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Identification and Biochemical Analysis of Mouse Mutants Deficient in Cytoplasmic Malic Enzyme†

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ABSTRACT: During the biochemical screening of mutant enzymes in mice, individuals with an apparent nonfunctional allele at the locus (*Mod-1*) responsible for cytoplasmic malic enzyme were identified by starch gel electrophoresis and by enzyme activity measurements. A series of matings and genetic analyses were made, and mice homozygous for the nonfunctional or null allele (*Mod-1*^{n}) were produced. The mutation appeared to occur spontaneously in the C57BL/6J strain. By double-immunodiffusion and enzyme immunoinactivation assays, the null mutants were shown to express no proteins that cross-react with the antiserum to cytoplasmic malic enzyme (CRM-negative). In liver homogenates of homozygous null mutants, lack of protein components that form complexes with IgG from the cytoplasmic malic enzyme specific antiserum was further demonstrated by passage of the original serum through a mutant liver homogenate–Sepharose column, where the postadsorbed serum retained its titer and specificity. The residual malic enzyme activity (<10% of the normal) observed in various tissue homogenates of the homozygous null mutants was attributed to that of mitochondrial isozyme of malic enzyme. Assays of enzymes from tissues of different genotypes revealed no significant differences in activities of six other enzymes in the related metabolic pathways. However, in liver from mutant mice, a lower NADPH/NADP$^+$ ratio was consistently observed in comparison to that from control mice. Both the mutant and the control mice of the same age were found to have comparable body weight and lipid content.

Malic enzyme [L-malate:NADP$^+$ [1] oxidoreductase (decarboxylating), EC 1.1.1.40] catalyzes the NADP$^+$–NADPH-dependent interconversion between L-malate and pyruvate (Ochoa et al., 1950). In mammalian species, two main isozymes have been identified and characterized by their localization in cytoplasm and in mitochondria (Henderson, 1966, 1968), respectively. The genetics and biochemistry of two isozymes of malic enzyme in mice have been well characterized (Shows et al., 1970; Lee et al., 1978; Bernstine, 1979). The structural genes of cytoplasmic (*Mod-1*) and mitochondrial (*Mod-2*) malic enzyme have been mapped to chromosomes 9 and 7 (Shows et al., 1970), respectively. Cytoplasmic malic enzyme has been purified in our laboratory by general ligand affinity chromatography (Lee et al., 1978). It was shown to be a tetramer with a native molecular weight of 27 000. Kinetic studies revealed that this enzyme has a much lower K_m for L-malate (K_m = 50 μM) than for pyruvate (K_m = 5 mM) (Lee et al., 1978). Judging from intracellular metabolite and coenzyme concentrations, this enzyme would appear to catalyze preferentially the oxidation of L-malate to pyruvate with a concomitant generation of NADPH (Guynn et al., 1972). The tissue levels of malic enzyme are increased by dietary carbohydrate and repressed by dietary lipid, paralleling the rate of lipogenesis (Geer et al., 1978, 1979; Silpananta & Goodridge, 1971; Li et al., 1975). Therefore, malic enzyme was postulated to be important for lipogenesis, but not for glucogenesis (Young et al., 1964; Wise et al., 1964; Geer et al., 1978).

Mice apparently having an inactive allele at the locus responsible for cytoplasmic malic enzyme were discovered in the course of screening for germinal mutations (F. M. Johnson, G. T. Roberts, R. K. Sharma, F. Chasalow, R. Zweidinger, A. Morgan, R. W. Hendren, and S. Lewis, unpublished experiments). The purpose of the present investigation was to provide detailed genetic analysis and to characterize the biochemical as well as immunological consequences of the deficiency of cytoplasmic malic enzyme in order to gain understanding of the impact of the mutation on the physiology and viability of this organism and to seek information regarding the function of the normal enzyme.

Materials and Methods

Chemicals. The following chemicals were purchased from Sigma Chemical Co.: NADP$^+$ (acid form), NADPH (sodium salt), 6-phosphogluconic acid, ascorbic acid, L-malate, pyruvate, NADH (sodium salt), oxaloacetate, oxidized glutathione, isocitrate, phosphoenolpyruvate, glucose 6-phosphate, α-ke-

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[1] Abbreviations used: NADH, reduced nicotinamide adenine dinucleotide; NADP, NAD phosphate; NADPH, reduced NADP; ADP, adenosine 5′-diphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid.

