

Valproic Acid Impairs Carnitine Uptake in Cultured Human Skin Fibroblasts. An *In Vitro* Model for the Pathogenesis of Valproic Acid-Associated Carnitine Deficiency

I. TEIN, S. DIMAURO, Z-W. XIE, AND D. C. DE VIVO

The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8 [I.T., Z-W.X.], and Columbia University, New York, New York, 10032 [S.D.M., D.C.D.V.]

ABSTRACT. The mechanisms of valproate-associated carnitine deficiency are controversial. The urinary excretion of valproylcarnitine is insufficient to account for tissue carnitine depletion. To explore this mechanism, we studied the effects of valproic acid (VPA) on carnitine uptake in cultured human skin fibroblasts by the method of Tein *et al.* (*Pediatr Res* 28:247-255, 1990). Fibroblasts were preincubated with varying concentrations (0-2000 μM) of VPA for 1, 3, 5, 7, 10, 14, 21, and 28 d and then incubated with fixed carnitine concentrations of 50 μM (normal physiologic concentration), 20 μM (as seen in secondary carnitine deficiency disorders), or 5 μM (as seen in the plasma membrane carnitine transport defect). There was an exponential dose-dependent decrease in carnitine uptake with increasing VPA concentrations, and the relative inhibitory effect was the same for all three carnitine concentrations. The mean percentages \pm SD ($n = 1$) of residual carnitine uptake for all combined preincubation periods (1-28 d) and combined carnitine concentrations (5, 20, and 50 $\mu\text{mol/L}$) with increasing concentrations of VPA varied from $83.4 \pm 2.6\%$ (10 μM VPA) to $56.7 \pm 0.1\%$ (500 μM) to $19.8 \pm 1.3\%$ (2000 μM). The degree of inhibition was directly proportional to the time of VPA preincubation and parallel for all three carnitine concentrations; the longer the preincubation period, the lower the toxic dose of VPA (to a minimum of 450 μM), resulting in a 50% suppression of carnitine uptake (TD_{50}). The mean TD_{50} of the combined carnitine concentrations for increasing preincubation periods of VPA varied from $1898 \pm 214 \mu\text{M}$ (1 d) to $447 \pm 9 \mu\text{M}$ (28 d), tapering toward an asymptote of 450 μM when the preincubation period exceeded 14 d. This *in vitro* TD_{50} value may be comparable to the *in vivo* therapeutic range of serum VPA concentrations (350-700 $\mu\text{mol/L}$) for anticonvulsant therapy. We conclude that one mechanism by which long-term VPA therapy induces serum and tissue carnitine depletion is through inhibition of plasmalemmal carnitine uptake, including decreased renal reabsorption of free carnitine. This effect is directly proportional to the duration of exposure and concentration of VPA. (*Pediatr Res* 34: 281-287, 1993)

Abbreviations

VPA, valproic acid
 TD_{50} , toxic dose of VPA resulting in 50% suppression of carnitine uptake

Low serum carnitine concentrations commonly accompany long-term VPA therapy for epilepsy (1-8). Less common but more serious side effects of VPA include a Reye-like syndrome, idiosyncratic and life-threatening hepatotoxicity (distinct from dose-related hepatotoxicity), and pancreatitis (8-16).

Carnitine deficiency in patients with epilepsy may be due to multiple causes, including nutritional deficiency, an underlying inborn error of metabolism, and VPA therapy. Numerous studies have shown a significant decrease in total and/or free serum carnitine concentrations in patients taking multiple anticonvulsants including VPA (3, 17-21) or VPA alone (3). However, in one study of 21 children on VPA, the mean serum carnitine concentration did not correlate with either oral VPA dose or serum VPA concentration (22). Even in the presence of normal serum carnitine concentrations, isolated muscle carnitine deficiency was documented in three children receiving chronic VPA therapy for intractable seizures (23). Given that 90% of body carnitine stores are contained within skeletal muscle (24), this finding suggests that serum carnitine concentrations are not always informative and that muscle carnitine concentrations may provide a more accurate reflection of the carnitine status of the patient. Again, no correlation was found in this study between VPA dose and degree of carnitine depletion, suggesting other mediating factors.

There may be multiple mechanisms of VPA-associated carnitine deficiency including a defect in hepatic biosynthesis, an increase in renal excretion, or a decrease in carnitine transport into tissues. Impairment of carnitine biosynthesis in liver (25, 26) would have to be accompanied by a nutritional deficiency to significantly diminish total body carnitine stores because approximately 75% of carnitine is obtained from the diet (24). Furthermore, carnitine deficiency can occur without significant hepatic dysfunction, implying that other mechanisms are involved.

The renal loss of carnitine as valproylcarnitine has been examined by a number of investigators (19, 22, 27). Several studies have reported an increased ratio of acylcarnitine to total carnitine in the urine of patients taking VPA, even though the total urinary carnitine excretion was not increased (19, 22). Millington *et al.* (27) documented decreased free carnitine and increased acylcarnitines in the urine of two children on long-term VPA therapy and reported that valproylcarnitine constituted less than 10% of the total urinary acylcarnitine pool. Conversely, a marked in-

Received December 16, 1992; accepted April 2, 1993.

Supported by an Operating Grant from the Medical Research Council of Canada and by a grant from the University of Toronto's Dean's Fund and from the Colleen Giblin Charitable Foundation for Pediatric Neurology Research. I.T. is a recipient of a Medical Research Council of Canada Scholarship.

Correspondence and reprint requests: Dr. Ingrid Tein, Division of Neurology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, M5G 1X8 Canada.

crease in urinary carnitine excretion has been documented in one patient with VPA-associated hepatotoxicity (28). However, it is still unproven whether long-term VPA therapy with continued urinary excretion of valproylcarnitine depletes total body stores of carnitine leading to a deficiency state.

