The Metabolic Fate of the Products of Citrate Cleavage

ADENOSINE TRIPHOSPHATE-CITRATE LYASE AND NICOTINAMIDE-ADENINE
DINUCLEOTIDE PHOSPHATE-LINKED MALATE DEHYDROGENASE IN FOETAL
AND ADULT LIVER FROM RUMINANTS AND NON-RUMINANTS

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1. Foetal rat liver slices incorporate the C-3 of aspartate and C-2 of glutamate into fatty acids at rates equal to those observed with adult rat liver slices. Incorporation of either of these labelled carbon atoms into fatty acids would require a functioning citrate-cleavage pathway which consists of the enzymes ATP-citrate lyase, NAD-malate dehydrogenase and NADP-malate dehydrogenase. However, NADP-malate dehydrogenase is present in foetal rat liver at only 5% of the activity detectable in adult rat liver. 2. From these findings and the effect of cofactors on the formation of $^{14}\text{CO}_2$ from $[1,5-^{14}\text{C}]$ citrate in liver supernatant fractions (100 000g), it is suggested that NADP-malate dehydrogenase limits the citrate-cleavage sequence. 3. Measurement of the citrate-cleavage pathway by incorporation studies with $[3-^{14}\text{C}]$ aspartate and $[\text{U-}^{14}\text{C}]$ glucose and by determining the activities of ATP-citrate lyase and NADP-malate dehydrogenase have shown that this sequence of reactions is present in the liver of the bovine foetus but not in the adult. However, C-2 of glutamate is not incorporated into fatty acids or non-saponifiable lipid by bovine liver slices. This finding as well as those presented above for the adult and foetal rat liver are interpreted on the basis of a competition between phosphoenolpyruvate carboxykinase and NAD-malate dehydrogenase for oxaloacetate produced by the cleavage of citrate in the cytosol.

Fatty acid synthesis from glucose in adipose tissue and liver involves the breakdown of glucose to pyruvate in the extramitochondrial space, the synthesis of acetyl-CoA from pyruvate in the mitochondria and the transport of acetyl-CoA from the mitochondria as citrate. The cleavage of citrate in the cytoplasm by ATP-citrate lyase is thought to be an important control point in fatty acid synthesis (Spencer & Lowenstein, 1962; Srere, 1965). Young, Shrago & Lardy (1964) and Wise & Ball (1964) suggested that the oxaloacetate produced by citrate cleavage is converted into malate via NAD-malate dehydrogenase and the malate is oxidatively decarboxylated by NADP-malate dehydrogenase. This sequence, termed the citrate-cleavage pathway, has been shown to be functional in adipose tissue and liver by studies with specifically labelled aspartate (Leveille & Hanson, 1966a) and glutamate (Madsen, Abraham & Chaikoff, 1964; D'Adamo & Haft, 1965; Leveille & Hanson, 1966b). It may be possible to generalize that tissues

which use glucose as a source of carbon for fatty acid synthesis require the citrate-cleavage pathway. This has been supported by previous studies in ruminants (Hanson & Ballard, 1967), where both adipose tissue and liver from sheep and cows synthesized fatty acids from glucose at a very low rate and had negligible activities of both ATP-citrate lyase and NADP-malate dehydrogenase.

An earlier study indicated that fatty acids were synthesized from glucose at a high rate in the liver of the 17–21-day rat foetus and that the rates of lipogenesis decreased sharply at birth (Ballard & Hanson, 1967a). ATP-citrate lyase activity followed the general pattern of fatty acid synthesis, but NADP-malate dehydrogenase was not detectable in the liver of foetal or newborn rats. This finding has also been reported by Taylor, Bailey & Bartley (1967). Thus, in the absence of NADP-malate dehydrogenase, the metabolic fate of the products of citrate cleavage, i.e. oxaloacetate and malate, remains uncertain. In the present study

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we have re-examined the problem by using specifically labelled intermediates to determine the carbon flow via the citrate-cleavage sequence.

MATERIALS AND METHODS

Chemicals. [U-¹⁴C]Glucose, [1-¹⁴C]acetate and [1,5-¹⁴C₂]-citrate were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.); DL-[3-¹⁴C]aspartate and DL-[4-¹⁴C]aspartate were from Mallinkrodt Nuclear Corp. (Orlando, Fla., U.S.A.); DL-[1-¹⁴C]glutamate, DL-[2-¹⁴C]-glutamate and DL-[5-¹⁴C]glutamate were from Volk Radiochemical Corp. (Burbank, Calif., U.S.A.); NADH, NADP⁺, CoA and ATP were from P-L Laboratories (Milwaukee, Wis., U.S.A.); NAD-malate dehydrogenase was from Boehringer Corp. (New York, N.Y., U.S.A.); malate, citrate, L-aspartate and L-glutamate were from Sigma Chemical Co. (St Louis, Mo., U.S.A.). The highly purified glucagon-free insulin used in this study was a gift from Dr O. K. Behrens (Eli Lilly Laboratories, Indianapolis, Ind., U.S.A.).

