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Carnitine metabolism in the vitamin B-12-deficient rat

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In vitamin B-12 (cobalamin) deficiency the metabolism of propionyl-CoA and methylmalonyl-CoA are inhibited secondarily to decreased L-methylmalonyl-CoA mutase activity. Production of acylcarnitines provides a mechanism for removing acyl groups and liberating CoA under conditions of impaired acyl-CoA utilization. Carnitine metabolism was studied in the vitamin B-12-deficient rat to define the relationship between alterations in acylcarnitine generation and the development of methylmalonic aciduria. Urinary excretion of methylmalonic acid was increased 200-fold in vitamin B-12-deficient rats as compared with controls. Urinary acylcarnitine excretion was increased in the vitamin B-12-deficient animals by 70%. This increase in urinary acylcarnitine excretion correlated with the degree of metabolic impairment as measured by the urinary methylmalonic acid elimination. Urinary propionylcarnitine excretion averaged 11 nmol/day in control rats and 120 nmol/day in the vitamin B-12-deficient group. The fraction of total carnitine present as short-chain acylcarnitines in the plasma and liver of vitamin B-12-deficient rats was increased as compared with controls. When the rats were fasted for 48 h, relative or absolute increases were seen in the urine, plasma, liver and skeletal-muscle acylcarnitine content of the vitamin B-12-deficient rats as compared with controls. Thus vitamin B-12 deficiency was associated with a redistribution of carnitine towards acylcarnitines. Propionylcarnitine was a significant constituent of the acylcarnitine pool in the vitamin B-12-deficient animals. The changes in carnitine metabolism were consistent with the changes in CoA metabolism known to occur with vitamin B-12 deficiency. The vitamin B-12-deficient rat provides a model system for studying carnitine metabolism in the methylmalonic acidurias.

INTRODUCTION

Carnitine, in addition to its well-established role as an obligate for the mitochondrial oxidation of long-chain fatty acids, interacts with other metabolic pathways by generating acylcarnitines from the corresponding acyl-CoAs [1]. Under normal physiological conditions, the generation of short-chain acylcarnitines (acyl-group chain length less than 10 carbon atoms) from short-chain acyl-CoAs operates near steady state [2,3], with the distribution of total carnitine between free (unesterified) carnitine and the short-chain acylcarnitines reflecting the metabolic state of the tissue [3]. Under pathophysiological conditions of acyl-CoA accumulation secondary to a metabolic defect, the generation of acylcarnitines provides a mechanism to remove the acyl group and liberate free CoA. This reaction may be critical for maintaining normal cellular function under conditions where organic acids (and the corresponding acyl-CoAs) accumulate [4]. This mechanism is thought to be in part responsible for the efficacy of carnitine in the treatment of some hereditary organic acidurias characterized by acyl-CoA accumulation secondary to an enzyme deficiency, such as in propionic acidaemia and the methylmalonic acidurias [4,5]. The resulting increase in urinary acylcarnitine excretion may lead to insufficient endogenous carnitine to meet metabolic requirements [5,6].

Understanding of the inter-relationship between the changes in endogenous carnitine metabolism, the consequences of the acyl-CoA buildup and overall cellular function is limited by a lack of experimental data. Evidence obtained *in vitro* demonstrates that high concentrations of unusual organic acids such as propionate can disrupt a number of metabolic pathways, including gluconeogenesis [7,8], fatty acid oxidation [8,9] and pyruvate oxidation [8–11]. For propionate, many of the cellular effects can be related to the accumulation of propionyl-CoA and methylmalonyl-CoA [12–15]. Under conditions where propionate interferes with cellular metabolism, addition of carnitine results in a partial normalization of metabolism associated with a large increase in propionylcarnitine production [8,9]. The use of carnitine in the treatment of patients with organic acidurias has resulted in clinical improvement of the patients and increased urinary excretion of the specific acylcarnitine corresponding to the accumulating acyl-CoA [4,5,16].