A defect in tissue carnitine transport could explain both serum depletion caused by decreased renal tubular reabsorption of free carnitine and muscle depletion caused by decreased muscle uptake. Evidence is emerging that muscle, kidney, and fibroblasts share a common plasmalemmal carnitine transporter (29, 30). Fibroblasts have become a useful model to examine the "muscle-kidney" carnitine transport system (30, 31). Therefore, we studied the effect of increasing VPA concentrations and increasing time periods of VPA preincubation on carnitine uptake in cultured human skin fibroblasts. To determine the relative severity of the VPA effect in different clinical conditions, we also varied the carnitine concentrations in the incubation medium to simulate serum carnitine concentrations seen in normal controls and in primary and secondary carnitine deficiency disorders.

MATERIALS AND METHODS

All studies were performed with the approval of the Institutional Review Board of The Hospital for Sick Children, Toronto. Carnitine uptake studies were performed in control cultured skin fibroblasts with documented normal carnitine uptake. Control fibroblasts were obtained from the Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ. The uptake of carnitine was investigated by the method of Tein *et al.* (30). Control fibroblasts (passage numbers 6–12) were plated onto 9.5-cm six-well plates (Gibco Laboratories, St. Lawrence, MA) and allowed to grow to confluence in RPMI 1640 medium supplemented with 10% FCS (medium total carnitine, 2 $\mu\text{mol/L}$). L-[methyl- ^3H]carnitine hydrochloride was purchased from Amersham, Arlington Heights, IL, and L-carnitine was a gift from Sigma-Tau Pharmaceuticals, Pomezia, Italy. Cell-bound radioactivity was determined in 800 μL of the final fibroblast hydrolysate using Aquasol-2 with a counting efficiency of 60%. Cell protein was measured in the remaining 200 μL of the hydrolysate for each individual plate by the method of Lowry *et al.* (32). VPA (2-propylpentanoic acid) was used as supplied from Sigma Chemical Co., St. Louis, MO. Experiments with VPA were run in parallel with a standard control carnitine uptake study without VPA. Nonspecific uptake was determined at 10 mmol/L carnitine. VPA concentrations in the preincubation medium of RPMI 1640 and 10% FCS were fixed at 0, 100, 200, 300, 500, 700, 900, 1100, 1300, 1500, 1700, and 2000 $\mu\text{mol/L}$. At the beginning of the uptake study, the plates were thoroughly washed four times with PBS to remove all excess FCS. The individual wells were then incubated with the same predetermined concentrations of VPA, in RPMI without FCS, and a fixed carnitine concentration of 5, 20, or 50 $\mu\text{mol/L}$, for the measurement of [^3H]-L-carnitine uptake *in vitro*. The VPA preincubation periods were varied from 4 h (actual incubation period of uptake study) to 1, 3, 5, 7, 10, 14, 21, and 28 d. Reproducibility of results was confirmed by repeat experiments using the identical VPA concentration, time period of VPA preincubation, and carnitine substrate incubation concentration, on three separate days. Specific uptake of radioactivity was used to calculate the rates of total carnitine uptake. Apparent K_m and V_{max} values for standard carnitine uptake studies were obtained by linear regression analysis of Lineweaver-Burk plots.

Carnitine uptake was examined against increasing VPA preincubation concentrations for each of the three carnitine incubation concentrations (5, 20, 50 μM) under eight fixed VPA preincubation periods (1–28 d) for triplicate experiments (72 analyses). The curves of residual carnitine uptake with increasing concentrations of VPA were plotted together for the different VPA preincubation periods. The mean of the residual carnitine uptake from all VPA incubation periods was determined for each of the

three carnitine concentrations and plotted against increasing concentrations of VPA to demonstrate the net effect of increasing VPA concentrations. The TD_{50} of VPA resulting in a 50% suppression of carnitine uptake was determined by nonlinear regression analysis of dose-response curves plotting carnitine uptake *versus* increasing VPA concentrations. The statistical nonlinear regression analysis program (Systat 5.0) was obtained from Systat Inc., Evanston, IL. These dose-response curves were individually analyzed for each of the three carnitine incubation concentrations (5, 20, 50 μM) under eight fixed VPA preincubation periods (1–28 d) for triplicate experiments (72 analyses). The mean TD_{50} (triplicate experiments) for the individual carnitine concentrations were then plotted against increasing time of VPA preincubation to establish the time period of maximum effect.

RESULTS

During the 4-h incubation period of the uptake study without any preincubation, VPA had no significant effect on carnitine uptake at the lower concentrations and only a minor effect at the highest concentration of 2000 $\mu\text{mol/L}$ (15% reduction in carnitine uptake). Significant effects were initially demonstrated with a preincubation period of 24 h (Fig. 1a). The curves of residual carnitine uptake with increasing concentrations of VPA (0 to 2000 $\mu\text{mol/L}$) were plotted for each of the three carnitine incubation concentrations (5, 20, 50 $\mu\text{mol/L}$) under each of the eight VPA preincubation periods (1–28 d) using nonlinear regression analysis.

To examine the rate of decline with increasing VPA preincubation periods, the individual curves from four preincubation periods for a fixed carnitine incubation concentration (*e.g.* 50 $\mu\text{mol/L}$) were collated on the same graph as shown in Figure 1a (VPA preincubation periods of 1, 3, 5, and 7 d) and Figure 1b (VPA preincubation periods of 10, 14, 21, and 28 d). To examine the isolated net effect of increasing VPA concentrations on carnitine uptake, the mean of the residual carnitine uptake from all VPA preincubation periods was subsequently determined for

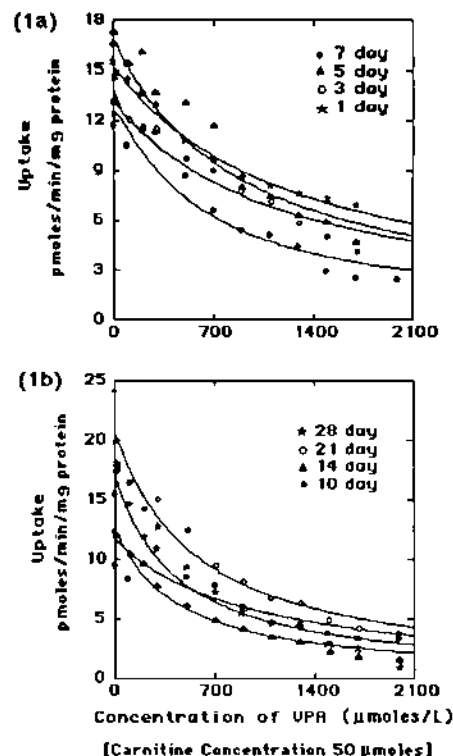


Fig. 1. Effect of increasing VPA concentration on carnitine uptake with carnitine substrate concentration of 50 $\mu\text{mol/L}$.