Animals. Sections of bovine liver from both adult and foetal animals in the last quarter of gestation were obtained from the abattoir. The tissue was immediately removed from the animals, transported directly to the laboratory and used within 20 min. after the death of the animal. The rats were males of the Wistar strain and were fed *ad libitum* on Purina Lab Chow; adults weighed 200–250 g.

Incubation studies. Liver slices were prepared for metabolic study as outlined by Ballard & Oliver (1964) and inserted in Krebs–Ringer bicarbonate buffer, pH 7.4 (Krebs & Henseleit, 1932). The slices were weighed and approx. 200 mg. portions were transferred to 25 ml. Erlenmeyer flasks containing 5.0 ml. of Krebs–Ringer bicarbonate buffer, pH 7.4. The flasks were equipped with a plastic bucket containing a 2 cm. × 3 cm. piece of filter paper. A rubber stopper served both as a cap to close the flasks and to hold the plastic buckets suspended into the flasks above the buffer. The substrates were dissolved in the buffer at the following concentrations: [¹⁴C]acetate, 50 mM (2.5 μC); [¹⁴C]glucose, 50 mM (2.5 μC); and 0.1 unit of insulin with the added glucose. When [3-¹⁴C]aspartate, [4-¹⁴C]aspartate, [1-¹⁴C]glutamate, [2-¹⁴C]glutamate or [5-¹⁴C]glutamate was used as substrate, it was present at a concentration of 5 mM (2.5 μC) with 5 mM-glucose plus 0.1 unit of insulin also added to the incubation medium. The tissue was incubated for 3 hr. at 37° in an atmosphere of O₂ + CO₂ (95:5) in a shaking water bath. At the end of the incubation period 0.15 ml. of 40% (w/v) KOH was injected through the plasma stopper on to the filter paper and 0.5 ml. of 1N-H₂SO₄ into the buffer. To ensure the complete liberation of CO₂, the flasks were shaken for an additional 30 min. The tissue was removed, rinsed in 0.9% NaCl and transferred to 10 ml. of chloroform–methanol (2:1, v/v). The lipid in the tissue was extracted and the radioactivity measurements were performed as outlined by Hanson (1965). The filter paper was removed from the flask, placed in a liquid-scintillation vial and dried in air overnight. The radioactivity was determined directly on the dried paper by the method of Buhler (1962). All radioactivity determinations were performed with a Nuclear–Chicago liquid-scintillation spectrometer. The efficiency of the system was 20–25%

for the filter paper and 60–70% for the lipid samples as determined by the channels-ratio method. The values were corrected to 100% efficiency on this basis.

As discussed in a previous paper (Hanson & Ballard, 1967), preliminary experiments with glucose and acetate at concentrations lower than 50 mM resulted in lower glucose or acetate utilization by the tissues. At 50 mM the incorporation rate of both substrates was approximately linear throughout the 3 hr. incubation.

Glutamate and aspartate labelling. A detailed discussion of the metabolic fate of the specifically labelled carbon atoms of both aspartate (Leveille & Hanson, 1966a) and glutamate (Leveille & Hanson, 1966b) has been published. Though the isotopically labelled aspartate and glutamate were DL-isomers, the metabolic fate of the D-amino acids has not been considered. Rates calculated from incorporation experiments have not been corrected for the D-amino acid present. The C-4 of aspartate is not incorporated into fatty acids by any known pathway and therefore no label is expected in the newly synthesized fatty acids. The C-3 of aspartate is incorporated into fatty acids via the citrate-cleavage sequence, and assuming that aspartate is converted into oxaloacetate in the cytosol the labelled intermediates would be: [3-¹⁴C]oxaloacetate, [3-¹⁴C]malate, [3-¹⁴C]pyruvate, [2-¹⁴C]acetyl-CoA, [2-¹⁴C]citrate and [2-¹⁴C]acetyl-CoA in the cytosol. Though this is the simplest labelling pattern, it is possible that [3-¹⁴C]malate would randomize with fumarate to yield [2,3-¹⁴C₂]malate with the final acetyl-CoA labelled as [1,2-¹⁴C₂]acetyl-CoA. It is also conceivable that the [3-¹⁴C]aspartate can be converted into [3-¹⁴C]oxaloacetate inside the mitochondria. In this case it would condense with acetyl-CoA to form [4-¹⁴C]citrate, which could diffuse out of the mitochondria into the cytosol and be cleaved to [3-¹⁴C]oxaloacetate and acetyl-CoA by ATP-citrate lyase. The C-3 of oxaloacetate, now in the cytosol, would be converted into acetyl-CoA as discussed above. It can thus be predicted that C-3 of aspartate will be incorporated into fatty acids only via the citrate-cleavage pathway.

The use of specifically labelled glutamate in these studies allows an alternative estimation of the functional significance of the citrate-cleavage sequence. Citrate is formed from glutamate-derived α-oxoglutarate via a reversal of the citric acid cycle that occurs outside the mitochondria (D'Adamo & Haft, 1965). The C-1 of glutamate cannot be incorporated into fatty acids by any known pathway and is evolved as ¹⁴CO₂. The C-5 of glutamate ultimately becomes C-1 of citrate and is incorporated into acetyl-CoA directly after the cleavage of citrate. This would occur entirely outside the mitochondria. The C-2 of glutamate becomes an inner carbon atom of citrate and after citrate cleavage becomes C-3 of oxaloacetate. It is then incorporated into fatty acids in the same way as was indicated above for [3-¹⁴C]aspartate. In the studies with glutamate, randomization of the labelled malate with fumarate is possible, and the differences in labelling pattern would be the same as those described above for aspartate. It should also be noted that if any of the α-oxoglutarate derived from glutamate enters the mitochondria it would be oxidized in the normal direction of the citric acid cycle so that C-1, C-2 or C-5 would eventually form radioactive CO₂. No labelled acetyl-CoA could be formed in this manner.

