Oxidation of *iso*Citrate and Associated Phosphorylations

By PAULETTE VIGNAIS*, P. VIGNAIS* AND W. BARTLEY

Medical Research Council Unit for Research in Cell Metabolism,

Department of Biochemistry, University of Oxford

(Received 21 June 1956)

No direct measurements have as yet been reported of the value of the phosphorylation quotient (P/O ratio) associated with the oxidation of *isocitrate* to oxalosuccinate. Ochoa (1947), studying the one-step oxidation of *isocitrate* to oxalosuccinate in the presence of arsenite, failed to obtain phosphorylation, probably owing to the uncoupling action of the inhibitor (Lehninger, 1949). Copenhaver & Lardy (1952) were unable to obtain the one-step oxidation of *isocitrate* in the presence of semicarbazide, but from experiments in which citrate was oxidized in the presence of malonate they calculated a P/O ratio of 3 for the reaction *isocitrate* $\rightarrow$ α -oxoglutarate.

P/O ratios based on measurements of the rate of incorporation of labelled phosphate into adenosine triphosphate (ATP) (Krebs, Ruffo, Johnson, Eggleston & Hems, 1953) were between 3.1 and 3.6 when citrate was the substrate, and correction for differential rate of exchange of the β phosphorus atom of ATP raised these values to between 3.4 and 4.2 (Whittam, Bartley & Weber, 1955).

This paper presents observations on the oxidation of *isocitrate* by rat-liver mitochondria under different experimental conditions.

EXPERIMENTAL

Tissue preparations. Rat-liver mitochondria were prepared by a modification of the method of Bartley & Davies (1954). The liver, after chilling in ice-cold 0.9% KCl, was homogenized for 45 sec. in 9 parts of 0.25M sucrose with a stainless-steel homogenizer of the Potter-Elvehjem type. The homogenate was centrifuged at 300 g for 10 min. at 0° to remove nuclei and unbroken cells. This rate of centrifuging, which is slower than that commonly used, minimizes the sedimentation of the large mitochondria (Novikoff, Podber, Ryan & Noe, 1953). Microscopic examination of the supernatant from this centrifuging showed very few nuclei. Potassium chloride (11.2%, w/v) was added to the supernatant to give a final concentration of 0.9%. The mixture was centrifuged at 2000 g for 15 min. The sedimented mitochondria were twice suspended in 0.9% KCl and centrifuged for 7 min. at 2000 g. They were finally suspended in the required volume of 0.9% KCl.

* Present address: Service de Biochimie, Faculté de Médecine et de Pharmacie, Algiers.

Special chemicals. Barium ATP (LePage, 1949) was converted into the sodium salt by treatment with Na_2SO_4 . In this product the ratio ADP/ATP was about 0.15.

Carrier-free $\text{KH}_2^{32}\text{PO}_4$ was obtained from the Atomic Energy Research Establishment, Harwell. It was diluted with water (1 mc/10 ml.) and neutralized if necessary.

Sodium (+)-*isocitrate* was obtained by alkaline hydrolysis of dimethyl*isocitric* lactone (Krebs & Eggleston, 1944; Hogeboom & Schneider, 1950).

Triphosphopyridine nucleotide (TPN) of 80% purity (LePage & Mueller, 1949) and diphosphopyridine nucleotide (DPN) of 60% purity (Kornberg & Pricer, 1951) together with preparations of DPN-*isocitric* dehydrogenase (Kornberg & Pricer, 1951) and TPN-*isocitric* dehydrogenase (Graffin & Ochoa, 1950) were gifts from Dr D. D. Davies.

Fumarase (Massey, 1952) was purified to the stage of the first $(\text{NH}_4)_2\text{SO}_4$ fractionation and potato apyrase was prepared according to Lee & Eiler (1951). Hexokinase (Berger, Slein, Colowick & Cori, 1946) was purified to stage 3.

All other chemicals were of analytical reagent grade.

Experimental procedures. Each Warburg vessel contained in the main compartment 2 ml. of particles suspended in 0.9% KCl, 0.2 ml. of substrate, 0.5 ml. of about 0.2M sodium ATP, 0.2 ml. of 0.1M potassium phosphate buffer, pH 7.4, and 0.2 ml. of 0.02M MgCl_2 . The side arm contained 0.1 ml. of the diluted solution of $\text{KH}_2^{32}\text{PO}_4$ and the centre well 0.2 ml. of 2N-NaOH with filter paper. The volume was brought to 4 ml. by the addition of water. The vessels were gassed with O_2 as described by Whittam *et al.* (1955). For anaerobic experiments a stick of freshly scraped yellow phosphorus was put in the centre well and the vessels were gassed with N_2 .

All the incubations were carried out at about 20°. When *isocitrate* was the substrate the radioactive phosphate was usually tipped into the main compartment at the start of incubation. When the sample of ATP contained more than 20% of ADP the isotope was tipped in after incubation for 3 min., when the ratio ATP/ADP had reached a steady-state value.

