Carboxylation of Pyruvate by Isolated Rat Adipose Tissue Mitochondria*

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SUMMARY

Mitochondria were isolated from rat epididymal adipose tissue and shown to be functionally intact as judged from both the P:O and respiratory control ratios, and structurally intact as determined by electron microscopy. In the presence of pyruvate, ATP, inorganic phosphate, and magnesium ions, these mitochondria incorporated $^{14}C_{4}$ into organic acids. Citrate and malate were the main radioactive products, with citrate containing about two-thirds of the radioactivity fixed. With the use of a rapid syringe filtration method the efflux of the newly formed intermediates from the mitochondria was shown. The observed high rates of pyruvate carboxylation and citrate formation have been further supported by the levels of pyruvate carboxylase and citrate synthase in mitochondria. The deletion of inorganic phosphate from the incubation medium reduced the $^{14}C$ fixation presumably due to the accumulation of ADP. Pyruvate carboxylation was also inhibited by ADP even in the presence of ATP. Mitochondria were shown to metabolize exogenously labeled citrate and malate, and pyruvate caused a 5-fold increase in the formation of $^{14}C$-citrate from $^{14}C$-malate. The results suggest that pyruvate carboxylation is regulated, in part, by the levels of pyruvate and ATP in adipose tissue mitochondria. Our observations lend experimental evidence supporting the role of the mitochondria in supplying the precursors for the citrate cleavage and glyceroneogenic pathways in rat adipose tissue, and emphasize the important role of pyruvate carboxylase in lipogenesis.

The mitochondrial carboxylation of pyruvate is an essential step in gluconeogenesis from pyruvate, lactate, and alanine and it has been intensively studied in mitochondria isolated from liver (1-3) and kidney (4, 5) of various species. Recently it has become evident that pyruvate carboxylation also plays an important role in lipogenesis in rat adipose tissue (6-9). In work carried out in this laboratory, we have shown pyruvate carboxylase to be present in adipose tissue at a level that exceeds the activity found in liver if it is expressed on a basis of milligrams of nitrogen (9). This high level of activity reflects the important role of oxalacetate at a metabolic crossroad of lipogenesis and glyceroneogenesis in adipose tissue. During fatty acid synthesis from glucose, pyruvate carboxylase replenishes the intramitochondrial oxalacetate that is lost when citrate leaves the mitochondria and is further metabolized via the citrate cleavage pathway (6, 10, 11). Adipose tissue is also capable of synthesizing $\alpha$-glycerophosphate from pyruvate via the dicarboxylic acid shuttle involving the coupling of pyruvate carboxylase and P-enolpyruvate carboxykinase (9, 12-15). Added to this are the recent reports from several laboratories of an alternative and potentially important function of pyruvate carboxylase in a transmemitochondrial shuttle of reducing equivalents to the cytosol of the adipose tissue cell to support the high rate of fatty acid synthesis from pyruvate (7-9).

The experimental evidence which supports the various functions of pyruvate carboxylase in adipose tissue, and in fact the entire sequence of transmitochondrial anion efflux in this tissue, has been largely based on studies of the intracellular compartmentation of various enzymes and incorporation of certain specifically labeled intermediates into end products such as fatty acids and glycerol. While recognizing the importance of studies of this type, it seemed to us necessary to isolate functional adipose tissue mitochondria and to measure these parameters directly. In the present paper, we have investigated the carboxylation of pyruvate, the formation in, and passage of, citric acid cycle intermediates from the mitochondria, as well as the metabolism of citrate, malate, and pyruvate by these mitochondria.

MATERIALS AND METHODS

Chemicals—Citrate-1,5-$^{14}$C, uniformly labeled malate-$^{14}$C, and NaH$^{14}$CO$_3$ were purchased from New England Nuclear. NaH$^{14}$CO$_3$ was diluted with unlabeled KHCO$_3$ to give the desired concentration. ATP, ADP, NAD$^+$, NADH, NADPH, and acetyl-CoA were obtained from P-L Laboratories. Malate dehydrogenase (EC 1.1.1.37), citrate lyase (EC 4.1.3.8), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), myokinase (EC 2.7.4.3), hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and phos-
available. All other reagents were of the highest purity commercially available.

Animals—Male Sprague-Dawley rats weighing about 180 g were used in all studies and were fed Purina laboratory chow ad libitum.

