Metabolism of Pyruvate and Malate by Isolated Fat-Cell Mitochondria

By B. R. MARTIN AND R. M. DENTON

Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(Received 25 May 1971)

1. Metabolism of pyruvate and malate by isolated fat-cell mitochondria incubated in the presence of ADP and phosphate has been studied by measuring rates of pyruvate uptake, malate utilization or production, citrate production and oxygen consumption. From these measurements calculations of the flow rates through pyruvate carboxylase, pyruvate dehydrogenase and citrate cycle have been made under various conditions. 2. In the presence of bicarbonate, pyruvate was largely converted into citrate and malate and only about 10% was oxidized by the citrate cycle; citrate and malate outputs were linear after lag periods of 6–9 min and 3 min respectively, and no other end products of pyruvate metabolism were detected. On the further addition of malate or hydroxymalonate, the lag in the rate of citrate output was less marked but no net malate disappearance was detected. If, however, bicarbonate was omitted then net malate uptake was observed. Addition of butyl malonate was found to greatly inhibit the metabolism of pyruvate to citrate and malate in the presence of bicarbonate. 3. These results are in agreement with earlier conclusions that in adipose tissue acetyl units for fatty acid synthesis are transferred to the cytoplasm as citrate and that this transfer requires malate presumably for counter transport. They also support the view that oxaloacetate for citrate synthesis is preferentially formed from pyruvate through pyruvate carboxylase rather than malate through malate dehydrogenase and that the mitochondrial metabolism of citrate in fat-cells is restricted. The possible consequences of these conclusions are discussed. 4. Studies on the effects of additions of adenine nucleotides to pyruvate metabolism by isolated fat-cell mitochondria are consistent with inhibition of pyruvate carboxylase in the presence of ADP and pyruvate dehydrogenase in the presence of ATP.

Studies of the total activities and intracellular distribution of enzymes in epididymal fat-cells and the permeability properties of their mitochondria (Martin & Denton, 1970a) were in full agreement with the views that the major pathway for the production of extramitochondrial acetyl-CoA for fatty acid synthesis is: acetyl-CoA_{mit.} → citrate_{mit.} → citrate_{cyt.} → acetyl-CoA_{cyt.} (Kornacker & Lowenstein, 1965a,b; Kornacker & Ball, 1965; Spencer, Corman & Lowenstein, 1964; Rognstad & Katz, 1968; Bartley, Abraham & Chaikoff, 1965; Watson & Lowenstein, 1970); and that in epididymal adipose tissue incubated with glucose and insulin the major pathway for the regeneration of mitochondrial oxaloacetate for further citrate synthesis from oxaloacetate formed in the cytoplasm by ATP citrate lyase is: oxaloacetate_{cyt.} → malate_{cyt.} → pyruvate_{cyt.} → pyruvate_{mit.} → oxaloacetate_{mit.} (Kornacker & Ball, 1965; Rognstad & Katz, 1966; Ballard & Hanson, 1967).

Our studies also lead to the suggestions that mitochondrial citrate transfer in fat-cells requires the counter transport of malate as in liver mitochondria (Chappell & Haarhoff, 1966; Chappell, Henderson, McGivan & Robinson, 1968), and that transfer of mitochondrial citrate to the cytoplasm in fat-cells metabolizing glucose to fatty acids may be facilitated by restricted mitochondrial metabolism of citrate. This could be attributed in part to the low mitochondrial activities of aconitase and NAD-isocitrate dehydrogenase. It was pointed out that restriction of the mitochondrial oxidation of malate would also appear to be necessary to prevent the excessive loss of cytoplasmic reducing power into mitochondria that would occur if a substantial proportion of the malate entering in exchange for citrate were to be converted into oxaloacetate.

In adipose tissue incubated with pyruvate alone, reducing power for cytoplasmic reactions (fatty acid, glycerol phosphate and lactate formation)

is necessarily formed by the dehydrogenase reactions of the citrate cycle and by pyruvate dehydrogenase rather than by the dehydrogenase reactions of the pentose phosphate pathway and glycolysis as in the presence of glucose. There is a greatly increased flux in the citrate cycle when pyruvate is the only substrate compared with the rate in the presence of glucose (Kneer & Ball, 1968; Schmidt & Katz, 1969). We have pointed out that slow rates of mitochondrial oxidation of isocitrate and malate would lead to the transport of these metabolites and favour the generation of cytoplasmic NADPH through NADP-isocitrate dehydrogenase and NADP-malate dehydrogenase. Thus it appears that when pyruvate is the only substrate much of the flow through the citrate cycle in the segments (citrate to 2-oxoglutarate) and (malate to oxaloacetate) is cytoplasmic (Martin & Denton, 1970a).