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toglutarate, bovine serum albumin, glutamate dehydrogenase, and glucose-6-phosphate dehydrogenase. ADP (sodium salt) was purchased from P-L Biochemicals.

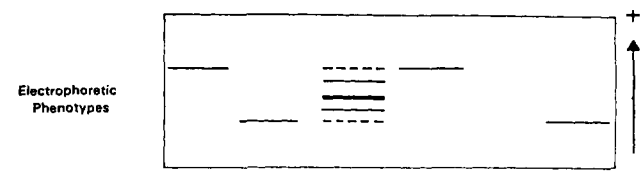
Electrophoresis. Electrophoresis was performed horizontally in starch gels (electrostarch, 12% w/v) by using a gel buffer of 0.016 M Tris and 3 mM citric acid. The runs were performed at 12 W for 4 h and cooled by plastic ice bags in contact with the gels. Sample material, adsorbed into 3 × 10 mm wicks of Whatman No. 1 filter paper, was introduced into a vertical slit cut in the starch gels near the cathodal end. The gel dimensions and the apparatus were essentially as described previously (Johnson & Shaffer, 1974). After electrophoresis, the gels were sliced horizontally and stained for malic enzyme activity in 50 mL of 0.1 M Tris-HCl, pH 8.5, to which was added 2 mL of 10 mg/mL solution of $NADP^+$, 2 mL of 10% $MgCl_2$, 1 mL of 50 mg/mL malic acid, 0.5 mL of nitroblue tetrazolium, and 0.25 mL of 6 mg/mL phenazine methosulfate. Sample material consisted of single whole kidneys removed by dissection or surgery. The kidneys were prepared for electrophoresis by homogenization in 0.6 mL of buffer (50 mM phosphate, pH 7.5, 1 mM EDTA, 2 mM dithiothreitol, and 0.2% v/v Triton X-100) followed by centrifugation at 9000*g* for 30 min at 4 °C. Surgery was used when it was necessary to keep the animals alive for subsequent mating.

Enzyme Assays. Enzyme assays were performed on a Beckman Model 25 spectrophotometer at 25 °C by monitoring the change in absorbance at 340 nm. All the enzyme assays followed the described procedures (Bergmeyer et al., 1974) with some modifications. Each enzyme was assayed in 1 mL of 0.1 M Tris-HCl, pH 8.0, with the addition of 0.25 mg/mL specified substrate, 0.5 mg/mL $NADP^+$ (or ADP) or 0.16 mM NADPH (or NADH), and 1 unit each of coupled enzyme(s), if necessary. Besides malic enzyme, the following enzymes from liver and kidney homogenates were analyzed: lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), isocitrate dehydrogenase (EC 1.1.1.42), glutathione reductase (EC 1.6.6.2), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and pyruvate kinase (EC 2.7.1.40). Kidney and liver homogenates from male mice of six different genotypes (age, 8 ± 1 weeks) were prepared to assay enzyme activities. The frozen tissues were homogenized in distilled water containing 10 mM α-thioglycerol (1:3 w/v). The supernatant was recovered by centrifugation at 27000*g* for 20 min and used for enzyme assays.

Preparation of Malic Enzymes and Specific Antisera. Cytoplasmic malic enzyme from DBA/2J mice was purified from mouse kidneys according to Lee et al. (1978). Mitochondrial malic enzyme was purified from mouse hearts according to Berstine (1979). The antisera specific to cytoplasmic and mitochondrial malic enzymes were prepared separately from rabbits according to Lopez-Barea and Lee (1979).

Immunological Procedures. Double-immunodiffusion experiments were performed according to the procedure of Ouchterlony (1958). Enzyme immunoinactivation experiments were carried out by incubating the pure enzymes or kidney homogenate (1:2 w/v, in 0.1 M Tris-HCl, pH 8.0) with increasing amounts of antisera specific to cytoplasmic or mitochondrial malic enzyme at 4 °C for 30 min in a final volume of 100 μL. Bovine serum albumin (60 mg/mL) was added to maintain a constant protein concentration. Residual enzyme activities were measured by enzyme assays. Normal nonimmune rabbit serum was employed as controls.