Isolation of Mitochondria from Adipose Tissue—Adipose cells were obtained essentially as described by Rodbell (18) from the epididymal adipose tissues pooled from about 20 rats. The fat pads were incubated in a 500-ml polypropylene bottle containing 100 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, in which were dissolved 4 g of bovine albumin, 90 mg of glucose, and 150 mg of bacterial collagenase. The gas phase in the bottle was 95% O₂-5% CO₂. The tissue was shaken rapidly in a metabolic shaker for about 40 min at 37°C. Following the incubation the stromal vascular components were removed with forceps and 150 mg of bacterial collagenase. The gas phase in the incubation medium with the use of the Swinnex-25 syringe filter assembly (Millipore Filter Corporation) equipped with a nylon prefilter and a 0.65 μ filter by a method described in detail by Garber and Ballard (25). Incubations were carried out in the small tubes (10 ml) which were shaken in a water bath at 30°C or 37°C. The compositions of the reaction mixtures are described in the legends of the tables and figures. The reaction was started by adding 0.2 ml of mitochondrial suspension after a temperature equilibrium period of about 5 min. After appropriate incubation periods, the reactions were stopped by adding perchloric acid to give a final concentration to 6%. The tubes were gassed with CO₂ for 4 min to remove residual labeled bicarbonate. An aliquot of the mixture was counted to determine H¹⁴CO₃⁻ fixation. Pyruvate (22), malate (23), and citrate (24) were measured enzymatically in the neutralized extracts.

Separation of Mitochondria from Incubation Medium—In the previous studies the incubations were terminated by adding perchloric acid in order to determine the total radioactivity fixed into organic acids. We wanted also to investigate the distribution of labeled organic acids in the mitochondria and the incubation medium, and two techniques were used; filtration and centrifugation. Mitochondria were isolated from the incubation medium with the use of the Spinmax-25 syringe filter assembly (Millipore Filter Corporation) equipped with a nylon prefilter and a 0.65 μ filter by a method described in detail by Garber and Ballard (25). Incubations were carried out in the small test tubes and at the end of the incubation period the reaction in one tube was stopped by adding 0.2 ml of 36% perchloric acid. The contents of another tube were transferred to a syringe connected to a multiheaded Luer-Lock syringe valve assembly. The medium was then filtered with the use of a liquid scintillation spectrometer. The radioactive spots were identified by comparing their migration with those of known radioactive organic acids. Fig. 1 shows a sample separation of standard malate, citrate, succinate, and fumarate compared with the separation of labeled compounds formed during the incubation of adipose tissue mitochondria with H¹⁴CO₃⁻ and pyruvate.

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Separation of Organic Acids—Labeled organic acids were separated as described by Walter, Paetkau, and Lardy (2) by high voltage electrophoresis (model L24, Shandon Scientific Company, Sewickley, Pennsylvania). An aliquot of the perchlorate-treated sample was adjusted to pH 3 with KOH, and 0.5 ml was spotted on a Whatman chromatography No. 3MM paper strip (4 × 61 cm). The strip was then soaked carefully with 2 M acetic acid buffer, pH 2.6, adjusted with pyridine, and the same buffer was placed in the tanks of the electrophoresis apparatus. Organic acids were separated on strips for 3 hours at 4500 volts yielding a current of 10 mA per strip. The strips were dried and cut into sections (4 × 1.2 cm) and radioactivity was determined in a Nuclear-Chicago liquid scintillation spectrometer. The radioactive spots were identified by comparing their migration with those of known radioactive organic acids. Fig. 1 shows a sample separation of standard malate, citrate, succinate, and fumarate compared with the separation of labeled compounds formed during the incubation of adipose tissue mitochondria with H¹⁴CO₃⁻ and pyruvate.

Fixation of Labeled Bicarbonate—Incubations were carried out in the small tubes (10 ml) which were shaken in a water bath at 30°C or 37°C. The compositions of the reaction mixtures are described in the legends of the tables and figures. The reaction was started by adding 0.2 ml of mitochondrial suspension after a temperature equilibrium period of about 5 min. After appropriate incubation periods, the reactions were stopped by adding perchloric acid to give a final concentration to 6%. The tubes were gassed with CO₂ for 4 min to remove residual labeled bicarbonate. An aliquot of the mixture was counted to determine H¹⁴CO₃⁻ fixation. Pyruvate (22), malate (23), and citrate (24) were measured enzymatically in the neutralized extracts.

Fig. 1. Separation of citric acid cycle intermediates by high voltage electrophoresis. The migration of standard ¹⁴C-labeled intermediates was compared with that of organic acids formed from H¹⁴CO₃⁻ and pyruvate by adipose tissue mitochondria. The sample experiment shown was for a 30-min mitochondrial incubation. The conditions used for the electrophoretic separation are described in detail under "Materials and Methods."
and the assembly were rapidly washed with 1 ml of cold 0.25 M sucrose. A second washing was carried out within a few seconds of the first with 1 ml of 6% perchloric acid from the syringe and was collected separately. To determine the acid-soluble radioactivity, an aliquot from the combined filtrate was acidified with perchloric acid, gassed with CO2, and the radioactivity was determined. The filter was also dried and the radioactivity was determined. Release of the mitochondrial matrix enzyme citrate synthase (EC 4.1.3.7) into the filtrate was used as a measure of mitochondrial breakage (25). Alternatively mitochondria were separated from the medium by centrifuging at 10,000 × g for 10 min (26). After removing the supernatant solution the mitochondria were washed with 1 ml of cold 0.25 M sucrose and then extracted with 1 ml of 6% perchloric acid. The supernatant and the washing were combined and an aliquot was acidified, gassed with CO2, and the radioactivity was determined.