In the present study, further evidence in support of the role of citrate in the transfer of acetyl units, the counter transport of citrate for malate and the restricted mitochondrial metabolism of malate and citrate has been obtained by investigating the metabolism of pyruvate and malate by isolated fat-cell mitochondria. Estimates of rates of pyruvate metabolism through pyruvate dehydrogenase, pyruvate carboxylase and the rate of citrate-cycle turnover have been calculated from measurements of pyruvate uptake, malate uptake (or output), citrate output and oxygen uptake; other possible end products of pyruvate (and malate) metabolism such as ketone bodies, 2-oxoglutarate, oxaloacetate, isocitrate, lactate, glutamate, fumarate, aspartate and acetaldehyde were not detected in significant amounts. The experimental design circumvented the use of ^{14}C -labelled pyruvate and the problems of radioactive purity and isotope exchange associated with it (Watson & Lowenstein, 1970; Kneer & Ball, 1968; Von Korff, 1964).

A preliminary account of part of this study has been published (Martin & Denton, 1970b).

EXPERIMENTAL

Materials

Rats. Epididymal fat-pads were obtained from male Wistar rats (130–150g) fed on a modified 41B diet (Oxoid Ltd., London S.E.1, U.K.). In all experiments the animals were allowed free access to food and water up to the time of killing (approx. 10 a.m.).

Chemicals. Except where otherwise stated, enzymes, nucleotides and other intermediates and triethanolamine hydrochloride were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. and other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K. Collagenase and L-malate were from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. L-Carnitine was from Koch-Light Laboratories Ltd., Colnbrook,

Bucks., U.K. Hydroxymalonate and butyl malonate were from Emmanuel & Co. Ltd., Alperston, Middx., U.K.

Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. and freed of fatty acids, citrate and other impurities as described by Denton & Halperin (1968).

Isolated fat-cell mitochondria. Isolated fat-cells were prepared as described by Rodbell (1964) with minor modifications (Martin & Denton, 1970a). Mitochondria were prepared from these cells as described by Martin & Denton (1970a) except that the sucrose medium was 0.25M-sucrose containing 7.5mM-GSH, 2mM-ethanedioxybis(ethylamine)tetra-acetate, 2% bovine serum albumin, 20mM-tris buffer, pH 7.4. Respiratory control and P/O ratios were similar to those reported by Martin & Denton (1970a).

Methods

Incubation of mitochondria. Mitochondria were incubated at 30°C in 'KCl medium', containing 0.125M-KCl, 2mM-MgCl₂, 2mM-ethanedioxybis(ethylamine)tetra-acetate, 2% bovine serum albumin and 20mM-tris-HCl buffer, pH 7.4, with other additions as indicated in text or legends to tables. This medium was bubbled with CO₂-free air for 15–30 min before use to ensure removal of dissolved CO₂ and saturation of the medium with air.

For the measurement of pyruvate uptake, malate uptake or output and citrate output, samples were removed at the indicated time and treated with 70% (w/v) HClO₄ to give a concentration of 5% (w/v). Assays of pyruvate were performed the same day without neutralization of the extracts. Assays of citrate and malate (and other intermediates) were performed after neutralization by addition of 1M-triethanolamine hydrochloride containing 3M-KOH. In some preliminary experiments, the incubation was terminated by removing the mitochondria by centrifugation at 16000g for 2 min at 4°C, decanting the supernatant and acidifying with HClO₄ as described above. No significant difference in citrate or malate could be found in extracts prepared before or after removing the mitochondria, indicating that the amounts of these intermediates contained within the mitochondria was negligible compared with that outside. This is to be expected since the intramitochondrial volume is probably less than 0.02% of the incubation volume under these conditions.

For the measurement of oxygen uptake, separate samples of mitochondria (0.1–0.2mg of protein) were incubated at 30°C in 0.6–0.8ml of KCl medium with additions as indicated and the oxygen uptake was continuously recorded by using a small Clark type oxygen electrode (Martin & Denton, 1970a).

Assay of pyruvate, malate, citrate and other intermediates. Pyruvate was assayed spectrophotometrically with lactate dehydrogenase (EC 1.1.1.27) as described by Bücher, Czok, Lamprecht & Latzko (1963) except that 0.2M-triethanolamine hydrochloride buffer was used and the assay was carried out in 4 cm light-path cuvettes in a volume of 3 ml. Citrate, malate, aspartate, glutamate, oxaloacetate, isocitrate, 2-oxoglutarate, fumarate, acetyl-carnitine, β -hydroxybutyrate and acetoacetate were assayed fluorimetrically by enzymic methods similar to those of Williamson & Corkey (1969). Lactate was

assayed fluorimetrically by the method of Hohorst (1963) and acetaldehyde by the method of Bergmeyer (1963).

Expression of results. Rates were expressed in terms of glutamate dehydrogenase in some experiments. This enzyme is exclusively mitochondrial in fat-cells (Martin & Denton, 1970a). Glutamate dehydrogenase was extracted from mitochondria by freezing and thawing three times in 0.1M-potassium phosphate buffer, pH 7.3, and assayed at 25°C as described by Martin & Denton (1970a). Mitochondrial protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS AND DISCUSSION

Metabolism of pyruvate by fat-cell mitochondria and the effects of addition of bicarbonate, hydroxymalonate and malate. Fig. 1(a) shows the time-courses of pyruvate uptake and of citrate and malate output by isolated fat-cell mitochondria incubated in potassium chloride medium containing pyruvate (1.5mM), potassium bicarbonate (12.5mM), ADP (1mM) and potassium phosphate (2mM). Time-courses under similar conditions but with the further addition of either hydroxymalonate (0.5mM) or malate (0.5mM) are shown in Figs. 1(b) and 1(c).

