Pathway of carbon flow during fatty acid synthesis from lactate and pyruvate in rat adipose tissue

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ABSTRACT The metabolism of pyruvate and lactate by rat adipose tissue was studied. Pyruvate and lactate conversion to fatty acids is strongly concentration-dependent. Lactate can be used to an appreciable extent only by adipose tissue from fasted–refed rats. A number of compounds, including glucose, pyruvate, aspartate, propionate, and butyrate, stimulated lactate conversion to fatty acids. Based on studies of incorporation of lactate-2-^{3}H and lactate-2-^{14}C into fatty acids it was suggested that the transhydrogenation sequence of the "citrate–malate cycle"[1] was not providing all of the NADPH required for fatty acid synthesis from lactate. An alternative pathway for NADPH formation involving the conversion of isocitrate to α-ketoglutarate via cytosolic isocitrate dehydrogenase was proposed. Indirect support for this proposal was provided by the rapid labeling of glutamate from lactate-2-^{14}C by adipose tissue incubated in vitro, as well as the demonstration that glutamate can be readily metabolized by adipose tissue via reactions localized largely in the cytosol. Furthermore, isolated adipose tissue mitochondria convert α-ketoglutarate to malate, or in the presence of added pyruvate, to citrate. Glutamate itself can not be metabolized by these mitochondria, a finding in keeping with the demonstration of negligible levels of NAD-glutamate dehydrogenase activity in adipose tissue mitochondria. Pyruvate stimulated α-ketoglutarate and malate conversion to citrate and reduced their oxidation to CO_2.

It is proposed that under conditions of excess generation of NADH malate may act as a shuttle carrying reducing equivalents across the mitochondrial membrane. Malate at low concentrations increased pyruvate conversion to citrate and markedly decreased the formation of CO_2 by isolated adipose tissue mitochondria. Malate also stimulated citrate and isocitrate metabolism by these mitochondria, an effect that could be blocked by 2-*n*-butylmalonate. This potentially important role of malate in the regulation of carbon flow during lipogenesis is underlined by the observation that 2-*n*-butylmalonate inhibited fatty acid synthesis from pyruvate, but not from glucose and acetate, and decreased the stimulatory effect of pyruvate on acetate conversion to fatty acids.

SUPPLEMENTARY KEY WORDS fasting–refeeding · glutamate · alanine aminotransferase · asparate aminotransferase · NADP-isocitrate dehydrogenase · mitochondrial metabolism · 2-*n*-butylmalonate.

THE SYNTHESIS of fatty acids from glucose by rat adipose tissue involves an integrated series of reactions which provide precursor acetyl CoA and ATP as well

[1] The reaction sequence involving the conversion of citrate via ATP-citrate lyase to oxaloacetate and acetyl CoA with the subsequent transhydrogenation of oxaloacetate to pyruvate via a coupling of the NAD- and NADP-malate dehydrogenases has been termed variously the "citrate cleavage pathway" (4), the "pyruvate cycle" (38), and the "malate–citrate cycle" (27). Recently, Flatt (41) has suggested that the conversion of oxaloacetate to pyruvate via the intermediary formation of malate be termed the "malate cycle," and the distinction between the "malate cycle" and the "malate–citrate cycle" has been pointed out. We shall adopt the terms "citrate–malate cycle" and, where applicable, "malate cycle" in place of "citrate cleavage pathway," formerly used in publications from this laboratory, since they more adequately describe the series of reactions being studied.

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as NADPH needed for the reductive steps in the process. Flatt and Ball (1) and Katz, Landau, and Bartsch (2) have calculated the amounts of reduced coenzymes produced and utilized by adipose tissue with glucose as substrate in the presence of insulin. Only about 60% of the NADPH required to support fatty acid synthesis in this tissue could be generated via the pentose pathway, so that the remainder required for lipogenesis is formed by the transhydrogenation of NADH to NADPH. The citrate cleavage pathway, which couples NAD-malate dehydrogenase and NADP-malate dehydrogenase in rat adipose tissue is sufficiently active to account for the required transhydrogenation (3–5). The situation is different, however, with pyruvate as a substrate since it enters the metabolic sequence at a point beyond the pentose and the glycolytic pathways and therefore does not generate extramitochondrial reducing equivalents directly. Pyruvate is converted to fatty acids by adipose tissue at a rapid rate even in tissue taken from fasted rats (6, 7). It is not clear, then, which reactions provide the NADPH for lipogenesis, although Kneer and Ball (5) have suggested that the NADP-malate dehydrogenase might generate NADPH by utilizing malate which has been formed by the reduction of oxaloacetate inside the mitochondria and then transported to the cytosol. Recently, Patel and Hanson (8), using mitochondria isolated from rat adipose tissue, demonstrated that malate synthesized from pyruvate was released by these mitochondria, but at a lower rate than citrate. These findings suggest that although malate efflux into the cytosol may provide some of the reducing equivalents it may not account for the total required for lipogenesis. We have thus sought some alternative mechanism for the generation of NADPH when pyruvate is the substrate.

Lactate, on the other hand, has been reported by several investigators (7, 9, 10) to be converted to fatty acids by adipose tissue from fasted–refed rats but not from fed or fasted animals. Schmidt and Katz (9) suggested that lipogenesis from lactate in adipose tissue is limited by the rate at which NADH, generated by lactate conversion to pyruvate via lactate dehydrogenase, can be reoxidized to NAD. In the present study we have measured the intermediates formed during the metabolism of lactate by rat adipose tissue and have determined directly the rate of metabolism, by adipose tissue mitochondria, of a number of intermediates of possible significance in the over-all conversion of lactate and pyruvate to fatty acids. Our findings strongly imply that in adipose tissue the pathway of carbon flow during lipogenesis involves the conversion of some fraction of the citrate transported into the cytosol from the mitochondria to α-ketoglutarate via that segment of the citric acid cycle present in the cytosol. We have also shown that malate can act as a shuttle for reducing equivalents between the cytosol and the mitochondria of the adipose tissue cell and that its entry into the mitochondria is associated with an increase in the rate of citrate formation.

MATERIALS AND METHODS

Materials

NAD-malate dehydrogenase (EC 1.1.1.37), citrate lyase (EC 4.1.3.8), and lactic dehydrogenase (EC 1.1.1.27) were purchased from Boehringer Mannheim Corp., New York. Malate, L-aspartate, pyruvate, L-glutamate, L-alanine, citrate, L-lactate, α-ketoglutarate, and DL-allo-isocitrate were from Calbiochem, Los Angeles, Calif., and NADH, NADP, ADP, and ATP were from P-L Biochemicals, Milwaukee, Wis. Glucose-U-^{14}C, pyruvate-3-^{14}C, malate-U-^{14}C, DL-lactate-2-^{3}H, L-lactate-3-^{14}C, DL-glutamate-5-^{14}C, DL-glutamate-1-^{14}C, α-ketoglutarate-5-^{14}C, malate-3-^{14}C, DL-isocitrate-5,6-^{14}C, citrate-1,5-^{14}C, and acetate-U-^{14}C were purchased from New England Nuclear Corp., Boston, Mass., and DL-lactate-2-^{14}C was from Amersham/Searle Corp., Arlington Heights, Ill. The highly purified porcine insulin was generously supplied by Dr. O. K. Behrens of the Lilly Research Laboratories, Indianapolis, Ind. 2-*n*-butylmalonate was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Animals

Male Spraque–Dawley rats weighing 180–200 g and maintained on Purina Lab Chow were used throughout this study.

