

REGULATION OF THE GLYOXYLATE
CYCLE IN *ESCHERICHIA COLI* K12

by

Mary Lynn Duckworth

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A thesis submitted to the Faculty of Graduate Studies of
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To Harry

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ABBREVIATIONS

| | |
|----------------|---|
| Acetyl CoA | Acetyl coenzyme A |
| AMP | Adenosine 5'-monophosphate |
| ATP | Adenosine 5'-triphosphate |
| CoA | Coenzyme A |
| Cyclic AMP | Cyclic 3',5'-adenosine monophosphate |
| DEAE cellulose | Diethylaminoethyl cellulose |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetracetic acid |
| EMS | Ethylmethane sulfonate |
| NAD (H) | Nicotinamide adenine dinucleotide (reduced) |
| NADP (H) | Nicotinamide adenine dinucleotide phosphate (reduced) |
| NEM | N-ethylmaleimide |
| PHD | Pyruvate dehydrogenase |
| PEP | Phosphoenolpyruvate |
| SDS | Sodium dodecylsulfate |
| TCA | Trichloroacetic acid |
| Tris | Tris (hydroxyl methyl) aminomethane |
| TTC | 2,3,5-Triphenyltetrazolium chloride |

ABSTRACT

ABSTRACT

In *Escherichia coli* K12 the level of isocitrate lyase, an enzyme of the glyoxylate cycle operon varies depending upon the carbon source. It is found to be low on glucose, intermediate on malate and succinate, higher on proline and α -ketoglutarate, and highest on acetate. When the induction of isocitrate lyase is followed after transfer of cells from glucose to different carbon sources, malate, succinate and α -ketoglutarate induce the enzyme significantly in less than an hour; acetate requires three hours or more to reach the same levels. Experiments with mutants lacking various TCA cycle enzymes, on different carbon sources, show that induction of isocitrate lyase occurs only if the carbon source can be metabolized through the oxidative energy-yielding steps of the cycle to C₄-intermediates beyond succinate. Acetate must be metabolized through both the TCA cycle and the glyoxylate cycle in order to induce isocitrate lyase fully. Changes in acetyl CoA and pyruvate levels in cells adapting to a new carbon source do not correlate with the induction of isocitrate lyase.

The enzyme, isocitrate dehydrogenase, is rapidly inactivated on acetate, proline and α -ketoglutarate, but is activated on glucose, pyruvate and succinate. The nature of these responses and the behaviour of mutants show that the regulation of this enzyme is different from that of the enzymes of the glyoxylate cycle operon. Cyclic AMP, however, does not appear to have a role in the regulation of either system.

Eleven independent glyoxylate cycle constitutive mutants, nine selected and two spontaneous, all map at the *icol R* locus, closely linked to the structural genes for isocitrate lyase (*ace A*) and malate synthase A (*ace B*) at 90 minutes on the *E. coli* map. Complementation studies using merodiploids show that these mutations fall into dominant and recessive classes.

INTRODUCTION

INTRODUCTION

When the facultative anaerobe *Escherichia coli* grows on glucose as its sole carbon source, it does so by the glycolytic pathway, and acetate is excreted into the medium. Glucose has a very strong repressing effect on many of the oxidative functions of the cell, and on a number of enzymes which are not required for its own metabolism. This catabolite repression is well understood in some cases, not in others, but the result is that while growing on glucose the cell is firmly adapted to an anaerobic fermentative type of metabolism.

Once the glucose is exhausted, *E. coli* begins to metabolize the acetate which had been excreted into the medium during the growth on glucose. Thus, at the point of glucose exhaustion, the cells shift from an anaerobic glycolytic type of metabolism to an oxidative gluconeogenic type of metabolism. This shift involves many changes in the metabolic pathways of the cell, the major one of which is the induction of the enzymes of the glyoxylate cycle so as to permit the use of acetate as sole carbon source for growth and energy. An understanding of the processes which regulate this shift from glucose to acetate as carbon source is basic to the understanding of *E. coli* metabolism.

The genes for two of the glyoxylate cycle enzymes, isocitrate lyase and malate synthase (A isozyme) lie side by side on the *E. coli* chromosome (Vanderwinkel and DeVlieghere 1968). The enzymes are

synthesized co-ordinately and they are thought to be regulated by repression by PEP or pyruvate (Kornberg 1966 , Dietrich and Henning 1970). The inhibition of isocitrate lyase by both these compounds has also been reported (Ashworth and Kornberg 1963). The regulation by PEP has been placed somewhat in doubt, however, by the experiments of Lowry *et al* (1971) in which levels of metabolites in cells growing on different carbon sources were determined. The concentrations of PEP were actually found to be higher on acetate than those on glucose.

Very little work has been done on the genetic regulation of the glyoxylate cycle operon. Mapping in close proximity to the genes for isocitrate lyase and malate synthase A is a locus, *icl R*, which is involved in the regulation of the operon (Brice and Kornberg 1968). Very few constitutive mutants of the operon have been mapped, however. Whether *icl R* is the only regulatory locus for the operon is therefore open to question, and the nature of the mutations at the *icl R* locus has never been thoroughly investigated.

The work in this thesis was undertaken to try to gain a better understanding of the regulation of the glyoxylate cycle in *E. coli* both at the physiological and the genetic levels.

CHAPTER 1

HISTORY

The Discovery of the Glyoxylate Cycle

The Tricarboxylic Acid Cycle (Figure 1)

By the early 1950s the work of Krebs (1943) had firmly established that the terminal respiration of carbohydrates in animal tissues is carried out by a series of enzymes which form a cycle - the tricarboxylic acid (TCA) cycle. With the discovery by Lynen *et al* (1951) of acetyl coenzyme A (acetyl CoA) it became clear that the purpose of the TCA cycle was to bring about the oxidation of acetate.

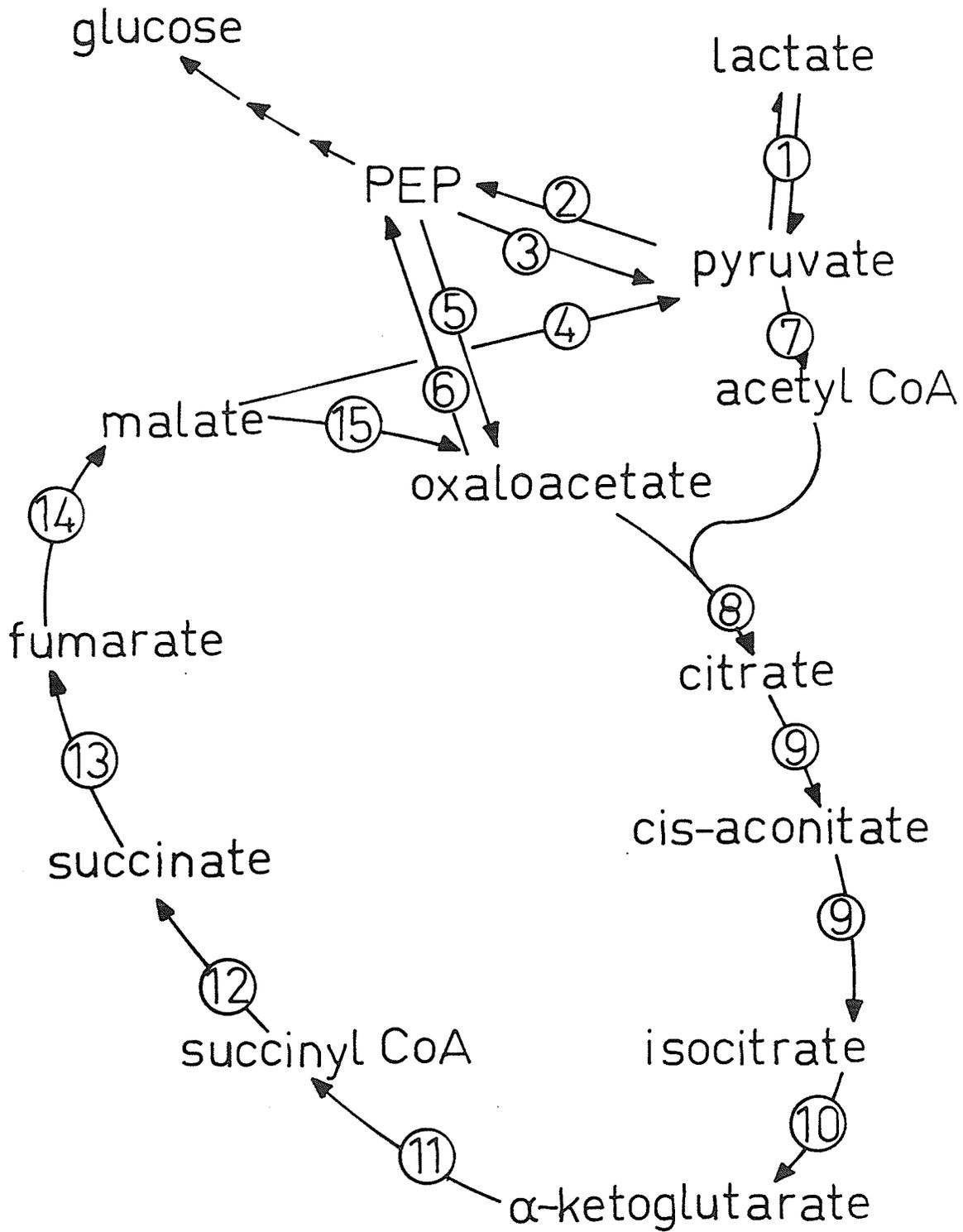
The hypothesis was advanced that the TCA cycle is also responsible for terminal respiration in microorganisms. Initially, there was great difficulty in proving this mainly because techniques similar to those for animal tissues were used, and permeability problems arose. The TCA cycle was finally firmly established as operating in microorganisms by the use of cell-free extracts, isotope experiments and auxotrophic mutants (Kornberg, 1959).

Krebs *et al* (1952) further suggested that the TCA cycle as well as taking part in terminal respiration, had a biosynthetic role in the cell, providing precursors for various cell components. It was not known what the metabolic processes were by which two carbon compounds such as acetate and ethanol could be converted to cell constituents in organisms which could meet all their carbon requirements from these compounds. The TCA cycle itself simply oxidizes C₂ compounds (acetate as its coenzyme A ester) to carbon dioxide and thus does not allow any production

Figure 1. The tricarboxylic acid cycle of *Escherichia coli* and its ancillary reactions. The enzymes and their mutants, where available, are numbered as follows: 1. lactate dehydrogenase; 2. PEP synthase (*pps*); 3. pyruvate kinase¹; 4. NAD-malic enzyme (*dne*); 5. PEP carboxylase (*ppc*); 6. PEP carboxykinase (*pck*); 7. pyruvate dehydrogenase (*ace E*, *ace F*); 8. citrate synthase (*glt A*); 9. aconitase; 10. isocitrate dehydrogenase (*icd*); 11. α -ketoglutarate dehydrogenase (*suc A*, *suc B*); 12. succinyl CoA synthase; 13. succinate dehydrogenase (*sdh*); 14. fumarase; 15. malate dehydrogenase (*mdh*).

¹Mutants lacking both of the pyruvate kinase isozymes have been reported (Perterra and Cooper 1977), but it appears that three letter locus designations have not been assigned, and the loci have not yet been mapped.

THE TRICARBOXYLIC ACID CYCLE



from these compounds of the reduced forms of carbon that are needed for biosynthesis. This focused attention on the one remaining gap in the knowledge of intermediary metabolism.

Thunberg and later Knoop (as cited by Kornberg and Krebs (1957)) had postulated a direct condensation of two acetates to form a succinate, but the true solution to the problem was not solved until the discovery of the enzymes isocitrate lyase and malate synthase.

Isocitrate Lyase

While doing work on *Pseudomonas aeruginosa* to try to establish the presence of the TCA cycle, Campbell *et al* (1953) found an activity which yielded glyoxylate as a product of citrate. Further results showed that *cis*-aconitate was readily dissimilated to glyoxylate but isocitrate was not. It is not clear why these workers found isocitrate inactive in their system. Reasoning that they might be getting a 4-2 split of the citrate, they looked for succinate as the other product, and found it. They found that the activity was reversible and that succinate plus glyoxylate yielded citrate always in the ratio one mole succinate/mole citrate.

Smith and Gunsalus (1954) also working with *Pseudomonas aeruginosa* purified this enzyme which they called isocitritase free of aconitase and found that it was dependent on isocitrate rather than citrate. Other workers rapidly confirmed and extended these findings. Saz (1954) and Saz and Hillary (1956) also working with *Pseudomonas* extracts found

that the naturally occurring D(+) isomer of isocitrate was the most active substrate for the enzyme and postulated that this would be the natural substrate.

Olson (1954) found a similar activity in *Penicillium chrysogenum* growing on acetate. He determined that in this organism also, D(+) isocitrate was the immediate precursor and that it was converted quantitatively to succinate and glyoxylate.

Wong and Ajl (1955) reported a similar activity in acetate-grown *E. coli*. Again the partially purified extract was found to be specific for isocitrate.

Malate Synthase

Initially, after the discovery of the isocitrate lyase activity, it was thought that the glyoxylate formed by this reaction was used in the synthesis of glycine and that succinate entered the TCA cycle. Olson had found that glycine accumulated in the medium of *Penicillium chrysogenum* growing on acetate.

However, another possibility was presented when Wong and Ajl (1956) discovered an activity in acetate-grown *E. coli* E-26 which converted equimolar concentrations of acetate and glyoxylate to malate. They were able to show a dependence for glyoxylate, acetyl phosphate, coenzyme A and phosphotransacetylase which suggested that glyoxylate and acetyl CoA were the components of the reaction. This activity was called malate synthase.

The Glyoxylate Cycle (Figure 2)

It remained for Kornberg and his co-workers to establish the presence of isocitrate lyase and malate synthase in the same extracts, and to formulate the concept of the glyoxylate cycle, which would allow cells to grow on acetate as a sole carbon source and replenish the intermediates of the TCA cycle.