In the absence of either hydroxymalonate or malate there was a marked lag of 6–9 min before the output of citrate became clearly linear. When hydroxymalonate was added the lag in citrate output was less marked. In the presence of malate linear rates of citrate output were again achieved more rapidly (3–6 min); the rate of citrate output was higher relative to that of pyruvate uptake even though there was no detectable net uptake or output of malate.

Under each of these conditions of incubation, the only major products of pyruvate metabolism appear to be citrate, when formed, malate and presumably CO_2 . No detectable rates (i.e. less than 5% of pyruvate uptake) of formation of the following have been observed: acetoacetate, β -hydroxybutyrate, lactate, acetate, 2-oxoglutarate, isocitrate, oxaloacetate, glutamate, fumarate, aspartate and acetaldehyde. Thus the metabolism of pyruvate by isolated fat-cell mitochondria would appear to be rather straightforward as indicated in Scheme 1 though significant conversion

Fig. 1. Time-courses of pyruvate uptake (○), malate output (△) and citrate output (●) by fat-cell mitochondria. The mitochondria (approx. 0.5 mg of protein) were incubated at 30°C in a total volume of 4 ml of KCl medium containing initially 2% bovine serum albumin, ADP (1 mM), KH_2PO_4 (2 mM), pyruvate (1.5 mM) and (a) KHCO_3 (12.5 mM) or (b) KHCO_3 (12.5 mM) and hydroxymalonate (0.5 mM) or (c) KHCO_3 (12.5 mM) and malate (0.5 mM). Samples (0.45 ml) were taken at 3 min intervals, HClO_4 was added to give a final concentration of 5% and assays for pyruvate, citrate and malate were performed as described in the Experimental section.

of pyruvate into succinate, which was not measured, has not been ruled out. However, if the model is correct then the rates of flux through pyruvate dehydrogenase, pyruvate carboxylase and citrate cycle can be calculated from the rates of output of citrate and malate together with the rate of oxygen uptake as indicated in the legend to Scheme 1. These rates can also be used to calculate the rate of pyruvate uptake and this provides a further check on the validity of the model. Overall, fair agreement was found between calculated and observed values of pyruvate uptake. For example,

in the time-courses shown in Fig. 1(a-c), the total oxygen uptakes were 34, 44 and 55 nmol respectively in the 9-18 min period, giving calculated values of pyruvate uptake over this period of 74, 71 and 92 compared with observed values of 76, 74 and 81 respectively. Further evidence of the close correspondence between observed and calculated values of pyruvate uptake are given in Tables 1 and 2. Overall the calculated values were $99 \pm 6\%$ (11 observations) of the observed values.

The concentrations of bicarbonate (12.5 mM) and phosphate (2 mM) used throughout this study were

Scheme 1. Calculation of flow rates of pyruvate metabolism by isolated fat-cell mitochondria. If the rate (as nmol/min) of pyruvate uptake = A, malate output = B, citrate output = C and oxygen consumption = D, then

$$A = B + 2C + 0.2(2D - C + D)$$

and the rate of flow through pyruvate dehydrogenase = $C + 0.2(2D - C + B)$, through pyruvate carboxylase = $B + C$ and through the citrate cycle = $0.2(2D - C + B)$.

Table 1. Effect of pyruvate concentration on the metabolism of pyruvate by isolated fat-cell mitochondria

Fat-cell mitochondria (approx. 50 µg of protein) were incubated for 9 min at 30°C in 0.3-1.2 ml of KCl medium containing 1 mM-ADP, 12.5 mM-KHCO₃, 2 mM-potassium phosphate plus pyruvate at stated concentration. Observed rates of citrate and malate production and pyruvate uptake are given as mean ± s.e.m. for observations on four separate samples of mitochondria; observed rates of oxygen uptake are those obtained by continuously recording the uptake of a single sample of the same batch of mitochondria during incubation under identical conditions. Calculated rates have been derived from the observed rates of citrate and malate production and pyruvate uptake as given in the legend to Scheme 1.

Initial concn. of pyruvate (mm)	Rates (µmol/9 min per unit of glutamate dehydrogenase)								
	Observed					Calculated			
						Flow			
	Citrate production	Malate production	Oxygen uptake	Pyruvate uptake	Pyruvate uptake	Through pyruvate dehydrogenase	Through pyruvate carboxylase	Through citrate cycle	
0.0	<0.1	<0.1	—	—	—	—	—	—	—
0.25	3.7±0.10	1.80±0.10	1.80	8.9±0.3	9.5	4.0	5.5	0.3	
0.75	3.4±0.17	0.85±0.07	1.25	9.5±0.2	7.7	3.3	4.4	0.0	
1.50	3.3±0.25	1.05±0.10	1.45	7.2±0.7	7.9	3.5	4.4	0.1	
5.00	3.5±0.05	0.48±0.06	1.75	—	7.6	3.6	4.0	0.3	

Table 2. *Effects of addition of malate, hydroxymalonate and KHCO_3 on the metabolism of pyruvate by isolated fat-cell mitochondria*

Mitochondria (approx. 50 μg of protein) were incubated for 18 min at 30°C in 300 μl of KCl medium containing ADP (1 mM) and potassium phosphate (2 mM) and, where appropriate, pyruvate (0.25 or 1.5 mM), malate (0.5 mM), hydroxymalonate (0.5 mM) and KHCO_3 (12.5 mM). Rates are for the 9–18 min period and are expressed as $\mu\text{mol}/9$ min per unit of glutamate dehydrogenase; malate uptakes are indicated as negative malate production. Other details as Table 1.

