SUMMARY

1. Mitochondrial fractions from rat liver have been analysed by means of density-gradient centrifuging. Two types of experiments were performed. In one, sedimentation boundaries and the derived sedimentation-coefficient distribution curves were determined after incomplete sedimentation in a stabilizing gradient. In the other, solutions of high density were used to make the gradient and the particles were allowed to approach density equilibrium with the medium by prolonged centrifuging at high speed. In each case, the distributions of up to nine different enzymes were studied simultaneously.

2. Cytochrome oxidase, glutamic dehydrogenase, malic dehydrogenase and alkaline deoxyribonuclease showed practically identical distributions under all conditions applied. The bulk of the proteins of the preparation showed the same distribution pattern, which undoubtedly was that of the true mitochondria. These particles appeared to be remarkably homogeneous with respect to their enzymic content.

3. The five lysosomal hydrolases, acid phosphatase, β -glucuronidase, cathepsin, acid ribonuclease and acid deoxyribonuclease, formed another distinct group, characterized by a lower range of sedimentation coefficients, a greater dispersion of density and a higher average density, especially in heavy water, than the mitochondria. The distribution curves of these enzymes were similar but not identical, indicating that if lysosomes form a single species, they are not enzymically homogeneous.

4. Uricase showed many properties in common with the lysosomal enzymes, but differed from them by having sharper distribution curves and a particularly large increase in equilibrium density in heavy water.

This work has been supported by grants from the Centre National de Recherches sur la Croissance normale et pathologique, the Centre National de Recherches Enzymologiques, the Rockefeller Foundation and the Lilly Research Laboratories. One of us (D.S.B.) is indebted to the Belgian Government for a scholarship and to the Administrators of the H.E. Durham Fund, King's College, Cambridge, for a grant. The authors also acknowledge gratefully the kind help and co-operation of Dr J. Berthet.

REFERENCES

- Anderson, N. G. (1955). *Exp. Cell Res.* **9**, 446.
 Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
 Appelmans, F., Wattiaux, R. & de Duve, C. (1955). *Biochem. J.* **59**, 438.
 Baudhuin, P. (1959). *Arch. int. Physiol. Biochim.* **67**, 106.
 Beaufay, H., Baudhuin, P. & Bendall, D. S. (1958). *Abstr. 4th int. Congr. Biochem., Vienna*, p. 71.
 Beaufay, H., Bendall, D. S., Baudhuin, P. & de Duve, C. (1959). *Biochem. J.* **73**, 623.
 Beaufay, H. & de Duve, C. (1959). *Biochem. J.* **73**, 604.
 de Duve, C. (1957). *Symp. Soc. exp. Biol.* **10**, 50.
 de Duve, C., Berthet, J. & Beaufay, H. (1959). *Progr. Biophys.* **9**, 325.
 de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
 Kuff, E. L., Hogeboom, G. H. & Dalton, A. J. (1956). *J. biophys. biochem. Cytol.* **2**, 33.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Straus, W. (1958). *J. biophys. biochem. Cytol.* **4**, 541.
 Tedeschi, H. & Harris, D. L. (1955). *Arch. Biochem. Biophys.* **58**, 52.
 Thomson, J. F. & Klipfel, F. L. (1957). *Arch. Biochem. Biophys.* **70**, 224.
 Thomson, J. F. & Klipfel, F. L. (1958). *Exp. Cell Res.* **14**, 612.
 Thomson, J. F. & Mikuta, E. T. (1954). *Arch. Biochem. Biophys.* **51**, 487.
 Thomson, J. F. & Moss, E. M. (1956). *Arch. Biochem. Biophys.* **61**, 456.
 Wattiaux, R. & de Duve, C. (1956). *Biochem. J.* **63**, 606.

Yield of Oxidative Phosphorylation Associated with the Oxidation of Succinate to Fumarate

By P. GREENGARD,* K. MINNAERT, E. C. SLATER AND I. BETEL
*Molteno Institute, University of Cambridge and Laboratory of Physiological Chemistry,
 University of Amsterdam*

(Received 4 May 1959)

Studies of oxidative phosphorylation with succinate as substrate have a special interest, since succinate enters the respiratory chain at a point closer to oxygen than other substrates, and pyridine nucleotide is not involved in the reaction. Unfortunately, since fumarate is readily oxidized by mitochondria

which carry out oxidative phosphorylation, it is often difficult to study phosphorylation coupled to the oxidation of succinate to fumarate without complications caused by the further oxidation of the fumarate.

Phosphorylation coupled with the oxidation of succinate to fumarate was first shown by Belitzer & Tsiabkova (1939), who added arsenite to prevent

* Present address: Geigy Research Laboratories, P.O. Box 430, Yonkers, New York, U.S.A.

the oxidation of fumarate (see also Colowick, Welch & Cori, 1940). Green *et al.* (1953) also found some phosphorylation in this reaction, using partially disrupted mitochondria which did not oxidize fumarate. However, either the addition of arsenite or the disruption of mitochondria impairs the phosphorylative activity, and neither treatment can be used to determine the yield of phosphorylation associated with the oxidation of succinate to fumarate by intact mitochondria.

A number of papers have reported P:O ratios approaching or even exceeding 2, with liver mitochondria oxidizing succinate. The most thorough study is that of Copenhaver & Lardy (1952), who found a mean P:O ratio of 1.7. However, it was not possible to determine from this work the proportion of the oxygen uptake associated with the further oxidation of fumarate, and it was therefore not possible to calculate the true P:O ratio for the step succinate $\rightarrow$ fumarate. Whittam, Bartley & Weber (1955) (see also Krebs, Ruffo, Johnson, Eggleston & Hems, 1953) were able to limit the reaction essentially to this step by using a high concentration of succinate, and found a P:O ratio of 2.4. However, since Whittam *et al.* measured the rate of phosphorylation by determining the rate of incorporation of ^{32}P from inorganic phosphate into adenosine triphosphate, there is a strong possibility that the calculated ratio exceeds the true P:O ratio (Korkes, 1952; Slater & Holton, 1954; Ernster, Ljunggren & Lindberg, 1954; Slater, 1956).

