Problem Set 3 (**Due February 4th**)

1. In 1896, Christiaan Eijkman famously stated that "White rice can be poisonous!" Research this quote and determine how it relates to the material covered in this unit. Be specific – which enzymes are affected and how? Please note the date – this quote has nothing to do with the 2012 discovery that some rice has elevated arsenic concentrations.

2. We know that pyruvate is ultimately oxidized to 3 equivalents of CO_2.
    a. Starting with the average oxidation number on each carbon in pyruvate, determine how many electrons are produced during the oxidation to CO_2. Does this make sense based on the number of NADH and $FADH_2$ that are produced?
    b. Start with pyruvate. Number each carbon (C1, C2, C3). Trace the fate of each of these carbons through a single TCA cycle.
    c. How many cycles does it take for these three carbons to be completely removed from the intermediate metabolites in the cycle (that is, at what point does C1 become CO_2, at what point does C2 become CO_2, etc.). Please state any assumptions that you make about symmetry or asymmetry in the intermediates/reactions.

3. Consider the conversion of Succinyl CoA to Succinate.
    a. What enzyme catalyzes this reaction?
    b. Draw a mechanism for this reaction. Make sure to include the isolatable phosphohistidine residue.
    c. What provides the energy for GTP synthesis?
    d. We often say that ATP is generated in this step. How can we make this statement when it is not part of the overall chemical reaction?

4. Oxaloacetate can by synthesized directly from pyruvate.
    a. This is an example of an anapleurotic reaction. What does this mean?
    b. Why is this not the optimal route for carbons from pyruvate to enter into the TCA cycle?
    c. Accumulation of Acetyl-CoA activates this enzyme. Why does this make sense in the metabolic scheme of the TCA cycle?
    d. Using the KEGG Pathway website, determine the name of the enzyme that catalyzes this reaction, the gene code in humans, and any cofactors that are necessary in humans.
    e. Propose a mechanism for this reaction (I encourage you to be creative and come up with a mechanism that makes sense based on your own chemical intuition – you will need to do this on exams).

5. The standard free energies of hydrolysis for three high energy phosphate compounds are listed below. Based on the structure or each molecule, justify the trend.

    Phosphoenolpyruvate → pyruvate ($\Delta G'^{o}$ = -61.9 kJ mol^{-1}), ATP → ATP ($\Delta G'^{o}$ = -30.5 kJ mol^{-1}), Phosphocreatine →creatine ($\Delta G'^{o}$ = -43.1 kJ mol^{-1})

6. The typical intracellular concentrations of ATP, ADP, and Pi in muscles are 5.0, 0.5, 1.0 mM, respectively. Using the values in problem 5, and assuming standard biological conditions:
    a. What is ΔG for the hydrolysis of ATP in these conditions?
    b. Calculate the equilibrium concentration ratio of phosphocreatine to creatine in the creatine kinase reaction

$$\text{Creatine} + \text{ATP} \rightleftharpoons \text{Phosphocreatine} + \text{ADP}$$

c. What concentration ratio of ATP to ADP would be required to yield equal concentrations of phosphocreatine and creatine?
d. Assuming the concentration of Pi does not change, calculate the free energy of hydrolysis of ATP under the conditions determined in 6c.

7. Recall that α-Ketoglutarate is converted to Succinyl-CoA in a reaction very similar to pyruvate decarboxylation. Please propose a mechanism for this reaction including important intermediate structures and all cofactors that are involved.

8. Noting that each equivalent of NADH produced in glycolysis and TCA Cycle leads to the production of 2.5 ATP and each $FADH_2$ produces 1.5 ATP (the topic of next week's lecture), determine how many equivalents of ATP are synthesized form one molecule of Glucose.

9. Mitochondrial Isocitrate Dehydrogenase relies on allosteric regulation through a regulatory subunit to activate or inhibit enzyme activity (the RCSB molecule of the month page does a good job at explaining this). *E. coli*, which lack mitochondria, rely on a different mechanism to regulate enzyme activity. Please read the attached paper and discuss how this *E. coli* enzyme is regulated and how this mechanism relates to the 'dietary' carbon source.

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Compensatory Phosphorylation of Isocitrate Dehydrogenase

A MECHANISM FOR ADAPTATION TO THE INTRACELLULAR ENVIRONMENT*

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When *Escherichia coli* grows on acetate, the flow of isocitrate through the glyoxylate bypass is regulated, in part, through the phosphorylation of isocitrate dehydrogenase. In addition to its role in adaptation to alternative carbon sources, this phosphorylation system responds to variation in the intracellular level of isocitrate dehydrogenase. This system can compensate for changes in the cellular level of isocitrate dehydrogenase in excess of 10-fold, maintaining a nearly constant activity for isocitrate dehydrogenase during growth on acetate.

