Biochemical Pharmacology, Vol. 36, No. 19, pp. 3147–3153, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

INHIBITION OF METABOLIC PROCESSES BY COENZYME-A-SEQUESTERING AROMATIC ACIDS

PREVENTION BY PARA-CHLORO- AND PARA-NITROBENZOIC ACIDS

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(*Received* 29 *August* 1986; *accepted* 2 *February* 1987)

Abstract—Octanoate, salicylate, valproic acid, *p*-octyl-, *p*-nitro-, and *p*-chlorobenzoic acids were effective inhibitors of benzoic acid activation to benzoyl-CoA by mitochondrial extracts. *p*-Aminobenzoic acid was much less effective. Of these compounds, only salicylate and *p*-nitrobenzoic acid were not activated to their respective CoA esters. Salicylate, *p*-chloro- and *p*-nitrobenzoic acids effectively prevented inhibition of glucose synthesis and α-keto[1-^{14}C]isovalerate oxidation by valproic acid, *p*-octyl-, and *p*-aminobenzoic acids. *p*-Octyl- and *p*-aminobenzoic acids greatly depleted hepatocyte free CoA and acetyl-CoA contents and increased the content of acid-insoluble and acid-soluble CoA esters respectively. *p*-Chloro- and *p*-nitrobenzoic acids prevented the sequestration of CoA as *p*-octylbenzoyl-CoA or *p*-aminobenzoyl-CoA in hepatocytes incubated with these compounds. *p*-Chlorobenzoic acid not only prevented but also reversed the inhibition of gluconeogenesis in hepatocytes incubated with *p*-octylbenzoic acid. These results suggest that *p*-chloro- or *p*-nitrobenzoic acids might be effectively used to reverse some of the hepatotoxic effects of the CoA esters of valproic acid or naturally-occurring organic acids, such as those which accumulate in Reye's Syndrome or organic acidemias.

Animals metabolize benzoic acid and its derivatives in the liver and kidney by activation to aryl-CoA esters and subsequent conjugation with glycine to form hippurates [1]. Activation of aromatic acids is catalyzed by enzymes classed as butyryl-CoA synthetases [EC 6.2.1.2, butyrate:CoA ligase (AMP-forming)]. Two aromatic acid activating enzymes have been partially purified and characterized [2]. They differ in their substrate specificity, e.g. only one activates salicylate and the other is inhibited by salicylate [3]. Rat liver does not metabolize salicylate to salicylurate, implying that it does not contain the salicylate-activating enzyme, whereas guinea pigs as well as humans, metabolize salicylate primarily in the liver [4–6].

That rat liver contains only the salicylate-sensitive enzyme for activating medium chain length fatty acids and aromatic acids has been exploited previously by this laboratory to investigate the mechanism by which valproic acid inhibits various metabolic processes [7]. Salicylate effectively prevents the inhibition of metabolic processes in hepatocytes incubated with valproic acid by preventing valproyl-CoA formation. In a separate report†, the varying abilities of para-substituted benzoic acids to inhibit metabolic processes of isolated hepatocytes were described. *p*-Chloro- and *p*-nitrobenzoic acids were unique in that they did not alter the metabolic processes investigated. Whereas *p*-chlorobenzoic acid was activated to a limited extent, activation of *p*-nitrobenzoic acid by isolated hepatocytes was not detected. Since it is known that these two compounds, like salicylate, can be metabolized to their respective hippurates by some species [8–11], the results suggested that these compounds may inhibit activation of other aromatic acids. This would prevent the inhibition of metabolic processes by the other aromatic acids if the inhibitory effects are a consequence of aryl-CoA ester accumulation. If effective, these compounds could be potentially useful in reversing some of the hepatotoxic effects of the CoA esters of valproic acids as well as in investigations of the link between Reye's Syndrome and valproic acid treatment [12] or salicylate ingestion [13].