Thus when this investigation began (in 1954), we did not feel that it had been established that the oxidation of succinate to fumarate was accompanied by two phosphorylation steps (see Slater, 1956). Our doubts were influenced by the fact that our preparations of rat-heart sarcosomes (mitochondria), which oxidized fumarate relatively slowly, gave a P:O ratio of about 1 with succinate. During the last five years, however, a number of observations reported in the literature made it increasingly likely that there were, in fact, two phosphorylation steps, and this has become widely accepted. The first convincing evidence was the demonstration by Nielsen & Lehninger (1954), later confirmed by Slater (1954), that phosphorylation occurred in the cytochrome *c* oxidase reaction. Nevertheless it appeared desirable to confirm directly that there were two phosphorylative steps in the oxidation of succinate to fumarate. Experiments carried out for this purpose are described in this paper.

EXPERIMENTAL

Materials

Chemicals and enzymes used in measurements of oxidative phosphorylation were the same as described in previous papers (Slater, 1953; Slater & Holton, 1954). In some

experiments, adenosine diphosphate (ADP) manufactured by the Sigma Chemical Co. (St Louis, Mo., U.S.A.) was used.

Guanosine diphosphate (GDP), cytidine diphosphate (CDP), uridine diphosphate (UDP), adenosine triphosphate (ATP) and adenosine monophosphate (AMP) were obtained from the Sigma Chemical Co.

Tissue preparations

Liver mitochondria. Mitochondria were isolated from the livers of male Wistar rats by the method of Hogeboom (1955), slightly modified as described by Myers & Slater (1957).

Heart sarcosomes (mitochondria). Two types of preparations were used. (1) Sarcosomes isolated from rat heart in 0.21 M-sucrose-10 mM-ethylenediaminetetra-acetate (EDTA), pH 7.4, by the method of Cleland & Slater (1953), and suspended in the same medium. These will be referred to as CS sarcosomes. (2) Sarcosomes isolated from mice, rat or guinea-pig hearts essentially according to the procedure of Holton, Hülsmann, Myers & Slater (1957).

Each preparation was made from the heart of one rat (male), or one guinea pig (male) or from six mouse hearts. The animal was killed by a blow on the head and the heart quickly removed. Auricular tissue and connective tissue and fat were removed and the heart was then placed in an ice-cold solution of 0.23 M-sucrose-5 mM-ATP, pH 7.4. The solution was decanted and replaced by fresh solution. The heart was finely minced with scissors and, in two to three batches, was homogenized for 10-15 sec. at moderately high speed in a Potter-Elvehjem homogenizer fitted with a polytetrafluoroethylene (Teflon) pestle and containing 5-10 ml. of the sucrose-ATP soln. The homogenizer tube was immersed in ice-water during this operation. The homogenate from each batch of heart was poured into cold centrifuge tubes and the combined homogenates were centrifuged for 3 min. at 600g at 0-2°. The supernatant was decanted into other centrifuge tubes, without contamination by the sediment, and centrifuged for 15 min. at 600g at 0-2°. The supernatant was completely decanted and the sediment suspended in an ice-cold solution of 0.24 M-sucrose-1 mM-ATP, pH 7.4 with the help of the homogenizer (hand-operated). The concentration of the sarcosomes in this suspension was usually about 3-10 mg. of sarcosomal protein/ml. This preparation will be referred to as HHMS sarcosomes.

Liver mitochondrial extract. This was prepared according to the method of Dianzani (1956).

Methods

Adenosine triphosphatase. Adenosine triphosphatase activity of isolated mitochondria or sarcosomes was determined at room temperature (18-22°) as described by Myers & Slater (1957), in the following reaction mixture: ATP, pH 7.4, 2 mM; KCl, 75 mM; EDTA, pH 7.4, 1 mM; MgCl_2 , 3 mM; sucrose, 10 mM, with or without 0.1 mM-2,4-dinitrophenol. The stimulation of the adenosine triphosphatase by dinitrophenol is expressed by the ratio, *r*, which equals (activity in the presence of dinitrophenol)/(activity in the absence of dinitrophenol).

P:O ratio. The method with oxygen as acceptor was the same as that described by Slater & Holton (1954). Differential manometers, with 6-ml. flasks, fluid vol. 1 ml., were used. Manometer constants were calculated according

to van Dorp & Slater (1959). The O_2 uptake during the equilibration period was determined by extrapolation as described by Slater & Holton (1954). This sometimes caused some difficulty, because of a rapid decline from the initial rate of O_2 uptake. More uniform rates were obtained in the presence of NaF and high concentrations of succinate. The standard reaction mixture contained potassium phosphate, pH 7.4, 30 mM; glucose, 30 mM; ADP, 0.6 mM; AMP, 0.6 mM; NaF, 20 mM; $MgCl_2$, 5 mM; EDTA, 2 mM; cytochrome *c*, 20 μ M; disodium succinate, 60 mM; hexokinase. The reaction mixture also contained 50 mM-sucrose introduced with the suspension of liver mitochondria, 20 mM-sucrose introduced with the suspension of CS sarcosomes or 20 mM-sucrose-0.1 mM-ATP introduced with the suspension of the HHMS sarcosomes. The reaction period was usually between 20 and 35 min. at 25°; it was found with heart sarcosomes (CS) that the P:O ratio was constant for at least 60 min. (Fig. 1). Respiratory activity is expressed as Q_{O_2} (μ l. of O_2 /mg. of mitochondrial protein/hr.); protein was determined by the biuret method (see Cleland & Slater, 1953).

