The citrate cleavage pathway and lipogenesis in rat adipose tissue: replenishment of oxaloacetate

F. J. BALLARD and RICHARD W. HANSON

Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

ABSTRACT Fatty acid synthesis via the citrate cleavage pathway requires the continual replenishment of oxaloacetate within the mitochondria, probably by carboxylation of pyruvate.

Malic enzyme, although present in adipose tissue, is completely localized in the cytoplasm and has insufficient activity to support lipogenesis. Pyruvate carboxylase was found to be active in both the mitochondria and cytoplasm of epididymal adipose tissue cells; it was dependent on both ATP and biotin. Alterations in dietary conditions induced no significant changes in mitochondrial pyruvate carboxylase activity, but the soluble activity was depressed in fat-fed animals.

The possible importance of the soluble activity in lipogenesis lies in its participation in a soluble malate transhydrogenation cycle with NAD malate dehydrogenase and malic enzyme, whereby a continual supply of NADPH is produced. Consequently, the pyruvate carboxylase in adipose tissue both generates mitochondrial oxaloacetate for the citrate cleavage pathway and supplies soluble NADPH for the conversion of acetyl-CoA to fatty acid.

KEY WORDS pyruvate carboxylase · adipose tissue · rat · intracellular distribution · dietary changes · oxaloacetate formation · lipogenesis

FATTY ACID SYNTHESIS from acetyl-CoA in adipose tissue and liver occurs in the cytoplasm of the cell but uses acetyl-CoA formed in the mitochondria (1, 2). However, Spencer and Lowenstein (3) have reported that the rate of diffusion of acetyl-CoA out of the mitochondria is too slow to meet the demands of rapid lipogenesis. Evidence is accumulating that the citrate cleavage enzyme (EC 4.1.3.8), described first by Srere (4), is supplying extramitochondrial acetyl-CoA (5, 6). Thus, citrate formed intramitochondrially can, after diffusion into the cytoplasm, be cleaved to acetyl-CoA and oxaloacetate. Recently, Young, Shrago, and Lardy (7) and Wise and Ball (8), in reports of studies on adipose tissue, proposed that oxaloacetate formed by citrate cleavage is reduced to malate via NAD malate dehydrogenase (EC 1.1.1.37), with subsequent decarboxylation to pyruvate via the NADP-linked malic enzyme (EC 1.1.1.40). An important feature of this proposal is the transhydrogenation of NADH to NADPH, which would augment the available extramitochondrial NADPH to support lipogenesis. These reactions are shown in the following equations:

1. oxaloacetate + acetyl-CoA + H_2O $\xrightarrow{\text{citrate synthase}}$ citrate + CoA

2. citrate + ATP + CoA $\xrightarrow[\text{citrate cleavage enzyme}]{Mg^{++}}$ ADP + oxaloacetate + acetyl-CoA + P_i

3. oxaloacetate + NADH $\xrightarrow[\text{dehydrogenase}]{\text{NAD-malate}}$ malate + NAD

4. malate + NADP $\xrightarrow[\text{enzyme}]{\text{malic}}$ pyruvate + CO_2 + NADPH

The functioning of the citrate cleavage pathway as shown above would result in a net loss in intramitochondrial oxaloacetate unless there is a reaction sequence for the regeneration of oxaloacetate. In liver, oxaloacetate is formed from pyruvate by the enzyme pyruvate carboxylase (EC 6.4.1.1.) (9). There has not, however, been a detailed study of the possible pathways of oxaloacetate synthesis in adipose tissue, although the presence of pyruvate carboxylase has been shown by Wise and Ball (8). In the present study we have measured the

intracellular distribution, cofactor requirements, methods of extraction, and effect of diet on pyruvate carboxylase from rat adipose tissue. We have also studied the possibility of the reversal of malic enzyme as a source of oxaloacetate in adipose tissue. The results of these investigations indicated that pyruvate carboxylase is present in adipose tissue and is active enough to supply the intramitochondrial oxaloacetate needed to support the citrate cleavage sequence. It therefore seems justifiable to complete the cycle of oxaloacetate utilization and reformation by adding the following equation to those listed above:

$$5.\ \text{pyruvate} + CO_2 + \text{ATP} \xrightarrow[\text{pyruvate carboxylase}]{\text{acetyl-CoA}} \text{oxaloacetate} + \text{ADP} + P_i$$

Our results also suggest the possibility of a separate "malate-transhydrogenation cycle" for the generation of extramitochondrial NADPH.