The purpose of the present study was to investigate the ability of *p*-nitro- and *p*-chlorobenzoic acids to prevent as well as to reverse *p*-octylbenzoic acid (OBA‡), *p*-aminobenzoic acid (ABA), and valproic acid sequestration of CoA and inhibition of glucose synthesis and flux through branched-chain α-ketoacid dehydrogenase (BCKDH) in isolated hepatocytes. In addition, the rate of activation of these aromatic acids by rat liver butyryl-CoA synthetase as well as the abilities of these compounds to inhibit benzoic acid activation were investigated.

METHODS

Isolation and incubation of hepatocytes. Hepatocytes were isolated from the livers of 48-hr fasted male Wistar rats by the method of Berry and Friend [14] with modifications from this laboratory [15].

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† M. S. Swartzentruber, S. A. McCune and R. A. Harris, unpublished results.

‡ Abbreviations: OBA, *p*-octylbenzoic acid; ABA, *p*-aminobenzoic acid; and BCKDH, branched-chain α-ketoacid dehydrogenase.

The hepatocytes were incubated in 2 ml of Krebs–Henseleit buffer containing 2.5% bovine serum albumin (charcoal-treated and dialyzed) in an atmosphere of 95% O_2/5% CO_2 (v/v) in stoppered, 25-ml Erlenmeyer flasks. Hepatocytes (25–30 mg wet weight/ml) were incubated with 10 mM lactate, 2 mM lysine, and other substrates, as indicated, for glucose synthesis and CoA and CoA ester measurement. Benzoic acid derivatives were added to the flasks as the sodium salt dissolved in water or were dissolved in acetone and added as described by Panek *et al.* [16]. Incubations were conducted in a shaking water bath (80–100 cycles/min) at 37° and, for most experiments, were terminated by the addition of 0.25 ml of 50% (w/v) $HClO_4$.

Metabolite assays. Glucose was assayed in KOH-neutralized $HClO_4$ extracts according to the method of Slein [17]. For the determination of CoA and CoA esters, incubations were terminated by centrifuging an aliquot of the hepatocyte incubation in an Eppendorf centrifuge for 30 sec. Supernatant fractions were discarded, and the hepatocytes were treated as described previously [18]. CoA and acetyl-CoA were determined according to Michal and Bergmeyer [19]; other acid-soluble CoA esters according to Williamson *et al.* [20]; and acid-insoluble CoA esters according to Garland [21].

Measurement of flux through BCKDH. Hepatocytes (12–15 mg wet weight/ml) were incubated in 25-ml Erlenmeyer flasks sealed with rubber serum caps fitted with hanging center wells. At 15 min, α-keto[1-^{14}C]isovalerate (85 cpm/nmol) was added to a final concentration of 0.2 mM, and the incubation was continued for an additional 10 min. The reaction was terminated, and $^{14}CO_2$ was collected and counted as described previously [22].

Aromatic acid activation assay. An extract of rat liver mitochondria, isolated by the method of Johnson and Lardy [23], was prepared by suspending 20 mg of mitochondrial protein in 1 ml of 100 mM Tris–HCl/0.5% Triton X-100, pH 8.0, on ice for 1 hr. This solution was then passed through a Sephadex G-25 column equilibrated with 100 mM Tris–HCl, pH 8.0. The void volume was collected and used as the source of aromatic acid activating enzyme. Benzoyl-CoA synthetase activity was assayed by a modification of an octanoyl-CoA synthetase assay described by Man and Brosnan [24]. The assay medium contained in a final volume of 1 ml at pH 8.0: 100 mM KCl, 50 mM Tris–Cl, 5 mM ATP, 5 mM $MgCl_2$, 0.5 mM CoA, 0.5 mM [8–^{14}C]sodium benzoate (250 cpm/nmol) and 0.3 to 0.5 mg mitochondrial protein. Aromatic acids were added as the sodium salts in neutralized aqueous solutions. The assay was performed at 30° and was initiated by addition of the mitochondrial extract. The reaction was terminated after 30 min by addition of 0.2 ml of 2 N HCl. After centrifugation, 1 ml of the supernatant fraction was transferred to a screw-top glass tube and extracted three times with 5 ml H_2O-saturated ether to remove the unreacted [7-^{14}C]benzoic acid. KOH (0.5 ml of 2 N) was then added, and the tubes were incubated at 55° for 45 min to hydrolyze the benzoyl-CoA. HCl (0.6 ml of 2 N) was added, and the [7-^{14}C]benzoic acid was extracted three times with 5 ml of H_2O-saturated ether. The ether extracts were evaporated from a scintillation vial at room temperature with a stream of air. Scintillation fluid (10 ml) and β-phenylethylamine/methanol (1/1, v/v) (1 ml) were added to each vial, and radioactivity was counted.