**Metabolism of Labeled Intermediates** The formation of 14CO2 from labeled citric acid cycle intermediates was initiated by incubation of mitochondria in 10-ml vials which were sealed with rubber serum stoppers having hanging polyethylene cups (Kontes Glass Company, Vineland, New Jersey). The composition of the reaction mixtures is described in the legend of Table V. At the end of the incubation period 0.3 ml of Hyamine was introduced into the cups and 0.2 ml of 36% perchloric acid was added to the incubation medium to stop the reaction and to release CO2. To ensure complete liberation and trapping of CO2, shaking was continued for another 40 min. The content of the cup was then transferred to a vial containing diotol (27) for the measurement of radioactivity. Labeled organic acids in the incubation medium were separated by high voltage electrophoresis as described previously.

**Assay of Mitochondrial Enzymes** Aliquots of liver and adipose tissue mitochondria suspended in 0.25 M sucrose were freeze-dried and then taken up in distilled water. Pyruvate carboxylase (EC 6.4.1.1) was assayed by a modification (6) of the method of Utter and Keen (28). Citrate synthase (29) and malate dehydrogenase (30) were measured by standard spectrophotometric assays at 37°C. A unit is the amount of enzyme which catalyzes the formation of 1 μmole of product per min.

### Table I
**Oxidative phosphorylation and respiratory control ratio of mitochondria from rat epididymal adipose tissue and liver**

The reaction medium contained, in a final volume of 2.5 ml, 0.225 M sucrose; 10 mM potassium phosphate, pH 7.4; 20 mM triethanolamine buffer, pH 7.4; 5 mM MgCl2; 20 mM KCl; and 25 mg of defatted bovine serum albumin. Succinate, 6.6 mM, and pyruvate, 10 mM, were added as indicated. The reaction was initiated by adding ADP (0.20 mM). Oxygen uptake was determined with a Gilson Oxygraph in the presence and absence of added ADP at 37°C. In Experiments 1 to 3, 1.1, 0.9, and 3.2 mg of mitochondrial protein were added, respectively.

| Experiment and mitochondrial source | Substrate | P:O ratio | Respiratory control ratio |
|------------------------------------|-----------|-----------|--------------------------|
| 1. Adipose tissue                  | Succinate + pyruvate | 2.40 | 3.5 |
| 2. Adipose tissue                  | Succinate  | 1.65 | 2.8 |
| 3. Liver                           | Succinate  | 1.70 | 3.7 |

**RESULTS**

**Integrity of Mitochondria**—An important requisite to a study of pyruvate metabolism in isolated mitochondria is to establish that the mitochondria are functionally intact. We have, therefore, measured several parameters, among them the P:O ratio, the respiratory control ratio, and the electron microscopic examination of the isolated mitochondria. The P:O ratios for adipose tissue mitochondria, as well as the observed respiratory control ratios shown in Table I agree well with values reported for mitochondria from rat liver (35, 36) and from other tissues (37-40). Since mitochondria can synthesize ATP from ADP either by oxidative phosphorylation of ADP when coupled, or by adenylate kinase when uncoupled, we have measured the net change in adenine nucleotide levels in isolated rat adipose tissue mitochondria incubated with ADP (Fig. 2). No significant change in the AMP concentration.
FIG. 3. Electron micrographs of mitochondria isolated from rat epididymal adipose tissue. The mitochondria were isolated and prepared for electron microscopy as described under “Materials and Methods.” The bar shown in the figure represents 0.1 μm.

(Fig. 2) was observed over the 20-min incubation period, whereas ATP was synthesized from ADP, indicating that adenylate kinase was not active in this mitochondrial preparation.

A composite photograph selected from several fields of mitochondria is shown in Fig. 3. The mitochondria appeared to be largely intact and some degree of swelling is apparent. Two types of mitochondria were evident (Fig. 3A); one has a more strongly condensed matrix with swollen cristae, whereas the other type appears similar to mitochondria in fixed, unfractionated adipose tissue having cristae which extend all the way across the organelle and a less dense matrix. It is not clear whether the difference between these types of mitochondria is due to factors introduced during their isolation from the fat cells or by fixation of the isolated mitochondria for electron microscopic examination.

