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Effect of Pent-4-enoic Acid, Propionic Acid and Other Short-Chain Fatty Acids on Citrulline Synthesis in Rat Liver Mitochondria

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1. The effect of pent-4-enoic acid, propionic acid and several other short-chain fatty acids on citrulline synthesis in rat liver mitochondria was studied. 2. Pent-4-enoate at 1mM inhibited mitochondrial citrulline synthesis by about 80–90%. It is concluded that pent-4-enoate inhibits citrulline synthesis by interfering with some aspect of mitochondrial energy metabolism. This results in impairment of mitochondrial ornithine uptake or depletion of mitochondrial ATP, which, in turn, impairs carbamoyl phosphate synthesis or both. Evidence in support of this conclusion includes: pent-4-enoate has no effect on citrulline synthesis supported by succinate or exogenous ATP; pent-4-enoate lowers the medium plus mitochondrial ATP concentration; finally, when glutamate is the oxidizable substrate, pent-4-enoate decreases the carbamoyl phosphate concentration in mitochondria incubated without ornithine to minimize citrulline synthesis and impairs the mitochondrial uptake of ornithine, but it has neither effect when succinate is the oxidizable substrate. 4. Propionate, butyrate and crotonate also inhibit mitochondrial citrulline synthesis, but much less than pent-4-enoate. 5. Acetate, pentanoate, pent-2-enoate, hexanoate, octanoate, isovalerate, tiglylate and α-methylbutyrate have little or no effect on mitochondrial citrulline synthesis.

Pent-4-enoic acid is an analogue of hypoglycin, the compound thought to cause Jamaican vomiting sickness (Sherratt, 1969). Reye's syndrome is an increasingly recognized acute encephalopathy of childhood (Reye *et al.*, 1963; Glasgow *et al.*, 1972), and pent-4-enoate will produce many of the features of this disorder in rats (Glasgow & Chase, 1975*a*). Pent-4-enoate is a potent inhibitor of fatty acid oxidation (Holland & Sherratt, 1973; Holland *et al.*, 1973), which may account for the ability of this compound to inhibit gluconeogenesis *in vitro* and produce hypoglycaemia *in vivo* (Ruderman *et al.*, 1970). It has also been shown to inhibit ureogenesis from ammonia in rat slices (Glasgow & Chase, 1975*b*). The mechanism of the inhibition of ureogenesis is unknown. The present paper reports experiments attempting to elucidate the mechanism.

The effects of propionic acid and several other short-chain fatty acids on citrulline synthesis have also been studied. Propionate is of particular interest with regard to ureogenesis because of the occurrence of unexplained hyperammonaemia in children with propionic acidaemia (Shih & Efron, 1972).

Materials and Methods

Animals

Adult male Sprague–Dawley rats, from Simonsen Laboratories, Gilroy, CA, U.S.A., were used in all these experiments and were allowed free access to Purina Rat Chow. Animals were killed by decapitation.

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Chemicals and enzymes

Pent-4-enoic acid was obtained from Research Organic/Inorganic Chemical Co., Sun Valley, CA, U.S.A. This was purified as described by Glasgow & Chase (1975*a*). Tigylic acid was obtained from Pfaltz and Bauer, Flushing, NY, U.S.A.; α-methylbutyric acid, pent-2-enoic acid, crotonic acid and hexanoic acid were from K & K Laboratories, Hollywood, CA, U.S.A., and octanoic acid was from Eastman Chemical Co., Rochester, NY, U.S.A. DL-[1-^{14}C]Ornithine (47 μCi/μmol) and Aquasol were obtained from New England Nuclear Corp., Boston, MA, U.S.A. All other organic chemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Measurement of citrulline synthesis

Mitochondria isolated by the procedure of Myers & Slater (1957) were incubated in 1ml of medium containing (additions and exceptions are indicated in the legends to the Tables) 15mM-KCl, 2mM-EDTA, 5mM-$MgCl_2$, 50mM-Tris/HCl buffer, 10mM-ornithine, 16.6mM-$KHCO_3$, 3mM-ATP, 5mM-potassium phosphate buffer and 25mM-sucrose. Incubation

was at 25°C at pH7.4 under O_2+CO_2 (95:5). The procedure of Charles *et al.* (1967) was followed except that the reaction mixture was not given a preliminary gassing, 0.1ml of 3M-$HClO_4$ was used to stop the reaction and citrulline was measured after 30min of heating at 100°C. A standard curve for citrulline was run daily.