In Vitro Metabolic Studies

In these experiments about 150–200 mg of epididymal adipose tissue was incubated in 5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, at 37°C in an atmosphere of 95% O_2–5% CO_2. Various substrates were added to the buffer at levels indicated in the legends of the appropriate tables. At the end of the incubation period CO_2, fatty acid, and glyceride-glycerol were isolated, and the radioactivity was measured as described previously (11).

Intermediates of Lactate Metabolism by Adipose Tissue In Vitro

About 3 g of adipose tissue from animals that were fasted for 24 hr or fasted for 24 hr and then refed for 24 hr was incubated in 30 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing approximately 5 mM L-lactate with 7.5 μCi of sodium L-lactate-3-^{14}C. At the end of the incubation period (15 or 30 min), the tissues were re-

moved from the buffer, rapidly rinsed with fresh Krebs-Ringer bicarbonate buffer, and frozen in liquid dichlorodifluoromethane. The frozen tissue was homogenized in 6% perchloric acid at 2°C, centrifuged at 700 *g* for 10 min, and the sediment was extracted once again. The resulting pooled supernatant solutions were adjusted to pH 3 with 2 N KOH; a fraction was taken for high voltage electrophoresis, the remainder was neutralized to pH 7 with 2 M $KHCO_3$ and centrifuged, and the supernatant was used for the analysis of metabolic intermediates.

Isolation of Adipose Tissue Mitochondria

Adipose tissue cells were obtained from epididymal fat pads essentially as described by Rodbell (12). The cells were ruptured by shaking them in a glass tube containing glass beads (13), and the mitochondria were isolated by differential centrifugation (14). During the entire isolation procedure care was taken to exhaustively remove fat, since the high concentration of triglyceride from the fat cell could severely alter the oxidative phosphorylating capacity of the mitochondria. A detailed description of the procedure for isolating adipose tissue mitochondria has been published previously (8). The isolated mitochondria (approximately 1.5 mg of protein/ml) were then suspended in 0.25 M sucrose prior to use in the incubation studies.

Metabolism of Isolated Adipose Tissue Mitochondria

Incubations were carried out in small tubes (10 ml) which were shaken in a water bath at 37°C. The tubes were capped with rubber serum stoppers having hanging polyethylene cups (Kontes Glass Co., Vineland, N.J.). The compositions of the reaction mixtures are described in the appropriate table legends. The reaction was started by the addition of 0.2 ml of mitochondrial suspension after a temperature equilibration period of about 5 min. The reaction was stopped after 30 min by adding perchloric acid to give a final concentration of 6%. In experiments in which $^{14}CO_2$ was measured, 0.3 ml of Hyamine was introduced into the cup at the end of the incubation. To ensure complete liberation and trapping of $^{14}CO_2$, shaking was continued for another 40 min. The content of the cup was then transferred to a liquid scintillation vial containing diotol (15) for the measurement of radioactivity. The radioactive products formed by the mitochondria were then separated by high voltage electrophoresis.

High Voltage Electrophoresis

An aliquot of the perchlorate-treated sample was adjusted to pH 3 with 2 N KOH, and 0.5 ml was spotted on a Whatman Chromatography No. 3MM paper strip (4 × 61 cm). The buffer used in this separation was 2 M acetic acid, adjusted to pH 2.6 with pyridine. The various amino acids and citric acid cycle intermediates were separated on the strips for 3.5 hr at 4500 v at a current of 10 mamp per strip using a Shadon high voltage electrophoresis unit. The strips were then dried and cut into 4 × 1.2 cm sections and radioactivity was determined in a Nuclear-Chicago liquid scintillation spectrometer. Radioactive spots were identified by comparing their migration with those of known radioactive compounds.

Measurement of Metabolic Intermediates

The concentrations of all intermediates were determined in perchloric acid extracts adjusted to pH 6 with $KHCO_3$. Pyruvate (16), lactate (17), and citrate (18) were measured spectrophotometrically. Alanine, aspartate, and glutamate were determined using a Beckman model 120 amino acid analyzer by the method of Moore, Spackman, and Stein (19).

Enzyme Assays

Epididymal adipose tissue was homogenized in 3 volumes of buffered sucrose (20) and the various subcellular particles were separated by differential centrifugation (14). The mitochondrial fraction was suspended in buffered sucrose, freeze-dried, and taken up in water. Aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) activity was determined in both the cytosol and mitochondrial fractions of adipose tissue by the methods outlined by Bergmeyer and Bernt (21); NADP-isocitrate dehydrogenase (EC 1.1.1.42) activity was measured as described by Ochoa (22), and NAD-glutamate dehydrogenase (EC 1.4.1.3.) was determined as described by Schmidt (23).

Nitrogen

Micro-Kjeldahl digestion of tissue samples was followed by nesslerization (24).

RESULTS

The effect of alterations in dietary pattern and substrate concentration on the rate of fatty acid synthesis from glucose, pyruvate, and lactate by rat adipose tissue is shown in Table 1. Fasting for 24 hr markedly reduced the rate of fatty acid synthesis from both glucose and pyruvate, whereas increasing the substrate concentration from 0.25 to 25 mM increased lipogenesis so that the rate of fatty acid synthesis found in adipose tissue from fasted animals was comparable to rates found in tissue from the fed animal. In fact, with nonphysiological concentrations of pyruvate (25 mM) as substrate, fatty acid synthesis in fat pads from fasted rats was equal to that observed in tissue from fed animals. Lactate, on

the other hand, was metabolized at a negligible rate by adipose tissue from fasted animals and at a relatively greater rate by tissues from fed rats. However, refeeding the fasted animals for 24 hr resulted in a 400–1000-fold increase in fatty acid synthesis from lactate by epididymal fat pads. At a substrate concentration of 25 mM, lactate was converted to fatty acids by tissue from fasted–refed rats at almost two times the rate of pyruvate and four times that of glucose.