Cofactor Requirements for \(^{14}C\)-bicarbonate fixation by mitochondria—The fixation of labeled bicarbonate into acid-soluble intermediates by adipose tissue mitochondria is shown in Table II. The omission of pyruvate, ATP, or MgCl\(_2\) resulted in a complete abolition or a drastic reduction in the fixation of labeled bicarbonate. The omission of sucrose from the medium increased the fixation of \(^{14}C\)-bicarbonate, presumably due to a swelling of the mitochondria in a hypotonic medium resulting in an increased permeability to the various substrates and cofactors. The fixation of bicarbonate by the mitochondria via pyruvate carboxylase is dependent upon acetyl-CoA (6, 41). As shown in Table II, \(^{14}C\)-bicarbonate fixation occurs at a rapid rate in the absence of added acetyl-CoA indicating that mitochondrial acetyl-CoA was endogenous or synthesized from pyruvate (or both). If pyruvate carboxylation were due to the solubilization of pyruvate carboxylase from adipose tissue mitochondria during incubation, the addition of avidin should inhibit \(^{14}C\)-bicarbonate fixation, as previously demonstrated by Ballard and Hanson (6). The lack of inhibition in the fixation of \(^{14}C\)-bicarbonate by avidin suggests that a significant number of mitochondria were not broken.

The optimum concentration of substrates and cofactors for \(^{14}C\)-bicarbonate fixation by mitochondria were pyruvate, 8 mM; ATP, 8 mM; MgCl\(_2\), 20 mM; and KHCO\(_3\), 40 mM (Fig. 4). The incorporation of \(^{14}C\)-bicarbonate continued to increase with an increased concentration of substrates or cofactors and no reduction in the fixation of \(^{14}C\)-bicarbonate was observed when any one component was added in excess of its optimum concentration. It is of interest to note that these optimum conditions of \(^{14}C\)-bicarbonate fixation by adipose tissue mitochondria are different from those reported for rat liver (1, 2) and kidney (4, 5), and guinea pig liver (3). For studies of \(^{14}C\)-bicarbonate incorporation defatted albumin was not added to the incubation medium since it was found that the rate of \(^{14}C\)-bicarbonate incorporation and the formation of organic acids were not changed, and the presence of albumin prevented the separation of citric acid cycle intermediates by high voltage electrophoresis. The omission of inorganic phosphate in the presence of 8 mM ATP reduced the fixation of \(^{14}C\)-bicarbonate by the mitochondria (Fig. 5A). This inhibition was completely overcome by the addition of 6.6 mM inorganic phosphate. We have noted that the addition of 10 mM ADP, in the presence and absence of 8 mM ATP, inhibited the fixation of \(^{14}C\)-bicarbonate by adipose tissue mitochondria by 50 and 86%.

### Table II

| Conditions          | Experiment I | Experiment II |
|---------------------|--------------|---------------|
| Complete system     | 0.983        | 0.888         |
| - sucrose           | 1.588        | 1.054         |
| - pyruvate          | 0            | 0             |
| - MgCl\(_2\)        | 0.185        | 0.091         |
| - ATP               | 0.041        | 0.009         |
| + Avidin            | 0.849        |                |
| + Biotin            | 0.945        |                |
| + Avidin + biotin   | 0.090        |                |

The reaction mixture contained, in a final volume of 1 ml, 0.25 M sucrose; 6.6 mM potassium phosphate, pH 7.4; 6.6 mM triethanolamine, pH 7.4; 10 mM potassium pyruvate; 40 mM potassium bicarbonate (10 μCi); 8 mM ATP; and 20 mM MgCl\(_2\). Where indicated, 2 mg of avidin and 37 μg of biotin were added. The incubation was carried out for 30 min at 30°C with about 0.3 mg of mitochondrial protein being added in each tube.
FIG. 4. Effect of MgCl₂, ATP, potassium bicarbonate, and pyruvate concentrations on the fixation of H¹⁴CO₃⁻ by rat adipose tissue mitochondria. Unless otherwise indicated, the reaction mixture was as described in Table II. Incubations were carried out at 37° for 20 min.

FIG. 5. Effect of phosphate, pH, and mitochondrial protein on the fixation of H¹⁴CO₃⁻ by rat adipose tissue mitochondria. Unless otherwise indicated, the reaction mixture was as described in Table II. When the effect of pH was studied, potassium phosphate buffer and triethanolamine buffer of indicated pH were added. Incubations were carried out at 37° for 20 or 30 min as indicated.

FIG. 6. Formation of organic acids from pyruvate and labeled bicarbonate by rat adipose tissue mitochondria. The reaction mixture was as described in Table II. Incubation time varied as indicated. Labeled organic acids were separated on high voltage electrophoresis as described under "Materials and Methods." Values are the means ± S.E. of four experiments.