The current studies were initiated to establish an animal model *in vivo* of impaired acyl-CoA utilization to permit controlled studies of the inter-relationships between carnitine metabolism, the underlying metabolic defect and overall cellular function. In the vitamin B-12 (cobalamin)-deficient rat, the activity of L-methylmalonyl-CoA mutase is decreased [17]. This results in the secondary accumulation of propionyl-CoA and methylmalonyl-CoA generated during the normal metabolism of several amino acids and odd-chain-length fatty acids [18]. This situation is directly analogous to the human methylmalonic acidurias [19]. The current studies demonstrate that in the vitamin B-12-deficient rat there is a marked increase in tissue short-chain acylcarnitine contents and in the urinary excretion of acylcarnitines, including significant amounts of propionylcarnitine,

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Table 1. Compositions of the experimental diets

The control and vitamin B-12-deficient diets contained the constituents listed below as determined by Teklad (g/kg of diet): * indicates constituent in control diet only.

L-Alanine	3.5
L-Arginine hydrochloride	12.1
L-Asparagine	6.0
L-Aspartic acid	3.5
L-Cystine	3.5
L-Glutamic acid	40.0
Glycine	23.3
L-Histidine hydrochloride,H_2O	4.5
L-Isoleucine	8.2
L-Leucine	11.1
L-Lysine hydrochloride	18.0
L-Methionine	8.2
L-Phenylalanine	7.5
L-Proline	3.5
L-Serine	3.5
L-Threonine	8.2
L-Tryptophan	1.8
L-Tyrosine	5.0
L-Valine	8.2
Sucrose	485.0
Corn starch	150.0
Corn oil	100.0
Cellulose	30.0
Calcium phosphate, dibasic	22.0
Succinylsulphathiazol	10.0
Ethoxyquin	0.02
Sodium chloride	2.59
Potassium citrate, monohydrate	7.7
Potassium sulphate	1.82
Magnesium oxide	0.84
Manganous carbonate	0.123
Ferric citrate	0.210
Zinc carbonate	0.056
Cupric carbonate	0.011
Potassium iodate	0.00035
Sodium selenite	0.00035
Chromium potassium sulphate	0.19
p-Aminobenzoic acid (97.5%)	0.110132
Ascorbic acid	1.0166
Biotin	0.000441
Calcium pantothenate	0.0661
Choline dihydrogen citrate	3.497
Folic acid	0.002
Inositol	0.1101
Menadione	0.0496
Niacin	0.0991
Pyridoxine hydrochloride	0.022
Riboflavin	0.022
Thiamin hydrochloride	0.022
Vitamin E acetate	0.0396
Vitamin A palmitate	0.0396
Vitamin D_3	0.0044
Vitamin B-12 (0.1% in mannitol)	0.029736*

associated with the metabolic abnormalities of vitamin B-12 deficiency.

MATERIALS AND METHODS

Animals

Male Fisher-strain weanling rats (52 ± 2 g; $n = 48$) were used in all studies. On arrival, animals were weighed and placed in individual wire-bottom metabolic cages. Animals assigned to the vitamin B-12-deficient group were fed on an amino acid-based vitamin B-12-deficient diet (Teklad Inc., Madison, WI, U.S.A.), which was nutritionally complete except for the absence of vitamin B-12 (Table 1). Food consumption for each rat given the deficient diet was recorded 3 times per week. On the basis of this food consumption, a control rat was pair-fed along with each rat in the vitamin B-12-deficient group. The control diet was identical with the deficient diet, except that vitamin B-12 was included (Table 1). All rats were allowed water *ad libitum*. The rats were weighed approximately biweekly.

Urine was collected into a 50 ml Erlenmeyer flask containing 10 μl of 1.2 M-HCl. The flask was held in a container holding solid CO_2, permitting the urine to be kept frozen during a 24 h collection period. Animals were killed by rapid decapitation, and blood was collected into chilled heparinized beakers. Plasma was prepared from the blood by centrifugation immediately after collection. Samples of liver and skeletal muscle were obtained by rapid freeze-clamping of the tissue in aluminium blocks cooled in solid CO_2/acetone.