The behavior of the phosphorylation system exhibited considerable strain-specific variation. This was most clearly demonstrated using mutants which lacked the ability to phosphorylate isocitrate dehydrogenase. In two strains, mutation of the gene for isocitrate dehydrogenase kinase/phosphatase rendered the cells unable to grow on acetate. In contrast, a third strain was relatively insensitive to a mutation in this gene. This lack of phenotypic expression appears to result from a lower cellular level of isocitrate dehydrogenase in this strain which renders the phosphorylation (and consequent inhibition) of isocitrate dehydrogenase less essential.

The gene for isocitrate dehydrogenase kinase/phosphatase (*aceK*) was located in the glyoxylate bypass operon, downstream from the genes for isocitrate lyase and malate synthase.

In a living organism, a regulatory system can be considered to serve two functions. The most obvious of these is to monitor the external environment and provide a suitable response when conditions change. However, this response is generated within a highly complex network of interlocking processes. The precise nature of this network will differ substantially between cells, depending on such factors as genetic composition and the history of the cell. Thus, the regulatory system must also monitor the internal environment and adjust the response to those specific conditions.

We have chosen to examine such responses using the branch point between the glyoxylate bypass and the Krebs cycle of *Escherichia coli*. The glyoxylate bypass, consisting of the enzymes isocitrate lyase and malate synthase, is essential for growth on acetate because it allows an alternate route from the carbon dioxide producing steps of the Krebs cycle (Fig. 1). Without the glyoxylate bypass, both of the carbons of acetate would be converted to CO_2 and no carbon would remain for synthesis of cellular constituents. The glyoxylate bypass enzymes are induced during growth on acetate but are suppressed in the presence of a carbon source such as glucose, since under these conditions such a diversion is unnecessary (1–4). The flow of isocitrate through the glyoxylate bypass is regulated, in part, via the phosphorylation of isocitrate dehydrogenase, the Krebs cycle enzyme which competes for a common substrate with isocitrate lyase (5–8). Phosphorylation of isocitrate dehydrogenase renders this enzyme inactive (9–11), decreasing the flow of isocitrate through the Krebs cycle and so forcing it through the glyoxylate bypass instead (12–14). This phosphorylation cycle is catalyzed by a bifunctional protein, the isocitrate dehydrogenase kinase/phosphatase (9, 15).

In this paper, we consider the ability of the isocitrate dehydrogenase phosphorylation system to adapt to differences in the intracellular environment. We have, in particular, focused on the system's ability to compensate for variations in the total level of isocitrate dehydrogenase during growth on acetate. This phenomenon was chosen because the flow of isocitrate through the glyoxylate bypass is ultrasensitive to the activity of this enzyme. The ultrasensitivity is a result of the branch point effect (12) and stems from the saturation of isocitrate dehydrogenase with isocitrate (16).

The behavior of the phosphorylated system was characterized in several K12 strains of *E. coli* during growth on acetate. In addition, the response of the system to the overproduction of isocitrate dehydrogenase was tested by introduction of a plasmid carrying the gene for that enzyme. Finally, the effects of mutations introduced into the isocitrate dehydrogenase kinase/phosphatase were examined.

EXPERIMENTAL PROCEDURES

Materials—[^{32}P]Orthophosphate was obtained from New England Nuclear. *N*-Methyl-*N'*-nitro-nitrosoguanidine was a product of Sigma.

Bacterial and Phage Strains—The principal strains of *E. coli* used in this study are listed in Table I. The Mud(*lac*) phage was that described by Casadaban and Cohen (17) and was a gift from J. Clark and S. Luvett (University of California, Berkeley, CA).

Media—L broth contained 1% Bacto-tryptone, 1% NaCl, and 0.5% Bacto-yeast extract. The minimal acetate medium was that described by Neidhardt *et al.* (18) containing 2% sodium acetate, 1 μg/ml thiamin, 0.5 mM L-methionine, and 0.5 mM L-histidine, in addition to the other components. Strains containing the plasmids pCK505 and pBR322 were grown in the presence of 500 μg/ml ampicillin.

Mutagenesis—A pool of random Mud(*lac*) phage lysogens in 23559 was prepared essentially as described by Casadaban and Cohen (17). Lysogens were grown initially on minimal succinate plates and then replicated onto minimal acetate. Colonies which grew on succinate but not on acetate were tested for the ability to phosphorylate isocitrate dehydrogenase *in vivo* after growth to stationary phase on

