ENZYME CHANGES ASSOCIATED WITH MITOCHONDRIAL MALIC ENZYME DEFICIENCY IN MICE

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Summary

A genetically determined absence of mitochondrial malic enzyme (EC 1.1.1.40) in c^{3H}/c^{6H} mice is accompanied by a four-fold increase in liver glucose-6-phosphate dehydrogenase and a two-fold increase for 6-phosphogluconate dehydrogenase activity. Smaller increases in the activity of serine dehydratase and glutamic oxaloacetic transaminase are observed while the level of glutamic pyruvate transaminase activity is reduced in the liver of deficient mice. Unexpectedly, the level of activity of total malic enzyme in the livers of mitochondrial malic enzyme-deficient mice is increased approximately 50% compared to littermate controls. No similar increase in soluble malic enzyme activity is observed in heart of kidney tissue of mutant mice and the levels of total malic enzyme in these tissues are in accord with expected levels of activity in mitochondrial malic enzyme-deficient mice. The divergence in levels of enzyme activity between mutant and wild-type mice begins at 19—21 days of age. Immunoinactivation experiments with monospecific antisera to the soluble malic enzyme and glucose-6-phosphate dehydrogenase demonstrate that the activity increases represent increases in the amount of enzyme protein. The alterations are not consistent with a single hormonal response.

Introduction

Animal models of inborn errors of metabolism are valuable for eliciting the pathophysiology of an enzyme defect as well as the interrelationships between metabolic pathways. A wide range of pathological and physiological effects

have been described in mice homozygous for radiation-induced deletions at the albino locus on chromosome 7 [1,2]. These radiation-induced mutations are known to be physical deletions of chromosomal material by both genetic [3] and cytological analyses [4,5]. Several of the deletions were found to include the mitochondrial malic enzyme locus, *Mod-2*, which maps 1 centimorgan from the albino locus. This locus is presumed to be the structural locus for mitochondrial malic enzyme (EC 1.1.1.40) as it controls electrophoretic variation of this enzyme [3]. It has recently been found that two different deletions which remove the mitochondrial malic enzyme locus can complement each other in heterozygotes to produce viable mice which still have a complete absence of mitochondrial malic enzyme although cytoplasmic malic enzyme, *Mod-1*, is present [6]. These mitochondrial malic enzyme-deficient mice survive to adulthood although their growth rate is reduced and both males and females are sterile [7]. In contrast to the *Mod-2*-deficient mice, *Mod-1*-deficient mice are fully viable and fertile and no alterations in the level of activity of several enzymes in related pathways were observed [8].

Malic enzyme catalyzes the oxidative decarboxylation of L-malate to pyruvate plus CO_2 with the concurrent generation of NADPH [9]. This generation of NADPH has been suggested to be important for lipid biosynthesis [9] and maintenance of reduced glutathione levels [10]. The activity of total liver malic enzyme in rats is responsive to several environmental stimuli. Malic enzyme is induced by high carbohydrate diets [11], low protein diets [9,12], thyroxine [13,14], and insulin [15,16]. Enzyme induction is inhibited by glucagon [9]. In light of the central role of malic enzyme we have examined the level of activity of several enzymes in liver, heart, and kidney tissue of mice with a complete absence of mitochondrial malic enzyme in a effort to understand further the possible mechanisms of metabolic interactions and pleiotropy.

Materials and Methods

Mice. As the radiation-induced deletions c^{3H} and c^{6H} are both lethal when homozygous, they must be maintained in the heterozygous state. Carriers of these deletions, c^{ch}/c^{3H} and c^{ch}/c^{6H} mice, can be distinguished from homozygous chinchilla (c^{ch}/c^{ch}) littermates by their non-dilute coat color. Interline crosses of c^{3H}/c^{ch} and c^{6H}/c^{ch} were used to produce partially complementing c^{3H}/c^{6H} double heterozygotes which are easily recognizable by their albino phenotype. Heterozygotes (c^{ch}/c^{3H}, c^{ch}/c^{6H}) or homozygous chinchilla (c^{ch}/c^{ch}) sibs were used for controls.