Enzyme assays. Livers from the various animals were homogenized in a buffered iso-osmotic sucrose solution

(Henning, Stumpf, Ohly & Seubert, 1966) in a coaxial homogenizer. The homogenates were centrifuged at 100000g for 30 min. in a Spinco model L ultracentrifuge at 2°. The supernatant was used to assay NADP-malate dehydrogenase [L-malate - NADP oxidoreductase (de-carboxylating), EC 1.1.1.40], ATP-citrate lyase [ATP-citrate oxaloacetate-lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8] and aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1). The particulate fraction was suspended in buffered sucrose, freeze-dried, resuspended in water and used for the assay of aspartate aminotransferase.

Spectrophotometric assay of NADP-malate dehydrogenase. The assay as described by Ochoa (1955) was used with the final concentrations of reagents reported by Ballard & Hanson (1967a).

Radioactive assay of NADP-malate dehydrogenase. The fixation of [^{14}C]bicarbonate in the presence of pyruvate, MgCl_2 , NAD^+ and NADH^+ was measured. The concentration of reactants in a final volume of 1.0 ml. were: tris-HCl buffer, pH 7.4, 25 mM; $\text{NaH}^{14}\text{CO}_3$, 5 mM (2 μC); sodium pyruvate, 10 mM; NADH^+ , 1.5 mM; NADP^+ , 1.5 mM. Tissue extract was added and the tubes were incubated for 5, 10 and 15 min. at 37°. The details of the assay as well as a proof of the identities of the reaction products were given in a previous paper (Ballard & Hanson, 1967b).

Assay of ATP-citrate lyase. Oxaloacetate formation from citrate, CoA and ATP was measured as the oxidation of NADH by coupling with NAD-malate dehydrogenase by the method of Srere (1962) as modified by Ballard & Hanson (1967a).

Assay of aspartate aminotransferase. This enzyme was measured spectrophotometrically by coupling with NAD-malate dehydrogenase as described by Bergmeyer & Bernt (1966).

Citrate metabolism by supernatant fractions. A 0.5 ml. sample of 100000g liver supernatant prepared as described under 'Enzyme assays' above was incubated at 37° for various times up to 30 min. with a solution containing in a total volume of 2.0 ml.: tris-HCl buffer, pH 7.4, 150 μmoles ; MgCl_2 , 10 μmoles ; MnCl_2 , 2 μmoles ; ATP (pH 7.4), 5 μmoles ; CoA, 1.5 μmoles ; NADH , 1 μmole ; NADP^+ , 2 μmoles ; citrate, 1 μmole , containing 0.5 μC of [$1,5\text{-}^{14}\text{C}$]citrate. This

medium with cofactors will, in the presence of ATP-citrate lyase, NAD-malate dehydrogenase and NADP-malate dehydrogenase, catalyse the formation of [^{14}C]acetyl-CoA, pyruvate and [^{14}C]bicarbonate. When the reaction was stopped by the addition of 1 ml. of 10% (w/v) trichloroacetic acid, $^{14}\text{CO}_2$ was liberated and collected on filter papers containing KOH (see under 'Incubation studies' above). The filter papers were dried and the radioactivity was determined. The reaction sequence:

involves the citrate-cleavage pathway, but if CoA or NADH or NADP^+ is omitted step (1), (2) or (3) is prevented respectively. As $^{14}\text{CO}_2$ will be liberated only when the complete sequence is functioning, no radioactive CO_2 is expected if any of the cofactors are omitted. However, when NADH is omitted, the sequence is stopped at oxaloacetate and this compound is converted into pyruvate and $^{14}\text{CO}_2$ in an acid solution.

RESULTS

The negligible activity of NADP-malate dehydrogenase in foetal liver demonstrated in an earlier study (Ballard & Hanson, 1967a) prompted us to examine the activity of the citrate-cleavage pathway during development, using as a tool [$3\text{-}^{14}\text{C}$]aspartate. As shown in Table 1, C-3 of aspartate is oxidized to $^{14}\text{CO}_2$ by liver slices from adult rats at three times the rate noted in slices from the 18-day rat foetus. The incorporation of C-3 of aspartate into fatty acids, however, is the same in both the 18- and 22-day foetal rat liver as in the adult rat liver slices. The 2-week-old animal, where the rate of lipogenesis in liver was previously shown to be very low, incorporated very little of C-3 of aspartate into fatty acids. That aspartate is

Table 1. Utilization of specifically labelled aspartate by rat liver slices during development

Values are the means $\pm$ s.e.m. for six animals. Liver slices were incubated in 5 ml. of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mM-glucose, 0.1 unit of insulin, 5 mM-L-aspartate and either 0.5 μC of DL-[$3\text{-}^{14}\text{C}$]aspartate or 0.5 μC of DL-[$4\text{-}^{14}\text{C}$]aspartate. The products were isolated as outlined in the Materials and Methods section.