Preparation of Sepharose-Bound Liver Homogenate. Ten grams of liver (frozen or fresh) from the homozygous null mutants (*Mod-1*^{n}/*Mod-1*^{n}) was homogenized with 20 mL of 0.2 M $NaHCO_3$, pH 7.8. After centrifugation at 27000*g* for 20 min, the supernatant was mixed with 30 mL of CNBr-activated Sepharose at 4 °C. After 5 h of vigorous shaking, the Sepharose-bound mutant liver homogenate was washed extensively with 0.2 M $NaHCO_3$, pH 7.8, to remove any uncoupled liver homogenate. The Sepharose-bound liver homogenate from DBA/2J mice (as a control) was prepared with the same procedure as that from the mutant mice.



Genotypes	*Mod-1*^{a}/*Mod-1*^{a}	*Mod-1*^{b}/*Mod-1*^{b}	*Mod-1*^{a}/*Mod-1*^{b}	*Mod-1*^{a}/*Mod-1*^{n}	*Mod-1*^{n}/*Mod-1*^{n}	*Mod-1*^{b}/*Mod-1*^{n}
Activities (♂♂)	1.10	1.10	1.10	0.60	< 0.10	0.60
Activities (♀♀)	0.50	0.50	0.50	0.25	< 0.10	0.25

FIGURE 1: Diagrammatic representation of electrophoretic phenotypes and relative enzyme activities (based on single kidney) for males and females of different genotypes with the previously designated *Mod-1*^{a} (DBA/2J) and *Mod-1*^{b} (C57BL/6J) alleles and with the new null allele *Mod-1*^{n}. This mutant allele was first identified in F_1 hybrid (C57BL/6J × DBA/2J) with the genotype of *Mod-1*^{a}/*Mod-1*^{n} (see text for further explanations).

Adsorption of Antiserum to Cytoplasmic Malic Enzyme by Liver Homogenate–Sepharose. Control (DBA/2J) and mutant liver homogenate–Sepharose were packed separately in two 50-mL syringe columns. The columns were equilibrated with 0.2 M $NaHCO_3$, pH 7.8. Two milliliters of antiserum to cytoplasmic malic enzyme was passed through the column containing DBA/2J liver homogenate–Sepharose. The unadsorbed serum was eluted from the column with 0.2 M $NaHCO_3$, pH 7.8. Fractions having absorbance at 280 nm were collected separately and pressure-concentrated until the protein concentration of the postadsorbed serum was identical with that of the preadsorbed serum. For comparisons, another batch of postadsorbed serum was prepared identically by passage of 2 mL of cytoplasmic malic enzyme specific antiserum through the column containing mutant liver homogenate–Sepharose.

Determinations of Tissue NADPH/$NADP^+$ Concentrations. After the animals were killed by cervical dislocation, the livers were removed, frozen immediately in liquid nitrogen, and stored at −80 °C. NADPH and $NADP^+$ in liver tissues were measured according to the procedures of Passonneau & Lowry (1974), except that the volume was increased threefold. Fluorescence was measured with a Turner Model 110 fluorometer.

Protein Determinations. Protein concentrations in tissue homogenates were determined by the fluorescamine assay of Böhlen et al. (1973).

Results

Genetic Analysis of a Null Mutation of Cytoplasmic Malic Enzyme in Mice. DBA/2J mice are characterized by the presence of a single, relatively fast migrating form of cytoplasmic malic enzyme controlled by the gene *Mod-1*^{a}. C57BL/6J animals are typically homozygous for the alternative allele, *Mod-1*^{b}, which results in an electrophoretically slow form of the enzyme. F_1 offspring of matings between the two strains have both parental forms plus three hybrid bands of intermediate mobility, which are usually not resolved upon electrophoresis (Figure 1). The results of the present