each of the three carnitine concentrations and plotted against increasing VPA concentrations (Fig. 2). The mean values are listed in Table 1 with the calculated percentages of residual uptake. There was no significant difference in the relative rates of decline of carnitine uptake with increasing VPA concentrations at the different carnitine concentrations. Although the percentage decrease of carnitine uptake was not affected by the initial carnitine concentration, the absolute values of carnitine uptake were lowest at 5 $\mu\text{mol/L}$ carnitine concentration, intermediate at 20 $\mu\text{mol/L}$, and highest at 50 $\mu\text{mol/L}$ concentration. The mean percentages [\pm SD ($n - 1$)] of residual carnitine uptake for the combined VPA preincubation periods and the combined carnitine concentrations (5, 20, 50 $\mu\text{mol/L}$) plotted against increasing concentrations of VPA demonstrated a

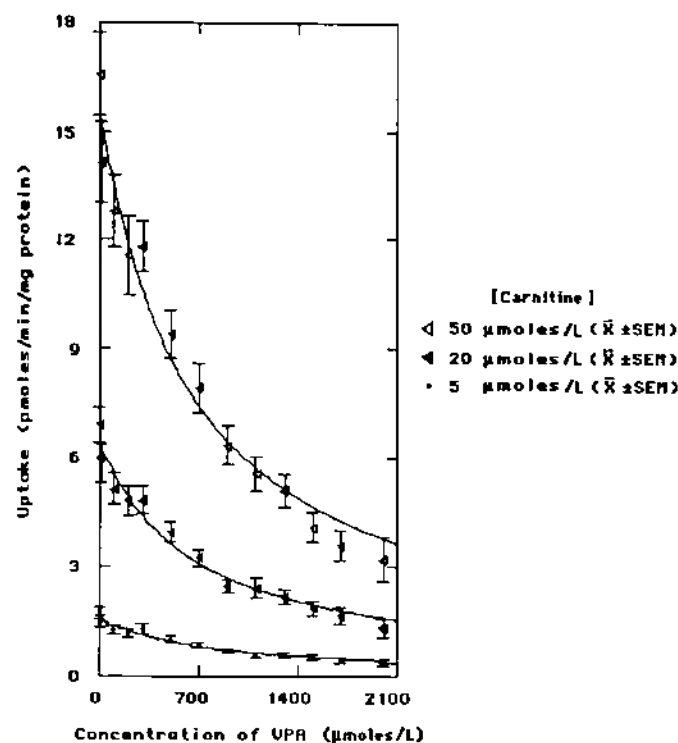


Fig. 2. Effect of increasing VPA concentrations on mean carnitine uptake from all combined VPA preincubation periods (1–28 d).

marked inverse dose-response effect with increasing VPA concentration (Table 1).

The dose-response curves of carnitine uptake *versus* increasing VPA concentration were individually analyzed for each of the three carnitine incubation concentrations (5, 20, 50 $\mu\text{mol/L}$) under the eight fixed VPA preincubation periods (1–28 d) for triplicate experiments (Fig. 3). The TD_{50} of VPA resulting in a 50% suppression of carnitine uptake was determined by nonlinear regression analysis of the dose-response curves for the individual carnitine concentrations and the means determined. The degree of inhibition of carnitine uptake was directly proportional to the time of VPA incubation, and parallel for all three carnitine concentrations (Fig. 4, Table 2); the longer the preincubation period, the lower the TD_{50} of VPA that tapered toward an asymptote of 450 μM when the preincubation period was ≥ 14 d. The maximum effect of VPA preincubation time on carnitine uptake, regardless of the carnitine incubation concentration, was reached by 10–14 d of VPA exposure, beyond which there was no significant additional effect on the TD_{50} . Of note, the mean *in vitro* TD_{50} values for VPA preincubations of 7–28 d, namely 560–450 $\mu\text{mol/L}$, may be comparable to the *in vivo* therapeutic range of serum VPA concentrations (350–700 $\mu\text{mol/L}$), which is used as a general guideline for VPA anticonvulsant therapy in epileptic patients. Preincubation periods of 1–28 d in standard control uptake plates without VPA (run in parallel with the VPA experiment plates) showed no significant decrease of carnitine uptake.

DISCUSSION

Carnitine concentrations in most tissues, except brain, are 20- to 50-fold higher than in serum (33). Carnitine uptake is mediated by a transport system, apparently driven by a large sodium potential across the plasma membrane (34, 35) and generally held to be energy-dependent (36–38). In studies of children with primary systemic carnitine deficiency, a genetically determined defect in plasmalemmal carnitine transport has been documented in cultured skin fibroblasts (29, 30). Clinically, these children present with hypotonia, weakness, lethargy, cardiomyopathy, fasting hypoglycemia, hypoketotic encephalopathy, and failure to thrive. Because these patients have a markedly decreased renal threshold for carnitine and are incapable of fully restoring muscle carnitine concentrations despite high-dose oral carnitine supplementation and almost complete restoration of normal serum carnitine concentrations, it has been postulated