Initial concn. of pyruvate (mM)	Other additions to incubation media	Rates ($\mu\text{mol}/9$ min per unit of glutamate dehydrogenase)				
		Observed		Calculated		
		Citrate production	Malate production	Oxygen uptake	Pyruvate uptake	Flow
						Through pyruvate dehydrogenase Through pyruvate carboxylase Through citrate cycle
1.5	None	0.3	<0.1	—	—	—
1.5	KHCO_3	3.64 ± 0.51	2.34 ± 0.26	2.7	13.0 ± 2.5	4.6 5.9 —
1.5	KHCO_3 , malate	8.85 ± 0.90	-0.3 ± 1.3	2.7	26.7 ± 3.1	8.8 8.9 0.8
1.5	KHCO_3 , hydroxymalonate	4.40 ± 0.70	2.14 ± 0.30	2.2	14.2 ± 1.5	4.9 6.5 0.0
1.5	Malate	5.40 ± 0.30	-3.80 ± 1.2	—	—	— — 0.4
0.25	KHCO_3 *	3.07	1.25	3.3	6.9	— — —
0.25	Malate*	2.50	-3.20	5.7	2.7	4.1 4.3 1.0
					3.0	3.6 0 1.1

* Mean of duplicate determinations on single sample of mitochondria.

found to be sufficient to give maximum rates of citrate output. Addition of each at half the stated concentration produced little or no decrease in citrate output. The effects of various pyruvate concentration on rates of citrate and malate production and rates of pyruvate and oxygen uptake are shown in Table 1. In the absence of pyruvate there was no detectable production of malate and citrate; between 0.25 mM- and 5.0 mM-pyruvate there was little difference in the rate of O_2 uptake or citrate production but the production of malate appears to decline at the higher pyruvate concentrations. Also shown in Table 1 are the flow rates through pyruvate dehydrogenase, pyruvate carboxylase and citrate cycle calculated as described in the legend to Scheme 1. The flow through the citrate cycle was never greater than 5% of the pyruvate uptake.

After 9 min, steady-state rates of pyruvate metabolism would appear to be achieved (Fig. 1). Table 2 shows the effects of additions of malate, hydroxymalonate and potassium bicarbonate on the rates of metabolism between 9 and 18 min expressed in terms of glutamate dehydrogenase activity. In the absence of potassium bicarbonate or malate the production of the citrate was very small. In the presence of bicarbonate, most of the pyruvate uptake could be accounted for by the metabolism of pyruvate through pyruvate dehydrogenase and pyruvate carboxylase to citrate and malate. As calculated from the oxygen consumption and rates of citrate and malate output little pyruvate was metabolized through the citrate cycle. In the presence of bicarbonate plus hydroxymalonate, the rates of pyruvate metabolism during this time-period were similar to those in the presence of bicarbonate alone. In the presence of bicarbonate plus malate, the rate of pyruvate uptake was greater and most of it was recovered in the acetate and oxaloacetate moieties of citrate (no net uptake or production of malate could be detected in these circumstances; see also Fig. 1c). Thus in the presence of bicarbonate, oxaloacetate for citrate synthesis appears to be formed almost exclusively from pyruvate and bicarbonate through pyruvate carboxylase. However, if bicarbonate is omitted, then malate uptake is observed and is sufficient to account for much of the oxaloacetate utilized in citrate synthesis.

Effects of butyl malonate on pyruvate metabolism by fat-cell mitochondria. In the presence of bicarbonate, there was a considerable lag in the rate of citrate production, which was less evident when hydroxymalonate or malate was also added (Fig. 1a–c). This would be in agreement with the proposal that citrate transport out of fat-cell mitochondria requires malate (or analogue) for counter transport. In the absence of added malate or

Table 3. *Effects of butyl malonate on citrate production in fat-cell mitochondria incubated in the presence of pyruvate and bicarbonate*

Fat-cell mitochondria were incubated for 18 min at 30°C in 0.3 ml of KCl medium containing 1 mM-ADP, 1.5 mM-pyruvate and 12.5 mM-KHCO₃. Results are means $\pm$ S.E.M. of four observations.