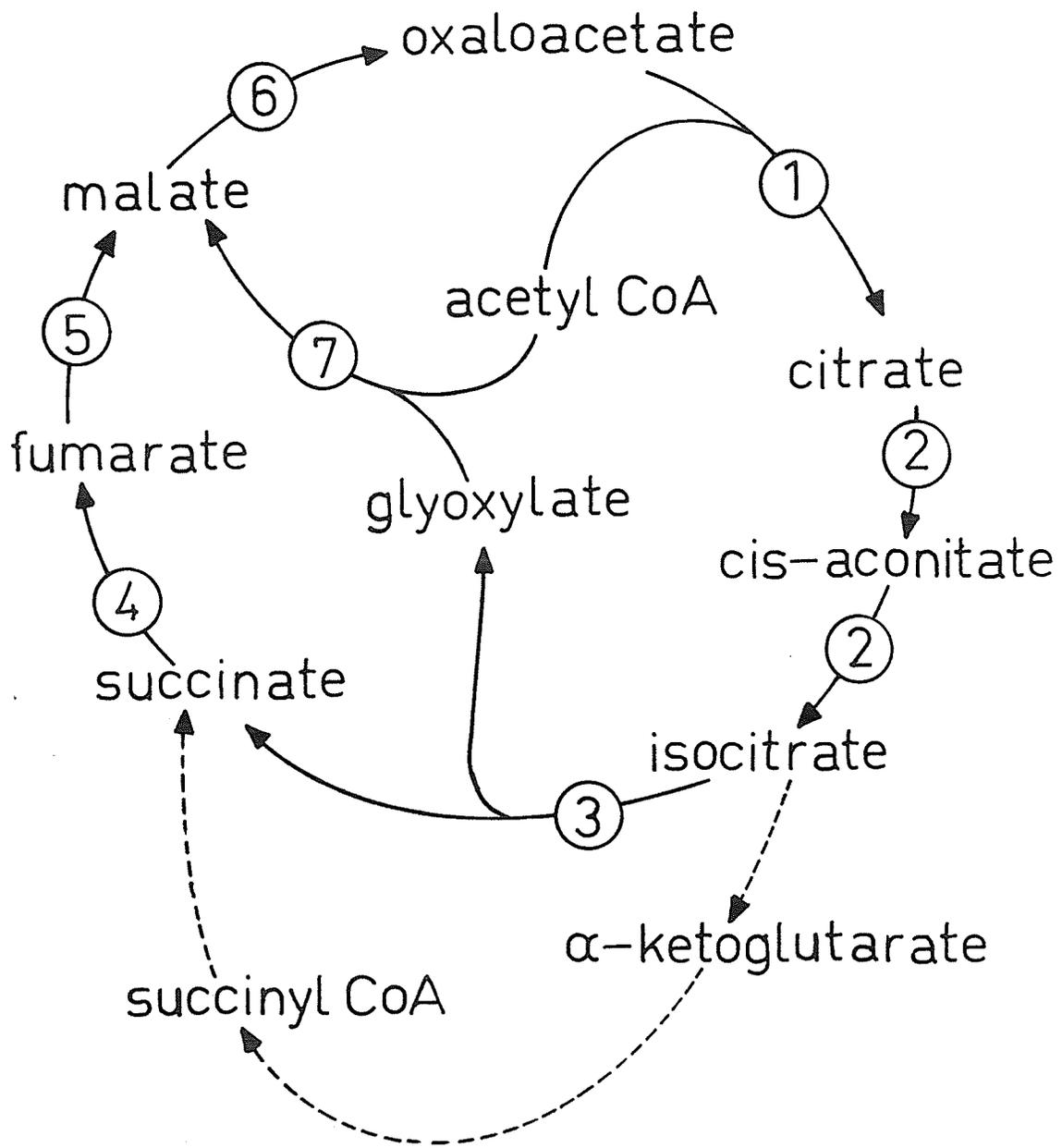
The first experiments which showed that these two activities could act in concert were performed by Kornberg and Madsen (1957a,b; 1958) on extracts of acetate-grown *Pseudomonas*. They demonstrated that such extracts were capable of forming labelled malate from isocitrate, ^{14}C -acetate, ATP, coenzyme A (CoA) and glutathione. Labelled malate was also formed if glyoxylate replaced isocitrate. Thus, the operation of these two enzymes could bring about the net synthesis of one molecule of C_4 -dicarboxylic acid from two molecules of acetate, and the oxaloacetic acid required to keep the TCA cycle operating could be replenished.

Kornberg and his co-workers then proceeded by means of isotope studies to look at the integrated metabolism of acetate in microorganisms. Although the possible existence of a glyoxylate cycle had been shown using extracts, it was important to demonstrate that this was indeed an integral part of acetate metabolism. The condensation reaction suggested by Thunberg and the possibility that carbon dioxide fixation reactions take part in acetate metabolism had to be excluded.

The first experiments with *Pseudomonas fluorescens* and a

Figure 2. The glyoxylate cycle of *Escherichia coli*. The enzymes and their mutants, where available are numbered as follows: 1. citrate synthase (*glt A*); 2. aconitase; 3. isocitrate lyase (*ace A*); 4. succinate dehydrogenase (*sdh*); 5. fumarase; 6. malate dehydrogenase (*mdh*); 7. malate synthase A (*ace B*) and malate synthase G (*glc*).

THE GLYOXYLATE CYCLE



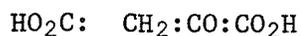
Corynebacterium sp. (Kornberg 1958) determined the distribution of label from ^{14}C -acetate in various TCA cycle intermediates at very early times. Within three seconds, 30% of the label was in citrate, 40% in malate, 10% in succinate and fumarate and 11% in aspartate. The steady-state was reached within thirty seconds and showed the distribution of 10% of the label in malate, 15% in citrate, 5% in succinate and fumarate and 40% in glutamate.

These experiments indicated that the label went to malate before it went to citrate, and that acetate could enter the TCA cycle at two sites to form citrate at one site and at the other either malate or a compound in close equilibrium with it.

Further experiments with *Pseudomonas* KB1 showed that a constant stream of carbon dioxide had little effect on the incorporation of ^{14}C -2-acetate into protein. This indicated that carbon dioxide fixation does not play a role in acetate metabolism in this organism.

The experiments which showed perhaps most decisively the role of the glyoxylate cycle in acetate metabolism were those of Kornberg and Quayle (1958). They looked at labelling patterns of aspartate and alanine in *Pseudomonas* growing on ^{14}C -2-acetate.

^{14}C -2-acetate recycled through the TCA cycle would lead to oxaloacetic acid labelled:



16.7: 33.3:33.3:16.7

and pyruvate derived from this:



40:40:20.

Kornberg and Quayle found less label in the carboxyl groups of both aspartate (derived by transamination from oxaloacetate) and alanine (derived by transamination from pyruvate) than would be predicted by the sole operation of the TCA cycle. This could be explained if a mechanism were operating in the cells in which there was an enhanced incorporation of the carboxyl group of acetate into the carboxyl groups of aspartate and alanine. The operation of the glyoxylate cycle would lead to such an effect because the unlabelled carboxyl group of the acetate would enter not only by condensation with oxaloacetic acid, but also by condensation with glyoxylate to produce malate.

^{14}C -2-acetate recycled through the glyoxylate cycle would lead to the distribution:

| | | | | |
|--------------|------------------------|----------------|-----------------------|-----------------------|
| oxaloacetate | $\text{HO}_2\text{C}:$ | $\text{CH}_2:$ | $\text{CO}:$ | CO_2H |
| | 0: | 50 : | 50: | 0 |
| and pyruvate | $\text{CH}_3:$ | $\text{CO}:$ | CO_2H | |
| | 50: | 50: | 0 | |

Kornberg and Quayle's results showed a distribution of label in the carboxyl groups which was higher than that expected for the operation of the glyoxylate cycle alone.

They concluded that in *Pseudomonas* growing on acetate, both the TCA cycle and the glyoxylate cycle were operating. This distinctive

pattern of labelling has also been found in other organisms which are able to grow on acetate as a sole carbon source.

Kornberg (1966) coined the term "anaplerotic" to describe sequences of which the glyoxylate cycle is the classic example, that play the role of replenishing intermediates which are consumed in biosynthetic reactions.

The discovery of the glyoxylate cycle and its important role in acetate metabolism led to an extensive search through the various phyla to try to determine how widespread the glyoxylate cycle was. Very early Kornberg and Krebs (1957) recognized the possible role the cycle could play in the conversion of fat to carbohydrate in oil rich seedlings such as *Ricinus*.

To date, the enzymes of the glyoxylate cycle have been found in many different bacteria and fungi, in yeast, algae, protozoa, metazoa, and plants, but never in higher animals, which are not known to be able to use fats for biosynthesis. Although the cycle itself is basically the same in all these organisms the variability of regulation of the cycle is almost as diverse as the organisms themselves. Some organisms have been studied extensively, others not so much, but the adaptation of the cycle to the organism's "life-style" is an interesting study in evolution.

The Distribution and Regulation of the Glyoxylate Cycle

Procaryotes

The original studies on the glyoxylate cycle enzymes, as we have seen, used bacteria as the source, notably *Pseudomonas* and *Escherichia coli*. The enzymes of the cycle have now been found in a wide variety of bacteria usually when they are growing on acetate, although there are exceptions even to this. The most extensively studied bacterium has been *E. coli*. For this reason, it will be discussed separately from the other procaryotes.

Pseudomonas was the first genus in which isocitrate lyase was detected. Once the existence of the glyoxylate cycle was established it became of interest to know under what conditions the enzymes might be present since it appeared that they were really required only during growth on acetate.

Working with *Pseudomonas ovalis* Chester, Kornberg *et al* (1958) looked at the levels of isocitrate lyase in cells growing on a variety of carbon sources. They found that acetate-grown cells contained three hundred times more isocitrate lyase than did glucose-grown cells, and ten times more than succinate cells. Cells grown in the presence of succinate and acetate showed no increase in enzyme levels above that seen on succinate alone. Further experiments showed that cells grown on succinate could utilize acetate without a lag, but only after succinate was exhausted did isocitrate lyase levels rise. These authors

postulated that, rather than acetate inducing the enzyme *per se*, there had to be a fall in the levels of a co-repressor before the glyoxylate enzymes could increase.

They noted that in this organism, there was a large increase in isocitrate lyase levels in cells on acetate before there was any increase in cell density.

Another *Pseudomonas* (Shaw Strain MA, Bellion and Hersh, 1972) was found to contain high levels of isocitrate lyase when cells were growing on acetate or methylamine. Cells growing on acetate or succinate were found to contain malate synthase, acetate giving higher levels than succinate, but cells growing on methylamine had only five percent of the malate synthase found in cells growing on acetate.

Further work (Bellion and Woodson, 1975) found that there were two quite distinct isocitrate lyases present in *Pseudomonas* (Shaw Strain MA) - one found only in cells growing on acetate, and one found only in cells growing on methylamine. The two enzymes were quite distinct in their elution profiles on DEAE cellulose, pH optima, Km values and sensitivity to heat.

The fact that malate synthase is high during growth on acetate but low during growth on methylamine implies that there may be co-ordinate regulation between malate synthase and only one of the isocitrate lyases. These two co-ordinately regulated enzymes would then constitute the glyoxylate cycle as it is defined in most organisms.

The isocitrate lyase found in *Pseudomonas* (Shaw Strain MA) growing

on methylamine seems not to be a part of this cycle at all. Rather, in the pathway worked out by Bellion and Hersh (1972) for the metabolism of methylamine, the glyoxylate is transaminated to glycine which then goes to serine, an essential intermediate in the pathway. Not only do cells growing on it not contain malate synthase, they have been found to contain a malate cleaving enzyme which can form more glyoxylate at the expense of ATP and CoA.

Thus in this organism, in spite of the fact that the two isocitrate lyases catalyze the same reaction which forms glyoxylate, their functions are quite different and the problem of regulation seems to have been solved by having the synthesis of two enzymes regulated separately.

Micrococcus denitrificans can grow autotrophically by assimilating carbon dioxide via the Calvin cycle, as well as heterotrophically on acetate and a number of other carbon sources. In this organism, all extracts showed citrate synthase and malate synthase activity but only cells grown on acetate had high levels of isocitrate lyase (Kornberg *et al* 1960). The presence of acetate however, was not sufficient to produce elevated levels. If fumarate, malate and especially succinate were added to cells growing on acetate synthesis of isocitrate lyase ceased until the C₄ acid had been utilized. These results were interpreted to mean that succinate acts as a co-repressor preventing the synthesis of isocitrate lyase. A shift from C₄ acids to acetate was regarded as causing depletion of intermediates and a lowering of levels of compounds like succinate.

It is interesting that Kornberg *et al* found malate synthase in all

extracts, a fact that implies that there is no co-ordinate regulation of the two enzymes. This could also imply that there is more than one malate synthase in this organism, but this was not investigated.

One organism in which acetate appears to have an inducing effect is an *Achromobacter* species d-15 (Rosenberger 1962). This organism does not grow on glucose or glycerol, but grows quite well on C₃ and C₄ organic acids. The synthesis of isocitrate lyase is not inhibited by the presence of succinate in cultures of *Achromobacter* growing on acetate, and the addition of acetate to cells growing on succinate causes an increase in enzyme synthesis. In addition, cells growing on lactate, alanine or pyruvate have high levels of isocitrate lyase. Kornberg who had postulated that PEP was the regulator of the glyoxylate cycle enzymes in *E. coli* speculated that *Achromobacter* was able to produce high levels of isocitrate lyase on C₃ compounds because they could not form PEP from pyruvate as *E. coli* can (Kornberg *et al* 1964). This organism is quite different in its metabolism from *E. coli*, however, and there is no reason why a common regulatory mechanism need be postulated for all organisms.

No mention was made of malate synthase in these organisms or if it is regulated co-ordinately with isocitrate lyase.

Another organism which is aerobic, does not grow on carbohydrates, but grows well on organic acids is *Acinetobacter* (formerly *Mima polymorpha*). In this organism also, acetate appears to have an inducing effect on isocitrate lyase levels in cells growing on succinate or L-malate.

Acinetobacter grows well on pyruvate, and is able to produce iso-

citrate lyase to a significant extent on that carbon source. This organism is known to contain PEP synthase in levels comparable to those found in *E. coli*, so Kornberg's argument as to why pyruvate does not repress would not hold true for this organism.

Sturm *et al* (1970) investigated the relationship between the glyoxylate cycle and the TCA cycle in *Acinetobacter*. They found that there appeared to be an inverse relationship between the levels of isocitrate lyase and α -ketoglutarate dehydrogenase. Growth on succinate produced a rise in α -ketoglutarate dehydrogenase levels. No increase in isocitrate lyase occurred until cells approached stationary phase when there was slight induction. Corresponding to the increase in isocitrate lyase levels was a decrease in levels of α -ketoglutarate dehydrogenase. Growth on malate was slow to initiate and produced an increase only in the level of α -ketoglutarate dehydrogenase. Acetate, however, induced high levels of isocitrate lyase, and produced only a slight rise in α -ketoglutarate dehydrogenase levels during early lag phase. These levels later fell. Sturm *et al* suggested that α -ketoglutarate might be the true inducer of the α -ketoglutarate dehydrogenase and that in *Acinetobacter*, there was a concurrent regulation of the glyoxylate and TCA cycles.

Work done on *Bacillus cereus* showed a rather different pattern of regulation from other bacteria. Megraw and Beers (1964) found that cells grown on acetate or glucose have much lower levels of isocitrate lyase than cells growing on glutamate or yeast extract as principal

carbon source. Glutamate-grown cells have about five times higher levels of isocitrate lyase, but ten to twenty-fold lower levels of malate synthase.

It would appear that the glyoxylate formed under these conditions is not metabolized through the glyoxylate cycle since malate synthase levels are so low. It is possible, although the authors give no data on this point, that the isocitrate lyase present in high levels on glutamate is not the same enzyme as is expressed on acetate, but as in *Pseudomonas* is a second enzyme synthesized for a specific function on glutamate. They postulate glyoxylate goes to tartaric semialdehyde, then to glycerate.

Megraw and Beers also found that isocitrate lyase was at its peak of activity just as sporogenesis began, and that the activity of the glyoxylate cycle seemed to decline as sporogenesis progressed. This is consistent with some data of Nakata and Halvorson (1960) who demonstrated that prior to sporulation of *Bacillus cereus* T in glucose medium, terminal respiration was inhibited and acetate accumulated. Subsequently at the onset of sporogenesis the acetate was utilized. Gallakota and Halvorson (1963) found that fluoroacetate, a known inhibitor of acetyl CoA synthase, inhibited sporulation although it had no effect on vegetative growth or the germination of spores. From this and other data they speculated that the metabolism of acetate, presumably via the glyoxylate cycle, was a necessary event for sporulation and that fluoroacetate could prevent the formation and also the utilization of the acetate metabolizing system.