Oxygen consumption was measured manometrically after an equilibration period of 10 min. As the rate of O_2 uptake with *isocitrate* or citrate as substrate remained fairly constant during the 10–30 min. incubation, it was possible to extrapolate to the zero time of incubation. After incubation, the vessels were placed in a dish of ice and water and the enzymic reactions stopped by the immediate addition of 0.5 ml. of 30% (w/v) trichloroacetic acid. After centrifuging, the trichloroacetic acid extracts were used for determination of phosphorus compounds and for other chemical estimations.

Table 1. *Oxygen uptake and incorporation of ^{32}P into ATP with varying amounts of substrate and mitochondria in the presence of (+)-isocitrate*

Each Warburg flask contained in the main compartment 2 ml. of rat-liver mitochondrial suspension in 0.9% (w/v) KCl, 0.5 ml. of 0.02M sodium ATP, 0.2 ml. of 0.02M-MgCl₂, 0.2 ml. of 0.1M phosphate buffer (pH 7.4), 0.2 ml. of sodium (+)-isocitrate (0.1M, 0.05M or 0.04M); in the side arm: 0.1 ml. of KH₂³²PO₄. Final vol. was brought up to 4 ml. with water. Gas phase O₂, temp. 20°. *v* was calculated by the method of Krebs *et al.* (1953) after 2 min. incubation.

Expt. no.	Final concn. of isocitrate (M)	Dry wt. of mitochondria added (mg.)	Q _{O₂} (μl. of O ₂ /mg. dry wt./hr.)	O ₂ consumption (μg.atoms/min.)	<i>v</i> (μg.atoms of P exchanged/min.)	P/O	Ratio (after 20 min.):*
1	0.005	46	11	0.75	2.07	2.76	0.56
2-3	0.005	37	11.8	0.65	1.87	2.94	0.56
4	0.005	31	12.3	0.57	1.30	2.29	0.50
5	0.005	24	13.1	0.47	0.84	1.80	0.53
6	0.0025	61	12.8	1.17	2.02	1.73	0.72
7	0.0025	40	10.2	0.60	0.84	1.40	0.35
8	0.0025	12	2.8				0.23
9	0.002	60	12.5	1.12	2.52	2.25	0.77

* Specific radioactivity of ATP (counts/min./μg.atom of labile P)

Specific radioactivity of inorganic P (counts/min./μg.atom of P)

Chemical estimations. Citrate was estimated (Taylor, 1953) on the neutralized trichloroacetic acid extract after incubation with crude aconitase. The sum of citrate, isocitrate and *cis*-aconitate was calculated according to Krebs & Eggleston (1944).

isocitrate was determined spectrophotometrically by TPN-isocitric dehydrogenase (Ochoa, 1948). The molecular extinction coefficient given by Horecker & Kornberg (1948) was used to calculate the amount of isocitrate present. Identification and estimation of α -oxoglutarate was carried out according to El Hawary & Thompson (1953). As α -oxoglutarate was the only keto acid found in the test solutions the phenylhydrazones were sometimes determined colorimetrically without prior chromatographic separation.

Phosphate esters were separated by paper chromatography with the formic acid-isopropyl ether solvent of Hanes & Isherwood (1949) or with the isobutyric acid-NH₃ solvent (Krebs & Hems, 1953) if ATP and ADP were to be separated. After wet-ashing of the chromatogram spots, the phosphorus was estimated according to Berenblum & Chain (1938) and Bartley (1953).

For measurement of radioactivity an M₄ liquid counter (Veall, 1948) was used. The results are corrected for isotope decay, dead time and background counts. The estimation of the radioactivity located in P β and P γ of ATP was made after action of apyrase at 0° according to Whittam *et al.* (1955).

Treatment of the data. When possible, P/O ratios were calculated as described by Whittam *et al.* (1955). In experiments where this was not technically possible or convenient, calculations were made by the formula of Stewart (1953) case 1, assuming that P β and P γ exchange at the same rate. The error in this case was minimized by calculating only for the short times of incubation.

RESULTS

Oxygen uptake and incorporation of ^{32}P into ATP with varying amounts of isocitrate and mitochondria

Table 1 shows the influence of substrate concentration and of the amount of tissue on the oxidation of

Table 2. *Comparison of the specific radioactivities of the P β and P γ atoms of ATP after incubation with (+)-isocitrate (0.0025M) and rat-liver mitochondria (40 mg. dry wt.)*

Determination of radioactivity of P β and P γ of ATP was made after apyrase action according to Whittam *et al.* (1955). Trichloroacetic acid extract (1 ml.) was chromatographed in order to separate ATP from inorganic P (Krebs & Hems, 1953). After elution by 0.02M-NaHCO₃, ATP was incubated for 80 min. at 0° with the following mixture: 0.1 ml. of 0.11M-CaCl₂, 0.1 ml. of M sodium succinate buffer (pH 6.5), 0.1 ml. of potato apyrase and water to bring the total vol. to 1.5 ml. Enzyme action was stopped by adding 0.2 ml. of 30% trichloroacetic acid. After centrifuging, 150 μl. of supernatant was used for determination of ADP and orthophosphate. (Results refer to Expt. 7, Table 1).