Assays

Carnitine was assayed by a modification of the radioenzymic assay of Cederblad *et al.* [20] as previously described [21]. Plasma and tissue samples were fractionated into acid-soluble and acid-insoluble fractions by precipitation in 3% (v/v) $HClO_4$. The acid-soluble fraction contained carnitine and short-chain acylcarnitines, and the acid-insoluble fraction contained long-chain acylcarnitines. Carnitine was generated from acylcarnitines by alkaline hydrolysis and then quantified as carnitine in the radioenzymic assay. Acid-soluble fractions were analysed twice, once without hydrolysis as a measure of carnitine, and again after hydrolysis to measure the sum of carnitine and short-chain acylcarnitines (this sum is termed total acid-soluble carnitine). Total carnitine refers to the sum of the carnitine, short-chain acylcarnitine and long-chain acylcarnitine concentrations. Urine samples were analysed for carnitine and short-chain acylcarnitines by the same procedure, except that the $HClO_4$ fractionation was omitted. The carnitine concentration in carnitine standards used was determined by the method of Marquis & Fritz [22].

Two techniques were used to study specific acylcarnitines present in samples obtained. Fast-atom-bombardment mass spectrometry permitted assessment of the acylcarnitine pool in samples and provided semi-quantitative information [9,23]; this analysis was performed as previously described [9], with octanoylcarnitine as an external standard. Acetylcarnitine and propionylcarnitine were quantified by a reverse-phase h.p.l.c. fractionation technique combined with post-chromatography application of the radioenzymic analysis for carnitine detailed above [24].

Urinary methylmalonic acid concentrations were measured by the g.l.c.–mass spectrometry assay previously described [25]. Liver samples were homogenized and the supernatants were assayed for holo-L-methylmalonyl-CoA mutase activity as described by Kovachy *et al.* [26]. This supernatant was also used to determine the hepatic vitamin B-12 content by using a commercially available radiodilution assay (Corning Immunophase, Medfield, MA, U.S.A.), which uses

purified hog intrinsic factor. β-Hydroxybutyrate concentrations were determined by the method of Olsen [27].

Reagents

Chemicals and solvents used were of reagent grade. Carnitine acetyltransferase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Acetyl-CoA and β-hydroxybutyrate dehydrogenase were purchased from Sigma Chemical Corp. (St. Louis, MO, U.S.A.). [^{3}H]Acetyl-CoA was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). L-Carnitine was a gift from Sigma Tau (Rome, Italy), and octanoylcarnitine was kindly provided by Dr. Charles Hoppel (Case Western Reserve University, Cleveland, OH, U.S.A.). Dowex 1 × 8 (Cl^- form; 200–400 mesh) ion-exchange resin used in the carnitine assay and to purify samples for propionylcarnitine analysis was purchased from Bio-Rad (Richmond, CA, U.S.A.)

Statistics

Values are means ± S.E.M.; n refers to the number of separate samples, each from a different experimental animal analysed to obtain the values presented. Data were analysed for statistical significance by Student's t test, with $P < 0.05$ considered significant.

RESULTS

Characterization of vitamin B-12-deficient rats

Rats fed on control or vitamin B-12-deficient diets maintained on the pair-feeding schedule gained weight at similar rates (Fig. 1). Urinary excretion of methylmalonic acid was monitored as an index of the metabolic defect associated with vitamin B-12 deficiency. After 11 weeks, methylmalonic acid excretion averaged 0.24 ± 0.03 and 10.3 ± 5.6 μmol/day in the control ($n = 11$) and vitamin B-12-deficient ($n = 11$) animals respectively. After 21 weeks on the vitamin B-12-deficient diet, the animals demonstrated a profound vitamin B-12 deficiency, as evidenced by a 230-fold increase in urinary methylmalonic acid excretion, a 74% decrease in hepatic vitamin B-12 content and a 21% decrease in hepatic holo-L-methylmalonyl-CoA mutase activity (Table 2).