Product	Age	Utilization (m μg .atoms of C/g. wet wt. of liver/3 hr.)	
		[$3\text{-}^{14}\text{C}$]Aspartate	[$4\text{-}^{14}\text{C}$]Aspartate
CO_2	Foetus (18 days)	834 $\pm$ 75	1466 $\pm$ 78
	Foetus (22 days)	1386 $\pm$ 72	3254 $\pm$ 529
	2 weeks	1923 $\pm$ 140	6113 $\pm$ 548
	Adult	2738 $\pm$ 175	9522 $\pm$ 576
Fatty acids	Foetus (18 days)	180 $\pm$ 24	2.7 $\pm$ 0.3
	Foetus (22 days)	120 $\pm$ 21	2.9 $\pm$ 1.3
	2 weeks	2.9 $\pm$ 0.9	0.4 $\pm$ 0.1
	Adult	155 $\pm$ 38	0.8 $\pm$ 0.2

Table 2. *Utilization of glutamate by liver slices from foetal and adult rats*

Values are the means $\pm$ s.e.m. for four animals. Liver slices were incubated in 5 ml. of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 5 mM-glucose, 0.1 unit of insulin, 5 mM-L-glutamate and 0.5 μ C of either DL-[1-¹⁴C]-glutamate, DL-[2-¹⁴C]glutamate or DL-[5-¹⁴C]glutamate. The products were isolated as outlined in the Materials and Methods section.

Product	Age	Utilization (m μ C atoms of C/g. wet wt. of liver/3 hr.)		
		[1- ¹⁴ C]Glutamate	[2- ¹⁴ C]Glutamate	[5- ¹⁴ C]Glutamate
CO ₂	Foetus	2022 $\pm$ 153	1333 $\pm$ 159	1382 $\pm$ 84
	Adult	3717 $\pm$ 395	2798 $\pm$ 322	2863 $\pm$ 369
Fatty acids	Foetus	0.65 $\pm$ 0.18	13.7 $\pm$ 4.5	94.1 $\pm$ 16.9
	Adult	0.27 $\pm$ 0.07	18.2 $\pm$ 8.0	108 $\pm$ 17.3
Non-saponifiable lipid	Foetus	1.50 $\pm$ 0.82	15.6 $\pm$ 3.3	121.9 $\pm$ 10.7
	Adult	0.50 $\pm$ 0.20	5.2 $\pm$ 1.05	34.2 $\pm$ 5.9

Table 3. *Activity of NADP-malate dehydrogenase in adult and foetal rat and bovine liver as measured spectrophotometrically*

Values are the means $\pm$ s.e.m. for five to eight values. All activities are expressed as μ moles/min./g. of tissue. The values are for liver supernatants prepared by centrifugation at 100 000g for 30 min. and assayed at 37° as outlined in the Materials and Methods section.

Animal	Age	Activity (units/g. wet wt. of liver)
Rat	Foetus	< 0.02
	Adult	1.94 $\pm$ 0.24
Ox	Foetus	0.250 $\pm$ 0.08
	Adult	0.037 $\pm$ 0.016

being utilized by rat liver at a rapid rate is readily attested to by the rapid oxidation of C-4 of aspartate to ¹⁴CO₂ (6 μ moles/g./3 hr.) in liver slices from 2-week-old rats. Also, as would be predicted on the basis of theoretical considerations, C-4 of aspartate is not incorporated into fatty acids at anything but negligible rates.

Because NADP-malate dehydrogenase activity was not detectable in foetal rat liver, we had not expected to find incorporation of C-3 of aspartate into fatty acids. We therefore tested for the presence of the citrate-cleavage pathway by using specifically labelled glutamate. The C-1 of glutamate (Table 2) is readily oxidized to ¹⁴CO₂ by liver slices from both foetal (22-day) and adult rats, but is not incorporated into fatty acids or non-saponifiable lipids. The C-2 of glutamate is oxidized to ¹⁴CO₂ at twice the rate in the adult as in liver slices from the foetal rat, but is incorporated into fatty acids at a similar rate in both animals. Synthesis of non-saponifiable lipid from [2-¹⁴C]-glutamate is three times as great in the foetal rat liver as in the adult. Liver slices from adult rats

Table 4. *Activity of NADP-malate dehydrogenase in adult and foetal rat and ox liver as measured by the radioisotopic assay*

Values are the means $\pm$ s.e.m. for five to eight values. All activities are expressed as μ moles/min./g. of tissue. The values are for liver supernatants prepared by centrifugation at 100 000g for 30 min. and assayed at 37° as outlined in the Materials and Methods section.