Period of incubation after addition of radioactivity (min.)	Specific radioactivity of P atoms of ATP (counts/min./μg.atom of P)		Ratio: specific activity of P γ specific activity of P β
	P β	P γ	
0	0	336	
2	524	4220	8.1
6	1364	6510	4.8
10	2170	7650	3.5
20	4240	9050	2.1
30	5260	8900	1.6

isocitrate and on the P/O ratio as measured by the rate of incorporation of ^{32}P into ATP. The phosphorylation rate (*v*) fell more rapidly than the O₂ uptake with decreasing concentrations of mitochondria. For example, in Expts. 2 and 5 the dry weight fell from 37 to 24 mg. but *v* was halved and the O₂ uptake diminished by about 30%. Thus the efficiency of the oxidative phosphorylation was dependent on the amount of mitochondria. When the concentration of mitochondria was greater than

about 37 mg./4 ml. the P/O ratio became constant, at a concentration of isocitrate of 0.005 M.

The concentration of ATP was maintained in spite of variation in the O₂ uptake, amount of mitochondria present and the variable phosphorylation rate.

When the concentration of isocitrate was lowered a larger quantity of mitochondria was required to maintain the same rate of incorporation of orthophosphate into ATP (Table 1).

In some experiments the ratio specific radioactivity of P^β and P^γ atoms of ATP/specific radioactivity of orthophosphate phosphorus remained low even after prolonged incubation. For instance, in Table 1 (Expt. 7) this ratio was only 0.35 after a period of incubation of 20 min. To test whether this low incorporation was due to lack of exchange between P^γ and P^β atoms of ATP, the relative amounts of radioactivity in these atoms was measured in this experiment according to Whittam *et al.* (1955). Table 2 shows that the exchange between P^β and P^γ was normal. Thus it is only the exchange between P^γ and orthophosphate which was lowered.

Effect of fluoride on the oxidation of isocitrate and on the rate of incorporation of ³²P into ATP

When phosphorylation is measured by coupling the synthesis of ATP with the hexokinase system (Slater & Holton, 1953), it is necessary to add fluoride to inhibit adenosine triphosphatase (ATPase) activity. The interpretation of the results obtained in the presence of fluoride depends on the validity of the assumption that inhibition of ATPase is the only action of fluoride in this system and, in particular, that it has no effect on phosphorylation. This assumption has been tested by measuring the effect of fluoride on the rate of incorporation of radioactive phosphorus into ATP in the presence of isocitrate.

As shown in Table 3 fluoride does not inhibit this reaction. However, as fluoride can inhibit the oxidation of isocitrate (see below) similar experiments were made with succinate as substrate. No effect of fluoride was found. The P/O ratios obtained, 2.12 with NaF and 2.31 without NaF, agree with the published figures of Krebs *et al.* (1953) and Whittam *et al.* (1955).

Slater & Bonner (1952) have shown that succinic oxidase is inhibited by fluoride and that this inhibition is enhanced by the addition of phosphate. Similar observations were made on rat-liver mitochondria. There was a slight inhibition of succinate oxidation (5–10 %) in the presence of 0.04 M fluoride and 0.005 M phosphate. This inhibition increased to about 40 % when the phosphate concentration was raised to 0.025 M. Inhibition of the rate of oxidation did not affect the P/O ratio. When isocitrate was the substrate, fluoride inhibited the O₂ uptake at 0.0025 M isocitrate concn., but not at 0.005 M isocitrate concn. (see Tables 3 and 6).

Fluoride is known to be an inhibitor of myokinase (Kotelnikova, 1950; Barkulis & Lehninger, 1951; Slater, 1953). The isotopic equilibration of the P^β and P^γ atoms of ATP is probably accounted for by the action of myokinase (Whittam *et al.* 1955) and thus the rate at which the P^β and P^γ atoms of ATP exchanged would be expected to decrease in the presence of fluoride. This expected decrease is illustrated in Table 3 by the fall, in the presence of fluoride, of the value of the slope *m*₂.

In the mathematical treatment by Whittam *et al.* (1955), *m*₁ and *m*₂ are constants obtained from a logarithmic plot of the time course of uptake of radioactivity into ATP. The rate of exchange of the P^γ atom of ATP with orthophosphate is measured by *m*₁ and the rate of exchange of the P^γ and P^β atoms of ATP by *m*₂. In Table 3 (Expt. 2), *m*₂ in the presence of fluoride was zero and thus myokinase must have been virtually completely inhibited.

