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Malate Dehydrogenase Mutants in *Escherichia coli* K-12

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Mutants devoid of malate dehydrogenase activity have been isolated in *Escherichia coli* K-12. They do not possess detectable malate dehydrogenase when grown aerobically or anaerobically on glucose as sole carbon source. All mutants revert spontaneously; a few partial revertants have been found with a malate dehydrogenase exhibiting altered electrophoretic mobility. Therefore, only one such enzyme appears to exist in the strains examined. No evidence could be obtained for the presence of a malate dehydrogenase not linked to nicotinamide adenine dinucleotide. Mutants deficient in both malate dehydrogenase and phosphoenol pyruvate carboxylase activities will grow anaerobically on minimal glucose plus succinate medium; also, malate dehydrogenase mutants do not require succinate for anaerobic growth on glucose. The anaerobic pathway oxaloacetate to succinate or succinate to aspartate appears to be accomplished by aspartase. Malate dehydrogenase is coded for by a locus somewhere relatively near the histidine operon, i.e., a different chromosomal location than that known for other citric acid cycle enzymes.

During an investigation on membrane-bound dehydrogenases not using pyridine nucleotides as cofactors, the results of a study by Cox, Snoswell, and Gibson (6) on membrane-associated malate oxidation indicated to us that a separate malate dehydrogenase could exist in *Escherichia coli*. Our search for a malate dehydrogenase not linked to nicotinamide adenine dinucleotide (NAD) was unsuccessful. Among other attempts to demonstrate such a type of malate dehydrogenase, we selected for mutants lacking the NAD-linked malate dehydrogenase. Here we report on the properties of these mutants and the evidence that, in *E. coli*, there appears to exist only one such enzyme which is coded for by a locus at a different chromosomal location than that known for other citric acid cycle enzymes.

MATERIALS AND METHODS

Bacterial strains, media, growth conditions, and preparation of extracts. The bacterial strains employed are listed in Table 1. Cells were grown at 37 C either in Antibiotic Medium No. 3 (Difco) or in minimal media (19) with glucose (0.4%), glycerol (0.5%), potassium succinate (0.5%), potassium fumarate (0.5%), or potassium D,L-malate (0.5%) as carbon sources. The organic acid carbon sources were neutralized with KOH to a *p*H of about 6 and were autoclaved as a 10 or 20% solution. Minimal media were supplemented where necessary with 20 μg of the required amino acids or thymine per ml. Thiamine was routinely added to all media to a final concentration of 1 μg/ml. Media were solidified with 1.5% agar (Difco) where required.

Cells were grown anaerobically in minimal medium with twice the salts concentration and 1% glucose in completely filled and tightly capped 1-liter bottles; the culture was stirred magnetically. Harvesting of cells was performed by centrifugation at 7,000 × *g* for 10 min at 4 C and washing once with 0.1 M potassium phosphate buffer, *p*H 7.0.

Extracts for enzyme assays were prepared by sonic disruption of cell suspensions (1 g wet weight of cells in 2 ml of 0.1 M potassium phosphate buffer, *p*H 7.0) for 2 min with a Branson Sonifier at an output of 6 to 7 amp. Crude extracts are the supernatants obtained by centrifugation at 4,000 × *g* at 4 C for 10 min. Alternatively, cells were disrupted by means of a Hughes press at a pressure of 10,000 psi.

Enzyme assays. (i) Aspartase (EC 4.3.1.1) was assayed by direct nesslerization. Assays were performed in 0.1 M potassium phosphate buffer (*p*H 7.2), 0.1 M potassium L-aspartate, and 3×10^{-4} M $Co(NO_3)_2$ in a total volume of 1.0 ml. The time-dependent formation of ammonia was determined by subtracting the ammonia present at zero time. Ammonia was measured by adding, to 1 ml of incubation mixture, 3.4 ml of 0.4 M KOH and 0.05 ml of Nessler reagent (Merck, Darmstadt, Germany). After 10 min, absorbance was read at 492 nm. A standard curve was prepared by using $Na(NH_4)HPO_4$ as ammonia source.