Animal	Age	Activity (units/g. wet wt. of liver)
Ox	Adult	0.036 $\pm$ 0.002
	Foetus	0.129 $\pm$ 0.02
Rat	Adult	1.96 $\pm$ 0.35
	3 weeks	1.23 $\pm$ 0.16
	2 weeks	0.088 $\pm$ 0.002
	1 week	0.150 $\pm$ 0.06
	Newborn	0.156 $\pm$ 0.06
	Foetus	0.094 $\pm$ 0.014

produce more ¹⁴CO₂ from [5-¹⁴C]glutamate than do slices from foetal rats, but they incorporate the same amount of radioactivity into fatty acids. Again, the foetal rat uses C-5 of glutamate for synthesis of non-saponifiable lipid at three to four times the rate noted in the adult. This no doubt reflects the high rates of cholesterol biosynthesis in foetal rat liver noted by Ballard & Hanson (1967a).

The studies with labelled aspartate and labelled glutamate both suggested most forcefully that the citrate-cleavage pathway or some sequence of reactions involving the conversion of a C₄ compound (oxaloacetate or malate) into a C₃ compound (pyruvate) was operative in foetal rat liver to an extent equal to that in the adult liver. In agreement with earlier findings (Ballard & Hanson, 1967a), NADP-malate dehydrogenase had negligible activity in liver from the foetal rat (Table 3). We have also measured the activity of

NADP-malate dehydrogenase in the foetal and adult ox liver. This seemed of particular importance since the adult ox liver and adipose tissue were previously shown (Hanson & Ballard, 1967) to have only very low activities of this enzyme. As shown in Table 3, the activity of NADP-malate dehydrogenase was seven times as great in the liver from the bovine foetus as in the adult.

To confirm the low activity of NADP-malate

dehydrogenase in foetal rat liver we assayed the activity of the enzyme by using a radioisotopic method that measures the rate of $^{14}\text{CO}_2$ incorporation into malate. As shown in Table 4, the activity of NADP-malate dehydrogenase in adult rat liver, as measured by the radioisotope assay, is 20 times as great as that noted in foetal rat liver. The activity in liver from newborn, 1-week-old and 2-week-old rats is not significantly different from that noted in the foetal liver, but increases to near the adult activity by 3 weeks after birth. NADP-malate dehydrogenase is three times as active in liver from the foetal ox as in liver from the adult.

ATP-citrate lyase, the first enzyme in the citrate-cleavage pathway, is twice as active in adult as in foetal rat liver. This is opposite to the situation in ox liver, where the enzyme is 20 times as active in the foetus as in the adult (Table 5). The very low activities of ATP-citrate lyase and NADP-malate dehydrogenase in adult ox liver are reflected in an almost total lack of incorporation of C-3 of aspartate into fatty acids by liver slices from the adult (Table 6). Bovine liver readily oxidizes aspartate to carbon dioxide, as shown by the high rate of $[4\text{-}^{14}\text{C}]\text{aspartate}$ conversion into $^{14}\text{CO}_2$. The C-3

Table 5. *Activity of ATP-citrate lyase in adult and foetal rat and ox liver*

Values are the means $\pm$ S.E.M. for five to eight values. All activities are expressed as $\mu\text{moles/min./g.}$ of tissue at 37° . All other conditions are as outlined in Table 3.

Animal	Age	Activity (units/g. wet wt. of liver)
Rat	Foetus	0.83 ± 0.11
	Adult	1.84 ± 0.41
Ox	Foetus	0.407 ± 0.03
	Adult	0.022 ± 0.005

Table 6. *Utilization of aspartate by liver slices from foetal and adult oxen*

Values are the means $\pm$ S.E.M. for four animals. Liver slices were incubated in 5 ml. of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mm-glucose, 0.1 unit of insulin, 5 mm-L-aspartate and either $0.5 \mu\text{C}$ of DL- $[3\text{-}^{14}\text{C}]\text{aspartate}$ or $0.5 \mu\text{C}$ of DL- $[4\text{-}^{14}\text{C}]\text{aspartate}$. The products were isolated as outlined in the Materials and Methods section.

Product	Age	Utilization ($\mu\text{g. atoms of C/g. wet wt.}$ of liver/3 hr.)	
		$[3\text{-}^{14}\text{C}]\text{Aspartate}$	$[4\text{-}^{14}\text{C}]\text{Aspartate}$
CO_2	Foetus	1609 ± 389	8923 ± 1697
	Adult	1151 ± 114	8322 ± 488
Fatty acids	Foetus	42 ± 6	1.9 ± 0.5
	Adult	1.8 ± 0.6	0.8 ± 0.3
Non-saponifiable lipid	Foetus	42 ± 10	1.9 ± 0.1
	Adult	2.1 ± 0.4	0.8 ± 0.2

Table 7. *Utilization of glutamate by liver slices from foetal and adult oxen*

Values are the means $\pm$ S.E.M. for four animals. Liver slices were incubated in 5 ml. of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mm-glucose, 0.1 unit of insulin, 5 mm-L-glutamate with $0.5 \mu\text{C}$ of either DL- $[1\text{-}^{14}\text{C}]\text{glutamate}$, DL- $[2\text{-}^{14}\text{C}]\text{glutamate}$ or DL- $[5\text{-}^{14}\text{C}]\text{glutamate}$. The products were isolated as outlined in the Materials and Methods section.