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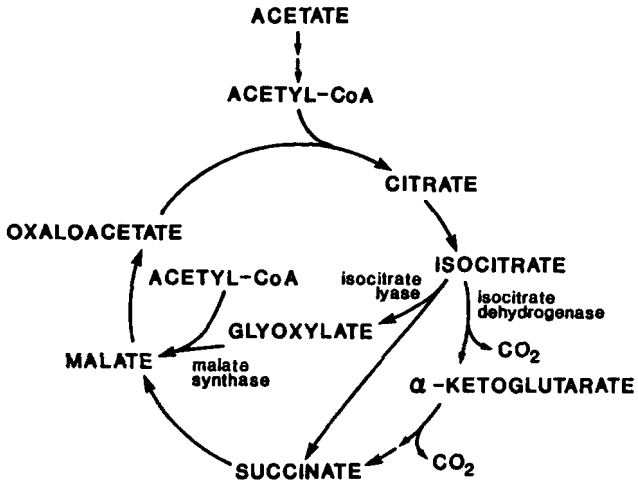

FIG. 1. **Krebs cycle and glyoxylate bypass.** During growth on acetate as the sole carbon source, isocitrate is diverted through the glyoxylate bypass in order to accumulate carbon for biosynthetic purposes.

TABLE I
Strains

Strain[a]	Relevant genotype[b]	Parent strain[c]
23559	*aceK*$^+$	
JC13510	*metA*	
5236	*ppc, aceB, glc*	
6024	*icd-11*	
DEK2000	*aceK2*::Mud(*lac*)	23559
DEK2001	*aceA1*::Mud(*lac*)	23559
DEK2002	*aceA2*::Mud(*lac*)	23559
DEK2003	*aceK2*::Mud(*lac*), pseudorevertant	DEK2000
DEK2010	*aceK*$^+$	JC13510
DEK2011	*aceK1*	JC13510
DEK2012	*aceK1*, pseudorevertant	DEK2011
DEK2015	*aceK*$^+$, *glc*	DEK2031
DEK2016	*aceK1, glc*	DEK2031
DEK2020	*aceK1*	23559
DEK2031	*metA, glc*	5236

[a] 23559 was from the American Type Culture Collection; JC13510 was a gift from A. J. Clark; and 5236 and 6024 were obtained from the *E. coli* Genetic Stock Center. All other strains were produced in the study.

[b] Only mutations which are relevant to this study are listed. Strains designated *aceK*$^+$ have the wild type allele for this gene. The *aceK*$^+$ and *aceK1* alleles were moved between strains by P1 transduction. Reversion of the *aceK*$^-$ phenotype is indicated by the designation "pseudorevertant."

[c] "Parent strain" refers to the strain from which derivatives are produced.

L-broth, as described previously (8). In addition, sonicated extracts of these isolates were assayed for the presence of isocitrate lyase and malate synthase. Nine lysogens were isolated which grew on minimal succinate but grew poorly or not at all on minimal acetate medium. Of these, three were found to be missing one or more of the enzymes specific to the glyoxylate bypass. Following initial characterization, these lysogens were stabilized by growth at 42 °C. The survivors which were obtained were indistinguishable from the original isolates with respect to failure to grow on acetate and the loss of the enzymatic activities.

A second method of mutagenesis used in this study employed MNNG[1] (19). One ml of 23559 was grown to midlog phase on L-broth, at which time MNNG was added to 30 μg/ml. The culture was incubated with shaking for 10 min at 37 °C and then diluted 25-fold into cold L-broth. The cells were harvested by centrifugation, resuspended in 5 ml of L-broth, and grown overnight. The procedure was repeated using 50 μg/ml MNNG. The mutagenized culture was then screened for kinase/phosphatase mutants as described above.

The mutant allele generated by MNNG was transduced from the original strain into clean genetic backgrounds using P1, essentially as described by Miller (19). The details are presented under "Results."

Characterization of Growth on Acetate—Cultures were grown to stationary phase on L-broth and then used to inoculate 5 ml of acetate minimal medium. These cultures were incubated at 37 °C with shaking until adapted to the acetate minimal medium. The samples were then diluted to 20 ml with fresh medium and shaken at 37 °C in 250-ml flasks for 90 min. Cells were harvested by centrifugation at ambient temperature, resuspended in 50 ml of fresh medium, and again incubated at 37 °C with shaking. The growth of the culture was monitored by light scattering determined at 650 nm. When the culture had shown exponential growth for about 1 doubling time, a 10-ml aliquot was withdrawn and quickly chilled to 0 °C. Bovine serum albumin was added to 1 mg/ml and the cells were immediately disrupted by sonication. Following centrifugation, samples were frozen in a dry ice/ethanol bath and stored at −80 °C. These samples were used within 2 days to determine the fractional phosphorylation of isocitrate dehydrogenase, as described below. (The freezing process was found not to affect the value determined.) The remainder of the culture was then harvested by centrifugation and stored at −20 °C. In order to determine the total isocitrate dehydrogenase activity, the pellet was thawed in 25 mM Mops, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol and the cells were disrupted by sonication. Following centrifugation, an aliquot was treated using the procedure for isocitrate dehydrogenase dephosphorylation (see below) and the total isocitrate dehydrogenase activity determined as described previously (10). Protein was estimated by the method of Lowry *et al.* (20) using bovine serum albumin as a standard.

