

Acetate represents a major product of heptanoate and octanoate β -oxidation in hepatocytes isolated from neonatal piglets

Xi LIN*, Sean H. ADAMS† and Jack ODLE*‡

Division of Nutritional Sciences and Department of Animal Science, University of Illinois, Urbana, IL 61801, U.S.A.

An experiment was conducted to explore the nature of the radiolabel distribution in acid-soluble products (ASPs) resulting from the oxidation of [$1-^{14}\text{C}$]C_{7:0} or C_{8:0} by isolated piglet hepatocytes. The differences between odd and even chain-length and the impacts of valproate and malonate upon the rate of β -oxidation and ASP characteristics were tested. A minor amount of fatty acid carboxyl carbon ($\leq 10\%$ of organic acids identified by radio-HPLC) accumulated in ketone bodies regardless of chain-length or inhibitor used. In all cases, acetate represented

the major reservoir of carboxyl carbon, accounting for 60–70% of radiolabel in identified organic acids. Cells given [$1-^{14}\text{C}$]C_{7:0} accumulated 85% more carboxyl carbon in Krebs cycle intermediates when compared with C_{8:0}, while accumulation in acetate was unaffected. The results are consistent with the hypothesis that anaplerosis from odd-carbon fatty acids affects the oxidative fate of fatty acid carbon. The piglet appears unique in that non-ketogenic routes of fatty acid carbon flow (i.e. acetogenesis) predominate in the liver of this species.

INTRODUCTION

Ketone bodies, i.e. β -hydroxybutyrate and acetoacetate, are considered to be the predominant end-products of accelerated hepatic mitochondrial β -oxidation of fatty acids. Studies with numerous animal models have shown that under conditions of fasting [1], diabetes [2], medium-chain-length triacylglycerol administration [3–5], and during suckling [6,7], fatty acid oxidation is brisk, and levels of ketone bodies in the blood rise concomitant with a high ketogenic rate. Based on data from adult fasted rats [8], it has been presumed that ketone bodies comprise the majority of the non-CO₂ carbon derived from oxidation of fatty acids with incubations of hepatocytes from piglets [9,10]. While this assumption may be true for species possessing significant hepatic ketogenic capacity, it may be questioned in animals whose capacity is low.

Neonatal hyperketonaemia is not characteristic of suckling piglets [11,12], suggesting that the rate of ketogenesis is minimal in newborn pigs relative to other neonates. Indeed, medium-chain fatty acid (MCFA: 6–12 carbons) administration to 24-h-old fasted piglets failed to increase the plasma β -hydroxybutyrate concentration above 40–60 μM [10,13,14], even though similar treatments induce marked ketonaemia in dogs, humans and rats [3–5]. The rate of acid-soluble product (ASP) generation from hepatocytes incubated with [$1-^{14}\text{C}$]octanoate or [$1-^{14}\text{C}$]oleate in piglets is 57% lower [15,16] than in neonatal rabbits [17,18], and is a fraction of that observed in preparations from fasted adult rats [19].

Accumulation of carboxyl carbon in ASP from piglet hepatocyte incubations is far greater (by 45%) for cells given odd-chain fatty acids (C_{7:0} and C_{9:0}) as substrate [15]; these fatty acids were expected to provide anaplerotic carbon to the Krebs cycle and thereby diminish ketogenesis. This prompted Odle et al. [10] to suggest that ASP from poorly ketogenic piglet hepatocytes may include organic acids other than ketone bodies, and that chain-length differences may exist with respect to the organic acid profile in the ASP. Herein, we examined these problems by

measuring β -oxidation and metabolic end-products of fatty acid oxidation in hepatocytes isolated from colostrum-deprived piglets. Attempts were made to alter carbon flow via inhibition of β -oxidation (inclusion of valproate) or blockage of the Krebs cycle (addition of malonate). The potential for carnitine to ameliorate the inhibitory effects of valproate on fatty acid metabolism [20–22] and to maintain cellular CoA status was addressed. HPLC/radiochromatographic characterization of radiolabelled organic acids resulting from MCFA β -oxidation led to the novel finding that the majority (i.e. > 70%) of identifiable carboxyl-carbon accumulation occurred in acetate. These results are consistent with our previous description of major radioactivity residing in an HPLC peak believed to represent acetate in ASP from $1-^{14}\text{C}$ -labelled fatty acid incubations with hepatocytes derived from 24-h-old newborn piglets [23].

MATERIALS AND METHODS

Animals and hepatocyte isolation

Yorkshire/Duroc piglets ($n = 5$, mass 811 ± 50 g) were obtained at farrowing and deprived of colostrum until hepatocyte isolation was carried out 24 h *post partum*. After animals were anaesthetized with pentobarbital (20 mg/kg), livers were cannulated, perfused with collagenase in retrograde fashion, and cells isolated as described previously [10]. Yields ranged from 1.2×10^9 to 2.3×10^9 hepatocytes/liver. Hepatocyte viability (Trypan Blue exclusion) was routinely > 95% using this method.