Product	Age	Utilization ($\mu\text{g. atoms of C/g. wet wt. of liver/3 hr.}$)		
		$[1\text{-}^{14}\text{C}]\text{Glutamate}$	$[2\text{-}^{14}\text{C}]\text{Glutamate}$	$[5\text{-}^{14}\text{C}]\text{Glutamate}$
CO_2	Foetus	4274 ± 814	2245 ± 604	2540 ± 491
	Adult	2582 ± 298	1475 ± 101	1730 ± 218
Fatty acids	Foetus	1.8 ± 1.0	1.2 ± 0.6	26.2 ± 12.1
	Adult	0.15 ± 0.1	0.13 ± 0.08	0.15 ± 0.07
Non-saponifiable lipid	Foetus	1.2 ± 0.7	1.1 ± 0.5	14.4 ± 7.8
	Adult	0.10 ± 0.01	0.26 ± 0.1	0.33 ± 0.09

of aspartate is incorporated into both non-saponifiable lipid and fatty acids by foetal ox liver slices at a rate 20 times that of the adult liver.

The aspartate labelling data imply an absence of the citrate-cleavage pathway from adult ox liver. This conclusion is supported by incorporation studies with specifically labelled glutamate (Table 7). The C-1 of glutamate is oxidized to $^{14}\text{CO}_2$ at twice the rate in foetal ox liver slices as in liver slices from adult animals and almost none of this carbon atom appears in fatty acids or non-saponifiable lipid. The C-2 of glutamate appears as $^{14}\text{CO}_2$, but is incorporated into fatty acids and non-saponifiable lipids at a negligible rate by both adult and foetal bovine liver slices. The C-5 of glutamate is also oxidized to $^{14}\text{CO}_2$ in foetal and adult liver, but is only incorporated into fatty acids and non-saponifiable lipids by liver slices from the foetal ox.

To demonstrate that the activity of aspartate aminotransferase was not limiting the rate of aspartate utilization in either rat or cow liver, we measured the activity of this enzyme and determined its intracellular distribution in adult and foetal liver (Table 8). In both foetal and adult rat

liver, aspartate aminotransferase activity is greater in the mitochondrial than in the extramitochondrial fraction. The total activity is higher in the adult than in the foetal rat liver. In the ox foetus the activity of aspartate aminotransferase is highest in the mitochondrial fraction, but, unlike the adult rat, the soluble and mitochondrial activities are the same in the adult ox liver. The total activity is again highest in the adult liver.

The activity of the overall citrate-cleavage pathway in converting citrate into pyruvate in the soluble fraction of the liver cell was measured by incubating a liver supernatant fraction with $[1,5-^{14}\text{C}_2]\text{citrate}$. The experimental procedure involves the addition of CoA, NADH and NADP^+ to the reaction mixture with the subsequent isolation of $^{14}\text{CO}_2$ at the end of the reaction. In the absence of CoA, but with NADH and NADP^+ added, relatively little radioactivity was found in $^{14}\text{CO}_2$ after addition of acid (line 1 of Table 9). In the presence of CoA and NADP the $[1,5-^{14}\text{C}_2]\text{citrate}$ is converted into $[1-^{14}\text{C}]\text{acetyl-CoA}$ and $[4-^{14}\text{C}]\text{oxaloacetate}$. The oxaloacetate is partially decarboxylated by the addition of acid and the radioactivity trapped on the filter paper containing potassium hydroxide. As shown in line 2 of Table 9, liver extracts from the adult rat cleave citrate to oxaloacetate at a rate greater than similar preparations from foetal rat liver. In the ox, the cleavage of citrate to oxaloacetate is greatest in extracts from foetal liver. With CoA and NADH present in the reaction mixture the radioactivity in carbon dioxide probably represents the amount of $[4-^{14}\text{C}]\text{oxaloacetate}$ that has not been converted into malate by malate dehydrogenase (line 3 of Table 9). Most of the radioactivity originally present in $[4-^{14}\text{C}]\text{oxaloacetate}$ (line 2 of Table 9) is not liberated as $^{14}\text{CO}_2$. This indicates that most of the oxaloacetate formed from citrate cleavage is converted into malate by liver extracts when CoA and NADH are supplied. When all three required cofactors are added to the incubation system, the rate of $^{14}\text{CO}_2$ production (line 4 of Table 9) indicates the con-

Table 8. *Activity of aspartate aminotransferase in adult and foetal rat and ox liver*

Values are means $\pm$ s.e.m. for the numbers of determinations shown in parentheses. All activities are expressed as $\mu\text{moles/min./g.}$ of tissue assayed at 37° . The procedure for the isolation of particulate and cytosol fractions from liver as well as the conditions of assay are outlined in the Materials and Methods section.

Animal	Age	Activity (units/g. wet wt. of liver)	
		Cytosol	Particulate
Rat	Foetus	22.2 ± 2.7 (6)	68.6 ± 9.8 (6)
	Adult	53.2 ± 6.0 (6)	145.1 ± 21.5 (7)
Ox	Foetus	13.2 ± 3.3 (4)	76.2 ± 13.7 (4)
	Adult	75.4 ± 2.5 (4)	72.1 ± 7.2 (4)

Table 9. *Metabolism of $[1,5-^{14}\text{C}_2]\text{citrate}$ by extracts of liver from foetal and adult rats and oxen*

A 0.5 ml. sample of mitochondria-free supernatant prepared from liver was incubated for 15 min. with cofactors and with 9.5×10^5 counts/min. ($1 \mu\text{mole}$) of $[1,5-^{14}\text{C}_2]\text{citrate}$ as described in the Materials and Methods section. $^{14}\text{CO}_2$ released by acidification of the solution was trapped on KOH-soaked filter papers and dried, and the radioactivity was determined.