The effect of a number of intermediates on the conversion of lactate and pyruvate to fatty acids in adipose tissue from fasted animals was tested (Table 2). Due to experimental variability adipose tissue from each rat was used as control in incubations to which no intermediate other than lactate or pyruvate was added. The most marked effect noted was when 5 mM glucose with insulin was added to the incubation medium with equimolar concentrations of lactate. Glucose increased lipogenesis approximately 1000-fold in adipose tissue from fasted rats, which is about the same magnitude observed when these animals were refed (Table 1). However, the oxidation of lactate to CO_2 by adipose tissue was not significantly changed by the addition of glucose. Pyruvate conversion to fatty acids was stimulated about 4-fold in the presence of glucose and insulin. A number of intermediates tested, including propionate, butyrate, L-aspartate, and pyruvate, stimulated lactate conversion to fatty acids by epididymal fat pads from fasted rats. Of these, only aspartate significantly increased lipogenesis from pyruvate. Both propionate and butyrate increased, by about 2-fold, the output of CO_2 from lactate, whereas β-hydroxybutyrate had no

TABLE 1 EFFECT OF DIETARY STATUS AND SUBSTRATE CONCENTRATION ON FATTY ACID SYNTHESIS FROM GLUCOSE, PYRUVATE, AND LACTATE BY RAT ADIPOSE TISSUE IN VITRO

Substrate Concentration	Dietary Status	Glucose + Insulin	Pyruvate	Lactate
mM			*μmoles incorporated/g/3 hr*	
0.25	Fed	0.24 ± 0.04	0.66 ± 0.25	0.35 ± 0.16
	Fasted	0.006 ± 0.002	0.02 ± 0.005	0.001 ± 0.000
	Fasted–refed	0.37 ± 0.06	2.03 ± 0.25	1.64 ± 0.41
1	Fed	1.84 ± 0.21	2.43 ± 0.69	0.71 ± 0.24
	Fasted	0.37 ± 0.09	0.28 ± 0.05	0.02 ± 0.01
	Fasted–refed	2.55 ± 0.32	4.68 ± 0.88	9.07 ± 2.00
5	Fed	6.33 ± 0.73	9.29 ± 2.00	2.13 ± 0.78
	Fasted	2.67 ± 0.37	3.40 ± 0.97	0.02 ± 0.007
	Fasted–refed	7.36 ± 0.69	15.52 ± 2.77	18.12 ± 2.39
25	Fed	7.19 ± 1.00	15.62 ± 4.61	3.08 ± 1.06
	Fasted	2.58 ± 0.37	12.79 ± 3.03	0.04 ± 0.005
	Fasted–refed	9.88 ± 1.89	21.06 ± 3.58	35.41 ± 2.88

Values are the means ± SEM for six animals. Where indicated, the animals were fasted for 24 hr or fasted for 24 hr and refed for 24 hr. Pieces of epididymal adipose tissue were incubated in 5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, which contained at the indicated substrate concentration 0.5 μCi per 5 ml of pyruvate-3-^{14}C, L-lactate-3-^{14}C, or D-glucose-U-^{14}C. At a substrate concentration of 25 mM, 2.5 μCi of isotope was used. When glucose was used as a substrate 0.1 unit of insulin was added.

TABLE 2 EFFECT OF VARIOUS INTERMEDIATES ON THE UTILIZATION OF PYRUVATE AND LACTATE BY ADIPOSE TISSUE FROM FASTED RATS

Experiment	Intermediate Added (5 mM)	Lactate		Pyruvate	
		CO_2	Fatty Acids	CO_2	Fatty Acids
		μmoles incorporated/g/3 hr			
1	None	2.39 ± 0.29	0.009 ± 0.005	14.67 ± 2.69	2.89 ± 0.78
	β-OH butyrate	3.72 ± 0.26	0.011 ± 0.006		
	Pyruvate	7.07 ± 0.65	2.77 ± 0.53		
2	None	5.70 ± 0.27	0.03 ± 0.02	33.23 ± 3.65	8.17 ± 1.63
	Glucose + 0.1 unit insulin	7.36 ± 0.81	29.20 ± 3.67	23.62 ± 2.79	26.38 ± 2.60
3	None	1.08 ± 0.09	0.008 ± 0.004	7.17 ± 1.02	4.73 ± 0.94
	L-Aspartate	0.97 ± 0.16	0.30 ± 0.05	11.03 ± 1.55	13.71 ± 2.89
4	None	0.81 ± 0.11	0.009 ± 0.002	3.50 ± 0.56	2.10 ± 0.38
	Propionate	1.35 ± 0.10	0.68 ± 0.22	4.24 ± 0.45	3.52 ± 0.60
	Butyrate	1.96 ± 0.14	1.07 ± 0.24	4.07 ± 0.31	2.68 ± 0.28

Values are the means ± SEM for six animals. In each experiment, sections of epididymal adipose tissue weighing about 100 mg from a single animal were incubated with 5 mM sodium L-lactate-3-^{14}C (0.5 μCi), 5 mM sodium pyruvate-3-^{14}C (0.5 μCi), and the indicated unlabeled intermediate.

TABLE 3 INCORPORATION OF LACTATE-2-^{3}H AND LACTATE-2-^{14}C INTO FATTY ACIDS AND GLYCERIDE-GLYCEROL BY RAT ADIPOSE TISSUE IN VITRO

Dietary Status	Intermediate Added	Metabolite Measured	Lactate-2-^{3}H	Lactate-2-^{14}C	^{3}H:^{14}C
				μmoles/g/90 min	
Fasted	5 mM Glucose + 0.1 unit insulin	Fatty acids	0.82 ± 0.12	7.99 ± 0.54	0.10
		Glyceride-glycerol	1.42 ± 0.29	0.24 ± 0.03	5.92
Fasted	5 mM Pyruvate	Fatty acids	0.39 ± 0.06	2.70 ± 0.64	0.14
		Glyceride-glycerol	0.46 ± 0.09	1.04 ± 0.08	0.44
Fasted–refed	None	Fatty acids	0.44 ± 0.12	5.59 ± 1.38	0.08
		Glyceride-glycerol	0.41 ± 0.06	0.21 ± 0.03	1.95

Values are the means ± SEM for six animals. Where indicated, the animals were fasted for 24 hr or fasted for 24 hr and refed for 24 hr. Pieces of adipose tissue (150–200 mg) from the same animal were incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mM sodium DL-lactate-2-^{3}H (2.5 μCi) or 5 mM sodium DL-lactate-2-^{14}C (0.5 μCi).

effect on lactate conversion to either CO_2 or fatty acids by adipose tissue from fasted animals.

