Biochemical Pharmacology, Vol. 41, No. 6/7, pp. 1015–1023, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

INHIBITION OF HEPATIC PROPIONYL-CoA SYNTHETASE ACTIVITY BY ORGANIC ACIDS

REVERSAL OF PROPIONATE INHIBITION OF PYRUVATE METABOLISM

STEPHAN KRAHENBUHL and ERIC P. BRASS*
Departments of Medicine and Pharmacology, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

(*Received* 21 *March* 1990; *accepted* 1 *October* 1990)

Abstract—Intracellular accumulation of propionyl-CoA is associated with impairment of important hepatic metabolic pathways. Since propionate absorbed from the intestine can be converted to propionyl-CoA in the liver, inhibition of propionyl-CoA synthesis from propionate and CoA may provide a strategy for decreasing toxicity from plasma propionate. Therefore, inhibition of propionyl-CoA formation by several organic acids was investigated. In isolated, solubilized mitochondria, octanoate, butyrate, salicylate and *p*-nitrobenzoate inhibited propionyl-CoA synthesis. Octanoate was the most potent inhibitor of propionyl-CoA synthetase activity and had a K_i of 58 μM. In isolated hepatocytes, octanoate inhibited propionate oxidation in a concentration-dependent manner. Consistent with previous studies, propionate (1.0 mM) inhibited the rates of $^{14}CO_2$ formation from [1-^{14}C]pyruvate (10 mM) to 55% of the control values in the hepatocyte system. Octanoate (0.8 mM) had no effect on [1-^{14}C]pyruvate oxidation under control conditions, but increased $^{14}CO_2$ formation from pyruvate to 88% of the control values in the presence of 1.0 mM propionate. Reversal of propionate inhibition of pyruvate oxidation by octanoate was associated with a 44% decrease in hepatocyte propionyl-CoA content. In contrast, while pyruvate oxidation rates were decreased to 53% of control rates in the presence of 10 mM propionylcarnitine, octanoate stimulated pyruvate oxidation under these conditions only to 67% of control levels. In conclusion, mitochondrial propionyl-CoA synthetase activity and hepatocyte propionyl-CoA accumulation can be inhibited by octanoate with consequent decreased propionate oxidation and toxicity in intact hepatocytes. The reversal by octanoate of propionate's inhibition of cellular metabolism may be useful in reducing tissue toxicity from circulating propionate.

Short-chain fatty acids may disrupt normal cellular metabolism, both *in vitro* [1] or when generated by intestinal bacteria in patients [2]. Studies with propionate and α-ketobutyrate in isolated hepatocytes suggest that toxicity associated with propionate is due to propionyl-CoA and methylmalonyl-CoA accumulation [3, 4]. Intracellular accumulation of these acyl-CoAs has been associated with decreased function of several hepatic metabolic pathways including gluconeogenesis [5, 6], ureagenesis [5–7], pyruvate oxidation [8, 9] and fatty acid oxidation [1, 4, 7]. Propionyl-CoA is generated in the liver either directly during the degradation of odd-chain fatty acids or amino acids, or by enzymatic activation of propionate absorbed from the intestine. In ruminants and guinea pigs, propionyl-CoA is synthesized from propionate and CoA by a specific propionate-activating enzyme [10, 11]. In rats the existence of a specific propionate-activating enzyme has not been established, and propionyl-CoA may be synthesized by one or more short-chain fatty acid-activating enzymes with broad substrate specificity [12–14]. Once generated, proponyl-CoA is normally metabolized to D-methylmalonyl-CoA by the biotin-dependent enzyme propionyl-CoA carboxylase [15]. D-Methylmalonyl-CoA, in turn, is isomerized to the L-form, and then converted to succinyl-CoA which can enter the citric acid cycle. The conversion from L-methylmalonyl-CoA to succinyl-CoA is catalyzed by the vitamin B12-dependent methylmalonyl-CoA mutase, and is the rate-limiting step for propionate metabolism [16, 17].

Accumulation of toxic acyl-CoAs can be reduced either by inhibition of their synthesis or increased removal. For some acyl-CoAs, increased removal can be achieved through acylcarnitine formation after treatment with carnitine [18, 19]. In isolated hepatocytes, carnitine partially reverses the inhibitory effect of propionyl-CoA accumulation on several metabolic pathways [1, 4, 20]. An alternative strategy to decrease propionate toxicity is to reduce synthesis of toxic CoA derivatives. Addition of salicylate or *p*-nitrobenzoate prevents the inhibition of gluconeogenesis and α-keto acid oxidation by valproic acid, an organic acid whose toxicity is dependent on acyl-CoA formation [21].

The present studies were undertaken to characterize the possible inhibition of propionyl-CoA synthetase activity by various organic acids. The results demonstrated that in isolated mitochondria, several organic acids are inhibitors of propionate activation. Octanoate was the most potent inhibitor of the organic acids studied. In isolated hepatocytes, octanoate inhibited propionyl-CoA formation from

* Correponsence: Eric P. Brass, M.D., Ph.D., Case Western Reserve University, Department of Medicine, Division of Clinical Pharmacology, Cleveland, OH 44106, U.S.A.

propionate and propionate oxidation, and almost completely reversed the inhibitory effects of propionate on pyruvate oxidation.