Measurement of ATP

Mitochondria were incubated as for the measurement of citrulline synthesis. Immediately after the addition of $HClO_4$ the samples were placed on ice and kept at 0–4°C until the final assay of ATP, which was done within 30min. The concentration of ATP was measured fluorimetrically (Lowry *et al.*, 1964) in sample of KOH-neutralized $HClO_4$ supernatant. A blank that did not contain $MgCl_2$ or glucose was subtracted for each sample. A standard curve was run simultaneously.

Measurement of carbamoyl phosphate

Mitochondria were incubated as for the measurement of citrulline synthesis, except that the volume of medium was 2ml, incubation was in a 25ml Erylenmeyer flask and ornithine was omitted. Immediately after the addition of $HClO_4$ the samples were placed on ice and maintained at 0–4°C until the final assay, which was done within 15min. Carbamoyl phosphate was measured by incubating, at 37°C for 10min, a 0.6ml portion of KOH-neutralized $HClO_4$ extract in a final volume of 1ml, containing the following (final concentrations given): 0.2M-Tris/HCl, pH8.0, 0.2mM-ornithine, 0.1μCi of DL-[1-^{14}C]ornithine and 400 units (μmol/h) of ornithine carbamoyltransferase. The reaction was stopped with 0.1ml of 90% (v/v) formic acid, and, after addition of carrier citrulline, 0.1ml of the mixture was spotted on Whatman paper (thickness 0.33mm, medium flow rate). This was run overnight by descending chromatography with butanol/acetic acid/water (12:3:5, by vol). After drying, the citrulline, located by staining a parallel run of standard, was cut out and placed in 15ml of scintillation mixture (1.6litre of toluene, 1.6litre of dioxan, 1litre of ethanol, 0.208g of 2-(1-naphthyl)-5-phenyloxazole, 0.208g of 2,5-diphenyloxazole and 322.8g of naphthalene). Radioactivity was measured with a Pickner nuclear liquid-scintillation counter, with 83–85% efficiency. Carbamoyl phosphate for standards was weighed, and, immediately before use, dissolved in an appropriate volume of ice-cold water. This procedure gave a straight-line standard curve up to 50nmol of carbamoyl phosphate/sample, but a value that was low by about 10% at 100nmol. At 50nmol of carbamoyl phosphate the conversion of [^{14}C]-ornithine into [^{14}C]citrulline was 96% of the theoretical value, assuming that D-[^{14}C]ornithine was unreactive.

Measurement of mitochondrial ornithine uptake

This procedure was modified from that of Gamble & Lehninger (1973). Mitochondria, prepared as for measurement of citrulline synthesis, were incubated in 1ml of medium containing (final concentrations): 0.25M-sucrose, 5mM-potassium buffer, final pH7.4, 10mM-glutamate or -succinate, 2mM-ornithine and 0.2μCi of DL-[1-^{14}C]ornithine. The mitochondria were incubated in centrifuge tubes for 15min under air at 25°C. The reaction was stopped by centrifugation, with 2min to reach maximum speed, then 1min at 18000*g* and 2min to stop. The supernatant was discarded and the tube and surface of the pellet were washed once with 1ml of ice-cold 0.25M-sucrose. The sides of the tube were blotted with a cotton swab. The tube was washed out with 3×1ml of Aquasol, the pellet being disrupted and transferred at the same time. The sample was counted in a final volume of 10ml of scintillation fluid. To allow for ornithine in the non-matrix water and perhaps some uptake during centrifugation a blank consisting of mitochondria that had been added to the medium and immediately centrifuged was subtracted. To calculate the uptake in mol it was assumed that only the L-ornithine of the DL-[1-^{14}C]ornithine was taken up, since Gamble & Lehninger (1973) did not find any uptake of D-ornithine by mitochondria. Counting efficiency was 90–91%, as determined by an internal [^{14}C]toluene standard.

Protein

Protein was measured by the biuret reaction after removing lipid (Cleland & Slater, 1953), with bovine serum albumin as standard.