Hepatocyte incubations

The hepatocyte incubations with $1-^{14}\text{C}$ -labelled fatty acids were conducted as described previously [10]. Hepatocytes [$(8-15) \times 10^6$ cells] were incubated in 3 ml of Krebs–Henseleit buffer (pH 7.4) at 37 °C. For those flasks in which radioactivity in CO₂ and ASP was of interest, incubations were terminated when 500 μl of 30% (w/v) trichloroacetic acid was injected into the medium

Abbreviations used: MCFA, medium-chain fatty acid; ASP, acid-soluble product.

* Present address: Department of Animal Science, North Carolina State University, Raleigh, NC 27695, U.S.A.

† Present address: Department of Internal Medicine, University of Texas, Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9135, U.S.A.

‡ To whom correspondence should be sent.

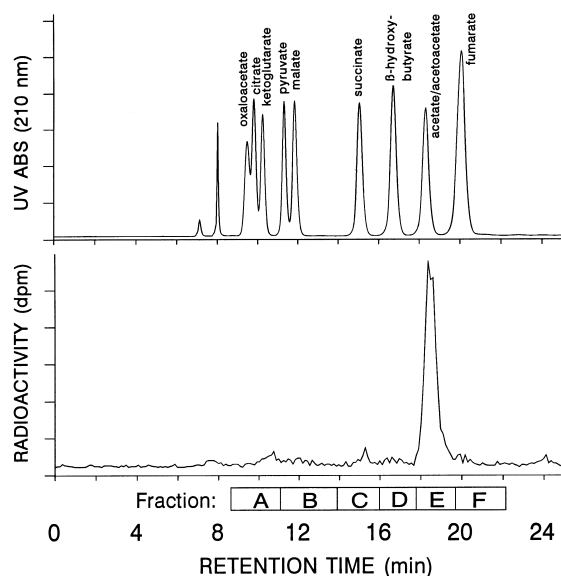


Figure 1 Ion-exchange chromatogram of organic acid standards (top panel) and radioactivity counts (bottom panel) from an ASP sample

The ASP sample was obtained from an incubation of piglet hepatocytes with $[1-^{14}\text{C}]C_{7:0}$ (see the Materials and methods section for incubation conditions and chromatography details). Fractions (from A to F) co-eluting with the organic acids were collected and radioactivity determined. (Ketoglutarate is referred to as oxoglutarate in the text.)

at 60 min. The contents of the flask were transferred to test tubes, centrifuged at 1200 *g* for 10 min, and the supernatant extracted six times with water-saturated hexane. Radioactivity in ethanolamine in centre wells (containing trapped $^{14}\text{CO}_2$) and in 150 μl aliquots of the acid-soluble fraction was determined using a scintillation counter after mixing with 10 ml of Biosafe II cocktail (Research Products International, Mt. Prospect, IL, U.S.A.). All radioactivity data were corrected for radioactivity in time zero acid-killed blanks. When CoA and CoA esters were to be assayed, incubations using non-radioactive substrate were terminated by submersion of flasks in a shaking water bath maintained at 0 °C (blanks were treatment-matched flasks submerged at time 0). The contents were subsequently transferred to preweighed polypropylene tubes, and centrifuged at 1200 *g* for 10 min at 4 °C. Tubes were re-weighed after removal of the

supernatant, wet cell weight was recorded, and cells were stored at -70 °C. Under these conditions, the relationship between cell wet weight and number averaged 5.18 ± 0.11 mg wet weight/ 10^6 cells.

Experimental design

Treatments flasks (run in duplicate) were assigned in a $2 \times 2 \times 3$ factorial, randomized complete block design, blocked by hepatocyte preparation. The experiment was replicated five times. Cells given either 1 mM $C_{7:0}$ or $C_{8:0}$ substrate were incubated in the presence or absence of valproate (1.5 mM) or malonate (10 mM), alone, or in combination with L-carnitine (3 mM), resulting in a total of 12 treatments. Valproate flasks were preincubated for 10 min at 37 °C prior to fatty acid addition. The accumulation of substrate $[1-^{14}\text{C}]$ carboxyl carbon in CO_2 , ASP and organic acids across treatments was monitored with addition of a tracer amount of $[^{14}\text{C}]$ MCFA (1.6 and 1.8 $\mu\text{Ci}/\mu\text{mol}$ for $C_{7:0}$ and $C_{8:0}$, respectively) in one set of flasks. A selected number of flasks were incubated for 20 min with non-radioactive MCFA/carnitine (control), MCFA/carnitine/malonate, and MCFA/carnitine/valproate. For these samples, O_2 consumption was then measured polarographically using water-jacketed (37 °C) Clark electrodes coupled to a YSI Model 5300 O_2 monitor (Yellow Springs, OH, U.S.A.). The xanthine oxidase method was used for electrode calibration [24].