Since lactate is more reduced than pyruvate, its entry into the metabolic pathway of lipogenesis via lactate dehydrogenase should provide both carbon for fatty acid synthesis and NADH in the cytosol. Lactate, therefore, should be an excellent precursor for lipogenesis provided the conditions for its oxidation to pyruvate are optimal. Shreeve, Lamdin, Oji, and Slavinski (25) reported that in mice injected with lactate-2-^{3}H and lactate-2-^{14}C the ^{3}H:^{14}C ratio in carcass lipids was far lower than in liver. Since the initial reaction in the metabolism of lactate involves the transfer of hydrogen from the 2-carbon of lactate to NAD to form NADH, these findings suggested that the transhydrogenation of NADH to NADPH in carcass depot fat is markedly less efficient than in liver. To test this possibility in incubated rat adipose tissue, we measured the rate of incorporation of lactate-2-^{3}H and lactate-2-^{14}C into fatty acids as well as into glyceride-glycerol (Table 3). In these experiments adipose tissue was taken from animals that were fasted for 24 hr and refed for 24 hr or from 24-hr-fasted animals. In the case of tissue from the fasted rats, equimolar concentrations of glucose (with insulin) or pyruvate were added to stimulate fatty acid synthesis. Under the three conditions tested the ^{3}H:^{14}C ratio in fatty acids was approximately 0.1. However, this same ratio in glyceride-glycerol was from 5.92 for adipose tissue from fasted animals incubated with glucose and lactate-2-^{3}H and lactate-2-^{14}C to 0.44 for tissue from fasted rats incubated with pyruvate and specifically labeled lactate. Glyceride-glycerol of adipose tissue from fasted–refed rats after incubation with lactate-2-^{3}H and lactate-2-^{14}C had a ^{3}H:^{14}C ratio of 1.95. Since fatty acid synthesis from lactate must proceed via intramitochondrial citrate formation followed by citrate cleavage in the cytosol in order to generate extramitochondrial acetyl CoA (3, 4, 26, 27), there will be available a ready source of cytosolic oxaloacetate. The NADH generated by lactate oxidation could in turn reduce this oxaloacetate to malate. If malate were then converted to pyruvate by NADP-malate dehydrogenase, the tritium from the 2-position of lactate should appear in NADPH and be available for the reductive synthesis of fatty acids. Since this is not in accord with the findings presented in Table 3 or with the work of Shreeve et al. (25), we sought other reaction sequences by which adipose tissue could convert lactate to fatty acids without the coupling of NAD- and NADP-malate dehydrogenases.

The products of lactate metabolism by incubated adipose tissue from fasted and fasted–refed animals are shown in Table 4. The major ^{14}C-labeled, perchloric acid-extractable intermediates which were detectable were glutamate, aspartate, and alanine. Analysis of the incubation medium indicated a negligible release of radioactive intermediates from the pads. Adipose tissue from fasted rats incubated for 15 or 30 min with lactate-2-^{14}C generally had a lower rate of isotope incorporation into glutamate and aspartate than tissue from fasted–refed animals. The labeling of the glutamate and aspartate pools is quite rapid; in tissue from both fasted and fasted–refed rats the specific activity of these intermediates was the same at 15 or 30 min of incubation. Alanine was rapidly labeled in adipose tissue from the fasted animal, whereas in tissue from fasted–refed rats the formation of ^{14}C-alanine from lactate decreased with increasing incubation time. In fat pads from fasted–refed animals the specific activity of glutamate, aspartate, and alanine isolated from the tissue after 15 min of incubation was 46, 14, and 26 dpm/nmole, respectively. The rate of labeling of glutamate is particularly striking; when expressed as a ratio of the specific activities of glutamate to that of the precursor lactate, the value is 0.37 (i.e., 46:123).

Glutamate is not only rapidly formed in rat adipose tissue but it is also rapidly metabolized. As shown in Table 5, glutamate-1-^{14}C is oxidized to CO_2 in fed, fasted, and fasted–refed animals at a rate of 2.72–4.21 μmoles per g per 3 hr. Glutamate-5-^{14}C is also converted to fatty acids, presumably via a reversal, in the cytosol,

of a portion of the citric acid cycle (28, 29). Interestingly, when glucose and insulin were added to the incubation medium there was an increase in the conversion of the C-5 of glutamate to fatty acids. This finding is consistent with previous studies from other laboratories (28, 30) and suggests that the generation of reducing equivalents from the oxidation of glucose is an important factor in stimulating lipogenesis.

From the data presented above it seems clear that in rat adipose tissue some portion of the lactate is rapidly converted to glutamate, which can be efficiently metabolized. NADH formed from the oxidation of lactate is not utilized efficiently in transhydrogenation to NADPH via the "citrate–malate cycle". There is, however, an alternative pathway by which NADPH can be formed directly, without transhydrogenation with NADH in the cytosol, and which has the capacity for the rapid incorporation of labeled lactate into glutamate. This sequence would involve the forward flow of citrate to α-ketoglutarate in the cytosol, with the subsequent formation of glutamate via alanine aminotransferase or aspartate aminotransferase. NADP-linked isocitrate dehydrogenase is present almost entirely in the cytosol of the rat adipose tissue (Table 6), as are both aspartate and alanine aminotransferases. NAD-glutamate dehydrogenase was detected in adipose tissue mitochondria, but at a level approximately 1/200 of that found in rat liver mitochondria (31). Based on the distribution of these enzymes in adipose tissue one can predict that both the formation and further metabolism of glutamate could proceed via transamination reactions involving α-ketoglutarate.

TABLE 4 EFFECT OF DIETARY STATUS ON THE SPECIFIC ACTIVITY OF AMINO ACIDS ISOLATED FROM ADIPOSE TISSUE INCUBATED WITH LACTATE-2-^{14}C

Dietary Status	Time	Glutamate		Aspartate		Alanine	
	min	*nmoles/g tissue*	*dpm/g tissue*	*nmoles/g tissue*	*dpm/g tissue*	*nmoles/g tissue*	*dpm/g tissue*
Fasted	15	113	2,046	19	877	60	1,793
	30	216	1,958	63	713	66	1,481
Fasted–refed	15	244	11,286	98	1,354	85	2,205
	30	334	11,564	73	1,520	51	611

Animals were fasted for 24 hr or fasted for 24 hr and refed for 24 hr. About 3 g of epididymal adipose tissue pooled from several rats was incubated in 30 ml of Krebs-Ringer bicarbonate buffer containing 135 μmoles of sodium L-lactate-3-^{14}C (7.5 μCi) with a specific activity of 123 dpm/nmole.

TABLE 5 METABOLISM OF SPECIFICALLY LABELED GLUTAMATE BY RAT ADIPOSE TISSUE IN VITRO

Dietary Status	Substrate	CO_2		Fatty Acids	
		Additions			
		None	Glucose + Insulin	None	Glucose + Insulin
		μmoles/g/3 hr			
Fed	Glutamate-1-^{14}C	2.72 ± 0.14			
	Glutamate-5-^{14}C	0.61 ± 0.09	0.81 ± 0.11	0.095 ± 0.019	0.58 ± 0.074
Fasted	Glutamate-1-^{14}C	4.21 ± 0.43			
	Glutamate-5-^{14}C	0.43 ± 0.11	0.89 ± 0.17	0.005 ± 0.001	0.48 ± 0.087
Fasted–refed	Glutamate-1-^{14}C	3.88 ± 0.81			
	Glutamate-5-^{14}C	0.82 ± 0.095	0.70 ± 0.16	0.23 ± 0.054	0.66 ± 0.10

Values are the mean ± SEM for five animals. Approximately 200 mg of tissue was incubated in 5 ml of Krebs-Ringer bicarbonate, pH 7.4, containing 10 mM glutamate (2.5 μCi, DL-glutamate-^{14}C) and, where indicated, 10 mM glucose with 0.1 unit of insulin.

