Measurement of Adipose-Tissue Metabolites *in vivo*

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1. The concentrations of glucose, pyruvate, lactate, citrate, glutamate, malate and aspartate were measured in epididymal adipose tissue from starved, fed and starved-re-fed rats. 2. To measure these intermediates it was necessary to correct for their concentration in the extracellular tissue space, which was considered to be most satisfactorily equated with the glucose space. This space *in vivo* was 7.42, 4.90 and 7.54 ml./100 g. wet wt. of tissue in adipose tissue taken from starved, fed and starved-re-fed rats respectively. After correction for the glucose space, the concentrations of metabolites (nmoles/g. of cells) in epididymal adipose tissue of fed rats were: pyruvate, 8.5; lactate, 50.3; citrate, 18.5; glutamate, 100.0; malate, 6.4; aspartate, 34.2. 3. Starvation for 72 hr. resulted in a fall in pyruvate and aspartate concentrations to 3.57 and 25.1 nmoles/g.; starvation for 72 hr. followed by re-feeding for 72 hr. caused an increase in glutamate and aspartate concentrations to 140 and 67.6 nmoles/g. 4. These changes are interpreted with regard to the simultaneous alteration in lipogenesis that occurs during the starvation-re-feeding cycle.

Starvation and other dietary alterations induce large changes in the rates of lipid synthesis in adipose tissue (Hollifield & Parson, 1962; Leveille & Hanson, 1966). This adaptive capacity implies a relationship between the activity of the overall pathway of lipid synthesis, the activities of individual key enzymes within the pathway and the intracellular concentrations of intermediates in this metabolic sequence. The enzymes involved in lipogenesis in adipose tissue have been studied in considerable detail under a number of hormonal and dietary conditions (Young, Shrago & Lardy, 1964; Leveille & Hanson, 1966; Wise & Ball, 1964; Kornacker & Ball, 1965; Ballard & Hanson, 1967a). There is, however, relatively little information on the concentrations of important metabolic intermediates in adipose tissue. Denton, Yorke & Randle (1966) have measured the concentrations of adenine nucleotides, citrate, L-glycerol 3-phosphate and glucose 6-phosphate in epididymal fat pads from normal and diabetic rats after incubation of the pads in the presence of glucose, but no measurements of metabolite concentrations *in vivo* have been reported. In the present work we have carried out such measurements.

MATERIALS AND METHODS

Chemicals. Lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), citrate lyase (EC 4.1.3.6), aspartate aminotransferase (EC 2.6.1.1.), hexokinase

(EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A.; NAD⁺, NADH, NADP⁺, 3-acetylpyridine-adenine dinucleotide and ATP were from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; *carboxyl-¹⁴C*-labelled inulin, [¹⁴C]sorbitol and [¹⁴C]glucose were from New England Nuclear Corp., Boston, Mass., U.S.A.

Animals. Sprague-Dawley rats weighing 280–320 g. were obtained from Carworth Farms, New City, N.Y., U.S.A. Animals in all experiments were either fed on Purina rat chow *ad libitum* (fed), starved for 72 hr. (starved) or starved for 72 hr. and then fed for 72 hr. (re-fed), and were killed between 9 and 10 a.m. The narrow weight range was chosen as the fat content, and thus the water content, of adipose tissue changes markedly with the age of the animals. In addition, both lipid synthesis (Gellhorn & Benjamin, 1965) and the adaptability of enzyme systems in adipose tissue (L. Reshef, R. W. Hanson & F. J. Ballard, unpublished work) decrease sharply with age. Preliminary experiments in the present study showed that in 200 g. rats several metabolites have a concentration two to three times the values reported here for 300 g. animals.