Organic acids

An aliquot of the acid-soluble fraction was subjected to ion-exchange HPLC/radiochromatographic analysis for organic acids as described previously [23] and modified as follows: flow rate was 0.65 ml/min and the ratio of β -flowmonitor cocktail and HPLC eluent was 5:1 (v/v). This method resolves organic acids into nine UV peaks, except that acetate co-elutes with acetoacetate (Figure 1) [23]. Radioactivity accumulated in organic acids was determined after collection of eluent into vials at appropriate times (see Figure 1) using a fraction collector (Isco Retriever II, Lincoln, NE, U.S.A.). The retention times of a number of acids were too close to allow for separate collection. In these cases (see Table 1), eluent was collected across the proximate organic acids (i.e. fraction A in Figure 1) and their radioactivity summed. For some samples (see the Results section), reversed-phase ion-pairing HPLC radiochromatography was used to characterize radioactivity accumulated in acetate and ketone bodies (Figure 2). This system employed a Beckman

Table 1 Accumulation of carboxyl carbon in CO_2 and ASPs from $1-^{14}\text{C}$ -labelled $C_{7:0}$ and $C_{8:0}$ plus carnitine and/or inhibitors incubated with hepatocytes isolated from 24-h-old colostrum-deprived piglets

Incubation conditions are given in the text ($n = 5$ piglets throughout Table). * $P < 0.0001$, significant difference between $C_{7:0}$ versus $C_{8:0}$. Means with different superscripts within a column are significantly different ($P < 0.01$ for CO_2 and $P < 0.0001$ for ASP).

Treatment/additions	Carboxyl carbon accumulation (natom of carboxyl-C/h per 10^6 hepatocytes)			
	CO_2		ASP	
	$C_{7:0}$ *	$C_{8:0}$	$C_{7:0}$ *	$C_{8:0}$
Substrate	13.1 ± 3.4^a	8.6 ± 2.1^a	19.5 ± 5.0^a	12.8 ± 3.1^a
+ Carnitine	12.5 ± 3.2^a	8.2 ± 2.1^a	18.8 ± 4.7^a	12.8 ± 3.0^a
+ Valproate	11.1 ± 2.9^b	7.7 ± 1.9^b	9.8 ± 2.7^b	7.9 ± 1.8^b
+ Carnitine and valproate	11.1 ± 2.5^b	6.7 ± 1.8^b	10.1 ± 2.2^b	7.1 ± 1.5^b
+ Malonate	12.7 ± 3.5^{ab}	8.0 ± 2.0^{ab}	19.9 ± 4.8^a	13.1 ± 3.2^a
+ Carnitine and malonate	11.9 ± 3.2^{ab}	7.9 ± 2.3^{ab}	18.8 ± 4.5^a	12.8 ± 2.9^a

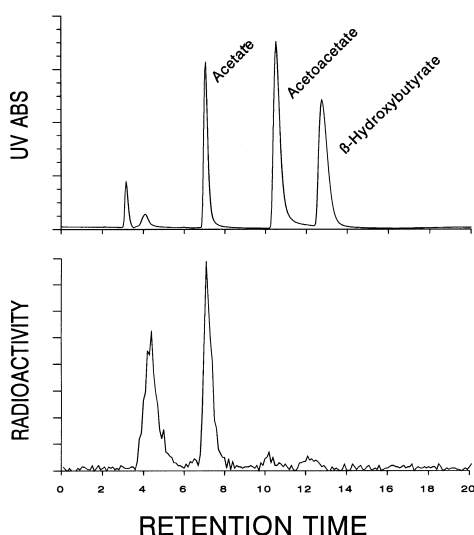


Figure 2 Reversed-phase chromatogram of UV absorbance (top panel: standards) and radioactivity counts (bottom: derived from an ASP sample)

The ASP sample was obtained from an incubation of piglet hepatocytes with $[1-^{14}\text{C}]_{\text{C}_{7:0}}$ and carnitine + malonate (see the Materials and methods section for details of HPLC procedure and incubation conditions). This HPLC method resolved Krebs cycle intermediates, acetoacetate, β -hydroxybutyrate, and acetate. The example shows the two major radioactive peaks present in every sample analysed from two replicates of the experiment. None of the organic acid standards tested co-eluted with the first radioactive peak; thus, this fraction remained unidentified. Only minor radioactivity could be attributed to the ketone bodies (see also Tables 3 and 4).

Ultrasphere IP column ($5\ \mu\text{m}$, $4.6\ \text{mm} \times 250\ \text{mm}$) with 0.3% (v/v) phosphoric acid at pH 2.1 (flow rate 0.65 ml/min, wavelength 210 nm). Organic acid peaks of interest were characterized by comparison with authentic standards, collected, and radioactivity determined in the same manner as for the ion-exchange procedure.