MATERIALS AND METHODS

Sodium pyruvate, oxaloacetic acid, malic acid, and Dowex-1 (200–400 mesh) were obtained from the Sigma Chemical Co., St. Louis, Mo. Acetyl-CoA, ATP, NADP, NAD, and NADH were obtained from P-L Biochemicals Inc., Milwaukee, Wis., and $NaH^{14}CO_3$ from New England Nuclear Corp., Boston, Mass. Propionyl-CoA used in these studies was prepared from propionic anhydride and CoA (10), and used immediately after synthesis.

Male Wistar albino rats from Carworth Farms, New City, N. Y., weighing between 350 and 400 g, were fed ad lib. on Purina Rat Chow or a high fat diet containing 45% vegetable oil, 29% sucrose, 18% casein, 4% brewer's yeast, 4% salt mixture, and supplementary vitamins.

Subcellular Fractionation of Adipose Tissue

In most experiments, epididymal fat pads were homogenized in a buffered isotonic sucrose medium, pH 7.5, containing 0.2 M sucrose, 0.02 M triethanolamine, 1 mM glutathione, and 1 mM EDTA (11), by the use of a coaxial glass homogenizer with Teflon pestle. The homogenate was centrifuged for 30 min at 100,000 *g;* the pellet was suspended in buffered sucrose and centrifuged for 15 min at 100,000 *g*. These steps were carried out at 0–5°C. Since pyruvate carboxylase was more stable at 25°C, soluble fractions used for assay of the enzyme were maintained at this temperature after isolation.

For the isolation of nuclei, mitochondria, microsomes, and soluble fraction, epididymal pads were treated with collagenase in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 3% bovine serum albumin (12), and the cells were harvested by centrifugation, washed several times with buffer, and suspended in 0.25 M sucrose. The fat cells were disrupted by mechanical agitation on a Vortex mixer and the bulk lipid was removed by centrifugation at 500 *g* for 15 sec at 2°C. Nuclei were sedimented from the lipid-free homogenate by centrifugation at 600 *g* for 12 min, mitochondria by centrifugation at 10,000 *g* for 15 min, and microsomes by centrifugation at 100,000 *g* for 30 min, all at 2°C. All fractions were suspended in sucrose and resedimented once. Particulate fractions suspended in sucrose were freeze-dried and dissolved in water for enzyme assay.

TABLE 1 PYRUVATE CARBOXYLASE ACTIVITY OF ADIPOSE TISSUE

All values are expressed as mμmoles incorporated per g wet weight of adipose tissue per min at 37°C. Enzyme was from the soluble fraction of adipose tissue. Each value is the mean ± SEM of four values.

Complete system	86.0 ± 3.5
−Pyruvate	6.5 ± 0.4
−Acetyl-CoA	5.6 ± 0.5
−ATP	5.8 ± 0.3
+Biotin	85.5 ± 5.1
+Avidin	5.9 ± 0.6
+Biotin + avidin	85.3 ± 3.6
+Phosphate	83.3 ± 4.6

Complete system contained enzyme, 25 μmoles of Tris pH 7.4, 10 μmoles of sodium pyruvate, 2.5 μmoles of sodium ATP, 0.75 μmole of acetyl-CoA, 50 μmoles of $NaH^{14}CO_3$ (2 μc), and 5 μmoles of $MgCl_2$ in a total volume of 1.0 ml. In those experiments in which other additions were present, the following amounts were added: 100 μg of biotin, 2 units of avidin, and 10 μmoles of sodium phosphate (pH 7.4).

Assay of Pyruvate Carboxylase

This assay is in principle similar to that used by Utter and Keech (13) for liver mitochondrial pyruvate carboxylase and measured the fixation of ^{14}C-labeled $NaHCO_3$ in the presence of ATP, pyruvate, $MgCl_2$, acetyl-CoA, and enzyme. The exact composition of the reaction mixture as well as the effects of deletions and additions to the complete system are shown in Table 1. After incubation, reactions were stopped by additions of trichloroacetic acid to 5%, the tubes were centrifuged, and the CO_2 remaining in the supernatant was removed by gassing vigorously for 3 min with unlabeled CO_2. A portion of this solution was counted in a Packard liquid scintillation counter model 314 EX, with Diotol (14) as solvent. The results in Table 1 illustrate the dependence of enzyme activity on acetyl-CoA, pyruvate, and ATP. Like the liver enzyme (13), adipose tissue pyruvate carboxylase is inhibited by avidin and this inhibition is relieved by biotin. The stimulation of the activity of the enzyme from rat liver by addition of phosphate recently reported by Walter, Paetkau, and Lardy (15) could not be confirmed for the enzyme from adipose tissue. In all assays the value for a blank without acetyl-CoA was sub-