Work with a pyruvate carboxylase mutant in a thermophilic *Bacillus* which has the property of being unable to grow on glucose or lactate yielded mutants which were found to be constitutive for isocitrate lyase (Sundaram, 1973). They were isolated as cells which could still grow on acetate in the presence of glucose or lactate. The wild type is inhibited under such conditions.

Similar mutants were isolated from *Bacillus stearothermophilus* and were found to be derepressed not only for isocitrate lyase but also for malate synthase on all media tested (Chell and Sundaram 1975).

These are among the few constitutive mutants known in organisms other than *E. coli*. The fact that there is an elevation in the levels of both glyoxylate cycle enzymes in these mutants implies that there may be a co-ordinate regulation.

The regulation of the glyoxylate cycle enzymes still is not really clear in *Bacillus* sp. As in some strains of *Pseudomonas* there may be more than one isocitrate lyase, only one of which takes part in the glyoxylate cycle. There does appear to be an isocitrate lyase which is regulated co-ordinately with malate synthase, possibly by acetate which seems to accumulate during growth on glucose. The switch from metabolizing glucose to metabolizing acetate may be an important one in triggering sporulation.

A rather different procaryote than those discussed so far is the soil bacterium, *Nocardia salmonicola* (Sarislani *et al* 1975). This organism prefers to grow on acetate and grows only poorly on glucose.

It produces isocitrate lyase in highest levels on acetate and DL-lactate. It produces low levels of the enzyme on TCA cycle intermediates and perhaps surprisingly on pyruvate and DL-alanine. Only succinate and fumarate completely prevent an increase in enzyme activity in the presence of acetate. Acetate when added to cells growing on the poor carbon source, glucose, causes an increase in activity of isocitrate lyase within three minutes. This increase can be prevented by chloramphenicol, the inhibitor of protein synthesis. The levels of isocitrate dehydrogenase also rise when acetate is added to glucose and this increase also seems to be due to *de novo* synthesis.

It has been postulated that acetate is the inducer of isocitrate lyase in this organism because the addition of amounts of acetate as small as 40 μ M can cause a rapid increase in levels of this enzyme. Fumarate appears to have a repressing effect which seems to overcome the inducing ability of acetate. Succinate also has a repressing effect, but this can be overcome by malonate, the specific inhibitor of succinate dehydrogenase.

It is possible that cyclic AMP may have a role in the regulation of isocitrate lyase synthesis in this organism (Westwood and Higgins 1976). The addition of 3 or 30 mM cyclic AMP simultaneously with fumarate to cells growing on acetate can slow the decline in the specific activity of the enzyme, but cannot prevent it. As the decline in enzyme activity is very rapid after the addition of fumarate it is not really clear what the mechanism of the decrease in activity is and what role the cyclic AMP is playing.

Nocardia is able to grow on hexadecane, 1-phenyldodecane and 1-phenylnonane (Trust and Miller 1970). Growth on these long chain phenylalkanes also produces high levels of isocitrate lyase. Whether this is the same, or a different enzyme from that found on acetate-grown cells was not reported.

Much of the work on the glyoxylate cycle in procaryotes has involved simply testing for the presence of the two characteristic enzymes. Isocitrate lyase is tested but malate synthase is not always assayed. It is obvious from some of the examples given, that the presence of isocitrate lyase is not sufficient to prove the existence of the cycle.

Fungi

Neurospora crassa has two different isocitrate lyases (Sjogren and Romano 1967). One ICL-2, is found even when cells are growing on glucose. The other, ICL-1 is produced in elevated amounts when the fungus is growing on acetate. The enzymes differ with respect to pH optima, Km values, and elution profiles on DEAE-cellulose. It has been speculated that ICL-2 might play a role in amino acid biosynthesis, and this is supported by the presence of an active alanine-glyoxylate transaminase in *Neurospora*.

Flavell and Fincham (1968a) isolated in *Neurospora crassa* probably the first acetate non-utilizing mutants to be identified in a fungus. They were able to identify seven complementation groups and assign

actual functions to five of these. They found mutants in isocitrate lyase (*acu-3*), acetyl CoA synthase (*acu-5*), α -ketoglutarate dehydrogenase (*acu-2*, *acu-7*) and PEP carboxykinase (*acu-6*).

The presence of PEP carboxykinase and α -ketoglutarate dehydrogenase mutants among the acetate non-utilizers indicates their important roles when this organism grows on acetate. PEP carboxykinase would be required to carry the immediate products of the glyoxylate cycle into gluconeogenesis, but the need for α -ketoglutarate dehydrogenase in acetate metabolism is not immediately obvious.

The lack of malate synthase mutants is interesting. Since isocitrate lyase and malate synthase activities vary proportionately on different carbon sources (Flavell and Fincham, 1968b), one might expect mutants lacking one of these enzymes to be equally common. Perhaps Flavell and Fincham did not screen enough mutants, or it may be that as in *E. coli* (Falmagne *et al* 1965) there is more than one malate synthase and both must be mutated in order to produce an acetate non-utilizer.

On transferring wild-type mycelium from sucrose to acetate there were considerable increases in the specific activities of isocitrate lyase, malate synthase, PEP carboxykinase and acetyl CoA synthase. Three-to four-fold increases were seen for citrate synthase, malate dehydrogenase, fumarate hydratase and NADP -isocitrate dehydrogenase. No increases were seen in the levels of α -ketoglutarate dehydrogenase or the NAD -isocitrate dehydrogenase.

Although none of the acetate mutants could grow after transfer from

sucrose to acetate, most were still able to synthesize new enzymes under these conditions. Only the *acu-3* mutants lacked isocitrate lyase. This gene has been established as the structural gene of ICL-1, the acetate induced enzyme (Leckie and Fincham 1971). These mutants were still able to produce considerable amounts of ICL-2. The *acu-5* mutants which were mutated in acetyl CoA synthase were still able to synthesize the glyoxylate cycle enzymes.

Studies done by two different groups who looked at the levels of the glyoxylate cycle enzymes in mycelia growing on different carbon sources and in a pyruvate carboxylase (*suc*) mutant have led to conflicting theories about the regulation of the cycle in *Neurospora* (Flavell and Woodward 1971; Beever 1975).

Both groups concluded that acetate was not acting directly as an inducer. Even though acetate can produce increased levels of isocitrate lyase and malate synthase in cells growing on sucrose, Beever argued that the levels of enzymes were not as high as would be expected if acetate acted as an inducer. Flavell and Woodward reasoned that the inducer-like effect of acetate stemmed from its ability to pull glycolytic intermediates efficiently into the TCA cycle and hence reduce their concentration. This is not at all compatible with the finding that an acetyl CoA synthase mutant which presumably cannot metabolize acetate at all, much less cause major metabolic shifts, still shows increases in levels of glyoxylate cycle enzymes on acetate.

Beever, working with a pyruvate carboxylase (*suc*) mutant which

requires a dicarboxylic acid supplement in order to grow on sucrose concluded that the glyoxylate cycle enzymes were repressed by a C₄ TCA cycle intermediate. His observation was that the pyruvate carboxylase mutant showed a lag before growing on sucrose plus succinate. During that time there was a partial derepression of isocitrate lyase and malate synthase. The initial growth lag was consistent with the idea that C₄ acids are limiting during this lag stage. Glycolytic intermediates on the other hand were evidently high as pyruvate was excreted into the medium.

An alternative explanation for the derepression of the isocitrate lyase and malate synthase might be that during the lag phase, succinate is not yet taken up, and that C₄ intermediates, including oxaloacetate are low. Acetyl CoA from pyruvate accumulates and cannot be metabolized since oxaloacetic acid is not being made from pyruvate in the absence of pyruvate carboxylase, and the acetyl CoA induces the enzyme.

Flavell and Woodward concluded that the metabolic repressor of ICL-1 and malate synthase was a glycolytic intermediate rather than a TCA cycle intermediate. They argued that there were high levels of glyoxylate cycle enzymes when a sole carbon source such as acetate or glutamate was metabolized through the TCA cycle but that levels were low when a carbon source such as sucrose was metabolized through glycolysis.

Isocitrate lyase-2, the enzyme which is present in sucrose grown

mycelia is regulated in a different way from ICL-1 (Flavell and Woodward 1971). High levels of ICL-2 appear to be correlated with low levels of both NADP and NAD -specific isocitrate dehydrogenases, a condition which is found in sucrose grown cells by comparison with acetate grown cells. It has been suggested that ICL-2 is induced by high levels of isocitrate. This cannot be tested directly because of the poor permeability of *Neurospora* to isocitrate.

There appears to be a further type of control imposed on the glyoxylate cycle enzymes in *Neurospora crassa*. Kobr *et al* (1960) isolated a particulate fraction, by sucrose density gradient centrifugation, which was distinct from mitochondria and contained the bulk of the isocitrate lyase, malate synthase, and malate dehydrogenase. Succinate dehydrogenase, however, was associated with a lighter particle tentatively identified as the mitochondrion. When mycelia were transferred from sucrose to acetate there was a twenty-fold increase in the isocitrate lyase. This new activity was initially found in the soluble fraction, but became progressively associated with the particulate fraction.

These particles are very similar to the glyoxysomes which will be discussed in detail in the section on oil seeds. Their existence suggests that at least in this fungus there is some separation of glyoxylate cycle enzymes from those of the TCA cycle which are located in mitochondria.

The glyoxylate cycle of the fungus *Aspergillus nidulans* has also

been studied with the aid of acetate non-utilizing mutants (Armitt *et al* 1976). The essential role of the glyoxylate cycle during growth on acetate was confirmed by finding mutants lacking isocitrate lyase (*acu* D) and malate synthase (*acu* E) among the acetate non-utilizers. Temperature sensitive gene products have been found at both these loci, confirming that they are the structural genes for the enzymes. The two genes are not linked. The two enzymes do, however, show co-ordinate regulation in this fungus (Armitt *et al* 1971). Enzyme levels on acetate plus glucose are about as high as on acetate alone. Levels of the enzymes on acetate plus sucrose are only about half of these, however, and on sucrose alone the levels are very low.

The *acu* D mutants are still able to induce malate synthase when grown on a mixture of sucrose plus acetate. This implies that the malate synthase is not induced sequentially by glyoxylate, the product of isocitrate lyase.

Mutants totally lacking isocitrate lyase were found to be unable to grow on L-glutamate as a sole carbon source. All malate synthase mutants were able to grow on glutamate or proline. This implies that isocitrate lyase may have another role in this organism, possibly the synthesis of glycine from glyoxylate.

A number of other acetate non-utilizing mutants were found, among them, mutants of PEP carboxykinase, malic enzyme and fructose-1,6-diphosphate phosphatase. None were found which lacked succinate dehydrogenase or fumarase. Among the acetate non-utilizing mutants were two groups *acu* A and *acu* C which were resistant to fluoroacetate in the presence of glucose. The *acu* A mutants lacked acetyl CoA synthase but

the function of *acu C* was not discovered. These correspond to the genes *fac A* and *fac C* isolated by Apirion (1965). A third class of fluoroacetate resistant mutants isolated by Apirion, *fac B*, whose function is also unknown, was not found among the acetate non-utilizers. The acetyl CoA synthase mutants and the other fluoroacetate resistant mutants showed only poor induction of the glyoxylate cycle enzymes on acetate. Armitt *et al* (1976) have interpreted this to mean that the true inducer of the glyoxylate cycle in *Aspergillus* may be acetyl CoA rather than acetate. This result is just the opposite of that seen with *Neurospora* which can still induce the glyoxylate cycle enzymes in the absence of acetyl CoA synthase.

Coprinus cinereus (formerly *lazopus*) is also a filamentous fungus like *Aspergillus* and *Neurospora*. It is able to use acetate as a sole carbon source, but in the presence of glucose or a glycolytic intermediate, levels of isocitrate lyase, malate synthase and acetyl CoA synthase are low.

Thirteen chromosomal loci have been located which affect acetate utilization by *Coprinus* (King and Casselton 1977). Mutants at only two loci are deficient in isocitrate lyase - *acu-1* the structural gene for acetyl CoA synthase and *acu-7* the structural gene for isocitrate lyase itself. A suppressor mutant of the *acu-1* mutation simultaneously restores the ability of the mutant to induce isocitrate lyase (Sealy-Lewis and Casselton 1978). This seems to imply that like *Aspergillus*, but unlike *Neurospora*, acetate must be metabolized at least to

acetyl CoA before it can have an inducing effect on the glyoxylate cycle enzymes.

A role for isocitrate lyase in sporulation has been postulated in several different fungi. Galbraith and Smith (1969) found that isocitrate lyase showed a marked increase in activity just prior to sporulation in *Aspergillus niger*. In *Blastocladiella emersonii* inhibition of the TCA cycle by the addition of bicarbonate, malonate or arsenite promotes the differentiation to male gametes (Cantino 1966). In *Allomyces*, similar treatments induce the formation of asexual spores (Turian 1966). In both cases, an increase in isocitrate lyase was correlated with the differentiation, but so was an increase in glycine-alanine transaminase which could mediate the transamination of glyoxylate to glycine. A similar correlation was also seen in *Neurospora crassa* but Turian found that conidiation could still occur when isocitrate lyase was low, and glyoxylate could be derived instead from pentoses. These findings imply that the role of isocitrate lyase which is important in sporulation is not that in the glyoxylate cycle, but rather the production of glyoxylate for glycine biosynthesis. The isocitrate lyase which increases in activity may not even be the enzyme involved in the glyoxylate cycle. At least in *Neurospora*, as we have seen, there is a second enzyme not co-ordinated with malate synthase which could perform this function.

Saccharomyces

In *Saccharomyces cerevisiae* the levels of TCA cycle enzymes and

glyoxylate cycle enzymes are low in cells growing on glucose, but as the sugar disappears from the medium there is a substantial rise in the enzymes of both cycles. This increase seems to occur when the sugar has been exhausted, and substantial amounts of acetate have accumulated in the medium (Polakis and Bartley 1965).

Duntze *et al* (1969) found that growth of *Saccharomyces cerevisiae* on acetate increases the specific activity of isocitrate lyase, malate synthase, and one specific isoenzyme of malate dehydrogenase, but that only small changes occur in citrate synthase, aconitase, succinate dehydrogenase and fumarase levels. The kinetics of derepression of isocitrate lyase, malate synthase and malate dehydrogenase showed no correlation among themselves or in relation to other enzymes.

Cells grown on acetate in the presence of glucose showed no increase in any of the enzymes, and if glucose was added to cells growing on acetate there was a rapid decline in the activity of malate dehydrogenase and a slower decline in isocitrate lyase activity. Malate synthase activity seemed not to be affected under these conditions.

When the cellular location of the various enzymes was investigated, all of the isocitrate lyase and malate synthase appeared to be located in the soluble fraction. Malate dehydrogenase, citrate synthase, aconitase, and fumarase were all found in both the cytoplasmic fraction and the mitochondrial fraction. Only succinate dehydrogenase was found in the mitochondrial fraction exclusively. Thus all the enzymes necessary for the complete functioning of the glyoxylate cycle are found in the cytoplasm, but the succinate formed must be oxidized in

the mitochondria which are the only source of succinate dehydrogenase. This is a very similar situation to that described in *Neurospora crassa*, except that there seems to be no evidence in *Saccharomyces* for a second organelle containing the glyoxylate cycle enzymes.