CoA and CoA esters

The CoA and acid-soluble CoA esters in the cells were extracted with trichloroacetic acid as described by Corkey et al. [25]. To each sample was added 0.3 nmol of isovaleryl-CoA (internal standard), followed by 750 μl of ice-cold trichloroacetic acid (0.6 M). Samples were vortexed, homogenized manually with a microcentrifuge pestle, and centrifuged for 10 min at 12000 g. The acid-insoluble precipitate was discarded, and the supernatant processed as follows. Supernatant was neutralized by removal of trichloroacetic acid by six ice-cold diethyl ether extractions, and the remaining sample evaporated to dryness using a Savant Speedvac (Model AS160, Farmingdale, NY, U.S.A.). The residue was redissolved in 250 μl of Buffer A (see below), and 50 μl analysed for CoA and acid-soluble CoA esters by HPLC. The separation of CoA and acyl-CoA esters was performed using a Beckman Ultrasphere IP column ($5\ \mu\text{m}$, $4.6\ \text{mm} \times 250\ \text{mm}$), and analysed with a UV detector at a wavelength of 242 nm. For the initial 2.5 min of each run, eluent consisted of 99% Buffer A [50 mM $(\text{NH}_4)_3\text{PO}_4$, pH 5.3] and 1% Buffer B (50:50 mixture of Buffer A and acetonitrile, pH 5.6). A gradient was initiated by successive changes in eluent composition as follows: eluent was changed to 7% B over 2.5 min, 10% B over 2.5 min, 17% B over 2.5 min, 36% B over 8 min, and 100% B over 40 min. The final composition (100% B) was maintained for 10 min and then returned back to initial conditions over 2 min. The initial flow rate (1 ml/min) was maintained for 28.5 min and then increased

to 1.2 ml/min over 15 min. CoA and acetyl-CoA in the cell pellet extracts were readily measured; however, other CoA esters could not be quantified using this method.

Chemicals

$[1-^{14}\text{C}]$ Heptanoate ($3.2\ \mu\text{Ci}/\mu\text{mol}$) and $[1-^{14}\text{C}]$ octanoate ($55\ \mu\text{Ci}/\mu\text{mol}$) were obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Carnitine was a gift from Lonza (Basel, Switzerland). Ethanolamine was from Eastman Kodak (Rochester, NY, U.S.A.). All other chemicals, including organic acid salts, CoA and its derivatives, sodium valproate, and sodium malonate, were purchased from Sigma (St. Louis, MO, U.S.A.).

Statistics

Data were analysed statistically using the general linear models procedure of SAS (SAS Institute, Cary, NC, U.S.A.), considering the effects of fatty acid substrate ($\text{C}_{7:0}$ versus $\text{C}_{8:0}$), carnitine, inhibitor (valproate or malonate), and interactions thereof. Differences in means for main effects or interactions were compared when found to be significant ($P < 0.05$) in the analysis of variance. The means \pm S.E.M. are reported unless otherwise noted. Statistical interactions between fatty acid-carnitine, carnitine-inhibitor, or fatty acid-carnitine-inhibitor were not significant for any variable tested ($P > 0.1$), and for brevity are not reported in the text or Figure/Table legends.

RESULTS

Accumulation of label in CO_2 and ASP

The accumulation of the carboxyl carbon of $\text{C}_{7:0}$ or $\text{C}_{8:0}$ in CO_2 was affected significantly by fatty acid chain-length and inhibitor used (Table 1). Across treatments, accumulation from $\text{C}_{7:0}$ was 54% greater than from $\text{C}_{8:0}$. Compared with substrate alone, there was no effect of malonate, and a 14% depression with valproate. There was no effect of carnitine across substrates or inhibitors ($P > 0.1$).

Carboxyl-carbon accumulation in ASP was also significantly altered by substrate chain-length and inhibitor. Accumulation was 46% higher with $\text{C}_{7:0}$ versus $\text{C}_{8:0}$. Valproate-inhibited cells accumulated carboxyl carbon in ASP to only 55% of that observed in controls or in cells incubated with malonate. No significant effects of malonate or carnitine were observed ($P > 0.1$).

Addition of inhibitor had a significant ($P < 0.0001$) impact on the pattern of carboxyl radiolabel partitioning between CO_2 and ASP. Relative accumulation in ASP, expressed as a percentage of total radiolabel accumulation in ASP + CO_2 (i.e. % of total oxidative flux), was lower in cells incubated with valproate (49%) when compared with controls (59%) or those incubated with malonate (62%; S.E.M. = 0.8%). The difference ($P = 0.02$) in relative ASP accumulation between $\text{C}_{7:0}$ (56%) and $\text{C}_{8:0}$ (58%) was small (S.E.M. = 0.6%). Across all treatments, the percentage of total accumulation of radiolabel into ASP ranged from 46 to 62% (results not shown). Again, no effect of carnitine was observed ($P > 0.1$).