The method with ferricytochrome *c* as acceptor was that described on p. 394 of Slater (1955) under the subheading '(iii) Spectrophotometric Thunberg tubes'. The reaction mixture contained potassium phosphate, pH 7.4, 30 mM; ADP, 0.19 mM; AMP, 0.19 mM; NaF, 20–40 mM; disodium succinate, 60 mM; glucose, 30 mM; $MgCl_2$, 5 mM; EDTA, 1 mM; hexokinase; sarcosomes (CS), 0.2–0.25 mg. of protein/ml.; 0.573 μ g.-atom O equiv. of ferricytochrome *c*. The reduction of the cytochrome *c*, which was followed by measuring the decrease of the extinction at 580 m μ , was complete in 11–20 min. at room temperature. The amount of ferricytochrome *c* reduced, calculated from $\Delta E_{580\text{ m}\mu}$, was 0.56–

0.58 μ g.-atom O equiv., practically identical with the amount added.

Malic dehydrogenase. In order to measure the activity of the pyridine nucleotide dehydrogenases in mitochondria, it is necessary to subject the latter to a preliminary hypo-osmotic treatment so that the pyridine nucleotide is readily accessible to the dehydrogenase (Christie & Judah, 1953). The mitochondria or sarcosomes were suspended in water at 0° for 3–5 hr. and, after brief treatment in a Potter-Elvehjem homogenizer in order to damage the mitochondria further and thereby facilitate the accessibility of the pyridine nucleotide to the dehydrogenase, a sample of the mitochondrial suspension was added to a spectrophotometric cuvette at 25° containing 80 μ M-reduced diphosphopyridine nucleotide (DPNH), prepared as in Slater (1953), 30 mM-2-amino-2-hydroxymethylpropane-1:3-diol-HCl buffer, pH 7.4; 3% of ethanol (derived from the DPNH and antimycin solutions) and 130–180 μ g. of antimycin/mg. of protein. (The antimycin was included to inhibit the DPNH oxidase system.) Measurements at 340 m μ were taken to confirm that no oxidation of DPNH occurred and then, 5 min. after the addition of the mitochondrial suspension, dipotassium oxaloacetate was added to a final concentration of 0.39 mM, and the rate of decrease of the extinction at 340 m μ was measured. Doubling the DPNH concentration had no effect on the activity. One unit of malic dehydrogenase is defined as the amount which caused a decrease of extinction of 0.01/min. at 25°, and specific activity as the number of units/mg. of protein in the reaction mixture.

Fumarate plus malate. The amount of (fumarate plus malate) present after oxidation of succinate by mitochondria or sarcosomes was determined by means of malic decarboxylase (Korkes, del Campillo & Ochoa, 1950; Nossal, 1951, 1952). In these experiments, oxidation of succinate was carried out according to the procedure and with the same reaction mixture as described above for measurements of the P:O ratio, except that two concentrations of succinate (6.6 mM and 66 mM) were used and the reaction volume was 1.5 ml. After oxidation for 20–30 min., the reaction was stopped by the addition of 0.1 ml. of 10% (w/v) acetic acid, and the contents of the manometer flask were transferred to a centrifuge tube, which was placed in a boiling-water bath for 2 min. After cooling, the precipitated protein was removed by centrifuging, and the clear supernatant (pH 5.0) was used for the estimation of the (fumarate + malate) content.

A sample (0.9 ml.) of this supernatant was added to the main compartment of a second 6 ml. manometer flask, which contained 15 units of fumarase [prepared from pig heart according to the method of Massey (1955) up to a specific activity of 27.5 units/mg. of protein], 2.1 μ moles of glucose, 1.87 μ moles of $MnCl_2$, 75 μ moles of KCl, 18.5 μ moles of sodium acetate, pH 5.0, and 1 μ mole of aniline. The side arm of the flask contained 2.1 μ moles of glucose, 1.87 μ moles of $MnCl_2$, 75 μ moles of KCl, 18.5 μ moles of sodium acetate, pH 5.0, and 0.1–0.2 ml. of a washed-cell suspension (20%, w/v) of malate-adapted *Lactobacillus arabinosus* (Nossal, 1951, 1952) in 0.1 M-KCl. The total fluid volume in the manometer flask was 1.5 ml. The gas phase was air. The amount of CO_2 liberated from the (malate + fumarate) was measured at 37°. The reaction was complete in 20–30 min.

For testing this method, the Keilin & Hartree heart-muscle preparation (Slater, 1949), which does not oxidize

Fig. 1. Stability of oxidative phosphorylation with rat-heart sarcosomes (CS). The standard reaction mixture (see under Methods) was used, with 0.37 mg. of sarcosomal protein/ml. and 366 units (Berger, Slein, Colowick & Cori, 1946) of hexokinase/mg. of sarcosomal protein. ●, Oxygen uptake (μ g.atoms); ○, Δ esterified P (Δ hexose monophosphate + Δ ~P; see Slater & Holton, 1954); □, P:O ratio.

malate, was used to oxidize the succinate. The reaction was continued until all the succinate was oxidized (Expt. 1, Table 1). There is close agreement between the amount of succinate used, the O_2 uptake and the amount of (fumarate + malate) found at the end of the reaction. In Expt. 2, in which the succinate was not completely oxidized, there was a good agreement between the O_2 uptake and the amount of fumarate + malate found.

RESULTS

Effects of change of the reaction mixture on the P:O ratio

Succinate concentration. The effects of varying the succinate concentration between 3 and 60 mM are shown in Table 2. The Q_{O_2} increased with increasing succinate concentration within this range, even though this was far above that normally necessary to saturate succinic dehydrogenase.

With liver mitochondria, the P:O ratio decreased with increasing succinate concentration whereas with heart sarcosomes variation of the succinate concentration between 6 and 60 mM had no effect on the ratio.

Table 3 shows that with 6-6 mM-succinate and liver mitochondria, the O_2 uptake is 1.66-1.83 times the amount of (fumarate + malate) found, showing that under these conditions oxidation proceeds past fumarate. With higher concentrations of succinate (66 mM), however, the O_2 uptake exceeded the (fumarate + malate) found by only 3-10%. With heart sarcosomes, there was little oxidation past fumarate, even with the lower concentrations of succinate.

Table 1. *Test of method of determining fumarate plus malate*

Heart-muscle preparation, 5 mg. of protein in Expt. 1, 0.5 mg. in Expt. 2; phosphate, pH 7.4, 40 mM; EDTA, 10 mM; vol., 1.5 ml. In Expt. 1 the reaction was run until all the succinate was oxidized (43 min.). In Expt. 2 the reaction was stopped after 17 min.