Table I: Segregation of the Null Allele at the *Mod-1* Locus

parental crosses, ♀ genotypes × ♂ genotypes	no. of litters	genotypes of progeny +/+	+/n	n/n	total offspring
Mod-1^b/*Mod-1*^{b} × *Mod-1*^{n}/*Mod-1*^{n}	6	0	47	0	47
Mod-1^a/*Mod-1*^{a} × *Mod-1*^{n}/*Mod-1*^{n}	4	0	13	0	13
Mod-1^a/*Mod-1*^{b} × *Mod-1*^{n}/*Mod-1*^{n}	2	0	21	0	21
pooled: +/+^{a} × n/n	12	0	71	0	71
Mod-1^a/*Mod-1*^{n} × *Mod-1*^{n}/*Mod-1*^{n}	12	0	21	27	48
Mod-1^b/*Mod-1*^{n} × *Mod-1*^{n}/*Mod-1*^{n}	5	0	10	16	26
pooled: +/n × n/n	17	0	31	43	74
Mod-1^b/*Mod-1*^{n} × *Mod-1*^{b}/*Mod-1*^{n}	5	13	19	8	40
Mod-1^a/*Mod-1*^{n} × *Mod-1*^{a}/*Mod-1*^{n}	11	18	27	17	62
Mod-1^b/*Mod-1*^{n} × *Mod-1*^{a}/*Mod-1*^{n}	4	15	12	3	30^b
pooled: +/n × +/n	20	46	58	28	132^b
Mod-1^a/*Mod-1*^{a} × *Mod-1*^{a}/*Mod-1*^{n}	4	10	3	0	13
Mod-1^b/*Mod-1*^{b} × *Mod-1*^{a}/*Mod-1*^{n}	8	5	28	0	33^b
Mod-1^b/*Mod-1*^{b} × *Mod-1*^{b}/*Mod-1*^{n}	2	10	8	0	18
Mod-1^a/*Mod-1*^{a} × *Mod-1*^{b}/*Mod-1*^{n}	1	3	1	0	4
pooled: +/+ × +/n	15	28	40	0	68
Mod-1^n/*Mod-1*^{n} × *Mod-1*^{n}/*Mod-1*^{n}	1	0	0	9	9

^{a} + = *Mod-1*^{a} or *Mod-1*^{b}. ^{b} $P \leq 0.01$ by χ^2.

work were the product of a screening program in which groups of DBA/2J male animals (mutagen-treated and controls) were mated repeatedly to C57BL/6J females. This was followed by electrophoretic analysis of samples from both the parents and the offspring (F. M. Johnson, G. T. Roberts, R. K. Sharma, F. Chasalow, R. Zweidinger, A. Morgan, R. W. Hendren, and S. Lewis, unpublished experiments).

By the electrophoretic method of detection, more than 10 000 F_1 offspring have been examined for several enzyme loci to date. In one family, three of eight F_1 offspring had what appeared to be the usual DBA/2J phenotype. That is, instead of having the typical zymogram pattern, these F_1 individuals showed only the fast electrophoretic band normally expected of DBA/2J parents. At a number of other loci there are different electrophoretically expressed alleles which distinguish the strains in a manner similar to the *Mod-1*^{a} and *Mod-1*^{b} alleles. Several of these [*Es-1*, *Es-3*, *Id-1*, *Hbb*, *Pgm-1*, *Dip-1*, *Gpd-1*, and *Gpi-1* (Hutton & Roderick, 1970; Roderick et al., 1971)] were checked, and, in all other respects, the F_1 mice with the unexpected cytoplasmic malic enzyme variation were normal, as were both parents.

For exploration of the transmission of the altered gene, an additional phenotypic criterion was added to electrophoretic analysis. This consisted of activity measurements for malic enzyme which were performed on aliquots of the same samples examined by electrophoresis. Activity comparisons revealed that the C57BL/6J parent of the variant animals had only about one-half normal values and, likewise, that the variant F_1 individuals were similarly reduced. Although sex-associated activity differences introduced a complicating factor (Figure 1), by scoring sex, activity, and electrophoretic pattern it was possible to understand the outcome of all matings in terms of a genetic model whereby reduced activity and/or missing electrophoretic bands are explained by the mutant gene *Mod-1*^{n}.

Table I shows the results of the matings which were made. In every case offspring and both parents were scored. The matings include the original cross which led to the discovery of the variation as well as variant F_1 individuals backcrossed in both directions and with one another. Insofar as ratios are concerned, results are genetically consistent.