Results

The effect of a number of short-chain fatty acids on mitochondrial citrulline synthesis is shown in Table 1. Pent-4-enoate at a concentration of 1mM and 5mM, but not at a concentration of 0.1mM, markedly inhibited citrulline synthesis. Three of the other short-chain fatty acids propionate, butyrate, crotonate also inhibited at a concentration of 5mM but the inhibition was much less than with pent-4-enoate. Acetate, isovalerate and pent-2-enoate at 5mM perhaps inhibited slightly but the effect was small. Tiglylate and methylbutyrate at 5mM apparently stimulated citrulline synthesis. Pentanoate, hexanoate and octanoate had no effect.

The time-course for citrulline synthesis with no inhibitor and with 1mM-pent-4-enoate and 5mM-

Table 1. *Effect of pent-4-enoate and other short-chain fatty acids on citrulline synthesis in rat liver mitochondria*

In addition to the basal medium the reaction mixture contained 10mM-NH_4Cl and 10mM-glutamate. Each flask contained 5.4, 3.6, 4.1 and 2.9mg of protein in Expts. 1, 2, 3 and 4 respectively. Incubation time was 60min. Results are expressed in μmol of citrulline produced/h per mg of mitochondrial protein. The two to four values for no addition and for duplicate incubations at each fatty acid concentration are given to show the reproducibility of the method.

				Citrulline synthesis		
	No addition	Fatty acid	Concn. (mM) ...	0.1	1	5
Expt. 1		Pent-4-enoate		1.13	0.17	0.14
	1.18			1.14	0.19	0.15
	1.24					
	1.26	Propionate		1.16	1.05	0.78
	1.11			1.26	1.01	0.80
		n-Butyrate		1.15	1.14	0.89
				1.14	1.14	0.87
Expt. 2		Acetate		1.65	1.67	1.45
	1.55			1.67	1.55	1.38
	1.64	n-Pentanoate		1.61	1.52	1.54
	1.55			1.62	1.42	1.45
	1.62	Isovalerate		1.55	1.52	1.39
				1.59	1.59	1.42
Expt. 3		Tiglylate		—	—	1.74
				—	—	1.79
		Methylbutyrate		—	—	1.75
				—	—	1.65
	1.44					
	1.42	n-Hexanoate		—	—	1.46
				—	—	1.26
		n-Octanoate		—	—	1.44
				—	—	1.43
Expt. 4		Pent-4-enoate		—	—	0.18
				—	—	0.17
	1.24	Pent-2-enoate		—	—	1.11
	1.28			—	—	1.19
		Crotonate		—	—	0.99
				—	—	0.93

propionate is shown in Fig. 1. Charles *et al.* (1967) found a similar initial lag in citrulline synthesis.

Table 2 shows the effect of pent-4-enoate on citrulline synthesis in the presence of various oxidizable substrates. Pent-4-enoate had no effect when succinate was the oxidizable substrate. The inhibition was similar with all other oxidizable substrates, including glutamate, pyruvate, citrate, fumarate and malate. There was no inhibition when both succinate and glutamate were present. Citrulline synthesis with 1mM-palmitate [in the presence of 3% (w/v) essentially fatty acid-free bovine serum albumin] was similar to that obtained in other experiments (not shown) when no oxidizable substrate was added. Pent-4-enoate at 1mM, but not at 0.1mM (not shown), inhibited citrulline synthesis when 1mM-palmitate was present.

Propionate (5mM) inhibited with all oxidizable substrates tested, including succinate (Table 3).

Malonate (Table 3) inhibited citrulline synthesis with succinate as substrate, showing that it is the oxidation of succinate that supports citrulline synthesis.

In the experiments on citrulline synthesis reported here ATP was added. Charles *et al.* (1967) have shown that exogenous ATP, although necessary for optimal citrulline synthesis, will not support it directly; ATP must be generated intramitochondrially by substrate oxidation. However, Graafmans *et al.* (1968) have shown that, in the presence of oligomycin and dinitrophenol, exogenous ATP can be utilized for citrulline synthesis; as shown in Table 4, pent-4-enoate had no effect on citrulline synthesis under these conditions. However, propionate was still inhibitory. In one part of this experiment mitochondria were preincubated with pent-4-enoate to allow for the possibility that the formation of the CoA derivative might be necessary for the

