to reach the active site of succinate dehydrogenase at a higher concentration.

As shown in Table 4 the rate of utilization of oxaloacetate by mitochondria from guinea-pig liver is relatively independent of the phosphorylating activity of the mitochondria or of the presence or absence of succinate. Thus at least some of the mitochondrial enzymes metabolizing oxaloacetate are freely available to their substrates and the effect of 2,4-dinitrophenol is not simply on the permeability of mitochondria to oxaloacetate. In the presence of succinate (Table 5) a considerable proportion of the metabolized oxaloacetate appeared as malate, indicating that malate dehydrogenase is available to the substrate. Because of the short time period of these experiments and the absence of oxygen uptake when oxaloacetate is the only added substrate, it is considered unlikely that the unlabelled malate could have been produced to any significant extent by a complete 'turn of the cycle' in the forward direction. Though this point needs further investigation, one would not expect succinate to stimulate such cycling. The K_m of malate dehydrogenase for oxaloacetate is low ($40 \mu\text{M}$) and it is possible that a concentration of oxaloacetate sufficient to produce a 60–70% inhibition of succinate dehydrogenase could act as an effective substrate for malate dehydrogenase. For this reason it is not essential to assign malate dehydrogenase and succinate dehydrogenase to separate 'compartments'.

In the presence of oxaloacetate and succinate, oxygen consumption equals or slightly exceeds that in the presence of succinate alone. This indicates that the further oxidation of oxaloacetate must be stimulated in some way as the oxygen consumption due to succinate itself is decreased (Table 5), and the increased reduction of oxaloacetate to malate will also lower overall oxygen consumption. Elucidation of the mechanism of this effect must await a more detailed examination of the fate of metabolized oxaloacetate.

As has been demonstrated in other systems, with the mitochondria from guinea-pig liver the addition of glutamate greatly stimulates the rate of oxidation of malate. It is therefore evident that undetectable concentrations (less than $30 \mu\text{M}$) of oxaloacetate are able to inhibit malate dehydrogenase. In our system the rate of oxidation of malate (Fig. 5) is apparently not affected by the oxidation of succinate, indicating that competition for the respiratory chain is not important. According to Greengard *et al.* (1959), with rat-liver mitochondria the oxidation of malate is completely suppressed with 66 mM-succinate, when the oxidation of succinate was nearly 5 times that with

6.6 mM-succinate. Greengard *et al.* (1959) suggested that respiratory-chain competition can occur at high succinate concentrations and that inhibition of the formation of oxaloacetate will stimulate the oxidation of succinate. A possible explanation for these findings could be competitive inhibition of fumarase and of malate dehydrogenase by high concentrations of succinate. The experiments reported in Table 2 show that the oxidation of isocitrate and of α -oxoglutarate is slightly inhibited by the simultaneous oxidation of succinate. This effect may be a direct competition for the respiratory chain or may be due to the decrease in the concentration of oxidized nicotinamide-adenine nucleotides caused by succinate.

In conclusion it appears that the oxidation of succinate by the mitochondria of guinea-pig liver is controlled by the rate of formation of ATP. The inhibition by oxaloacetate is only important during the oxidation of succinate by mammary-gland mitochondria, which dispose of this product more slowly than do liver mitochondria. The inhibition by added oxaloacetate of the oxidation of succinate bears no simple relation to the overall rate of removal of oxaloacetate. Liver mitochondria metabolize oxaloacetate at approximately the same rate during oxidative phosphorylation and in the presence of 2,4-dinitrophenol, yet added oxaloacetate does not inhibit respiration and only partially inhibits the oxidation of succinate during 'state 3' phosphorylation, whereas it completely inhibits respiration and the oxidation of succinate under uncoupled conditions.

The rate of metabolism of oxaloacetate appears to be independent of a wide variety of conditions, though the products obtained will no doubt vary considerably. Preliminary experiments (E. A. Jones & H. Gutfreund, unpublished work) show that the addition of succinate to mitochondria metabolizing oxaloacetate in 'state 4' stimulates the reduction of oxaloacetate to malate. This indicates that the reductive potential of succinate can be used to reduce NAD^+ directly (Chance & Hollinger, 1961) and the resulting NADH will reduce oxaloacetate to malate. The presence of succinate in 'state 3' has little effect on the total removal of oxaloacetate, but only determines its distribution among various products. The fate of oxaloacetate, a substrate for many mitochondrial enzymes, remains a problem. The competition for this substrate under various metabolic conditions is likely to be an important control phenomenon.

SUMMARY

1. Several conditions that control the rate of the oxidation of succinate by mitochondria from guinea-pig liver, mammary gland and kidney were

investigated in studies of the kinetics of oxygen uptake and analyses of the interconversion of succinate, fumarate, malate and oxaloacetate.

2. Kinetic studies of oxygen uptake revealed that in intact liver mitochondria the rate-determining step of the oxidation of succinate is the rate of phosphorylation of ADP to ATP, whereas in mammary-gland mitochondria the oxidation of succinate is controlled by the rate of removal of oxaloacetate.

3. The overall rate of the removal of oxaloacetate added to liver mitochondria was not affected by a variety of conditions such as the simultaneous oxidation of succinate, or the addition of ADP or 2,4-dinitrophenol. During oxidative phosphorylation of ADP the addition of oxaloacetate inhibited the oxidation of succinate only to a limited extent, but, on disruption of the energy supply by the addition of 2,4-dinitrophenol, oxaloacetate completely inhibited the oxidation of succinate. Mammary-gland mitochondria metabolize oxaloacetate more slowly than do liver mitochondria, and in this system the oxidation of succinate is more readily inhibited by oxaloacetate.

4. The time-course of the inhibition of the oxidation of succinate by the addition of oxaloacetate and 2,4-dinitrophenol, as well as the stimulation of respiration on the addition of oxaloacetate to mitochondria in 'state 4', indicates that there is an energy requirement for the local removal of oxaloacetate in the vicinity of the succinate-oxidase sites.

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Studies in the Biosynthesis of Fungal Metabolites

5. THE UTILIZATION OF SULPHUR-CONTAINING AMINO ACIDS AS PENICILLIN SIDE-CHAIN PRECURSORS*

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During a preliminary survey of the influence of various amino acids on the biosynthesis of penicillins by *Penicillium chrysogenum*, it was observed that methionine stimulated the formation of a new antibiotic that was chromatographically distinct from the penicillins normally produced by this mould. In addition, the methionine analogues

ethionine, *S*-methyleysteine and *S*-ethylcysteine yielded a corresponding series of antibacterial metabolites that, on the basis of their relative R_f values, appeared to be homologically related.

Evidence is now presented that these amino acids are incorporated via a pathway involving oxidative decarboxylation, the resulting acids then serving as penicillin side-chain precursors in the normal manner.

To facilitate a radioautographic study, a convenient procedure, requiring minimum amounts of

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