TABLE 6 INTRACELLULAR DISTRIBUTION OF SEVERAL ENZYMES INVOLVED IN THE SYNTHESIS AND METABOLISM OF GLUTAMATE IN RAT ADIPOSE TISSUE

Cellular Fraction	NAD-Glutamic Dehydrogenase	NADP-Isocitric Dehydrogenase	Aspartate Aminotransferase	Alanine Aminotransferase
	units/g tissue			
Cytosol	<0.001	1.01 ± 0.005	0.365 ± 0.005	1.60 ± 0.60
Mitochondria	0.021 ± 0.001	<0.001	0.015 ± 0.001	<0.001

Values are the mean ± SEM for four animals. Activities are expressed as μmoles/min/g tissue at 37°C.

TABLE 7 METABOLISM OF α-KETOGLUTARATE, GLUTAMATE, AND PYRUVATE BY RAT ADIPOSE TISSUE MITOCHONDRIA

Labeled Substrate	Additions	Citrate	Malate	CO_2
	nmoles of substrate converted/mg of protein/30 min			
α-Ketoglutarate-5-^{14}C	None	22	377	45
	Malate	1	343	2
	Pyruvate	180	143	18
	Pyruvate + malate	41	127	1
Glutamate-5-^{14}C	None	27	2	3
	Pyruvate	27	7	6
Pyruvate-3-^{14}C	None	1,504	280	
	Malate	1,394	703	
	Aspartate	1,784	309	
	Glutamate	1,700	322	
Malate-U-^{14}C*	None	408		131
	Pyruvate	2,008		16

The incubation mixture contained, in a volume of 1 ml: 6.6 mM potassium phosphate, pH 7.4; 6.6 mM triethanolamine, pH 7.4; 0.25 M sucrose; 8 mM ATP; 20 mM $MgCl_2$; and 40 mM $KHCO_3$. Where indicated, glutamate, malate, pyruvate, and aspartate were added to give a concentration of 10 mM, whereas α-ketoglutarate was added to give a 5 mM concentration. In these experiments, 0.5 to 2 μCi of various labeled substrates was added. The incubation was carried out for 30 min at 37°C.

* Values from Patel and Hanson (8).

As shown in Table 7, α-ketoglutarate can be utilized by isolated adipose tissue mitochondria and can be converted to malate, citrate, and CO_2. The addition of pyruvate to the incubation medium shifts the relative distribution of α-ketoglutarate carbon sharply in favor of citrate formation at the expense of malate. Glutamate, on the other hand, was not readily used by the mitochondria either when present alone or in combination with pyruvate. This probably reflects the low levels of the enzymes of glutamate metabolism in adipose tissue mitochondria (Table 6).

Since lactate is readily converted to fatty acids under certain metabolic conditions such as fasting–refeeding, some mechanism must be available for the reoxidation of the NADH generated via lactate dehydrogenase. During these periods of rapid lipogenesis from lactate, there will be one molecule of oxaloacetate formed by ATP-citrate lyase for every molecule of acetyl CoA formed in the cytosol. In view of the high activity of extramitochondrial NAD-malate dehydrogenase in adipose tissue (3, 4), this oxaloacetate could be rapidly reduced to malate. If a large portion of the malate is not converted to pyruvate via NADP-malate dehydrogenase, as is suggested by the low level of incorporation of lactate-derived tritium into fatty acids (Table 3), it probably enters the mitochondria for further metabolism. We have tested this possibility directly with isolated adipose tissue mitochondria and have measured the metabolism of malate-^{14}C as well as the effect of pyruvate on malate-^{14}C utilization. Pyruvate markedly increased the conversion of malate carbon to citrate while reducing $^{14}CO_2$ output (Table 7). Malate also altered the metabolic fate of pyruvate-^{14}C by increasing the synthesis of citrate and diminishing the rate of $^{14}CO_2$ formation (Table 8). This effect was noted at the lowest malate concentration used (0.25 mM), even with pyruvate-^{14}C present at a concentration of 2.5 mM. The appearance of pyruvate carbon in malate was also noted after the addition of malate. This labeling of malate presumably occurs via pyruvate carboxylation rather than via the citric acid cycle, since little radioactivity was found in CO_2. Conversely, when malate was labeled and the pyruvate concentration maintained at 2.5 mM, a similar pattern of product formation was noted (Table 8). Increasing concentrations of malate resulted in an

TABLE 8 EFFECT OF MALATE ON THE METABOLISM OF PYRUVATE BY RAT ADIPOSE TISSUE MITOCHONDRIA

Radioactive Substrate	Additions	Citrate Formed	Citrate-^{14}C	Malate-^{14}C	$^{14}CO_2$
		μmoles	*μmoles substrate converted/mg of protein/30 min*		
Pyruvate-3-^{14}C (2.5)*	None	0.79	1.27	0.15	0.027
(2.5)	Malate (0.25)	1.42	1.93	0.23	0.005
(2.5)	(0.63)	1.50	2.23	0.41	0.005
(2.5)	(1.25)	1.88	1.99	0.62	0.002
(2.5)	(2.50)	1.65	2.76	0.72	<0.001
(2.5)	(5.00)	1.20	1.87	0.71	<0.001
Malate-3-^{14}C (0.25)	Pyruvate (2.5)	1.56	0.28		<0.001
(0.63)	(2.5)	1.66	0.66		<0.001
(1.25)	(2.5)	1.57	0.94		<0.001
(2.50)	(2.5)	1.41	1.51		<0.001
(5.00)	(2.5)	1.41	1.03		<0.001

The reaction mixture contained, in a total volume of 1 ml: 6.6 mM potassium phosphate, pH 7.4; 6.6 mM triethanolamine, pH 7.4; 0.25 M sucrose; 8 mM ATP; 20 mM $MgCl_2$; and 20 mM $KHCO_3$. Where indicated, 1 μCi of pyruvate-3-^{14}C or 0.5 μCi of malate-3-^{14}C was added. Additions of malate at various concentrations were as indicated. The incubation was carried out for 30 min at 37°C.

* Numbers in parentheses are the mM concentrations.

enhancement of citrate-^{14}C formation by the mitochondria and an almost complete suppression of $^{14}CO_2$ formation.