MATERIALS AND METHODS

Animals. Male, Sprague–Dawley rats purchased from Charles River Laboratories (Portage, MI) were used for all experiments. Rats were housed with free access to food (Lab Chows, Purina Mills Inc., St. Louis, MO) and water. Animals were allowed to acclimate in the animal facility for at least 1 week before the preparation of hepatocytes or the isolation of mitochondria.

Isolation of mitochondria and hepatocytes. Rat liver mitochondria were prepared using the method described by Hoppel *et al.* [22]. The concentration of the final preparation was adjusted to approximately 75 mg mitochondrial protein/mL and the preparation was stored at −20°.

Hepatocytes were prepared from fed animals by the method of Berry and Friend [23] with the modifications described previously [24]. All hepatocyte preparations were started at 7:00 a.m. The body weights of the animal at the time of hepatocyte preparations were 380 ± 90 g (N = 18). The isolated hepatocytes were 97 ± 2% (N = 18) viable based on trypan blue exclusion, had a wet weight of 16.2 ± 3.4 mg/10^6 hepatocytes (N = 18) and a protein content of 3.2 ± 0.9 mg/10^6 hepatocytes (N = 15).

Propionyl-CoA synthetase assay. Mitochondria were dissolved in an equal volume of cholate 5% (w/v) for use in the propionyl-CoA synthetase assay. The dissolved mitochondria were diluted with 0.1 M potassium phosphate buffer (pH 7.2) to 5 mg mitochondrial protein/mL.

Propionyl-CoA synthetase activity was measured by a modification of the methods described by Man and Brosnan [25] and Swartzentruber and Harris [21]. The assay medium contained in a final volume of 1 mL at pH 8.0; 50 mM KH_2PO_4, 5 mM $MgCl_2$, 3 mM ATP, 1.2 mM CoA, 2.5 μg oligomycin and 100 μL mitochondrial preparation. Oligomycin was included to avoid depletion of ATP by the action of mitochondrial ATPase. Inhibitors of propionyl-CoA synthetase were added at the concentrations indicated, the mixture was preincubated for 5 min at 37°, and the reaction was started by addition of [1-^{14}C]propionate (1.1×10^5 dpm/assay). The reaction was stopped by addition of 0.2 mL of 2 N HCl, and the reaction tubes were centrifuged (2000 *g* for 5 min) to pellet the precipitated protein. To remove unreacted propionate from propionyl-CoA formed, 1 mL of the acid supernatant was transferred to a 15-mL glass tube, and extracted four times with 4 mL of water-saturated diethyl ether (extraction efficiency for propionate >99.8%). ^{14}C-Radioactivity was directly counted in the remaining water phase. Reactions stopped at time 0 were used as blanks. After subtraction of the blank (typically less than 200 dpm), product formed was calculated by dividing the net dpm by the specific activity of [1-^{14}C]propionate. The reactions (reaction time 10 min) were carried out in duplicate and the mean of the duplicates was used for calculation. The assay was linear with respect to time (0–30 min) and protein (0–1 mg/assay). Since other water-soluble products than propionyl-CoA may be formed from propionate and contribute to ^{14}C-radioactivity in the water phase, the specificity of the assay was verified in selected incubations. For this purpose, 500 μL of 2 N KOH was added to 1 mL of the water phase after ether extraction, and the mixture was heated to 55° for 1 h to hydrolyze propionyl-CoA formed. After hydrolysis, 600 μL of 2 N HCl was added to acidify the mixture, and propionate formed was extracted four times with water-saturated diethyl ether. As compared to direct counting of the water phase, the recovery of ^{14}C-radioactivity in the ether phase after alkaline hydrolysis was 96 ± 4%, showing that the direct counting of the water phase overestimated the reaction velocity by not more than 5%.

Kinetics of the inhibition of propionyl-CoA synthesis. K_I values for organic acids inhibiting the formation of propionyl-CoA were obtained by the method of Dixon [26]. Two propionate concentrations were chosen, one below and one above the published K_m value for propionate activation. Seven inhibitor concentrations were tested which bracketed the K_I value estimated in pilot experiments for the inhibition by each compound of propionyl-CoA synthetase activity. Regression analysis of the plots was performed by the method of least squares [27]. The mean K_I for each inhibitor was calculated by averaging the K_I values obtained in individual experiments. Assuming that inhibition of propionyl-CoA synthesis by the organic acids added was competitive (assumption based on linearity of the plots), the Dixon plot method allowed the calculation of K_m and V_{max} for propionate for all inhibitors studied and for each substrate concentration. These values, which should be equal for each substrate concentration for all inhibitors studied, were used as an additional quality control for the Dixon transformation and as a verification of competitive inhibition.