A further type of regulation also operates in *Saccharomyces*. This is called catabolite inactivation and is known to affect several enzymes in yeast, not just some of those involved in the glyoxylate cycle. The phenomenon involves the rapid inactivation of specific enzymes when yeast cells are transferred to medium which does not support growth, or medium containing glucose, fructose, mannose or sucrose. It was originally described for a galactose metabolizing system in yeast (Spiegelman and Reiner 1947) but has since been described for α -glucoside permease (Robertson and Halvorson 1957) fructose-1,6-diphosphatase (Gancedo 1971), PEP carboxykinase (Gancedo and Schwerzmann 1976), and cytoplasmic malate dehydrogenase (Ferguson *et al* 1967).

The very rapid inactivation of malate dehydrogenase and isocitrate lyase described by Duntze *et al* after the addition of glucose to cells growing on acetate is thought to be an example of this catabolite inactivation. It had long been known that the ratio of isocitrate lyase and malate synthase varied widely on different carbon sources. Since there is no indication that malate synthase is subject to catabolite inactivation, this, rather than the fact that the enzymes are not co-ordinately induced, might be the explanation for the differences in levels.

At least in the cases of PEP carboxykinase and fructose-1,6-diphosphatase, catabolite inactivation seems to occur in the absence of *de novo* protein synthesis. The exact nature of the phenomenon is not clear. It has been speculated that it could be a modification, or degradation by a protease (Molano and Gancedo 1974).

A mutant called *hex-1* has been isolated which shows reduced phosphorylation of glucose and at the same time strongly reduced catabolite inactivation (Entian 1977). This mutant seems to indicate that in some as yet unknown way, the hexose phosphorylation step in glycolysis is involved in triggering the catabolite inactivation response. The *hex-1* mutant still shows normal catabolite repression of isocitrate lyase and malate synthase by glucose, so these two phenomena seem not to be related.

Algae

Very few algae have been examined for the presence of glyoxylate cycle enzymes, and of these, two, which were different strains of the same organism, *Chlorella vulgaris*, showed rather different regulation.

Harrop and Kornberg (1967) studying *Chlorella vulgaris* Brannon No. 1 strain, found that isocitrate lyase was present under all conditions of growth. These included aerobic growth on ethanol in the dark, growth on glucose in the dark, and growth on carbon dioxide in the light.

There seemed to be only one isocitrate lyase in the cells. The physical and kinetic properties of the enzyme were the same no matter what the carbon source or growth conditions were. Studies with ^{14}C -1-

acetate, however, indicated that the glyoxylate cycle did not actually function when cells were growing in the dark on glucose, or in the light on carbon dioxide.

Further investigation revealed that in cells growing on acetate or ethanol where the glyoxylate cycle was functioning, the isocitrate lyase was associated with a particulate fraction of the cell. Malate synthase, which was not found to be constitutive, but to increase on acetate or ethanol, was also found associated with this fraction, as were citrate synthase and malate dehydrogenase. No special attempt was made in this study to differentiate between this particulate fraction and the mitochondrial fraction, so it is not clear if these are the same type of structures seen in *Neurospora* and germinating oil seedlings.

An earlier study using Pearsall's strain of *Chlorella vulgaris* (Syrett *et al* 1963) showed only low levels of isocitrate lyase and malate synthase under all conditions except growth on acetate in the dark. When the cells were given acetate as a sole carbon source, there was a twenty-four hour lag before cell division during which time isocitrate lyase and to a lesser extent malate synthase increased. If glucose was added as well as acetate, there was no increase in the enzymes.

Similar results were seen for *Chlamydomonas reinhardtii* (Kornberg and Harrop 1967) which showed low isocitrate lyase and malate synthase on photoautotrophically growing cultures but a preferential synthesis

of these enzymes on acetate.

Since so few algae have been studied, it is not possible to decide whether the compartmentation of the glyoxylate cycle enzymes into special organelles might be an important general control mechanism in these organisms.

Higher Plants

At the time that the glyoxylate cycle was formulated, Kornberg and Krebs (1957) recognized the important role that the cycle might have in the conversion of fats to carbohydrates, particularly during the germination of oil-rich seeds. It was known that the germination of fat containing seeds was accompanied by a decrease in fat content and an increase in carbohydrate.

Kornberg and Beevers (1957) found the enzymes of the glyoxylate cycle in cell-free extracts of germinating castor beans (*Ricinus*). There seemed to be enough activity to account for the rate of conversion of fats to carbohydrates. Beevers (1957) was also able to show that ^{14}C -acetate was very efficiently converted to sucrose in the intact castor bean cotyledon.

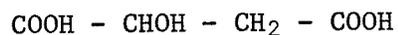
Other studies using germinating peanuts and castor beans revealed that malate synthase and isocitrate lyase showed a marked lag in appearance after germination suggesting that they are induced by a compound either synthesized or released during the early phase of germination. The appearance of the glyoxylate cycle enzymes seemed

to coincide with the period of rapid fat breakdown, however.

Bradbeer and Stumpf (1959) studied the distribution of ^{14}C -acetate in sunflower cotyledons and found that essentially no label went into glutamate. This seemed to indicate that the pathway of acetate assimilation by-passes α -ketoglutarate almost completely. This was in spite of the fact that isocitrate dehydrogenase levels were comparable to those of isocitrate lyase.

More extensive labelling experiments were carried out by Canvin and Beevers (1961) using the germinating castor bean system. These workers did labelling experiments with ^{14}C -1-acetate, ^{14}C -2-acetate, ^{14}C -1-succinate, ^{14}C -2-succinate, and ^{14}C -malate. Results confirmed that the entire acetate molecule was initially incorporated into organic acids. Malate, citrate and succinate were the earliest labelled compounds formed from either C1 or C2 labelled acetate.

Unlike Kornberg and Quayle (1958) who had found in *Pseudomonas* a labelling pattern which was consistent with the operation of both the TCA cycle and the glyoxylate cycle, Canvin and Beevers found that castor bean seedlings gave a labelling pattern which was consistent with the operation of the glyoxylate cycle alone.



50 : 50

Even after two hours, 90% of the ^{14}C -acetate label was retained in the two centre carbons.

Evidence that acetate was metabolized almost exclusively via the

glyoxylate cycle in this tissue which also contained TCA cycle enzymes led to investigations of the distributions of these two cycles in the cell.

Preliminary studies had indicated that the glyoxylate cycle enzymes seemed to sediment with mitochondria. Breidenbach and Beevers (1967) ran a crude mitochondrial fraction on sucrose density gradients and found that the glyoxylate cycle enzymes were actually associated with a distinct particulate component of higher density than mitochondria. This particle also contained citrate synthase and malate dehydrogenase, but no succinate dehydrogenase, fumarase, NADH oxidase, or α -ketoglutarate dehydrogenase. They called these structures glyoxysomes and speculated that since they contained all the enzymes for the operation of the glyoxylate cycle they were the site of the cycle *in vivo*.

The confinement of the glyoxylate cycle enzymes to a distinct organelle which lacks several of the oxidative enzymes of the TCA cycle simplifies the problem of the regulation of the flow of carbon at isocitrate. The subsequent finding by Cooper and Beevers (1969) that glyoxysomes also contain the enzymes necessary for the β -oxidation of fatty acids seems to indicate that all of the acetyl CoA generated endogenously in this tissue is channeled into the glyoxylate cycle and escapes oxidation by the mitochondria.

Since the glyoxysome does not contain succinate dehydrogenase it would appear that the succinate produced by the isocitrate cleavage

must be transported into the mitochondria before it can be oxidized to malate. Labelling patterns indicate however, that succinate is not extensively metabolized by the TCA cycle.

The evidence therefore seems to indicate that fatty seedlings have two entirely different and spatially separated pathways for handling acetyl CoA and that during a period of fat utilization virtually all the acetyl CoA is metabolized via the glyoxylate cycle. This is borne out by the fact that mature castor bean plants which are not utilizing fat do not have glyoxysomes (Gerhardt and Beevers 1970).

Hormones which affect germination in these plants also seem to have an effect on the induction of glyoxylate cycle enzymes. Gibberellic acid stimulates the induction of isocitrate lyase (Marriot and Northcote 1975). Abscisic acid which is a potent inhibitor of seed germination also delays the induction of isocitrate lyase (Marriot and Northcote 1977). Both effects can be overcome by gibberellic acid. This is compatible with an early observation that there was a lag in the appearance of glyoxylate cycle enzymes during early germination perhaps because of the requirement for the synthesis of some inducing compound (Marcus and Velasco 1960).

Protozoa

Tetrahymena pyriformis has been found to have glyoxylate cycle enzymes whose activities are affected by the composition of the medium, although they seem not to be controlled in a co-ordinate way (Hogg and Kornberg 1963).

Malate synthase was found in high levels in cells grown on proteose peptone. There was no further increase in activity when acetate was added to this medium, but glucose was able to repress enzyme levels strongly. Cells growing on acetate as a sole carbon source also had high levels of malate synthase which could be repressed by glucose.

Isocitrate lyase, on the other hand, was produced only in low amounts in cells growing on proteose peptone. Acetate, either in the presence or absence of glucose, could bring about a fifteen-to twenty-fold increase in activity.

It would appear that these enzymes may have functions in the cell apart from their role in the glyoxylate cycle and they are therefore regulated differently. No attempt was made to see if there was more than one isozyme of each of these enzymes.

In *Tetrahymena* capable of gluconeogenesis from fats, the glyoxylate cycle enzymes were found in a particulate fraction of the cell. In cells not utilizing fats, the enzymes were not concentrated in one intracellular location, but were found more in the soluble fraction.

These particles could be separated from mitochondria by sucrose density centrifugation (Müller *et al* 1968). Like glyoxysomes they had a higher density than mitochondria. As well as the glyoxylate cycle enzymes, these particles were found to contain glyoxylate oxidase, catalase, D-amino acid oxidase, and a large proportion of NADP isocitrate dehydrogenase. Essentially all of the citrate synthase, succinate dehydrogenase, fumarase, malate dehydrogenase and a large part of the

aconitase were found in the mitochondrial fraction. Because they contain catalase as do the peroxisomes of mammals, these particles from *Tetrahymena* are called peroxisomes.

The metabolic interactions between *Tetrahymena* peroxisomes and mitochondria must be more complex than those envisaged for the glyoxysomes and mitochondria of castor bean seedlings. Since the peroxisomes have no citrate synthase, and little aconitase, isocitrate for the glyoxylate cycle must be formed in the mitochondria, and diffuse into the peroxisomes, while the further lack of succinate and malate dehydrogenases means that succinate and malate must diffuse back. Since this organism can synthesize glycogen from fat there must be some further control mechanism, still undiscovered, to prevent total oxidation of the malate and succinate. The *Tetrahymena* system seems to show an interesting position between the highly specialized glyoxysomes of plants and the situation in higher animals which have no glyoxylate cycle but do have highly evolved mitochondria, and peroxisomes only in liver and kidney.

Metazoa

Caenorhabditis briggsae was perhaps the first multicellular animal in which an enzyme of the glyoxylate cycle was detected (Rothstein and Mayoh 1964). Isocitrate lyase was found in this free-living nematode, but no malate synthase. A later report confirmed the presence of malate synthase in four free-living nematodes, *Caenorhabditis briggsae*,

Panagrellus redivivus, *Rhabditis anomala* and *Turbatrix aceti* (Rothstein and Mayoh 1966). Colonna and McFadden (1975) were able to demonstrate both isocitrate lyase and malate synthase in the adult form of another free-living nematode *Caenorhabditis elegans*.

The purpose of a glyoxylate cycle in these organisms is generally unclear. These nematodes normally feed on bacteria, except for *Turbatrix aceti* which lives in the presence of high concentrations of acetic acid.

The glyoxylate cycle enzymes of *Caenorhabditis elegans* appear to be found in organelles similar to plant glyoxysomes (Pakel and McFadden 1977). These particles also contain citrate synthase, and catalase. The early larval stage of *Caenorhabditis elegans* has a greater amount of isocitrate lyase than the adult.

A correlation between glyoxylate cycle enzymes and the synthesis of carbohydrates from fats was demonstrated in the eggs of the intestinal roundworm *Ascaris lumbricoides* (Barrett *et al* 1970). Stored trehalose and glycogen were metabolized during the first ten days of development of *Ascaris* eggs. During the next fifteen days of development these carbohydrates were completely resynthesized from triglyceride fatty acids. Increases in the specific activities of the glyoxylate cycle enzymes closely paralleled increases in the rates of carbohydrate resynthesis and increases in the rates of incorporation of label from U-¹⁴C-palmitic acid into trehalose and glycogen. The developing eggs were also found to contain the key enzymes for β -oxidation of fatty acids.

No isocitrate lyase or malate synthase was detected in adult organisms.



Higher Animals

The glyoxylate cycle enzymes have never been detected in the tissues of higher animals. Shortly after the discovery of the glyoxylate cycle in microorganisms and in oil-seed plants, Madsen (1958) looked at animal tissues in which a net conversion of fats to carbohydrates might be expected to occur. The livers, hearts and kidneys of rats fed on high fat diets were examined. In no cases were either isocitrate lyase or malate synthase detected. There was no enhancement of the labelling of malate by ^{14}C -acetate if glyoxylate or isocitrate was added as would have occurred if the glyoxylate cycle were operating. The inhibition of succinate dehydrogenase by malonate prevented label from acetate appearing in malate.

The evidence all supported the conclusion that acetate was metabolized only through the TCA cycle in these cells. Madsen found that rats fed an all fat diet with no protein lost weight. It is now known that animals do not carry out the net synthesis of carbohydrates from fats. Rather, fats serve as a source of energy only. In keeping with this is the finding that the enzymes for the β -oxidation of fats are found in the mitochondria, in higher animals, the same organelles in which the TCA cycle enzymes are located (Green and Allman 1968).

The Glyoxylate Cycle in *Escherichia coli*

The most extensively studied glyoxylate cycle from the point of view

of regulation is that of the facultative anaerobe *Escherichia coli*. Kornberg *et al* (1960) demonstrated that *E. coli* W, and Crookes strain of *E. coli* utilized acetate in a manner that was consistent with the simultaneous operation of the TCA cycle and the glyoxylate cycle. Isocitrate lyase seemed to be present in the highest amount when cells were growing on acetate. Malate synthase was also elevated, but in addition it was also present in high amounts when cells were growing on glycollate, a carbon source which was known to be metabolized via the glycerate pathway.

Although acetate produced the highest levels of isocitrate lyase, there appeared to be a range of specific activities for the enzyme depending on the carbon source. Glucose gave the lowest levels and acetate the highest, and there was a wide range of activities between these (Kornberg 1963). Kornberg's data suggested that the greater the generation time, the higher the level of isocitrate lyase.

When acetate was added to cultures growing on glucose or another glycolytic intermediate, or a utilizable TCA cycle intermediate, there was no increase in the specific activities of the glyoxylate cycle enzymes. Only when proline, glutamate or γ -aminobutyrate served as the carbon or nitrogen source did acetate have a stimulatory effect on the synthesis of isocitrate lyase (Kornberg 1966).

Ashworth and Kornberg (1964) were able to isolate isocitrate lyase mutants from among acetate non-utilizers. Revertants were again able to grow on acetate and some were found to have altered heat stability

properties, thus identifying the mutants as being in the structural gene of isocitrate lyase. These mutants were found to grow quite well on glutamate and glycollate at rates comparable to the wild-type.