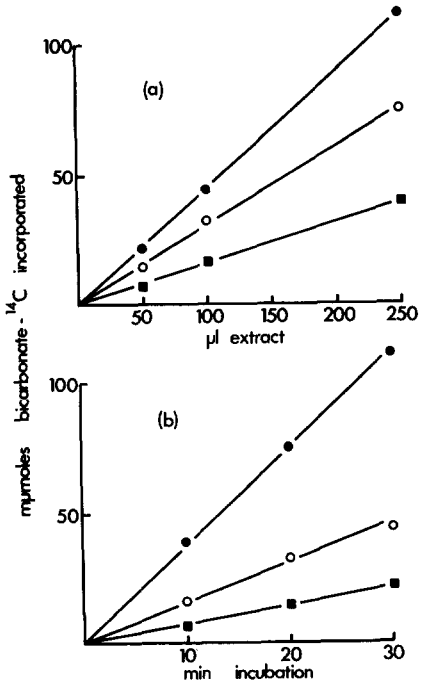

FIG. 1. (a) Linearity of adipose tissue pyruvate carboxylase activity with increasing amounts of the soluble enzyme preparation. Incubation times: 10 min (■), 20 min (○), and 30 min (●). (b) Linearity of pyruvate carboxylase activity with increasing incubation times, when 50 μl (■), 100 μl (○), or 250 μl (●) of the soluble enzyme preparation was used.

tracted from the value for incorporation in the complete system. The linearity of product formation with increasing time or amount of enzyme is shown in Fig. 1.

In one experiment (Fig. 2a) a portion of the radioactive product of the reaction, after being gassed with unlabeled CO_2, was chromatographed with unlabeled citrate and malate on a column of Dowex-1 (formate) and eluted with a 0–6 N formic acid gradient (16). Determinations of radioactivity, malate (17), and citrate (18) on the eluent showed that most of the bicarbonate-^{14}C incorporated in this assay appeared in citrate. That oxaloacetate was not the principal product was checked by treatment of another portion of the final reaction product with aniline citrate (19), collection of the CO_2 evolved on filter paper saturated with 7 N NaOH, and determination of the radioactivity directly on these papers by the method of Buhler (20). This CO_2, obtained from the decarboxylation of the oxaloacetate formed, contained only 1.6% of the radioactivity incorporated. Citrate, the final product of the pyruvate carboxylase assay, was probably formed by condensation of oxaloacetate with acetyl-CoA, an added cofactor, in the presence of citrate synthase (EC 4.1.3.7). The possibility that the citrate was the product of a carboxylation reaction independent of any pyruvate carboxylase activity was eliminated by the experiment described in Table 2. In this experiment acetyl-CoA was replaced as cofactor by propionyl-CoA. Although propionyl-CoA is an active cofactor (21), it cannot combine with oxaloacetate to form citrate and we found that essentially all the radioactivity fixed was in oxaloacetate.

TABLE 2 PRODUCT OF THE PYRUVATE CARBOXYLASE REACTION

The assay solution was identical with that described in Table 1, with propionyl-CoA replacing acetyl-CoA where indicated. Blanks without pyruvate have been subtracted from the total activity. Enzyme was from the soluble fraction of adipose tissue.

Cofactor, Concentration	Bicarbonate Incorporated	Oxaloacetate Formed	Per Cent of Product as Oxaloacetate
	mμmoles/min/g tissue		
Acetyl-CoA, 0.75 mM	82.4	1.3	1.6
Propionyl-CoA, 0.75 mM	19.2	17.3	90.0
Propionyl-CoA, 1.5 mM	28.2	25.1	89.6

Assay of Malic Enzyme

The spectrophotometric assay described by Ochoa (22) was used unless otherwise noted. The final concentrations