Hepatocyte metabolism. $^{14}CO_2$ formation from [1-^{14}C]propionate and [1-^{14}C]pyruvate by isolated hepatocytes was measured in 25-mL Erlenmeyer flasks equipped with sidearms and center wells. Incubation procedures were as detailed elsewhere [3]. The incubations contained hepatocytes (6.4 ± 4.9 × 10^6 cells/mL; N = 15), 1% defatted bovine serum albumin (w:v), 124 mM NaCl, 3.8 mM KCl, 0.95 mM KH_2PO_4, 1.9 mM $MgSO_4$, 0.90 mM $CaCl_2$, 20 mM $NaHCO_3$, 0.8 mM Tris(hydroxymethyl) aminomethane, 3.2 mM glucose, and other compounds as detailed in the individual experiments. The incubation volume was 2.5 mL, and the incubation pH was 7.4. The incubations were conducted under 95% O_2/5% CO_2 at 37°. After a preincubation of 15 min, substrates and inhibitors (except for salicylate) as specified in the individual experiments were added at time 0. When included, salicylate was added to the incubations at the start of the preincubation. The specific activities of the substrates were determined for each experiment, and averaged 38 ± 8 dpm/nmol for 1.0 mM [1-^{14}C]propionate and 8.2 ± 1.6 dpm/nmol for 10.0 mM [1-^{14}C]pyruvate. The reactions were terminated by

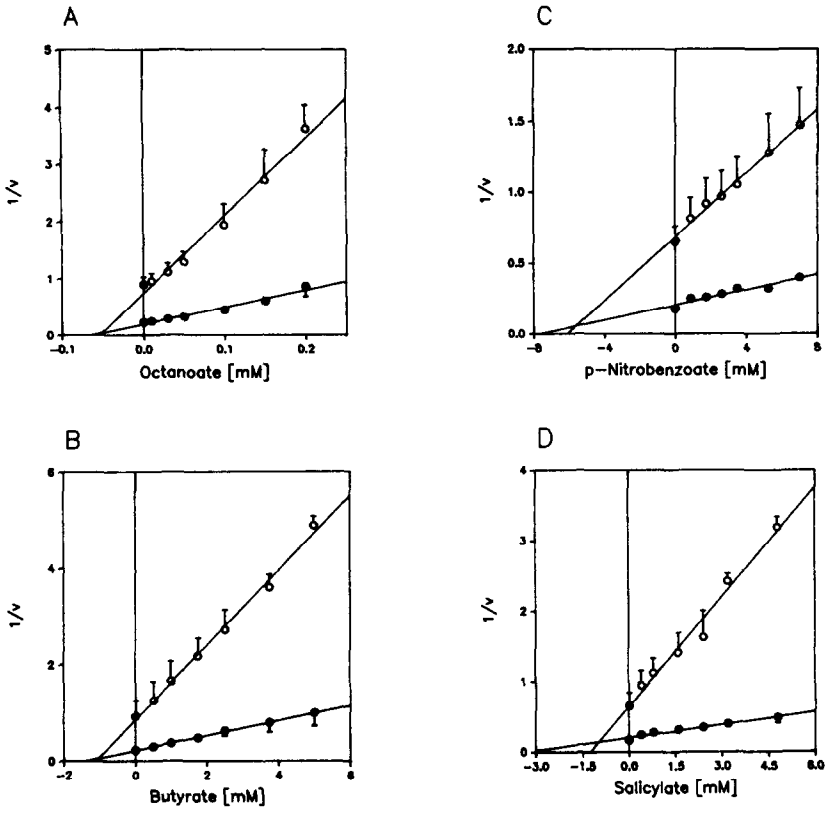

Fig. 1. (A–D) Dixon plots for octanoate (A), butyrate (B), *p*-nitrobenzoate (C) and salicylate (D) as inhibitors of propionyl-CoA synthetase activity. The reciprocal velocity of propionyl-CoA formation [min · (mg mitochondrial protein)$^{-1}$ · nmol^{-1}] is indicated on the ordinate and inhibitor concentrations [mM] on the abscissa. The priopionate concentrations were 0.5 mM (○) and 5 mM (●) respectively. The incubations contained 0.5 mg mitochondrial protein, and the composition of the incubation medium was as described under Materials and Methods. Each point is the mean ± SD of three to four determinations using different mitochondrial preparations. Where only points are shown, the standard deviation was too small to be displayed.

the addition of perchloric acid to yield a final concentration of 3% (v:v) in the incubations. The high pyruvate concentration used in the hepatocyte incubation experiments (10 mM) was chosen to ensure full activation of pyruvate dehydrogenase [28–30].

$^{14}CO_2$ was collected in the flask center wells containing 0.3 mL ethanolamine:ethylene glycol monomethyl ether (1:2, v:v). ^{14}C collected from incubations terminated at time 0 was subtracted to provide net $^{14}CO_2$ production. The amount of 1-^{14}C-substrate converted to CO_2 was calculated as $^{14}CO_2$ production (net dpm) divided by the specific activity of the substrate used.

Analysis of the hepatocyte CoA-pool. Hepatocytes ($9.1 \pm 2.6 \times 10^6$ cells/mL; N = 3) were incubated under the same conditions as detailed above. After a preincubation period of 15 min, substrates and inhibitors were added to the incubations as specified in the individual experiments. Ten minutes after addition of substrates and inhibitors, 900 μL of the hepatocyte suspensions was added to 100 μL of cold 30% perchloric acid (v:v) containing 100 mM dithiotreitol and centrifuged for 1 min at 10,000 *g*. The pellet which contains long-chain acyl CoAs was washed once with 3% perchloric acid (v:v) and stored at −80° after addition of 20 μL of 0.2 M dithiothreitol. To 450 μL of the supernatant (containing CoA and short-chain acyl CoAs) 150 μL of cold 2 N $KHCO_3$ was added and the uncapped tubes were kept for 30 min on ice before centrifugation (1 min at 10,000 *g*). The neutralized supernatant was frozen in acetone/dry ice and stored at −80°.