Only on the specific radioactivity of the added bicarbonate and does not take into account any CO₂ produced during the incubation or CO₂ dissolved in the incubation medium. Furthermore, some of the fixed H¹⁴CO₃⁻ is lost again during the metabolism of labeled citrate in the tricarboxylic acid cycle. Adipose tissue mitochondria fixed 0.95 µmole of H¹⁴CO₃⁻ per mg of protein into citrate over the 30-min incubation period, compared with 0.53 µmole converted to malate (Fig. 6). We also detected small amounts of radioactivity in fumarate (0.041 µmole per mg of protein) and succinate (0.056 µmole per mg of protein).

The utilization of pyruvate and the formation of citrate and malate by adipose tissue mitochondria were linear over a 30-min incubation period (Fig. 6). About 4.1 µmoles of pyruvate were used by mitochondria, and 0.99 µmole of citrate and 0.21 µmole of malate were formed in 30 min. No attempt was made to determine fumarate and succinate in the medium. When varying concentrations of pyruvate (5, 10, 20, and 30 mM) or KHCO₃ (8, 20, 40, and 60 mM) were added the distribution of the radioactivity in citrate and malate was not altered. This contrasts with rat liver (1, 2) and kidney (4, 5), and guinea pig liver (3) mitochondria in which marked alterations in the distribution of radioactivity in citrate and malate have been noted when the concentration of pyruvate or bicarbonate was altered.

Efflux of Labeled Organic Acids from Adipose Tissue Mitochondria—Previous studies with mitochondria isolated from rat (26) and guinea pig livers (25) had indicated the efflux of various citric acid cycle intermediates into the incubation medium. Since there is indirect evidence, based on labeling studies with whole fat pads (7, 8, 15, 42, 43), that both citrate and malate also move across the mitochondrial membrane in adipose tissue, we have attempted to measure the distribution of newly synthesized organic acids after an incubation with H¹⁴CO₃⁻ and pyruvate (Table III). Two techniques were used to separate the mitochondria from the incubation medium; syringe filtration with the use of Millipore filters and centrifugation at 10,000 × g for 10

respectively.¹ This finding is consistent with the observed inhibition of pyruvate carboxylation in rat liver mitochondria by ADP (4) and suggests that an inhibition of this enzyme by ADP also occurs in adipose tissue mitochondria. Since the fixation of H¹⁴CO₃⁻ was maximal over a wide pH range from 7 to 8.2 (Fig. 5D) we used pH 7.4 in all experiments. The fixation of H¹⁴CO₃⁻ was linear up to 0.40 mg of mitochondrial protein (Fig. 5C) and in most of the studies reported in this paper 0.30 to 0.45 mg of mitochondrial protein was used in each incubation.

Products of Fixation of H¹⁴-C-Bicarbonate—The incorporation of H¹⁴CO₃⁻ into acid-soluble intermediates by adipose tissue mitochondria was linear over the 30-min incubation, and about 2 µmoles of H¹⁴CO₃⁻ per mg of protein were incorporated in 30 min under the experimental conditions used (Fig. 6). The fixation of H¹⁴CO₃⁻ represents a minimum value since it is based

¹ M. S. Patel, unpublished observations.
Incubations were carried out at 37° for 30 min as described in Table II. At the end of the incubation period, the reaction in the control tube was stopped by adding perchloric acid. The amount of H^4CO_3^- fixed in this tube was taken as 100%. The filtration and centrifugation were carried out as described under "Materials and Methods." To determine the activity of citrate synthase added, an aliquot of the mitochondrial suspension was freeze-dried to release the enzyme and this activity (control) was expressed as 100%.

| Treatment                  | H^4CO_3^- fixed during incubation | Release of citrate synthase |
|----------------------------|----------------------------------|-----------------------------|
|                            | Experiment I | Experiment II | Experiment I | Experiment II |
| Filtration with syringe    |              |               |              |               |
| Control                    | 100          | 100           | 100          | 100           |
| Filtrate + sucrose wash    | 94.0         | 95.0          | 15           | 14            |
| Acid wash + mitochondria   | 4.5          | 3.2           |              |               |
| Centrifugation             |              |               |              |               |
| Control                    | 100          | 100           | 100          | 100           |
| Supernatant + sucrose wash | 98.5         | 99.0          | <1           | <1            |
| Acid wash + mitochondria   | 0.3          | 0.2           |              |               |

The high rate of pyruvate conversion to citrate by adipose tissue mitochondria occurs at a low rate limited perhaps by the availability of intermediates such as pyruvate and fatty acids which can be converted to acetyl-CoA. The addition of pyruvate overcomes this limitation and markedly stimulates the rate of citrate formation.