Table 3. Influence of fluoride on oxygen uptake and on rate of incorporation of ³²P into ATP in rat-liver mitochondria in the presence of isocitrate

Incubation in Warburg vessels at 20° in O₂. The incubation medium (4 ml.) contained 2 ml. of tissue suspension in 0.9 % KCl, 0.5 ml. of about 0.02 M sodium ATP, 0.2 ml. of 0.02 M-MgCl₂ and 0.2 ml. of 0.1 M phosphate buffer (pH 7.4). Solution (0.1 ml. of KH₂³²PO₄) in the side arm was tipped when the vessels were put into the bath. The centre well contained 0.2 ml. of 2N-NaOH and filter paper. Sodium fluoride, when present: 0.04 M.

Expt. no.	Dry wt. of mitochondria (mg.)	Final concn. of isocitrate (M)	NaF	O ₂ uptake (μg. atom/min.)	<i>v</i> * (Krebs <i>et al.</i> 1953)	P/O	<i>m</i> ₂ †	<i>m</i> ₁ †	<i>v</i> (Whittam <i>et al.</i> 1955)	P/O
1	46	0.005	–	0.75	2.07	2.76	–0.022	–0.482	2.55	3.4
	46	0.005	+	0.76	2.16	2.85	–0.0115	–0.406	2.52	3.3
2	61	0.0025	–	1.17	2.02	1.73	–0.035	–0.517	2.76	2.38
	61	0.0025	+	0.74	1.81	2.45	.	.	.	.

* *v* calculated after a period of incubation of 2 min. (μg. atoms of P exchanged/min.).

† *m*₁ and *m*₂ are constants used in calculating *v* (see Whittam *et al.* 1955). *m*₁ measures the rate at which the P^γ atom of ATP exchanges with orthophosphate and *m*₂ the rate at which the P^β and P^γ atoms of ATP exchange with each other.

Consumption of isocitrate and the effect of fluoride thereon

Table 4 shows the changes in amounts of the tricarboxylic acids when (+)-isocitrate was incubated with rat-liver mitochondria. As expected, the aconitase reaction rapidly removed isocitrate.

Table 4. *Consumption of isocitrate by rat-liver mitochondria*

isoCitrate was determined spectrophotometrically according to Ochoa (1947). Citrate was estimated (Taylor, 1953) on the neutralized trichloroacetic acid extract after incubation with crude aconitase. The sum of citrate, isocitrate and cis-aconitate was calculated according to Krebs & Eggleston (1944). (Results obtained from Expt. 7, Table 1.)

Period of incubation (min.)	Total tricarboxylic acids found (μmoles)	isoCitrate found (μmoles)	cis-Aconitate + citrate calculated (μmoles)
0	9.4	9.7	0
6	6.65	2.4	4.25
30	3.34	0.4	2.94

The relation between the O₂ uptake and the quantity of tricarboxylic acid consumed is given in Table 5. The ratio μg. atoms of O₂ used/μmoles of tricarboxylic acid used rose with time; even during the earliest period of incubation it was greater than 1 (Table 5), which indicates that the oxidation went beyond the stage of α-oxoglutarate.

When fluoride was added, the consumption of isocitrate was increased whilst the O₂ uptake remained the same or decreased. This reduced the above ratio in some cases to values less than 1 (Table 6, Expt. 1). This means that tricarboxylic acids have been removed by a non-oxidative reaction. The determination of α-oxoglutarate in these experiments conclusively showed that oxidation to α-oxoglutarate was not the only reaction, as the removal of 5.4 μmoles of tricarboxylic acids was accompanied by the production of only 1 μmole of α-oxoglutarate. The observation suggests an anaerobic removal of tricarboxylic acids, a point which will be discussed below.

Table 5. *Variation of the ratio oxygen uptake/tricarboxylic acids consumed with time of incubation and concentration of substrate*

The standard content of Warburg vessels has been given in Table 1.

Expt. no.	Dry wt. of tissue (mg.)	isoCitrate concentration in flask (M)	Time of incubation (min.)	Tricarboxylic acids used (μmoles)	O ₂ used (μg.atoms)	Ratio: O ₂ (μg.atoms) tricarboxylic acid used (μmoles)
1	46	0.005	6	2.6	4.5	1.7
	46	.	30	8.2	22.5	2.7
4	31	0.005	20	8.3	11.4	1.4
5	24	0.005	30	10.0	14.1	1.4
6	61	0.0025	4	2.0	4.7	2.3
	61	.	10	2.6	11.7	4.5
	61	.	30	5.7	35.1	6.2
7	40	0.0025	6	2.75	3.6	1.3
	40	.	30	6.06	18.0	3.0
9	60	0.002	20	4.0	22.4	5.6
10	75	0.002	30	4.23	36.0	8.5

Table 6. *Influence of fluoride on consumption of isocitrate by rat-liver mitochondria*

Standard incubation conditions. Fluoride, when present: 0.04 M.