CoA and acyl-CoA contents were quantified in the neutralized supernatants using a high performance chromatography (HPLC) technique based on the method described by Corkey *et al.* [31]. On the same day as the hepatocyte incubations, supernatants were thawed and 10 μL of 100 μM isovaleryl-CoA external standard was added to 190 μL of supernatant. This sample (20 μL) was then injected onto a Beckman (San Ramon, CA) HPLC Gold System equipped with a Beckman 160 UV detector (254 nm) and a Hewlett–Packard (Avondale, PA) model 3390A integrator. A Nova-Pak 15 cm C_{18} column (3.9 mm diameter, 4 μm particle size; Waters Associates, Milford, MA) equipped

Table 1. K_I values for inhibitors of hepatic mitochondrial propionyl-CoA synthetase activity

Inhibitor	K_I [mM]
p-Nitrobenzoate	4.7 ± 0.1
Salicylate	1.5 ± 0.6
Butyrate	0.91 ± 0.52
Octanoate	0.058 ± 0.024

The K_I values were obtained from Dixon plots shown in Fig. 1, as described in the text. The values are means ± SD; N = 3 for butyrate and *p*-nitrobenzoate and N = 4 for octanoate and salicylate.

with a Bondpak C_{18} precolumn (Waters Associates) was used. The elution was started with 5% methanol in 50 mM phosphate buffer, pH 5.3 The methanol concentration was linearly increased to 8% over 10 min, and then to 50% over 30 min where it was maintained for an additional 10 min. These conditions allowed the quantification (typical retention times in minutes in parentheses) of CoA (11.2), methylmalonyl-CoA (13.2), succinyl-CoA (15.6), acetyl-CoA (19.5), propionyl-CoA (23.7), butyryl-CoA (27.8), isovaleryl-CoA (31.9) and octanoyl-CoA (47.7). Summation of the contents of CoA and the individual acyl-CoAs yielded the total HPLC-CoA content.

The total hepatocyte CoA content was verified independently (to ensure that all CoA species were measured) by the enzymatic recycling assay of Allred and Guy [32] with the modifications previously described [33]. The enzymatic recycling assay, which was performed in the neutralized supernatant after hydrolysis at pH 12.5, 55° for 1 hr, measures the total acid-soluble CoA content. The long-chain acyl CoA content was measured with the enzymatic recycling assay in the initial perchloric acid pellet after alkaline hydrolysis (0.5 N KOH, 55° for 1 hr). Coenzyme A standard solutions were calibrated by the method of Michal and Bergmeyer [34]. The true isovaleryl-CoA content in the HPLC external standard solution was verified in the recycling assay against the coenzyme A standards.

Reagents. [1-^{14}C]Propionate and [1-^{14}C]pyruvate were purchased from New England Nuclear (Boston, MA). Propionyl-L-carnitine was provided by Sigma Tau (Rome, Italy). Collagenase (Type II) used for the isolation of hepatocytes was from Worthington Biochemicals (Freehold, NJ). Defatted bovine serum albumin, oligomycin and CoA were purchased from Sigma Chemicals (St. Louis, MO). All other chemicals were reagent grade.

Statistics. Data are presented as mean ± SD with N referring to the number of observations, each made using a separate mitochondrial or hepatocyte preparation. Data were analyzed using Student's *t*-test [27]. The paired *t*-test was used to compare means from paired observations. $P < 0.05$ was considered to be statistically significant.

RESULTS

Propionyl-CoA formation is an obligate first step in propionate metabolism. Propionyl-CoA synthetase activity in the presence of other organic acids was studied in rat liver mitochondrial preparations. Inhibition of propionyl-CoA synthesis was characterized using Dixon plots for each inhibitor (Fig. 1, A–D). Consistent with competitive antagonism versus propionate, all inhibitors studied resulted in linear Dixon plots with positive $1/v$ values for the intersections of the two lines representing individual substrate concentrations. The K_I for the inhibition by octanoate of propionyl-CoA synthetase activity was 58 ± 24 μM (N = 4), and was 15–100 times lower than the corresponding values for butyrate, salicylate and *p*-nitrobenzoate (Table 1). Assuming competitive inhibition, values for K_m and V_{max} for propionate could be calculated. The K_m value for propionate obtained from the four plots (each plots yields two estimates) was 4.9 ± 3.0 mM which is similar to the value previously reported for rat liver [35]. The V_{max} for propionyl-CoA synthetase activity obtained from the same four plots averaged 16.5 ± 6.3 nmol/min/mg mitochondrial protein.

The effects of salicylate, butyrate and octanoate on propionate oxidation were characterized using intact rat hepatocytes. Octanoate caused a significant

Table 2. Propionate metabolism in isolated hepatocytes

Inhibitor	N	Rate of propionate oxidation (nmol $^{14}CO_2$/10^6 cells/30 min)	
		− Inhibitor	+ Inhibitor
Salicylate, 1 mM	5	60.5 ± 4.9	61.7 ± 8.9
Salicylate, 4 mM	5	60.5 ± 4.9	55.9 ± 11.8
Butyrate, 0.8 mM	4	72.7 ± 33.5	70.0 ± 32.7
Butyrate, 3.2 mM	4	72.7 ± 33.5	67.3 ± 28.7
Octanoate, 0.2 mM	4	57.5 ± 7.7	46.1 ± 11.6*
Octanoate, 0.8 mM	4	57.5 ± 7.7	26.2 ± 5.2*

Isolation of hepatocytes and incubations were conducted as described in the text. N indicates the number of experiments per inhibitor, each in different hepatocyte preparations. Means ± SD are given.