(ii) Malate dehydrogenase (EC 1.1.1.37) was assayed in the forward direction by following the in-

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TABLE 1. *Bacterial strains*

Strain	Relevant genotype[a]	Source
E. coli B	Prototroph	C. Yanofsky
E. coli K-12		
CR63	Prototroph	R. Hertel
W945T1	*thi thr leu lac gal trp mtl str*^{R}, F^-	*See* Clowes and Hayes[b]
HfrH	Prototroph, *Hfr* (*O*, *thrleu* .. *gal* .. *trp* ..)	H. Kneser
AB313	*thi thr leu lac str*^{R}, *Hfr* (*O*, *mtl* .. *str* .. *his* ..)	E. A. Adelberg
AB3290	*trp*, F^+	F. Gibson
CR63-2	*ppc*	This study
CR63-2-1 through 2-5	*ppc mdh*, the last number indicating *mdh* mutants of different origin	This study
W945T1-1	W945T1 *ppc*	This study
W945T1-1-1 through 1-8	W945T1 *ppc mdh*, last number as above	This study
W945T1-2	W945T1 *mdh*	This study

[a] Symbols: *thi*, thiamine; *thr*, threonine; *leu*, leucine; *trp*, tryptophan; *his*, histidine; *lac*, lactose; *gal*, galactose; *mtl*, mannitol; *str*^{R}, streptomycin resistance; *ppc*, phosphoenol pyruvate carboxylase; *mdh*, malate dehydrogenase; *O*, origin of Hfr transfer.

[b] W945T1 is a derivative of W945, the genealogy of which is described by Clowes and Hayes (5).

crease in absorbance at 546 nm in the following reaction mixture: potassium phosphate buffer (*p*H 7.8), 200 μmoles; KCN, 30 μmoles; phenazine methosulfate (PMS; Serva, Heidelberg, Germany), 1.63 μmoles; 3-(4,5-dimethyl thiazolyl-2-)2,5-diphenyl tetrazolium bromide (MTT, Serva, Heidelberg, Germany) 0.12 μmole; NAD, 1 μmole; potassium D,L-malate, 37.5 μmoles; in a total volume of 2.4 ml. The reverse reaction was assayed in a total volume of 2.4 ml with potassium phosphate buffer (*p*H 7.8), 200 μmoles; potassium oxaloacetate, 1.4 μmoles; reduced NAD (NADH), 0.6 μmole. The disappearance of NADH was followed spectrophotometrically at 334 nm. Blanks were run without malate or oxaloacetate. Although nicotinamide adenine dinucleotide phosphate (NADP) may serve as a cofactor, the rate of the reaction observed was less than 2% of the rate with NAD.

(iii) Succinate dehydrogenase was assayed by a modification of the procedure of Arrigoni and Singer (2). The reaction mixture contained 200 μmoles of potassium phosphate buffer (*p*H 7.8), 30 μmoles of KCN, 1.63 μmoles of PMS, 0.12 μmole of MTT, and 40 μmoles of potassium succinate, in a total volume of 2.4 ml. The reaction was followed by the increase in absorbance at 546 nm. The tetrazolium assay system was calibrated with the malate dehydrogenase assay by using limiting amounts of NADH. It was found that 11.2 nmoles of NADH caused an increase in absorbance of 0.10 at 546 nm. For all assays, the light path was 1 cm and the temperature was 25 C (Eppendorf photometer or Zeiss spectrophotometer).

(iv) Pyruvate dehydrogenase complex (pyruvate dehydrogenase, EC 1.2.4.1; plus dihydrolipoamide transacetylase, EC 2.3.1.12; plus dihydrolipoamide dehydrogenase, EC 1.6.4.3) was measured by the method of Reed, Leach, and Koike (17) with catalytic amounts of coenzyme A and phosphotransacetylase in the assay. Specific activity is expressed as micromoles of acetylphosphate produced per minute and per milligram of protein at 30 C.