For the measurements *in vivo*, rats were lightly anaesthetized with ether and the peritoneal cavity was exposed with a longitudinal cut in the abdomen. Care was taken to avoid cutting major blood vessels. The epididymal adipose tissue was held up with forceps, rinsed with 0.15 M-NaCl, blotted lightly and frozen *in situ* with a fine spray of dichlorodifluoromethane. This freezing took 1–2 sec. The frozen tissue from each pad (1–2 g.) was removed and placed in liquid dichlorodifluoromethane. Venous blood was obtained from the vena cava (approx. 5 ml.) and a portion was placed in a tared tube containing 6% (w/v) HClO₄ at 0°. The remaining blood was centrifuged at 100 000 g for 5 min. to

obtain plasma. A portion of this plasma was pipetted into 6% HClO_4 at 0° . The times between commencing the ether anaesthesia and obtaining adipose tissue, blood and plasma were approx. 3, 4 and 15 min. respectively.

Preparation of extracts. The samples of blood, plasma and adipose tissue in 6% HClO_4 were homogenized at 0° . After centrifugation, the supernatants were removed and the residues were re-extracted with 6% HClO_4 at 0° and centrifuged again. The combined supernatants were neutralized to approx. pH 3 with 4M-KOH and then to pH 6 with 2M- KHCO_3 . The precipitate of KClO_4 was removed by centrifuging at 2° and the supernatants were stored frozen.

Spectrophotometric assays of glucose and lactate. Glucose was measured spectrophotometrically with hexokinase, glucose 6-phosphate dehydrogenase, ATP, MgCl_2 and NADP^+ (Slein, 1965). Lactate was measured as described by Hohorst (1965), with NAD^+ and lactate dehydrogenase at high pH and in the presence of hydrazine.

Fluorescence measurements. A Turner model 111 fluorimeter with attached Bausch and Lomb VOM-5 recorder was used in all experiments. This fluorimeter has the advantage of an excitation light with low energy, a property that minimizes the photodegradation of NADH. The instrument was fitted with a constant-temperature door and the temperature of the samples was maintained at 37° . Temperature stability was essential as the fluorescence of NADH is strongly temperature-dependent. A Corning 7-60 primary filter and a Wratten 8 secondary filter were used in all experiments described in this paper, although many other combinations of primary filters (to pass light at 360 nm.) or secondary filters (to pass light at 460 nm.) were found suitable and could be varied to change the sensitivity. For these and other fluorimetric assays, the fluorescence blank should be low and kept as constant as possible. With NADH-linked assays at nucleotide concentrations below $0.5 \mu\text{M}$, appreciable blanks were contributed by both the cuvettes and water. To minimize these, the cuvettes used were quartz (75 mm. $\times$ 10 mm.) (Farrand Optical Co., Mount Vernon, N.Y., U.S.A.) and not scratched. Double-distilled water was passed through a mixed-bed ion-exchange resin before use and all buffers were prepared in this water. One hundred fluorescence units are defined as full-scale deflexion on the fluorimeter and recorder under the conditions described for each assay.

The amounts of enzymes added in each assay were chosen so that the reactions were completed within 5 min. Additional enzyme was a disadvantage owing to the fluorescence of some enzyme solutions.

Measurement of oxaloacetate, pyruvate and citrate. This multiple assay combines the specific enzymic determinations of oxaloacetate (Hohorst & Reim, 1965) and pyruvate (Bücher, Czok, Lamprecht & Latzko, 1965) with an equally sensitive measurement of citrate. The availability of stable preparations of citrate lyase has permitted the development of spectrophotometric assays for citrate (Dagley, 1965; Moellering & Gruber, 1966) that have some advantages over the aconitase-isocitrate dehydrogenase assay (Siebert, 1965). These advantages result from the low stability of aconitase preparations and the necessity to activate aconitase with cysteine and Fe^{2+} (Siebert, 1965).

The method described below is a fluorimetric adaptation of the technique described by Moellering & Gruber (1966) and measures the amounts of oxaloacetate, pyruvate and

citrate by the sequential addition of the enzymes malate dehydrogenase, lactate dehydrogenase and citrate lyase.