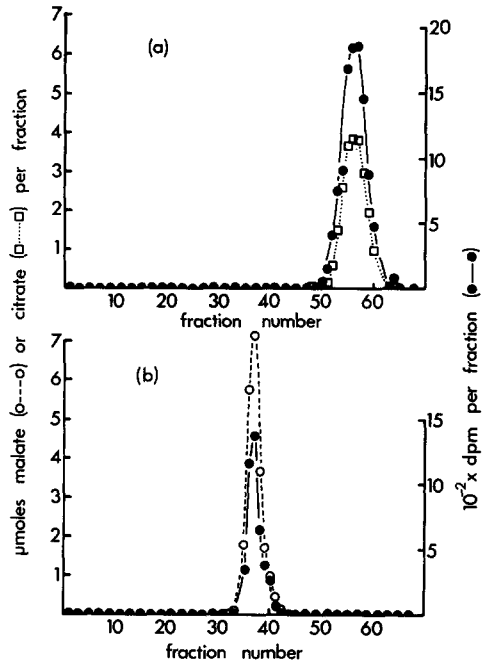

FIG. 2. Identification of citrate as the product of the pyruvate carboxylase assay (a), or malate as the product of the malic enzyme assay (b).

$NaH^{14}CO_3$ was incubated in an assay system with the supernatant fraction from adipose tissue, and a portion of the inactivated reaction mixture, after being gassed with unlabeled CO_2, was chromatographed with 30 μmoles of citrate or malate on a 0.8 cm^2 × 15 cm column of Dowex-1 (formate) (200–400 mesh) and eluted with a 0–6 N formic acid gradient (16). Each 4 ml fraction was dried in vacuo, the residue was dissolved in 3 ml of water, and portions were taken for the determination of citrate (18), malate (17), and radioactivity.

of reactants at 37°C were Tris (adjusted to pH 7.4), 30 mM; $MnCl_2$, 1.5 mM; NADP, 0.3 mM; sodium malate, 0.75 mM; and tissue extract. Extinction changes without malate were subtracted from the rates in the complete system.

In the radioactivity assay of malic enzyme the fixation of bicarbonate-^{14}C in the presence of pyruvate, $MgCl_2$, NAD, and NADPH was measured. The concentration of reactants in a final volume of 1.0 ml were Tris (adjusted to pH 7.4), 25 mM; sodium bicarbonate-^{14}C, 50 mM (2 μc); sodium pyruvate, 10 mM; NAD, 1.5 mM; and NADPH, 1.5 mM. Tissue extract was added and the tubes were incubated for 5, 10, and 15 min at 37°C. The reaction was stopped by the addition of trichloroacetic acid, CO_2 was liberated, and incorporation was measured as for pyruvate carboxylase.

The effect of deletions or additions in the complete malic enzyme system are shown in Table 3. Negligible activity occurred in the absence of NADPH or pyruvate, and omission of NAD produced a 20% decrease in activity. This effect of NAD was probably caused either by inhibition of the conversion of pyruvate substrate to lactate or by removal of malate product as oxaloacetate. The addition of biotin or avidin had no effect. The radioactive product of the malic enzyme assay was shown by chromatographic separation on Dowex-1 (formate) to be malate (Fig. 2b).

Determination of Nitrogen

Nitrogen was determined in tissue homogenates by micro-Kjeldahl digestion followed by nesslerization (23).

RESULTS

Preliminary experiments indicated that adipose tissue isolated by differential centrifugation contained pyruvate carboxylase activity in both particulate and soluble fractions. The activity of pyruvate carboxylase measured in particulate fractions that had been frozen and thawed several times was greater than in the untreated particles (Table 4). However, the greatest activity could be detected in freeze-dried preparations in which about 75% of this activity had been solubilized. Sonication was found to reduce the enzyme activity below that of the untreated pellet. In all subsequent experiments, freeze-dried, particulate fractions were used for the determination of pyruvate carboxylase activity. The particulate pyruvate carboxylase had the same cofactor and substrate requirements as reported for the soluble enzyme in Table 1.

Wagle (24) has reported that homogenization and centrifugation of liver at room temperature release pyruvate carboxylase from mitochondria. This method was used to determine the degree to which pyruvate carboxylase leaches out of particles from adipose tissue. As shown in Table 5, no difference could be found between the activity of either soluble or particulate enzyme when the separation was carried out at 2° or 25°C.

In order to ensure a minimum destruction of subcellular components, we measured the activities of pyruvate carboxylase and malic enzyme in subcellular fractions prepared from collagenase-released adipose tissue cells. The results in Table 6 show that 30% of the total pyruvate carboxylase activity was in the soluble fraction and most of the remainder was mitochondrial. The malic enzyme activity was in the soluble fraction. This collagenase method of cell separation was necessarily slow, and pyruvate carboxylase and malic enzyme activities were lower than those found in preliminary experiments in which

TABLE 3 REVERSAL OF MALIC ENZYME IN ADIPOSE TISSUE

Values are expressed as mμmoles of bicarbonate-^{14}C incorporated per g of tissue per min at 37°C, with the soluble enzyme fraction from untreated animals. Each value is the mean of three determinations.