Additions to incubation medium	Citrate production (nmol/18 min)
None	147 $\pm$ 2.5
Butylmalonate (1 mM)	31 $\pm$ 2.5

Table 4. *Production of acetyl-L-carnitine by fat-cell mitochondria in the presence of L-carnitine*

Fat-cell mitochondria (approx. 100 μ g of protein) were incubated for 12 min at 30°C in 0.3 ml of KCl medium containing pyruvate (1.5 mM), KHCO₃ (12.5 mM), ADP (1 mM) in the presence or absence of L-carnitine (1 mM). Rates are expressed as nmol produced in 12 min and are the means $\pm$ S.E.M. of observations on four samples of mitochondria.

Additions	Production (nmol)		
	Citrate	Malate	Acetyl-L-carnitine
None	257 $\pm$ 8	151 $\pm$ 10	<5
L-Carnitine	257 $\pm$ 21	150 $\pm$ 7	88 $\pm$ 5

hydroxymalonnate, malate would be required to be transported from the mitochondria before citrate. In liver mitochondria there is evidence for a dicarboxylate carrier, which catalyses the counter transport of malate-phosphate and this carrier is inhibited by butyl malonate (Chappell & Robinson, 1968). Fat-cell mitochondria appear to contain this carrier since addition of phosphate is necessary before fat-cell mitochondria swell in iso-osmotic solutions of ammonium malate (Martin & Denton, 1970a). If the sequence of events when fat-cell mitochondria are incubated in pyruvate plus bicarbonate is that malate must be transported from the mitochondria on the carrier catalysing counter transport of malate-phosphate before citrate can be transported out on the carrier catalysing citrate-malate transport, then citrate production should be strongly inhibited by butyl malonate and this is, indeed, found to be the case (Table 3).

Production of acetyl-L-carnitine by fat-cell mitochondria in the presence of L-carnitine. Fat-cell mitochondria contain carnitine acetyltransferase but the activity of this enzyme is less than 10% of that of citrate synthase. It has also been demonstrated that fat-cell mitochondria will oxidize acetyl L-carnitine in the presence of malate (Martin & Denton, 1970a). It is therefore to be expected

that, on addition of carnitine to fat-cell mitochondria incubated with pyruvate, bicarbonate, ADP and phosphate, some acetyl-L-carnitine will be formed. In the presence of 1 mM-carnitine the output of acetyl-L-carnitine can amount to about one-third of that of citrate (Table 4).

Effects of addition of ADP, ATP and atracyloside on pyruvate metabolism by fat-cell mitochondria. Table 5 shows the effects of varying ADP and ATP concentration on the metabolism of pyruvate in the presence of bicarbonate and phosphate. In the rest of this study, 1 mM-ADP has been used to give the maximum opportunity for malate oxidation and flow through the citrate cycle to occur. If the concentration of ADP is increased to 5 mM the rate of pyruvate metabolism especially to citrate is decreased. On omission of ADP the rate of pyruvate metabolism to both citrate and malate is increased. A similar effect was seen on addition of atracyloside in the presence of 1 mM-ADP. Atracyloside inhibits the carrier in rat liver mitochondria that catalyses the counter transport of ATP/ADP (Klingenberg, 1970).

On addition of ATP (5 mM), the output of citrate was greatly decreased and that of malate perhaps slightly increased when compared with the outputs seen with no addition of adenine nucleotide. Also shown in Table 5 are the calculated flow rates through pyruvate dehydrogenase and pyruvate carboxylase. The inhibitory effects of ADP on pyruvate utilization appear to be more marked on flux through pyruvate carboxylase than through pyruvate dehydrogenase: the percentage inhibitions in flux at 5 mM-ADP were 58 and 35 respectively. In contrast, the inhibitory effects of 5 mM-ATP on fluxes through pyruvate carboxylase and pyruvate dehydrogenase were 33 and 43% respectively.

The effects of ATP are in conflict with those reported by Patel & Hanson (1970). They found that in the absence of added ATP the incorporation of [¹⁴C]bicarbonate into citrate and malate by fat-cell mitochondria was almost abolished whereas the flux through pyruvate carboxylase is increased by omission of ATP in our hands. The capacity of the mitochondria used in this present study to generate ATP required for pyruvate carboxylation in the absence of added nucleotide or in the presence of ADP would appear to be good evidence that the mitochondria are well coupled.

General discussion

Products of pyruvate metabolism. The method which has been used in this study to calculate rates of flow through pyruvate dehydrogenase, pyruvate carboxylase and the citrate cycle depends on the assumption that citrate and malate are the

Table 5. *Effects of addition of ADP, ATP and atractyloside on pyruvate metabolism by isolated fat-cell mitochondria*

Mitochondria (approx. 25 μ g of protein) were incubated for 18 min at 30°C in 300 μ l of KCl medium containing pyruvate (1.5 mm), KHCO₃ (12.5 mm), potassium phosphate (2 mm) and additions of ADP, ATP and atractyloside as indicated. Rates are for the 9–18 min period and are expressed as total nmol/9 min. Other details are as for Table 2. Results are means $\pm$ S.E.M. of four observations.