Determination of Isocitrate Dehydrogenase Fractional Phosphorylation—Fractional phosphorylation of isocitrate dehydrogenase was determined by comparison of the isocitrate dehydrogenase activities of cellular extracts incubated with or without isocitrate dehydrogenase phosphatase. The incubation mixture contained 25 mM Mops, pH 7.5, 100 mM NaCl, 5 mM MgCl, 1 mM dithiothreitol, 2 mM ADP, 5 mM 3-phosphoglycerate, 1 mM isocitrate, and 100 μl of cellular extract, in a final volume of 150 μl. Parallel samples were incubated with or without isocitrate dehydrogenase phosphatase and were assayed periodically for isocitrate dehydrogenase to ensure the reaction had reached completion. The endogenous isocitrate dehydrogenase phosphatase had no effect on the phosphorylation state of isocitrate dehydrogenase due to extreme dilution.

Isolation of a Plasmid Carrying the Gene for Isocitrate Dehydrogenase—A clone of the isocitrate dehydrogenase gene (*icd*) was selected from the Clarke-Carbon clone bank (21) by complementation of the glutamate auxotrophy. (Mutations in *icd* result in the inability to synthesize glutamate.) The clone bank was transferred from its F$^+$ host to 6024 (*icd*$^-$) by conjugation. The mating mixture was then plated on minimal glucose plates supplemented with tryptophan. Plasmid DNA was isolated from the resulting clones and was found to transform 6024 to glutamate prototrophy at a high efficiency. The presence of the *icd* gene was confirmed by the direct assay of isocitrate dehydrogenase activity in a sonicated extract. This plasmid was subcloned by placing an 8-kilobase pair *Eco*RI-*Eco*RV restriction fragment into pBR322. This plasmid, pCK505, conveyed the same phenotypic properties to *E. coli* 6024 as did the original Clark-Carbon plasmid.

Enzyme Assays—Isocitrate dehydrogenase was assayed by monitoring the reduction of NADP at 340 nm. The reaction mixture contained 25 mM Mops, pH 7.5, 250 μM NADP, 500 μM DL-isocitrate, 5 mM $MgCl_2$. Assays were performed at 37 °C.

Isocitrate dehydrogenase kinase was assayed by monitoring the ATP-dependent inhibition of isocitrate dehydrogenase activity, as described (9). The assays were performed at 37 °C and contained 25 mM Mops, pH 7.5, 1 mM dithiothreitol, 100 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 50 μg/ml isocitrate dehydrogenase, 1 mM EDTA, 1 mM ATP, 4 mM phosphocreatine, and 40 units/ml of creatine phosphokinase.

Isocitrate dehydrogenase phosphatase was assayed by monitoring the release of ^{32}P from phosphoisocitrate dehydrogenase (9). The assays were performed at 37 °C and contained 25 mM Mops, pH 7.5, 1 mM dithiothreitol, 100 mM NaCl, 5 mM $MgCl_2$, 2 mg/ml bovine serum albumin, 10 μg/ml phosphoisocitrate dehydrogenase (~20,000 cpm/assay), 1 mM EDTA, 1 mM ATP, 4 mM phosphocreatine, 40

[1] The abbreviations used are: MNNG, *N*-methyl-*N'*-nitro-nitrosoguanidine; Mops, 3-(*N*-morpholino)propanesulfonic acid.

units/ml of creatine phosphokinase, 5 mM pyruvate, and 1 mM D-isocitrate.

Malate synthase was assayed by monitoring cleavage of the thioester bond at 232 nm (22). The assays were performed at 37 °C and contained 25 mM Mops, pH 7.5, 1 mM dithiothreitol, 100 mM NaCl, 5 mM $MgCl_2$, 50 μM acetyl-CoA, and 400 μM glyoxylate.

Isocitrate lyase was assayed at 37 °C by the method of Roche *et al.* (23). The reaction contained 20 mM Mops, pH 7.5, 2 mM DL-isocitrate, 200 mM KCl, 1 mM $MgCl_2$, and 56 mM 2-mercaptoethanol.

RESULTS

Adaptation to Differing Levels of Isocitrate Dehydrogenase—If the glyoxylate bypass is to function efficiently, its regulatory system must adapt to the changes of condition exhibited by different strains of *E. coli*. Of particular importance is the system's ability to compensate for variation in the level of isocitrate dehydrogenase, since the distribution of carbon flow through the branch point is highly sensitive to this parameter (12). When three strains of *E. coli* were examined, the level of total isocitrate dehydrogenase (phospho- and dephospho-) was found to vary over a 2-fold range (Table II). The phosphorylation system efficiently compensated for these differences by adjusting the phosphorylation state of isocitrate dehydrogenase. The result was a nearly constant isocitrate dehydrogenase activity during growth on acetate.