Immunological Studies. Double-immunodiffusion experiments were employed to examine if the homozygous null

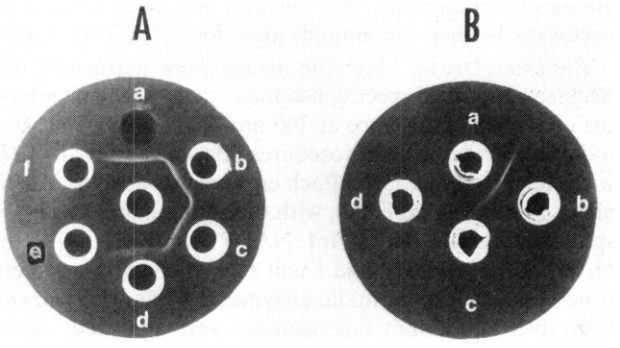


FIGURE 2: Double-immunodiffusion plates showing (A) the absence of the precipitin lines between the antiserum to cytoplasmic malic enzyme and the tissue homogenates of the homozygous null mutant and (B) the specificity of the antisera to cytoplasmic as well as to mitochondrial malic enzyme. In plate A, well a contained 25 μL of DBA/2J liver homogenate; well b, 25 μL of DBA/2J kidney homogenate; well c, 25 μL of C57BL/6J liver homogenate; well d, 25 μL of C57BL/6J kidney homogenate; well e, 50 μL of kidney homogenate from the homozygous null mutant; and well f, 50 μL of liver homogenate from the homozygous null mutant. The center well held 25 μL of the antiserum to cytoplasmic malic enzyme. The protein concentration was about 35 mg/mL for each well. In plate B, well a contained 25 μL of antiserum to cytoplasmic malic enzyme; well b, 0.1 unit of cytoplasmic malic enzyme; well c, 25 μL of antiserum to mitochondrial malic enzyme; and well d, 0.05 unit of mitochondrial malic enzyme.

mutants produce proteins that cross-react (CRM) with the antiserum to cytoplasmic malic enzyme. As shown in Figure 2A, liver and kidney homogenates of DBA/2J and C57BL/6J mice showed clear fusable precipitin lines with the antiserum to cytoplasmic malic enzyme. The homozygous null mutants showed no precipitin line even in high concentrations. In the control shown in Figure 2B, we found no cross-reactivity between the antiserum to cytoplasmic malic enzyme and mitochondrial malic enzyme or between the antiserum to mitochondrial enzyme and the cytoplasmic isozyme.

Lack of CRM expression in the homozygous null mutants was also confirmed by enzyme immunoinactivation studies. As shown in Figure 3, malic enzyme in kidney homogenate of the parental strains and the F_1 hybrids was inhibited to the same extent by the antiserum to cytoplasmic malic enzyme. In each case, ~30% of the original activity remained after incubation in the presence of excess antiserum to cytoplasmic

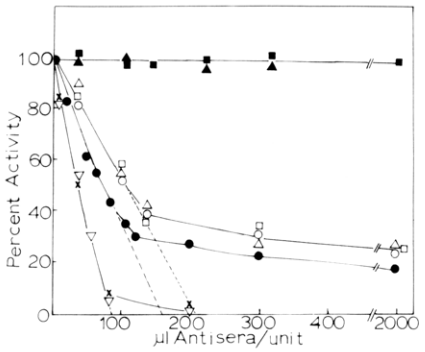


FIGURE 3: Enzyme immunoinactivation study demonstrating the absence of cytoplasmic malic enzyme in the homozygous null mutants. The percent residual malic enzyme activity was plotted as a function of microliters of antiserum per unit of enzyme. Kidney homogenates (1:2 w/v in 0.1 M Tris-HCl, pH 8.0) from DBA/2J (○), C57BL/6J (△), F_1 hybrid (□, DBA/2J × C57BL/6J), and homozygous null mutant mice (■) were incubated with increasing amounts of antiserum to cytoplasmic malic enzyme at 4 °C for 30 min. ● is the percent residual activity of pure cytoplasmic malic enzyme after incubation with antiserum to cytoplasmic malic enzyme. ▲ is the percent residual activity of pure mitochondrial malic enzyme upon incubation with antiserum to cytoplasmic malic enzyme. Symbols ▽ and X are respectively percent residual activity of pure mitochondrial malic enzyme and that in the kidney homogenate of the homozygous null mutant after incubation with variable amounts of antiserum to mitochondrial malic enzyme. Male mice of different genotypes were employed in this study. The initial malic enzyme activity for kidney homogenates of DBA/2J, C57BL/6J, and their F_1 hybrid was 1.4 unit/mL and that of the homozygous null mutant was 0.1 unit/mL.