Enzyme assays. Enzyme assays were performed utilizing the Miniature Centrifugal Fast Analyzer [17]. The enzymes glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.43), hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40), glutathione reductase (EC 1.6.4.2), malate dehydrogenase (EC 1.1.1.37), and glutamic-oxaloacetic transaminase (EC 2.6.1.1) were assayed as described by Fielek and Mohrenweiser [18]. Other enzymes assayed utilizing modified methodology as noted were malic enzyme (EC 1.1.1.40) [19], serine dehydratase (EC 4.2.1.13) [20], isocitrate dehydrogenase (EC 1.1.1.42) [21], fatty acid synthetase (EC 2.3.1.38) [22], adenosine triphosphatase (EC

3.6.1.3) [23], and glutamic-pyruvate transaminase (EC 2.6.1.2) [24] (Feuers, R. et al., unpublished data). All assays were performed at saturating substrate and cofactor concentrations and enzyme activity was a linear function of protein concentration with the assay conditions employed in this study. Tissues were collected and homogenates prepared for enzyme analysis as previously described [25]. The mitochondrial fraction was isolated by differential centrifugation [26]. Mitochondrial and soluble malic enzyme was examined by starch gel electrophoresis [3].

All substrates and coupling enzymes for the enzyme assays were obtained from Calbiochem. Other components were of analytical/reagent quality.

Immunoinactivation studies of glucose-6-phosphate dehydrogenase. The preparation of the specific antiserum to mouse glucose-6-phosphate dehydrogenase has previously been described [27]. Liver extracts were prepared as described for enzyme analyses; erythrocyte hemolysates were prepared by freezing a 3% solution of heparinized blood in glucose-6-phosphate dehydrogenase buffer (0.05 M Tris-HCl, pH 7.5, 10^{-5} M NADP; 0.1% β-mercaptoethanol; 0.05% bovine serum albumin). These extracts were diluted to an activity of about 0.1 I.U./ml, and 200 μl were incubated for 30 min at 24°C with up to 10 μl of normal serum or antiserum. After centrifugation at 1400 × *g* for 15 min, the supernatants were assayed by the standard spectrophotometric technique [8]. The remaining activity is expressed as percent of controls incubated with the same ratio of normal sera.

Preparation of antisera to soluble malic enzyme. Purified soluble malic enzyme was the gift of Dr. Chi Yu Lee and had been prepared by affinity chromatography [29]. The enzyme was electrophoresed on 6.0% acrylamide gels, with 0.2 M Tris-HCl buffer, pH 8.6, in the gels and 0.4 M Tris-HCl, pH 8.3, as the tank buffer. After staining for activity [30], the gels were frozen. The major protein band was found by staining a longitudinal, tangential slice with amidoschwarz; this band corresponded to the activity band. Gel slices (corresponding to the enzyme and containing about 20 μg protein) were pulverized in 1 ml of phosphate-buffered saline, and homogenized with equal volume of Freund's adjuvant, complete for the first injection and incomplete thereafter. Injections were continued at weekly intervals and the sera used were obtained at 6 weeks. A single precipitation arc was found on double diffusion with homogenates of liver, testes, and heart.

Immunoinactivation studies of the soluble malic enzyme. Liver extracts were prepared as for enzyme analyses and diluted to about 0.1 I.U./ml with 0.2 M glycylglycine buffer, pH 8.0. 200 μl were incubated for 30 min at 24°C with up to 80 μl of normal sera or antisera diluted 1:100 in the buffer. They were then centrifuged at 1400 × *g* for 15 min and the supernatants were assayed by the standard spectrophotometric technique [31]. The remaining activity is expressed as percent of controls incubated with the same ratio of normal sera.