Each assay cuvette contained, in a total volume of 2.0 ml., 300 μmoles of tris buffer, pH 7.5, extract or standards, and NADH. The NADH solution was freshly prepared at a concentration of approx. $60 \mu\text{M}$ and the exact NADH content was measured in a spectrophotometer at 340 nm. Each cuvette was preincubated at 37° for 5 min. before being placed in the fluorimeter and then the fluorescence of the solution was adjusted to between 5 and 10 units on the 100-division scale. Then 40–60 μl . of the NADH solution (equivalent to 2–3 nmoles of NADH) was added to the cuvettes and mixed by inversion. The NADH added to the cuvette served both as a standard and as substrate. The addition of malate dehydrogenase (2 μg . of protein containing 1.5 units) converted oxaloacetate into malate and the decrease in fluorescence of NADH was proportional to the amount of oxaloacetate present. The standard curve showed linearity of fluorimeter response between 0.25 and 3 nmoles of oxaloacetate.

After the measurement of oxaloacetate, pyruvate was measured by addition of lactate dehydrogenase (10 μg . of protein containing 3.6 units). The decrease in fluorescence was proportional to the amount of pyruvate in the sample at amounts between 0.25 and 3 nmoles. Additional known amounts of NADH could be added to the cuvette if large amounts of pyruvate were present.

Citrate was measured by the addition of citrate lyase (40 μg . of protein) after oxaloacetate and pyruvate had been determined. Malate and lactate dehydrogenase are both required for this assay: malate dehydrogenase to measure the oxaloacetate formed and lactate dehydrogenase to account for any oxaloacetate decarboxylase present in the citrate lyase. The citrate assays were linear between 0.25 and 3 nmoles.

Two assays of oxaloacetate, pyruvate and citrate in an extract of rat blood are shown in Fig. 1. In these extracts and in extracts of adipose tissue, oxaloacetate was never detected.

Measurement of aspartate. L-Aspartate was measured fluorimetrically by coupling the aspartate aminotransferase and malate dehydrogenase reactions (Pfleiderer, 1965). Each assay cuvette contained, in a total volume of 2.0 ml., 300 μmoles of tris buffer, pH 7.5, extract or standards, and 5 μmoles of α -oxoglutarate. Tubes were preincubated and standard NADH was added as described for the assay of oxaloacetate, pyruvate and citrate. Malate dehydrogenase (2 μg . containing 1.5 units) was added either after the NADH standard if oxaloacetate was in the sample, or during the preincubation if no oxaloacetate was expected. After addition of aspartate aminotransferase (2 μg ., containing 0.4 unit) the decrease in fluorescence was proportional to the amount of aspartate present, at least between 0.25 and 3 nmoles. As with the other fluorimetric assays described, additional known amounts of NADH could be added if aspartate was present in amounts larger than the 2–3 nmoles of NADH added as standard. Assays of aspartate in an extract of blood are shown in Fig. 2.

Measurement of malate. Malate was measured by a fluorimetric adaptation of the spectrophotometric technique described by Holzer & Söling (1965). The assay measures reduction of 3-acetylpyridine-adenine dinucleotide by malate in the presence of malate dehydrogenase and a glycine buffer at pH 9.5. The assay cuvette contained

Fig. 1. Tracings of fluorimeter recordings during assays of oxaloacetate, pyruvate and citrate in an extract of blood. The arrows numbered 1-9 indicate addition of: 1, 5 and 9, NADH; 2 and 6, malate dehydrogenase; 3 and 7, lactate dehydrogenase; 4 and 8, citrate lyase. Twice the amount of extract was present in (b) as in (a). Other experimental details are given in the text.

Fig. 2. Tracings of fluorimeter recordings during assays of aspartate (a and b) or malate (c) in an extract of blood. The arrows numbered 1-6 indicate addition of: 1 and 3, NADH; 2 and 4, aspartate aminotransferase; 5, malate dehydrogenase; 6, malate standard. Twice the amount of extract was present in (b) as in (a). Other experimental details are given in the text.