Electrophoresis. Electrophoresis was performed in 1% agarose (L'Industrie Biologique Française, Gennevilliers, Seine, France) gels in 0.045 M tris(hydroxymethyl)aminomethane borate-ethylenediaminetetraacetate buffer (*p*H 8.7) by the procedure of Ursprung and Leone (18). Gels were stained for enzymatic activity by immersing them into a solution of the same composition as that used in the respective MTT-linked enzyme assays.

Identification of products of the aspartase reaction. Aspartate was identified as the product of the fumarate plus ammonia reaction by incubation of about 50 μg of protein of a crude extract with 0.45 μmoles of potassium *2,3-*^{14}*C*-fumarate (2.22 μc/μmole; New England Nuclear Corp., Boston, Mass.) and 7.55 μmoles of ammonium sulfate in 0.1 M potassium phosphate buffer (*p*H 7.2) in the presence of 3×10^{-4} M $Co(NO_3)_2$. To 5 or 10 μliters of the incubation mixture were added 0.2 μmole each of potassium aspartate, potassium D,L-malate, and sodium fumarate. The mixture was applied to Whatman no. 3MM chromatography paper and was developed by ascending chromatography, first in ethanol:water (70:30) and in the second direction with *n*-butyl alcohol saturated with 2 N formic acid. The acids were identified with bromocresol purple (0.04 mg in 100 ml of 50% ethanol, adjusted to *p*H 10.5 with NaOH) and ninhydrin. The fumarate separates from the aspartate and malate in the first solvent system and the malate from the aspartate in the second system. The chromatograms were cut into strips, counted in a gasflow Packard Radiochromatogram Scanner-7200, and then sprayed for identification of components.

Fumarate as reaction product was identified as follows. On incubation of 50 μg of protein (crude extract) with aspartate under the conditions described above,

an increase in absorbance at 240 nm was detected. Sufficient quantities of the absorbing compound were obtained after 3 to 4 hr of incubation to be detected as an ultraviolet-absorbing region after two-dimensional chromatography. The substance was co-chromatographed with ^{14}C-fumarate; radioactivity and ultraviolet absorption showed perfect coincidence.

Selection of mutants devoid of phosphoenol pyruvate carboxylase (EC 4.1.1.31) and malate dehydrogenase. *Ppc* mutants (3) can easily be identified by phenotype since this is the only known mutation conferring, with glucose as sole carbon source, an absolute requirement for succinate during *both* aerobic and anaerobic conditions. Other potential succinate requirers (e.g., α-ketoglutarate dehydrogenase, succinyl-coenzyme A thiokinase mutants) will not display the requirement anaerobically for reasons detailed by Hirsch et al. (13). *Ppc* mutants were thus simply isolated (in strains CR63 and W945T1) as succinate auxotrophs by plating cells surviving diethylsulfate mutagenesis (followed by penicillin selection in minimal medium with glucose in the absence of succinate) onto minimal glucose plus succinate (0.1%) medium. Succinate requirers were identified by replica plating and were tested for this requirement during anaerobic growth in minimal glucose medium.

Malate dehydrogenase mutants were selected in both *ppc*$^+$ and *ppc* CR63 and W945T1 as follows. After mutagenesis with diethylsulfate, the cells were grown on minimal glycerol medium supplemented with aspartate (500 μg/ml). Penicillin selection was then performed in minimal glycerol plus aspartate medium. Colonies were replica plated onto both minimal malate and minimal glycerol plus malate (0.1%) medium. Colonies not growing on malate and poorly on glycerol plus malate were purified and tested for malate dehydrogenase activity.

Screening for electrophoretic variants of revertants of mdh mutants. Revertants were selected on minimal malate plates. After purification, single-colony isolates were grown in 10 ml of Antibiotic No. 3 medium (Difco) with aeration. Cells were collected by centrifugation, washed twice with 0.1 M potassium phosphate buffer (*p*H 7.0), and resuspended in 2 ml of this buffer containing 2 μmoles of dithiothreitol (Calbiochem, Zürich, Switzerland). The cells were disrupted by sonic treatment, and cell debris was removed by centrifugation. Samples of about 2 μliters were electrophoresed as described above. Revertants apparently possessing a malate dehydrogenase with altered electrophoretic mobility were grown in gram quantities. The extracts were mixed with equal amounts (based on malate dehydrogenase activity) of wild-type extract and subjected again to electrophoresis. Electrophoretic mobilities are expressed as the fraction: distance from origin revertant/distance from origin wild type.