Results

The level of activity of 14 enzymes in liver, heart, and kidney tissues of c^{3H}/c^{6H} and control mice is shown in Table I. The most significant result is the unexpectedly higher level of liver malic enzyme in the mitochondrial malic

TABLE I

ENZYME ACTIVITY IN TISSUES OF MITOCHONDRIAL MALIC ENZYME-DEFICIENT AND CONTROL MICE

Mice and enzyme assays are as decribed in Materials and Methods. N is 10 for the $c^{3H}/c6H$ group and 6 for the control group. Activity is expressed as μM product formed/g tissue per min, mean ± S.E.

Enzyme	Liver		Heart		Kidney	
	$c^{3H}/c6H$	Control	$c^{3H}/c6H$	Control	$c^{3H}/c6H$	Control
Malic enzyme	10.0 ± 0.6	6.7 ± 0.8	1.2 ± 0.4	2.8 ± 0.4	8.3 ± 0.6	11.7 ± 1.8
Glucose-6-phosphate dehydrogenase	0.9 ± 0.1	0.2 ± 0.1	0.3 ± *	0.3 ± *	1.4 ± 0.1	1.5 ± 0.1
6-Phosphate gluconate dehydrogenase	0.5 ± *	0.2 ± *	0.1 ± *	0.1 ± *	0.4 ± *	0.5 ± 0.1
Hexokinase	1.2 ± 0.1	0.7 ± 0.1	0.6 ± *	0.8 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Serine dehydratase	20.0 ± 2.6	13.5 ± 0.5	—	—	0.1 ± *	0.1 ± *
Isocitrate dehydrogenase	38.2 ± 0.8	39.0 ± 1.3	88.4 ± 3.6	78.2 ± 6.9	42.3 ± 1.1	44.8 ± 2.6
Fatty acid synthetase	1.3 ± 0.1	1.5 ± 0.1	0.2 ± *	0.3 ± *	0.8 ± 0.3	0.8 ± 0.1
Lactate dehydrogenase	332.9 ± 18.4	330.8 ± 16.9	97.4 ± 5.7	131.6 ± 6.4	177.4 ± 6.3	184.6 ± 8.6
Pyruvate kinase	15.4 ± 0.9	18.6 ± 1.3	37.7 ± 1.6	48.8 ± 1.3	33.4 ± 1.1	32.4 ± 2.5
Citrate cleavage enzyme	1.2 ± 0.3	1.1 ± 0.1	0.2 ± *	0.3 ± *	0.4 ± 0.1	0.7 ± 0.1
Adenosine triphosphatase	26.4 ± 1.1	27.5 ± 1.1	17.3 ± 1.1	21.3 ± 1.3	39.5 ± 1.7	48.2 ± 5.1
Glutathione reductase	9.9 ± 0.3	10.4 ± 0.7	1.3 ± 0.1	1.8 ± 0.1	9.1 ± 0.2	13.9 ± 1.3
Glutamic-oxaloacetic transaminase	222.5 ± 9.9	197.7 ± 15.7	58.8 ± 3.5	76.6 ± 5.8	237.2 ± 7.8	273.2 ± 20.3
Glutamic-pyruvate transaminase	29.1 ± 0.9	40.3 ± 3.0	2.4 ± 0.2	3.3 ± 0.2	5.3 ± 0.2	8.1 ± 0.8

* S.E. < 0.05.

enzyme-deficient mice. In contrast to liver, the level of malic enzyme in heart and kidney of c^{3H}/c^{6H} mice is lower than observed for the sibling controls. This level of reduction is consistent with the complete absence of mitochondrial malic enzyme in heart and kidney of these mice. The first two enzymes of the hexose monophosphate pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are markedly increased in the liver of c^{3H}/c^{6H} mice but are normal in kidney and heart. A smaller increase in hexokinase is also observed. The level of activity of the gluconeogenic enzyme, serine dehydratase, is also increased in the liver of deficient mice. The level of glutamic-oxaloacetic transaminase is increased in liver while glutamic-pyruvate transaminase is reduced. No differences in the activity levels of seven other enzymes which are associated with lipogenesis, energy metabolism or glycolysis were detectable. In contrast to liver, kidney and heart have decreased levels of glutamic oxaloacetic transaminase in c^{3H}/c^{6H} mice, a pattern similar to that observed for malic enzyme. The activities of glutathione reductase and adenosine triphosphatase are reduced in both the heart and kidney of the mitochondrial malic enzyme-deficient mice. The levels of total malic enzyme and glutathione reductase have been previously shown to change in a coordinated manner in response to dietary manipulation [10]. Another transaminase, glutamic-pyruvate transaminase, has a unique pattern in that c^{3H}/c^{6H} mice have reduced activities in all three of the tissues examined.