1.5 m-moles of glycine (containing 15 μ moles of EDTA) at pH 9.5, extract or standards, and 0.1 μ mole of 3-acetylpyridine-adenine dinucleotide in a total volume of 2.0 ml. The cuvettes were preincubated at 37° and, after the fluorescence had been set between 5 and 10 scale units, malate dehydrogenase (25 μ g., containing 17 units) was added. The reaction was generally completed in 5 min. A standard amount of malate was added and the malate concentration in the extract was calculated from the increase in fluorescence produced by the standard malate. The assays were linear between 0.25 and 2 nmoles of malate. A sample assay of malate in an extract of rat blood is shown in Fig. 2.

Stability of intermediates. For most measurements of

intermediates, samples were first homogenized or suspended in 5% (w/v) HClO₄. As the time that elapsed before neutralization varied depending on the number of samples, it was decided to measure the stability of the intermediates at acid pH. Solutions of the intermediates (20 nmoles/ml.) were kept in 5% HClO₄ at 0° for various times. After neutralization and removal of the precipitate of KClO₄, the intermediate concentration was measured. With the exception of oxaloacetate, all the intermediates were stable for 1 hr. in 5% HClO₄. About 20-30% of the oxaloacetate was degraded under the acid conditions and corresponding amounts of pyruvate were formed. Similar experiments to measure the stability of glucose, lactate, glutamate, pyruvate, citrate, oxaloacetate, aspartate and malate in

neutralized solutions showed that, with the exception of oxaloacetate, all these substances were stable at 0° for 48 hr. Over this period 63% of the oxaloacetate remained and the amount degraded was present as pyruvate.

Recovery of metabolic intermediates. The recoveries of glucose, pyruvate, lactate, citrate, glutamate, malate and aspartate when added to homogenates of adipose tissue in

6% HClO₄ are shown in Table 1. The metabolites were added at approximately the concentration that was expected in the tissue and were quantitatively recovered in the final neutralized extracts.

Measurements of haemoglobin space and inulin space. Haemoglobin was measured in blood, and in adipose tissue that had been frozen *in situ*, by lysis of the erythrocytes in water followed by spectrophotometric measurement at 414 nm. The haemoglobin space:

$$\frac{E_{414}/\text{g. of adipose tissue}}{E_{414}/\text{g. of blood}} \times 100,$$

was taken to be equivalent to the blood content of adipose tissue.

Inulin space was determined *in vitro* by incubating pieces of adipose tissue for 1 hr. in a medium containing [¹⁴C]inulin and measuring the radioactivity in both the pads and the medium. The 1 hr. incubation time coincided with the plateau of incorporation of inulin.

Table 1. *Recoveries of metabolites added to adipose-tissue homogenates*

Metabolites were added to adipose tissue homogenized in 6% HClO₄. The solutions were centrifuged and the residues re-extracted with HClO₄ as described in the Materials and Methods section.

Metabolite	Concentration (nmoles/g. wet wt. of tissue)			Percentage recovered
	Content	Added	Recovered	
Glucose	280	260	524	97
		520	826	103
		780	1107	105
Pyruvate	10.5	12.8	21.0	90
		25	37.0	104
		37.8	43.8	91
Lactate	220	225	450	101
		450	690	103
		675	850	95
Citrate	20.7	12.5	35.5	109
		25	44.5	98
		37.5	50.5	87
Glutamate	120	110	198	90
		220	308	91
		330	507	112
Malate	10.7	10	24.8	120
		20	30.5	99
		30	35.7	88
Aspartate	59	25	87	104
		50	107	98
		75	133	99

RESULTS

The concentrations of glucose, pyruvate, lactate, citrate, glutamate, malate and aspartate in adipose tissue in starved, fed and starved-re-fed rats are shown in Table 2. Glucose is present at a higher concentration in tissue from re-fed than in tissue from fed rats, and both aspartate and glutamate concentrations increase from starved to fed and again from fed to re-fed animals.

Preliminary experiments indicated that glucose and all the other metabolites were present in significant amounts in blood. It was therefore necessary to correct the results in Table 2 to obtain the true intracellular concentrations. Three methods of correction were considered: (1) correction for the blood content; (2) correction for the total extracellular space as measured *in vivo*; (3) correction for the total extracellular space as measured *in vitro*.