Expt. no.	Initial amount of succinate (μmoles)	O_2 uptake (μg.atoms)	Fumarate + malate at end of reaction (μmoles)
1	7.0	7.2	7.05
2	50.0	7.55	7.34

Table 2. *Effect of succinate concentration of P:O ratio*

Values given are the means of a number of experiments, in which the concentrations of succinate were varied.

	Concn. of succinate (mM)				
	3	6	15	30	60
Rat-liver mitochondria					
No. of expts.	3	6	2	1	9
Q_{O_2}	15	23	34	56	109
P:O	2.20	2.17	2.14	1.94	1.62
Rat-heart sarcosomes (CS)					
No. of expts.		5	3	3	6
Q_{O_2} (mean)		43	56	66	165
P:O (mean)		0.95	0.93	0.90	0.93

Table 3. *Relationship between oxygen consumed and (fumarate plus malate) formed by rat-liver and -heart mitochondria oxidizing succinate*

Expt. no.	Mitochondria	Protein (mg.)	Time of expt. (min.)	Concn. of succinate (mM)	Concn. of Amytal (mM)	ΔO (μg.atoms)	Δ (fumarate + malate) formed (μmoles)	ΔO / Δ (fumarate + malate)
1	Liver	2.1	29.5	6.6	—	5.83	3.20	1.83
			31	6.6	1.8	6.49	6.24	1.04
			26.5	66	—	7.70	6.95	1.10
			28.5	66	1.8	7.45	7.35	1.01
2	Liver	1.6	32.5	6.6	—	1.64	0.99	1.66
			33	6.6	1.8	1.49	1.40	1.06
			30	66	—	4.80	4.67	1.03
			30.5	66	1.8	4.35	4.35	1.00
3	Heart (HHMS)	0.7	26.5	6.6	—	1.00	0.98	1.02
			26.5	6.6	1.8	1.60	1.65	0.97
			22.5	66	—	2.90	2.94	0.99
			25	66	1.8	4.78	4.99	0.96
4	Heart (HHMS)	0.57	31	6.6	—	1.02	0.98	1.04
			32	6.6	1.8	2.14	1.97	1.09
			27.5	66	—	3.12	3.11	1.00
			28	66	1.8	4.98	4.59	1.09

Table 4. *Effect of Amytal on P:O ratio with liver mitochondria*

Means of three expts., each in duplicate.

Concn. of succinate (mm)	P:O		
	Without Amytal	With 1.8 mm-Amytal	Δ
6	1.92	1.36	-0.56
60	1.44	1.28	-0.16
Δ	-0.48	-0.08	—

Table 5. *Malic dehydrogenase activity of rat-liver mitochondria and rat-heart sarcosomes*

Malic dehydrogenase activity was measured as described under Methods. The succinic oxidase activity of the intact mitochondria or sarcosomes was measured with the standard reaction mixture.

	Mitochondria	Sarcosomes (HHMS)
Malic dehydrogenase specific activity (a)	870	350
Succinic oxidase Q_{O_2} (b)	85	105
(a)/(b)	10.2	3.3

Attempts to inhibit the oxidation of malate by the acetylpyridine or pyridine-3-aldehyde analogues of diphosphopyridine nucleotide (DPN), kindly supplied by Dr N. O. Kaplan, were unsuccessful. These compounds slightly stimulated the oxidation of malate by liver mitochondria, held for 3.5 hr. at 0° in order to increase the permeability of the mitochondria to these compounds.

Jalling, Lindberg & Ernster (1955) found that 1.8 mm-Amytal (5-amino-5-ethylbarbituric acid) completely inhibited the oxidation by liver mitochondria of substrates dependent upon DPN, without affecting the oxidation of succinate. In agreement with these findings, added malate was not oxidized by our preparations of liver mitochondria in the presence of Amytal of this concentration, and Table 3 shows that the addition of Amytal caused a decrease of the ratio $\Delta O/\Delta$ (fumarate + malate) from 1.83 and 1.66 to 1.04 and 1.06 respectively.

From these results it appears likely that the higher P:O ratio obtained with liver mitochondria at low succinate concentrations is due to the fact that under these conditions a considerable proportion of the O_2 uptake is associated with the oxidation of malate, which has a higher P:O ratio. This interpretation is supported by the results given in Table 4, which show that in the presence of Amytal, the P:O ratio becomes largely independent of the concentration of succinate, and that Amytal has much less effect on the P:O ratio obtained with 60 mm-succinate than with 6 mm-succinate.

Even with low concentrations of succinate, there is little oxidation past fumarate-malate with heart sarcosomes (Table 3). A partial explanation is the

relatively lower malic dehydrogenase activity of the sarcosomes (Table 5).

Concentration of mitochondria or sarcosomes. Variation of the concentration of liver mitochondria between 0.4 and 6.65 mg. of protein/ml. or of the concentration of heart sarcosomes (HHMS) between 0.16 and 1.7 mg. of protein/ml. had no appreciable effect on the Q_{O_2} or on the P:O ratio. The addition of serum albumin (5 mg./ml.) also had no effect.

Hexokinase concentration. The P:O ratio with liver mitochondria was unaffected by varying the concentration of hexokinase between 10 and 600 units (Berger, Slein, Colowick & Cori, 1946)/mg. of mitochondrial protein. With heart sarcosomes (CS), in the absence of fluoride, the following values were obtained with different concentrations of hexokinase: 40 units/mg. of protein, 0.68; 120 units/mg. of protein, 0.90; 400 units/mg. of protein, 0.88; 800 units/mg. of protein, 0.95; extrapolated to infinite hexokinase concentration, 0.96. The value for infinite hexokinase concentration was calculated from the straight line obtained by plotting $1/P:O$ against $1/[\text{hexokinase}]$ (see Lewis & Slater, 1954). It can also be calculated from this straight line that, in the absence of fluoride, 400 units of hexokinase/mg. of protein (the usual concentration with the CS sarcosomes) underestimates by only 3% the P:O ratio obtained with an infinite concentration of hexokinase.