Table 1. Effect of increasing VPA concentrations on mean carnitine uptake*

VPA μM	Carnitine incubation concentration						Combined mean of 5, 20, and 50 μM [% Residual uptake \pm SD ($n - 1$)]
	5 μM Carnitine		20 μM Carnitine		50 μM Carnitine		
	Uptake†	% Residual uptake	Uptake	% Residual uptake	Uptake	% Residual uptake	
0	1.750	100.0	6.930	100.0	16.592	100.0	100.0
10	1.408	80.5	5.854	84.5	14.155	85.3	83.4 \pm 2.57
100	1.239	70.8	5.144	74.2	12.747	77.1	74.0 \pm 3.15
200	1.144	65.4	4.792	69.1	11.565	69.7	68.1 \pm 2.33
300	1.285	73.4	4.824	69.6	11.804	71.1	71.4 \pm 1.91
500	0.993	56.7	3.933	56.7	9.420	56.8	56.7 \pm 0.06
700	0.852	48.7	3.220	46.5	7.944	47.9	47.7 \pm 1.11
900	0.673	38.4	2.453	35.4	6.344	38.2	37.3 \pm 1.68
1100	0.576	32.9	2.388	34.4	5.563	33.5	33.6 \pm 0.87
1300	0.564	32.2	2.138	30.8	5.086	30.6	31.2 \pm 0.87
1500	0.507	29.0	1.852	26.7	4.074	24.6	26.8 \pm 2.20
1700	0.442	25.3	1.628	23.5	3.565	21.5	23.4 \pm 1.90
2000	0.372	21.3	1.303	18.8	3.182	19.2	19.8 \pm 1.34

* Combined means for eight incubation periods (1–28 d) and triplicate experiments ($n = 24$).

† Uptake expressed in pmol/min/mg fibroblast protein.

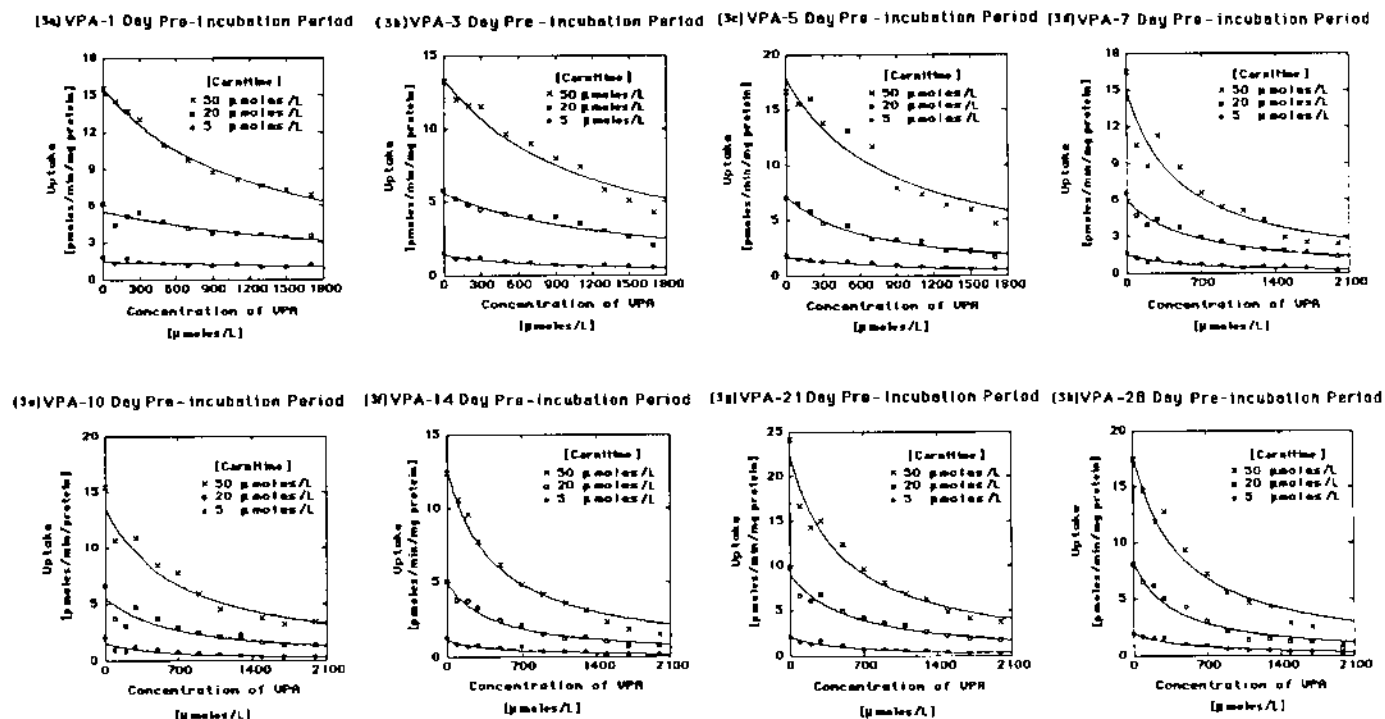


Fig. 3. Effect of increasing VPA preincubation periods on carnitine uptake.

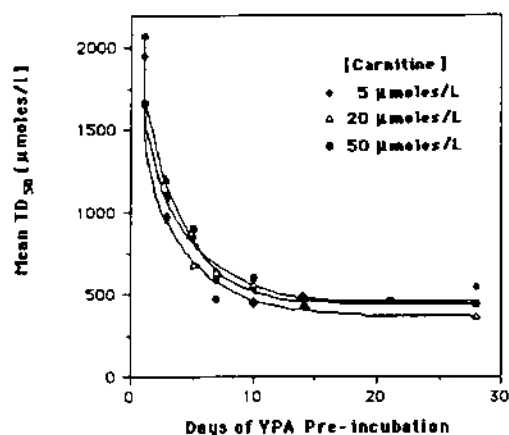


Fig. 4. Effect of increasing VPA preincubation periods on mean toxic dose of VPA causing 50% decrease (TD_{50}) of carnitine uptake.

that muscle, kidney, and fibroblasts share a common plasmalemmal carnitine transporter (29, 30). This concept is supported by the similar K_m values for carnitine uptake in cultured muscle ($1.90 \pm 1.38 \mu M$) (39), human heart cells ($4.8 \pm 2.2 \mu M$) (40), and skin fibroblasts ($5.5 \pm 0.58 \mu M$) (30), which are very different from the K_m values observed in human liver ($500 \mu M$) and brain ($> 1000 \mu M$) (41). A VPA-associated defect in carnitine transport could mimic the genetically determined defect in plasmalemmal carnitine transport and could result in decreased renal tubular reabsorption of free carnitine and explain the isolated deficiency of muscle carnitine documented in three patients on long-term VPA therapy (23).