The high rate of pyruvate carboxylation in adipose tissue mitochondria, together with the high ratio of citrate to malate and CO_2, respectively, in the first turn of the citric acid cycle. We have noted about 0.35 μmole of 1^4CO_2 and 0.74 μmole of malate-1^4C formed from citrate-1,5,1^4C per mg of protein in 30 min. The lower amount of 1^4CO_2 noted in this experiment may be due to a fixation of CO_2 by mitochondria during the course of the incubation. The addition of pyruvate further lowered the output of 1^4CO_2 from citrate-1,5,1^4C without affecting malate formaion, presumably due to the carboxylation of pyruvate. Adipose tissue mitochondria oxidized uniformly labeled malate-1^4C to 1^4CO_2 and citrate-1^4C even in the absence of added pyruvate, but the addition of pyruvate caused a 5-fold increase in citrate formation. Thus, malate conversion to citrate by adipose tissue mitochondria occurs at a low rate limited perhaps by the availability of intermediates such as pyruvate and fatty acids which can be converted to acetyl-CoA. The addition of pyruvate overcomes this limitation and markedly stimulates the rate of citrate formation.
formed by the carboxylation of pyruvate noted in the present study, prompted us to measure the rate of several enzymes involved in the synthesis of these intermediates in both adipose tissue and liver mitochondria (Table VI). Pyruvate carboxylase was about 3-fold higher and citrate synthase was about 7-fold higher in mitochondria isolated from rat adipose tissue than observed in liver mitochondria. The activity of NAD-malate dehydrogenase is the same in mitochondria from both liver and adipose tissue.

**DISCUSSION**

Studies of metabolic regulation in adipose tissue have been largely limited to the measurement of the end products of various reaction sequences, or the relative distribution and activities of enzymes at key points in these sequences. With the availability of more sensitive techniques, attempts at the measurements of some important parameters have been made (44–47) but all of these beginning efforts have been limited by the fact that adipose tissue is about 85% fat with only a 1.4% intracellular water content (44). One example is the total lack of information on the metabolism of adipose tissue mitochondria, despite a number of recent publications indicating an important role for the mitochondria of this tissue in the compartmentalization of key intermediates in lipogenesis (7, 8) and glycero-neogenesis (9). Studies on oxidative phosphorylation in mitochondria from brown fat have been published, but they are contradictory, and illustrate the difficulties inherent in working with subcellular particles isolated from a tissue containing large amounts of fat. Mitochondria from brown fat of cold-adapted rats (48) and from newborn rabbits (49) were reported to be unable to catalyze electron transport-linked phosphorylation of ADP. Studies of the latter by Guillory and Racker (37) and Aldridge and Street (40) demonstrated that if proper care were taken to exhaustively remove fat during the incubation, mitochondria from brown fat were able to carry out oxidative phosphorylation with a P:O ratio comparable to that of other tissues. Our own studies on the metabolism of mitochondria from white adipose tissue were begun as an attempt to provide a workable procedure for the isolation of coupled mitochondria from this tissue and to determine the optimum conditions for their incubation in vitro.

In the course of this study we found that three relatively minor, but important, modifications in the normal procedure were necessary for the isolation of coupled mitochondria from adipose tissue. First, we used relatively young animals weighing 140 to 180 g as a source of tissue, since at this age the nitrogen to fat ratio in the epididymal fat pad is relatively high (50). Second, we found it necessary to isolate fat cells from the pads by the collagenase method of Rodbell (18) before rupturing the cells on glass. All our attempts to homogenize the tissue directly in a Potter-Elvehjem homogenizer resulted in severe restriction of oxidative phosphorylation. Third, it is very important to remove as much fat as possible by repeated centrifugation during the isolation of mitochondria. The functional integrity of mitochondria prepared taking these precautions has been established by measurements of the P:O ratio and the respiratory control ratio, both of which agree well with values obtained for rat liver mitochondria in our laboratory (Table I) and values reported by others (35, 36).

In a study of pyruvate metabolism by rat adipose tissue Kneer and Ball (7) reported the incorporation of $\text{H}^4 \text{CO}_2$ into keto acids. Although the keto acids were not identified, it was estimated that 14.8 $\mu$moles of $\text{H}^4 \text{CO}_2$ were fixed per g of tissue per 4 hours. With the use of isolated adipose tissue mitochondria we have found a rate of 66 $\mu$moles of $\text{H}^4 \text{CO}_2$ fixed per mg of mitochondrial protein per min (Fig. 6). This rate is of the same general magnitude found in mitochondria from rat liver, 56 $\mu$moles (2), and somewhat higher than found in rat kidney, 28 $\mu$moles (5), and guinea pig liver, 32 $\mu$moles (3). Isolation of the intermediates formed in the fixation of $\text{H}^4 \text{CO}_2$ indicates that adipose tissue mitochondria form mainly citrate and malate, with traces of succinate and fumarate when pyruvate and ATP were added to the incubation medium. Since any $\text{H}^4 \text{CO}_2$ fixed by pyruvate carboxylase in the mitochondria will not appear in succinate, fumarate, or malate via the forward direction of the citric acid cycle, the radioactivity observed in these 4-carbon acids results from the reduction of $\text{H}^4 \text{C}-\text{oxalacetate}$ to malate and the equilibrium of malate with fumarate and succinate.