Electrophoretic studies had indicated that no mitochondrial malic enzyme was detectable in liver, heart or kidney of c^{3H}/c^{6H} mice [6]. In order to con-

TABLE II

CELLULAR DISTRIBUTION OF SEVERAL ENZYMES IN CONTROL AND MITOCHONDRIAL MALIC ENZYME-DEFICIENT (c^{3H}/c^{6H}) MICE

A mitochondrial fraction was isolated from tissues of two control and two variant mice as described in Materials and Methods (hearts from two mice were pooled). Mice and assays are described in Materials and Methods. Activity is the individual value for each mouse expressed as μmol product formed/g tissue per min.

Cell fraction	Liver		Kidney		Heart	
	c^{3H}/c^{6H}	Control	c^{3H}/c^{6H}	Control	c^{3H}/c^{6H}	Control
Malic enzyme						
Total	4.3, 4.4	2.9, 3.9	1.2, 4.6	3.0, 5.0	0.8	1.55
Soluble	4.2, 4.6	2.5, 3.0	1.3, 3.7	1.8, 4.4	0.7	1.32
Mitochondria	0.2, 0.3	0.34, 0.40	0.14, 0.54	0.65, 0.75	0.1	0.4
Malic dehydrogenase						
Total	376, 474	389, 393	360, 544	466, 546	640	627
Soluble	295, 395	246, 374	306, 474	394, 514	495	535
Mitochondria	84, 100	87, 93	53, 177	98, 126	162	114
Lactate dehydrogenase						
Total	230, 364	235, 347	170, 270	200, 340	193	220
Soluble	230, 390	247, 353	170, 275	218, 302	195	240
Mitochondria	11, 13	7, 14	13, 26	17, 23	3	4
Glutamic-oxaloacetic transaminase						
Total	150, 270	164, 166	76, 85	71, 89	221	215
Soluble	39, 105	13, 42	21, 22	20, 28	109	121
Mitochondria	115, 171	138, 141	40, 65	40, 60	96	76

firm that the increase in total malic enzyme activity in the liver of c^{3H}/c^{6H} mice was due to an increased level of *MOD-1* in the absence of the mitochondrial form, tissue fractionation experiments were conducted with two pair of mice. Only small amounts of malic enzyme activity could be detected in the isolated mitochondrial preparation (Table II) and upon starch gel electrophoresis all the activity had an electrophoretic mobility associated with malic enzyme from the cytosol, further confirming that the increase in activity was associated with *MOD-1*. The lactate dehydrogenase distribution would suggest that about 5% of the soluble enzyme activity in liver and 10% in heart and kidney is trapped in the mitochondrial pellet. Thus it is assumed that all of the malic enzyme activity in the mitochondrial pellet is the result of cytosolic contamination, which is consistent with other electrophoretic data [6] that the c^{3H}/c^{6H} mice are totally deficient in mitochondrial malic enzyme. The increased level of glutamic-oxaloacetic transaminase in liver may also result from an increased level of soluble enzyme (glutamic-oxaloacetic transaminase-1).

No alterations in the ratio of soluble to mitochondrial activity is noted for malate dehydrogenase.