* $P < 0.05$ between incubations with and without inhibitor.

inhibition of propionate oxidation, whereas 4 mM salicylate and 3.2 mM butyrate showed no effect (Table 2). Higher concentrations of salicylate or butyrate could not be evaluated due to toxicity in the hepatocyte system (data not shown). Octanoate inhibited propionate oxidation in isolated hepatocytes in a concentration-dependent fashion, averaging 20% inhibition at an octanoate concentration of 0.2 mM and 54% at 0.8 mM. In contrast, and in agreement with other studies in isolated hepatocytes [30], 0.8 mM octanoate showed no inhibition of pyruvate oxidation (Table 3), demonstrating that the inhibition of propionate metabolism was not due to a nonspecific effect.

As propionyl-CoA formation has been hypothesized to be responsible for the metabolic toxicity of propionate, inhibition of propionyl-CoA synthetase activity should decrease the inhibitory effect of propionate on pyruvate oxidation. To test this hypothesis, pyruvate oxidation in isolated hepatoctyes was measured in the absence and presence of 0.8 mM octanoate and 0.8 mM butyrate. Neither butyrate nor octanoate affected pyruvate oxidation rates in the absence of propionate. In contrast, both octanoate and butyrate significantly reversed the inhibitory effect of propionate on pyruvate oxidation (Table 3). The reversal by octanoate averaged 74 ± 22% and was significantly higher than the 36 ± 13% reversal induced by butyrate.

The specificity of the reversal by octanoate of the inhibitory effect of propionate on pyruvate oxidation was further investigated by using propionylcarnitine, which can generate intramitochondrial propionyl-CoA independent of propionyl-CoA synthetase activity, as an inhibitor of pyruvate oxidation. Propionylcarnitine (10 mM) inhibited pyruvate oxidation by 47% (Table 3), a magnitude of inhibition similar to that observed with 1 mM propionate (Table 3). Octanoate reversed 34 ± 11% of this inhibition, a value significantly lower than the reversal by octanoate of propionate inhibition (Table 3).

To challenge propionyl-CoA accumulation as the basis for the octanoate–propionate interaction, the hepatocyte coenzyme A pool was quantified under the experimental conditions of Table 3. Addition of 1 mM propionate to hepatocyte incubations increased the propionyl-CoA content from 0 (control incubation containing no propionate) to 0.27 ± 0.03 nmol/10^6 cells, and the methylmalonyl-CoA content from 0.07 ± 0.06 to 1.48 ± 0.20 nmol/10^6 cells. Addition of propionate also resulted in a decrease of CoA, acetyl-CoA and succinyl-CoA contents, leaving the total CoA (i.e. CoA and all acyl-CoAs) content unchanged (Table 4). Addition of octanoate (0.8 mM) in the presence of propionate decreased hepatocyte propionyl-CoA and methylmalonyl-CoA contents by 44 and 23%, respectively, while total hepatocyte CoA content was unaffected. Consistent with the formation of octanoyl-CoA (which is precipitated in 3% perchloric acid), the long-chain acyl CoA content was higher in the incubations containing octanoate as compared to octanoate-free incubations. In contrast with

Table 3. Reversal of the inhibitory effect of propionate on pyruvate metabolism in isolated hepatocytes

Propionyl-CoA source	Propionyl-CoA synthetase inhibitor	N	Rate of pyruvate oxidation				% Reversal of inhibition of pyruvate metabolism
			Pyruvate	Pyruvate + propionyl-CoA synthetase inhibitor	Pyruvate + propionyl-CoA source	Pyruvate + propionyl-CoA source + propionyl-CoA synthetase inhibitor	
Propionate, 1 mM	Octanoate, 0.8 mM	6	153 ± 59	158 ± 57	84.7 ± 44.5*	135 ± 50†	74 ± 22
Propionate, 1 mM	Butyrate, 0.8 mM	5	141 ± 23	138 ± 26	66.0 ± 12.7*	92.2 ± 11.7†	36 ± 13‡
Propionylcarnitine, 10 mM	Octanoate, 0.8 mM	4	128 ± 21	123 ± 14	67.3 ± 11.6*	86.3 ± 11.5†	34 ± 11‡

Isolation of hepatocytes and incubations were conducted as described in the text. $^{14}CO_2$ production rates are given as nmol/10 min/10^6 cells of [1-^{14}C]pyruvate (10 mM) used. N indicates the number of experiments per inhibitor in different hepatocyte preparations. Means ± SD are given.

* $P < 0.05$ vs pyruvate oxidation rates with only pyruvate present in incubations.

† $P < 0.05$ vs pyruvate oxidation rates with pyruvate and propionyl-CoA source.

‡ $P < 0.05$ vs reversal of the inhibition by propionate of pyruvate oxidation by octanoate.