Vanderwinkel *et al* (1963) working with a glucose negative mutant later shown to be mutated in PEP carboxylase found that growth on acetate but not on glycollate was completely inhibited by the addition of glucose. This agreed with the observation that the activity of malate synthase was not repressed by glucose when cells were growing on glycollate.

They were able to isolate a further mutant that was resistant to glucose inhibition and could grow on glucose, although it was not a revertant to the wild-type. Both enzymes of the glyoxylate cycle were high in this mutant irrespective of the carbon source. This suggested to them that the mutant had lost the regulation of the glyoxylate cycle by glucose, leading to a simultaneous derepression of both enzymes.

The activity of malate synthase was, however, much higher on glycollate than on any other carbon source. This observation coupled with the inability of glucose to repress growth on glycollate led them to postulate the presence of two malate synthases in *E. coli*, one regulated as part of the glyoxylate cycle, and one regulated by glycollate.

Falmagne *et al* (1965) were able to separate on a DEAE-cellulose column two malate synthases from *E. coli* growing on acetate. Cells growing on glycollate showed only one of these. The two enzymes could also be differentiated on the basis of their heat stability. Malate

(10 mM) was found to protect specifically the glycollate induced malate synthase (designated G) from heat inactivation, and glycollate (10 mM) had a similar stabilizing effect on the acetate-specific malate synthase (designated A).

These properties became important when Vanderwinkel and DeVlieghere (1968) examined further the regulation of isocitrate lyase and the two malate synthases. They were able to isolate mutants of isocitrate lyase (*ace A*) as acetate non-utilizers, but they were only able to isolate a malate synthase A mutant (*ace B*) in a strain lacking malate synthase G (*gle*). This *gle* mutation was isolated as a mutant unable to grow on glycollate.

In strains with wild-type genes, 60% of the malate synthase in cells growing on acetate was malate synthase A, the remainder being malate synthase G. This would explain why *ace B* mutants fail to grow on acetate only if the strain is also *gle*. It would seem that malate synthase G is readily inducible, probably by glyoxylate, the product of isocitrate lyase.

Isocitrate lyase and malate synthase A activities were always found to be present in similar relative amounts through different growth conditions. Mapping experiments showed *ace A* and *ace B* to be closely linked in a region at 90 minutes on the *E. coli* map between *met A* and *mal B*, in the order *met A, ace B, ace A, mal B*.

The *gle* locus was not linked to *ace A* and *ace B*, but was located at 64 minutes near *ser A*.

The mapping data for the isocitrate lyase gene were confirmed by Brice and Kornberg (1968).

Regulatory Mutants

The first glyoxylate cycle constitutive mutant to be isolated was that described by Vanderwinkel *et al* (1963). It was obtained as a glucose positive PEP carboxylase (*ppc*) negative mutant as we have seen. Since then many glyoxylate cycle constitutive mutants (*icl R*) have been isolated by this and other methods. Interestingly, they have all been isolated as suppressors of other mutations.

A *ppc* mutant cannot convert glucose to oxaloacetate and thus cannot replenish TCA cycle intermediates as they are drawn off for use in various biosynthetic processes. A *ppc* mutant which is also constitutive for the glyoxylate cycle enzymes will be able to produce oxaloacetic acid from acetyl CoA and thus will regain its ability to grow on glucose alone. Therefore, the derepressed glyoxylate cycle would serve the anaplerotic function normally played by PEP carboxylase.

Vanderwinkel and DeVlieghere discovered another interesting fact about these *ppc* mutants: they are able to grow in limiting glucose in chemostat cultures. Under these conditions, there is an increase in the levels of both isocitrate lyase and quite specifically malate synthase A. Growth on limiting succinate and acetate also produces higher levels of these enzymes than is seen when these mutants grow on non-limiting concentrations of these carbon sources.

A second method of isolating constitutive mutants of the glyoxylate cycle enzymes was discovered by Kornberg (1966). Mutants in the *pps* locus, which lack the enzyme PEP synthase, cannot grow on lactate or pyruvate, but Kornberg found that a secondary mutation leading to constitutive levels of the glyoxylate cycle enzymes restored the ability of *pps* mutants to grow on lactate. Surprisingly, these secondary mutants still failed to grow on pyruvate, with or without the addition of acetate.

On lactate, these *pps* mutants, like the *ppe* mutants on glucose, would be unable to produce oxaloacetic acid for the functioning of the TCA cycle. It would appear that the constitutive glyoxylate cycle could again play an anaplerotic role, providing oxaloacetic acid and TCA cycle intermediates for biosynthesis.

It is not clear why pyruvate and pyruvate plus acetate should not serve as carbon sources in these constitutive mutants. Lactate is known to be metabolized through pyruvate and one might suppose the cell would treat them as being essentially the same. This appears not to be the case.

Brice and Kornberg (1968) mapped one of the constitutive mutants obtained in a *pps* mutant and found that it mapped in the same location as the structural genes for the glyoxylate cycle, and very close to the isocitrate lyase gene. They designated the locus *icl R*, since it was concerned with the regulation of isocitrate lyase.

The third mutation which has led to the isolation of constitutive glyoxylate cycle mutants is a rather unusual one. Vinopal and Fraenkel

(1974) found that they could isolate *icl* R mutants in strains that were mutated at *pfk* A, the locus for phosphofructokinase. These *pfk* A mutants were characterized by their impaired growth on carbon sources which entered glycolysis above fructose-6-phosphate. The degree of impairment seemed to depend on the carbon source: the growth rate on glucose-6-phosphate and lactose was reduced three- to four-fold as compared to the wild-type, but the growth rate on glucose and mannose was very much lower. Vinopal and Fraenkel were able to isolate mutants which could grow as rapidly on glucose as they did on glucose-6-phosphate. These mutants were found to be constitutive for the glyoxylate cycle enzymes. It appeared that the hexose monophosphate shunt was not necessary to produce this suppression, but that there had to be some residual activity of phosphofructokinase present. Mutants totally lacking this enzyme would not produce *icl* R mutants.

It is rather more difficult to visualize how a constitutive glyoxylate cycle could be advantageous to a *pfk* A mutant.

To try to arrive at an explanation of this phenomenon, two facts should be kept in mind: the selection procedure was carried out on glucose, and the mutant had to have some residual phosphofructokinase activity.

Glucose is transported into *E. coli* via the phosphotransferase system which requires PEP specifically as an energy source (Kundig and Roseman 1971). Neither glucose-6-phosphate nor lactose is taken up

by this system in *E. coli* and neither utilizes PEP as an energy source for transport. When growing on glucose, *E. coli* obtains its supply of PEP via glycolysis which in the case of the *pfk A* mutant is operating at a much reduced level.

Although the TCA cycle is nominally catalytic, its intermediates are constantly being tapped off as starting materials for various synthetic routes. During growth on glucose, the replenishment of the TCA cycle intermediates is by way of the PEP carboxylase reaction, a reaction which is in direct competition with the phosphotransferase system for PEP. In cells with low levels of phosphofructokinase this competition could lead to a serious reduction in the ability to take up glucose, and glucose uptake might actually be rate-limiting for growth.

When the glyoxylate cycle is induced on glucose, as in the *icl R* mutants, the replenishment of the TCA cycle intermediates can occur using acetyl CoA rather than PEP. The acetyl CoA can be derived from pyruvate, which in turn is obtained from PEP when the latter is used for glucose uptake via the phosphotransferase system. Thus in an *icl R* mutant when levels of PEP are low, all the PEP available can be used to support glucose uptake and the pyruvate produced can be used for the replenishment of the TCA cycle intermediates.

In *pfk A*, *icl R* mutants, the rate limiting step for growth is probably at phosphofructokinase, since its levels are low. This limitation would be the same for glucose or for glucose-6-phosphate or

lactose, so for *pfk A*, *icl R* mutants, the growth rate on any of the three substrates would be the same. In a wild-type cell where glycolysis is proceeding at a normal rate, a constitutive glyoxylate cycle would not be any advantage, but in a cell where the energy supply for substrate transport is low, it could make the difference between starvation and growth at a slow rate.

The *icl R* mutants obtained from *pfk A* mutants also mapped in close proximity to the *met A*, *ace A* loci.

Metabolic Regulation of the Glyoxylate Cycle in *E. coli*

Kornberg's Mutant Experiments

The high levels of the glyoxylate cycle enzymes in cells growing on acetate, and the rapid appearance of the enzymes when cells were transferred into medium containing acetate suggested that acetate or possibly acetyl CoA might be an inducer of the cycle. The finding that acetate could not induce in the presence of glucose and most TCA cycle intermediates made this less likely.

The search for the metabolic regulator of the cycle inspired Kornberg (1966) to carry out a large series of experiments using various mutants of enzymes closely related to the metabolism of acetate.

In order to clarify the role of acetate, he looked at its effect in a citrate synthase mutant, which required glutamate for growth on glucose, but could grow on glutamate alone at rates comparable to a wild-

type cell. Acetate did not stimulate isocitrate lyase in these cells growing on glutamate which it was able to do in a wild-type strain. Since this strain could still activate acetate to acetyl CoA it appeared that neither of these compounds could be acting as a direct inducer. It seemed more likely that citrate or a compound derived from it was the true inducer, or that acetate was having its inducing effect by removing a co-repressor metabolite which was close to oxaloacetic acid.

Since *E. coli* does not grow on citrate, it was not possible to test that compound directly. However, because pseudomonads and *Salmonella typhimurium* are able to grow on citrate, and produce very little isocitrate lyase under these conditions, Kornberg concluded that this was not a likely inducer in *E. coli* either. This left the possibility that acetate was able to reduce the level of a co-repressor in the cell.

Kornberg (1966) found that when a mutant of *E. coli* M, which lacked a component of the pyruvate dehydrogenase complex, was transferred from a medium containing acetate as a sole carbon source to one which contained glyoxylate as well as acetate, there was a virtual cessation of isocitrate lyase synthesis. The specific activity decreased by 50% for each generation of growth. Transfer of these cells from a medium containing glyoxylate plus acetate to acetate as a sole carbon source resulted in rapid synthesis of isocitrate lyase.

These results were in contrast to those with a citrate synthase

(*glt A*) mutant which under similar conditions showed a marked decrease in the rate of synthesis of isocitrate lyase although a small amount of synthesis was still carried on. Kornberg suggested that the compound responsible for the repression of isocitrate lyase was either pyruvate, the metabolite which could not be oxidized by the pyruvate dehydrogenase mutant, or a precursor of pyruvate which also could not be oxidized. Pyruvate was found to accumulate in the medium of the pyruvate dehydrogenase mutant during growth on glyoxylate and acetate, but not on acetate alone.

A similar mutant of *E. coli* K12 which lacked the first enzyme of the pyruvate dehydrogenase complex synthesized isocitrate lyase while growing on acetate with aspartate as a nitrogen source at a rate only slightly less than that seen on acetate alone. Since no keto-acid accumulated in the medium, it appeared that the carbon skeleton of aspartate was being utilized. This was interpreted as suggesting that a C₄ intermediate was not responsible for repression of the glyoxylate enzymes since aspartate had little effect on isocitrate lyase levels.

To test this further, Kornberg studied a PEP carboxylase mutant of *E. coli* B (*ppc*). This mutant could grow on acetate, producing high levels of isocitrate lyase, but when pyruvate was added to cells growing on acetate as sole carbon source and aspartate or glutamate as a nitrogen source there was a sharp decrease in the rate of isocitrate lyase synthesis. This repression of synthesis continued until the

pyruvate was consumed. Since a *ppc* mutant presumably cannot convert pyruvate to C₄ acids, this observation seems to eliminate C₄-intermediates of the TCA cycle from consideration as metabolic co-repressors of the glyoxylate cycle. Rather, Kornberg concluded that the co-repressor is pyruvate or some closely related metabolite such as PEP.

He was not satisfied, however, with pyruvate as the co-repressor. He felt that his experiments did not rule out a substance catabolized to pyruvate. As well, he knew that an *Achromobacter* sp., which like *E. coli* produced only low amounts of isocitrate lyase on C₄ compounds, was able to produce large amounts of this enzyme during growth on lactate, alanine or pyruvate (Rosenberger 1962). As pyruvate was unlikely to be a co-repressor in that organism and assuming that the regulatory mechanisms governing the synthesis of anaplerotic enzymes in one micro-organism would be similar to those in others, he concluded that pyruvate was probably not the co-repressor in *E. coli*.

There was however, an alternative open. Cooper and Kornberg (1967) had discovered in *E. coli* B an enzyme PEP synthase which was able to convert pyruvate and ATP to PEP and AMP and phosphate. They were able to isolate mutants which could not grow on pyruvate although they could still utilize glucose and acetate, and these mutants, at a locus designated *pps*, lacked PEP synthase. Thus, pyruvate can be metabolized very readily to PEP. When mutants lacking both PEP synthase and PEP carboxylase were grown on acetate, with either ammonium chloride or

aspartate as a nitrogen source, isocitrate lyase was synthesized at a high rate. The addition of pyruvate to such cultures essentially abolished isocitrate lyase synthesis, but only if aspartate were the nitrogen source. If the C_4 acid was omitted there was a decline in the rate of synthesis of isocitrate lyase, but synthesis still continued at a significant rate. Kornberg interpreted this as an amelioration of the pyruvate effect by the PEP synthase mutation.

Further studies with this *pps*, *ppc* mutant showed that it grew rather slowly on acetate if pyruvate were present. Mutants which could grow normally in the presence of pyruvate were isolated from this strain and they showed no repression of isocitrate lyase by pyruvate. Glucose, however, could cause the cell to stop growing, but growth resumed if glyoxylate was supplied. Levels of isocitrate lyase did not however recover. From these results, Kornberg concluded that growth in the presence of glucose or the C_4 acids presumably derived from the interaction of acetate and glyoxylate completely stopped the synthesis of isocitrate lyase and thus the repressor metabolite must be PEP or a metabolite (not pyruvate) which was closely related to it.

It was subsequently discovered by Kornberg and Smith (1967) that the growth of PEP synthase mutants on acetate plus pyruvate could select for pyruvate transport mutants which were no longer sensitive to pyruvate. This probably explains why the pyruvate-resistant mutants which Kornberg used in his experiments were no longer sensitive to pyruvate but showed an effect with glucose which can act as an endogenous source of pyruvate.

Kornberg considered that this regulation of enzyme levels by PEP was a "coarse" control. He had also done experiments which showed that pyruvate and PEP were inhibitors of the enzyme isocitrate lyase (Ashworth and Kornberg 1963). He postulated that the inhibition of isocitrate lyase *in vitro* by PEP, which was stronger than that seen for pyruvate, would serve as a "fine" control of the rate of anaplerosis achieved through the glyoxylate cycle, acting more quickly than regulation of isocitrate lyase synthesis could do.

An example of this fine control of anaplerosis could be seen, he reasoned, in the case of the mutant lacking PEP synthase and PEP carboxylase whose growth on acetate was stopped by the addition of glucose, but restored if glyoxylate was also added. The fact that glyoxylate relieved the glucose effect suggested that glucose acted by reducing the flow of carbon through the glyoxylate cycle, and Kornberg attributed this to an accumulation of PEP from glucose metabolism, which inhibited isocitrate lyase.