Oxygen consumption

Oxygen consumption was not affected by inclusion of valproate or malonate in the medium (results not shown). However, fatty acid chain-length affected ($P < 0.0001$) the rate of O_2 utilization across treatments, being 7% greater for $\text{C}_{7:0}$ (323 nmol of O_2/h per 10^6 cells) than $\text{C}_{8:0}$ (301 nmol of O_2/h per 10^6 cells).

Table 2 Free CoA and acetyl-CoA concentrations in hepatocytes isolated from 24-h-old colostrum-deprived piglets before incubation (blank) and after incubation with non-radioactive C_{7:0} or C_{8:0} substrate in the absence or presence of carnitine and/or inhibitors

Incubation conditions are given in the text ($n = 5$). * $P \leq 0.05$, significant difference between C_{7:0} versus C_{8:0}. Means with different superscripts within a column are significantly different ($P < 0.05$). All treatments are different from blank value ($P < 0.0001-0.01$).

Treatment/additions	Free CoA (nmol/g wet wt of cells)		Acetyl-CoA (nmol/g wet wt of cells)	
	C _{7:0} *	C _{8:0}	C _{7:0} *	C _{8:0}
Blank	15.2 ± 4.3	12.6 ± 4.1	24.3 ± 4.6	24.4 ± 3.7
Substrate	6.6 ± 3.4 ^a	6.6 ± 1.7 ^a	12.3 ± 4.0 ^a	16.4 ± 6.3 ^a
+ Carnitine	7.3 ± 3.4 ^a	6.2 ± 2.4 ^a	10.2 ± 3.8 ^a	15.0 ± 4.5 ^a
+ Valproate	3.9 ± 2.1 ^b	4.6 ± 2.2 ^b	11.3 ± 4.5 ^{ab}	12.9 ± 4.6 ^{ab}
+ Carnitine and valproate	5.0 ± 2.4 ^b	4.9 ± 2.1 ^b	5.9 ± 0.9 ^{ab}	12.6 ± 4.6 ^{ab}
+ Malonate	8.9 ± 2.8 ^a	5.7 ± 2.1 ^a	18.1 ± 5.1 ^b	18.8 ± 3.2 ^b
+ Carnitine and malonate	6.2 ± 3.3 ^a	6.0 ± 2.1 ^a	8.9 ± 1.9 ^b	17.1 ± 5.7 ^b

Table 3 Carboxyl carbon accumulation in organic acids derived from the ASP fraction of newborn piglet hepatocyte incubations

Cells were incubated with [1-¹⁴C]C_{7:0} or [1-¹⁴C]C_{8:0}, and the organic acids separated by ion-exchange HPLC. Values in the far right column represent the percentage of total ASP radioactivity which could be accounted for in the metabolites listed, and is calculated thus: (summed d.p.m. in all identified metabolite peaks) ÷ (total ASP d.p.m.) × 100. Acetate values represent a combination of acetate + acetoacetate accumulation. However, accumulation in acetoacetate was negligible (see the text and Table 4).

Treatment/additions	Carboxyl carbon accumulation (natom/h per 10 ⁶ hepatocytes)						
	Citrate/ α -oxoglutarate/ oxaloacetate	Pyruvate/ malate	Succinate	β -Hydroxy- butyrate	Acetate	Fumarate	%
C _{7:0} substrate	0.68 ± 0.09*†	0.73 ± 0.08*†	0.20 ± 0.09†‡	0.57 ± 0.12*†	5.96 ± 1.59†	0.20 ± 0.04†‡	46.7*†
+ Carnitine	0.72 ± 0.08	0.83 ± 0.10	0.21 ± 0.11	0.71 ± 0.08	6.38 ± 1.59	0.27 ± 0.04	53.9
+ Valproate	0.33 ± 0.05	0.39 ± 0.11	0.14 ± 0.06	0.06 ± 0.03	1.76 ± 0.46	0.01 ± 0.01	31.4
+ Carnitine and valproate	0.47 ± 0.10	0.51 ± 0.13	0.16 ± 0.08	0.15 ± 0.08	2.21 ± 0.48	0.06 ± 0.04	38.1
+ Malonate	0.69 ± 0.12	0.60 ± 0.16	0.71 ± 0.08	0.65 ± 0.10	6.08 ± 1.53	0.14 ± 0.05	49.4
+ Carnitine and malonate	0.66 ± 0.09	0.57 ± 0.14	0.75 ± 0.14	0.62 ± 0.12	6.15 ± 1.44	0.14 ± 0.04	51.4
C _{8:0} substrate	0.27 ± 0.05	0.31 ± 0.05	0.31 ± 0.08	0.44 ± 0.08	5.51 ± 1.37	0.17 ± 0.05	56.0
+ Carnitine	0.29 ± 0.04	0.31 ± 0.05	0.31 ± 0.04	0.47 ± 0.09	5.31 ± 1.24	0.19 ± 0.04	56.6
+ Valproate	0.18 ± 0.05	0.22 ± 0.06	0.23 ± 0.05	0.09 ± 0.03	2.80 ± 0.63	0.10 ± 0.04	46.1
+ Carnitine and valproate	0.16 ± 0.03	0.17 ± 0.03	0.27 ± 0.07	0.14 ± 0.04	2.38 ± 0.58	0.15 ± 0.07	46.1
+ Malonate	0.28 ± 0.05	0.30 ± 0.08	0.52 ± 0.07	0.44 ± 0.08	5.80 ± 1.38	0.18 ± 0.03	61.7
+ Carnitine and malonate	0.30 ± 0.04	0.28 ± 0.06	0.42 ± 0.08	0.42 ± 0.09	5.64 ± 1.31	0.19 ± 0.04	59.7