The high rate of pyruvate carboxylation by rat adipose tissue mitochondria correlates well with the high activity of pyruvate carboxylase in these mitochondria. Pyruvate carboxylase plays an important role in gluconeogenesis in liver, but the activity of this enzyme is 3-fold greater in adipose tissue than in liver mitochondria (Table VI), despite the fact that adipose tissue does not synthesize glucose (51). This finding underlines the importance of pyruvate carboxylase in lipogenesis and glycero-neogenesis in adipose tissue. In an earlier paper from this laboratory (6) we suggested that pyruvate carboxylase functions as the initial enzyme in the citrate cleavage pathway, replenishing the mitochondrial oxalacetate lost during citrate cleavage in the cytosol. In fact, tissues which do not synthesize fatty acid from glucose, such as adipose tissue from the sheep and cow, have a negligible pyruvate carboxylase activity (52). The synthesis of glyceric glycerol from pyruvate via the dicarboxylic acid shuttle also requires the presence of pyruvate carboxylase and this enzyme undoubtedly contributes to the increased activity of the glyceroenic pathway observed during fasting and diabetes (53).

In view of the importance of pyruvate carboxylase in the metabolism of adipose tissue, we have studied factors which regulate the carboxylation of pyruvate within the mitochondria. Acetyl-CoA is an obligatory cofactor for adipose tissue pyruvate carboxylase activity (6). With pyruvate present in the incubation medium, the mitochondrial concentration of acetyl-CoA is probably adequate to saturate the activation effect (2), and it seems likely that the changes in pyruvate carboxylase observed
with increasing pyruvate concentration are due, only in part, to an increase in mitochondrial acetyl-CoA formation. Changes in the levels of pyruvate over a concentration range of 0 to 6 mM result in a sharp increase in HClO\textsubscript{3} fixation. The apparent \textit{K_m} for the carboxylation of pyruvate by adipose tissue mitochondria calculated from Fig. 1D is about 2 mM. The actual concentration of pyruvate in the intracellular water of adipose tissue is approximately 0.5 mM (47). Considering the difficulties in measuring the concentration of intermediates in adipose tissue, pyruvate levels are within the range where a small change in the concentration of pyruvate can significantly alter its carboxylation by the mitochondria. Although not shown in Fig 4D, we have extended the concentration curve to 200 mM pyruvate and have observed no significant change in the rate of pyruvate carboxylation above that noted at 8 mM. This finding contrasts with the results of similar experiments with mitochondria from rat kidney in which pyruvate carboxylation is reduced by 60% when the concentration of pyruvate is increased from 6 to 33 mM (4); and with guinea pig liver mitochondria where the rate of carboxylation of pyruvate is increased by 30% over the same concentration range (3). In a previous study of the synthesis of fatty acids from pyruvate by adipose tissue in vitro (9), we demonstrated that raising the concentration of pyruvate in the incubation medium from 0.25 to 25 mM increased the rate of lipogenesis in tissues from fasted rats to a level almost equal to that observed in epididymal fat pads from fasted-rexed animals. It is most likely that these changes in fatty acid synthesis with increasing pyruvate concentration are related to the increased carboxylation of pyruvate by adipose tissue mitochondria illustrated in Fig. 4D.

Another aspect of the regulation of lipogenesis in adipose tissue is the rate at which high energy phosphate is generated within the mitochondria. One acetyl-CoA moiety formed from pyruvate and incorporated into fatty acids requires 3 molecules of ATP. The data presented here indicate that adipose tissue mitochondria have the capacity to generate about 0.175 \mu mol of ATP per min per mg of protein with pyruvate and malate as substrates (Fig. 2) compared with 0.066 \mu mol of pyruvate carboxylated (Fig. 6) under similar experimental conditions. The inhibition of pyruvate carboxylation by ADP (compare “Results” and Reference 2) even in the presence of ATP suggests that the ATP:ADP ratio directly affects the rate of pyruvate conversion to oxalacetate. Recently, Rognstad and Katz (8) reported that the incubation of rat adipose tissue with dinitrophenol stimulated pyruvate oxidation to CO\textsubscript{2} but inhibited its conversion to fatty acids. These results were attributed by the authors to a dinitrophenol-induced decrease in ATP concentration within the fat cell, which reduced the level of pyruvate carboxylation by the mitochondria, thereby inhibiting lipogenesis.