Ethylenediaminetetra-acetate. When purified reagents were used, the addition of 2 mm-EDTA had no effect on the Q_{O_2} or on the P:O ratio with liver mitochondria oxidizing α -oxoglutarate, succinate, glutamate or β -hydroxybutyrate. The effect of EDTA on heart sarcosomes has been reported by Slater & Cleland (1953).

Magnesium and phosphate. Variation of the concentration of magnesium and phosphate, with 2 mm-EDTA and 20 mm-NaF, showed that the highest P:O ratios with heart sarcosomes were obtained with 5 mm-magnesium and 30 mm-phosphate. However, a considerable departure from the optimum conditions had only a small effect on the P:O ratio.

pH. The effect of pH on the P:O ratio with heart sarcosomes has been studied by Hülsmann & Slater (unpublished work). The maximum P:O ratio, which was found at 6.5, was in the experiments of these authors 19% (guinea pig) and 16% (rat) greater than that at 7.4, the pH used in the present study.

Fluoride. Table 6 shows that fluoride up to 0.04M had no effect on the P:O ratio with heart sarcosomes, in agreement with Slater & Bonner (1952). Its effect on liver mitochondria was variable. Fluoride increased the P:O ratio with both high and low succinate concentrations, in Table 6, Expt. 1. Since at the same time the Q_{O_2} was lowered, this

result could be explained by the fact that fluoride, in the presence of phosphate, is a competitive inhibitor of succinate dehydrogenase (Slater & Bonner, 1952). Thus fluoride might be expected to have the same effect as decreasing the succinate concentration. On the other hand, in Expt. 2, Table 6, fluoride had little effect on the P:O ratio, although it markedly decreased the Q_{O_2} .

These results give no support to the suggestion (Whittam *et al.* 1955) that fluoride, in the concentrations often used in studying oxidative phosphorylation, might lower the P:O ratio.

Cytochrome c. Variation of the cytochrome *c* concentration between 0 and 0.1 mM had no effect on the P:O ratio with heart sarcosomes. However, the Q_{O_2} is in some preparations slightly enhanced by the addition of cytochrome *c*. A concentration of 2 μ M-cytochrome *c* was sufficient for the maximum stimulation. This is much less than is required by

the succinic oxidase system of the Keilin & Hartree heart-muscle preparation (sarcosomal fragments) (see Slater, 1949).

Adenosine diphosphate. Variation of the ADP concentration between 0.1 and 1.2 mM had no effect on the ratio with rat-heart sarcosomes (CS).

Addition of possible cofactors. Neither the P:O ratio nor the Q_{O_2} was affected by the addition of UDP, CDP or GDP, alone or in combination with ADP. Similarly, no effect was found by adding an extract of liver mitochondria prepared according to the method of Dianzani (1956), who found that the P:O ratio of aged liver mitochondria was increased by the addition of this extract.

The effect of adding Mn^{2+} ions was also studied, in view of the finding by Ernster, Lindberg & Löw (1955) that this addition decreased the amount of ATP required to restore phosphorylation associated with the oxidation of succinate after a pre-incubation of liver mitochondria with Ca^{2+} ions. Table 7 shows that Mn^{2+} ions did not increase the P:O ratio with rat-heart sarcosomes. Low concentrations slightly increased the Q_{O_2} , without affecting the P:O ratio, whereas higher concentrations inhibited both the oxidation and the phosphorylation.

P:O ratio for oxidation of succinate to fumarate

The mean values of different preparations are shown in Table 8. In our hands, isolated heart sarcosomes have appreciably lower P:O ratios than isolated liver mitochondria. They also have a higher adenosine triphosphatase activity in the presence of Mg^{2+} ions and a lower degree of stimulation of the adenosine triphosphatase by DNP. The isolation medium used in preparing the HHMS sarcosomes in this paper was selected out of 15 media tried by Holton *et al.* (1957) as giving the greatest stimulation of adenosine triphosphatase by dinitrophenol. The highest P:O ratio and the greatest degree of stimulation by dinitrophenol was

Table 6. *Effect of fluoride*

The P:O values for heart sarcosomes are the means of five experiments. The Q_{O_2} values for heart sarcosomes are means of five to eight experiments. The two experiments with liver are each single experiments.

Preparation	Concn. of succinate (mM)	Concn. of fluoride (mM)	Q_{O_2}	P:O
Liver (Expt. 1)	6	0	48.5	1.91
	6	0.02	24	2.31
	60	0	154	1.37
	60	0.02	83	1.94
Liver (Expt. 2)	6	0	85	2.03
	6	0.02	31	1.95
	60	0	177	1.43
	60	0.02	135	1.47
Heart (CS)	60	0	317	1.01
	60	0.01	—	1.09
	60	0.02	182	1.04
	60	0.04	121	1.08
	60	0.08	—	0.59

Table 7. *Effect of manganese*

CS rat-heart sarcosomes were used in Expt. 1; in Expt. 2, the CS rat-heart sarcosomes were washed with and suspended in 0.25 M-sucrose to remove the EDTA. 20 mM-Succinate was used instead of the standard 60 mM-succinate. (Free Mn) = (Total Mn) - (EDTA).