Bacterial matings. Matings were performed at 37 C in Antibiotic No. 3 medium by using 2×10^8 to 4×10^8 cells per ml of each parent. All crosses mentioned were incubated for 30 min and interrupted by shaking on a Vortex mixer; appropiate dilutions were plated onto selective media.

RESULTS

Malate dehydrogenase activity in extracts. The 4,000 $\times$ *g* supernatant of a crude extract of malate-grown *E. coli* strain CR63 was prepared as described. This extract was fractionated by centrifugation at 25,000 $\times$ *g* for 30 min. The pellet was washed once by resuspending in 0.1 M potassium phosphate buffer (*p*H 7.0) and centrifuging once more at 25,000 $\times$ *g* for 30 min; it was finally resuspended in a volume of buffer equal to that of the supernatant. Malate and succinate oxidation were examined in supernatant and pellet fractions by means of the spectrophotometric assays described in Materials and Methods. Although succinate dehydrogenase activity is present in both fractions, no NAD-independent malate dehydrogenase activity could be demonstrated (Table 2). The malate-dependent reduction of MTT occurs only when NAD^+ is added to the assay mixture and about 96% of this activity is found in the non-particulate fraction. Activity of the pyruvate dehydrogenase complex was also followed by the fractionation procedure; only about 2% of this activity remained in the particulate fraction (Table 2).

No malate-dependent reduction of MTT in the absence of NAD^+ could be demonstrated in crude or fractionated extracts of *E. coli* strains W945T1, CR63, or AB3290. Furthermore, no malate dehydrogenase activity independent of NAD was found in extracts of anaerobically or aerobically grown cells; the same results were obtained when cells were disrupted in a Hughes press.

Electrophoresis of malate dehydrogenase. Extracts of *E. coli* K-12 strains W945T1, CR63, AB3290, or *E. coli* B, were electrophoresed on agarose gels and subsequently stained for malate or succinate dehydrogenase activities as described. When extracts of either aerobically or anaerobically grown cells were electrophoresed and the gels were developed for malate dehydrogenase activity, only a single zone of formazan deposition could be detected; furthermore, there was no difference in the electrophoretic mobility of the enzyme obtained from these aerobically or anaerobically grown cultures. A broad smear near the origin was observed when succinate was supplied as substrate.

Mutants devoid of malate dehydrogenase activity. Mutants of strains CR63 and W945T1 unable to grow on malate as sole carbon source were obtained after penicillin selection. The properties of these mutants and their respective parent strains are listed in Table 3.

Crude extracts of glycerol- or glucose-grown mutant cells were assayed for malate dehydro-

TABLE 2. *Malate, succinate, and pyruvate oxidations in fractionated extracts*[a]

Substrate	NAD in assay	Supernatant		Precipitate		Fraction in precipitate
		Specific activity	Total units	Specific activity	Total units	
						%
Malate	−	<0.008		<0.015		
Malate	+	0.475	9.1	0.037	0.384	4.0
Succinate	−	0.32	6.2	0.76	7.9	55.4
Pyruvate	+	1	117	0.05	2.5	2.1

[a] CR63 cells were grown on minimal malate medium. Pyruvate dehydrogenase complex was measured in extracts from cells grown on sodium pyruvate (0.5%) to induce synthesis of the enzyme as much as possible (9).