The ability of the phosphorylation system to compensate for more profound changes in the total level of isocitrate dehydrogenase was tested by the overproduction of this enzyme. To accomplish this, a plasmid carrying the gene for isocitrate dehydrogenase, pCK505, was introduced into DEK2010. As a control, pBR322 was also introduced into DEK2010. Growth and phosphorylation levels of isocitrate dehydrogenase were determined for DEK2010 containing either pCK505 or pBR322. (These strains are not, of course, completely isogenic since there are other genes carried by pCK505.) The increase in gene dosage led to a 15-fold overproduction of isocitrate dehydrogenase (Table II). Although this increase was much greater than might be expected from normal strain to strain variations, the phosphorylation system could still respond efficiently. It did so by decreasing the fraction of isocitrate dehydrogenase in the active, dephospho-form from 0.26 to 0.02. This resulted in an isocitrate dehydrogenase activity that was essentially identical with the pBR322 control that contained normal levels of isocitrate dehydrogenase.

TABLE II

Isocitrate dehydrogenase during growth on acetate as sole carbon source

Strain	Plasmid	Total enzyme[a] present	Fraction in dephospho-form[b]	Activity during growth on acetate[c]	Doubling time
		units/mg		*units/mg*	*h*
DEK2010		2.3	0.26	0.6	3.6
23559		2.2	0.36	0.8	3.8
DEK2015		1.3	0.54	0.7	3.4
DEK2010	pBR322	2.3	0.26	0.6	3.6
DEK2010	pCK505[d]	34.5	0.02	0.6	6.3[e]

[a] Isocitrate dehydrogenase activity of cellular extract after dephosphorylation *in vitro* (see "Experimental Procedures"). Isocitrate dehydrogenase was assayed by monitoring its ability to reduce NADP. The reaction mixture contained 25 mM Mops, pH 7.5, 0.5 mM DL-isocitrate, 0.25 mM NADP, and 5 mM $MgCl_2$. One unit of isocitrate dehydrogenase will reduce 1 μmol of NADP per min.

[b] Determined during log phase growth on acetate, as described under "Experimental Procedures."

[c] Calculated from values in the third and fourth columns.

[d] pCK505 is a derivative of pBR322 carrying the gene for isocitrate dehydrogenase, *icd*.

[e] An *icd*-bearing plasmid has been found to increase the doubling time of a strain a proportional amount during growth on glucose as is seen with growth on acetate.

Elimination of the Phosphorylation System—Theoretical analysis of this branch point has indicated that relatively modest variations in the activity of isocitrate dehydrogenase would be amplified to yield profound effects on the distribution of carbon flux at the branch point (12). In order to demonstrate this experimentally, we chose to isolate mutants which had lost the ability to phosphorylate isocitrate dehydrogenase. The immediate effect of such a mutation would be a 2- to 4-fold increase in isocitrate dehydrogenase activity since no inactive phosphoenzyme would be formed.

Mutant alleles of the gene coding for isocitrate dehydrogenase kinase/phosphatase (*aceK*) were isolated from the parent 23559 as described under "Experimental Procedures." Cultures of 23559 were mutagenized either with nitrosoguanidine or by lysogeny with the Mu phage. Mutants which had lost functions specific for growth on acetate were then identified by their ability to grow on succinate but not on acetate. Sonicated extracts of these strains were assayed for the glyoxylate bypass-specific enzymes: isocitrate dehydrogenase kinase/phosphatase, isocitrate lyase, and malate synthase. Mutagenesis with nitrosoguanidine produced an allele, *aceK1*, which had lost both kinase and phosphatase activities. This allele was transduced from DEK2020 into clean genetic backgrounds using the tight linkage between *aceK* and *metA* (see below). A second allele was generated by lysogeny of the phage Mud(*lac*) into the *aceK* locus of 23559.

When isocitrate dehydrogenase kinase/phosphatase was eliminated from 23559, the resulting strain, DEK2000, was unable to grow on acetate (Table III). A similar result was obtained when the mutant allele *aceK1* was transduced into a strain isogenic to DEK2010 to yield DEK2011 (*aceK1*). The immediate effect of the mutational loss of isocitrate dehydrogenase kinase/phosphatase in these strains is a 3- to 4-fold increase in isocitrate dehydrogenase activity. This prevents growth on acetate presumably because insufficient isocitrate can enter the glyoxylate bypass under these conditions.