Expt. no.	Dry wt. of mitochondria (mg.)	Final concn. of isocitrate (M)	NaF	Period of incubation at 20° (min.)	O ₂ uptake (μg.atoms)	Tricarboxylic acids used (μmoles)	Ratio: O ₂ used (μg.atoms) tricarboxylic acid used (μmoles)	α-Oxo-glutarate formed (μmoles)
1	46	0.005	-	6	4.5	2.6	1.7	0.4
				30	22.5	8.2	2.7	.
	46	0.005	+	6	4.55	5.4	0.85	1.0
				30	22.8	8.3	2.7	.
2	61	0.0025	-	4	4.7	2.0	2.3	.
				10	11.7	2.6	4.5	.
				30	35.0	5.7	6.1	.
	61	0.0025	+	4	2.96	2.0	1.5	.
				10	7.4	4.4	1.7	.
				30	22.2	8.8	2.5	.

Oxidation of citrate in the presence of semicarbazide

Semicarbazide (0.04M) was found to have no inhibitory action on the incorporation of ^{32}P into ATP after 20 min. when β -hydroxybutyrate was the substrate.

When citrate was the substrate, semicarbazide inhibited the incorporation of ^{32}P into ATP in liver homogenates or mitochondrial suspensions without affecting the O_2 uptake.

When fluoride and semicarbazide were added the value of v was increased, the O_2 uptake was slightly inhibited and the P/O ratio was about twice that observed with semicarbazide alone (0.73 instead of 0.33, Table 7, Expt. 4). The quantity of citrate removed was increased when fluoride as well as semicarbazide was added (13 μmoles instead of 10 μmoles after incubation for 60 min.). This is analogous to the observation that the removal of isocitrate is increased by fluoride. However, in all cases the ratio $\mu\text{g. atoms of O}_2 \text{ used}/\mu\text{moles of tri-carboxylic acid used}$ was still higher than 1 (Table 8), which indicates that semicarbazide was ineffective as a trapping agent for α -oxoglutarate.

In spite of the extremely low rate of incorporation of radioactivity into ATP the total amount of ATP did not diminish. Semicarbazide thus stabilized ATP, possibly by inhibition of ATPase activity. A test was therefore made of the effect of semicarbazide on the ATPase activity of the mitochondria under anaerobic conditions in the absence of substrate. Under these conditions only a slight (10–15%) inhibition of the ATPase activity occurred on the addition of semicarbazide. This suggests that in the intact mitochondria, metabolizing in O_2 , ATPase activity is in some way linked with the rate at which P is incorporated into ATP.

Rate of turnover of ATP under anaerobic conditions

Krebs *et al.* (1953) and Whittam *et al.* (1955) showed that anaerobic phosphorylation was low under the conditions used for the study of turnover of ^{32}P into ATP, being at most 10% of that obtained aerobically. Table 9 shows anaerobic experiments in which the rate of turnover of ATP was determined in the presence of isocitrate. After 6 min. incubation at 20° , the concentration of ATP had fallen, particularly when 2:4-dinitrophenol (DNP) was present, but in all cases an appreciable incorporation of ^{32}P into ATP occurred. The incorporation was approximately halved by fluoride and DNP (Table 9, Expt. 1). In Expt. 2 the concentration of ATP remained constant during the time of incubation, which made it possible to calculate v . The average value obtained (0.78) was approximately one-third of that found under aerobic conditions with a similar amount of mitochondria.

Table 7. Influence of fluoride on the oxidation of citrate in the presence of semicarbazide

Besides the standard additions (see Table 1) each Warburg flask contained 0.02M citrate and 0.04M semicarbazide.

Expt. no.	Fluoride added (final concn.) (M)	Tissue preparation	Dry wt. (mg.)	O_2 uptake ($\mu\text{g. atoms/min.}$)	v (Krebs <i>et al.</i> 1953)	P/O	v (Whittam <i>et al.</i> 1955)	P/O	Ratio (after 30 min.)*	Citrate used (μmoles)
1	.	Whole homogenate	62	0.44	0.15	0.34	.	.	0.12	5 (30 min. incubation)
2	.	Whole homogenate	59	0.42	0.17	0.40	.	.	0.27	9 (80 min. incubation)
3	.	Mitochondria	58	0.67	0.22	0.33	.	.	0.20	10 (60 min. incubation)
4	0.04	Mitochondria	58	0.56	0.41	0.73	0.56	1.06	0.51	13 (60 min. incubation)

* Specific radioactivity of ATP (counts/min./ $\mu\text{g. atom of labile P}$)
Specific radioactivity of inorganic P (counts/min./ $\mu\text{g. atom}$)

Table 8. Citrate consumption by rat-liver mitochondria in the presence of semicarbazide

Results from Expt. 2, Table 7. The vessels initially contained 80 μ moles of citrate and 0.04M semicarbazide; other additions as in Table 1. Tricarboxylic acids were estimated after 60 min. incubation.