* Significant difference between C_{7:0} and C_{8:0} across treatments ($P < 0.01$).

† Significant effect of valproate/malonate inhibitors ($P < 0.01$).

‡ Significant interaction of fatty acid and inhibitor ($P < 0.05$).

CoA and acetyl-CoA levels

Across fatty acid substrates, the treatments resulted in a substantial 52% (substrate alone or malonate) to 67% (valproate) drop in CoA levels relative to the 13.9 nmol/g wet wt of cells measured in time-zero blanks (Table 2). The drop in CoA was unaffected by carnitine or chain-length of fatty acid.

The slight decrease in acetyl-CoA concentration with the addition of carnitine was not statistically significant. The difference between C_{7:0} and C_{8:0} was significant (Table 2). Acetyl-CoA levels across treatments were just 55% (substrate only), 64% (malonate), and 45% (valproate) of that determined for blanks (24.3 nmol/g wet wt of cells).

HPLC/radiochromatography of organic acids

The radioactivity accumulated in the organic acid peaks resolved by ion-exchange HPLC/radiochromatography is presented in Table 3. Of the organic acids identified, which included the ketone bodies, accumulation of carboxyl carbon in acetate was

predominant across all treatments. Relative to acetate, the contribution of acetoacetate to the carboxyl accumulation in the acetate/acetoacetate HPLC fraction was presumed to be small because (a) accumulation in β -hydroxybutyrate was a small fraction of identifiable organic acid accumulation (Table 3), (b) ketogenesis from *in vitro* piglet liver preparations incubated with fatty acids has been shown to be low [16,19], and (c) *in vivo* assessments of ketogenic capacity in piglets have repeatedly underscored their low ketogenic potential (i.e. [10,13,14]). Nevertheless, it was necessary to develop a method which resolved these metabolites in order to quantify radioactivity in acetoacetate. Reversed-phase ion-pairing HPLC was thus employed on ASP samples from two replicates. As judged by elution of radioactive compounds through in-line radiochromatography (Figure 2), and by quantification of radioactivity associated with eluent corresponding to these metabolites (Table 4), only minor amounts of carboxyl carbon accumulated in acetoacetate or β -hydroxybutyrate relative to acetate. Across treatments or fatty acid chain-length, acetoacetate accounted for a maximum of only 3% of ASP radioactivity (6% of identifiable

Table 4 Distribution of ASP radioactivity in acetoacetate, acetate and unidentified compound(s) from hepatocytes isolated from 24-h-old colostrum-deprived piglets and incubated with [1-¹⁴C]C_{7:0} or [1-¹⁴C]C_{8:0} in the presence or absence of carnitine and/or inhibitors

Aliquots of ASP across all treatments from two replicates were subjected to reversed-phase ion-pairing HPLC (see the Materials and methods section), and peaks eluting with retention times corresponding to acetoacetate, acetate and an unidentified radioactive entity (Figure 2) were collected and counted for radioactivity. Radioactivity in each peak was corrected for d.p.m. determined at equivalent retention times from ASP blanks (acid-killed at time 0 incubation). * $P < 0.0003$, significant difference between C_{7:0} and C_{8:0}. Means with different superscripts within a column are significantly different ($P < 0.05$).