Unlike its role in 23559 and DEK2010, isocitrate dehydrogenase kinase/phosphatase does not appear to play as essential a role in another wild type, DEK2015. When *aceK1* was transduced into the parent of DEK2015 to yield DEK2016 (*aceK1*), the resulting strain still grew on acetate. The relative lack of phenotypic expression of *aceK1* in DEK2016 appears to result from the 2-fold lower level of total isocitrate dehydrogenase expressed in this background. Thus, isocitrate de-

TABLE III

Effects of mutations in the isocitrate dehydrogenase kinase/phosphatase gene

Strain[a]	Relevant genotype	Doubling time[b]	Isocitrate dehydrogenase during growth on acetate	
			Total activity	Activity during growth on acetate
		h	*units/mg*	*units/mg*
23559	*aceK*$^+$	3.8	2.2	0.8
DEK2000	*aceK2*::Mud(*lac*)	No growth		
DEK2010	*acek*$^+$	3.6	2.3	0.6
DEK2011	*aceK1*	No growth		
DEK2015	*acek*$^+$	3.4	1.3	0.7
DEK2016	*aceK1*	5.8	1.0	1.0

[a] Strains are presented as isogenic pairs.

[b] Growth on minimal acetate medium, monitored by light scattering at 650 nm.

TABLE IV

Phenotypic reversions of aceK mutants

Strain[a]	Phenotype	Relevant genotype	Doubling time	Isocitrate dehydrogenase during growth on acetate	
				Total enzyme	Activity during growth on acetate
				units/mg	*units/mg*
23559	Wild type	*aceK*$^+$	3.8	2.2	0.8
DEK2003	Revertant	*aceK2*::Mud(*lac*), pseudorevertant[b]	3.8	0.02	0.02
DEK2010	Wild type	*aceK*$^+$	3.6	2.3	0.6
DEK2012	Revertant	*aceK1*, pseudorevertant[b]	3.8	2.6	2.6

[a] Strains are presented as isogenic pairs.

[b] Phenotypic reversion of aceK mutants by a secondary mutation are indicated by the designation "pseudorevertant."

hydrogenase kinase is apparently not essential when the level of isocitrate dehydrogenase expression is already relatively low. The loss of isocitrate dehydrogenase kinase/phosphatase did, however, result in a substantially slower growth rate. Thus, even in this low isocitrate dehydrogenase background, the kinase/phosphatase is required for greatest efficiency.

Isolation of Phenotypic Revertants—Identification of secondary mutations that suppress a mutant phenotype has often revealed relationships between the elements of the living system. In addition to phenotypic revertants which had recovered isocitrate dehydrogenase kinase activity, we had expected to isolate pseudorevertants which had a reduction in isocitrate dehydrogenase activity. Five pseudorevertants were, in fact, isolated which had reduced isocitrate dehydrogenase activity. In two of these cases, however, the extent of loss of isocitrate dehydrogenase activity was much greater than expected. An example of this is provided by DEK2003, in which isocitrate dehydrogenase activity was decreased by a factor of 100 relative to wild type (Table IV). Despite this reduction, DEK2003 showed the same growth rate on acetate as a nearly isogenic wild type strain, 23559. It remains to be determined whether the isocitrate dehydrogenase activity assayed *in vitro* accurately reflects this enzyme's activity *in vivo*.

To confirm that DEK2003 had reverted by a secondary mutation in the gene for isocitrate dehydrogenase, *icd*, a strain was constructed in which the transposon Tn10 (24) was linked to a wild type allele of *icd*. P1 phage grown on this strain were used to transduce DEK2003 to tetracycline resistance. When these transductants were tested for growth on acetate, approximately 50% had recovered the *aceK*$^-$ phenotype, *i.e.* the inability to grow on acetate. This indicates that the secondary mutation was near the Tn10 and so was very probably in *icd*.

A third class of pseudorevertants was isolated that was completely unanticipated. An example of this class is given by DEK2012, which exhibits the same growth rate on acetate as the isogenic wild type DEK2010. The revertant had not, however, recovered isocitrate dehydrogenase kinase activity, nor did the activity of isocitrate dehydrogenase appear to be affected. It seemed possible that this reversion arose from an alteration in the gene for isocitrate dehydrogenase which was not apparent from the *in vitro* assay. This possibility was ruled out by transducing a wild type gene for isocitrate dehydrogenase into DEK2012, as described above. In contrast to the results obtained with DEK2003, all of the tetracycline-resistant transductants retained the pseudorevertant phenotype. We have also considered the possibility that the secondary mutation in DEK2012 might have occurred in one of the genes coding for the glyoxylate bypass enzymes: isocitrate lyase (*aceA*) or malate synthase (*aceB*). This was tested by transducing the *ace* operon (consisting of *aceA*, *aceB*, and *aceK*; see below) from the pseudorevertant into a clean genetic background, JC13510. To accomplish this, we took advantage of the tight linkage between the *ace* operon and *met*A. On the *E. coli* chromosome, *aceA* and *aceB* fall in between *met*A and *aceK*. Thus, if the secondary mutation mapped to *aceA* or *aceB*, it would require a double crossover for a met$^+$ transductant to acquire the *aceK*$^-$ phenotype. Such a double crossover should occur at a very low frequency. When the met$^+$ transductants were tested for growth on acetate, approximately 50% exhibited the *aceK*$^-$ phenotype. This is similar to the frequency which was observed when the *aceK*$^-$ strain DEK2011 (the parent of DEK2012) was used as the donor. Thus, the secondary mutation did not occur in *aceA* or *aceB*.