Complete system	40.7
−NADPH	3.6
−Pyruvate	3.5
−NAD	34.1
+Biotin	42.1
+Avidin	39.5
+Biotin + avidin	40.3

Complete system contained enzyme, 25 μmoles of Tris pH 7.4, 10 μmoles of sodium pyruvate, 50 μmoles (2 μc) of $NaH^{14}CO_3$, 5 μmoles of $MgCl_2$, 1.5 μmoles of NaDPH, and 1.5 μmoles of NAD. Other additions: 100 μg of biotin, and 2 units of avidin.

TABLE 4 ASSAY OF MITOCHONDRIAL PYRUVATE CARBOXYLASE ACTIVITY

Activity is expressed as mμmoles bicarbonate-^{14}C incorporated per min per g of adipose tissue (assuming quantitative yield of particles) at 37°C. Particles were suspended in buffered sucrose.

Treatment	Activity
Untreated	29.4
Sonicated, 1 min	10.8
Frozen and thawed 3 times	81.5
Frozen and thawed 6 times	114
Freeze-dried, suspended in water	216

TABLE 5 ASSAY OF PYRUVATE CARBOXYLASE AFTER ISOLATION OF ENZYME IN THE COLD OR AT ROOM TEMPERATURE

Activity is expressed as mμmoles bicarbonate-^{14}C incorporated per min per g of adipose tissue. In each experiment one fat pad was homogenized, and centrifuged at 25°C, the other at 2°C. Values are the means of two experiments.

Treatment	Enzyme	Activity
Isolated at 25°C	Soluble	94.9
"	Mitochondrial	135
Isolated at 2°C	Soluble	91.6
"	Mitochondrial	133

TABLE 6 INTRACELLULAR DISTRIBUTION OF PYRUVATE CARBOXYLASE AND MALIC ENZYME

Pyruvate carboxylase activities are expressed as mμmoles bicarbonate-^{14}C incorporated per min per g of tissue with the assumption that yields of subcellular particles were quantitative. Malic enzyme activity is in mμmoles of NADPH formed per min per g of tissue. In experiment 1, cells were separated with collagenase, isolated and ruptured on a Vortex mixer and subcellular fractions isolated as described in the text. In the second experiment tissue was homogenized directly in a coaxial homogenizer and centrifuged at 100,000 *g*. In this experiment the pellet was resuspended, homogenized, and centrifuged to give the washings.

Experiment	Subcellular Fraction	Activity	
		Pyruvate Carboxylase	Malic Enzyme
1	Supernatant	61.2	122
	Microsomes	3.6	0
	Mitochondria	120.8	2.5
	Nuclei	19	0
2	Supernatant	92.0	242
	Unwashed particles	253	7.2
	First washings	15.5	3.2
	Second washings	12.0	2.0
	Washed particles	188	0.8

tissue was homogenized with the coaxial homogenizer. We therefore conducted an additional experiment to determine whether direct homogenization could be routinely used. This experiment (Table 6) showed a similar pyruvate carboxylase distribution between particles and soluble fraction. In addition, there was little activity in the supernatant fraction when the isolated particles were homogenized and centrifuged by the same methods used with intact tissue. As was found with the collagenase separation, all the malic enzyme activity was in the 100,000 *g* supernatant solution.

The results of changes in dietary conditions on the activities of soluble or mitochondrial pyruvate carboxylase are given in Table 7. These experiments were designed to determine whether pyruvate carboxylase activity was altered under conditions which favored or depressed lipogenesis in adipose tissue. Fasting the rats for 96 hr and then refeeding them lab chow for 96 hr produced no elevation of either the soluble or particulate activity. Fasting the rats for 96 hr followed by refeeding them a high fat diet significantly depressed the pyruvate carboxylase activity of the soluble enzyme below that found in untreated animals. The mean value of the mitochondrial activity was also lower, but this fall was not statistically significant.