The increased soluble malic enzyme activity in liver could result from increased enzyme protein or enzyme with increased specific activity. As seen in Fig. 1, liver extracts from a group of uniformly aged inbred mice (normal controls) were rapidly inactivated to 10% of initial values and were not further
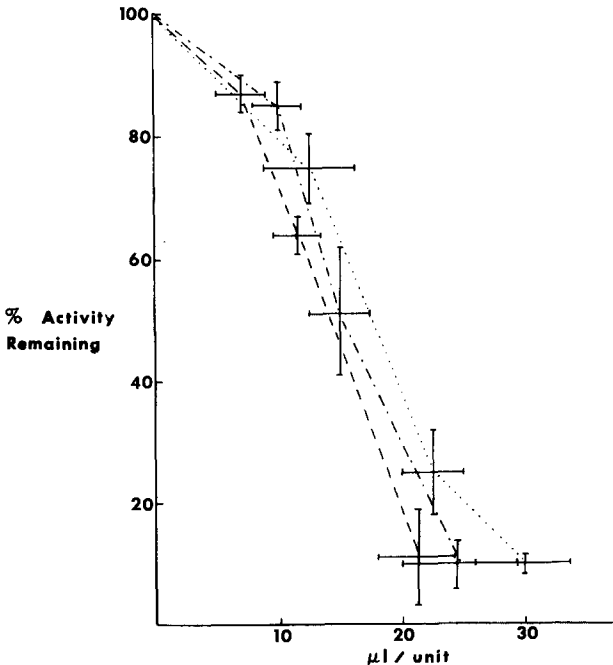

Fig. 1. Immunoprecipitation of liver soluble malic enzyme. Supernatant fractions from liver of c^{3H}/c^{6H} (- - - - - -) and sibling (— · — ·) control mice were prepared and immunoprecipitations were conducted as described in Materials and Methods. Each curve represents the data from three mice. The variation in the procedures was determined by utilizing liver supernatants from control mice of a uniform age (· · · · · ·). The horizontal bars represent the interval for which the vertical bars (●) represent ±1 S.E.
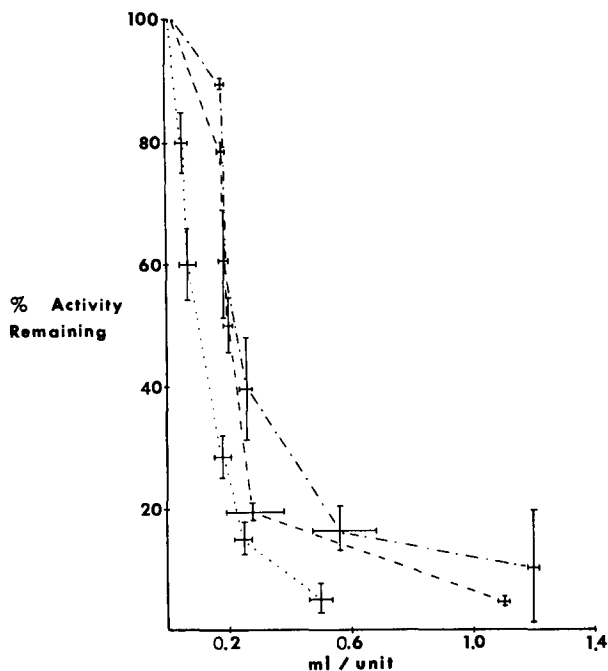
Fig. 2. Immunoprecipitation of liver glucose-6-phosphate dehydrogenase. Symbols and procedures are as described in Fig. 1 except that erythrocyte glucose-6-phosphate dehydrogenase was utilized in the control experiment (· · · · · ·).

inactivated when incubated with a large excess of antibody. The residual activity presumably represents mitochondrial malic enzyme. The immunoinactivation of malic enzyme in variously aged deficient mice and their sibs (from several litters) were considerably more variable but there was considerable over-
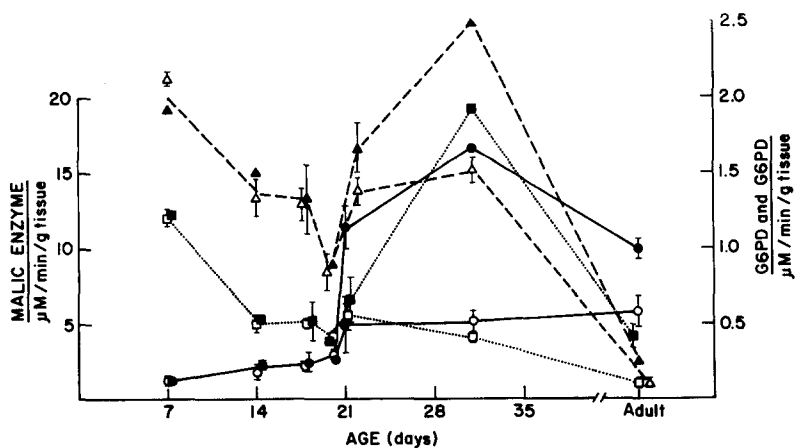