Additions to incubation media	Rates (nmol/min)						
	Observed			Calculated			
				Pyruvate uptake	Flow		
	Citrate production	Malate production	Oxygen uptake		Through pyruvate dehydrogenase	Through pyruvate carboxylase	Through citrate cycle
ADP (5 mm)	0.94 $\pm$ 0.08	0.45 $\pm$ 0.06	2.3	3.2	1.8	1.4	0.9
ADP (1 mm)	1.34 $\pm$ 0.07	0.42 $\pm$ 0.03	2.9	4.1	2.3	1.8	1.0
None	2.70 $\pm$ 0.30	0.87 $\pm$ 0.04	0.9	6.1	2.8	3.3	0.1
Atractyloside (0.03 mm)	2.10 $\pm$ 0.14	1.13 $\pm$ 0.01	1.1	6.2	2.7	3.5	0.2
+ ADP (1 mm)							
ATP (5 mm)	0.80 $\pm$ 0.13	1.36 $\pm$ 0.08	1.1	3.8	1.6	2.2	0.8

only significant products (other than CO₂) of pyruvate metabolism. This assumption appears to be valid as no other end products of pyruvate metabolism were detected. Succinate was not assayed but Patel & Hanson (1970) found in their study of incorporation of [¹⁴C]bicarbonate into citrate-cycle intermediates by fat-cell mitochondria that 95% of the radioactivity incorporated appeared in citrate and malate and that the remaining 5% appeared mostly in succinate and fumarate. The validity of the model used is further supported by the fact that observed and calculated rates of pyruvate uptake were in good agreement.

In the presence of ADP and bicarbonate, pyruvate was found to be metabolized by fat-cell mitochondria mainly to citrate and malate and, in most experiments, less than 10% was oxidized by the citrate cycle. A similar pattern of metabolism was found in the studies by Patel & Hanson (1970) and is in marked contrast with that in mitochondria from heart, liver or kidney.

With rat heart mitochondria incubated in the presence of pyruvate and ADP, oxaloacetate for citrate synthesis appears to be derived from aspartate initially and there is a small amount of net production of citrate and 2-oxoglutarate. However, by 6 min of incubation there is little further net production of these intermediates and nearly all the pyruvate taken up is oxidized by the citrate cycle; under these conditions oxaloacetate for citrate synthesis is regenerated by the cycle (LaNoue, Nicklas & Williamson, 1970). This pattern of metabolism is, of course, in line with the primary function of heart mitochondria in supplying ATP and the very low activity of pyruvate carboxy-

lase in these mitochondria (Böttger, Wieland, Brdiczka & Pette, 1969).

With rat liver and kidney mitochondria, citrate and malate are major products of pyruvate metabolism in the presence of bicarbonate, but even when ATP is added, production of citrate and malate accounts for only about 30% of pyruvate utilized, the remainder apparently being mainly oxidized in the citrate cycle (Walter, Paetkau & Lardy, 1966; Mehlmán, Walter & Lardy, 1967).

Varying the concentration of pyruvate between 0.25 and 5.0 mm was found to have little effect on the rate of pyruvate uptake and citrate output by fat-cell mitochondria (Table 1). This would suggest that the overall K_m for pyruvate uptake is considerably below 0.25 mm, and this is comparable with the K_m for pyruvate of both pyruvate dehydrogenase (70 μ M; Coore, Denton, Martin & Randle, 1971) and pyruvate carboxylase (100 μ M; A. P. Halestrap & R. M. Denton, unpublished work).

In an earlier study (Martin & Denton, 1970a) we concluded on the basis of the cytoplasmic activity of carnitine acetyltransferase in fat-cells that only 5% of the acetyl units for fatty acid synthesis in cells incubated in glucose and insulin could be generated from acetyl-L-carnitine (unfortunately the figure was given in this paper as 50% in error). In this study acetyl-L-carnitine production by fat-cell mitochondria incubated with added L-carnitine was about one-third of that of citrate. Since the activity of the cytoplasmic L-carnitine acetyltransferase is one-quarter of that of the mitochondrial enzyme, the transfer of acetyl-carnitine from mitochondria would not appear to

be an important means of generating cytoplasmic acetyl-CoA when compared with the transfer of citrate. The rate of fatty acid synthesis in fat-pads incubated in glucose and insulin is in the range $0.15\text{--}0.30\text{ }\mu\text{mol}$ of acetyl units/min per g of tissue at 37°C , which is equivalent to approx. $0.45\text{--}0.90\text{ }\mu\text{mol}$ of acetyl units/min per unit of glutamate dehydrogenase. This is similar to the range of rates of citrate production by fat-cell mitochondria seen in this study (Tables 1 and 2).

The very low rates of citrate cycle found are in agreement with the low activities of mitochondrial aconitase and NAD-isocitrate dehydrogenase in fat-cells (see the introduction).

Role of malate in citrate production by fat-cell mitochondria. In a previous study (Martin & Denton, 1970a) malate was shown to be required for citrate to enter fat-cell mitochondria. In the present study, the observations of the effects of malate, hydroxymalate and butyl malonate on citrate production by fat-cell mitochondria are further evidence that malate is required as the counter-ion for the transport of citrate out of fat-cell mitochondria.