DISCUSSION

The main aim of the present study was to investigate the possible mechanisms of oxaloacetate synthesis in adipose tissue. This process in liver is currently being intensively investigated (9, 11, 15) since gluconeogenesis from pyruvate involves the initial carboxylation of pyruvate to oxaloacetate and subsequent conversion to glucose via phosphoenolpyruvate. There now seems little doubt that pyruvate carboxylase plays a central role in gluconeogenesis, but the importance of this enzyme in adipose tissue remained, until recently, of little interest. As evidence accumulates for the citrate cleavage pathway as an important metabolic route for supplying extramitochondrial acetyl-CoA, the synthesis of oxaloacetate becomes as important for lipogenesis in adipose tissue as it is for gluconeogenesis in liver.

The citrate cleavage pathway, shown in equations 1–4, provides for the adipose tissue cell a mechanism for overcoming the mitochondrial impermeability to acetyl-CoA (3) as well as for generating NADPH to support lipogenesis (7, 8). The functioning of such a pathway demands, however, that the synthesis of citrate inside the mitochondria be at a rate sufficient to supply the cytoplasmic acetyl-CoA required for lipogenesis. This in turn requires a continual and equivalent supply of oxaloacetate for citrate formation via its condensation with acetyl-CoA.

There are several possible ways in which mitochondrial oxaloacetate could arise, by known reaction sequences, in adipose tissue, as follows. (*a*) Oxaloacetate formed extramitochondrially may itself pass into the mitochondria. This is not likely (3). (*b*) The oxaloacetate formed from the cleavage of citrate in the cytoplasm can be converted to malate by NAD malate dehydrogenase and the malate may enter the mitochondria. If only systems *a* and (or) *b* were functional in adipose tissue, the citrate cleavage pathway would require the quantitative

TABLE 7 EFFECT OF DIET ON PYRUVATE CARBOXYLASE ACTIVITY IN RAT ADIPOSE TISSUE

Enzyme activities are expressed as mμmoles of bicarbonate-^{14}C incorporated per min per g of tissue or mg of nitrogen at 37 °C, and shown as the mean ± SEM for the number of animals in parentheses.

Activity	Fasted 96 Hr; Refed Chow 96 Hr	Normal Chow-Fed	Fasted 96 Hr; Refed High Fat 96 Hr
Soluble, *per g tissue*	89.9 ± 25.2 (6)	79.0 ± 10.3 (5)	49.8* ± 9.1 (6)
" , *per mg tissue nitrogen*	31.6 ± 4.7 (6)	33.2 ± 3.4 (5)	13.7* ± 2.2 (5)
Particulate, *per g tissue*	149.3 ± 29.8 (6)	140.4 ± 22.7 (6)	109.1 ± 10.5 (6)
" , *per mg tissue nitrogen*	56.8 ± 6.1 (6)	61.4 ± 11.0 (6)	42.2 ± 11.1 (6)

* A significant difference from the normal fed value at the 5% level.

conversion of extra- to intramitochondrial oxaloacetate. Leveille and Hanson (25) have demonstrated, by using specifically labeled aspartate-^{14}C as a source of oxaloacetate, that some of the cytoplasmic oxaloacetate is converted, via malate, to pyruvate. This would rule out a direct *quantitative* conversion of either oxaloacetate or malate to mitochondrial oxaloacetate. (*c*) The action of malic enzyme (see equation 4) could be reversed and the malate dehydrogenated via reaction 3. Although we have demonstrated a reversal of this enzyme in adipose tissue (Table 3), this mechanism probably does not account for the quantity of oxaloacetate needed to support lipogenesis. Besides, a reversal of malic enzyme would result in a loss of NADPH, which is already at a premium for the reductive synthesis of fatty acids (26), and would, moreover, occur in the cytoplasm rather than the mitochondria. (*d*) Finally, pyruvate could be carboxylated by pyruvate carboxylase.

We have confirmed the presence of pyruvate carboxylase in rat adipose tissue and have demonstrated its distribution in both the mitochondrial and cytoplasmic fractions of the fat cell. Since there can be no doubt that a mitochondrial pyruvate carboxylase is present, it is necessary to consider whether the observed soluble activity could possibly be due to leaching from the mitochondria. The experiments designed to test this possibility (gentle and severe homogenization, warm and cold preparation of cellular fractions, and repeated rehomogenization of isolated particles) all showed that the soluble activity was indeed a cytoplasmic enzyme and not an artifact of preparation. Henning, Stumpf, Ohly, and Seubert (11) have also reported a soluble and mitochondrial pyruvate carboxylase in rat liver and kidney, a result which differs from the earlier work of Utter and Keech (21) and Wagle (24). Henning et al. also noted (11) that changes in dietary conditions which favor gluconeogenesis in liver and kidney are accompanied by an increased pyruvate carboxylase activity in both the soluble and mitochondrial fractions. These changes of hepatic pyruvate carboxylase under different dietary conditions have also been reported by Wagle (24) and Freedman and Kohn (27) but could not be found by Krebs (28) or by Shrago and Lardy (29). In the present study we have shown a decrease in the activity of the soluble enzyme in adipose tissue of rats that were fasted and then fed a high fat diet, a process which greatly enhances hepatic gluconeogenesis. These findings suggest a different metabolic function of pyruvate carboxylase in liver from that in adipose tissue.