Rates of aryl-CoA ester synthesis were determined in incubations similar to the benzoyl-CoA synthetase assay. Aromatic acids (0.5 mM) were substituted for [7-^{14}C]benzoic acid, and the incubation temperature was 37°. Disappearance of CoA due to aromatic acid activation was followed by the reaction of CoA with 5,5′-dithiobis-(2-nitrobenzoic acid) [25].

Statistical analysis. The difference between conditions was analyzed for significance by Student's *t*-test for paired samples.

Sources of materials. Radioactive compounds were obtained from NEN Research Products (Boston, MA). *p*-Nitro- and *p*-chlorobenzoic acids were obtained from the Aldrich Chemical Co. (Milwaukee, WI). *p*-Octylbenzoic acid was obtained from the Fluka Chemical Corp. (Ronkonkoma, NY). Other enzymes and most biochemicals were from the Sigma Chemical Co. (St. Louis, MO).

RESULTS

Inhibition of benzoic acid activation by various aliphatic or aromatic acids. Aromatic acids inhibited activation of benzoic acid by mitochondrial extracts to varying degrees (Table 1). *p*-Chlorobenzoic acid, salicylate, OBA, and octanoate were very effective inhibitors of benzoic acid activation, whereas ABA was relatively ineffective. Although *p*-nitrobenzoic acid and salicylate were fairly potent inhibitors, they were virtually not activated to their respective aryl-CoA esters (Table 1). *p*-Chlorobenzoic acid, although activated at a rate similar to that of benzoic acid, was a potent inhibitor of benzoic acid activation. OBA was activated at a rate approximately double that of benzoic acid. This correlated well with its ability to inhibit benzoic acid activation. ABA was activated more slowly than benzoic acid and was not a very effective inhibitor of benzoic acid activation. Valproic acid was also not readily activated. Octanoate was much more rapidly activated than was benzoic acid, which is consistent with studies using the purified enzyme [2, 3].

Effects of various compounds on inhibition of glucose synthesis in hepatocytes incubated with OBA, ABA, or valproic acid. As determined previously, OBA*, ABA*, and valproic acid [7] were effective inhibitors of glucose synthesis by hepatocytes isolated from 48-hr fasted rats (Table 2). *p*-Chloro- and *p*-nitrobenzoic acids, even at 1 mM, were ineffective (Table 2). To elucidate the mechanism by which inhibition by OBA, ABA, and valproic acid occurred, several compounds with potential to prevent inhibition were tested (Table 2). Carnitine (5 mM) had no effect on the ability of either OBA or ABA to inhibit glucose synthesis, suggesting that carnitine esters cannot be formed from the CoA esters of these compounds. Carnitine also did not reverse valproic acid inhibition of metabolic pro-

* M. S. Swartzentruber, S. A. McCune and R. A. Harris, unpublished results.

Table 1. I_{50} for inhibition of benzoic acid activation and rates of activation of various acids by rat liver mitochondrial extracts

Acid	I_{50} for inhibition of benzoic acid activation* [μM]	Rate of acid activation† [nmol·min^{-1}·(mg protein)$^{-1}$]
Benzoic acid		4.7 ± 0.6
p-Chlorobenzoic acid	50	4.5 ± 0.1
p-Nitrobenzoic acid	175	ND‡
Salicylate	80	ND
p-Octylbenzoic acid	45	8.7 ± 0.5
p-Aminobenzoic acid	2000	3.3 ± 0.4
Valproic acid		3.8 ± 1.5
Octanoate	75	38.5 ± 0.9

* Values are from one experiment which was reproduced with another mitochondrial preparation.

† Values are means ± SE of three determinations.