The first correction gives an accurate measure of intracellular concentrations only if either the

Table 2. *Metabolites in adipose tissue in vivo*

Adipose tissue, frozen *in situ*, was extracted and the metabolites were measured as described in the Materials and Methods section. Values are the means $\pm$ s.e.m. with the numbers of animals in parentheses. Those values that are significantly different from values in fed animals at the 5% probability level are indicated by an asterisk (*) and those significantly different from values in starved animals by a dagger (†).

Metabolite	Concentration (nmoles/g. wet wt. of tissue)		
	Starved	Fed	Starved-re-fed
Glucose	552 $\pm$ 91 (9)	378 $\pm$ 32 (10)	552 $\pm$ 47* (9)
Pyruvate	9.24 $\pm$ 1.79 (9)	13.6 $\pm$ 1.59 (10)	15.1 $\pm$ 3.2 (9)
Lactate	180 $\pm$ 56 (8)	146 $\pm$ 25 (9)	177 $\pm$ 31 (9)
Citrate	21.9 $\pm$ 4.1 (9)	23.9 $\pm$ 2.5 (10)	25.4 $\pm$ 3.8 (9)
Glutamate	86.5 $\pm$ 9.4 (11)	97.4 $\pm$ 12.0 (10)	134 $\pm$ 7.6*† (11)
Malate	7.24 $\pm$ 1.88 (5)	6.42 $\pm$ 0.19 (5)	11.1 $\pm$ 1.97 (6)
Aspartate	24.3 $\pm$ 2.4* (11)	33.4 $\pm$ 3.6 (11)	63.6 $\pm$ 6.0*† (11)

Table 3. *Blood and extracellular space in adipose tissue in vivo*

Space determinations were calculated from blood, plasma and adipose-tissue measurements carried out as described in the Materials and Methods section. Values are the means $\pm$ s.e.m. with the numbers of animals in parentheses. Values that are significantly different from those in fed animals at the 5% probability level are indicated by an asterisk (*).

	Space (% of total tissue space)		
	Starved	Fed	Starved-re-fed
Haemoglobin space	1.95 $\pm$ 0.37* (11)	0.69 $\pm$ 0.16 (10)	1.31 $\pm$ 0.19* (9)
Inulin space	17.5 $\pm$ 1.18 (6)	16.2 $\pm$ 0.52 (6)	17.5 $\pm$ 1.52 (6)
Glucose space	7.42 $\pm$ 0.81* (9)	4.90 $\pm$ 0.38 (10)	7.54 $\pm$ 0.69* (9)

Table 4. *Metabolites in blood and plasma*

Venous blood was removed from rats under ether anaesthesia and the plasma obtained by centrifugation. The metabolites were extracted and measured as described in the Materials and Methods section. Values are the means $\pm$ s.e.m. with the numbers of animals in parentheses. Values that are significantly different from those in fed animals at the 5% probability level are indicated by an asterisk (*) and values significantly different from those in starved animals by a dagger (†).

Metabolite	Blood or plasma	Concentration (nmoles/g. wet wt. of tissue)		
		Starved	Fed	Starved-re-fed
Glucose	Blood	4364 $\pm$ 275 (9)	5404 $\pm$ 410 (9)	5026 $\pm$ 261 (9)
	Plasma	7439 $\pm$ 721 (9)	7704 $\pm$ 808 (10)	7318 $\pm$ 565 (9)
Pyruvate	Blood	52.8 $\pm$ 6.5* (6)	85.5 $\pm$ 5.7 (14)	59.2 $\pm$ 8.0* (6)
	Plasma	78.2 $\pm$ 10.2* (9)	112 $\pm$ 9.9 (10)	77.7 $\pm$ 10.0* (9)
Lactate	Blood	600 $\pm$ 80 (4)	948 $\pm$ 106 (7)	776 $\pm$ 77.1 (6)
	Plasma	2252 $\pm$ 618 (8)	1992 $\pm$ 372 (10)	1831 $\pm$ 184 (9)
Citrate	Blood	73.9 $\pm$ 4.7 (7)	90.4 $\pm$ 5.9 (16)	53.2 $\pm$ 6.3*† (6)
	Plasma	129 $\pm$ 10.0 (9)	129 $\pm$ 9.8 (10)	88.3 $\pm$ 7.9*† (9)
Glutamate	Blood	136 $\pm$ 15.5 (10)	103 $\pm$ 16.9 (9)	130 $\pm$ 22.6 (10)
	Plasma	56.5 $\pm$ 12.5 (10)	43.8 $\pm$ 13.0 (8)	47.0 $\pm$ 5.9 (10)
Malate	Blood	13.5 $\pm$ 2.15 (5)	14.7 $\pm$ 0.83 (12)	10.9 $\pm$ 2.07 (6)
	Plasma	8.26 $\pm$ 1.89 (5)	6.65 $\pm$ 0.74 (6)	5.78 $\pm$ 1.25 (6)
Aspartate	Blood	15.7 $\pm$ 0.77* (6)	29.5 $\pm$ 2.30 (12)	21.8 $\pm$ 2.49 (6)
	Plasma	13.3 $\pm$ 3.59 (6)	17.5 $\pm$ 2.75 (6)	13.2 $\pm$ 2.67 (6)