TABLE V

Effect of Mu phage insertion on expression of ace operon products

The ace operon was induced by growing these strains to stationary phase in L-broth containing 2% sodium acetate. The cultures were harvested by centrifugation, resuspended in buffer A (25 mM Mops, pH 7.5, 1 mM EDTA, 2 mM 2-mercaptoethanol), and disrupted by sonication. The samples were then dialyzed for 3 h against buffer A, following which the enzyme activities were assayed as described under "Experimental Procedures" and protein was determined by the method of Lowry *et al.* (20).

Strain	Malate synthase[a]	Isocitrate lyase[a]	Isocitrate dehydrogenase kinase[a]	Isocitrate dehydrogenase phosphatase[a]
23559	1.00	1.00	1.00	1.00
DEK2000	0.94	0.76	0.02	0.04
DEK2001	0.29	0.06	0.00	0.04
DEK2002	0.41	0.02	0.05	0.00

[a] Enzyme activities are normalized relative to parental wild type strain 23559.

Mapping the Gene for Isocitrate Dehydrogenase Kinase/Phosphatase—The glyoxylate bypass is regulated by a variety of mechanisms, including control of gene expression. The genes coding for isocitrate lyase (*aceA*) and malate synthase (*aceB*) have been shown to form an operon with *aceB* first followed by *aceA* (4). In preliminary studies, we had found that the regulation of *aceK* expression paralleled that of *aceA* and *aceB*, suggesting that *aceK* might also be in this operon.[2] Some additional support was provided by the observation that *aceK* was linked to *met*A, a gene which is found immediately adjacent to the *ace* operon. In order to obtain conclusive evidence, we have isolated several polar mutants which have Mu phage inserted into the *ace* operon (see "Experimental Procedures").

[2] D. C. LaPorte, K. Walsh, and D. E. Koshland, Jr., unpublished results.

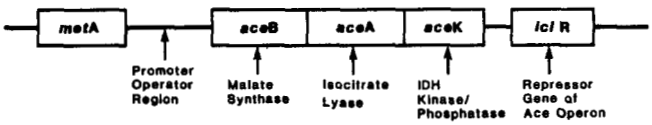

FIG. 2. **Organization of the glyoxylate bypass operon.** The assignment of the positions of the promoter/operator, malate synthase gene (*aceB*), and isocitrate lyase gene (*aceA*) are taken from Malloy and Nunn (4). The position of the kinase/phosphatase gene (*aceK*) is deduced from the data in Table V. The gene designated *iclR* codes for the repressor protein which regulates the *ace* operon (4).

Three Mu lysogens of 23559 have been isolated which have lost one or more of the glyoxylate bypass specific enzyme activities (Table V). Two of these strains, DEK2001 and DEK2002, have simultaneously lost isocitrate lyase, isocitrate dehydrogenase kinase, and isocitrate dehydrogenase phosphatase activities. In addition, they exhibit reduced levels of malate synthase. Although we have assigned these as insertions in *aceA*, this assignment is not unambiguous. The uncertainty arises because there are two genes coding for malate synthase, *aceB* and *glc*, both of which are expressed during growth on acetate (25). This ambiguity is of no consequence in the mapping of *aceK*, however, since the simultaneous loss of isocitrate lyase and isocitrate dehydrogenase kinase/phosphatase clearly places *aceK* in the *ace* operon. The third lysogen, DEK2000, has lost only isocitrate dehydrogenase kinase/phosphatase and so appears to result from an insertion into *aceK*. Since DEK2000 has lost only isocitrate dehydrogenase kinase/phosphatase, it follows that *aceK* is downstream from *aceA* and *aceB*. Therefore, the *ace* operon has the structure shown in Fig. 2.

The inclusion of the gene for isocitrate dehydrogenase kinase/phosphatase in the *ace* operon provides a mechanism for coordinating its expression with that of glyoxylate bypass enzymes. It might be noted, however, that the kinase/phosphatase is present at a cellular level that is approximately 1000-fold less than that of isocitrate lyase.[2] This is not surprising in view of fundamentally different roles that these two enzymes play in the functioning of the glyoxylate bypass. The precise mechanism that yields this profound downshifting in expression between *aceA* and *aceK* remains to be determined.

DISCUSSION

The environment inside a living organism is a complex function of the organism's genetic makeup and its environmental history. Because of this, the intracellular environment may exhibit variations between individuals of the same species and even between genetically identical organisms which have been subjected to differing external conditions. Examples of such variations might include the presence or absence of a major pathway, the level of key enzymes, or the availability of intracellular metabolite pools. A successful regulatory system must have the flexibility to detect these differences and adapt to the specific conditions which exist in the cell. For example, a specific pathway or regulatory system may be called upon to function efficiently in hepatocytes and in muscle cells even though the intracellular environments will be quite different.