Urinary carnitine excretion

Over the course of 21 weeks on the special diet, the vitamin B-12-deficient group developed an increase in urinary excretion of short-chain acylcarnitines as compared with the control animals (Table 3). This difference was demonstrated by both a higher daily elimination of acylcarnitines and an increase in the percentage of total urinary carnitine present as acylcarnitine. The increase in urinary acylcarnitines associated with vitamin B-12 deficiency was evident after 11 weeks on the deficient diet, and after 21 weeks acylcarnitines accounted for 30% of total urinary carnitine in the deficient animals, compared with 15% in the control group. In contrast, urinary excretion of total carnitine was not different in the two groups. The increased urinary excretion of acylcarnitines in the vitamin B-12-deficient animals was correlated with the urinary methylmalonic acid excretion (Fig. 2).

Acylcarnitines are generated from the corresponding acyl-CoA, and therefore the specific acylcarnitines excreted in the vitamin B-12-deficient rats should correspond to the acyl-CoAs which accumulate secondarily to the metabolic defect in vitamin B-12 deficiency. Acetylcarnitine and propionylcarnitine were therefore specifically quantified in the urine of control and vitamin B-12-deficient rats after 21 weeks on the special diets. A h.p.l.c. method was used to resolve acetylcarnitine and propionylcarnitine for post-chromatographic quantification [24]. In control rats, acetylcarnitine accounted for most (58%) of the measured total urinary acylcarnitine, with very little

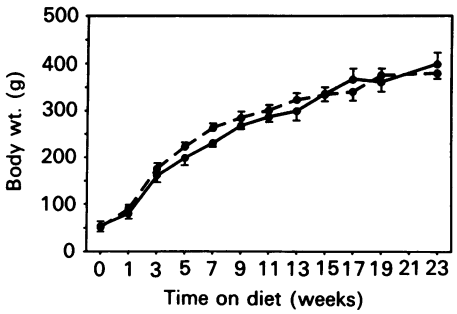

Fig. 1. Weight gain of experimental animals

Weanling rats were begun at week 0 on control (——) or vitamin B-12-deficient diets (– – – –) on a pair-feeding schedule as described in the text. Values are means ± S.E.M., with n ranging from 5 to 24 at individual time points.

Table 2. Vitamin B-12 status in the experimental animals

Animals were maintained on vitamin B-12-deficient or control diets for 24 weeks as described in the text. After 21 weeks on the diet, 24 h urine collections were obtained for quantification of methylmalonic acid excretion. Liver samples obtained at the time of killing were analysed for vitamin B-12 content and holo-L-methylmalonyl-CoA mutase activity. Values are means ± S.E.M., with n shown in parentheses: *$P < 0.05$ for vitamin B-12-deficient versus control.

	Urinary methylmalonic acid excretion (μmol/24 h)	Hepatic vitamin B-12 content (ng/g wet wt. of liver)	Hepatic holo-L-methylmalonyl-CoA mutase activity (units/g wet wt. of liver)
Control	0.21 ± 0.02 (11)	90 ± 10 (5)	34 ± 3 (5)
Vitamin B-12-deficient	49.4 ± 22.5* (12)	23 ± 5* (6)	27 ± 2* (6)

Table 3. Urinary carnitine excretion in vitamin B-12-deficient rats

At various times after initiation of the experimental diet feedings, 24 h urine collections were obtained as described in the text. The urinary excretions of carnitine, short-chain acylcarnitines and total carnitine were determined by the radioenzymic assay as described in the text. Values are means ± S.E.M., with n shown in parentheses. *$P < 0.05$ for vitamin B-12-deficient versus control.

Time of collection	Rats	Excretion (μmol/day)			
		Carnitine	Short-chain acylcarnitine	Total carnitine	Percentage of total as acylcarnitine
Week 7	Control (16)	0.540±0.069	0.270±0.070	0.810±0.110	32±4
	B-12-deficient (18)	0.546±0.066	0.401±0.130	0.945±0.170	37±3
Week 11	Control (11)	1.83±0.32	0.404±0.090	2.23±0.31	19±4
	B-12-deficient (12)	1.38±0.15	0.819±0.150*	2.20±0.18	35±6*
Week 16	Control (15)	1.44±0.17	0.400±0.081	1.84±0.19	23±4
	B-12-deficient (15)	1.46±0.14	0.663±0.114*	2.12±0.17	29±4
Week 21	Control (11)	2.23±0.28	0.418±0.080	2.65±0.33	15±2
	B-12-deficient (12)	1.77±0.19*	0.719±0.110*	2.49±0.13	30±5*