TABLE 3. *Growth characteristics of mutants*

Medium	Strain (genotype)						
	CR63	CR63-2 (*ppc*)	CR63-2-1 (*ppc, mdh*)	W945T1	W945T1-1 (*ppc*)	W945T1-1-6 (*ppc, mdh*)	W945T1-2 (*mdh*)
Aerobic							
Glucose	+	−	−	+	−	−	+
Glucose + succinate	+	+	+	+	+	+	+
Glucose + aspartate	+	+	+	+	+	+	+
Glycerol	+	−	−	+	−		
Glycerol + succinate	+	+	±[a]	+	+	−	∓[a]
Glycerol + aspartate	+	+	+	+	+	±[a]	+
Malate	+	+	−	+	+	+	+
Succinate	+	+	±[a]	+	+	−	−
						±[a]	±[a]
Anaerobic							
Glucose	+	−	−	+	−	−	+
Glucose + succinate	+	+	+	+	+	+	+
Glucose + aspartate	+	+	+	+	+	+	+

[a] Very slow growth, colonies become visible only after 30 to 36 hr of incubation at 37 C.

genase activity by means of both the forward and reverse reactions. No enzymatic activity could be detected in any of the mutants examined (Table 4), although wild-type extracts clearly possess activity for both the forward and reverse reactions. No malate dehydrogenase activity could be demonstrated in extracts of anaerobically grown cells or on agarose gels after electrophoresis. Mixed extracts of mutant and wild type showed no inhibition of wild-type malate dehydrogenase activity.

Revertants of mdh mutants. The frequencies of spontaneous reversion for different *mdh* mutants was about 10^{-6} to 10^{-8}. A number of independent isolates of *mdh* revertants were obtained from five different mutants and qualitatively checked for malate dehydrogenase electrophoretic mobility. A small proportion (3/50) of the revertants were found to possess malate dehydrogenase with altered electrophoretic mobility. This altered mobility in revertants from three different *mdh* mutants is expressed as the fraction of wild-type enzyme mobility when revertant and wild type are mixed and co-electrophoresed (Table 5).

Aspartase activity in aerobically and anaerobically grown wild-type and mutant cell extracts. The mutants lacking malate dehydrogenase will grow anaerobically on glucose without any further supplementation, e.g., with malate or succinate. Succinate cannot be produced under this growth condition from α-ketoglutarate because no α-ketoglutarate dehydrogenase is synthesized (1). A mutant devoid of phosphoenol pyruvate carboxylase activity, on the other hand, has an absolute succinate requirement for both aerobic and anaerobic growth on glucose. Moreover, the *mdh ppc* double mutant will grow anaerobically on glucose plus succinate. Thus, it must be possible to produce aspartate from succinate in the absence of malate dehydrogenase.

TABLE 4. *Malate dehydrogenase- and aspartase-specific activities*

Strain	Specific activities of reactions[b]					
	Malate → oxaloacetate		Oxaloacetate → malate		Aspartate → fumarate	
	Aerobic growth	Anaerobic growth	Aerobic growth	Anaerobic growth	Aerobic growth	Anaerobic growth
W945T1	0.35	0.06	6.5	1.6	0.015	0.073
W945T1-2 (*mdh*)	0	0	0	0	0.021	0.089
W945T1-1-6 (*mdh, ppc*)	0	0	0	0	—[c]	—[c]
CR63	0.29	0.072	5	1.19	0.015	0.108
CR63-2-3 (*mdh, ppc*)	0	0	0	0	0.061	0.22
CR63-2-5 (*mdh, ppc*)	0	0	0	0	0.108	0.142

[a] All cells were grown on minimal glucose medium.

[b] Expressed as: micromoles of MTT reduced (malate → oxaloacetate), micromoles of NADH oxidized (oxaloacetate → malate), and micromoles of ammonia produced (aspartate → fumarate) per milligram of protein per minute at 25 C.

[c] Not determined.

TABLE 5. *Relative electrophoretic mobilities of mdh revertants*

Strain	Relative mobility
CR63 (wild type)	1.0
CR63-2-2 R3[a]	0.65
CR63-2-6 R5[a]	0.80
CR63-2-4 R1[a]	0.75

[a] Number following R designates the revertant isolate.