Fluoride added (final concn.) (M)	Total tricarboxylic acid found (μ moles)	Citrate found (μ moles)	isoCitrate found (μ moles)	cis-Aconitate calculated (μ moles)	Ratio: O ₂ uptake (μ g.atoms) tricarboxylic acid removed (μ moles)
.	68.5	40.8	6.4	21.3	4.0
0.04	65.5	62.4	2.2	0.9	2.4

Table 9. Rate of turnover of ATP under anaerobic conditions

isoCitrate (final concn. 0.004M) was incubated anaerobically with rat-liver mitochondria. The flasks were pre-incubated for 2 min. before tipping in $\text{KH}_2^{32}\text{PO}_4$; 46 mg. dry wt. of mitochondria was used in Expt. 1 and 40 mg. in Expt. 2. Otherwise, conditions were as in Table 3.

Expt. no.	Additions (final concn.) (M)	Time of incubation after addition of ^{32}P (min.)	ATP in vessel (μ moles)	v [μ g.atoms of P exchanged/min. (Krebs <i>et al.</i> 1953)]	Ratio:*
1	.	0	5.7	.	0
	.	6	4.8	.	0.201
	Fluoride (0.04)	6	4.9	.	0.096
	DNP (0.0001)	6	1.3	.	0.078
2	.	0	9.4	.	.
	.	1.75	9.0	0.73	0.072
	.	3.25	9.2	0.86	0.138
	.	4.25	9.0	0.74	0.174
	Fluoride (0.04)	1.25	9.0	0.20	0.012

* Specific activity of ATP (counts/min./ μ g.atom of labile P)
Specific activity of inorganic P (counts/min./ μ g.atom of P)

Table 10. Anaerobic phosphorylation when glucose and hexokinase was used as trapping agent

Rat-liver mitochondria (43 mg. dry wt.) were incubated with isocitrate at 20°. The main compartment of the Warburg vessels contained: 2 ml. of mitochondrial suspension in 0.9% KCl or 2 ml. of 0.9% KCl, 0.1 ml. of 0.02M sodium adenosine monophosphate, 0.1 ml. of 0.02M sodium ADP, 0.2 ml. of 0.1M phosphate buffer, 0.4 ml. of 0.4M-NaF, 0.2 ml. of 0.02M-MgCl₂ and 0.1 ml. of 0.15M sodium (+)-isocitrate when added. The side arm contained 0.2 ml. of hexokinase solution (20 mg./ml.) and 0.1 ml. of 0.25M glucose. The gas phase was N₂ (a stick of phosphorus was put in the centre well). Vessels were shaken for 2 min. at 20° before tipping in hexokinase + glucose. Incubation time, 6 min.

Addition	Glucose phosphate formed/vessel (μ mole)
Mitochondria + isocitrate	0.38
Mitochondria	0.21
None	0.06

The incorporation of phosphate into ATP could be due to an exchange reaction accounted for by the reversibility of the system. This would not require energy (Boyer, Falcone & Harrison, 1954; Swanson, 1956). To check this point an experiment was carried out with the same preparation in which the hexo-

kinase system served to trap the ATP formed (Table 10). This showed that some net synthesis occurred under anaerobic conditions, but at a rate no more than 4% of that calculated from the rate of incorporation. After allowing for the synthesis of glucose phosphate by the myokinase reaction from the added ADP and AMP there remains 0.17 μ g.-atom of P fixed, compared with about 4.5 μ g.atoms of P measured from the rate of incorporation. Thus the anaerobic incorporation was mainly due to an exchange reaction.

Effect of added DPN and TPN on the incorporation of phosphate into ATP in the presence of isocitrate

It is known that mitochondria transfer H₂ from isocitrate either to TPN (Grafflin & Ochoa, 1950) or to DPN (Plaut & Sung, 1954). The relative magnitude of the phosphorylation associated with the two pathways is not known.

To test this, DPN or TPN, and a DPN- or TPN-specific isocitric dehydrogenase were added to phosphorylating mitochondrial suspensions. Added DPN does not readily enter native mitochondria but does so after treatment of mitochondria with water (Lehninger, 1951). In a control experiment the effect of water treatment on the ability of the mitochondria to oxidize and phosphorylate was tested. One-half of a sedimented mitochondrial preparation was suspended in water and allowed to stand for

Table 11. *Effect of fluoride on the phosphorylation quotient for the oxidation of reduced DPN and reduced TPN*

Warburg flasks contained in the main compartment: 2 ml. of rat-liver mitochondria (dry wt. 42 mg.) in 0.9% KCl, 0.5 ml. of approx. 0.02M ATP, 0.2 ml. of phosphate buffer (pH 7.4), 0.2 ml. of 0.02M-MgCl₂, 0.1 ml. of 0.1M isocitrate, 0.1 ml. of 0.5M nicotinamide and either 0.2 ml. of 0.008M DPN solution and 0.2 ml. of DPN-specific isocitric dehydrogenase or 0.2 ml. of 0.002M TPN solution and 0.2 ml. of TPN-specific isocitric dehydrogenase. Water added to 4 ml. The side arm contained 0.1 ml. of KH₂³²PO₄. NaF, when present, 0.04M.