‡ None detected. Limit at which activation could have been detected was approximately 0.5 nmol/min/mg protein.

cesses in a previous study [7]. Dichloroacetate (5 mM) slightly stimulated the rate of glucose synthesis in isolated hepatocytes, but it had no effect on the rate of glucose synthesis by isolated hepatocytes incubated with OBA. The inhibition caused by ABA at 0.1 mM was prevented by dichloroacetate, but at this concentration ABA is not a very effective inhibitor. At 1 mM ABA, the inhibition of glucose synthesis was virtually unaffected by dichloroacetate.

The inhibition by OBA was also unaffected by 10 mM glycine, whereas inhibition by ABA (0.1 mM) was reversed completely (data not shown). Reversal by glycine was only partial at 1 mM ABA (82 ± 16% of control with 10 mM glycine versus 57 ± 11% of control without glycine, means ± SE of six hepatocyte preparations). No conjugation of valproic acid to glycine could be demonstrated in a previous study [7].

Although salicylate (1 mM) slightly inhibited the rate of glucose synthesis by isolated hepatocytes, it effectively prevented the inhibition by OBA (0.075 and 0.25 mM) and ABA (0.1 and 1 mM) (Table 2). Likewise, *p*-nitro- and *p*-chlorobenzoic acids (1 mM) prevented the inhibition of glucose synthesis in hepatocytes incubated with OBA, ABA, or valproic acid.

Effects of salicylate, p-nitro- and p-chlorobenzoic acids on the inhibition of flux through BCKDH in hepatocytes incubated with OBA, ABA, or valproic acid. OBA (0.075 mM), ABA (1 mM), and valproic acid (1 mM) inhibited flux through BCKDH in isolated hepatocytes, whereas salicylate, *p*-nitro- and *p*-chlorobenzoic acids at 1 mM did not (Table 3). Valproic acid was the most effective, whereas OBA was the least effective inhibitor at the concentrations tested. Salicylate, *p*-nitro- and *p*-chlorobenzoic acids at 1 mM prevented the inhibition of flux through BCKDH in hepatocytes incubated with OBA, ABA, or valproic acid.

Effects of p-nitro- and p-chlorobenzoic acids on CoA and CoA ester distribution in hepatocytes incubated with OBA or ABA. OBA (0.075 mM) and ABA (1 mM) greatly depleted the free CoA and acetyl-CoA content of isolated hepatocytes, whereas neither *p*-nitrobenzoic acid (1 mM) nor *p*-chlorobenzoic acid (1 mM) had any significant effect (Table 4). Both *p*-nitro- and *p*-chlorobenzoic acids effectively prevented depletion of free CoA and acetyl-CoA content by OBA (0.075 mM) or ABA (1 mM). OBA-CoA is acid-insoluble; therefore OBA sequesters CoA as an acid-insoluble ester (Table 4). On the other hand, ABA-CoA is acid-soluble; therefore ABA sequesters CoA as an acid-soluble ester (Table

Table 2. Reversal by salicylate, *p*-chlorobenzoic acid and *p*-nitrobenzoic acid of the inhibition of glucose synthesis in hepatocytes incubated with *p*-octylbenzoic acid, *p*-aminobenzoic acid, or valproic acid

Addition (mM)	Rate of glucose synthesis [μmol·min^{-1}·(g wet weight)$^{-1}$]					
	Control	*p*-Octylbenzoic acid (0.075 mM)	*p*-Octylbenzoic acid (0.25 mM)	*p*-Aminobenzoic acid (0.1 mM)	*p*-Aminobenzoic acid (1 mM)	Valproic acid (1 mM)
None	1.22 ± 0.06	0.76 ± 0.05*	0.39 ± 0.05*	0.95 ± 0.03*	0.65 ± 0.02*	0.44 ± 0.05*
Carnitine (5)	1.21 ± 0.10	0.68 ± 0.12	0.35 ± 0.06	1.00 ± 0.08	0.62 ± 0.02	
Dichloroacetate (5)	1.41 ± 0.06*	0.70 ± 0.10	0.29 ± 0.06	1.39 ± 0.06†	0.62 ± 0.04	
Salicylate (1)	1.12 ± 0.08*	0.99 ± 0.11†	0.79 ± 0.06†	1.17 ± 0.08†	1.15 ± 0.06†	
p-Nitrobenzoic acid (1)	1.20 ± 0.06	1.14 ± 0.05†	0.86 ± 0.05†	1.27 ± 0.10†	1.11 ± 0.05†	0.98 ± 0.08†
p-Chlorobenzoic acid (1)	1.16 ± 0.05	1.06 ± 0.05†	0.82 ± 0.04†	1.08 ± 0.05†	1.08 ± 0.04†	0.94 ± 0.05†