We therefore investigated the effect of increasing concentrations and exposure periods of VPA on plasmalemmal carnitine uptake in cultured control human skin fibroblasts at three carnitine concentrations. There was an exponential dose-dependent decrease in carnitine uptake with increasing VPA concentration. The relative inhibitory effect of increasing VPA concentrations on carnitine uptake was the same for all three carnitine concentrations. The degree of inhibition was directly proportional to

the time of VPA exposure and parallel for all three carnitine concentrations. The mean TD_{50} tapered toward a minimum asymptote of $450 \mu mol/L$ of VPA when the preincubation period was ≥ 14 d and did not decrease further thereafter. This *in vitro* VPA concentration may be comparable to the *in vivo* therapeutic range of serum VPA concentrations (350 – $700 \mu mol/L$) for anti-convulsant therapy. At 1 d of incubation, the TD_{50} was well above "therapeutic range" (TD_{50} of $1898 \mu mol/L$) but decreased dramatically by 3 d (TD_{50} of $1096 \mu mol/L$) approaching high therapeutic levels. No significant effect on carnitine uptake was noted after 4 h of incubation with VPA, with the exception of a minor decrease (15%) in uptake at the highest concentration of $2000 \mu mol/L$, which is three times the normal therapeutic range. Thus, significant VPA inhibition of carnitine uptake appears to require a period of several days and reaches maximum effect by approximately 10–14 d. This could be compatible with the observation that 75% of cases of idiosyncratic fatal hepatotoxicity occur within the first 3 mo of therapy (42).

There are at least four different mechanisms by which VPA can interfere with carnitine uptake. One mechanism could be the direct inhibition of carnitine uptake by valproylcarnitine. VPA (2-propylpentanoic acid), an eight-carbon fatty acid, is assumed to pass freely through the mitochondrial membrane in a carnitine-independent manner (43). VPA is then probably converted into its activated intermediate, valproyl-CoA (2-propyl-pentanoyl-CoA), as demonstrated in rat liver mitochondria (43). As proposed by Millington *et al.* (27), valproyl-CoA is probably converted into valproylcarnitine by one or more of the carnitine acyltransferases, predominantly carnitine octanoyl-transferase, at the inner surface of the inner mitochondrial membrane. We suggest that valproylcarnitine may then be transported out of the mitochondrion, leaving behind free CoA within the mitochondria. The valproylcarnitine may diffuse across the plasmalemma and then interfere with tissue free carnitine transport or renal tubular reabsorption of free carnitine. Depressed renal tubular reabsorption of free carnitine has been documented by Matsuda and Ohtani (28) in children receiving VPA therapy. Stanley *et al.* (44) examined the renal free carnitine threshold (plasma level at which urinary free carnitine fell below 5% of the filtered load) and also found that it was below the normal range

Table 2. Effect of increasing VPA preincubation time on mean TD_{50} of VPA for carnitine uptake*

	VPA preincubation time (d)							
	1	3	5	7	10	14	21	28
Carnitine incubation concentration								
5 μ M	1954	978	849	589	454	492	450	535
20 μ M	2079	1209	674	624	569	449	459	366
50 μ M	1662	1100	895	467	590	430	457	433
Combined mean of 5, 20, and 50 μ M \pm SD ($n - 1$)	1898 \pm 214	1096 \pm 115	806 \pm 116	560 \pm 82	538 \pm 73	437 \pm 32	456 \pm 5	447 \pm 9

* Each TD_{50} is a mean of three experiments ($n = 3$).

of plasma free carnitine (36–50 μ mol/L) in medium-chain acyl-CoA dehydrogenase deficiency (17 μ mol/L) and in long-chain acyl-CoA dehydrogenase deficiency (40 μ mol/L) compared with controls (57 μ mol/L). In a subsequent report, Stanley *et al.* (31) found that the "muscle-kidney carnitine transport system" expressed in cultured skin fibroblasts was strongly inhibited by acylcarnitines (50% inhibition at 3.05 μ mol/L L-carnitine, 4.6 μ mol/L acetylcarnitine, 2.9 μ mol/L octanoylcarnitine, and 0.37 μ mol/L palmitoylcarnitine). It was therefore suggested that the secondary carnitine deficiency seen in intramitochondrial β -oxidation defects may reflect inhibition of free carnitine transport by medium- or long-chain acylcarnitines rather than excessive excretion of the acylcarnitines themselves (31). This concept is also supported by the work of Nishida *et al.* (45) in VPA-treated rats.

An indirect mechanism by which VPA could interfere with carnitine uptake into tissues would be through the formation of VPA metabolites, *e.g.* 4-en valproate (46), with secondary inhibition of β -oxidation and accumulation of excessive acyl-CoA derivatives. VPA is almost entirely cleared through β -oxidation and glucuronidation (12). In mitochondria, VPA is converted to 2-propylpentanoyl-CoA, which is dehydrogenated to 2-propyl-2-pentanoyl-CoA by 2-methyl-branched-chain acyl-CoA dehydrogenase (47) and subsequently hydrated by enoyl-CoA hydratase to 3-hydroxy-2-propylpentanoyl-CoA (43). Li *et al.* (43) have demonstrated that the latter compound is dehydrogenated by a novel NAD⁺-specific 3-hydroxyacyl-CoA dehydrogenase associated with the mitochondrial membrane to 3-keto-2-propylpentanoyl-CoA, which is then slowly degraded most likely by hydrolysis. Kesterson *et al.* (46) studied the hepatotoxic effects of VPA and its metabolites in rats and found that 4-PA, 4-en-VPA, and 2,4-dien-VPA were potent inducers of microvesicular steatosis in young rats. 4-en-VPA produced ultrastructural changes characterized by myeloid bodies, lipid vacuoles, and mitochondrial abnormalities. In other studies, VPA was shown to be toxic to rat liver mitochondria both *in vitro* (48) and *in vivo* (49). The morphologic swelling of rat liver mitochondria exposed to VPA could be prevented by simultaneously giving L-carnitine (49). In mice injected with VPA, hepatic concentrations of CoA and free carnitine decreased, whereas acylcarnitine concentrations increased (50). In isolated rat hepatocytes, VPA inhibited oxidation of [¹⁴C]palmitate and decreased ketogenesis and acetyl-CoA levels (51).