Addition to medium			Radioactivity in CO_2 (counts/min.)			
			Rat		Ox	
CoA	NADH	NADP^+	Foetus	Adult	Foetus	Adult
—	+	+	149	—	206	292
+	—	+	1975	29960	2997	542
+	+	—	50	780	253	177
+	+	+	310	9216	530	300

Table 10. *Utilization of glucose and acetate by liver slices from foetal and adult rats and oxen*

Values are the means $\pm$ S.E.M. for four animals. Liver slices were incubated in 5 ml. of Krebs–Ringer bicarbonate buffer, pH 7.4, containing either 50 mM-[U- 14 C]glucose (2.5 μ C) or 50 mM-[1- 14 C]acetate (2.5 μ C) and with the glucose 0.1 unit of insulin. Isolation procedures are as outlined in the Materials and Methods section.

Product	Animal	Age	Utilization	
			[U- 14 C]Glucose (m μ moles/g. wet wt. of liver/3 hr.)	[1- 14 C]Acetate (m μ g.atoms of C/g. wet wt. of liver/3 hr.)
CO ₂	Ox	Foetus	4976 $\pm$ 2293	6009 $\pm$ 885
		Adult	1444 $\pm$ 326	6729 $\pm$ 767
Fatty acids	Rat	Foetus	550 $\pm$ 50*	420 $\pm$ 25
		Adult	608 $\pm$ 165	1689 $\pm$ 570
	Ox	Foetus	51.1 $\pm$ 18.2	243 $\pm$ 61
		Adult	9.6 $\pm$ 1.8	31.9 $\pm$ 3.1
Non-saponifiable lipid	Rat	Foetus	250 $\pm$ 22*	1100 $\pm$ 308
		Adult	200 $\pm$ 32	278 $\pm$ 59
	Ox	Foetus	43.6 $\pm$ 19	137 $\pm$ 32
		Adult	2.7 $\pm$ 0.9	49 $\pm$ 18

* Values taken from Ballard & Hanson (1967a).

version of C-1 of citrate into 14 CO₂ via the citrate-cleavage sequence and gives a measure of the activity of the total pathway. In extracts of adult rat liver the activity of the entire citrate-cleavage sequence is about 30 times as great as in the foetus. The total activity of the citrate-cleavage pathway was about twice as great in extracts of foetal ox liver as in similar extracts from adult ox liver.

The difference in the activity of the citrate-cleavage pathway in the foetal as compared with the adult ox is reflected in the differences in the rate of glucose incorporation into lipid in these animals. In liver slices from the adult ox, fatty acid and non-saponifiable lipid synthesis from glucose is markedly lower than found in slices of foetal bovine liver. Oxidation of glucose to carbon dioxide is also more rapid in liver slices from bovine foetuses than from the adult animals (Table 10). In the rat, liver slices from the foetus synthesize lipids from glucose at a rate equal to that with slices from the adult. Incorporation of acetate into fatty acids and non-saponifiable lipid is greater in liver slices from the foetal cow than in liver slices from the adult. However, liver from the adult rat synthesizes four times as much fatty acids as does liver from the foetal rat. The oxidation of acetate to carbon dioxide is similar in bovine liver slices from adult and foetal animals.

DISCUSSION

We were surprised to note that the rate of fatty acid synthesis from [3- 14 C]aspartate was the same in the adult and foetal rat liver. This finding was confirmed by using as a label [2- 14 C]glutamate.

These results suggest either that the low activity of NADP-malate dehydrogenase in foetal rat liver was sufficient to sustain carbon flow over the citrate-cleavage pathway at a rate equal to adult liver, or that the decarboxylation of the intermediate to pyruvate occurred by some reaction other than NADP-malate dehydrogenase.

It is possible to explain the results of these labelling studies on the basis of a competition for oxaloacetate between NAD-malate dehydrogenase and phosphoenolpyruvate carboxykinase. The latter enzyme is absent from the cytosol of rat foetal liver and appears at birth, increasing 25-fold in the first 2 days (Ballard & Hanson, 1967c; Vernon & Walker, 1968). It is this increase in phosphoenolpyruvate carboxykinase that initiates gluconeogenesis in liver from the newborn rat and, in doing so, markedly alters the competition for oxaloacetate. Thus in the foetal rat liver, where the concentration of NADH is high (H. Philippidis & F. J. Ballard, unpublished work; Burch, 1965) and phosphoenolpyruvate carboxykinase activity in the cytosol is negligible, the flow of carbon over the citrate-cleavage pathway can be supported despite low activities of NADP-malate dehydrogenase.