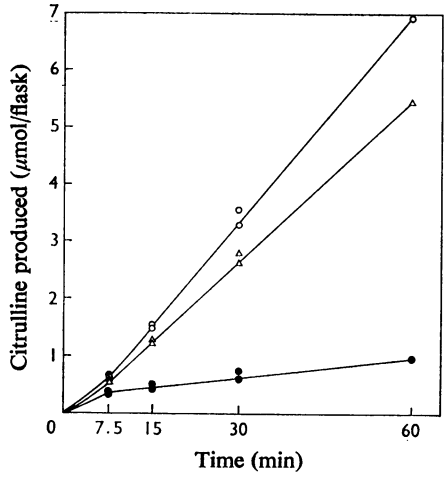

Fig. 1. *Time-course of citrulline production by rat liver mitochondria with no inhibitor* (○), *5mM-propionate* (△), *or 1mM-pent-4-enoate* (●)

Conditions were the same as in Table 1. Each point represents a separate flask in which the incubation was stopped at the indicated time. Each flask contained 3.8mg of protein.

Table 2. *Effect of pent-4-enoate on mitochondrial citrulline synthesis in the presence of various oxidizable substrates*

Conditions were the same as in Table 1 except that other oxidizable substrates (all 10mM except palmitate) replaced glutamate where indicated. The albumin was essentially fatty acid-free bovine serum albumin. Each flask contained 3.5 or 2.0mg of protein in Expts. 1 and 2 respectively. Results are the means from duplicate incubations in μmol of citrulline produced/h per mg of mitochondrial protein.

	Substrate	No addition	1mM-Pent-4-enoate
Expt. 1	Glutamate	1.46	0.24
	Succinate	1.00	1.05
	Pyruvate (no 'sparker')	0.69	0.16
	Citrate	1.10	0.25
	Glutamate + succinate	1.16	1.13
Expt. 2	Fumarate	0.63	0.28
	Malate	0.47	0.29
	1mM-palmitate + 3% albumin	0.27	0.07

inhibition of citrulline synthesis. Pent-4-enoate will not inhibit fatty acid oxidation unless the CoA derivative is formed, and dinitrophenol will prevent the formation of the CoA derivative (Holland & Sherratt, 1973).

Table 5 shows the effect of pent-4-enoate on ATP concentrations in the medium plus mitochondria. Citrulline synthesis was somewhat less with the 1mM-ADP used in this experiment than with the usual 3mM-ATP, but was more than with no adenine nucleotide. Pent-4-enoate at 1mM decreased ATP

Table 3. *Effect of propionate and malonate on mitochondrial citrulline synthesis in the presence of various oxidizable substrates*

Conditions were the same as in Table 1 except that other oxidizable substrates (all 10mM) replaced glutamate where indicated. Each flask contained 4.0mg of protein in Expt. 1 and 2.8mg in Expt. 2. Results are the means from duplicate incubations in μmol of citrulline produced/h per mg of mitochondrial protein.

	Substrate	No addition	5mM-Propionate	10mM-Malonate
Expt. 1	Glutamate	1.25	0.72	—
	Succinate	0.59	0.49	—
	Citrate	1.06	0.47	—
	Fumarate	0.50	0.39	—
Expt. 2	Succinate	0.99	—	0.24
	Glutamate	1.75	—	1.05

Table 4. *Effect of pent-4-enoate and propionate on citrulline synthesis in the presence of oligomycin and dinitrophenol*

Each flask contained the basal medium plus 10mM-NH_4Cl, 10mM-glutamate and 1% (v/v) ethanol. Each flask contained 4.7mg of protein in Expt. 1, and 3.5mg of protein in Expt. 2. Incubation time was 30min. For the preincubation with pent-4-enoate mitochondria were incubated for 10min at 25°C in medium from which ornithine had been omitted. During this incubation the pent-4-enoate concentration was 1.18mM. Then oligomycin, dinitrophenol and ornithine were added so that the final concentration of pent-4-enoate was 1mM, and a further 30min incubation was performed.

	Additions	Citrulline produced (μmol/h per mg of mitochondrial protein)
Expt. 1	None	1.22
	Oligomycin (10μg)	0.04
	2,4-Dinitrophenol (0.04mM)	0.05
	Pent-4-enoate (1mM)	0.17
	Oligomycin + pent-4-enoate	0.01
	Oligomycin + dinitrophenol	0.40
	Oligomycin + dinitrophenol + pent-4-enoate	0.41
	Oligomycin + dinitrophenol + pent-4-enoate*	0.50
Expt. 2	None	1.33
	Oligomycin	0.12
	Dinitrophenol	0.24
	Propionate (5mM)	1.15
	Oligomycin + dinitrophenol	0.60
	Oligomycin + dinitrophenol + propionate	0.35