malic enzyme. When pure cytoplasmic malic enzyme was employed for the inactivation studies, a similar inhibition profile was observed except that ~20% of the original activity remained in the presence of excess serum and that the apparent antiserum titer was slightly different. Under identical experimental conditions, the residual malic enzyme activity in the kidney homogenate of the homozygous null mutants was not appreciably inhibited by excess antiserum to cytoplasmic malic enzyme. Mitochondrial malic enzyme was not inhibited by the antiserum to cytoplasmic malic enzyme but was inactivated specifically by the antiserum to mitochondrial malic enzyme. The residual malic enzyme activity found in the kidney homogenate of the null mutant was also similarly inactivated by the antiserum specific to mitochondrial isozyme. In homogenates from other tissues of the homozygous null mutants, such as heart, testis, brain, and spleen, the observed malic enzyme activity was inactivated only by the mitochondrial isozyme specific antiserum but not by that specific to cytoplasmic isozyme (data not shown).

When the cytoplasmic malic enzyme specific antiserum was passed through the liver homogenate column prepared from liver of homozygous null mutants, the postadsorbed and the preadsorbed antiserum showed identical antiserum titer with and specificity to cytoplasmic malic enzyme. On the contrary, the postadsorbed antiserum that had been passed through the liver homogenate–Sepharose column of DBA/2J mice could no longer form a precipitin line with cytoplasmic malic enzyme nor inhibit the enzyme activity upon incubation. The results of this experiment are presented in Figure 4.

Comparison of NADPH/NADP⁺ Concentrations in Normal and Mutant Mice. The concentrations of NADPH and NADP⁺ (separately and combined) were determined for the liver tissue of the homozygous null mutants and the control (D2B6 F_1 hybrid) mice. The results are presented in Table II, although the mutant mice showed an ~15% lower NADPH/NADP⁺ ratio in liver compared to the control mice (3.33 vs. 2.78). However, both the mutant and the control

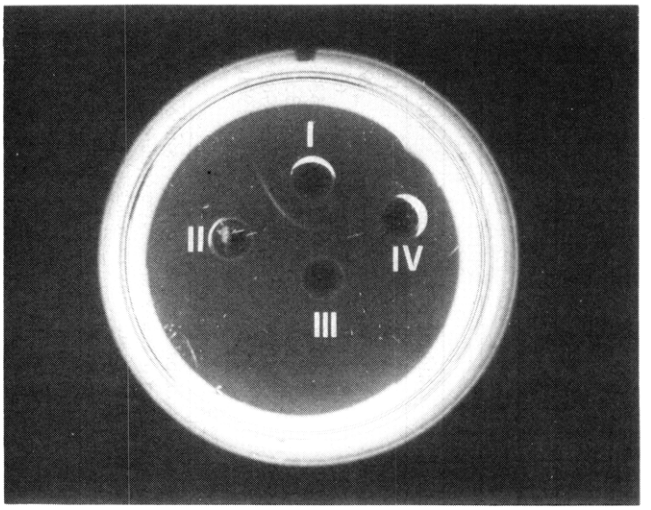


FIGURE 4: Double-immunodiffusion plate showing the effect of adsorption of antiserum to cytoplasmic malic enzyme by liver homogenate–Sepharose. Well I, 0.1 unit of cytoplasmic malic enzyme; well II, 25 μL of original (preadsorbed) antiserum to cytoplasmic malic enzyme; well III, 25 μL of postadsorbed antiserum prepared by passage of the original serum through the mutant liver homogenate–Sepharose column; well IV, 25 μL of postadsorbed antiserum prepared by passage of the original serum through the DBA/2J (control) liver homogenate–Sepharose column.

Table II: Comparison of NADPH/NADP⁺ Levels in Liver Tissue of Normal and Homozygous Mutant Mice

coenzymes	coenzyme concn (nmol/g of tissue)	
	control mice[a]	mutant mice[b]
total (NADPH + NADP⁺)	373 ± 30[c]	350 ± 25
NADPH	309 ± 5	272 ± 4
NADP⁺	93 ± 3	98 ± 2
NADPH/NADP⁺ ratio	3.33 ± 0.15	2.78 ± 0.10

[a] Control mice consisted of a mixture of five equal-size liver samples from five F_1 hybrid males (C57BL/6J × DBA/2J) of 8 ± 1 weeks old. The determinations were performed in triplicate; averages are presented. [b] Mutant mice included a mixture of five equal-size live samples from five homozygous null mutant males of 8 ± 1 weeks old. The values are averages of triplicate determinations. [c] ± represents the average deviation from the mean for each set of determinations.

mice have a comparable amount of combined NADPH and NADP⁺.