At the time Kornberg was formulating these ideas on the regulation of the glyoxylate cycle in *E. coli*, the phosphotransferase system which is responsible for the transport of glucose and some other sugars into *E. coli* had not been discovered. We now know because of the work of Roseman (1969) and others that PEP is the high energy compound required in glucose transport. For every molecule of glucose transported, one molecule of PEP is hydrolyzed to pyruvate. Thus, although cells growing on glucose have the lowest level of glyoxylate cycle enzymes, it would seem unlikely that the levels of PEP in these cells would be

unusually high.

Measurement of Metabolic Intermediates in *E. coli*

This suspicion was confirmed when Lowry *et al* (1971) examined the levels of glycolytic and TCA cycle intermediates in *E. coli*, under conditions of logarithmic growth on a variety of carbon and nitrogen sources. They found that intracellular PEP levels were actually half as high on glucose as they were in acetate. On all carbon sources, when glucose was added to the medium there was a drop in PEP levels. They tentatively ascribed this to an activation of pyruvate kinase by fructose-1,6-diphosphate, but it is more likely due to the fact that PEP is consumed during glucose transport.

These workers also looked at the changes in the levels of metabolites very early after glucose was added to cells growing on acetate. They tested two strains, a wild-type, and a mutant of Kornberg's which as *pps*, *ppc* and *icl R*, similar to the mutant discussed above but also constitutive for the glyoxylate cycle.

During the first few minutes the changes in the levels of the metabolites were the same for both the mutant and the wild-type. PEP levels did not increase in either case, and pyruvate levels changed only slightly over the thirty minutes of the experiment. In both the strains there was a decrease in levels of isocitrate. Initially there were increases in glutamate levels for both strains, but within ten minutes in the mutant there was a decline in the intermediates of the TCA cycle. After thirty minutes on glucose the mutant had stopped

growing and the levels of malate and isocitrate were undetectable. They interpreted the decline in isocitrate and the increase in glutamate as an activation of the isocitrate dehydrogenase when glucose is added.

The results of Lowry *et al* contradict Kornberg's hypothesis that PEP is either a "coarse" or "fine" control of the glyoxylate cycle. PEP levels are highest on the carbon source (acetate) which has the highest levels of the enzymes. Lowry *et al* point out that neither the PEP nor the pyruvate levels appear to be high enough to inhibit the activity of isocitrate lyase. The *pps*, *ppe* mutant seems to stop growing not because there is an inhibition of the glyoxylate cycle, but because there is an activation of isocitrate dehydrogenase, which leads to an exhaustion of TCA cycle intermediates by way of the decarboxylating steps of the TCA cycle.

Although Lowry's measurements of PEP levels show that this compound cannot be the co-repressor of glyoxylate cycle enzymes in *E. coli* Kornberg's less preferred metabolite, pyruvate, is still a possibility as the regulating metabolite in *E. coli*. Lowry *et al* did not do extensive pyruvate determinations in cells grown on different carbon sources but those they did showed that carbon sources such as acetate, which give high levels of glyoxylate cycle enzymes, have low levels of pyruvate (0.03 μ moles/g dry weight) while glucose with the lowest levels of these enzymes gives highest levels (0.9 μ moles/g dry weight) among the carbon sources tested. Glycerol (0.4 μ moles/g dry weight) and succinate (0.22 μ moles/g dry weight) which give

intermediate levels of isocitrate lyase showed higher levels of pyruvate than acetate, but lower levels than glucose.

Pyruvate as a Regulatory Metabolite

Henning and his coworkers who have done extensive studies on the regulation of pyruvate dehydrogenase in *E. coli* have postulated that pyruvate is the regulating metabolite for both pyruvate dehydrogenase and the glyoxylate cycle. The synthesis of the enzymes of the pyruvate dehydrogenase complex appears to be regulated, the amount of complex produced varying by a factor of ten depending on the carbon source. Levels of the complex are highest on pyruvate and lowest on acetate.

To test the effect of pyruvate on the synthesis of the pyruvate dehydrogenase complex, Dietrich and Henning (1970) constructed a strain which was mutated in PEP synthase and in the dihydrolipoamide transacetylase (*ace F*) component of the complex. This strain was still able to synthesize the pyruvate dehydrogenase component in an uncomplexed but still active form. In order to observe a range of induced enzyme synthesis as large as possible they wanted to add pyruvate to cells growing on acetate, the carbon source which had the lowest level of enzyme complex. Since a *pps*, *ace F* double mutant growing on acetate was inhibited by pyruvate, they made the strain constitutive for the glyoxylate cycle. Pyruvate no longer had an inhibitory effect on this strain growing in acetate.

Pyruvate addition had an immediate and marked inducing effect on the synthesis of the pyruvate dehydrogenase component of the complex. α -ketobutyrate showed the same inducing effect on the enzyme, but higher chain ketoacids did not.

Further experiments were done with a thiamine requiring strain starved for thiamine on various carbon sources. Any enzyme complex produced under such conditions would be completely inactive because of the absence of thiamine pyrophosphate. Extracts of these cells grown on glucose could be assayed for pyruvate dehydrogenase complex after the addition of thiamine pyrophosphate and proved to contain even higher levels of the complex than did pyruvate-grown cells. Pyruvate was excreted into the medium under these growth conditions. There was very little difference seen in the levels of the enzyme complex in cells grown on acetate with and without thiamine.

Dietrich and Henning interpreted these experiments as showing that pyruvate was the inducer of the pyruvate dehydrogenase complex. Pyruvate, without further metabolism, appeared to have an inducing effect, even in the presence of a carbon source like acetate. This, they said, excluded the alternate explanation that the enzyme complex synthesized a repressor metabolite.

In view of the reverse correlation between the induction of the pyruvate dehydrogenase complex and that of the glyoxylate cycle, they postulated that pyruvate was the inducer of the former complex and the repressor of the latter. They felt that some of Kornberg's data

pointed to pyruvate, as much as to PEP, as the repressor metabolite for the glyoxylate cycle and questioned the validity of his arguments based upon evidence obtained from different genera.

Flatgaard *et al.* (1971) were able to isolate constitutive mutants of the pyruvate dehydrogenase complex. Taking advantage of the fact that PEP synthase mutants growing on acetate are very sensitive to small amounts of lactate or pyruvate, they isolated cells able to grow on acetate medium plus 15 μ M lactate. Several of these lactate resistant mutants were found to be constitutive for the enzymes of the pyruvate dehydrogenase complex.

Such constitutive mutants were also found to have somewhat elevated levels of isocitrate lyase on glucose, succinate and even acetate. However, the levels of isocitrate lyase in a strain which was lacking the pyruvate dehydrogenase component but produced the active transacetylase constitutively were the same for all carbon sources as those for an inducible strain. Flatgaard *et al.* argued that in a constitutive pyruvate dehydrogenase strain, the levels of pyruvate in the cell would be lower, and that this would lead to elevated levels of the glyoxylate cycle enzymes if pyruvate was the co-repressor. In a mutant that was constitutive for the transacetylase component of the complex, but mutated in the pyruvate dehydrogenase component, pyruvate could not be metabolized, levels would be higher, and this would explain the lower levels of isocitrate lyase in these cells. This also implied that the regulatory gene was not the same for

pyruvate dehydrogenase and the glyoxylate cycle enzymes. Mapping experiments show that the regulatory element responsible for the control of the PDH complex, unlike that for the glyoxylate cycle, maps very close to the *ace E* gene which specifies pyruvate dehydrogenase.

The Role of the TCA Cycle in Acetate Metabolism

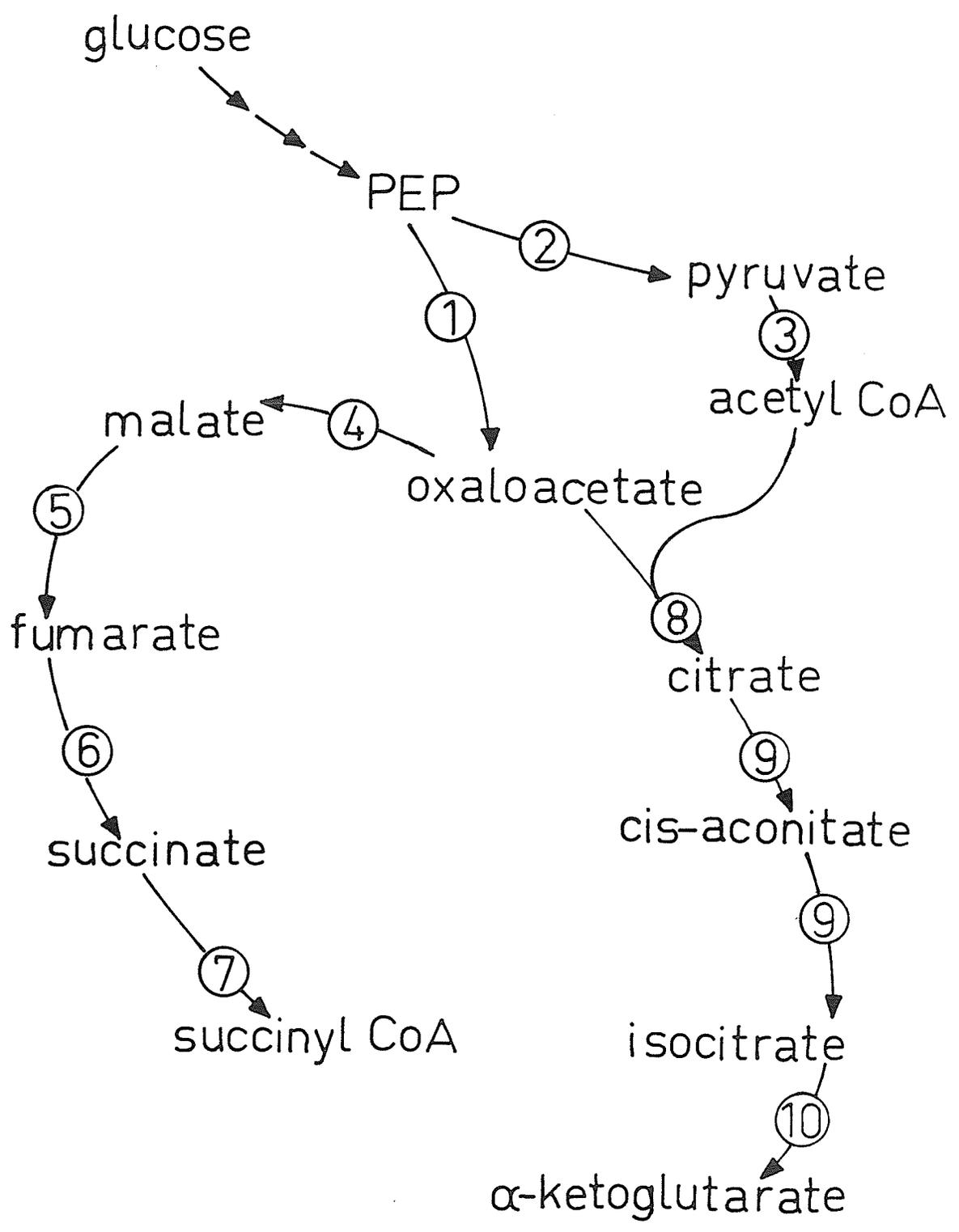
Under aerobic conditions, succinate is formed via the TCA cycle by the oxidation of α -ketoglutarate. Various organisms are able to make it anaerobically however, by way of the reduction of fumarate. It has been shown that under anaerobic conditions, *E. coli* forms an inducible fumarate reductase which is distinct from succinate dehydrogenase. Mutants lacking this enzyme can no longer grow anaerobically on glycerol plus fumarate, but can still grow aerobically on succinate (Hirsch *et al* 1963).

It appears that while the TCA cycle enzymes do form a true cycle under aerobic conditions, under anaerobic conditions they provide a branched non-cyclic pathway instead (Figure 3).

The right branch is an oxidative pathway leading to α -ketoglutarate for biosynthetic purposes, and the left branch is a reductive pathway leading to succinate. α -Ketoglutarate dehydrogenase provides the connection between the two branches, but does not operate under

Figure 3. The TCA cycle as it operates in *Escherichia coli* growing on glucose. The enzymes are numbered as follows: 1. PEP carboxylase; pyruvate kinase and PEP-dependent phosphotransferase system; 3. pyruvate dehydrogenase; 4. malate dehydrogenase; 5. fumarase; 6. fumarate reductase; 7. succinyl CoA synthase; 8. citrate synthase; 9. aconitase; 10. isocitrate dehydrogenase.

THE TCA CYCLE ON GLUCOSE



anaerobic conditions and seems not to be synthesized.

Enteric bacteria such as *E. coli* appear to live largely by anaerobic glycolysis even when they are growing aerobically on glucose. Glucose has been found to repress such crucial TCA cycle enzymes as succinate dehydrogenase, succinic thiokinase and the cytochromes. Amarsingham and Davis (1965) also found that α -ketoglutarate dehydrogenase was not present in cells growing aerobically in glucose if they were harvested during exponential growth. α -Ketoglutarate dehydrogenase was produced in cells growing on glucose only after substantial metabolism of the glucose had occurred. A similar result was seen for lactate, but α -ketoglutarate dehydrogenase appeared more rapidly after growth on this carbon source. They postulated that the rise in levels of α -ketoglutarate dehydrogenase was related to the accumulation of one or more metabolites in the medium.

As acetate was known to accumulate in the medium of cells growing on glucose (Britten 1954) Amarsingham and Davis tested the effect of acetate on the levels of α -ketoglutarate dehydrogenase. They found that 2% acetate added to glucose medium at pH 7.0 was effective in inducing the enzyme at an early phase of growth. At pH 6.0 0.2% acetate in medium was equally as effective. Presumably this means that acetate is taken up as the neutral acetic acid rather than as the anion. Their results also showed that cells growing on acetate alone contained high levels of α -ketoglutarate dehydrogenase.

High levels of the enzyme were also found in cells growing on

glutamate as sole carbon source. In the presence of glucose, glutamate and α -ketoglutarate were actually more effective inducers than was acetate. Amarsingham and Davis reasoned that since α -ketoglutarate must be metabolized through the dehydrogenase before it could produce acetate, acetate was probably not required *per se* for the induction. They argued that although acetate would be of great importance as a nutritional inducer of α -ketoglutarate dehydrogenase, because it is a natural product of glucose metabolism in *E. coli*, it might be acting indirectly by causing the accumulation of the direct physiological inducer, α -ketoglutarate.

Unlike the induction of the glyoxylate cycle enzymes on acetate, the adaptation of acetate terminal respiration as measured by α -ketoglutarate dehydrogenase seems to show only a weak repression by glucose and glycolytic intermediates. Although under aerobic conditions this enzyme is repressed by glucose and lactate this repression is easily overcome by sufficient inducer. It is known that *E. coli* growing on a limiting supply of glucose exhibits a gradual fall in growth rate as it approaches stationary phase. There is no *diauxie* or abrupt change in growth rate. This observation indicates that acetate can begin to be utilized via the TCA cycle before growth slows to the rate observed on acetate as sole carbon source. Early experiments of Kornberg *et al*, already cited, indicated that *E. coli* growing on acetate as sole carbon source utilized both the TCA cycle and the glyoxylate cycle.

The Regulation of Isocitrate Dehydrogenase

Upon exhaustion of glucose, *E. coli* cultures can metabolize acetate through the TCA cycle, but they cannot begin to grow on acetate until the glyoxylate cycle has been induced. Once the glyoxylate cycle is also operating, a competition will develop between the two cycles, at the branch point of isocitrate, the substrate for both isocitrate lyase and isocitrate dehydrogenase.