Expt. no.	EDTA (mM)	Mn		ΔO	Δ Esterified P	P:O
		Total (mM)	Free (mM)			
1	1	0	0	4.40	3.42	0.80
	1	1	0	3.78	2.74	0.72
	1	1.5	0.5	3.28	2.35	0.71
	1	2	1	3.17	1.96	0.62
2	0	0	0	4.24	2.68	0.63
	2	0	0	4.81	3.00	0.62
	0	0.05	0.05	4.69	2.92	0.63
	0	0.1	0.1	3.74	2.25	0.60
	0	1	1	3.79	1.86	0.49
	2	1	0	4.24	—	—

Table 8. P:O ratios of different mitochondrial preparations

Preparations	No. of determinations	P:O (mean $\pm$ s.e.m.)
Rat-heart sarcosomes (CS)	19	1.01 $\pm$ 0.04
Rat-heart sarcosomes (HHMS)	14	0.94 $\pm$ 0.05
Mouse-heart sarcosomes (HHMS)	1	0.99
Guinea-pig sarcosomes (HHMS)	10	1.31 $\pm$ 0.08
Rat-liver mitochondria	12	1.57 $\pm$ 0.07

Fig. 2. Relationship between the P:O ratio and the adenosine triphosphatase activity (expressed as 1/*r*) of different preparations. This graph includes all our preparations on which both measurements were made, including a few in which the isolation procedure was purposely altered to give damaged mitochondria. ○, Rat-heart sarcosomes (HHMS); ●, guinea-pig-heart sarcosomes (HHMS); ×, mouse-heart sarcosomes; ▲, ▼, rat-liver mitochondria.

found with some preparations isolated from the hearts of young guinea pigs by the method of Holton *et al.* (1957).

The quantitative relationship between the adenosine triphosphatase activity and the P:O ratio of various preparations of heart sarcosomes and liver mitochondria is shown in Fig. 2. The adenosine triphosphatase activity is expressed as 1/*r* (see under Methods), which is a measure of the fraction of the potential adenosine triphosphatase which is active in the absence of dinitrophenol. It is significant that all values, both for liver and heart, lie around the same curve.

Phosphorylation coupled with the reduction of ferricytochrome c by succinate

Table 9 summarizes an experiment demonstrating phosphorylation coupled with the reduction of ferricytochrome *c* by succinate. The P:O ratio is small (0.2), but well outside the experimental error. Other experiments are summarized in Table 10. Hypo-osmotic pretreatment of the sarcosomes (CS), which causes increased phosphorylation with ferrocytochrome *c* as hydrogen donor, brought

Table 9. Phosphorylation coupled with reduction of ferricytochrome *c* by succinate

Rat-heart sarcosomes (CS). Phosphorylation was measured (method II) as described by Slater (1955). HMP, Hexose monophosphate; ~P, 2 ATP + ADP (see Slater, 1953).

Ferricytochrome <i>c</i> (μg.atoms O equiv.)	0.573	0.573
~P (μmole)	{ initial	0.727
	{ final	0.661
	{ Δ	-0.089
HMP (μmole)	{ initial	0.047
	{ final	0.231
	{ Δ	0.184
Δ HMP + Δ ~P	0.09	0.12
P:O	0.16	0.21

Table 10. Comparison of phosphorylation ratios with succinate as hydrogen donor and either oxygen or ferricytochrome *c* as hydrogen acceptor

Normal preparation refers to rat-heart sarcosomes isolated in 0.21M-sucrose-10 mm-EDTA, pH 7.4. Hypo-osmotic preparation refers to sarcosomes treated as in Table 9 of Slater (1955). Method II (see Slater, 1955) was used to measure phosphorylation in Expt. 1 (see Table 9); method I was used in Expts. 2 and 3.

Expt. no.	Preparation	P:O	
		With O ₂	With ferricytochrome <i>c</i>
1	Normal CS	0.99	0.18
2	Normal CS	1.11	0.21
3	{ Normal CS	1.44	0.20
	{ Means	1.18	0.20
	{ Hypo-osmotic CS	—	0.11

about a decrease of the phosphorylation with ferricytochrome *c* as acceptor (cf. Slater, 1955).

In Expt. 2 of Table 10, a control was carried out with fumarate, in a concentration equivalent to that of the ferricytochrome *c* concentration, in the place of the succinate. Only 0.06 μmole of ferricytochrome *c* (0.03 μg.atom O equiv.) was reduced (calc. from Δ*E*_{580 mμ}), with negligible phosphorylation. It is clear that under the conditions of the experiments with ferricytochrome *c* as acceptor and succinate as donor, virtually all the phosphorylation is associated with the reduction of ferricytochrome *c* by succinate.

The P:O ratio with ferrocytochrome *c* as substrate and unswollen rat-heart sarcosomes is 0.12 (Slater, 1956). The experimental results with different donors and acceptors are given as figures above the arrows in the following reaction schemes:

This illustrates the relative inefficiency of added ferricytochrome *c* as an acceptor or of added ferrocytochrome *c* as a donor.

DISCUSSION

Succinate is often used in studies of oxidative phosphorylation in order to bypass that phosphorylation step in the respiratory chain which involves DPN. Since the further oxidation of fumarate, the product of oxidation of succinate, involves DPN, it is important to select conditions under which the further oxidation does not proceed to any appreciable extent. Table 3 shows that these conditions are not fulfilled with liver mitochondria oxidizing 6 mM-succinate, the concentration mostly used in studies reported in the biochemical literature. The further oxidation of fumarate by liver mitochondria can be made negligible by the addition of Amytal (1.8 mM), or by increasing the succinate concentration to 60 mM. With heart sarcosomes, the further oxidation of fumarate is not appreciable even with low concentrations of succinate.

The effect of increasing the concentration of succinate from 6 to 60 mM on the proportion of the total oxygen uptake which is associated with the further oxidation of the fumarate can be understood by a consideration of the Q_{O_2} values given in Table 2. The Q_{O_2} of liver mitochondria with 60 mM-succinate was 4.75 times that with 6 mM-succinate, and the relative rate of the step succinate $\rightarrow$ fumarate must have been even greater, since oxidation past fumarate was greater with the lower concentration of succinate (Table 3). This shows that the K_m for succinate in mitochondria is much larger than that found in succinic oxidase preparations, e.g. 0.5 mM found by Slater & Bonner (1952) for the Keilin & Hartree heart-muscle preparation. This suggests that mitochondria contain a competitive inhibitor of succinic dehydrogenase. The effect of increasing the succinate concentration ten-fold will be to increase the velocity of the succinate $\rightarrow$ fumarate step relative to that of the further oxidation of fumarate, even if the latter reaction remains unaffected. [In fact, it is not unlikely that an increased rate of oxidation of succinate through the respiratory chain would cause an inhibition of the oxidation of other substrates; cf. the inhibition by

succinate of DPNH oxidation, studied by Wu & Tsou (1955).]