	O ₂ uptake (μg.atoms/min.)	^v [μg.atoms of P exchanged/min. (Krebs <i>et al.</i> 1953)]	P/O ratio
DPN + DPN-specific isocitric dehydrogenase	0.77	2.18	2.83
DPN + DPN-specific isocitric dehydrogenase + fluoride	0.21	0.56	2.68
TPN + TPN-specific isocitric dehydrogenase	1.00	2.36	2.36
TPN + TPN-specific isocitric dehydrogenase + fluoride	0.54	0.85	1.56

10 min. at 0°. Concentrated KCl solution was then added to bring the final concentration to 0.9%. The second half of the preparation was suspended directly in the same volume of 0.9% KCl solution. Water treatment increased the O₂ uptake from 0.64 to 1.02 μg.atoms/min. but somewhat diminished the phosphorylation quotient (2.92 instead of 3.08). Fluoride reduced the O₂ consumption in the presence of the DPN-specific isocitric dehydrogenase but did not affect the P/O ratio; in the presence of the TPN-specific enzyme both O₂ consumption and the P/O ratio were reduced by fluoride (Table 11). These results are in agreement with the assumption that the oxidation of reduced TPN is not as effectively coupled with phosphorylation as is the oxidation of reduced DPN. However, further work is necessary to assess the effect of fluoride on the phosphorylation associated with the oxidation of the two reduced coenzymes.

DISCUSSION

The main purpose of this investigation was to establish the phosphorylation quotient for the oxidation of isocitrate to α-oxoglutarate. On addition of isocitrate to liver mitochondria phosphorylation quotients of between 1.4 and 3.4 were observed (Tables 1 and 3). However, these values do not refer to conditions where the conversion of isocitrate into α-oxoglutarate was the only reaction. In most cases there was further oxidation of the α-oxoglutarate and even when the ratio (μg.atoms of oxygen uptake)/(μmoles of tricarboxylic acid used) was below 1 the amount of α-oxoglutarate found was only about one-fifth of that expected, which indicates a non-oxidative removal of tricarboxylic acid. This might be one of the two dissimilation reactions known to occur in bacteria (but not so far

demonstrated in animal tissues), namely citrate → oxaloacetate + acetate (Wheat & Ajl, 1955), isocitrate → glyoxylate + succinate (Saz & Hillary, 1956), or a fission to oxaloacetate and acetyl-coenzyme A.

The calculation of the phosphorylation quotient from the rate of incorporation of ³²P into ATP was complicated by the fact that phosphate was incorporated into ATP anaerobically when isocitrate was present. The anaerobic rate was about 25% of the aerobic rate. This incorporation was essentially due to an exchange reaction not utilizing energy, as shown by the virtual absence of a net synthesis of ATP in the presence of the hexokinase system.

Under a variety of conditions, especially in the presence of semicarbazide and oxygen, the concentration of ATP was maintained, although little or no synthesis of ATP took place, as indicated by non-incorporation of ³²P (e.g. in Table 7). This can only mean that the ATPase activity was low under these conditions. Anaerobically, under otherwise identical conditions, ATP was hydrolysed relatively rapidly. Thus oxygen can, in the presence of semicarbazide, reduce the ATP-splitting activity of mitochondria. It follows that ATP can be maintained as a result of respiration in two ways, either by resynthesis from ADP and orthophosphate, or by inhibition of ATPase activity. When the second mechanism operates, low P/O ratios are found. This represents an 'uncoupling' of respiration from phosphorylation, which differs from that observed in the presence of nitrophenols in that it does not cause a net ATP breakdown.

It is evident that the ATPase activity of mitochondria is highly variable under aerobic conditions. It should be appreciated that 'ATPase' activity may be the result of several different kinds of enzyme systems. In the present context ATPase

activity is defined as any reaction which converts ATP into ADP. When the concentration of ATP remains constant the rate of this reaction can be measured by the rate of ^{32}P incorporation.

The rate of anaerobic incorporation of ^{32}P into ATP seems to depend on the nature of the substrate present. With isocitrate it was 25 % of the aerobic rate; with succinate, under comparable conditions, 3 % (Whittam *et al.* 1955) and with α -oxoglutarate 13 % of the aerobic rate (Krebs *et al.* 1953). Thus although the incorporation of ^{32}P into ATP may be expected to measure the rate of true ATP synthesis under some conditions, this is not necessarily correct for all conditions.

SUMMARY

1. Rat-liver mitochondria were incubated with isocitrate (and other substrates) under a variety of conditions. The rates of oxygen uptake, substrate removal and incorporation of ^{32}P into ATP were measured. Phosphorylation quotients between 1.4 and 3.4 were found when isocitrate was substrate.