Potentially, malate may also act as a source of oxaloacetate for citrate synthesis but it would appear that in fat-cell mitochondria incubated in the presence of pyruvate and bicarbonate the most important source is pyruvate. Patel & Hanson (1970) and Patel, Jomain-Baum & Hanson (1971) have found that when fat-cell mitochondria are incubated with pyruvate, ATP, bicarbonate and $[3\text{-}^{14}\text{C}]\text{malate}$ there is appreciable incorporation of radioactivity into citrate. In fact, this is not inconsistent with our conclusion since these workers also found nearly as much incorporation of radioactivity from $[3\text{-}^{14}\text{C}]\text{pyruvate}$ into malate; thus most of the radioactivity incorporated from malate into citrate appears to be occurring by exchange rather than net flow. It can be calculated from their results that the net flow from malate through oxaloacetate into citrate accounts for about 5% of the total citrate production when 0.25 mM -malate was used and about 20% when 5.0 mM -malate was used.

The mitochondrial oxidation of malate would thus appear to be restricted in the presence of pyruvate and bicarbonate. This may simply be the result of the production of oxaloacetate from pyruvate through pyruvate carboxylase and the equilibrium position of malate dehydrogenase, which is greatly in favour of malate.

In agreement with our previous conclusion this restriction in the utilization of the malate which enters mitochondria in exchange for citrate would prevent loss of cytoplasmic reducing power. In addition, in adipose tissue incubated in the presence of pyruvate alone, the restriction together with a low

mitochondrial metabolism of citrate could lead to a high proportion of the flux in the two spans of the citrate cycle (citrate to 2-oxoglutarate; malate to oxaloacetate) occurring in the cytoplasm. This would allow cytoplasmic production of NADPH for fatty acid synthesis through the NADP-linked malate and isocitrate dehydrogenases and NADH for lactate and glycerol phosphate formation through NAD-dependent malate dehydrogenase (Martin & Denton, 1970a).

Effects of adenine nucleotides on pyruvate metabolism by isolated fat-cell mitochondria. The effects of adding ADP or ATP on the metabolism of pyruvate in mitochondria would be expected to be rather complex. On the one hand, addition of ADP may lead to inhibition of pyruvate carboxylase. Walter & Stucki (1970), from studies on the effect of added ADP to intact rat liver mitochondria and mitochondrial extracts, have concluded that pyruvate carboxylase in liver is inhibited by ADP. This has been confirmed by the extensive kinetic studies of Wimbhurst & Manchester (1970) and Seufret, Herlemann, Albrecht & Seubert (1971), and has also been shown for the enzyme prepared from rat adipose tissue (A. P. Halestrap & R. M. Denton, unpublished work). On the other hand, addition of ATP may lead to inhibition of pyruvate dehydrogenase. This enzyme in liver, kidney, heart and brain has been shown to exist in two interconvertible forms, an inactive phosphorylated form and an active non-phosphorylated form (Linn, Pettit, Hucho & Reed, 1969). Recently we have obtained evidence suggesting that the enzyme from adipose tissue has similar properties (Coore *et al.* 1971). In the absence of added ADP, an additional and important factor is that the extent of oxidation by the respiratory chain of NADH produced by pyruvate dehydrogenase and the citrate cycle will be limited by the rate of endogenous production of ADP. In well-coupled fat-cell mitochondria the only obvious source of ADP is pyruvate carboxylase; presumably excess of NADH formed by pyruvate dehydrogenase may be reconverted into NAD under these conditions only by increased conversion of oxaloacetate into malate.

The effects of adenine nucleotides reported in Table 5 are explicable in the above terms. However, it should be pointed out that in the absence of information on the actual intramitochondrial concentrations of adenine nucleotides, conclusions must remain tentative. The inhibition of pyruvate metabolism in the presence of ADP may be the result primarily of the inhibition of pyruvate carboxylase. This may lead in turn to the observed fall in flux through pyruvate dehydrogenase by allowing accumulation of acetyl-CoA. Acetyl-CoA is an end-product inhibitor of the

heart-muscle enzyme (Garland & Randle, 1964; Wieland, von Jagow-Westermann & Stukowski, 1969), but this inhibition has not been demonstrated for the adipose tissue enzyme. In contrast, the inhibition of pyruvate metabolism in the presence of ATP is consistent with an increase in the proportion of pyruvate dehydrogenase present in the inactive phosphorylated form. This in turn may lead to a fall in acetyl-CoA concentration and thus to the observed diminished flow through pyruvate carboxylase. Acetyl-CoA activates pyruvate carboxylase from adipose tissue, as found with the enzyme from other sources (Ballard & Hanson, 1967; A. P. Halestrap & R. M. Denton, unpublished work). However, on addition of ATP it is to be expected that the NADH/NAD concentration ratio in the mitochondria would be increased; this might be an explanation of the high rate of malate production and also in part of the inhibition of pyruvate dehydrogenase.

In adipose tissue, flux through pyruvate carboxylase and pyruvate dehydrogenase must alter in the main in parallel. Opposing effects of both adenine nucleotides and probably also acetyl-CoA on the two enzymes may be important in achieving this end.