The results of these studies suggest that in the presence of malate pyruvate carbon is preferentially converted to citrate with a concomitant reduction in CO_2 formation via the citric acid cycle. It is possible that the effect of malate is due not only to the supply of oxaloacetate for combination with acetyl CoA, but also to an alteration in the transport of citrate across the mitochondrial membrane. Such a transport system for citrate has been demonstrated in rat liver mitochondria (32) and in adipose tissue mitochondria (33) under different experimental conditions. The data in Table 9 clearly indicate that malate stimulates the oxidation of exogenous citrate-1,5-^{14}C to $^{14}CO_2$ by rat adipose tissue mitochondria. This stimulation can be partially blocked at lower malate (2 mM) concentrations by the addition of 5 mM malonate. However, when malate was present at 5 mM, malonate at the same concentration had no effect. Increasing the level of malate caused a stimulation of citrate-1,5-^{14}C oxidation to $^{14}CO_2$ by the isolated mitochondria. At a malate concentration of 10 mM, 2-*n*-butylmalonate (10 mM) reduced by 50% the normally observed enhancement of citrate oxidation caused by malate. Pyruvate, on the other hand, had no effect on citrate metabolism by these mitochondria. Comparable experiments with isocitrate-5,6-^{14}C (Table 10) clearly show the effect of malate on $^{14}CO_2$ production and the removal of this stimulation by 2-*n*-butylmalonate, an inhibitor of the malate-stimulated citrate carrier.

Since 2-*n*-butylmalonate inhibited citrate oxidation by isolated adipose tissue mitochondria, we tested the effect of this compound, using isolated fat pads, on fatty acid synthesis from a number of substrates, some requiring citrate efflux from the mitochondria to the cytosol. As shown in Table 11, 2-*n*-butylmalonate at a concentration of 10 mM caused a decrease in fatty acid synthesis from pyruvate (5 mM) to a level one-third of that found in the controls. With glucose as substrate (5 mM) inhibition of fatty acid synthesis by 2-*n*-butylmalonate was less marked, whereas CO_2 output was decreased to about the same extent as noted with pyruvate. Interestingly, 2-*n*-butylmalonate depressed the synthesis of glyceride-glycerol from pyruvate but not from glucose, suggesting that glyceroneogenesis in adipose tissue is similar to gluconeogenesis in rat liver, in that it requires malate efflux from the mitochondria when pyruvate is the substrate. Acetate (5 mM), which forms acetyl CoA directly in the cytosol and therefore does not require the efflux of citrate to translocate acetyl CoA from the mitochondria, was utilized for fatty acid synthesis at a low rate which was unaffected by 2-*n*-butylmalonate. Both glucose and pyruvate stimulated acetate conversion to fatty acids, but 2-*n*-butylmalonate inhibited lipogenesis only in the presence of pyruvate. The oxidation of acetate to CO_2 was unaffected by 2-*n*-butylmalonate either in the presence or absence of glucose and pyruvate.

TABLE 9 EFFECT OF MALATE ON THE METABOLISM OF CITRATE BY RAT ADIPOSE TISSUE MITOCHONDRIA

Additions	Concentration	$^{14}CO_2$
	mM	*nmoles oxidized/mg protein/30 min*
None		43
Pyruvate	5	46
Malate	2	151
Malate	5	220
Malate	10	213
Malonate	5	54
Malate + malonate	2 + 5	94
Malate + malonate	5 + 5	204
Malate + 2-*n*-butylmalonate	10 + 10	105

The incubation mixture contained, in a volume of 1 ml: 6.6 mM potassium phosphate, pH 7.4; 6.6 mM triethanolamine, pH 7.4; 0.25 M sucrose; 4 mM ATP; 10 mM $MgCl_2$; 20 mM $KHCO_3$; and 5 mM citrate-1,5-^{14}C (0.5 μCi). Various intermediates were added at the concentrations indicated. The mitochondria were incubated for 30 min at 37°C.

TABLE 10 EFFECT OF MALATE ON THE METABOLISM OF ISOCITRATE BY RAT ADIPOSE TISSUE MITOCHONDRIA

Additions	Concentration	$^{14}CO_2$
	mM	*dpm/mg protein/30 min*
None		6,222
α-Ketoglutarate	5	5,077
Malate	2	25,786
Malate	5	29,085
Malate + malonate	5 + 10	17,470
Malate + 2-*n*-butylmalonate	5 + 10	5,051
2-*n*-butylmalonate	10	4,274
Butyrate	5	6,803

The incubation mixture contained, in a volume of 1 ml: 6.6 mM potassium phosphate, pH 7.4; 6.6 mM triethanolamine, pH 7.4; 0.25 M sucrose; 8 mM ATP; 20 mM $MgCl_2$; 20 mM $KHCO_3$; and 6.36 mM DL-allo-isocitrate (0.84 μCi, DL-isocitrate-5,6-^{14}C). Various intermediates were added at the concentrations indicated. The mitochondria were incubated for 30 min at 37°C.

DISCUSSION

Lipogenesis from Pyruvate

It is well known from earlier studies on the metabolism of pyruvate by rat adipose tissue that this compound is an excellent precursor for lipogenesis (5–7, 9). In fact, even after starvation periods of up to 48 hr the rates of fatty acid synthesis from pyruvate are the same as in adipose tissue from fed animals, provided that the pyruvate concentration is 5 mM or above. This rapid rate of pyruvate metabolism by adipose tissue poses several

TABLE 11 EFFECT OF 2-*n*-BUTYLMALONATE ON THE METABOLISM OF DIFFERENT SUBSTRATES BY RAT ADIPOSE TISSUE

Substrate	2-*n*-Butylmalonate (10 mM)	Metabolite Measured		
		CO_2	Fatty Acids	Glyceride-Glycerol
			μmoles/g tissue/3 hr	
Pyruvate-3-^{14}C	−	8.99 ± 0.70 (10)	10.87 ± 0.82 (7)	1.51 ± 0.31 (6)
	+	5.38 ± 0.47 (10)	3.81 ± 0.53 (7)	0.70 ± 0.06 (6)
Glucose-U-^{14}C	−	11.77 ± 1.40 (8)	9.08 ± 1.27 (8)	1.88 ± 0.23 (8)
	+	7.53 ± 0.89 (8)	6.45 ± 0.82 (8)	1.84 ± 0.15 (8)
Acetate-U-^{14}C	−	3.54 ± 0.32 (5)	0.166 ± 0.04 (5)	
	+	3.84 ± 0.52 (5)	0.151 ± 0.04 (5)	
Acetate-U-^{14}C + glucose and insulin	−	2.60 ± 0.23 (8)	16.09 ± 0.63 (8)	
	+	2.26 ± 0.11 (9)	17.91 ± 1.53 (8)	
Acetate-U-^{14}C + pyruvate	−	2.27 ± 0.18 (8)	2.82 ± 0.27 (8)	
	+	2.14 ± 0.14 (8)	1.81 ± 0.18 (8)	