* Preincubated with pent-4-enoate.

concentrations about 40%, an effect less than that of oligomycin but similar to that of dinitrophenol; and pent-4-enoate inhibited citrulline synthesis to roughly the same extent as dinitrophenol. It should be noted that, in this experiment, medium plus mitochondrial ATP was measured, whereas the mitochondrial concentration is important in at least one step of citrulline synthesis, the synthesis of carbamoyl phosphate. In another experiment (not shown) pent-4-enoate decreased medium plus mitochondrial ATP concentrations by about 20% in mitochondria incubated for 30min with 3mM-ATP and with glutamate as the oxidizable substrate, but not with succinate.

Table 5. *Effect of pent-4-enoate on medium plus mitochondrial ATP concentrations*

Conditions were the same as in Table 1 except that where indicated 1mM-ADP replaced 3mM-ATP or ATP was omitted. The incubation time was 30min. Each flask contained 3.2mg of mitochondrial protein. Duplicate incubation values are given for ATP.

	Citrulline formation (μmol/h per mg of protein)	ATP (μmol/flask)
3mM-ATP	1.08	—
No adenine nucleotide	0.45	—
1mM-ADP	0.81	1.00, 0.90
1mM-ADP+1mM-pent-4-enoate	0.15	0.54, 0.62
1mM-ADP+10μg of oligomycin	0.03	0.27, 0.27
1mM-ADP+0.04mM-2,4-dinitrophenol	0.10	0.62, 0.68

Pent-4-enoate at 1mM resulted in a lower concentration of carbamoyl phosphate in mitochondria, incubated without ornithine to minimize citrulline synthesis, when glutamate was the oxidizable substrate, but not with succinate (Table 6). Propionate at 5mM also may have caused a slightly decreased concentration when glutamate was the oxidizable substrate (Table 6).

Pent-4-enoate at 1mM also inhibited the mitochondrial uptake of ornithine when glutamate was the oxidizable substrate, but not with succinate (Table 7). Propionate at 5mM had little, if any, effect on the mitochondrial uptake of ornithine when glutamate was the oxidizable substrate.

Discussion

Two types of evidence suggest that the inhibition of ureogenesis by pent-4-enoate occurs at a stage preceding the synthesis of citrulline. Pent-4-enoate inhibits ureogenesis from citrulline and aspartate much less than from ammonia (Glasgow & Chase, 1975*b*), and it inhibits citrulline synthesis when this process is isolated.

The synthesis of citrulline could be inhibited by one of several mechanisms: inhibition of carbamoyl phosphate synthetase or ornithine carbamoyltransferase, impairment of the transport of ornithine or citrulline across the mitochondrial membrane or substrate or cofactor depletion.

Pent-4-enoate has no direct effect on carbamoyl phosphate synthetase or ornithine carbamoyltransferase assayed in rat liver homogenates (Glasgow & Chase, 1975*b*). Although an enzyme inhibition by a metabolic derivative of pent-4-enoate has not been

Table 6. *Effect of pent-4-enoate and propionate on mitochondrial carbamoyl phosphate concentration*

Mitochondria were incubated in 2ml of basal medium without ornithine to which 10mM-NH_4Cl and 10mM oxidizable substrate were added. Each flask contained 4.5mg of protein in Expt. 1 and 5.9mg of protein in Expt. 2. Incubation was for 15min. The results from duplicate incubations show the carbamoyl phosphate concentration in μmol/mg of mitochondrial protein.

	Substrate	No addition	1mM-Pent-4-enoate	5mM-Propionate
Expt. 1	Glutamate	0.032, 0.034	0.020, 0.020	
	Succinate	0.042, 0.046	0.043, 0.041	
Expt. 2	Glutamate	0.042, 0.038		0.029, 0.034

Table 7. *Effect of pent-4-enoate and propionate on mitochondrial ornithine uptake*

Mitochondria were incubated in 1ml of medium containing 0.25M-sucrose, 5mM-potassium phosphate buffer, pH7.4, 10mM-glutamate or -succinate and 2mM-ornithine. Each flask contained 3.7 or 3.6mg of protein in Expts. 1 and 2 respectively. Incubation was for 15min at 25°C. Results from duplicate incubations are in nmol of ornithine uptake/flask.