extracellular space that is not blood is insignificant or a very sharp concentration gradient exists between the blood and the cells. Although these possibilities are unlikely, a correction based on the haemoglobin content of adipose tissue *in vivo* was calculated.

The haemoglobin-space measurements are shown in Table 3. They indicate that the blood content of the epididymal fat pad, and hence the contribution of the blood metabolites to the measured tissue concentrations, increase markedly on starvation and remain high during re-feeding. These changes coincide with conditions of increased fatty acid mobilization and synthesis respectively.

The concentrations of the metabolites in the blood in the different dietary states are shown in Table 4. Starvation depressed the blood concentrations of pyruvate and aspartate, whereas the

citrate concentration was depressed in the re-fed animals.

From a knowledge of the concentrations of the metabolites in the blood and of the amount of blood in adipose tissue it was possible to adjust the adipose-tissue concentrations of metabolites given in Table 2 to take account of the contribution made by the blood in the tissue. The corrected values are lower, but the differences between the dietary conditions remained unchanged (Table 5).

Attempts were made to measure the total extracellular adipose-tissue space *in vivo* by injecting either [^{14}C]sorbitol or [^{14}C]inulin and, after equilibration, measuring the amount of label in the plasma and in the adipose tissue after the latter had been frozen *in situ*. These experiments were not successful, as the label was absorbed directly into the epididymal adipose tissue after intraperitoneal

injection. Moreover, sorbitol was rapidly metabolized in the liver so that the radioactivity in the blood was only partially that of sorbitol. Measurement of the extracellular space *in vitro* in isolated fat pads with either [^{14}C]sorbitol or [^{14}C]inulin was also unsuccessful. Expansion of the extracellular space under the conditions *in vitro* gave large values (see inulin spaces in Table 3). By using such values as measures of the extracellular space, negative concentrations of several metabolites were obtained.

Correction for the concentration of extracellular metabolites in adipose tissue may also be made from measurements of the glucose space (see the Discussion section). Since the concentrations of glucose

and other metabolites in plasma are known (Table 4), the adipose-tissue values can be corrected from the relationship:

$$\text{Corrected concentration} = \frac{(A - PS)}{1 - S}$$

where A is the observed concentration in adipose tissue, P is the observed concentration in plasma and S is the glucose space, defined as the ratio of the glucose concentration in adipose tissue (nmoles/g. wet wt. of tissue) to the glucose concentration (nmoles/g.) in plasma. This calculation gives the concentration of a metabolite/g. of adipose-tissue cells. The corrected values are reported in Table 6. Like the uncorrected (Table 2), the aspartate and glutamate concentrations are decreased on starvation and increased when the animals are re-fed. The corrected pyruvate concentrations are also lower in the starved animals. The correction applied to the lactate concentrations is so great that a wide scatter of values is obtained. This is indicated by the large standard errors.