Values are means ± SE of four to eight hepatocyte preparations.

* Significantly different from the no addition control ($P < 0.05$).

† Significantly different from the *p*-octylbenzoic acid, *p*-aminobenzoic acid, or valproic acid controls ($P < 0.05$).

Table 3. Effects of *p*-chlorobenzoic acid, *p*-nitrobenzoic acid, and salicylate on the inhibition by *p*-octylbenzoic acid, *p*-aminobenzoic acid, or valproic acid of α-ketoisovalerate oxidation by isolated hepatocytes

	Rate of α-Ketoisovalerate oxidation [nmol·min^{-1}·(g wet weight)$^{-1}$]			
Addition (1 mM)	Control	*p*-Octyl benzoic acid (0.075 mM)	*p*-Amino benzoic acid (1 mM)	Valproic acid (1 mM)
None	561 ± 68	324 ± 32*	155 ± 27*	78 ± 4*
Salicylate	448 ± 49	504 ± 67†	418 ± 42†	444 ± 58†
p-Nitrobenzoic	541 ± 65	497 ± 74†	463 ± 27†	376 ± 65†
p-Chlorobenzoic	503 ± 62	520 ± 70†	510 ± 66†	464 ± 48†

Values are means ± SE of four hepatocyte preparations.
* Significantly different from the no addition control ($P < 0.05$).
† Significantly different from the *p*-octylbenzoic acid, *p*-aminobenzoic acid, or valproic acid controls ($P < 0.05$).

4). In conjunction with the prevention of depletion of free CoA and acetyl-CoA, the accumulation of these aryl-CoA esters was also prevented by *p*-nitrobenzoic acid (1 mM) and *p*-chlorobenzoic acid (1 mM). Although *p*-chlorobenzoic acid was more effective than *p*-nitrobenzoic acid at preventing the accumulation of acid-soluble CoA esters in incubations of hepatocytes with ABA, both compounds were similarly effective at preventing accumulation of acid-insoluble CoA esters in incubations of hepatocytes with OBA.

Reversal by p-*chlorobenzoic acid of OBA inhibition of glucose synthesis.* *p*-Chlorobenzoic acid was found effective at both preventing and reversing OBA inhibition of gluconeogenesis (Fig. 1). Striking inhibition by OBA was apparent at the first time point (15 min) and continued over the entire time course of the experiment. As also shown in Table 2, addition of *p*-chlorobenzoic acid at time zero neutralized much of the inhibitory effect of OBA (Fig. 1), decreasing the inhibition from 68% with OBA alone to 33% when *p*-chlorobenzoic acid was also present. Adding *p*-chlorobenzoic acid at either 15 or 30 min after OBA also affected reversal of OBA inhibition, stimulating the rate between 45 and 60 min to 175% of the rate in the presence of OBA alone. Indeed, similar rates of gluconeogenesis were obtained regardless of the time of addition of *p*-

Table 4. Effect of *p*-chlorobenzoic acid and *p*-nitrobenzoic acid on CoA and CoA ester content of hepatocytes incubated with *p*-octylbenzoic acid or *p*-aminobenzoic acid