Fig. 3. Changes in enzyme activity in liver of c^{3H}/c^{6H} and sibling control mice between 7 days and 45 days of age. Tissue preparations and enzyme assays were performed as described in Materials and Methods. ●,■, c^{3H}/c^{6H} mice, and ○,□, control mice. ●,○, Malic enzyme; ■,□, glucose-6-phosphate dehydrogenase (G6PD); ▲,△, 6-phosphogluconate dehydrogenase (6PGD).

TABLE III

ENZYME ACTIVITY IN LIVER OF CONTROL AND DEFICIENT MICE AT THREE AGES

Mice and enzyme assays are as described in Materials and Methods. N = 9, 6 and 6 for the ocntrol groups and 4, 4 and 10 for the deficient groups. Data from animals killed at 14 and 16 days were combined as were the data from 19 and 21-day-old mice. Activity is expressed as in Table I.

Age (days)	Glutamic oxaloacetic transaminase		Serine dehydratase	
	c^{3H}/c^{6H}	Control	c^{3H}/c^{6H}	Control
14—16	128 ± 0.5	121 ± 6	5.2 ± 0.4	6.2 ± 0.8
19—21	163 ± 6	140 ± 8	10.4 ± 0.9	7.8 ± 1.3
35	222 ± 10	198 ± 16	20.2 ± 2.6	13.5 ± 0.5
	Hexokinase		Lactate dehydrogenase	
14—16	0.5 ± 0.1	0.4 ± 0.1	522 ± 21	466 ± 20
19—21	0.8 ± 0.1	0.5 ± 0.1	401 ± 30	380 ± 20
35	1.2 ± 0.1	0.7 ± 0.1	332 ± 18	330 ± 17

lap between the two groups, indicating similar ratios of enzyme protein to enzyme activity.

As seen in Fig. 2, glucose-6-phosphate dehydrogenase in erythrocyte extracts was reproducibly inactivated by small amounts of antisera. Liver extracts required larger and more variable amounts of antisera and inactivation was usually greater than 90%. The immunoinactivation curves from the malic enzyme-deficient mice and their unaffected sibs were not distinguishable suggesting that the differences in enzyme activity were indeed due to differences in amount of enzyme protein.

The developmental profile of eight liver enzymes was determined in the c^{3H}/c^{6H} and control mice. As seen in Fig. 3, differences in the level of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme between the two groups became evidence at 3 weeks of age, which is near the time of weaning. Similar changes were observed for serine dehydratase, glutamic-oxaloacetic transaminase and hexokinase (Table III), although the differences were not as dramatic as observed for the three enzymes in Fig. 3. No difference between groups at any age was noted for malate dehydrogenase or lactate dehydrogenase.

Discussion

The c^{3H}/c^{6H} mice respond to the absence of mitochondrial malic enzyme (and possibly a reduction in the levels of mitochondrial NADPH) by increasing the level of liver cytosolic malic enzyme approximately 50%, although less than 2% of the malic enzyme activity in rat liver is associated with the mitochondrial fraction [32] if any is present [9] and only 4% in mouse liver [32]. They also increase the levels of two other cytoplasmic enzymes capable of generating NADPH (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) 4.5- and 2.5-fold, respectively. It is doubtful whether these enzyme increases would be sufficient to increase the cytoplasmic NADPH levels suffi-