Values are the mean ± the SEM for the number of animals in parentheses and are expressed as μmoles of substrate converted to the various products measured/g tissue/3 hr. Pieces of epididymal adipose tissue from fed rats were incubated in 5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing where indicated: 5 mM pyruvate-3-^{14}C (0.5 μCi), 5 mM glucose-U-^{14}C (0.5 μCi); and 5 mM acetate-U-^{14}C (0.5 μCi), with either 5 mM glucose or 5 mM pyruvate added. Insulin (0.1 unit/5 ml) was added with the glucose.

interesting questions concerning the mechanisms by which reducing equivalents are balanced within the fat cell. As was first pointed out by Kneer and Ball (5), all of the reducing equivalents required to support lipogenesis from pyruvate must be generated inside the mitochondria and transported out. Since the mitochondrial membrane is impermeable to pyridine nucleotides (34) some mechanism other than the direct transport of reduced coenzymes into the cytosol must be sought. One probable pathway for the production of reducing equivalents from pyruvate is the coupling of three reactions involving (*a*) pyruvate conversion to oxaloacetate via pyruvate carboxylase within the mitochondria, (*b*) oxaloacetate reduction to malate, and (*c*) passage of malate from the mitochondria and its conversion to pyruvate by NADP-malate dehydrogenase. This would generate NADPH in the cytosol. The NADH necessary to drive this "transhydrogenation" from mitochondria to cytosol is derived from the oxidative decarboxylation of some of the pyruvate substrate via the pyruvate dehydrogenase reaction, and its metabolism in the tricarboxylic acid cycle. It is also possible that a portion of the citrate which normally leaves the mitochondria is not cleaved by ATP-citrate lyase but is instead converted to α-ketoglutarate via an abbreviated sequence of the citric cycle known to be present in the cytosol (28, 30). Strong support for such a pathway occurring during lipogenesis in adipose tissue of the mouse has been provided by Lamdin, Shreeve, Slavinski, and Oji (35). These workers have compared the rate of incorporation of malate-2-^{3}H-3-^{14}C and isocitrate-2-^{3}H-5,6-^{14}C into lipids in order to determine which of the NADP-linked dehydrogenases contributes to a greater extent in mouse liver and adipose tissue. In liver, tritium from malate 2-^{3}H is readily incorporated into fatty acids, and the rate exceeds that in adipose tissue. In contrast to findings with malate-2-^{3}H, the transfer of ^{3}H from isocitrate-2-^{3}H to fatty acids was more extensive for the carcass fat than for the liver, indicating that in mouse carcass depot fat there is a substantial contribution of reducing hydrogen for lipogenesis from the NADP-isocitrate dehydrogenase reaction. If this occurs in rat adipose tissue, then lipogenesis from pyruvate probably includes the conversion of a portion of the citrate to α-ketoglutarate via NADP-isocitrate dehydrogenase.

Earlier studies by Patel and Hanson (8) have shown that adipose tissue mitochondria incubated with pyruvate release more citrate than malate. If studies with isolated mitochondrial preparations even approximate metabolic events within the cell, then NADPH formed from the conversion of this malate to pyruvate via NADP-malate dehydrogenase in the cytosol cannot account for the reducing equivalents required to support fatty acid synthesis if all the citrate leaving the mitochondria were cleaved and the acetyl CoA converted to fatty acids. It is possible that part of the citrate released from the mitochondria was converted to α-ketoglutarate via aconitase and NADP-isocitrate dehydrogenase forming additional NADPH. The rapid labeling of glutamate after incubation with lactate-^{14}C indicates that a portion of the lactate carbon was converted to α-ketoglutarate. In the absence of detectable glutamate dehydrogenase in adipose tissue the labeled glutamate could only be formed by transamination of α-ketoglutarate. Evidence has been presented in this paper that isolated rat adipose tissue mitochondria metabolize exogenous α-ketoglutarate and convert it primarily to malate and to citrate (Table 8). The data are consistent

with the passage of most of the citrate formed in the mitochondria to the cytosol where a portion is cleaved to yield oxaloacetate and acetyl CoA, and the remainder is metabolized to α-ketoglutarate. This intermediate then enters the mitochondria and is further metabolized to malate and citrate (Table 7). It is possible that this malate leaves the mitochondria to be converted to pyruvate via NADP-malate dehydrogenase, thereby forming NADPH. Such a pathway permits the generation of a substantial amount of NADPH and also suggests that a finely tuned mechanism may exist for control of the direction of citrate metabolism. Recently, Martin and Denton (36) proposed a similar pathway in rat adipose tissue which is at variance with the scheme suggested above in one major respect. Instead of the α-ketoglutarate formed from the isocitrate dehydrogenase reaction in the cytosol being metabolized by the forward flow of the citric acid cycle, in the mitochondria, Martin and Denton (36) suggested that in mitochondria there is a recycling of α-ketoglutarate to isocitrate via mitochondrial NADP-isocitrate dehydrogenase. The data presented in Table 7 do not support this suggestion since in the absence of pyruvate, α-ketoglutarate-5-^{14}C is converted to malate, thus indicating a forward flow of α-ketoglutarate carbon in the citric acid cycle rather than a reversal to isocitrate. Carbon from α-ketoglutarate does appear in citrate, but only if pyruvate, an acetyl CoA source, is added to the incubation medium, indicating that the labeled citrate was formed via citrate synthase.

Another problem posed by the rapid conversion of pyruvate to fatty acids in adipose tissue is the metabolic fate of oxaloacetate generated in the cytosol from the cleavage of citrate. Since pyruvate forms NADH by intramitochondrial oxidative reactions there is no obvious source of reduced coenzymes to convert oxaloacetate in the cytosol to malate for reentry into the mitochondria or conversion to pyruvate. Based on enzymatic reactions known to metabolize oxaloacetate in adipose tissue, we can speculate that oxaloacetate is removed from the cytosol by: (*a*) conversion to *P*-enolpyruvate via *P*-enolpyruvate carboxykinase, (*b*) decarboxylation to pyruvate via cytosol pyruvate carboxylase, (*c*) transamination to aspartate by aspartate aminotransferase, or (*d*) passage directly across the mitochondria to contribute to citrate formation. Both (*a*) and (*b*) are probably not important under these conditions since *P*-enolpyruvate carboxykinase, although present in rat adipose tissue, increases in activity during fasting when lipogenesis is normally depressed (6), while pyruvate carboxylase, even if present in the cytosol of the fat cell, would probably not be reversible under conditions of the low ADP, low oxaloacetate, and high bicarbonate concentrations. Transamination via aspartate aminotransferase would require stoichiometric quantities of glutamate which, although glutamate is present in a relatively high concentration in rat adipose tissue (37), are not sufficient to effectively remove the large amounts of oxaloacetate formed during periods of rapid lipogenesis. A fourth possibility is that oxaloacetate reenters the mitochondria. We have shown, using isolated adipose tissue mitochondria (8), that exogenous oxaloacetate (5 mM) can inhibit pyruvate carboxylation to an extent almost equal to that of malate, indicating oxaloacetate penetration into the mitochondrial matrix. We have also determined that the rate of aspartate-1-^{14}C oxidation to $^{14}CO_2$ by epididymal fat pads from 48-hr-fasted rats is 4.0 μmoles per g tissue per 3 hr.[2] Since the maximum activity of aspartate aminotransferase detectable in adipose tissue mitochondria is 0.015 μmole per min per g of tissue (Table 6), or 2.7 μmoles of aspartate utilized per g tissue per 3 hr, some fraction of the aspartate must have been transaminated to oxaloacetate in the cytosol. With aspartate as substrate and in the absence of a source of NADH in the cytosol it seems probable that oxaloacetate entered the mitochondria to be directly oxidized to CO_2. However, experiments in which the swelling of adipose tissue mitochondria was measured with various intermediates suggested that oxaloacetate is a poor penetrant of mitochondria (33). Therefore, at the present time, it is not possible to directly answer the question of the metabolism of oxaloacetate generated from pyruvate during lipogenesis.