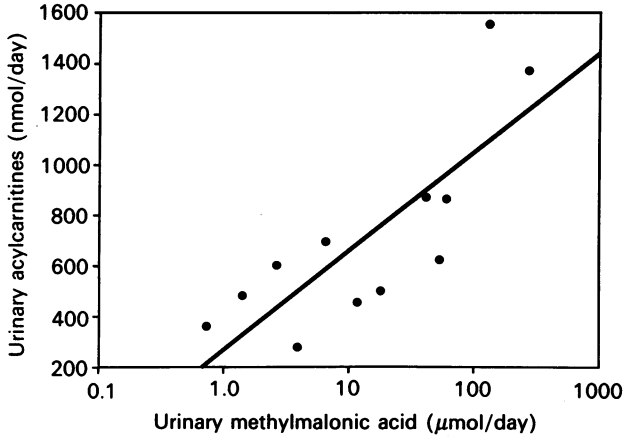

Fig. 2. Correlation between urinary methylmalonic acid and acylcarnitine excretion in vitamin B-12-deficient rats

After 21 weeks on the vitamin B-12-deficient diet, 24 h urine collections were obtained from the rats, and methylmalonic acid and acylcarnitine contents were determined. Each point on the Figure represents an individual animal. Note the logarithmic scale for the methylmalonic acid excretion. The line shown is the linear least-squares regression fit [acylcarnitine excretion = 392 × log (methylmalonic acid excretion) − 269; $r = 0.802$].

propionylcarnitine (Table 4). Urine from the vitamin B-12-deficient rats contained significant amounts of propionylcarnitine as compared with controls, with daily excretion of propionylcarnitine reaching 120 nmol in the vitamin B-12-deficient rats as compared with 11 nmol in the control group. Acetylcarnitine accounted for only 28% of the total urinary acylcarnitine in the vitamin B-12-deficient rats. The increased content of propionylcarnitine in the urine of the vitamin B-12-deficient rats was verified by using fast-atom-bombardment mass spectrometry (Table 4), in both absolute (comparison with external standard) and relative (comparison with acetylcarnitine) terms.

Plasma and tissue carnitine pools

Groups of vitamin B-12-deficient and control animals were killed after 24 weeks on the diet, and their plasma, liver and skeletal-muscle carnitine pools were measured (Table 5). The vitamin B-12-deficient animals demonstrated decreased plasma concentrations of carnitine and total carnitine as compared with the control animals. No differences in the concentrations of plasma short- or long-chain acylcarnitines were seen. Thus the percentage of total acid-soluble carnitine (the sum of carnitine and short-chain acylcarnitine) present as acylcarnitines was increased in the vitamin B-12-deficient rats (22%) as compared with the control rats (18%).

Comparison of the liver carnitine pools in the two groups of rats showed decreases in carnitine and total carnitine content in the vitamin B-12-deficient rats. The percentage of hepatic total acid-soluble carnitine present as acylcarnitine was increased in the vitamin B-12-deficient rats (59%) as compared with the control animals (48%). The skeletal-muscle contents of carnitine, short- and long-chain acylcarnitine and total carnitine were equivalent in the two groups. Propionylcarnitine contents in skeletal muscle and liver of control rats were low (12±3 and 16±4 nmol/g respectively; $n = 6$). Skeletal-muscle propionylcarnitine content was 51±29 nmol/g ($n = 7$) in the vitamin B-12-deficient rats, accounting for 32% of the muscle short-chain acylcarnitines. Hepatic propionylcarnitine content in the vitamin B-12-deficient rats was 12±3 nmol/g ($n = 7$), and not different from control values.