Adaptation by Compensatory Phosphorylation—The glyoxylate bypass of *E. coli* provides an attractive system for the study of the adaptation of a regulatory system to the intracellular environment. *E. coli* offers a variety of experimental advantages, not the least of which is the potential for manipulation with the techniques of molecular genetics. Within this broadly defined context, we have initially focused on the response of the system to variation in the level of isocitrate dehydrogenase.

The flow of isocitrate through the glyoxylate bypass is regulated, in part, by the "reversible" phosphorylation of isocitrate dehydrogenase. Phosphorylation of this enzyme decreases its activity and so diverts isocitrate into the glyoxylate bypass. This phosphorylation system also provides the cell with a convenient mechanism with which to compensate for variations in the total level of isocitrate dehydrogenase. When the total level of isocitrate dehydrogenase varied over a 2-fold range in different strains of *E. coli*, the phosphorylation system efficiently compensated for this variation by maintaining a nearly constant activity for isocitrate dehydrogenase during growth on acetate. Even when the isocitrate dehydrogenase level was raised more than 10-fold, the phosphorylation system could still compensate efficiently. Thus, this system appears to have a remarkable capacity for adaptation to at least one aspect of the intracellular environment.

There are two possible but not mutally exclusive mechanisms by which the phosphorylation extent of isocitrate dehydrogenase could be varied to keep the amount of active isocitrate dehydrogenase constant. The first would involve modulating the activities of the isocitrate dehydrogenase kinase and phosphatase with allosteric effectors that are sensitive to the total level of isocitrate dehydrogenase and hence the metabolic state of the cell. Known effectors of the kinase and phosphatase, such as 3-phosphoglycerate and isocitrate (10, 13), would be good candidates for the process. The second mechanism for maintaining a constant level of isocitrate dehydrogenase activity rests upon the inherent kinetic parameters of the modifying enzymes. During log phase growth on acetate, the kinase is operating essentially in the first order region and the phosphatase is saturated with its substrate (10). As a result, the velocity of the phosphatase is independent of substrate concentration over a wide range. Consequently, the steady state concentration of the phosphatase's substrate, phosphoisocitrate dehydrogenase, will vary but the substrate of the kinase, isocitrate dehydrogenase, will remain nearly constant. The relative contribution of these mechanisms in maintaining the level of isocitrate dehydrogenase phosphorylation during steady state growth on acetate is currently being investigated.

The Loss of Kinase/Phosphatase Activity—The importance of this adaptation is emphasized by examining the effect of the loss of isocitrate dehydrogenase kinase/phosphatase. This loss results in a relatively modest 2- to 4-fold increase in isocitrate dehydrogenase activity. The effect on cellular metabolism is, however, greatly amplified because this branch point exhibits ultrasensitivity to this control mechanism (12). In the normal cell, the glyoxylate bypass is regulated indirectly via changes in the phosphorylation of isocitrate dehydrogenase and in the rate of isocitrate production. The control of the bypass results from the effect that these events have on the steady state level of isocitrate. This control exhibits ultrasensitivity because the Michaelis-Menten constant of isocitrate dehydrogenase for isocitrate (8 μM) is much less than that of isocitrate lyase (600 μM). In a normal cell, this allows for a very sensitive balancing of the flow of isocitrate through the competitive pathways. Under the pathological conditions which result from a mutation in *aceK* (the kinase/phosphatase gene), however, these control mechanisms will magnify the deleterious effects. In a previous study, we have analyzed the regulatory sensitivity of this branch point in *E. coli* strain 23559 (12). During growth on acetate, a 3-fold increase in isocitrate dehydrogenase activity resulting from an *aceK* mutation (see Table II) would, by itself, cause a

decrease by a factor of 9 in the flux through the bypass. The decrease in this flux rate should produce a precipitous drop in the level of the Krebs cycle intermediates. A possible consequence of this decrease in intermediate level would be to slow the rate of isocitrate production. The branch point was also found to be ultrasensitive to this rate and so further magnification of the pathological effects would result (12).

Pseudorevertants—Mutants which lacked isocitrate dehydrogenase kinase/phosphatase were capable of spontaneously reverting to growth on acetate by second site mutations. One particularly intriguing class of phenotypic revertants is exemplified by DEK2012. These revertants have undergone second site mutations which do not map to the genes for any of the enzymes known to be involved at this branch point: isocitrate lyase, isocitrate dehydrogenase, isocitrate dehydrogenase kinase/phosphatase, or malate synthase A. (The possibility that the mutation involves malate synthase G, expressed from *glc*, has not been ruled out, but seems unlikely.) Although other explanations must be considered as well, this mechanism of reversion may be indicative of a previously unidentified mechanism of control.

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