Table 4. Hepatocyte CoA pool

Additions to incubation	Hepatocyte CoA pool (nmol/10^6 cells) CoA	Acetyl-CoA	Succinyl-CoA	Propionyl-CoA	Methylmalonyl-CoA	Total HPLC-CoA	Total acid-soluble CoA	Long-chain acyl-CoA	Total CoA
Pyruvate, 10 mM	0.93 ± 0.01	0.63 ± 0.16	0.58 ± 0.09	0	0.07 ± 0.06	2.20 ± 0.25	2.43 ± 0.19	0.97 ± 0.10	3.40 ± 0.28
Pyruvate, 10 mM + propionate, 1 mM	0.39 ± 0.09*	0.37 ± 0.09*	0.10 ± 0.09*	0.27 ± 0.03*	1.48 ± 0.20*	2.61 ± 0.28	2.49 ± 0.10	0.89 ± 0.1	3.38 ± 0.11
Pyruvate, 10 mM + propionate, 1 mM + butyrate, 0.8 mM	0.43 ± 0.05*	0.39 ± 0.05*	0.14 ± 0.05*	0.24 ± 0.03*	1.41 ± 0.12*	2.60 ± 0.10	2.52 ± 0.08	0.97 ± 0.05	3.49 ± 0.12
Pyruvate, 10 mM + propionate, 1 mM + octanoate, 0.8 mM	0.45 ± 0.06*	0.40 ± 0.14	0.15 ± 0.08*	0.15 ± 0.02*†	1.14 ± 0.19*†	2.30 ± 0.20	2.47 ± 0.28	1.08 ± 0.08*†	3.55 ± 0.19

Isolation of hepatocytes and incubations were conducted as described in Materials and Methods. CoA and individual acyl-CoA concentrations were determined by HPLC, and total acid-soluble and long-chain acyl-CoA concentrations by the CoA recycling assay as described in Materials and Methods. Total HPLC-CoA represents the sum of the CoA and the individual acyl-CoA concentrations measured by HPLC, and total CoA represents the sum of total acid-soluble CoA and long-chain acyl CoA concentrations. N = 3. Values are means ± SD.

* $P < 0.05$ vs incubation with pyruvate as substrate.

† $P < 0.05$ vs incubation with pyruvate and priopionate as substrate.

octanoate, butyrate had no effect on the hepatocyte coenzyme A pool in the presence of propionate.

DISCUSSION

The formation of propionyl-CoA from propionate and CoA is the first step in propionate metabolism. The present studies demonstrated that octanoate, butyrate, *p*-nitrobenzoate and salicylate were inhibitors of hepatic mitochondrial propionyl-CoA synthetase activity. Octanoate was the most potent inhibitor of propionyl-CoA synthetase activity of the four organic acids investigated, and the only one which significantly impaired propionate oxidation in isolated hepatocytes. Octanoate reversed the inhibitory effect of propionate on pyruvate metabolism and decreased propionyl-CoA accumulation from propionate, consistent with an inhibition of propionyl-CoA formation as the mechanism of octanoate's beneficial effect on pyruvate metabolism in the presence of propionate.

The magnitude of the inhibitory effects by the organic acids studied on mitochondrial propionyl-CoA formation (measured with the propionyl-CoA synthetase assay) was greater than their inhibition of propionate oxidation in isolated heptocytes. This discordance is consistent with the observation that propionate activation is not the rate-limiting step for propionate metabolism [17]. The activity of methylmalonyl-CoA mutase, which catalyzes the formation of succinyl-CoA from methylmalonyl-CoA, is approximately 20% that of propionyl-CoA synthetase per g rat liver [16]. Therefore, in intact hepatocytes, the inhibition of propionyl-CoA synthesis must exceed 80% before an effect on propionate oxidation would be predicted. Based on the K_I for octanoate of 58 μM obtained in isolated mitochondria, 0.2 and 0.8 mM octanoate should inhibit propionyl-CoA synthesis with 1.0 mM propionate as substrate by approximately 80 and more than 90% respectively. The observed rates of propionate oxidation in the presence of octanoate by isolated hepatocytes (Table 2) are in agreement with these estimates. By contrast, no significant effect on propionate oxidation was seen with 4 mM salicylate and 3.2 mM butyrate, which would be expected to inhibit propionyl-CoA synthesis by less than 80%.

The inhibitory effect of propionate on pyruvate metabolism has been hypothesized to be mediated by intracellular buildup of propionyl-CoA [3, 4]. Consistent with the propionyl-CoA synthetase activity data obtained in isolated mitochondria, octanoate reversed the inhibitory effect of propionate on pyruvate oxidation more efficiently than butyrate, when both inhibitors were used at the same concentration (Table 3). Importantly, butyrate and octanoate are metabolized to ketone bodies at equal rates in the hepatocyte system [1]. This may be important as mitochondrial acetoacetate formation may be critical for pyruvate transport into mitochondria under certain metabolic conditions [30]. Thus, the effect of octanoate is not secondary to the addition of a fuel substrate, but represents a specific interaction with propionate.

The specificity of the octanoate–propionate interaction was further tested with studies in which propionate was replaced with propionylcarnitine as the inhibitor of pyruvate metabolism. Propionylcarnitine enters the mitochondrial matrix via the carnitine translocase located in the inner mitochondrial membrane [36]. In the mitochondrial matrix propionylcarnitine is converted to propionyl-CoA by carnitine acetyltransferase [37], a reaction independent of propionyl-CoA synthetase activity. The observed inhibition of pyruvate oxidation by propionylcarnitine supports the hypothesis that propionyl-CoA generation is responsible for the impairment of pyruvate metabolism by propionate. The observation that octanoate reversed the inhibition by propionate of pyruvate oxidation more efficiently than the inhibition induced by propionylcarnitine suggests a specific interaction between octanoate and propionate, and that octanoate reverses the inhibitory effect of propionate on pyruvate metabolism by impairing propionyl-CoA formation.