ciently to restore the mitochondrial NADPH levels to normal because of the impermeability of the mitochondrial membrane. Hexokinase levels are also increased in the c^{3H}/c^{6H} mice. The alterations observed when three additional enzymes, serine dehydratase, glutamic-oxaloacetic transminase, and glutamic-pyruvate transaminase are studied are less easily explained. These three enzymes usually respond similarly to hormonal and dietary manipulation and in a pattern opposite from the three carbohydrate-metabolizing enzymes, since these latter enzymes are induced by glucagon and hydrocortisone as well as high protein/low carbohydrate diets [24,25]. In the liver of malic enzyme-defifient mice, glutamic-pyruvate transaminase is reduced as would be expected in mice with increased carbohydrate-metabolizing enzymes. The dichotomy between glutamic-pyruvate transaminase on the one hand and glutamic-oxalo-acetic transaminase and serine dehydratase on the other, that is two amino acid-metabolizing enzymes responding similarly to glycolytic enzymes, cannot be explained by a simple hormonal-dietary mechanism. Thus, six of 13 other enzymes in the liver were altered in response to the absence of mitochondrial malic enzyme. Interestingly, another source of mitochondrial reducing equivalent, isocitrate dehydrogenase, was not elevated in the c^{3H}/c^{6H} mice although it normally responds to dietary and hormonal stimulation in a manner similar to malic enzyme [33]. The enzymatic alterations associated with mitochondrial malic enzyme deficiency are in contrast to the observations with mice deficient in soluble malic enzyme where the level of other enzymes is unchanged [8]. This would suggest mitochondrial malic enzyme has a key role in cellular homeostasis possibly via the regulation of the levels of NADPH and/or glutathione.

29% of the malic enzyme activity in mouse kidney is intramitochondrial [32] and mitochondrial malic enzyme accounts for 50—80% of the activity in heart tissue [26,32]. Thus, in contrast to the situation in liver, the levels of malic enzyme in heart and kidney are as expected in tissues simply void of mitochondrial malic enzyme. The activities of the two transaminase enzymes are reduced in the c^{3H}/c^{6H} mice as might be expected in animals with reduced growth rates which are storing less fat than normal. The c^{3H}/c^{6H} mice are notably deficient in adipose tissue.

As previously reported by others, changes in the activity of malic enzyme and glucose-6-phosphate dehydrogenase are associated with increases in the amount of enzyme protein and are not merely due to alterations in levels of cofactors or activators.

Although previous data indicated that no electrophoretically detectable mitochondrial malic enzyme was present, they could not completely eliminate the possibility that the increased liver activity was the result of a new variant MOD-2 enzyme with a fortuitous mobility similar to MOD-1. However, the immuno-precipitation experiments suggest that all of the malic enzyme in the c^{3H}/c^{6H} mice is antigenically similar to MOD-1. Additionally, in the fractionation experiment, all of the increased malic enzyme activity is associated with the supernatant fraction and only minimal activity (probably due to contamination from the cytosol) is located in the mitochondrial fraction. Thus, the data are conclusive that the viable complementer mouse, c^{3H}/c^{6H}, is mitochondrial malic enzyme deficient but more interestingly has approximately a 50% eleva-

tion in cytoplasmic malic enzyme in liver but not kidney or heart.

It was of interest to determine the stage of development at which the differences in enzyme activity were first detectible in an effort to identify factors responsible for the alterations. No significant differences were noted prior to weaning, that is, in mice less than 19—21 days of age. Except for the deviation between the groups at the time of weaning, the developmental profile for both groups is as expected. These differences are also larger than would be expected simply by the reduced growth rate of the c^{3H}/c^{6H} mice [34]. The enzyme developmental profile in the control mice is similar to that previously reported for C57BL/6 mice [35].

The increase in both glycolytic and transaminase enzymes would suggest that more than a single regulatory mechanism is responsible at the protein level for the differences between the c^{3H}/c^{6H} and control mice or that perturbations of enzyme pathways can have wide-ranging pleiotropic effects. The possible involvement of NADP/NADPH and glutathione is being examined.

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