Such a pathway is also indicated by the fact that *mdh* mutants will, albeit very slowly, grow on succinate as carbon source, whereas strains devoid of succinate dehydrogenase activity cannot do so at all (*unpublished data*). The long-known aspartase was envisioned as an obvious candidate for this pathway.

Aspartase activity in wild type and mutant cell extracts was examined by three different procedures. Cell extracts were incubated with aspartate for varying lengths of time and then tested for ammonia production by direct Nesslerization of samples. The specific activity of both mutant and wild-type cell extracts is comparable after aerobic as well as anaerobic growth (Table 4). About fourfold higher aspartase activities were usually found after anaerobic than after aerobic growth. The specificity of the aspartase reaction was checked by the identification of either fumarate or aspartate as products after incubation of extracts with aspartate or fumarate plus ammonia, respectively. In the first case, fumarate formation was confirmed by the time-dependent increase in absorbance at 240 nm. The substance absorbing at 240 nm was identified as fumarate as judged by the fact that it co-chromatographed with added *2,3-*^{14}*C*-fumarate. Conversely, when *2,3-*^{14}*C*-fumarate and ammonium sulfate were incubated with wild-type or mutant cell extracts from 1 hr, ^{14}C-aspartate could be identified as a reaction product after two-dimensional paper chromatography. The fact that ^{14}C-aspartate can be obtained in mutant extracts argues against any aspartate formation via the malate-oxaloacetate pathway.

Preliminary localization of the malate dehydrogenase locus. So far, three loci concerning citric acid cycle enzymes are known: citrate synthase (4), α-ketoglutarate dehydrogenase (10–12), and succinate dehydrogenase (Shipp and Henning, *unpublished data*). All of them are located near the *gal* operon. It therefore was of interest to see whether the *mdh* locus could also be found in this region of the chromosome.

Two orienting crosses, with HfrH and AB313 as donors, showed that the *mdh* locus is somewhere relatively near the *his* operon. Two *mdh* mutants of W945T1 were used; they were made *his* (diethylsulfate mutagenesis followed by penicillin selection) and, for crosses with AB313, *leu*$^+$ (spontaneous reversion). The results of one experiment were as follows. With HfrH, 0 of 1,000 *thr*$^+$*leu*$^+$ and 17 of 129 (13%) *gal*$^+$ recombinants were *mdh*$^+$. With AB313, 60 of 219 (27%) *mtl*$^+$, and 254 of 458 (55%) *mtl*$^+$*his*$^+$ were *mdh*$^+$; when *mdh*$^+$ was the selective marker, 127 out of 165 (77%) of them were also *his*$^+$. Very similar data were obtained with the other *mdh* mutant as recipient. Although we have not located the gene more precisely, it is clear that it is not near the other known loci for citric acid cycle enzymes.

DISCUSSION

The results of this study show that the *E. coli* strains used possess only one NAD-linked malate dehydrogenase. This conclusion appears to be unescapable. Mutants which have no detectable malate dehydrogenase activity are freely reverting and some revertants were found exhibiting altered electrophoretic mobilities of the enzyme. Although different forms of malate dehydrogenase have been reported to be separable on diethylaminoethyl cellulose (Amarasingham, Fed. Proc. 1964, p. 487), this result should perhaps be interpreted with caution, since Murphy et al. (16) have shown that multiple forms of malate dehydrogenase could be generated by freezing and thawing partially purified preparations. Although no pyridine nucleotide-independent malate dehydrogenase activity could be detected, this evidence cannot be taken as absolute proof for the nonexistence of some other type of malate-oxaloacetate oxidoreductase. It is conceivable that such an enzyme, in contrast to the succinate dehydrogenase, may be unable to utilize the artifical electron acceptors used. However, it is rather unlikely that this hypothetical malate dehydrogenase is a flavine-linked dehydrogenase since both metal (e.g., succinate dehydrogenase) and nonmetal-containing (e.g., dihydrolipoamide dehydrogenase) flavine-linked dehydrogenases can reduce phenazine methosulfate or ferricyanide. Furthermore, the pyruvate dehydrogenase component of the *E. coli* pyruvate dehydrogenase complex, which normally reduces enzyme-bound lipoyl residues without the participation of a flavine, can react with artificial electron acceptors (ferricyanide; 7), including the phenazine methosulfate MTT-system (*unpublished data*). In summary, the existence of a malate dehydrogenase not linked to pyridine nucleotides has not been excluded but is highly improbable. Our data do not exclude, however, that at least part of the malate dehydrogenase in vivo is associated with the cytoplasmic membrane. In experiments not reported here, we have studied the relevant behavior of the membrane-bound succinate and L-α-glycerophosphate dehydrogenases. Almost no enzymatic activity is solubilized by a fractionation procedure similar to that used here for malate dehydrogenase. The succinate dehydrogenase present in the supernatant (Table 2) also is not truly in a soluble state, as evidenced by its behavior on electrophoresis; in addition, on a 5 to 20% linear sucrose density gradient, it will not sediment as a symmetrical peak but as a broad smear present almost all over the gradient. As judged by the latter criterion, the pyruvate dehydrogenase complex is completely soluble. Although we have not followed this question in any detail for malate dehydrogenase, its apparent higher "affinity" than that of the pyruvate dehydrogenase complex to the particulate fraction may indicate complete or partial membrane association in vivo.