As pyruvate carboxylase from both tissues requires acetyl-CoA for activity, alterations in pyruvate carboxylase activity in vivo could be due as well to changes in the level of acetyl-CoA as to enzyme synthesis or degradation. It is possible that dietary or hormonal changes which result in an increase in the hepatic acetyl-CoA concentration, such as fat feeding (30), will also produce a decrease in the concentration of acetyl-CoA in adipose tissue. If the effective activity of the enzyme were controlled in this manner, alteration in the activity in vivo of pyruvate carboxylase would not be detected since the assay used in the present study is carried out at saturating levels of acetyl-CoA. Such changes at levels below the saturation concentration of pyruvate carboxylase for this coenzyme would simultaneously stimulate hepatic gluconeogenesis and depress adipose tissue lipogenesis.

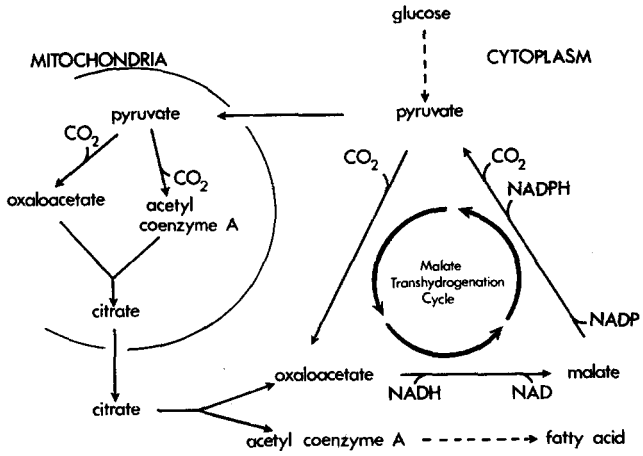

FIG. 3. The citrate cleavage pathway in rat adipose tissue, including the proposed "short circuiting" or cytoplasmic pyruvate to oxaloacetate to form the "malate transhydrogenation cycle." The reactions within the mitochondria are abbreviated to include only those involved with citrate formation.

Flatt and Ball (26) and more recently Katz, Landau, and Bartsch (31) measured the flow of glucose carbon over the various pathways in adipose tissue under conditions of enhanced lipogenesis (fasting–refeeding and insulin), and found that under these conditions the NADPH formed via the pentose pathway provides only 59–88% of the reducing equivalents required for fatty acid synthesis. Moreover, Winegrad and Renold (32) reported a synthesis of fatty acids from pyruvate, which would effectively bypass the pentose pathway as a source of NADPH. An alternative source of NADPH was suggested by Young et al. (7), Wise and Ball (8), and Lowenstein (33); it involves a transhydrogenation of NADH to NADPH by the coupling of cytoplasmic NAD malate dehydrogenase and malic enzyme (Fig. 3). If such a transhydrogenation sequence were part of the citrate cleavage pathway, as suggested by Young et al. (7), the synthesis of oxaloacetate within the mitochondria would be essential. On the other hand, if the transhydrogenation sequence were a separate "short circuit" of the citrate cleavage pathway, as implied in the sequence suggested by Wise and Ball (8), pyruvate carboxylase must be cytoplasmic. Our finding of a soluble as well as a

mitochondrial pyruvate carboxylase activity in rat adipose tissue supports both possibilities. As shown in Fig. 3, our data are consistent with the functioning of a "malate transhydrogenation cycle" for the generation of NADPH in the cytoplasm when NADH is available. This cycle might operate separately from the citrate cleavage pathway even though the intramitochondrial pyruvate carboxylase present would provide the necessary oxaloacetate for citrate formation.