Holms and Bennett (1971) investigated the levels of isocitrate dehydrogenase in *E. coli* during and after its growth on limiting glucose, and also on limiting glycerol, as a carbon source on which cells do not accumulate acetate, though they contain levels of isocitrate dehydrogenase comparable to glucose.

They found that during growth on glucose acetate accumulated in the medium. Shortly after cessation of growth on glucose acetate began to disappear from the medium and the activity of isocitrate dehydrogenase fell until, after about two hours, 80% of the activity had been lost. When acetate was exhausted the activity rose again and after four hours was at 75% of its original level. Throughout the same period, malate dehydrogenase and α -ketoglutarate dehydrogenase levels remained constant. The glycerol grown cells showed no acetate accumulation or changes in the levels of any of the enzymes after growth ceased. If acetate was added to the medium after glycerol exhaustion, isocitrate dehydrogenase activity fell and then partially recovered.

The activity of isocitrate dehydrogenase decreased and subsequently recovered after growth on glucose-6-phosphate, mannitol or pyruvate. All of these substrates should be metabolized to give acetate. No loss in activity was seen after growth on galactose, fructose, α -ketoglutarate, succinate or malate unless 1 mM acetate was added.

The loss of isocitrate dehydrogenase activity could be prevented after growth on glucose if malate, succinate, fumarate or oxaloacetate were added. In these cases, growth resumed and when it stopped there was no loss of activity. Pyruvate could also prevent activity loss, but when growth stopped for the second time, isocitrate dehydrogenase activity declined. α -Ketoglutarate was not able to prevent loss of the enzyme activity, but activity recovered more rapidly. They suggested that this negative result might have been due to the presumed inability of α -ketoglutarate to penetrate the cell quickly.

If chloramphenicol was added simultaneously with acetate after growth on glucose had ceased there was no loss of isocitrate dehydrogenase activity or increase in isocitrate lyase. If acetate was added to cells which had recovered after the initial acetate adaptation, chloramphenicol did not prevent the loss of isocitrate dehydrogenase activity. This suggested that *de novo* protein synthesis was not required for the inactivation.

Pyruvate at 1 mM added to cells which were adapted to growing on acetate, produced an immediate three- to four-fold increase in isocitrate dehydrogenase activity. Chloramphenicol added simultaneously with the

pyruvate did not prevent this activation, confirming that the inactivation-activation of isocitrate dehydrogenase did not require *de novo* protein synthesis (Bennett and Holms 1975).

Similar activations were seen for glucose, malate and succinate, but these were inhibited by chloramphenicol. Bennett and Holms speculated that this might have been due to transport difficulties, since cells no longer contained the transport systems for these carbon sources after extended growth on acetate.

They investigated the isocitrate dehydrogenases isolated under conditions of maximal, minimal and recovered activity for heat sensitivity, optimum pH, and substrate and cofactor requirements. They found no differences among the extracts, and thus concluded that they were not looking at different forms of the enzyme. Although there was some evidence for three electrophoretically distinct NADP isozymes of isocitrate dehydrogenase in *E. coli* (Reeves *et al* 1968), Helling and Kukora (1971) have isolated mutants of *E. coli* which are lacking NADP-isocitrate dehydrogenase and will not grow on glucose or acetate without a glutamate or proline supplement. This suggests that there is only one enzyme.

Bennett and Holms concluded that as long as the glyoxylate cycle was operating isocitrate dehydrogenase was maintained in a partially inactive state, and that in cells with an active glyoxylate cycle the activity of this enzyme reflected the availability of acetate. The mechanism by which isocitrate dehydrogenase activity was regulated did

not involve *de novo* synthesis nor did it appear to involve a low molecular weight effector. Dialysis of inactive enzyme preparations did not restore activity. They suggested that the mechanism might be a covalent modification such as has been described for mammalian glycogen phosphorylase (Brown and Cori 1961), and *E. coli* glutamine synthase (Shapiro and Stadtman 1971).

Although Bennett and Holms found this inactivation-activation phenomenon in *E. coli* strains B, B/r, C, Crookes and ML 308 as well as *Salmonella typhimurium*, *Klebsiella aerogenes* and *Serratia marcescens*, there were unable to demonstrate it in two *E. coli* K12 strains they tested.

The inactivation-activation is not missing in all K12 strains, however, for Garnak and Reeves (1979) have presented evidence for the partial inactivation of isocitrate dehydrogenase in a K12 strain. Furthermore, they have presented experiments which suggest that a covalent modification, possibly a phosphorylation, is indeed involved in the inactivation.

Garnak and Reeves grew cells on limiting glycerol, and when the carbon source was exhausted they added rifampicin to prevent further RNA synthesis. After ten minutes ^{32}P was added and after a further five minutes acetate was added to one of the cultures. The culture containing acetate showed rapid inactivation of isocitrate dehydrogenase. Autoradiography of polyacrylamide slab gels run with extracts of the various cultures showed a band of radioactivity which corresponded to isocitrate dehydrogenase, only for the culture which had acetate added.

Treatment of the extract with proteinase K destroyed this band, but ribonuclease and deoxyribonuclease had no effect on it. They concluded that under the conditions of the experiment, isocitrate dehydrogenase was the major protein phosphorylated. The phosphorylated protein co-chromatographed on a Sephadex G150 column with isocitrate dehydrogenase. The pH stability of the phosphorylated isocitrate dehydrogenase was apparently characteristic of a phosphoserine or a phosphothreonine - stable at low or neutral pH, unstable at high pH.

One interesting difference between the experiments of Garnak and Reeves and those of Bennett and Holms was that Bennett and Holms found no inactivation of isocitrate dehydrogenase if the cells were prevented from adapting to acetate (inducing the glyoxylate cycle enzymes) by chloramphenicol. Once cells had adapted to acetate, chloramphenicol no longer had an effect. Garnak and Reeves, however, added rifampicin fifteen minutes before they added acetate. Although they did not assay for isocitrate lyase, if the rifampicin was having an effect it would seem unlikely that the glyoxylate cycle enzymes would be induced. These conflicting results leave in some doubt whether acetate *per se* is able to bring about the partial inactivation of isocitrate dehydrogenase, or whether acetate must be metabolized via the glyoxylate cycle to have its effect. It has been argued by Bennett and Holms, and by Garnak and Reeves, that the partial inactivation of isocitrate dehydrogenase in cells which are growing on acetate is a useful mechanism whereby isocitrate can be channeled into the glyoxylate cycle instead of the TCA cycle. It is interesting that the inactivation is partial,

because isocitrate must still be metabolized via isocitrate dehydrogenase in order to supply glutamate for biosynthesis. The mechanism also seems to allow for a very rapid restoration of the full capacity of the TCA cycle when nutritional conditions change.

Bennett and Holms also confirmed that the NADP-specific isocitrate dehydrogenase was strongly inhibited by a mixture of 3 mM glyoxylate plus 3 mM oxaloacetate. This had been observed by other workers (Marr and Weber 1969) and had been postulated as the reason for low levels of isocitrate dehydrogenase in cells utilizing the glyoxylate cycle. This inhibition seems to have no relationship to the inactivation-activation mechanism, as it could be overcome by increasing the concentration of isocitrate, or by dialysis of the extract. Whether this inhibition phenomenon has a significance *in vivo* is not known.

Cyclic AMP as a Regulator of Aerobic Metabolism

The repressive effect of glucose on several enzymes of the TCA cycle and on cytochromes has been linked in some cases to the low levels of cyclic AMP present in glucose-grown cells. Cyclic AMP has been known for some time to be required for the synthesis of certain inducible catabolite systems in *E. coli* such as those needed for the metabolism of lactose, galactose, maltose and arabinose to name only a few. Perlman and Pastan (1969) isolated a mutant which exhibited a

pleiotropic deficiency in the utilization of these carbon sources unless cyclic AMP was added to the medium. This mutant was found to be deficient in adenylate cyclase, and although it could grow on glucose, it did so slowly compared to the wild-type.

Broman *et al* (1974) found that an adenylate cyclase mutant which grew more slowly on glucose had reduced levels of cytochrome b_1 and cytochrome oxidase. The addition of exogenous cyclic AMP restored growth on glucose to a normal rate and at the same time stimulated cytochrome synthesis. They concluded that cyclic AMP had some role in regulating the levels of cytochromes in the *E. coli* membrane. These workers also found that wild-type *E. coli* growing on succinate and acetate contained higher levels of cytochromes than cells growing on glucose glycerol or mannitol. This finding is in keeping with the fact that these carbon sources are metabolized aerobically through the TCA cycle rather than through glycolysis as the latter substrates are. The addition of 2.5 mM cyclic AMP to cells growing on succinate and acetate did not appreciably increase the levels of cytochromes in wild-type cells, a fact that suggests that cytochrome levels are already fully induced on these carbon sources.

Hempfling (1970) showed that the efficiency of oxidative phosphorylation in glucose-grown *E. coli* B cells was less than ten percent of that found in organisms growing on non-carbohydrate medium. Later work (Hempfling and Beeman 1971) showed that the repressive effect of glucose on oxidative phosphorylation could be overcome if 2.5 - 5 mM cyclic AMP was added to the medium.

The effect of cyclic AMP on the synthesis of succinate dehydrogenase and isocitrate lyase, both of which are known to be repressed on glucose, was investigated by Takahashi (1975). He grew *E. coli* K12 on buffered nutrient broth with and without glucose.

The repressed respiration of cells grown in the presence of 2% glucose was derepressed, as measured by oxygen uptake, when cells were incubated in a buffer containing Casamino acids. The glucose repressed cells were deficient in succinate dehydrogenase and isocitrate lyase, but both these activities increased on incubation in the buffer plus Casamino acids. If glucose was added, there was no increase in either activity, but the succinate dehydrogenase activity did increase somewhat if cyclic AMP were also added. Cyclic AMP had no effect on isocitrate lyase activity in the presence of glucose. The addition of chloramphenicol or puromycin prevented succinate dehydrogenase recovery from glucose repression.

Takahashi (1975) interpreted these results to mean that cyclic AMP had no role in the repression of isocitrate lyase synthesis by glucose, although it was involved in the glucose repression of succinate dehydrogenase.

Gluconeogenesis from Acetate as Sole Carbon Source

Davis (1961) coined the term "amphibolic" to describe systems such as the TCA cycle which are used both for catabolic energy yielding

processes and for anabolic biosynthetic purposes. Three factors would appear to influence the biosynthesis of the enzymes of the TCA cycle: the presence or absence of molecular oxygen, the repressive effect of glucose, and the balance between the catabolic and anabolic demands of the cycle which are dictated by the carbon source.

Gray *et al* (1966) have suggested that the TCA cycle can be divided into three sectors which are under individual control: enzymes metabolizing the tricarboxylic acids, those metabolizing the C₅-dicarboxylic acids, and those metabolizing the C₄-dicarboxylic acids.

When glutamate must be synthesized the enzymes such as isocitrate dehydrogenase which lead to its formation are elevated even in the presence of glucose. Only α -ketoglutarate dehydrogenase, whose substrate is required for the synthesis of glutamate, is repressed.

Experiments have shown that when cells adapt to growing on acetate, there are changes not only in the levels of the glyoxylate cycle enzymes but also in the functioning of the TCA cycle itself. Some changes, such as the increase in the level of α -ketoglutarate dehydrogenase, seem to occur gradually, in a smooth transition as cells change from growth exclusively on glucose to growth exclusively on acetate. Other changes, such as the inactivation of isocitrate dehydrogenase, occur rather abruptly and coincide with the actual adaptation of cells to acetate as the sole carbon source. Citrate synthase has also been shown to rise in cells growing exclusively on acetate (Gray *et al* 1966). Thus when cells grow on acetate as a sole source of carbon and energy, they do so by means of a carefully regulated balance between the TCA

cycle and the glyoxylate cycle.

When *E. coli* grows on a C₄-dicarboxylic acid, and by extension on acetate which is metabolized via the anaplerotic glyoxylate cycle to produce C₄ acids, it must have a means of producing PEP which is required for gluconeogenesis. PEP carboxykinase had generally been considered to be the enzyme responsible for the net synthesis of PEP during growth on C₄ carbon sources (Hsie and Rickenberg 1966). This enzyme was extensively studied by Utter and Kurahashi (1954) and was shown to decarboxylate oxaloacetic acid to PEP in the presence of ATP.

Hansen and Juni (1974) found, however, that a mutant lacking PEP carboxykinase could still grow slowly on C₄ acids, but that a double mutant lacking both PEP carboxykinase and PEP synthase was unable to grow on these carbon sources.

Starting with a PEP synthase mutant which could not grow on lactate or pyruvate, they isolated the further double mutant by selecting for cells which were unable to grow on malate plus acetate, as well as being pyruvate negative. They obtained two types of revertants from these double mutants. One, which was able to grow on malate, but not lactate, had regained only PEP carboxykinase activity, and the other, which could grow on both malate and pyruvate, had regained PEP synthase activity. It was this finding that strongly suggested to Hansen and Juni that one route to gluconeogenesis was via pyruvate.

PEP synthase had been discovered in *E. coli* K12 by Cooper and Kornberg (1967) as an enzyme which could reverse the pyruvate kinase

reaction by splitting two energy rich bonds of ATP to form PEP. It had long been recognized on energetic grounds that the reaction catalyzed by pyruvate kinase was not likely to be reversible *in vivo* and this left open the question of how microorganisms could grow on lactate, pyruvate or alanine as sole carbon source. With the discovery of PEP synthase, and a mutant lacking this enzyme which was unable to grow on lactate or pyruvate (Cooper and Kornberg 1967), it appeared that this was the key enzyme in the metabolism of C₃ compounds.

The work of Hansen and Juni however, indicated that PEP synthase might also play a role in gluconeogenesis when cells were growing on C₄ carbon sources. *E. coli* was known to have both the NAD and NADP-specific malic enzymes which could catalyze the decarboxylation of malate to pyruvate (Katsuki *et al* 1967). Together, these enzymes would constitute a route from C₄ dicarboxylic acids to PEP.

In an attempt to decide whether both malic enzymes have a role in PEP synthesis, Hansen and Juni (1975) isolated mutants in both the NAD and NADP-specific malic enzymes. Starting with a PEP carboxykinase mutant they isolated a mutant which grew slowly on malate plus acetate, but still contained PEP synthase. This mutant was found to be lacking the NAD-specific malic enzyme, although it still contained NADP-specific malic enzyme. Some spontaneous revertants of this strain, which could now grow on malate, contained elevated levels of the NADP-malic enzyme. A further mutant was isolated which again could grow only slowly on malate. This strain was found to lack PEP carboxykinase and both NAD- and NADP-malic enzymes. They postulated that the enzymatic mechanism which enabled these

mutants to grow slowly on malate might be due to leakiness in one or more of the mutations, or to the presence of low levels of oxaloacetic decarboxylase which although never reported in *E. coli* has been found in the closely related genus *Salmonella* (Rosenberger 1966).

From these results Hansen and Juni concluded that it was probably the NAD -malic enzyme which played a role in gluconeogenesis. Even in the presence of normal levels of NADP -malic enzyme cells were able to grow only very slowly on malate, probably for one of the above reasons, and a mutant in both malic enzymes showed similar growth rates on malate. They postulated that the main role of the NADP -malic enzyme was to provide NADPH for biosynthetic purposes when cells were growing on C₄ dicarboxylic acids.