Metabolite	Acid addition (1 mM)	Control	*p*-Octyl benzoic acid (0.075 mM)	*p*-Amino benzoic acid (1 mM)
			(nmol/g wet weight)	
Free CoA	None	226 ± 47	64 ± 16*	18 ± 8*
	p-Nitrobenzoic	211 ± 30	130 ± 14†	82 ± 21†
	p-Chlorobenzoic	177 ± 21	164 ± 39†	179 ± 36†
Acetyl-CoA	None	83 ± 7	34 ± 3*	22 ± 2*
	p-Nitrobenzoic	97 ± 9	61 ± 6†	55 ± 6†
	p-Chlorobenzoic	74 ± 11	67 ± 3†	58 ± 14†
Acid-soluble CoA esters	None	20 ± 11	61 ± 6	280 ± 14*
	p-Nitrobenzoic	25 ± 11	44 ± 6	164 ± 23†
	p-Chlorobenzoic	42 ± 21	28 ± 15	63 ± 12†
Acid-insoluble CoA esters	None	58 ± 7	214 ± 7*	52 ± 4
	p-Nitrobenzoic	44 ± 2	111 ± 7†	48 ± 5
	p-Chlorobenzoic	48 ± 3	110 ± 8†	52 ± 9

Values are means ± SE of four hepatocyte preparations.
* Significantly different from no addition control ($P < 0.05$).
† Significantly different from the *p*-octylbenzoic acid or *p*-aminobenzoic acid control ($P < 0.05$).

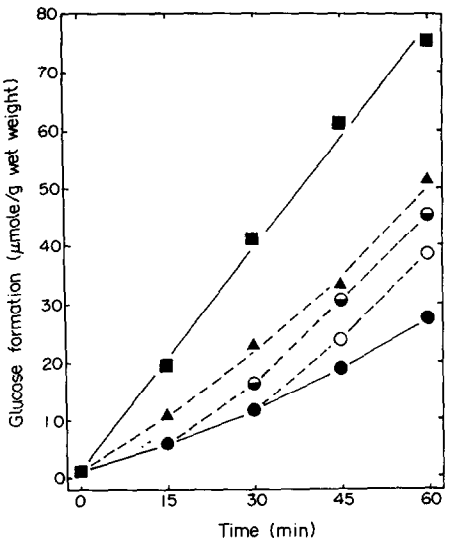

Fig. 1. Reversal by *p*-chlorobenzoate acid of the inhibition of glucose synthesis in hepatocytes incubated with OBA. Key: (■) no addition control; (●) OBA (0.25 mM) present from $t = 0$; (▲) OBA (0.25 mM) and *p*-chlorobenzoic acid (1 mM) both added at $t = 0$; (◒) OBA added at $t = 0$, *p*-chlorobenzoic acid at $t = 15$; (○) OBA added at $t = 0$, *p*-chlorobenzoic acid at $t = 30$. Dashed lines indicate samples containing OBA with *p*-chlorobenzoic acid added at 0, 15, or 30 min. Data are from one experiment which was reproduced with another hepatocyte preparation.

chlorobenzoic acid, indicating that *p*-chlorobenzoic acid was as effective at reversing as it was at preventing OBA inhibition of the process.

DISCUSSION

Salicylate, *p*-nitro- and *p*-chlorobenzoic acids prevented the inhibition of glucose synthesis by hepatocytes incubated with OBA, ABA, or valproic acid. Inhibition of glucose synthesis by hepatocytes incubated with OBA, ABA, or valproic acid was concurrent with a fall in free CoA and acetyl-CoA contents and a rise in the acid-insoluble (with OBA) or acid-soluble (with ABA and valproic acid) CoA esters. The alteration of the distribution of CoA and esters was also prevented by *p*-nitro- and *p*-chlorobenzoic acids. That OBA, ABA, and valproic acid sequester CoA as the respective CoA esters, thereby depleting the cell of acetyl-CoA, an activator of pyruvate carboxylase [26], suggests that inhibition of pyruvate carboxylase may be the mechanism by which these compounds inhibit glucose synthesis from lactate. That salicylate and *p*-nitrobenzoic acid, which are virtually not activated by rat liver mitochondrial extracts, prevent the inhibitory effects of OBA, ABA, and valproic acid and also prevent their activation to CoA esters support the hypothesis that activation of the latter compounds is required for inhibition of glucose synthesis to occur.