The high level of pyruvate conversion to fatty acids in adipose tissue (7–9) raises the question of the source of reducing equivalents required to support the over-all process. The pentose pathway provides about 60% of the NADPH used in the reductive synthesis of fatty acids (54, 55) but pyruvate enters the metabolic sequence at a point beyond the pentose pathway. Knorr and Ball (7) and Schmidt and Katz (56) have suggested that the NADPH required to support lipogenesis from pyruvate is derived from a series of reactions involving the formation of oxalacetate from pyruvate by pyruvate carboxylation in the mitochondria, followed by the reduction of oxalacetate to malate by NAD-malate dehydrogenase. The malate then passes from the mitochondria to the cytosol and is converted to pyruvate via NADP-malate dehydrogenase. The NADH required for the formation of malate from oxalacetate in the mitochondria comes from the fixation of pyruvate. The level of pyruvate carboxylase activity in adipose tissue was shown by Reshef, Hanson, and Ballard (9) to be more than sufficient to account for the required rate of oxalacetate synthesis. This reaction sequence resembles the initial steps of gluconeogenesis in liver (1, 2) but in adipose tissue it contributes to glyceroenogenesis from pyruvate during fasting (9, 12, 53). It is important to remember, however, that lipogenesis from pyruvate requires the formation of citrate and its subsequent efflux from the mitochondria to provide acetyl-CoA in the cytosol. Thus, any oxalacetate formed by pyruvate carboxylation is at a metabolic crossroads, and subject to the competition of NAD-malate dehydrogenase and citrate synthase. It is also probable that the conversion of oxalacetate to malate as opposed to citrate is influenced in part by the oxidation-reduction state of the adipose tissue mitochondria. This may explain the observation of Reshef, Niv, and Shapiro (12) that the addition of butyrate stimulated the conversion of pyruvate to \alpha-glycerophosphate by adipose tissue in vitro. In that case the intramitochondrial generation of NADH caused by the oxidation of butyrate might shift oxalacetate toward malate, which could then diffuse into the cytosol and subsequently be converted to P-enolpyruvate via the same reaction sequence as occurs in gluconeogenesis in rat liver (1, 2). Butyrate would also increase the acetyl-CoA levels in adipose tissue mitochondria which could in turn stimulate pyruvate carboxylase activity.

Adipose tissue mitochondria have been shown in this study to form 3 to 4 times more citrate than malate. This contrasts with mitochondria from gluconeogenetic tissues such as liver (1–3) and kidney (4, 5) where pyruvate conversion to malate is equal to or greater than its conversion to citrate. The high ratio of citrate to malate formation from pyruvate in isolated rat adipose tissue mitochondria raises a question of how many of the reducing equivalents can be supplied by the intramitochondrial formation of malate. From the data presented in Fig. 6 it is improbable that malate could provide all of the reducing equivalents for lipogenesis, since each acetyl-CoA moiety formed from citrate and incorporated into fatty acids, requires 2 molecules of NADPH. As an alternative pathway, some of the citrate leaving the mitochondria could, instead of being cleaved by ATP-citrate lyase, be converted to \alpha-ketoglutarate, thereby generating NADPH via isocitrate dehydrogenase. This enzyme is present in the cytosol of adipose tissue (10) and the over-all functioning of this extramitochondrial portion of citric acid cycle has been established in the reverse direction by measuring glutamate conversion to fatty acids (43, 57). It is thus possible that some mechanism other than the formation of malate from oxalacetate within the mitochondria accounts for the total reducing equivalents required to support the conversion of pyruvate carbon to fatty acids. Since citrate efflux from the mitochondria has been proposed as a mechanism for the transport of acetyl-CoA, the data shown in Table III offer the first direct evidence for citrate efflux across the adipose tissue mitochondrial membrane. This is further supported by the fact that citrate-1,5-\textsuperscript{14}C is oxidized to \textsuperscript{14}CO\textsubscript{2} and converted to malate-\textsuperscript{14}C by the mitochondria (Table V). In view of the importance of citrate in lipogenesis it is of interest to note that in adipose tissue where the rate of fatty acid
synthesis is high relative to gluconeogenesis, the formation of citrate from pyruvate exceeds by about 5-fold the formation of malate.

It is becoming increasingly apparent that the carboxylation of pyruvate is an important regulatory step in lipogenesis in adipose tissue. Our results indicate that the activity of pyruvate carboxylase in mitochondria of rat adipose tissue is greater than is found in mitochondria from rat liver, and that the over-all rate of pyruvate carboxylation proceeds at a high level in adipose tissue mitochondria. Two major points of regulation of pyruvate carboxylation appear to be the availability of ATP and the concentration of pyruvate. A third factor, acetyl-CoA availability within the mitochondria, which is undoubtedly important, has not been assessed in this study. Our findings with isolated adipose tissue mitochondria agree well with the results of studies with whole tissue, in which alterations in the levels of ATP (8) or of pyruvate (9) resulted in major changes in the over-all level of lipogenesis.

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