In terms of mechanisms, it has been suggested that VPA may inhibit β -oxidation in humans and rats either by sequestering CoA or by increasing the acyl-CoA:free CoA ratio (46, 52, 53). Secondly, Li *et al.* (43) have suggested that trapping of a finite pool of CoA also may deplete the reduced CoA needed for the thiolytic cleavage of 3-ketoacyl-CoA thereby inhibiting β -oxidation. A third possible mechanism of inhibition suggested by Li *et al.* (43) would be the inhibition of one or more β -oxidation enzymes by 3-keto-2-propylpentanoyl-CoA. This compound, serving as substrate or product analogue, could bind to 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase, without being acted on by these enzymes, thereby functioning as an inhibitor (43). A related metabolite, 4-pentenol, when metabolized by β -oxidation to 3-keto-4-pentenoyl-CoA, has been

shown to be a potent inhibitor of 3-ketoacyl-CoA thiolase and of acetoacetyl-CoA thiolase (54). VPA and its metabolites could therefore inhibit enzymes of β -oxidation and have potent and long-term effects. Carnitine could be used for the formation of acylcarnitines to buffer these potentially toxic acyl-CoA derivatives that accumulate proximal to the block in fatty acid β -oxidation. The increasing inhibitory effect on carnitine uptake that we observed with increasing preincubation periods of VPA may therefore relate to the accumulation, over time, of acylcarnitines including valproylcarnitine that may then diffuse into the incubation medium and interfere with free carnitine uptake into the cell. The lack of further inhibition after 14 d of VPA preincubation may relate to severe inhibition of intramitochondrial β -oxidation with no further increase in acylcarnitine formation or to saturation of the inhibitory effect of acylcarnitines at the carnitine plasmalemmal transport site.

Finally, a decrease in intracellular ATP due to inhibition of β -oxidation, pyruvate metabolism, and gluconeogenesis could account for decreased efficiency of the energy-dependent carnitine transporter. Sequestration of free CoA through formation of valproyl-CoA (52, 53) would decrease ATP production. VPA also interferes with pyruvate uptake by rat brain mitochondria (55) and inhibits pyruvate oxidation as well as the activities of pyruvate carboxylase and pyruvate dehydrogenase (53). In addition, VPA may lead to uncoupling of mitochondrial oxidation (55) and inhibition of oxidative phosphorylation (48, 56, 57), possibly in part through inhibition of cytochrome *aa₃* activity (58). Furthermore, VPA inhibits gluconeogenesis in rat liver (52, 53, 59) possibly through inhibition of pyruvate carboxylase (53). These several observations, in the aggregate, suggest that VPA may compromise cellular energetics by a variety of mechanisms.

Clinically, there has been much interest in the role of the carnitine deficiency in predisposing a child on VPA therapy to the idiosyncratic, potentially life-threatening Reye-like syndrome (13). Several patients with a Reye-like syndrome caused by VPA were shown to have carnitine deficiency (8, 17, 28) but others did not (3). Some patients may have a preexisting carnitine deficiency due to an inborn error of metabolism (60); however, this is not true for all (3, 17). The patients at greatest risk for VPA hepatotoxicity (1 of 500) are children under the age of 2 y with neurologic disabilities who are receiving multiple anticonvulsants (42). The clinical scenario for fatal hepatotoxicity may be as follows. Seizures may be the clinical signature of a genetically determined metabolic error such as "Alpers syndrome" (progressive infantile poliodystrophy with cirrhosis) or an intramitochondrial β -oxidation disorder, which also may be associated with secondary carnitine deficiency. This metabolic condition may be further exacerbated by VPA. Young children may be more prone to decreased carnitine stores because of decreased carnitine biosynthesis and decreased intake. Other anticonvulsants may increase potentially hepatotoxic VPA metabolites, having a synergistic effect. When VPA leads to carnitine deficiency, this interferes with long-chain fatty acid oxidation. VPA also may reduce the available free CoA for β -oxidation, which could lead to the accumulation of potentially toxic short-chain fatty acids (7). In every case, a thorough investigation should be conducted to determine whether the epileptic patient has an

associated inborn metabolic error predisposing him or her to this idiosyncratic complication (24, 61).

In summary, the carnitine deficiency associated with VPA therapy may be due to several different mechanisms. One mechanism is through inhibition of plasmalemmal carnitine uptake into tissues, which could include decreased renal tubular reabsorption of free carnitine. Our studies show that this effect is directly proportional to the duration of exposure and concentration of VPA. We propose that the inhibition may be due to increasing competition between free carnitine and acylcarnitines, including valproylcarnitines and short-chain acylcarnitines, at the plasmalemmal transporter site. We suggest that L-carnitine supplementation may decrease the apparent impairment of plasmalemmal free carnitine uptake into tissues and of free carnitine reabsorption in the kidneys through an increase in the free carnitine concentration at the transport site. Additional studies are necessary to support this hypothesis. As well, increased free carnitine would provide a greater buffering capacity for excessive potentially toxic acyl-CoA including valproyl-CoA, thereby decreasing secondary inhibition of fatty acid oxidation, pyruvate oxidation, and gluconeogenesis and increasing the intramitochondrial free CoA concentration. Because there is no evidence that L-carnitine administration adversely alters the anticonvulsant properties of VPA (62) or lowers the serum VPA concentration, our data suggest that carnitine should be administered to all children on VPA who have laboratory or clinical evidence of serum or tissue carnitine deficiency.