For these reasons it seems likely that the P:O ratios obtained with 60 mM-succinate are more representative of the step succinate $\rightarrow$ fumarate than those obtained with lower succinate concentrations. Packer (1958) has suggested that succinate might cause loss of ATP by the reaction

This reaction cannot be operating in our experiments, since the hexokinase which was present in great excess would trap the ATP formed. That the higher concentrations of succinate do not have an uncoupling effect is shown by the lack of effect of increasing the succinate concentration to 60 mM on the P:O ratio of heart sarcosomes (Table 2) and of liver mitochondria in the presence of Amytal (Table 4).

The competitive inhibitor of succinic dehydrogenase formed in mitochondria has not been positively identified in this study, but it appears rather likely that it is oxaloacetate. This conclusion is not necessarily in conflict with the fact that succinic dehydrogenase is inhibited even in heart sarcosomes where only a small proportion of the oxygen uptake is associated with the further oxidation of fumarate, since only a very small amount of oxaloacetate is necessary to inhibit succinic dehydrogenase (K_i 1.5 μM). For example, in the presence of 6 mM-succinate, only 70 μM -oxaloacetate is necessary to inhibit succinic dehydrogenase by 80 %. The same concentration of oxaloacetate would inhibit succinic dehydrogenase by 29 % in the presence of 60 mM-succinate. If the inhibitor in mitochondria is oxaloacetate, the degree of inhibition will be determined by the steady-state concentration of oxaloacetate, which will depend upon the rate of decomposition of oxaloacetate as well as upon the rate of its formation, and will not necessarily bear a simple relationship to the extent of oxidation past fumarate. However, quantitative measurements of the small concentrations of oxaloacetate expected are required before it can be accepted with certainty that this is the major competitive inhibitor in mitochondria.

The P:O ratios for rat-liver and heart sarcosomes oxidizing succinate found in this study are in general agreement with most reports in the literature. The ratio with 6 mM-succinate and liver mitochondria which we find (2.17) is not lower than the value (1.70) found by Copenhaver & Lardy (1952) with the same concentration of succinate. Our results for rat-heart sarcosomes (0.98 for all preparations in Table 8) are similar to the value of 1.1 recently reported by Packer (1958). Both our results and those of Packer are considerably lower than that

(1.64) reported by Maley & Plaut (1953, 1954) for rat-heart sarcosomes. The cause of this discrepancy does not lie in the composition of the reaction mixture used by Maley & Plaut, which gave the same P:O ratio as our standard reaction mixture with our sarcosome preparation.

The larger difference between the P:O values with succinate of rat-liver and rat-heart mitochondria reported in the literature is explained by our experiments. With 6 mM-succinate, which is about the concentration used in most studies, the average P:O ratios in Table 2 are 2.17 for liver and 0.95 for heart. About one-half of this difference is due to the fact that under these conditions a considerable proportion of the oxygen uptake with liver mitochondria is associated with the oxidation of fumarate, which yields a higher P:O ratio. This has the effect of exaggerating the true difference between the two types of mitochondria, which we believe is better reflected by the ratios obtained with 60 mM-succinate.

The remaining difference (1.57 with rat-liver mitochondria, 0.98 with rat-heart sarcosomes; see Table 8) is due to the fact that in the latter preparation hydrolytic side reactions, at pH 7.4 in the presence of Mg^{2+} ions, compete with inorganic phosphate for the energy-rich intermediates of oxidative phosphorylation. A quantitative measure of these side reactions is given by $1/r$, where r is the ratio of the adenosine triphosphatase activity in the presence and absence of the uncoupling agent dinitrophenol (Mg^{2+} ions present in both cases). Fig. 2 shows a negative correlation between the P:O ratios of individual preparations of mitochondria (from rat liver and rat, mouse and guinea-pig heart) and the values of $1/r$.

Heart sarcosomes isolated from the guinea pig gave appreciably higher ratios (mean 1.31) than those obtained from the rat, and this difference is correlated with a lower activity of the side reactions in the guinea-pig preparation. Hülsmann & Slater (unpublished work) have found a mean P:O ratio of 1.47 with guinea-pig-heart sarcosomes at pH 6.5. Hatefi & Lester (1958) have reported a P:O ratio of 1.93 with 'heavy' mitochondria isolated from ox heart obtained from a slaughterhouse. They suggest that the lower P:O ratios reported by Cleland & Slater (1953) might be due to the presence of both light and heavy particles in our sarcosomal preparation. However, this does not seem a likely explanation of our present results, since the sarcosomes were isolated by sedimentation at relatively low speed (15 min. at 600 g).

Thus there appears to be no fundamental difference between the mechanism of oxidative phosphorylation in liver and heart mitochondria. A P:O ratio substantially above 1.0 for the step succinate $\rightarrow$ fumarate in rat-liver and guinea-pig-

heart mitochondria shows that there are two phosphorylating steps in this reaction in these preparations. There is evidence that this is also the case even with our preparations of rat-heart sarcosomes in which the P:O ratio does not exceed 1.0. With this preparation, phosphorylation has been demonstrated between substrate and ferricytochrome *c* (this paper) and between ferrocycytochrome *c* and oxygen (Slater, 1954). Further evidence is provided by a study of the pH-activity curve of the P:O ratio (Hülsmann & Slater, 1957).

SUMMARY

1. Factors affecting the P:O ratio with succinate as substrate were studied, both with rat-liver mitochondria and heart sarcosomes of the rat, mouse and guinea pig. The concentrations of all components of the reaction mixture except succinate could be varied widely, without appreciable effect.

2. Higher P:O ratios were obtained with liver mitochondria with low concentrations of succinate. It is believed that this is because, under these conditions, a large proportion of the total oxygen uptake is associated with oxidation of fumarate, a reaction which gives a higher yield of oxidative phosphorylation. With higher concentrations of succinate there was no appreciable oxidation of the fumarate formed. With heart sarcosomes there was no appreciable oxidation of the fumarate, with either high or low concentrations of succinate.