2. Fluoride (0.04M) inhibited the oxygen uptake in the presence of succinate (up to 40 %) or isocitrate (up to 30 %), but did not change the phosphorylation quotient.

3. Fluoride inhibited the exchange of P^{β} and P^{γ} atoms of adenosine triphosphate (ATP) presumably by inhibition of myokinase.

4. Fluoride reduced the ratio atoms of oxygen consumed/tricarboxylic acid consumed to values less than 1. The tricarboxylic acid removal was not accounted for by the appearance of an equivalent amount of α -oxoglutarate.

5. Semicarbazide (0.04M) had no effect on phosphorylation when β -hydroxybutyrate was the substrate, but almost completely inhibited phosphorylation when citrate was the substrate. The oxygen uptake was not affected. In spite of non-phosphorylation the concentration of ATP was maintained. Anaerobically, semicarbazide did not appreciably inhibit adenosine triphosphatase activity.

6. Incorporation of ^{32}P into ATP occurred anaerobically when isocitrate was substrate, the rate being about 25 % of the aerobic rate. This rate is much higher than that observed in the presence of succinate or α -oxoglutarate. The anaerobic incorporation was lowered by fluoride and 2:4-dinitrophenol.

7. The P/O ratio associated with the activity of the triphosphopyridine nucleotide-specific isocitric dehydrogenase was 2.36; the ratio was reduced to 1.56 by fluoride (31 % inhibition). The P/O ratio associated with the activity of the diphosphopyridine nucleotide-specific isocitric dehydrogenase

was 2.83; fluoride reduced this to 2.68 (6 % inhibition).

We wish to thank Professor H. A. Krebs, F.R.S., for his interest and advice, and Dr D. D. Davies for the gift of enzymes. This work was aided by a grant from the Rockefeller Foundation.

REFERENCES

- Barkulis, S. S. & Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 339.
- Bartley, W. (1953). *Biochem. J.* **54**, 677.
- Bartley, W. & Davies, R. E. (1954). *Biochem. J.* **57**, 37.
- Berenblum, I. & Chain, F. (1938). *Biochem. J.* **32**, 295.
- Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **29**, 379.
- Boyer, P. D., Falcone, A. B. & Harrison, W. H. (1954). *Nature, Lond.*, **174**, 401.
- Copenhaver, G. H. & Lardy, H. A. (1952). *J. biol. Chem.* **195**, 225.
- El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
- Grafflin, A. L. & Ochoa, S. (1950). *Biochim. biophys. Acta*, **4**, 205.
- Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
- Hogebloom, G. H. & Schneider, W. C. (1950). *J. biol. Chem.* **186**, 417.
- Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
- Kornberg, A. & Pricer, W. E. (1951). *J. biol. Chem.* **189**, 123.
- Kotelnikova, A. V. (1950). *Biokhimiya*, **15**, 371.
- Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
- Krebs, H. A. & Hems, R. (1953). *Biochim. biophys. Acta*, **12**, 172.
- Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V. & Hems, R. (1953). *Biochem. J.* **54**, 107.
- Lee, K. H. & Eiler, J. J. (1951). *Science*, **114**, 393.
- Lehninger, A. L. (1949). *J. biol. Chem.* **178**, 625.
- Lehninger, A. L. (1951). *J. biol. Chem.* **190**, 345.
- LePage, C. A. (1949). *Biochem. Prep.* **1**, 5.
- LePage, C. A. & Mueller, G. C. (1949). *J. biol. Chem.* **180**, 975.
- Massey, V. (1952). *Biochem. J.* **51**, 490.
- Novikoff, A. B., Podber, E., Ryan, J. & Noe, E. (1953). *J. Histochem. Cytochem.* **1**, 27.
- Ochoa, S. (1947). *Ann. N.Y. Acad. Sci.* **47**, 835.
- Ochoa, S. (1948). *J. biol. Chem.* **174**, 233.
- Plaut, G. W. E. & Sung, Shan-Ching (1954). *J. biol. Chem.* **207**, 305.
- Saz, H. J. & Hillary, E. P. (1956). *Biochem. J.* **62**, 563.
- Slater, E. C. (1953). *Biochem. J.* **53**, 521.
- Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.
- Slater, E. C. & Holton, F. A. (1953). *Biochem. J.* **55**, 530.
- Stewart, C. A. (1953). *Biochem. J.* **54**, 117.
- Swanson, M. A. (1956). *Biochim. biophys. Acta*, **20**, 85.
- Taylor, T. G. (1953). *Biochem. J.* **54**, 48.
- Veall, N. (1948). *Brit. J. Radiol., N.S.*, **21**, 347.
- Wheat, R. W. & Ajl, S. J. (1955). *J. biol. Chem.* **217**, 897.
- Whittam, R., Bartley, W. & Weber, G. (1955). *Biochem. J.* **59**, 590.