Although salicylate, *p*-nitro- and *p*-chlorobenzoic acids are potent inhibitors of benzoic acid activation by mitochondrial extracts, only *p*-chlorobenzoic acid was activated at a detectable rate. This is consistent with our previous finding that small amounts of *p*-chlorobenzoyl-CoA were present in hepatocytes incubated with *p*-chlorobenzoic acid.* That the CoA and CoA ester distribution of hepatocytes incubated with *p*-chlorobenzoic acid was not altered significantly suggests that some mechanism exists to prevent extensive CoA sequestration by this compound. That *p*-chlorobenzoyl-CoA apparently accumulates in incubations of mitochondrial extracts but does not accumulate to high concentrations in isolated hepatocytes indicates a major difference in regulation of CoA sequestration in these two systems. Any mechanism that prevents excessive sequestration of mitochondrial matrix space CoA would be of physiological significance. Indeed, benzoic acid also sequesters CoA poorly in hepatocytes [18] relative to the ability of mitochondrial extracts to form benzoyl-CoA (present study). It has been proposed that non-specific acyl-CoA hydrolases partially protect mitochondria against sequestration of CoA by carboxylic acids [27]. Whether the futile cycle established by hydrolase activity prevents extensive *p*-chlorobenzoyl- and benzoyl-CoA accumulation cannot be determined from the data available at this time.

p-Chlorobenzoic acid both prevented and reversed the inhibitory effects of OBA on gluconeogenesis. Prevention can be explained by simple inhibition of OBA-CoA formation. Reversal demands a process by which CoA can be recycled from OBA-CoA. This could be accomplished by the non-specific hydrolase discussed above. The hydrolase may not have sufficient activity towards OBA-CoA to prevent CoA sequestration in hepatocytes incubated with this aromatic acid. However, the inhibition of the aromatic acid activating enzyme by *p*-chlorobenzoic acid would increase the effectiveness of the hydrolase and allow the escape of sequestered CoA.

Dichloroacetate did not prevent the inhibition of glucose synthesis in hepatocytes incubated with OBA or ABA. Dichloroacetate dramatically inhibits oxidation of octanoate in heart but is much less effective in liver [24], suggesting that different enzymes are involved in octanoate activation in the two tissues. The data of the present study indicate that the aromatic acid activating enzyme in liver, which is probably the same enzyme responsible for octanoate activation in this tissue, is not inhibited by dichloroacetate.

Two different butyryl-CoA synthetases have been shown to activate aromatic acids to aryl-CoA esters. One is sensitive to inhibition by, and does not activate, salicylate. The other activates salicylate as well as many other aromatic acids [2, 3]. *p*-Nitrobenzoic acid was found to be similar to salicylate in that it inhibits but is not activated by the rat liver mitochondrial enzyme. There is evidence that *p*-nitrobenzoic acid is excreted as the glycine conjugate by rat [10], perhaps reflecting the capacity of the salicylate-activating enzyme of rat kidney to activate

* M. S. Swartzentruber, S. A. McCune and R. A. Harris, unpublished results.

this compound. *p*-Chlorobenzoic acid is apparently activated by the salicylate-sensitive enzyme, but it, or its CoA ester, potently inhibits the activation of other aromatic acids. *p*-Chlorobenzoic acid is also excreted as its hippurate in some species [8, 11]. Indeed, rat liver mitochondrial extracts will synthesize *p*-chlorohippurate.*

p-Nitrobenzoic acid is metabolized to *p*-nitrohippurate in humans [9]. No information on the metabolism of *p*-chlorobenzoic acid by humans is available. The butyryl-CoA synthetase which activates salicylate has been little studied. Whether these compounds will inhibit activation of carboxylic acids by this enzyme is not known. Since human liver contains both aromatic acid activating enzymes [4, 5], studies of the salicylate-activating enzyme, particularly in humans, are needed. Guinea pig liver apparently contains primarily the salicylate-activating enzyme [6] and might be a better model than rat liver for studies of the conjugation of aromatic acids in the human liver.