Effect of fasting on carnitine metabolism in the vitamin B-12-deficient rat

During fasting, significant changes occur in carnitine metabolism in the rat, including a redistribution of the total carnitine pool into acylcarnitines [21,28,29]. After 24 weeks of pair-feeding, a group of control and vitamin B-12-deficient rats were fasted for 48 h. Over the 48 h fast, the plasma β-hydroxybutyrate concentration rose from 0.12±0.01 mM in the fed state ($n = 6$) to 0.80±0.07 mM ($n = 7$) in the control rats, and from

Table 4. Urinary acetylcarnitine and propionylcarnitine excretion in vitamin B-12-deficient rats

After 21 weeks on the pair-feeding schedule, 24 h urine collections were obtained from control and vitamin B-12-deficient rats. Acetylcarnitine and propionylcarnitine were measured after h.p.l.c. resolution by using the radioenzymic assay as described in the text. Total acylcarnitine content was determined by direct analysis of the urine. Urinary propionylcarnitine content was also analysed by fast-atom-bombardment mass spectrometry (FAB-MS) for the seven pairs of rats in which methylmalonic acid excretion was above 10 μmol/day in the vitamin B-12-deficient animal. The propionylcarnitine content was characterized by comparing the signal intensity at $m/z = 218$ (propionylcarnitine) with that at $m/z = 288$ (octanoylcarnitine external standard) or $m/z = 204$ (acetylcarnitine). FAB-MS values are not corrected for background. Values are means ± S.E.M.; $*P < 0.05$ for control versus vitamin B-12-deficient.

	H.p.l.c. separation						
	24 h excretion (nmol/day)			Percentage of total as acetyl-carnitine	Percentage of total as propionyl-carnitine	FAB-MS	
	Acetyl-carnitine	Propionyl-carnitine	Total acylcarnitine			218/288	218/204
Control	218±63	11±2	418±80	58±14%	3.2±0.8%	0.131±0.013	1.73±0.14
B-12-deficient	198±47	121±70*	719±110*	28±5%*	10.2±4.9%*	0.207±0.002*	2.11±0.07*

Table 5. Plasma and tissue carnitine pools in vitamin B-12-deficient rats

Rats were maintained on control and vitamin B-12-deficient diets as described in the text. After 24 weeks, groups of rats were killed; plasma, liver and skeletal-muscle samples were collected and the contents of carnitine, short-chain acylcarnitines, long-chain acylcarnitines and total carnitine (the sum of carnitine, short-chain acylcarnitines and long-chain acylcarnitines) determined. Total acid-soluble carnitine refers to the sum of carnitine and short-chain acylcarnitine. Values are means ± S.E.M.; n is shown in parentheses; $*P < 0.05$ for vitamin B-12-deficient versus control.

	Carnitine	Short-chain acylcarnitine	Percentage of total acid-soluble as acylcarnitine	Long-chain acyl-carnitine	Total carnitine
Plasma (μM)					
Control (6)	68.9±4.5	14.5±0.7	18±1%	6.8±0.6	88.1±3.8
B-12-deficient (7)	56.2±5.6*	14.8±1.5	22±3%*	6.3±0.5	77.3±5.8*
Liver (nmol/g wet wt.)					
Control (6)	112±9	103±11	48±2%	13±1	228±21
B-12-deficient (7)	72±11*	97±5	59±4%*	11±1	180±14*
Skeletal muscle (nmol/g wet wt.)					
Control (6)	626±17	147±21	19±2%	33±2	806±30
B-12-deficient (7)	625±42	161±27	21±4%	35±1	808±19

0.11±0.01 mM ($n = 7$) to 1.13±0.34 mM ($n = 5$) in the vitamin B-12-deficient rats. With fasting, the urinary excretion of carnitine, acylcarnitines and total carnitine decreased (Table 6) as compared with the fed state, with an increase in the percentage of total carnitine as acylcarnitine. In the vitamin B-12-deficient rats the larger excretion of acylcarnitines as compared with controls seen in the fed state was maintained, and resulted in an increased total carnitine excretion during fasting. In plasma, the free carnitine concentration fell by 18% in control animals during fasting, but it rose by 17% in the vitamin B-12-deficient rats. Plasma short-chain acylcarnitines increased by 86% in control rats and by 136% in the vitamin B-12-deficient group. Increases in short-chain acylcarnitines were also seen in liver during fasting. The percentage of hepatic total acid-soluble carnitine present as short-chain acylcarnitine was higher in the vitamin B-12-deficient rats (53%) as compared with control animals (43%). In skeletal muscle from control rats, the short-chain acylcarnitine content fell with fasting by 22%. In contrast, an increase of 68% was seen in the short-chain acylcarnitine content of skeletal muscle in vitamin B-12-deficient rats during the 48 h fast. Thus fasting accentuated the redistribution of total carnitine towards short-chain acylcarnitines in the vitamin B-12-deficient rats.