Treatment/additions	Distribution of ASP radioactivity (% of total ASP radioactivity)					
	Unknown		Acetate		Acetoacetate	
	C _{7:0} *	C _{8:0}	C _{7:0} *	C _{8:0}	C _{7:0} *	C _{8:0}
Substrate	52.1 ± 1.4 ^a	41.2 ± 6.9 ^a	32.3 ± 2.4 ^a	48.6 ± 1.7 ^a	0.2 ± 0.2 ^a	0.8 ± 0.1 ^a
+ Carnitine	50.4 ± 4.3 ^a	33.8 ± 3.0 ^a	38.7 ± 0.8 ^a	43.1 ± 5.2 ^{ab}	2.5 ± 0.4 ^b	0.3 ± 0.3 ^{ab}
+ Valproate	76.0 ± 11.8 ^b	47.2 ± 5.1 ^a	19.3 ± 1.6 ^b	37.0 ± 6.4 ^b	0.0 ± 0.0 ^a	0.0 ± 0.0 ^b
+ Carnitine and valproate	72.6 ± 4.5 ^b	43.5 ± 3.2 ^a	26.5 ± 0.3 ^b	36.0 ± 6.0 ^b	0.6 ± 0.5 ^a	0.0 ± 0.0 ^b
+ Malonate	56.5 ± 7.2 ^a	36.4 ± 5.1 ^a	35.1 ± 0.3 ^a	46.6 ± 4.1 ^{ab}	1.6 ± 0.3 ^c	1.5 ± 0.3 ^c
+ Carnitine and malonate	53.5 ± 5.7 ^a	32.2 ± 3.8 ^a	38.0 ± 0.1 ^a	44.7 ± 4.3 ^{ab}	3.0 ± 0.3 ^b	0.8 ± 0.4 ^a

organic acid radioactivity). The primacy of acetate in the ion-exchange acetate/acetoacetate peak was suggested in an earlier study [23]. Substrate chain-length significantly affected the distribution pattern of carboxyl carbon accumulated in ASP organic acids (see Table 3). Across treatments, accumulation in citrate/ α -oxoglutarate/oxaloacetate and pyruvate/malate was ≥ 2 -fold higher from incubations with C_{7:0} versus those with C_{8:0}. On average, valproate significantly lowered accumulation in citrate/ α -oxoglutarate/oxaloacetate and pyruvate/malate by about 40%, and in fumarate by 30% to 84% (C_{8:0} and C_{7:0}, respectively). Although carboxyl carbon accumulation in β -hydroxybutyrate was similar for C_{7:0} and C_{8:0} in the presence of valproate, accumulation was 1.5-fold higher for C_{7:0} across other treatments. Valproate addition markedly lowered accumulation in β -hydroxybutyrate to levels only 16% (C_{7:0}) and 25% (C_{8:0}) of controls ($P < 0.0001$). Relative to controls, malonate had no effect upon accumulation in β -hydroxybutyrate ($P > 0.1$). Accumulation of carboxyl carbon in acetate dropped by an average of 61% when cells were incubated with valproate ($P < 0.0001$), while malonate had no effect (Table 3). Carnitine had no effect across all treatments ($P > 0.1$).

Interestingly, the summated radioactivity contained in the organic acids identified and separated by ion-exchange HPLC could account for just 31–62% of the radioactivity residing in ASP (Table 3, last column). With samples subjected to reversed-phase ion-pairing separation, 80–90% of ASP radioactivity was recovered in acetate, acetoacetate and an unidentified radioactive peak (Figure 2 and Table 4). The percentage of radioactivity in identified organic acids was significantly lower from C_{7:0} than C_{8:0} (mean across treatments = 45% and 54%, respectively, Table 3). Compared with controls (53%) or malonate-treated cells (56%), a smaller fraction (40%) of total carboxyl-carbon accumulation in ASP could be accounted for in identifiable organic acids when cells were incubated with valproate (Table 3).

DISCUSSION

Organic acid profile

Numerous studies [11–14,19] have illustrated that neonatal pigs display a minimal ketogenic capacity. These studies prompted the development of an ion-exchange HPLC/radiochromatographic method for analysis of organic acids in ASP from piglet hepatocytes incubated with 1-¹⁴C-labelled fatty acids which

showed that major carboxyl carbon radioactivity was in a peak corresponding to acetate/acetoacetate [23]. However, radioactivity in other organic acids was not obtained, and the complete characterization of β -oxidative end-products derived from piglet hepatocytes remained unclear. The current study extends the previous experiment [23], showing major accumulation of radiolabel in acetate and an unidentified entity (Table 4, Figure 2). Under all conditions studied the ketone bodies were a minor reservoir of carboxyl carbon. The finding that acetogenesis far surpasses ketogenesis in piglet hepatocytes appears unique. Nevertheless, substantial endogenous (non-fermentative) production of acetate has been described in rat and sheep liver [26,27]. Plasma acetate doubled in humans fasted for 4.5 days [28], and is elevated in diabetic humans, rats and sheep [26,27,29], suggesting that endogenous acetogenesis occurs concomitant with ketogenesis. β -Oxidative end-products not arising from ketogenesis (i.e. acetyl-carnitine) have been described in rat muscle and liver under various conditions [30,31].

It is intriguing that when ASP samples were subjected to reversed-phase ion-pairing HPLC, a large fraction (up to 70%, Table 4) of radiolabel accumulated in a peak as yet unidentified (Figure 2). It is notable that the percentage of unidentified ASP radioactivity was significantly affected by differences in fatty acid and by the presence of valproate (see Tables 3 and 4), supporting the view that this fraction is related to cellular metabolism. The unidentified fraction could, in theory, represent such metabolites as acetyl-carnitine, glucose, glutamate, etc. Clarification of this phenomenon will be required.