The effects of propionate, octanoate and butyrate on the hepatocyte CoA pool were studied directly in isolated hepatocytes during pyruvate oxidation. Consistent with previous observations [1, 9, 31], addition of propionate resulted in accumulation of propionyl- and methylmalonyl-CoA at the expense of CoA and acetyl-CoA (Table 4). Addition of octanoate to the hepatocyte incubations in the presence of propionate decreased propionyl-CoA content by 44%, consistent with inhibition of propionyl-CoA formation as the mechanism by which octanoate decreases the inhibition by propionate of pyruvate oxidation. In contrast, addition of butyrate to hepatocyte incubations containing propionate had no effect on propionyl-CoA content. This is consistent with the lack of effect of butyrate on propionate oxidation (Table 2), the minimal response of butyrate on propionate's inhibition of pyruvate oxidation (Table 3) and the failure of butyrate to inhibit propionyl-CoA synthetase activity by greater than 80% (see above).

The current findings may have clinical importance in the management of patients with metabolic or liver disease when short-chain fatty acids absorbed from the gastrointestinal tract are potentially toxic [1, 2]. Since propionyl-CoA appears to be a mediator of propionate toxicity, the inhibition of propionyl-CoA formation may be beneficial in these situations. An alternative strategy to prevent propionate toxicity in these clinical situations has been oral administration of antibiotics to block bacterial production of short-chain fatty acids, but with the associated risks and expense of antibiotic therapy [2]. The low octanoate concentration necessary to reverse the inhibitory effect of propionate on pyruvate oxidation suggests that octanoate may be useful in these conditions at doses that minimize the known risk of side-effects from octanoate [38]. Alternatively, medium chain triglycerides, which are well tolerated by patients [39, 40], may be an efficient method for delivery of medium chain fatty acids to the liver [41].

Acknowledgements—We thank Dr. Charles L. Hoppel and

Dr. John Mieyal for their helpful comments on this manuscript and Ms. Laurie Nadler for performing important preliminary experiments. This work was supported by Grant DK 36069 from the National Institutes of Health. Eric P. Brass is a Burroughs Wellcome Scholar in Clinical Pharmacology and Stephan Krahenbuhl was supported by a grant of the Swiss National Science Foundation.