Table 5. *Metabolites in adipose tissue in vivo after correction for blood content*

Values were calculated from the means of the metabolite concentrations in adipose tissue (Table 2) and in blood (Table 4) and from the haemoglobin space (Table 3) according to the relationship:

$$\text{Corrected value} = \frac{(A - BH)}{1 - H}$$

where A is the observed concentration in adipose tissue, B is the observed concentration in blood and H is the haemoglobin space defined as the ratio of the haemoglobin concentration in adipose tissue to the haemoglobin concentration in blood.

Metabolite	Concentration (nmoles/g. corrected wt.)		
	Starved	Fed	Starved-re-fed
Glucose	476	344	493
Pyruvate	8.83	13.1	14.5
Lactate	172	140	169
Citrate	20.9	23.5	25.0
Glutamate	85.5	97.5	134
Malate	7.13	6.38	11.2
Aspartate	24.5	33.5	64.2

DISCUSSION

Space calculations. The use of glucose distribution in adipose tissue to correct for the extracellular space requires that there shall be a negligible intracellular concentration of glucose and that there shall be no significant glucose gradient from the plasma to the cell wall. Although information with respect to the latter is not available, with respect to the former Denton *et al.* (1966) found no difference between the glucose space and the sorbitol space. Since sorbitol is believed not to penetrate adipose-tissue cells, this suggests that the intracellular concentration of glucose is low or insignificant. We have not detected glucose in isolated adipose-tissue cells. If glucose is not present in the cell, but is distributed uniformly in extracellular water and plasma, then the glucose-space determinations are accurate measurements of

Table 6. *Metabolites in adipose tissue in vivo after correction for glucose content*

Values have been calculated as described in the Materials and Methods section and are expressed as means $\pm$ s.e.m. with the numbers of animals in parentheses. Those values that are significantly different from the values in fed animals at the 5% probability level are indicated by an asterisk (*) and those significantly different from the values in starved animals by a dagger (†).

Metabolite	Concentration (nmoles/g. of cells)		
	Starved	Fed	Starved-re-fed
Pyruvate	3.57 $\pm$ 1.30* (9)	8.50 $\pm$ 1.64 (10)	9.94 $\pm$ 3.29 (9)
Lactate	7.1 $\pm$ 55.7 (8)	50.3 $\pm$ 22.6 (9)	42.0 $\pm$ 19.1 (9)
Citrate	17.5 $\pm$ 3.4 (9)	18.5 $\pm$ 2.3 (10)	20.2 $\pm$ 3.5 (9)
Glutamate	88.9 $\pm$ 9.8 (11)	100.0 $\pm$ 12.2 (10)	140 $\pm$ 8*† (11)
Malate	7.16 $\pm$ 1.92 (5)	6.40 $\pm$ 0.16 (5)	11.6 $\pm$ 2.3 (6)
Aspartate	25.1 $\pm$ 2.7* (11)	34.2 $\pm$ 3.6 (11)	67.6 $\pm$ 6.3*† (11)

the total extracellular space. However, it should be mentioned that Crofford & Renold (1965) did find differences between the glucose space and the sorbitol spaces and they suggested that free glucose might be present intracellularly under some conditions. Gellhorn & Benjamin (1965) reported that the fat-free wet weight of adipose tissue from 300g. rats was 10% of the total weight of the tissue. This value includes the intracellular water, and will therefore be greater than the glucose space reported in Table 3.

A possible source of error in using the glucose space as a measure of the extracellular space is the higher concentration of glucose in plasma than in erythrocytes. However, the erythrocyte content of adipose tissue is only 10% or less of the glucose space and the error in the correction factor is therefore small. An additional small error is introduced since glutamate, aspartate and malate are present at higher concentrations in erythrocytes than in plasma; for these metabolites little correction is necessary, as all are present at high concentrations in adipose tissue.