Amarasingham and Davis (1) pointed out that in *E. coli* the citric acid cycle operates in a cyclic manner only under aerobic conditions, whereas anaerobically it is modified to serve as a branched pathway. Our study shows that the reaction oxaloacetate to malate anaerobically does not require malate dehydrogenase. Therefore, analogous to fumarate reductase (13), either a pyridine nucleotide-independent oxaloacetate reductase is present—an activity we have not looked for—or, more likely in view of the data of Table 4, the branched anaerobic pathway operates as suggested in Fig. 1. Phosphoenol pyruvate carboxylase has been reported to be allosterically inhibited by aspartate (14), a fact which also appears to favor aspartate as an intermediate not only in amino acid biosynthesis.

Another problem which we have not investigated is posed by the "malic enzyme" (EC 1.1.1.40) catalyzing the reductive carboxylation of pyruvate using reduced NADP as cofactor. It has been shown by Kornberg (15) that phosphoenol pyruvate carboxylase-negative mutants

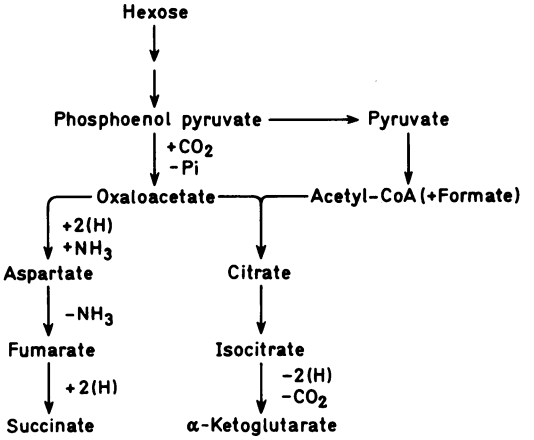

FIG. 1. *Modification of the anaerobic oxaloacetate branched pathway in mdh mutants. Anaerobically, the conversion of pyruvate to acetyl-coenzyme A (CoA) is achieved mainly by the pyruvate-formate lyase (8). The reaction oxaloacetate to aspartate will probably be effected by transamination with glutamate; aspartase yields fumarate and fumarate reductase succinate. From the data of Table 4, it appears likely that also in wild type a major fraction of succinate is synthesized anaerobically via aspartate.*

with their absolute requirement for a C_4-dicarboxylic acid do contain the malic enzyme. In vivo, therefore, the malic enzyme does not function in the malate direction. If present in our strains, there is no obvious reason why via this activity, and provided the *ppc*$^+$ allele, an *mdh* mutant should not grow on malate.

Finally, the preliminary genetic evidence produced excludes a "citric acid cycle operon," but otherwise cannot contribute anything more to the understanding of the regulation of the synthesis of these enzymes. For this reason, a more precise localization of the *mdh* locus has not been attempted.

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