	Substrate	No addition	1mM-Pent-4-enoate	5mM-Propionate
Expt. 1	Glutamate	35.1, 37.4	10.1, 11.6	29.8, 35.6
Expt. 2	Succinate	14.1, 13.9	16.6, 16.9	

directly excluded it seems unlikely that both succinate and oligomycin plus dinitrophenol would reverse an enzyme inhibition. It also seems unlikely that both succinate and oligomycin plus dinitrophenol would reverse a direct (not energy-related) effect on a transport process.

NH_4Cl, CO_2 and ornithine were added so that the only substrates (or cofactor) that could be depleted in the basic system for measuring citrulline synthesis are *N*-acetylglutamate or ATP. Addition of *N*-acetylglutamate had no effect on the inhibition of citrulline synthesis by pent-4-enoate (results not shown). However, *N*-acetylglutamate may not cross the mitochondrial membrane (Charles *et al.*, 1967). Thus by a process of elimination it seems likely that the block is caused by an interference with energy metabolism, which, in turn, either inhibits the mitochondrial uptake of ornithine [the transport of citrulline is not energy-dependent (Gamble & Lehninger, 1973)] or depletes mitochondrial ATP, impairing carbamoyl phosphate synthesis or both.

The effect of pent-4-enoate on carbamoyl phosphate concentration and ornithine uptake was measured at only one point in time with conditions that certainly did not reflect the rates of carbamoyl phosphate synthesis or ornithine uptake that occurred in the complete system for citrulline synthesis. Calculated as a rate, the data for carbamoyl phosphate would give a rate of synthesis of about 0.15 μmol/h per mg of protein, and the rate of ornithine uptake would be even less, whereas rates of citrulline synthesis higher than 1 μmol/h per mg of protein were consistently observed. Nevertheless, the data are consistent with a block in the synthesis of carbamoyl phosphate and ornithine uptake, which, in turn, is consistent with the suggestion that the block is caused by an impairment in energy metabolism. Three other lines of experimental evidence support the conclusion that energy metabolism is impaired. (1) Pent-4-enoate had no effect on mitochondrial citrulline synthesis when succinate was the oxidizable substrate. It seems likely that succinate maintains energy metabolism. (2) Under conditions in which exogenous ATP could support citrulline synthesis pent-4-enoate had no effect. (3) Although total medium plus mitochondrial ATP was measured and not that at the site of carbamoyl phosphate synthetase, pent-4-enoate did decrease ATP as much as dinitrophenol.

The mechanism of interference with energy metabolism is unknown. A major known effect of pent-4-enoate is the inhibition of fatty acid oxidation. However, the inhibition of fatty acid oxidation by pent-4-enoate probably does not account for the inhibition of citrulline synthesis. The effect of pent-4-enoate on palmitate oxidation in rat liver mitochondria is close to maximal at concentrations as low as 0.01 mM (Senior *et al.*, 1968) yet 0.1 mM-pent-4-enoate had little effect on citrulline synthesis. In addition, pent-4-enoate inhibited citrulline synthesis with a number of non-fatty acid-oxidizable substrates when the only fatty acids present were those that contaminated the mitochondrial preparation.

The present investigation shows that citrulline synthesis was inhibited by pent-4-enoate when supported by a number of substrates that are oxidized via the reduction of NAD^+, but not with succinate which results in the reduction of FAD. This suggests that pent-4-enoate may somehow interfere with oxidative phosphorylation when NAD^+ is involved.

Pent-4-enoate at a dose of 200 mg/kg results in about a 4-fold elevation of plasma ammonia in rats after 20–25 min (Glasgow & Chase, 1975*a*). The explanation may be that the effects of pent-4-enoate observed in the present experiments also occur *in vivo*, at least with this large dose.

The major ultrastructural finding in the liver in Reye's syndrome is swollen pleomorphic mitochondria (Partin *et al.*, 1971). For this reason it has been suggested that mitochondrial injury may be instrumental in the evolution of Reye's syndrome. The demonstration that pent-4-enoate will produce in rats many of the features of Reye's syndrome and the data presented here, which suggest that pent-4-enoate impairs important mitochondrial functions, are consistent with this hypothesis.

Since this study was completed, it has been reported that incubation of rat liver homogenates with pent-4-enoate for 15 min results in about 50% inhibition of carbamoyl phosphate synthetase, alters the K_m of ornithine carbamoyltransferase for carbamoyl phosphate and decreases the $V_{max.}$ (Sinatra *et al.*, 1975).

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