Surveys of Activities of Related Enzymes. Six other enzymes were assayed to see if there were any major alterations in total activities as a consequence of the null mutation of cytoplasmic malic enzyme. The enzymes were lactate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, glutathione reductase, glucose-6-phosphate dehydrogenase, and pyruvate kinase. The activities of these enzymes from liver and kidney homogenates of mice with six different genotypes were compared (Table III). In both liver and kidney homogenates, the malic enzyme activity of the homozygous null mutants was <10% of that found in the control mice. The activities of the other enzymes, however, did not show significant variation. Variable amounts of residual malic enzyme activity were found in heart (sp act. 8 milliunits/mg), brain (2.7 milliunits/mg), testis (3.2 milliunits/mg), and spleen (1.4 milliunits/mg) of homozygous mutants, but it was consistently less than the activity found in the control mice (17, 7.1, 33, and 4.4 milliunits/mg, respectively). By the specific enzyme immunoinactivation assay, the residual malic enzyme activity

Table III: Comparison of Enzyme Activities in Related Metabolic Pathways from Tissues of Mice with Different Genotypes

enzymes[c]	genotypes:[a] tissues:	specific activities[b] [(unit × 10^3)/mg]											
		o/o		*a/o*		*b/o*		*a/a*		*a/b*		*b/b*	
		liver	kidney	liver	kidney	liver	kidney	liver	kidney	liver	kidney	liver	kidney
Me		0.8	1.8	4.9	25	5.7	21	13	38	12	43	11	41
G6PD		7.5	30	7.1	32	8.3	33	7.8	32	7.3	34	8.5	29
GR		33	65	33	69	35	62	32	71	28	65	31	62
IDH		273	527	260	580	296	547	245	484	250	491	261	538
PK		116	336	115	284	127	279	142	294	122	322	131	337
MDH		6830	13000	6550	13600	6600	13100	7370	12800	5880	14800	6700	14900
LDH		750	2130	710	1900	640	1960	710	2320	780	2320	680	1970
body weight (g)		20 ± 1		22 ± 2		20 ± 1		21 ± 2		22 ± 3		22 ± 2	

[a] Genotype *o/o* represents homozygous null (*Mod-1*^{n}/*Mod-1*^{n}), and *a/o* and *b/o* represent heterozygous null with DBA/2J (*a* allele) and C57BL/6J (*b* allele) as the background. *a/a*, *a/b*, and *b/b* represent that of DBA/2J, F_1 (DBA/2J × C57BL/6J), and C57BL/6J mice, respectively. [b] The data presented are averages of triplicate assays of samples which consisted of a mixture of five equal-size livers or kidneys from male mice (8 ± 1 weeks old) of each genotype. [c] ME, malic enzyme; G6PD, glucose-6-phosphate dehydrogenase; GR, glutathione reductase; IDH, isocitrate dehydrogenase; PK, pyruvate kinase; MDH, malate dehydrogenase; LDH, lactate dehydrogenase.

found in the mutants was exclusively that of mitochondrial isozyme as mentioned in the previous section.

Discussion

The approach that was described for the biochemical screening of null mutants of cytoplasmic malic enzyme in mice has the potential of detecting germinal mutations reflected by altered electrophoresis patterns. For example, if either *Mod-1* parental gene product were absent or electrophoretically altered in the isolated F_1 individual (and if the F_1 individual had phenotypically normal parents), then this would constitute evidence of a newly occurring mutation originating in one particular parental germ line. Thus, each event detected in this way can be assigned to a given parental animal. Mutations arising in stock animals, which result in heterozygous parental generation animals, can also be recognized by repeated transmission of variant bearing gametes from such individuals.

Judging from our observations as illustrated in Figure 1, we found that the first null variant phenotype was of maternal (C57BL/6J) origin and she was heterozygous for a variant gene. Because at other loci the animal was typical of the strain, contamination is not a reasonable explanation.