DISCUSSION

Methylmalonic aciduria in the vitamin B-12-deficient rat was associated with a redistribution of urine and tissue total carnitine content towards short-chain acylcarnitines, and away from free carnitine. Propionylcarnitine became a significant constituent of the carnitine pool in vitamin B-12 deficiency. The increase in short-chain acylcarnitines seen in vitamin B-12 deficiency was

Table 6. Effect of fasting on carnitine metabolism in the vitamin B-12-deficient rat

After 24 weeks on the pair-feeding protocol, food was removed from control and vitamin B-12-deficient rats for 48 h. At the conclusion of the 48 h, the rats were killed and plasma, liver and skeletal muscle collected; 24 h urine collections were obtained before the fast, and during the last 24 h of the fast. Values are means ± S.E.M.; with $n = 5$; $*P < 0.05$ for vitamin B-12-deficient versus control. Percentage changes from the mean values measured in fed animals are shown in parentheses.

	Carnitine	Short-chain acylcarnitine	Percentage of total acid-soluble as acylcarnitine	Long-chain acylcarnitine	Total carnitine
Plasma (μM)					
Control	56.0±2.3 (−18)	27.0±1.9 (+86)	32±2	8.9±0.2 (+31)	92.5±1.5 (+5)
B-12-deficient	65.5±2.2* (+17)	34.9±2.7* (+136)	35±2	10.1±0.4 (+60)	110.5±4.5* (+43)
Liver (nmol/g wet wt.)					
Control	239±20 (+113)	182±10 (+77)	43±2	41±3 (+215)	462±26 (+103)
B-12-deficient	160±17* (+122)	181±13 (+87)	53±4*	38±2 (+246)	379±15* (+110)
Skeletal muscle (nmol/g wet wt.)					
Control	617±44 (−1)	115±19 (−22)	16±2	45±4 (+36)	777±59 (−4)
B-12-deficient	717±107 (+15)	271±34* (+68)	29±5*	36±6 (+3)	1024±107* (+27)
Urine (μmol/day)					
Control	0.280±0.079 (−92)	0.325±0.052 (−49)	55±10	–	0.605±0.030 (−85)
B-12-deficient	0.417±0.088 (−85)	0.718±0.210* (−41)	58±9	–	1.135±0.170* (−71)

maintained or accentuated under the metabolic stress of starvation ketosis.

In the rat, vitamin B-12 is a required cofactor for two enzymes. The first, methionine synthase, is responsible for methionine generation from homocysteine. The second, L-methylmalonyl-CoA mutase, converts L-methylmalonyl-CoA into succinyl-CoA for further metabolism. In the vitamin B-12-deficient rat L-methylmalonyl-CoA and propionyl-CoA accumulate secondarily to the decreased L-methylmalonyl-CoA mutase activity [18]. This accumulation in turn leads to a large increase in urinary methylmalonic acid excretion [30,31]. This is qualitatively identical with the defects in the methylmalonic acidurias [19]. In the current studies urinary excretion of methylmalonic acid was used as a marker of the metabolic defect with the development of vitamin B-12 deficiency. After 21 weeks on the experimental diet, the methylmalonic acid excretion in the vitamin B-12-deficient rats was 230 times the control value. Hepatic vitamin B-12 contents and holo-L-methylmalonyl-CoA mutase activity also confirmed the vitamin B-12 deficiency.