3. Our preparations of rat-heart sarcosomes gave appreciably lower P:O ratios than liver mitochondria. The lower ratios with sarcosomes are quantitatively correlated with the higher adenosine triphosphatase activities (in the presence of Mg^{2+} ions) of these preparations.

4. Oxidative phosphorylation (with low ratios) was detected, coupled with the reduction of ferricytochrome *c* by succinate.

5. The results support the view generally held that the oxidation of succinate to fumarate by mitochondrial preparations is accompanied by two phosphorylating steps per pair of electrons, even in preparations of rat-heart sarcosomes which give a P:O ratio of 1.0.

REFERENCES

- Belitzer, V. A. & Tsiabakowa, E. T. (1939). *Biokhimiya*, **4**, 516.
- Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
- Christie, G. S. & Judah, J. D. (1953). *Proc. Roy. Soc. B*, **141**, 420.
- Cleland, K. W. & Slater, E. C. (1953). *Biochem. J.* **53**, 547.
- Colowick, S. P., Welch, M. S. & Cori, C. F. (1940). *J. biol. Chem.* **133**, 359.
- Copenhaver, J. H. & Lardy, H. A. (1952). *J. biol. Chem.* **195**, 225.

- Dianzani, M. U. (1956). *Biochim. biophys. Acta*, **22**, 389.
- Ernster, L., Lindberg, O. & Löw, H. (1955). *Nature, Lond.*, **175**, 168.
- Ernster, L., Ljunggren, M. & Lindberg, O. (1954). *Acta chem. scand.* **8**, 658.
- Green, D. E., Beinert, H., Fuld, M., Goldmann, D., Paul, M. H. & Sarkar, N. K. (1953). *Exp. Cell Res.* **4**, 222.
- Hatefi, Y. & Lester, R. L. (1958). *Biochim. biophys. Acta*, **27**, 83.
- Hogeboom, G. H. (1955). In *Methods in Enzymology*, vol. 1, p. 16. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Holton, F. A., Hülsmann, W. C., Myers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 579.
- Hülsmann, W. C. & Slater, E. C. (1957). *Nature, Lond.*, **180**, 372.
- Jalling, O., Lindberg, O. & Ernster, L. (1955). *Acta chem. scand.* **9**, 198.
- Korkes, S. (1952). In *Phosphorus Metabolism*, vol. 2, p. 618. Ed. by McElroy, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
- Korkes, S., del Campillo, A. & Ochoa, S. (1950). *J. biol. Chem.* **187**, 891.
- Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V. & Hems, R. (1953). *Biochem. J.* **54**, 107.
- Lewis, S. E. & Slater, E. C. (1954). *Biochem. J.* **58**, 207.
- Maley, G. F. & Plaut, G. W. E. (1953). *J. biol. Chem.* **205**, 297.
- Maley, G. F. & Plaut, G. W. E. (1954). *Biochim. biophys. Acta*, **14**, 443.
- Massey, V. (1955). In *Methods in Enzymology*, vol. 1, p. 729. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Myers, D. K. & Slater, E. C. (1957). *Biochem. J.* **67**, 558.
- Nielsen, S. O. & Lehninger, A. L. (1954). *J. Amer. chem. Soc.* **76**, 3860.
- Nossal, P. M. (1951). *Biochem. J.* **49**, 407.
- Nossal, P. M. (1952). *Biochem. J.* **50**, 349.
- Packer, L. (1958). *Exp. Cell Res.* **15**, 551.
- Slater, E. C. (1949). *Biochem. J.* **45**, 1.
- Slater, E. C. (1953). *Biochem. J.* **53**, 157, 521.
- Slater, E. C. (1954). *Nature, Lond.*, **174**, 1143.
- Slater, E. C. (1955). *Biochem. J.* **59**, 392.
- Slater, E. C. (1956). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 264.
- Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.
- Slater, E. C. & Cleland, K. W. (1953). *Biochem. J.* **55**, 566.
- Slater, E. C. & Holton, F. A. (1954). *Biochem. J.* **56**, 28.
- van Dorp, A. & Slater, E. C. (1959). *Biochim. biophys. Acta*, **33**, 559.
- Whittam, R., Bartley, W. & Weber, G. (1955). *Biochem. J.* **59**, 590.
- Wu, C. Y. & Tsou, C. L. (1955). *Sci. Sinica*, **4**, 137.

Some Properties of the Malic Enzyme of Pigeon Liver

1. CONVERSION OF MALATE INTO PYRUVATE

By R. G. STICKLAND*

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

(Received 14 April 1959)

The malic enzyme, first described in pigeon liver by Ochoa, Mehler & Kornberg (1948), catalyses the reaction:

where TPN^+ and TPNH stand for the oxidized and reduced forms of triphosphopyridine nucleotide. The enzyme is thought to play a role in the synthesis of glycogen from lactate and pyruvate and to be one of the factors controlling the supply of oxaloacetate in the liver (Krebs, 1954; Hiatt, Goldstein, Lareau & Horecker, 1958). Although the coenzyme requirements of the enzyme are firmly established relatively little is known about the kinetics and the effects of inhibitors. Rutter & Lardy (1958) have examined the effects of pH and cations on the conversion of malate into pyruvate.

* Present address: John Innes Horticultural Institution, Bayfordbury, Hertford, Herts.

A number of carboxylic acids, such as malonic acid, tartronic acid, mesoxalic acid, D- and meso-tartaric acid, oxalic acid, fluoromalic acid and fluorocitric acid, cause an accumulation of acetoacetate in respiring liver preparations (Edson, 1936; H. A. Krebs, unpublished work). Since ketogenesis may be related to the supply of oxaloacetate, the action of the above-mentioned acids might be due to the inhibition of either the 'oxaloacetate-synthesizing enzyme' (Utter & Kura-hashi, 1955), which catalyses the reaction

(where GDP and GTP stand for guanosine di- and tri-phosphate and IDP and ITP for inosine di- and tri-phosphate), or malic enzyme, both of which lead to the formation of oxaloacetate from the products of glycolysis. The action of the ketogenic