Citrate concentrations. Citrate is thought to be involved in the transport of acetyl-CoA from the mitochondria to the cytosol (for review see Srere, 1965), and the presence of ATP citrate lyase (EC 4.1.3.8) in the cytosol of tissues such as liver and adipose tissue provides a mechanism for the formation of acetyl-CoA from citrate. The concentration of citrate should therefore reflect the balance between the synthesis of citrate and its utilization in the tricarboxylic acid cycle and for acetyl-CoA formation. In the present study we have shown that in adipose tissue from re-fed rats, where lipogenesis is occurring at a rapid rate, the concentration of citrate does not differ from that in starved rats. Denton *et al.* (1966) found that under conditions of depressed lipogenesis, as in diabetes, the concentration of citrate in epididymal fat pads incubated in glucose was slightly increased, although insulin had no effect on the concentration of citrate in adipose tissue from normal rats. Thus there appears to be no direct relationship between the total citrate concentration in epididymal fat pads *in vivo*, or in those incubated *in vitro*, and the rate of fatty acid synthesis going on within the tissue.

Pyruvate, malate and lactate concentrations. The changes in pyruvate concentration reported in Table 6 suggest that either the formation of pyruvate is lower in adipose tissue of starved animals or its utilization is more rapid. Experiments with isolated epididymal fat pads have shown that both the rate of synthesis of pyruvate from glucose (Cahill, Leboeuf & Renold, 1959) and its utilization for lipid synthesis (Ballard & Hanson, 1967b) are depressed during starvation.

The formation of oxaloacetate, after cleavage of citrate by ATP citrate lyase, is considered to be an essential step in the synthesis of fatty acid from glucose. Should this oxaloacetate be converted into malate and then into pyruvate during periods of active lipogenesis, high steady-state concentrations of malate and pyruvate might be expected. This postulate is consistent with the raised cellular concentrations of malate and pyruvate in adipose tissue from starved-re-fed rats that are shown in Table 6.

Changes in lactate concentration have been reported in livers from animals under different dietary and hormonal regimes (Williamson, Lund & Krebs, 1967; Williamson, Lopes-Vieira & Walker, 1967). However, the high concentration of lactate in the extracellular fluid in adipose tissue, relative to its concentration within the cell, precludes any meaningful interpretation of the apparent alterations in cellular lactate concentration with changes in diet that were observed in the present study.

Glutamate and aspartate concentrations. Glutamate may play an important role in lipogenesis. Lardy, Shrago, Young & Paetkau (1964) have suggested that glutamate derived from α -oxoglutarate, rather than citrate, may diffuse out of the mitochondria into the cytosol. This glutamate could then be converted into citrate, which would be subsequently cleaved to yield acetyl-CoA. It has been demonstrated that specifically labelled glutamate is converted into fatty acids via this pathway and that the rate of lipogenesis from this intermediate is markedly increased by factors that increase lipogenesis from other substrates (Madsen, Abraham & Chaikoff, 1964; Leveille & Hanson, 1966). The present paper shows that the concentration of glutamate increases in adipose tissue taken from starved-re-fed rats and suggests a possible relationship with lipogenesis.

Both glutamate and aspartate may act as stores of oxaloacetate for citrate synthesis within the mitochondria. If citrate is cleaved in the cytosol and the oxaloacetate formed is converted into malate and then pyruvate (Young *et al.* 1964; Wise & Ball, 1964), a relative deficiency of mitochondrial oxaloacetate will result. This could be made up by carboxylation of pyruvate (Ballard & Hanson, 1967a), by transamination of aspartate or by metabolism of glutamate via α -oxoglutarate in the mitochondria. The last two processes may be especially significant since the concentrations of aspartate and glutamate are so great *in vivo*. Using the value for the intracellular water content of adipose tissue found by Denton *et al.* (1966), we find aspartate and glutamate concentrations in the intracellular water of 4.5mM and 9.3mM in adipose tissue of re-fed animals. These concentrations are far in excess of those of any other

metabolites measured in adipose tissue *in vivo*. Moreover, from the concentrations of aspartate and glutamate in plasma (0.013mM and 0.047mM respectively), it is clear that a sharp concentration gradient occurs from the plasma to the adipose-tissue cytoplasm. This evidence of active transport, or rapid intracellular synthesis, further supports the importance of these two amino acids in adipose-tissue metabolism.

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