Lipogenesis from Lactate

Previous studies on lactate metabolism in adipose tissue have indicated that the rate at which the NADH generated from lactate conversion to pyruvate is reoxidized controls the rate of lactate utilization in this tissue (7, 9). Recently, Halperin and Robinson (10) have suggested that fasting and refeeding stimulates lactate metabolism by supplying glucose from glycogenolysis which can in turn provide dihydroxyacetone phosphate. This would utilize NADH for the formation of α-glycerophosphate, thereby balancing the redox state in the cytosol. This theory also explains the stimulatory effect of glucose on lactate metabolism by adipose tissue shown by a number of investigators (7, 9, 10) and is supported by the fact that 2-deoxyglucose inhibits by 50% the normal stimulatory effect of fasting–refeeding on lactate metabolism (10). However, some pathway must exist for the removal of α-glycerophosphate. Schmidt and Katz (9) have reported that glucose does not cause an increase in lactate utilization; they have attributed the effect of both glucose and pyruvate on the conversion of lactate to fatty acids to a pyruvate–lactate exchange. Also, as

[2] Hanson and Ballard, unpublished observation.

shown in Table 2, intermediates such as aspartate, propionate, and butyrate greatly stimulate lipogenesis from lactate. Interestingly, they affect only fatty acid synthesis and not $^{14}CO_2$ formation, presumably by increasing the synthesis and release of intramitochondrial citrate. Propionate and butyrate are capable of forming CoA derivatives within the mitochondria and as such could activate pyruvate carboxylase. This would result in an increase in the intramitochondrial formation of oxaloacetate. Aspartate could reoxidize NADH since it would provide oxaloacetate via transamination in the cytosol. It therefore seems possible that the various intermediates which stimulate lactate metabolism by rat adipose tissue do so by affecting different regulatory sites.

The cytosolic NADH generated by the oxidation of lactate may alter the metabolism of malate. During the normal functioning of the "citrate–malate cycle" much of the oxaloacetate formed from citrate is converted to malate and malate is converted to pyruvate. The results of Shreeve et al. (25) and those presented in this paper (Table 3) indicate that not much malate is converted to pyruvate when lactate is the substrate. This conclusion is supported by the low $^{3}H:^{14}C$ ratio of fatty acids synthesized from lactate-2-^{3}H and lactate-2-^{14}C. It is probable that some portion of the excess reducing equivalents formed during lactate oxidation is transported directly into the mitochondria for oxidation. Malate is readily metabolized by isolated rat adipose tissue mitochondria and is converted largely to citrate when pyruvate is also present in the incubation medium (Table 7). Malate, therefore, acts as a shuttle between the mitochondria and the cytosol with the function of reoxidizing cytosolic NADH. Interestingly, Rognstad and Katz (38) have suggested a similar mechanism based on experiments with adipose tissue incubated with various concentrations of dinitrophenol.

Malate transport into the mitochondria of adipose tissue may also have an effect on the passage of citrate or isocitrate into the cytosol. Earlier work by Chappell and Haarhoff (32) has established the existence, in rat liver mitochondria, of a vectoral transport system for citrate or isocitrate dependent on malate and phosphate. Recently, Halperin, Robinson, Martin, and Denton (33), using isolated adipose tissue mitochondria, have shown a similar dependence of citrate oxidation on added malate and phosphate with a rate of citrate transport more rapid than that observed by England and Robinson (39) with rat heart mitochondria. The results presented in Tables 9 and 10 confirm the observations of Halperin et al. (33) and indicate that citrate and isocitrate transport into isolated adipose tissue mitochondria is dependent on malate. Malonate can successfully compete with malate at lower malate concentrations (5 mM), but at higher concentrations (10 mM) the effect of malate on citrate oxidation is not altered. The transport site for malate in rat liver mitochondria is sensitive to 2-*n*-butylmalonate (40), the presence of which inhibits the transport of citrate into the mitochondria. A similar effect is found in rat adipose tissue mitochondria, suggesting a similar type of transport in the two tissues. Such a mechanism could have far-reaching effects on our understanding of the regulation of fatty acid synthesis in adipose tissue. Since citrate is the principal carrier of acetyl CoA across the mitochondrial membrane any intermediate which influences this translocation could influence lipogenesis. In fact, 2-*n*-butylmalonate caused a marked reduction in fatty acid synthesis from both pyruvate and glucose (Table 11) as well as an inhibition of glyceroneogenesis from pyruvate. It does not seem likely that 2-*n*-butylmalonate inhibits lipogenesis by affecting the available coenzyme A, since this compound does not alter the rate of fatty acid synthesis from acetate. Furthermore, since 2-*n*-butylmalonate diminished the pyruvate-induced stimulation of fatty acid synthesis from acetate, it seems probable that the NADPH required for fatty acid synthesis from acetate is derived from reactions involving the transport across the mitochondrial membrane of either citrate or malate (or both).

Malate is rapidly converted to citrate if pyruvate is available, and this citrate appears largely in the incubation medium as measured by rapid syringe filtration experiments (8). Together with the stimulation in citrate formation there is a concomitant decrease in CO_2 formation (see Table 8). At malate concentrations similar to those found by Ballard and Hanson (37) in quick-frozen adipose tissue (0.5 mM), the formation of CO_2 is decreased by 80% and citrate formation is stimulated 2-fold. The effect of malate in these experiments may be to activate the citrate transport system of the mitochondria and also to block the NAD-dependent isocitrate dehydrogenase by generating excess NADH. Further work is required to clarify the nature of this interaction. It does seem clear, however, that the regulation of citrate formation and efflux from the mitochondria probably are dependent upon the availability of malate. With lactate as a substrate, malate could be generated from the reduction of oxaloacetate, an obligatory product of citrate cleavage. Thus, under favorable redox conditions the reaction which produces acetyl CoA in the cytosol could also provide the malate required for citrate efflux from the mitochondria.

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