We are encouraged in this view by the results of the study reported in Table 9, where the overall activity of the citrate-cleavage pathway is measured with a supernatant fraction of liver from adult and foetal rats. In the absence of competing reactions, the conversion of C-5 of citrate into carbon dioxide via the citrate-cleavage sequence is 30 times as great in the adult as in foetal rat liver. If the assumption is made, based on the evidence presented in line 3 of Table 9, that almost all of the

oxaloacetate formed from citrate cleavage is converted into malate, then the difference between lines 2 and 4 of Table 9 suggests that in foetal rat liver the limiting step in the sequence from oxaloacetate is the NADP-dependent decarboxylation of malate to pyruvate, presumably via NADP-malate dehydrogenase. This system measures the potential of the citrate-cleavage pathway without any competition from phosphoenolpyruvate carboxykinase for the oxaloacetate formed as an intermediate.

Additional evidence for the suggestion that oxaloacetate may be converted into either phosphoenolpyruvate or malate is provided by the differential labelling experiments with [3-¹⁴C]-aspartate and [2-¹⁴C]glutamate. [3-¹⁴C]Oxaloacetate can be formed by transamination of [3-¹⁴C]aspartate, and the intracellular distribution of aspartate aminotransferase demonstrates that the potential exists to form labelled oxaloacetate both in the mitochondria and in the cytosol. On the other hand, the formation of [3-¹⁴C]oxaloacetate from [2-¹⁴C]glutamate must involve at least one extramitochondrial reaction, i.e. ATP-citrate lyase.

Extramitochondrial phosphoenolpyruvate carboxykinase would only compete for that oxaloacetate produced in the cytosol, so that at least part of the oxaloacetate formed from aspartate would not be subject to this competition. This probably explains the experimental finding that, though radioactivity from [3-¹⁴C]aspartate is incorporated into fatty acids in foetal ox liver, no incorporation of radioactivity from [2-¹⁴C]glutamate could be detected. The oxaloacetate formed from [3-¹⁴C]aspartate in the mitochondria probably produces malate for passage into the cytosol (Haynes, 1965), which could then form pyruvate, acetyl-CoA and finally fatty acids without the intermediate formation of extramitochondrial oxaloacetate. The lack of incorporation of radioactivity from [2-¹⁴C]glutamate into fatty acids in foetal ox liver indicates that phosphoenolpyruvate carboxykinase is competing effectively with NADP-malate dehydrogenase for any oxaloacetate produced in the cytosol. Previous work (Ballard & Oliver, 1965) had indicated that the liver of the foetal ruminant has an active gluconeogenesis, and more recent experiments (A. Garber, personal communication) have confirmed that phosphoenolpyruvate carboxykinase is present in the cytosol of foetal ox liver at an activity that is at least ten times that of the foetal rat liver. It should be noted that, in the other tissues that contain the citrate-cleavage pathway, label from both [3-¹⁴C]aspartate and [2-¹⁴C]glutamate appears in fatty acids (Leveille & Hanson, 1966a; Abraham, Madsen & Chaikoff, 1964; D'Adamo & Haft, 1965). This would indicate that there is either an extremely active NADP-

malate dehydrogenase as in adult rat liver, or an absence of the competing phosphoenolpyruvate carboxykinase as in foetal rat liver.

Liver slices from the adult ox have negligible ATP-citrate lyase and NADP-malate dehydrogenase activities and therefore incorporate virtually no aspartate, glutamate or glucose into fatty acids. During lactation, gluconeogenesis is very rapid in the liver of these animals and high activities of phosphoenolpyruvate carboxykinase and pyruvate carboxylase have been reported (Ballard, Hanson & Kronfeld, 1968). This combination of low citrate-cleavage-pathway activity and high rates of gluconeogenesis would direct most of the oxaloacetate formed from amino acids in the direction of phosphoenolpyruvate.

There are several alternative explanations for the unimpaired functioning of the citrate-cleavage sequence, as measured by aspartate and glutamate labelling, that occurs despite the low activity of NADP-malate dehydrogenase. Aspartate may be converted into oxaloacetate inside the mitochondria and then into phosphoenolpyruvate by the intramitochondrial phosphoenolpyruvate carboxykinase that is present in the foetal rat liver (Ballard & Hanson, 1967c). In this case the phosphoenolpyruvate would first have to form pyruvate to be converted into acetyl-CoA for incorporation into fatty acids, and we have been unable to demonstrate the presence of pyruvate kinase in the mitochondria. It is also possible that the oxaloacetate formed from aspartate is decarboxylated spontaneously in the presence of metal ions or by oxaloacetate decarboxylase, which may be present in liver (Corwin, 1959).

The foetal ox liver, unlike that of the adult, can synthesize fatty acids from glucose. The presence of hepatic ATP-citrate lyase and NADP-malate dehydrogenase in the bovine foetus when they are absent from the adult supports our earlier suggestion that the citrate-cleavage sequence is required in tissues that synthesize fatty acids from glucose (Hanson & Ballard, 1967). The bovine foetus is supplied with ample glucose from the maternal circulation and, by utilizing the ATP-citrate lyase and NADP-malate dehydrogenase present in the liver, the animal is able to synthesize fatty acids and non-saponifiable lipids from this glucose. After birth, when dietary glucose is no longer available owing to bacterial fermentation in the rumen, the need for these two enzymes terminates and the citrate-cleavage pathway is no longer present.

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