This study has demonstrated the utility of *p*-chloro- and *p*-nitrobenzoic acids as inhibitors of a butyryl-CoA synthetase activity in rat liver. Although salicylate has been a useful inhibitor for studying this enzyme, the broad effects that salicylate has on the metabolism of liver [28–30], particularly uncoupling of oxidative phosphorylation, complicate its use with isolated hepatocytes. In contrast, *p*-nitro- and *p*-chlorobenzoic acids inhibit this enzyme without significantly affecting any of the hepatocyte metabolic processes studied to date.

Valproic acid, as well as salicylate, have been linked to the development of a Reye's-like syndrome in children [12, 13]. 4-Pentenoate and methylene cyclopropylacetate, both medium chain fatty acids that yield CoA esters, also cause a Reye's-like syndrome [31]. It has been proposed that Reye's Syndrome is precipitated by some, as yet unknown, toxic agent which causes acute damage to the liver, thereby limiting the ability of this organ to oxidize fatty acids and to maintain the blood glucose level during fasting [32]. In response to hypoglycemia, lipids are released into the bloodstream. Short and medium chain fatty acids are elevated both in the blood and liver of these patients [32, 33]. The primary fatty acids appearing in the blood are propionic, butyric, isobutyric, and isovaleric acids [34]. Butyryl-CoA and isovaleryl-CoA have been shown to inhibit pyruvate carboxylase from pig liver [31] and, as isobutyryl-CoA, propionyl-CoA, and isovaleryl-CoA accumulate in rat hepatocytes, the rate of glucose synthesis falls [35]. Thus, accumulation of CoA esters of these fatty acids further aggravates the hypoglycemia and consequently the release of fatty acids in these patients. Some of these fatty acyl-CoA esters have also been shown to inhibit carbamoyl phosphate synthase I, the rate-limiting step in urea synthesis [35]. Therefore, the hyperammonemia of Reye's Syndrome patients is also aggravated by the metabolism of the fatty acids to their CoA esters.

We propose that *p*-nitro- or *p*-chlorobenzoic acids may prove useful clinically to prevent and reverse the accumulation of toxic levels of acyl- and aryl-CoA esters. If the hepatic damage in Reye's Syndrome is due to the dead-end formation of the CoA esters of acyl- and aryl-CoA esters, then *p*-chloro- or *p*-nitrobenzoic acids may block their formation, thereby relieving the inhibitions of gluconeogenesis and urea synthesis and preventing some of the damage to the liver. That *p*-chlorobenzoic acid can reverse the inhibition once it has begun suggests that such compounds may be useful in reversing toxic effects of CoA-sequestering compounds. A cautionary note, however, must be added. Infusions of butyrate, valerate, and octanoate have been shown to cause coma, seizures, and EEG abnormalities in rabbits [36]. Whether these effects are due to the acids themselves or to their CoA esters is not known. Indeed, it is important to note that no evidence of the formation of valproyl-CoA from valproic acid in rat brain mitochondria was found in a previous study [7]. If the effects on the brain are due to the acids themselves, the proposed treatment of patients with these compounds could restore the liver and relieve the hyperammonemia and hypoglycemia while aggravating direct effects on the brain. Recovery from Reye's Syndrome has been correlated with the disappearance of short chain fatty acids from the blood [37]. Since this treatment would prevent activation of these fatty acids by the liver, their removal would be dependent upon metabolism by other tissues (such as the heart), excretion as the sodium salt or glucuronide, and formation of triglyceride in fat tissue. On the other hand, if the effects on the brain are due at least in part to the accumulation of CoA esters in this tissue or are primarily due to the hyperammonemia or hypoglycemia, use of these compounds could relieve the damage in both tissues. The ability of benzoic acid to relieve the hyperammonemia of patients with urea cycle [38] or pyruvate dehydrogenase defects [39] has already been demonstrated. Clearly, a better understanding of the etiology of Reye's Syndrome and of the enzymology of the aromatic and aliphatic acid activating enzymes is required.

Acknowledgements—Excellent technical assistance was provided by Ms. Pat Jenkins, and expert typing by Ms. Peggy Smith. This research was supported in part by grants from the U.S. Public Health Service (AM19259 and 21178), the Grace M. Showalter Residuary Trust, and the Abbott Laboratories (North Chicago, IL) Fund.

* M. S. Swartzentruber and R. A. Harris, unpublished results.

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