REFERENCES

- Winter SC, Szabo-Aczel S, Curry CJ, Hutchinson HT, Hogue R, Shug A 1987 Plasma carnitine deficiency. Clinical observations in 51 pediatric patients. *Am J Dis Child* 141:660-665
- Stumpf DA, Parker Jr WD, Angelini C 1985 Carnitine deficiency, organic acidemias, and Reye's syndrome. *Neurology* 35:1041-1045
- Laub MC, Paezke-Brunner J, Jaeger G 1986 Serum carnitine during valproic acid therapy. *Epilepsia* 27:559-562
- Ohtani Y, Endo F, Matsuda I 1982 Carnitine deficiency and hyperammonemia associated with valproic acid therapy. *J Pediatr* 101:782-785
- Matsuda I, Ohtani Y, Ninomiya N 1986 Renal handling of carnitine in children with carnitine deficiency and hyperammonemia associated with valproate therapy. *J Pediatr* 109:131-134
- Coulter DL 1984 Carnitine deficiency: a possible mechanism for valproate hepatotoxicity. (letter) *Lancet* 1:689
- Coulter DL 1991 Carnitine, valproate, and toxicity. *J Child Neurol* 6:7-14
- Böhles H, Richter K, Wagner-Thiessen E, Schäfer H 1982 Decreased serum carnitine in valproate induced Reye syndrome. *Eur J Pediatr* 139:185-186
- Addison GM, Gordon NS 1980 Sodium valproate and acute hepatic failure. *Dev Med Child Neurol* 22:248-249
- Coulter DL, Allen RJ 1980 Pancreatitis associated with valproic acid therapy for epilepsy (letter) *Ann Neurol* 7:92
- Dreifuss FE, Langer DH, Moline KA, Maxwell JE 1989 Valproic acid hepatic fatalities. II. US experience since 1984. *Neurology* 39:201-207
- Eadie MJ, Hooper WD, Dickinson RG 1988 Valproate-associated hepatotoxicity and its biochemical mechanisms. *Med Toxicol Adverse Drug Exp* 3:85-106
- Gerber N, Dickinson RG, Harland RC, Lynn RK, Houghton D, Antonias JI, Schimschock JC 1979 Reye-like syndrome associated with valproic acid therapy. *J Pediatr* 95:142-144
- Suchy FJ, Balistreri WF, Buchino JJ, Sondheimer JM, Bates SR, Kearns GL, Stull JD, Bove KE 1979 Acute hepatic failure associated with the use of sodium valproate. *N Engl J Med* 300:962-966
- Wyllie E, Wyllie R, Cruse RP, Erenberg G, Rothner AD 1984 Pancreatitis associated with valproic acid therapy. *Am J Dis Child* 138:912-914
- Zimmerman HJ, Ishak KG 1982 Valproate-induced hepatic injury: analyses of 23 fatal cases. *Hepatology* 2:591-597
- Murphy JV, Marquardt KM, Shug AL 1985 Valproic acid associated abnormalities of carnitine metabolism. (letter) *Lancet* 1:820-821
- Morita J, Yuge K, Yoshino M 1986 Hypocarnitinemia in the handicapped individuals who receive a polypharmacy of antiepileptic drugs. *Neuropediatrics* 17:203-205
- Melegh B, Kerner J, Kispál G, Acsádi G, Acsádi G, Dani M 1987 Effect of chronic valproic acid treatment on plasma and urine carnitine levels in children: decreased urinary excretion. *Acta Paediatr Hung* 28:137-142
- Rodríguez-Segade S, de la Peña CA, Tutor JC, Paz JM, Fernández MP, Rozas I, Del Río R 1989 Carnitine deficiency associated with anticonvulsant therapy. *Clin Chim Acta* 181:175-181
- Beghi E, Bizzi A, Codegani AM, Trevisan D, Torri W 1990 Valproate, carnitine metabolism, and biochemical indicators of liver function. Collaborative Group for the Study of Epilepsy. *Epilepsia* 31:346-352
- Murphy JV, Marquardt KM, Shug A 1984 Plasma and urine carnitine concentrations in patients receiving valproic acid. *Pediatr Res* 18:380A(abstr)
- Shapira Y, Gutman A 1991 Muscle carnitine deficiency in patients using valproic acid. *J Pediatr* 118:646-649
- De Vivo DC, Tein I 1990 Primary and secondary disorders of carnitine metabolism. *Int Pediatr* 5:134-141
- Rebouche CJ, Engel AG 1980 *In vitro* analysis of hepatic carnitine biosynthesis in human systemic carnitine deficiency. *Clin Chim Acta* 106:295-300
- Englard S 1979 Hydroxylation of gamma-butyrobetaine to carnitine in human and monkey tissues. *FEBS Lett* 102:297-300
- Millington DS, Bohan TP, Roe CR, Yergey AL, Liberato DJ 1985 Valproyl-carnitine: a novel drug metabolite identified by fast atom bombardment and thermospray liquid chromatography-mass spectrometry. *Clin Chim Acta* 145:69-76
- Matsuda I, Ohtani Y 1986 Carnitine status in Reye and Reye-like syndromes. *Pediatr Neurol* 2:90-94
- Treem WR, Stanley CA, Finegold DN, Hale DE, Coates PM 1988 Primary carnitine deficiency due to a failure of carnitine transport in kidney, muscle, and fibroblasts. *N Engl J Med* 319:1331-1336
- Tein I, De Vivo DC, Bierman F, Pulver P, De Meirleir LJ, Cvitanovic-Sojat L, Pagon RA, Bertini E, Dionisi-Vici C, Servidei S, DiMauro S 1990 Impaired skin fibroblast carnitine uptake in primary systemic carnitine deficiency manifested by childhood carnitine-responsive cardiomyopathy. *Pediatr Res* 28:247-255
- Stanley CA, DeLeruw S, Coates PM, Vianey-Liaud C, Divry P, Bonnefont J-P, Saudubray J-M, Haymond M, Trefz FK, Breningstall GN, Wappner RS, Byrd DJ, Sansaricq C, Tein I, Grover W, Valle D, Rutledge SL, Treem WR 1991 Chronic cardiomyopathy and weakness or acute coma in children with a defect in carnitine uptake. *Ann Neurol* 30:709-716
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275
- Stanley CA 1987 New genetic defects in mitochondrial fatty acid oxidation and carnitine deficiency. *Adv Pediatr* 34:59-88
- Bremer J 1983 Carnitine: metabolism and functions. *Physiol Rev* 63:1420-1480
- Rebouche CJ, Mack DL 1984 Sodium gradient-stimulated transport of L-carnitine into renal brush border membrane vesicles: kinetics, specificity, and regulation by dietary carnitine. *Arch Biochem Biophys* 235:393-402
- Huth PJ, Thomsen JH, Shug AL 1978 Carnitine transport by rat kidney cortex slices: stimulation by dibutyryl cyclic AMP. *Life Sci* 23:715-722
- Huth PJ, Shug A 1980 Properties of carnitine transport in rat kidney cortex slices. *Biochim Biophys Acta* 602:621-634
- Kispál G, Melegh B, Alkonyi I, Sandor A 1987 Enhanced uptake of carnitine by perfused rat liver following starvation. *Biochim Biophys Acta* 896:96-102
- Rebouche CJ, Engel AG 1982 Carnitine transport in cultured muscle cells and skin fibroblasts from patients with primary systemic carnitine deficiency. *In Vitro (Rockville)* 18:495-500
- Bahl JJ, Bressler R 1987 The pharmacology of carnitine. *Annu Rev Pharmacol Toxicol* 27:257-277
- Bieber LL 1988 Carnitine. *Annu Rev Biochem* 57:261-283
- Dreifuss FE, Santilli N, Langer DH, Sweeney KP, Moline KA, Menander KB 1987 Valproic acid hepatic fatalities: a retrospective review. *Neurology* 37:379-385
- Li J, Norwood DL, Mao L-F, Schulz H 1991 Mitochondrial metabolism of valproic acid. *Biochemistry* 30:388-394
- Stanley CA, Berry GT, Treem WR 1990 Differences in the evolution of carnitine deficiency among secondary carnitine deficiency disorders. Proceedings V International Congress Inborn Errors of Metabolism. Asilomar, CA, June 1-5, W 17:10(abstr)
- Nishida N, Sugimoto T, Araki A, Woo M, Sakane Y, Kobayashi Y 1987 Carnitine metabolism in valproate-treated rats: the effect of L-carnitine supplementation. *Pediatr Res* 22:500-503
- Kesterson JW, Granneman GR, Machinist JM 1984 The hepatotoxicity of valproic acid and its metabolites in rats. I. Toxicologic, biochemical and histopathologic studies. *Hepatology* 4:1143-1152
- Ito M, Ikeda Y, Arnez JG, Finocchiaro G, Tanaka K 1990 The enzymatic basis for the metabolism and inhibitory effects of valproic acid: dehydrogenation of valproyl-CoA by 2-methyl-branched-chain acyl-CoA dehydrogenase. *Biochim Biophys Acta* 1034:213-218
- Haas R, Stumpf DA, Parks JK, Eguen L 1981 Inhibitory effects of sodium valproate on oxidative phosphorylation. *Neurology* 31:1473-1476
- Sugimoto T, Araki A, Nishida N, Sakane Y, Woo M, Takeuchi T, Kobayashi Y 1987 Hepatotoxicity in rat following administration of valproic acid: effect of L-carnitine supplementation. *Epilepsia* 28:373-377
- Thurston JH, Carroll JE, Hauhart RE, Schiro JA 1985 A single therapeutic dose of valproate affects liver carbohydrate, fat, adenylate amino acid, coenzyme A, and carnitine metabolism in infant mice: possible clinical significance. *Life Sci* 36:1643-1651
- Coudé FX, Grimber G, Pelet A, Benoit Y 1983 Action of the antiepileptic drug, valproic acid, on fatty acid oxidation in isolated rat hepatocytes. *Biochem Biophys Res Commun* 115:730-736
- Becker CM, Harris RA 1983 Influence of valproic acid on hepatic carbohydrate and lipid metabolism. *Arch Biochem Biophys* 223:381-392
- Turnbull DM, Bone AJ, Bartlett K, Koundakjian PP, Sherratt HS 1983 The effects of valproate on intermediary metabolism in isolated rat hepatocyte and intact rats. *Biochem Pharmacol* 32:1887-1892