We are very grateful to Professor P. J. Randle and Dr H. G. Coore for helpful advice and discussions and to Mrs R. Low for very competent technical assistance. The work was supported in part by grants from the British Diabetic Association, the Medical Research Council, The Royal Society and the Percival Wait Salmond Bequest to Professor P. J. Randle. B.R.M. held a Medical Research Council Studentship.

REFERENCES

- Ballard, F. J. & Hanson, R. W. (1967). *J. Lipid Res.* **8**, 73.
- Bartley, J., Abraham, S. & Chaikoff, I. L. (1965). *Biochem. biophys. Res. Commun.* **19**, 770.
- Bergmeyer, H.-U. (1963). In *Methods of Enzymatic Analysis*, p. 266. Ed. by Bergmeyer, H.-U. Berlin: Verlag Chemie.
- Böttger, I., Wieland, O., Brdiczka, D. & Pette, D. (1969). *Eur. J. Biochem.* **8**, 113.
- Bücher, Th., Czok, R., Lamprecht, W. & Latzko, E. (1963). In *Methods of Enzymatic Analysis*, p. 253. Ed. by Bergmeyer, H.-U. Berlin: Verlag Chemie.
- Chappell, J. B. & Haarhoff, K. N. (1966). In *Biochemistry of Mitochondria*, p. 75. Ed. by Slater, E. C., Kanuiga, Z. & Wojtezak, L. London: Academic Press.
- Chappell, J. B., Henderson, P. J. F., McGivan, J. D. & Robinson, B. H. (1968). In *The Interaction of Drugs and Subcellular Components of Animal Cells*, p. 71. Ed. by Campbell, P. N. London: Churchill.
- Chappell, J. B. & Robinson, B. H. (1968). *Biochem. Soc. Symp.* **27**, 123.
- Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971). *Biochem. J.* **125**, 115.
- Denton, R. M. & Halperin, M. L. (1968). *Biochem. J.* **110**, 27.
- Garland, P. B. & Randle, P. J. (1964). *Biochem. J.* **91**, 6c.
- Hohorst, H.-J. (1963). In *Methods of Enzymatic Analysis*, p. 266. Ed. by Bergmeyer, H.-U. Berlin: Verlag Chemie.
- Klingenberg, M. (1970). *FEBS Lett.* **6**, 145.
- Kneer, P. & Ball, E. G. (1968). *J. biol. Chem.* **243**, 2863.
- Kornacker, M. S. & Ball, E. G. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 123.
- Kornacker, M. S. & Lowenstein, J. M. (1965a). *Biochem. J.* **94**, 209.
- Kornacker, M. S. & Lowenstein, J. M. (1965b). *Biochem. J.* **95**, 832.
- LaNoue, K., Nicklas, W. J. & Williamson, J. R. (1970). *J. biol. Chem.* **245**, 102.
- Linn, T. M., Pettit, F. H., Hucho, F. & Reed, L. J. (1969). *Proc. natn. Acad. Sci. U.S.A.* **64**, 227.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Martin, B. R. & Denton, R. M. (1970a). *Biochem. J.* **117**, 861.
- Martin, B. R. & Denton, R. M. (1970b). *Biochem. J.* **119**, 38p.
- Mehlman, M. A., Walter, P. & Lardy, H. A. (1967). *J. biol. Chem.* **242**, 4594.
- Patel, M. S. & Hanson, R. W. (1970). *J. biol. Chem.* **245**, 1302.
- Patel, M. S., Jomain-Baum, M. & Hanson, R. W. (1971). *J. Lipid Res.* **12**, 179.
- Rodbell, M. (1964). *J. biol. Chem.* **239**, 375.
- Rognstad, R. & Katz, J. (1966). *Proc. natn. Acad. Sci. U.S.A.* **55**, 1148.
- Rognstad, R. & Katz, J. (1968). *Archs Biochem. Biophys.* **127**, 437.
- Schmidt, K. & Katz, J. (1969). *J. biol. Chem.* **244**, 2125.
- Seufret, D., Herlemann, E.-M., Albrecht, E. & Seubert, W. (1971). *Hoppe-Seyler's Z. physiol. Chem.* **352**, 459.
- Spencer, A. F., Corman, L. & Lowenstein, J. M. (1964). *Biochem. J.* **93**, 378.
- Von Korff, R. W. (1964). *Analyt. Biochem.* **8**, 171.
- Walter, P., Paetkau, V. & Lardy, H. A. (1966). *J. biol. Chem.* **241**, 2523.
- Walter, P. & Stucki, J. W. (1970). *Eur. J. Biochem.* **12**, 508.
- Watson, J. A. & Lowenstein, J. M. (1970). *J. biol. Chem.* **245**, 5993.
- Wieland, O., von Jagow-Westermann, B. & Stukowski, B. (1969). *Hoppe-Seyler's Z. physiol. Chem.* **350**, 329.
- Williamson, J. R. & Corkey, B. E. (1969). In *Methods in Enzymology*, vol. 13, p. 434. Ed. by Lowenstein, J. M. New York: Academic Press.
- Wimhurst, J. M. & Manchester, K. L. (1970). *Biochem. J.* **120**, 79.