Carboxyl-carbon accumulation in CO₂ and ASP

Odle et al. [10] reported a higher (by 45%) accumulation of carboxyl carbon in oxidative flux from cells given odd-chain MCFA. Consistent with this finding, we observed that C_{7:0} significantly increased radioactivity accumulation in both CO₂ and ASP relative to C_{8:0} (Table 1). Furthermore, results from radio-HPLC analysis established that the carboxyl-carbon accumulation in Krebs cycle intermediates was 85% higher in cells incubated with C_{7:0} versus C_{8:0} while flux to β -hydroxybutyrate increased by a mere 12% (Table 3) and accumulation in acetate was unaffected. These results support the hypothesis that anaplerosis from odd-chain MCFA expands the pool of Krebs cycle intermediates, thereby resulting in more complete com-

bustion of acetyl-CoA units to CO₂ with less acetyl-CoA flux into ketone bodies or acetate.

The capability of valproate to lower β -oxidative flux and ketogenesis has been documented repeatedly *in vivo* [22,32] and *in vitro* [20,21], and is thought to be due to a combination of competition with fatty acid substrate for a limited mitochondrial CoA pool [20,22,31], and to some degree an effect upon β -oxidative enzymes [33]. Indeed, CoA was lowered by 32% in cells incubated with valproate versus those incubated with substrate alone or with malonate (Table 2), consistent with CoA sequestration by valproate or its metabolites. There is evidence that carnitine may help to relieve some negative effects of valproate *in vivo*, presumably through formation of acyl-carnitine esters which may liberate CoA. In support of this notion, patients under valproate therapy excrete valproyl-carnitine and other medium-chain acyl-carnitines [34]. However, carnitine addition to isolated piglet hepatocytes failed to increase hepatocyte CoA concentration (Table 2), and had no effect upon carboxyl-carbon accumulation in ASP or CO₂ under any condition (Table 1). Kempen and Odle [35], in studies employing constant infusion of C_{8:0} in piglets *in vivo*, found that carnitine co-infusion with valproate did not affect liver or muscle CoA profiles relative to animals infused with C_{8:0} + carnitine only. However, initiation of carnitine co-infusion did raise C_{8:0} oxidation to CO₂ by approx. 7% *in vivo*, and depressed C_{8:0}-induced dicarboxylic acid excretion by approx. 50% in animals given C_{8:0} + valproate [35]. Thus, the precise role of carnitine in alleviating the negative effects of valproate upon β -oxidation is not entirely clear.

Inclusion of malonate at 10 mM failed to affect carboxyl-carbon accumulation in ASP, CO₂, or β -hydroxybutyrate. Evidence of succinate dehydrogenase inhibition by malonate was obtained (Table 3), in that addition of malonate increased accumulation in succinate by 3.6- and 1.5-fold (C_{7:0} and C_{8:0}, respectively), and depressed accumulation in pyruvate/malate by 25% (C_{7:0} only) and in fumarate by 39% (C_{7:0} only); however, the Krebs cycle organic acids were a minor pool of carboxyl carbon. The lack of strong malonate effects upon total ASP and CO₂ in this study might be related to the use of hepatocytes rather than mitochondria.

Free CoA and acetyl-CoA

The oxidation of fatty acids, which requires free CoA, should theoretically increase the level of acetyl-CoA and lower the content of free CoA in hepatocytes. Consistent with this, neonatal piglet hepatocytes incubated with MCFA displayed a free CoA content much lower than that in blank preparations (Table 2), and liver acetyl-CoA concentration increased with gastric administration of long-chain fatty acids in piglets [36]. Valproate further lowered the free CoA concentration, probably via formation of valproyl- and other acyl-CoA esters. However, with addition of 1 mM MCFA, cellular acetyl-CoA content was diminished relative to blanks (Table 2). Qualitatively similar results were obtained by Kempen and Odle [35], who observed a drop in liver acetyl-CoA and free CoA after *in vivo* infusion of C_{8:0} in neonatal piglets. It is constructive to emphasize that the concentration of acetyl-CoA was extremely low from neonatal piglets (this study and [35,36]) compared with rats [37]. Explanations for this finding are only speculative, but it is possible that (1) in the presence of MCFA, CoA becomes bound in other acyl-CoA moieties (i.e. butyryl- and hexanoyl-CoA for

cells given C_{8:0}) which were not quantifiable with the HPLC analysis employed, and (2) piglets fail to accumulate acetyl-CoA which could be related to a high concentration of acetate generated from acetyl-CoA in the cells. If so, a high activity of acetyl-CoA hydrolase in pig hepatocytes may be expected.

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