REFERENCES

1. Brass EP and Beyerinck RA, Effects of propionate and carnitine on the hepatic oxidation of short- and medium-chain-length fatty acids. *Biochem J* **250**: 819–825, 1988.
2. Bain MD, Jones M, Borriello SP, Reed PJ, Tracey BM, Chalmers RA and Stacey TE, Contribution of gut bacterial metabolism to human metabolic disease. *Lancet* **I**: 1078–1079, 1988.
3. Brass EP and Beyerinck RA, Interaction of propionate and carnitine metabolism in isolated hepatocytes. *Metabolism* **36**: 781–787, 1987.
4. Brass EP, Effect of alpha-ketobutyrate on palmitic acid and pyruvate metabolism in isolated rat hepatocytes. *Biochim Biophys Acta* **888**: 18–24, 1986.
5. Martin-Roquero A, Corkey BE, Cordan S, Walajtys-Rode E, Parilla RL and Williamson JR, Interactions between alpha-ketoisovalerate metabolism and the pathways of gluconeogenesis and urea synthesis in isolated hepatocytes. *J Biol Chem* **258**: 3673–3681, 1983.
6. Walajtys-Rode E, Coll KE and Williamson JR, Effects of branched-chain alpha-ketoacids on the metabolism of isolated rat liver cells. II. The interactions with gluconeogenesis and urea synthesis. *J Biol Chem* **254**: 11521–11529, 1979.
7. Glasgow AM and Chase HP, Effect of propionic acid on fatty acid oxidation and ureagenesis. *Pediatr Res* **10**: 683–686, 1976.
8. Walajtys-Rode E and Williamson JR, Effects of branched-chain alpha-ketoacids on the metabolism of isolated rat liver cells. *J Biol Chem* **255**: 413–418, 1980.
9. Patel TB, DeBuysere MS and Olson MS, The effect of propionate on the regulation of pyruvate dehydrogenase complex in the rat liver. *Arch Biochem Biophys* **220**: 405–415, 1983.
10. Ash R and Baird GD, Activation of volatile fatty acids in bovine liver and rumen epithelium. Evidence for control by autoregulation. *Biochem J* **136**: 311–319, 1973.
11. Groot PH and Scheek LM, Acyl-CoA synthetase in guinea-pig liver mitochondria. Purification and characterization of distinct propionyl-CoA synthetase. *Biochim Biophys Acta* **441**: 260–267, 1976.
12. Aas M, Organ and subcellular distribution of fatty acid activating enzymes in the rat. *Biochim Biophys Acta* **231**: 32–47, 1971.
13. Barth C, Sladek M and Decker K, Dietary changes of cytoplasmatic acetyl-CoA synthetase in different rat tissues. *Biochim Biophys Acta* **260**: 1–9, 1972.
14. Scholte HR and Groot PH, Organ and intracellular localization of short-chain acyl-CoA synthetases in rat and guinea-pig. *Biochim Biophys Acta* **409**: 283–296, 1975.
15. Flavin M, Castro-Mendoza H and Ochoa S, Metabolism of propionic acid in animal tissues. II. Propionyl-CoA carboxylation system. *J Biol Chem* **229**: 981–996, 1957.
16. Beck W, Flavin M and Ochoa S, Metabolism of propionic acid in animal tissues. III. Formation of succinate. *J Biol Chem* **229**: 997–1010, 1957.
17. Kaziro Y and Ochoa S, The metabolism of propionic acid. *Adv Enzymol* **26**: 283–379, 1964.
18. Roe CR, Hoppel CL, Stacey TE, Chalmers RA, Tracey BM and Millington DS, Metabolic response to carnitine in methylmalonic aciduria. *Arch Dis Child* **58**: 916–920, 1983.
19. Brass EP and Ruff L, Effect of carnitine on propionate metabolism in the vitamin B12 deficient rat. *J Nutr* **119**: 1196–1202, 1989.
20. Brass EP, Fennessey PV and Miller LV, Inhibition of oxidative metabolism by propionic acid and its reversal by carnitine in isolated hepatocytes. *Biochem J* **236**: 131–136, 1986.
21. Swartzentruber MS and Harris RA, Inhibition of metabolic processes by coenzyme-A-sequestering aromatic acids: Prevention by para-chloro- and para-nitrobenzoic acid. *Biochem Pharmacol* **36**: 3147–3153, 1987.
22. Hoppel C, DiMarco J and Tandler B, Riboflavin and rat hepatic cell structure and function. *J Biol Chem* **254**: 4164–4170, 1979.
23. Berry MN and Friend DS, High-yield preparation of isolated rat parenchymal cells. *J Cell Biol* **43**: 506–520, 1969.
24. Brass EP, Garrity M and Robertson RP, Inhibition of glucagon-stimulated hepatic glycogenolysis by E-series prostaglandins. *FEBS Lett* **169**: 293–296, 1984.
25. Man KC and Brosnan JT, Inhibition of medium and short-chain fatty acid oxidation in rat heart mitochondria by dichloroacetate. *Metabolism* **31**: 744–748, 1982.
26. Dixon M, The determination of enzyme inhibitor constants. *Biochem J* **55**: 170–171, 1953.
27. Armitage P and Berry G, *Statistical Methods in Medical Research*. Blackwell Scientific Publications, Oxford, 1987.
28. Pratt ML and Roche TE, Mechanism of pyruvate inhibition of kidney pyruvate dehydrogenase, kinase and synergistic inhibition by pyruvate and ADP. *J Biol Chem* **254**: 7191–7196, 1979.
29. Bremer J, Pyruvate dehydrogenase, substrate specificity and product inhibition. *Eur J Biochem* **8**: 535–540, 1969.
30. Agius L and Alberti KGMM. Regulation of flux through pyruvate dehydrogenase and pyruvate carboxylase in rat hepatocytes. Effects of fatty acids and glucagon. *Eur J Biochem* **152**: 699–707, 1985.
31. Corkey BE, Brandt M, Williams RJ and Williamson JR, Assay of short-chain acyl coenzyme A intermediates in tissue extracts by high-pressure liquid chromatography. *Anal Biochem* **118**: 30–41, 1981.
32. Allred JB and Guy DG, Determination of coenzyme A and acetyl-coenzyme A in tissue extracts. *Anal Biochem* **29**: 293–299, 1969.
33. Brass EP, Effect of propionyl-CoA on enzymic methods for the quantification of acetyl-CoA. *Biochem J* **256**: 1062–1063, 1988.
34. Michal G and Bergmeyer HU, Coenzyme A. In: *Methods of Enzymatic Analysis* (Ed. Bergmeyer HU), pp. 1972–1975. Academic Press, New York, 1974.
35. Aas M and Bremer J, Short-chain fatty acid activation in rat liver. A new assay procedure for the enzymes and studies on their intracellular localization. *Biochim Biophys Acta* **164**: 157–166, 1968.
36. Pande SV, A mitochondrial carnitine acylcarnitine translocase system. *Proc Natl Acad Sci USA* **72**: 883–887, 1975.
37. Hoppel CL and Brady L, Carnitine palmitoyltransferase. In: *The Enzymes of Biological Membranes* (Ed. Martonosi AM), pp. 139–175. Plenum Press, New York, 1985.
38. McCandless DW, Octanoic acid-induced coma and reticular formation energy metabolism. *Brain Res* **335**: 131–137, 1985.
39. Bach AC and Babayan VK, Medium-chain triglycerides: An update. *Am J Clin Nutr* **36**: 950–962, 1982.

40. Gracey M, Burke U and Anderson CM, Assessment of medium chain triglyceride feeding in infants with cystic fibrosis. *Arch Dis Child* **44**: 401–403, 1969.
41. Brass EP, Tserng KY and Eckel RH, Urinary organic acid excretion during feeding of medium-chain or long-chain triglyceride diets in patients with non-insulin dependent diabetes mellitus. *Am J Clin Nutr* **52**: 923–926, 1990.