Increased generation of acylcarnitines from acyl-CoAs may be reflected as either an increase in the absolute amount of acylcarnitine or as a redistribution of the carnitine pool (regardless of total carnitine content) towards acylcarnitines [3,21,29]. The changes observed in the carnitine pool with the development of vitamin B-12 deficiency were consistent with both of these concepts. Urinary excretion of acylcarnitines was doubled in the vitamin B-12-deficient rats as compared with controls. The percentage of total acid-soluble carnitine present as acylcarnitine was also higher in urine, plasma and liver from the vitamin B-12-deficient animals as compared with the controls. Overall, the increased urinary excretion of acylcarnitines correlated well with the degree of metabolic impairment resulting from the vitamin B-12 deficiency as assessed by methylmalonic acid excretion (Fig. 2). The use of the pair-feeding schedule eliminated energy intake or age, both of which alter carnitine metabolism [21,29,32], as possible etiologies for the differences observed in the two groups of animals.

The nature of the acyl-CoA accumulation is also reflected in the specific acyl moieties present in the acylcarnitine pool. Propionylcarnitine excretion was increased 11-fold in vitamin B-12-deficient rats as compared with control animals. Propionylcarnitine has also been identified as a major acylcarnitine in the urine of patients with methylmalonic aciduria [5]. The increase in urinary acylcarnitine excretion with vitamin B-12 deficiency was approximately 300 nmol/day (Table 3), or which 100 nmol/day could be accounted for by propionylcarnitine. This is consistent with the production of other, as yet unidentified, acylcarnitines in vitamin B-12 deficiency. Acyl-CoAs other than propionyl-CoA or methylmalonyl-CoA might also accumulate and form acylcarnitines secondarily to disruption of normal intermediary metabolism by the propionyl-CoA build-up. Burton & Frenkel [33] identified propionylcarnitine in the liver of vitamin B-12-deficient rats. In the most severely vitamin B-12-deficient animals in the current study, propionylcarnitine was estimated to represent 45% and 30% of total short-chain acylcarnitine in skeletal muscle and liver respectively. The large variability in the degree of methylmalonic aciduria attained in the current studies minimized the changes in mean tissue propionylcarnitine content. Despite the large degree of methylmalonic aciduria in our animals, and the dramatic changes in the CoA pool previously described in vitamin B-12 deficiency [18], the magnitude of the redistribution of tissue carnitines towards acylcarnitines was relatively small (Table 5). Although the reasons for this are unclear, the poor conversion of branched-chain acyl-CoAs (such as methylmalonyl-CoA, a major acyl-CoA in vitamin B-12 deficiency) into the corresponding acylcarnitine [34], may have minimized the changes in the carnitine pool.

Starvation is associated with an increase in short-chain

acylcarnitines [21,28,29]. This phenomenon was maintained or enhanced in the vitamin B-12-deficient rats. For example, although skeletal-muscle short-chain acylcarnitine content fell in the control animals with fasting, consistent with previous observations [21], short-chain acylcarnitine content of skeletal muscle increased by 68 % with fasting in the vitamin B-12-deficient group. This increase in short-chain acylcarnitines may be associated with the increased utilization of branched-chain amino acids by muscle during starvation [35].

The biosynthesis of carnitine requires the methylation of lysine contained in protein to form trimethyl-lysine [36,37]. The impairment of methionine formation from homocysteine in vitamin B-12 deficiency might therefore interfere with carnitine biosynthesis. As most of the rat's carnitine is in skeletal muscle [21,29], the normal total carnitine content in the skeletal muscle of the vitamin B-12-deficient rats after 24 weeks on the diets, along with the normal total urinary carnitine elimination, suggests that there is no major impairment in carnitine biosynthesis in these animals. Thus the increase in short-chain acylcarnitines seen in vitamin B-12 deficiency reflect changes in the CoA pool rather than a broad disturbance in carnitine homoeostasis.

The vitamin B-12-deficient rat demonstrates changes in carnitine metabolism in both the fed and fasted states as compared with control animals. These changes correspond both qualitatively and quantitatively to predictions based on the accumulation of specific acyl-CoAs in these animals. The vitamin B-12-deficient rat provides a potential model system for studying the effects of changes in acylcarnitine generation on intermediary metabolism in the methylmalonic acidurias, with possible extrapolation to other organic acidaemias and diseases associated with acyl-CoA accretion.

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