The phenotype of the F_1 offspring, observed as an absence of the maternal band, suggested a null or inactive allele as the responsible factor. Thus, a mutation which occurred spontaneously in the production of the C57BL/6J animals at the Jackson Laboratory would appear to be the basis of the variant phenotype. The maternal parent of the variants, therefore, would be expected to be heterozygous for a null allele, here provisionally designated *Mod-1*^{n}. For confirmation of this hypothesis, two matings were initially established. First, the parental female was mated with one of her male offspring, and, second, the remaining male and female offspring were mated with one another to produce F_2 generation. Additional crosses were made subsequently with the products of these matings. As shown in Table I, the results of our genetic analysis are completely consistent with the explanation that the variation originally observed is due to a mutant gene, *Mod-1*^{n}.

The immunological analysis indicated that the homozygous null mutants (*Mod-1*^{n}/*Mod-1*^{n}) do not have proteins capable of forming a precipitable complex with antiserum to cytoplasmic malic enzyme. The double-immunodiffusion and enzyme immunoinactivation studies show an absence of cross-reactivity between the antiserum to cytoplasmic malic enzyme and its mitochondrial isozyme. From the specific inhibition of the mitochondrial malic enzyme by its antiserum, one can show that the apparent malic enzyme activity detected in tissues of homozygous null mutants would seem to be best explained as the result of contamination of the soluble homogenate by the mitochondrial components. The deviation of the enzyme immunoinactivation curves between the pure cytoplasmic malic enzyme and the kidney homogenate (Figure 3) is probably an indication of a minor amount of mitochondrial malic enzyme in the kidney homogenate of control mice.

In liver homogenate of the homozygous null mutants, lack of protein components that form complexes with cytoplasmic malic enzyme specific IgG was further demonstrated by the antiserum adsorption experiments. The postadsorbed antiserum prepared by passage through the mutant liver homogenate–Sepharose column was identical with the preadsorbed serum in terms of titer and specificity.

The nature of CRM-negative mutation at the DNA and chromosome levels is unknown. Structural (frameshift, nonsense, missense, or deletion) or regulatory gene mutation are both possible explanations (Lee et al., 1979). However, if the mutation is a deletion, the deleted segment does not extend

beyond the very closely linked *pgm-3* locus (F. M. Johnson, G. T. Roberts, R. K. Sharma, F. Chasalow, R. Zweidinger, A. Morgan, R. W. Hendren, and S. Lewis, unpublished experiments) since homozygous *Mod-1*^{n}/*Mod-1*^{n} individuals were examined and found to have normal *pgm-3* phenotypes. Further studies by recombinant DNA techniques, including the isolation and characterization of malic enzyme determining mRNA, might be considered to investigate the fundamental basis of the mutational event.

The establishment of the first mouse mutants lacking cytoplasmic malic enzyme is important and interesting, particularly with respect to its potential for aiding in the analysis of the regulation of malic enzyme concentration and the relationship of malic enzyme activity to the flux of metabolites in various metabolic pathways.

CRM-negative null mutants of malic enzyme have been reported for *Drosophila melanogaster* (Geer et al., 1979; Lee et al., 1979). Geer et al. (1979) found that as much as 41% of the total NADPH might be generated by malic enzyme in early third instar larvae of *Drosophila* in vivo. However, in the present study, mutant mice deficient in cytoplasmic malic enzyme seem to have only a slightly lower NADPH/NADP$^+$ ratio compared to that of the normal mice (Table II). Therefore, when the null mutant is homozygous, there might be no dramatic imbalance in intracellular oxidation–reduction states for biosynthesis, such as lipogenesis. This is consistent with our observation that there is no significant difference in body weight and lipid content (as judged by the size of the anatomical body fat deposits observed in dissection) between the normal and the mutant mice of the same age (Table III). Our observation also raised the question regarding the relative importance of this enzyme in lipogenesis in vivo (Wise et al., 1964; Geer et al., 1978).

On the other hand, we might expect a certain imbalance among the related intermediate metabolites in mutant mice lacking cytoplasmic malic enzyme, unless it can be compensated by other related metabolic pathways. The results of our preliminary analysis seemed to indicate that null mutation in cytoplasmic malic enzyme caused no significant alterations of activities for six other enzymes in the related metabolic pathways. Further analysis on the related metabolites in tissues of normal and mutant mice is now in progress in order to assess the real metabolic role of this enzyme.

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