54. Schulz H, Fong JC 1981 4-Pentenoic acid. *Methods Enzymol* 72:604-610
55. Benavides J, Martin A, Ugarte M, Valivieso F 1982 Inhibition by valproic acid of pyruvate uptake by brain mitochondria. *Biochem Pharmacol* 31:1633-1636
56. Haas R, Stumpf DA, Bergen BJ, Parks JK, Eguren L 1980 Inhibition of oxidative phosphorylation by sodium valproate. *Neurology* 30:420(abstr)
57. Rumbach L, Warter JM, Rendon A, Marescaux C, Micheletti G, Wakeman A 1983 Inhibition of oxidative phosphorylation in hepatic and cerebral mitochondria of sodium valproate-treated rats. *J Neurol Sci* 61:417-423
58. Hayasaka K, Takahashi I, Kobayashi Y, Inuma K, Narisawa K, Tada K 1986 Effects of valproate on biogenesis and function of liver mitochondria. *Neurology* 36:351-356
59. Rogiers V, Vandenberghe Y, Verbruyse A 1985 Inhibition of gluconeogenesis by sodium valproate and its metabolites in isolated rat hepatocytes. *Xenobiotica* 15:759-765
60. Kay JD, Hilton-Jones D, Hyman N 1986 Valproate toxicity and ornithine carbamoyltransferase deficiency. (letter) *Lancet* 2:1283-1284
61. Appleton RE, Farrell K, Applegarth DA, Dimmick JE, Wong LT, Davidson AG 1990 The high incidence of valproate hepatotoxicity in infants may relate to familial metabolic defects. *Can J Neurol Sci* 17:145-148
62. Ater SB, Swinyard EA, Tolman KG, Madsen JA, Matsuo F 1984 Effects of SKF525A, phenobarbital, fasting, and carnitine on the anticonvulsant activity and neurotoxicity of valproate in mice. *Epilepsia* 25:599-604