Available data on the production and consumption of pyridine nucleotide coenzymes in adipose tissue during lipogenesis from glucose allow an approximate calculation of the coenzyme balance. Flatt and Ball (26) have reported that the pentose pathway would produce 0.39 μmole of NADPH per min per g of adipose tissue with glucose and insulin, whereas fatty acid synthesis requires 0.62 μmole of NADPH. An additional 0.43 μmole of reducing equivalents is potentially available as NADH from triose-*P* oxidation. Our data indicate that the total pyruvate carboxylase activity of 0.22 μmole/g of adipose tissue per min could supply sufficient oxaloacetate to provide the required 0.23 μmole of NADPH by transhydrogenation from NADH via this malate transhydrogenation cycle.

The authors are indebted to Doctors Sidney Weinhouse and Gilbert A. Leveille for helpful discussions during the course of this work. The expert technical assistance of Miss Joan Krassenstein is also acknowledged.
This work was supported by National Institutes of Health Grants (AM 5487 and CA 7174) and American Cancer Society Grant (P202), and in part by a National Institute of Health fellowship to one of us (R.W.H.).

Manuscript received 10 August 1966; accepted 20 October 1966.

References

1. Wakil, S. J., E. B. Tilchener, and D. M. Gibson. 1958. *Biochim. Biophys. Acta.* **29:** 225.
2. Srere, P. A. 1965. *Nature.* **205:** 766.
3. Spencer, A. F., and J. M. Lowenstein. 1962. *J. Biol. Chem.* **237:** 3640.
4. Srere, P. A. 1959. *J. Biol. Chem.* **234:** 2544.
5. Spencer, A., L. Corman, and J. M. Lowenstein. 1964. *Biochem. J.* **93:** 378.
6. Spencer, A. F., and J. M. Lowenstein. 1966. *Biochem. J.* **99:** 760.
7. Young, J. W., E. Shrago, and H. A. Lardy. 1964. *Biochemistry.* **3:** 1687.
8. Wise, E. M., Jr., and E. G. Ball. 1964. *Proc. Nat. Acad. Sci. U.S.* **52:** 1255.
9. Utter, M. F., and D. B. Keech. 1963. *J. Biol. Chem.* **238:** 2603.
10. Stadtman, E. R. 1957. *Methods Enzymol.* **3:** 931.
11. Henning, H. V., B. Stumpf, B. Ohly, and W. Seubert. 1966. *Biochem Z.* **344:** 274.
12. Rodbell, M. 1964. *J. Biol. Chem.* **239:** 375.
13. Utter, M. F., and D. B. Keech. 1960. *J. Biol. Chem.* **235:** PC17.
14. Herberg, R. J. 1960. *Anal. Chem.* **32:** 42.
15. Walter, P., V. Paetkau, and H. A. Lardy. 1966. *J. Biol. Chem.* **241:** 2523.
16. Busch, H., R. B. Hurlbert, and V. R. Potter. 1952. *J. Biol. Chem.* **196:** 717.
17. Hohorst, H. J. 1963. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, New York. 328.
18. Saffran, M., and O. F. Denstedt. 1948. *J. Biol. Chem.* **175:** 849.
19. Krebs, H. A., and L. V. Eggleston. 1945. *Biochem. J.* **39:** 408.
20. Buhler, D. R. 1962. *Anal. Biochem.* **4:** 413.
21. Keech, D. B., and M. F. Utter. 1963. *J. Biol. Chem.* **238:** 2609.
22. Ochoa, S. 1955. *Methods Enzymol.* **1:** 699.
23. Johnson, M. J. 1941. *J. Biol. Chem.* **137:** 575.
24. Wagle, S. R. 1964. *Biochem. Biophys. Res. Commun.* **14:** 533.
25. Leveille, G. A., and R. W. Hanson. 1966. *J. Lipid Res.* **7:** 46.
26. Flatt, J. P., and E. G. Ball. 1964. *J. Biol. Chem.* **239:** 675.
27. Freedman, A. D., and L. Kohn. 1964. *Science.* **145:** 58.
28. Krebs, H. A. 1965. *Abstracts, Federation European Biochem. Soc.* Vienna. 351.
29. Shrago, E., and H. A. Lardy. 1966. *J. Biol. Chem.* **241:** 663.
30. Wieland, O., and L. Weiss. 1963. *Biochem. Biophys. Res. Commun.* **10:** 333.
31. Katz, J., B. R. Landau, and G. E. Bartsch. 1966. *J. Biol. Chem.* **241:** 727.
32. Winegrad, A. I., and A. E. Renold. 1958. *J. Biol. Chem.* 1958. **233:** 267.
33. Lowenstein, J. M. 1961. *J. Biol. Chem.* **236:** 1213.