It therefore would appear that when *E. coli* is growing on C₄-dicarboxylic acids, or on acetate which generates these acids via the glyoxylate cycle, it uses two routes to produce PEP for gluconeogenesis. One involves the ATP-dependent decarboxylation of oxaloacetic acid by PEP carboxykinase, and the other the sequential action of NAD -malic enzyme and PEP synthase.

Goldie and Sanwal (1980) also produced a double mutant of PEP carboxykinase and PEP synthase. This mutant showed impaired growth on succinate, malate, fumarate, acetate and pyruvate, but not on glucose or glycerol. PEP carboxykinase levels were very similar in wild-type cells growing on any of succinate, malate, glycerol, α -ketoglutarate or acetate as sole carbon source. Glucose had a

repressive effect on the levels of PEP carboxykinase, but 5 mM cyclic AMP was able to overcome this effect. A strain which was deleted for adenylate cyclase and the cyclic AMP receptor protein had lower levels of PEP carboxykinase on all carbon sources, and added cyclic AMP had no effect on the levels. They therefore concluded that PEP carboxykinase synthesis is regulated at the transcription level by cyclic AMP and its receptor protein.

Murai *et al* (1972) have found that the NAD⁺-malic enzyme is repressed by growth on glucose and induced by growth on malate. Addition of glucose to cells growing on malate caused a suppression of NAD⁺-malic enzyme synthesis, but this could be overcome by the addition of greater amounts of malate.

The NADP⁺-malic enzyme level was repressed not only by glucose, but to lesser extents by glycerol, lactate and acetate in that order. A combination of pyruvate and acetate had a marked repressive effect on the NADP⁺ malic enzyme.

The repressive effect of acetate suggests that the NADP⁺-malic enzyme plays no role in gluconeogenesis from this carbon source. The effects of cyclic AMP on the synthesis of the two malic enzymes appear not to have been investigated as yet.

In vitro experiments on purified preparations of the two malic enzymes showed that the NADP⁺-specific enzyme could be inhibited by acetyl CoA, oxaloacetic acid, NADPH, NADH and cyclic AMP (Sanwal and Smando 1969) and that the NAD⁺-specific enzyme was inhibited by ATP and coenzyme A, and activated by aspartate (Sanwal 1970).

CHAPTER 2

METHODS

METHODS

E. coli K12 Strains

All the strains used are listed on pages 84 - 88 with their relevant genotypes.

MediaMedium A

This is the minimal salts medium of Davis and Mingioli (1950) with the citrate omitted. Four litres of 20X medium A contained of K_2HPO_4 (840 g), KH_2PO_4 (360 g), $(NH_4)_2SO_4$ (120 g) and $MgSO_4$ (4 g). It was supplemented with amino acids and other growth factors as required.

Carbon sources were kept as sterile 20% solutions, (w/v) which were added to a final concentration of 0.4% or 1% as specified.

Amino acids were kept as sterile 1% solutions and added to a final concentration of 100 μ g/ml.

Thiamine - HCl was kept as a sterile 1% solution and added to a final concentration of 10 μ g/ml. This was added routinely to all cultures.

When required, streptomycin was added at 2 ml per litre of a stock solution prepared by dissolving 1 g of streptomycin sulfate in 17.5 ml of sterile water.

When medium A was used for making plates, the salt solution and the agar (1.5%) were autoclaved separately.

Medium A with no supplements was used for washing cells.

The following media are as described by Miller (1972).

Enriched Medium A

This medium consisted of medium A supplemented per 100 ml with 0.5 ml 1% thiamine -HCl, 2 ml 20% glucose, 0.1 ml 20% MgSO₄ and 2 ml LB broth.

LB Medium

This medium consisted of 1% Bacto-Tryptone, 0.5% yeast extract, 1% NaCl, and 0.1 ml per 100 ml of 1N NaOH.

LB agar was LB medium solidified with 1.5% agar.

LB top agar for phage plates was LB medium supplemented with 0.7% agar. After autoclaving, 0.2 ml of sterile 1 M CaCl₂ and 0.5 ml of sterile 20% glucose were added.

Tetrazolium Plates (TTC)

This medium was made by using either 25.5 g per litre of Difco Antibiotic Medium No. 2 or 23 g of Difco nutrient agar and 1 g of NaCl per litre. To this was added 50 mg per litre of 2,3,5-triphenyl-tetrazolium chloride (TTC) before autoclaving.

After autoclaving 50 ml of a sterile 20% solution of the appropriate sugar was added.

Growth of Cultures

For genetic experiments and routine growth of cultures, cells were grown in 5 ml of medium in large screw cap tubes in a Dubnoff shaking water bath at 37°C.

Larger volumes of cells were grown on a gyratory shaker at 37°C. Routinely, 100 ml of medium in a 500 ml flask or 500 ml of medium in a 2 litre flask were used to ensure good aeration.

Storage of Cultures

Cultures were stored in 40% glycerol at -15°C. Usually 1 ml of a fresh LB culture was mixed with 1 ml of 80% sterile glycerol in a 1-dram glass vial with a rubber-lined plastic screw cap (Wheaton Glassware).

F' cultures were grown up in minimal medium A with appropriate supplements and stored as above.

Cultures were recovered from storage by inoculating a few drops of the glycerol culture into fresh medium.

New strains, or cultures recovered from storage were always checked for the expected phenotype before use. This was usually done by replica plating onto a series of plates with and without the required growth supplements.

E. coli K12 Strain List

All strains listed are *E. coli* K12. Many strains were obtained from the *E. coli* Genetic Stock Center (CGSC) at Yale University, New Haven, Connecticut. The original strain designation and reference are given for these strains, as well as the CGSC number. Strains will be referred to in the text by the CGSC designation.

The three letter code to denote genotype is according to the convention of Demerec *et al* (1966). Most of the loci listed can be found on the latest *E. coli* K12 linkage map (Bachmann and Low 1980).

"P₁." before a strain designation signifies that a P₁ stock of the given strain was used as a donor in a transduction with the given recipient, selecting for the phenotype indicated.

| <u>Strain</u> | | <u>Genotype</u> | <u>Source</u> |
|---------------|-------------|-----------------|-------------------------------------|
| CSH60 | Hfr (Ra-2) | <i>icl</i> R-1 | Cold Spring Harbor (Miller 1971) |
| CSH78 | Hfr (KL209) | wild-type | Cold Spring Harbor (Miller 1971) |
| MLD 1 | Hfr | <i>pps</i> | EMS mutagenesis of CSH 60 |
| MLD 2 | Hfr | <i>pps</i> | EMS mutagenesis of CSH 78 |
| MLD 3 | Hfr | <i>pps</i> | EMS mutagenesis of CSH 78 |

| <u>Strain</u> | <u>Genotype</u> | <u>Source</u> |
|------------------------|---|---|
| MLD 4 | Hfr <i>pps</i> | EMS mutagenesis of CSH 78 |
| MLD 5 | Hfr <i>pps, icl R-2</i> | ICR 191 mutagenesis of MLD 1 |
| MLD 6 | Hfr <i>pps, icl R-3</i> | ICR 191 mutagenesis of MLD 1 |
| MLD 7 | Hfr <i>pps, icl R-4</i> | ICR 191 mutagenesis of MLD 2 |
| MLD 8 | Hfr <i>pps, icl R-5</i> | ICR 191 mutagenesis of MLD 3 |
| MLD 9 | Hfr <i>pps, icl R-6</i> | ICR 191 mutagenesis of MLD 3 |
| MLD 10 | Hfr <i>pps, icl R-8</i> | ICR 191 mutagenesis of MLD 3 |
| MLD 11 | Hfr <i>pps, icl R-9</i> | EMS mutagenesis of MLD 4 |
| CGSC 5859 (JRG 548) | F ⁻ <i>sdh, chl, trp A, trp R, icl R-7gal, rps L</i> | Creaghan and Guest (1972) |
| CGSC 1927 (AB 1927) | Hfr (KL14) <i>met A, arg H, pur F, xyl, sup E</i> | G. Eggertsson |
| CGSC 5236 (DV21A05) | Hfr (DV21) <i>ace B, ppc, glc, thi, rel A, lac Z</i> | Vanderwinkel and DeVlieghere (1968) |
| MLD 12 | Hfr as CGSC 1927 except <i>arg H⁺ ppc</i> | P1-CGSC 5236 X CGSC 1927 → <i>arg</i> + |
| MP 180 | Hfr H wild-type | Pearson (1972) |
| MP 258 | Hfr H <i>crp</i> | Spontaneous from MP 180 Pearson (1972) |
| MP 259 | Hfr H <i>cya</i> | Spontaneous from MP 180 Pearson (1972) |
| MLD 13 | Hfr as MLD 12 except <i>icl R-10</i> | EMS mutagenesis of MLD 12 |

| <u>Strain</u> | <u>Genotype</u> | <u>Source</u> |
|-----------------------|--|--|
| MLD 14 | Hfr as MLD 13 except <i>met A</i> ⁺ | P ₁ ·CSH 78 X MLD 13 → <i>met</i> ⁺ |
| CGSC 4456 (JRG465) | F ⁻ <i>suc A, trp A, trp R, gal,</i> <i>λ⁻, icl R-10</i> | Herbert and Guest (1969) |
| CGSC 4457 (JRG153) | F ⁻ <i>suc B, λ⁻</i> | Herbert and Guest (1969) |
| CGSC 4206 (KL16-99) | Hfr <i>thi, rec A, rel A, deo B</i> | Low (1968) |
| CGSC 4869 (PA505-1-5) | F ⁻ <i>pro A, pps, arg H, thi,</i> <i>met A, ace A, str A, λ⁻</i> | |
| MLD 15 | F ⁻ as CGSC 4869 except <i>pro A</i> ⁺ | P ₁ ·CSH 78 X CGSC 4856 → <i>pro</i> ⁺ |
| MLD 16 | F ⁻ as CGSC 4869 except <i>thy A</i> | aminopterin selection |
| CGSC 5134 (DF11) | F ⁻ <i>his, met A, pgi, rps L</i> | Fraenkel (1967) |
| MLD 17 | F ⁻ as CGSC 5134 except <i>thy A</i> | aminopterin selection |
| MLD 18 | F ⁻ as CGSC 5134 except <i>rec A</i> | mating CGSC 4206 X MLD 17 → <i>thy</i> ⁺ |
| MLD 19 | F' <i>met A</i> ⁺ , <i>icl R-4, pgi</i> ⁺ / <i>his</i> <i>met A, pgi, rps L, rec A</i> | mating MLD 7 X MLD 18 → <i>met</i> ⁺ <i>glucose</i> ⁺ |
| MLD 20 | F' <i>met A</i> ⁺ <i>icl R-1, pgi</i> ⁺ / <i>his</i> <i>met A, pgi, rps L, rec A</i> | mating CSH 60 X MLD 18 → <i>met</i> ⁺ , <i>gluc</i> ⁺ |
| MLD 21 | F' <i>met A</i> ⁺ , <i>icl R</i> ⁺ , <i>pgi</i> ⁺ / <i>his</i> <i>met A, pgi, rps L, rec A</i> | mating CSH 78 X MLD 18 → <i>met</i> ⁺ , <i>gluc</i> ⁺ |
| MLD 22 | F' <i>met A</i> ⁺ , <i>icl R</i> ⁺ , <i>pgi</i> ⁺ / <i>his</i> , <i>met A, pgi, rps L, rec A</i> | mating CSH 78 X MLD 18 → <i>met</i> ⁺ , <i>gluc</i> ⁺ |
| MLD 23 | F' <i>met A</i> ⁺ , <i>icl R-5, pgi</i> ⁺ / <i>his</i> <i>met A, pgi, rps L, rec A</i> | mating MLD 8 X MLD 18 → <i>met</i> ⁺ , <i>gluc</i> ⁺ |
| MLD 24 | F' <i>met A</i> ⁺ , <i>icl R-6, pgi</i> ⁺ / <i>his</i> , <i>met A, pgi, rps L, rec A</i> | mating MLD 9 X MLD 18 → <i>met</i> ⁺ , <i>gluc</i> ⁺ |
| MLD 25 | F' <i>met A</i> ⁺ , <i>icl R-8, pgi</i> ⁺ / <i>his</i> <i>met A, pgi, rps L, rec A</i> | mating MLD 10 X MLD 18 → <i>met</i> ⁺ , <i>gluc</i> ⁺ |

| <u>Strain</u> | <u>Genotype</u> | <u>Source</u> |
|---------------|---|---|
| MLD 26 | F ⁺ met A ⁺ , <i>icl</i> R-1, <i>pgi</i> ⁺ / <i>his</i> , <i>met</i> A, <i>pgi</i> , <i>rps</i> L, <i>rec</i> A | mating CSH 60 X MLD 18 → met +, gluc + |
| MLD 27 | F ⁺ met A ⁺ , <i>icl</i> R-3, <i>pgi</i> ⁺ / <i>his</i> , <i>met</i> A, <i>pgi</i> , <i>rps</i> L, <i>rec</i> A | mating MLD 6 X MLD 18 → met +, gluc + |
| MLD 28 | F ⁺ met A ⁺ , <i>icl</i> R-2, <i>pgi</i> / <i>his</i> <i>met</i> A, <i>pgi</i> , <i>rps</i> L, <i>rec</i> A | mating MLD 5 X MLD 18 → met +, gluc + |
| MLD 29 | F ⁻ as MLD 16 except <i>icl</i> R-9 | P ₁ ·MLD 11 X MLD 16 → ace + |
| MLD 30 | F ⁻ as MLD 16 except <i>icl</i> R-11 | P ₁ ·CGSC 4456 X MLD 16 → ace + |
| MLD 31 | F ⁻ as MLD 16 except <i>icl</i> R-10 | P ₁ ·MLD 14 X MLD 16 → ace + |
| MLD 32 | F ⁻ as MLD 16 except <i>icl</i> R-7 | P ₁ ·CGSC 5859 X MLD 16 → ace + |
| MLD 33 | F ⁻ as MLD 29 except <i>rec</i> A | mating CGSC 4206 X MLD 29 → thy + |
| MLD 34 | F ⁻ as MLD 30 except <i>rec</i> A | mating CGSC 4206 X MLD 30 → thy + |
| MLD 35 | F ⁻ as MLD 31 except <i>rec</i> A | mating CGSC 4206 X MLD 31 → thy + |
| MLD 36 | F ⁻ as MLD 32 except <i>rec</i> A | mating CGSC 4206 X MLD 32 → thy + |
| MLD 37 | F ⁺ met A ⁺ , <i>icl</i> R ⁺ , <i>pgi</i> ⁺ / <i>met</i> A, <i>icl</i> R-9, <i>pro</i> A, <i>pps</i> , <i>arg</i> H, <i>thi</i> , <i>rps</i> L | mating MLD 22 X MLD 33 → met + |
| MLD 38 | F ⁺ met A ⁺ , <i>icl</i> R ⁺ , <i>pgi</i> ⁺ / <i>met</i> A, <i>icl</i> R-10, <i>pro</i> A, <i>pps</i> , <i>arg</i> H, <i>thi</i> , <i>rps</i> L | mating MLD 22 X MLD 35 → met + |
| MLD 39 | F ⁺ met A ⁺ , <i>icl</i> R ⁺ , <i>pgi</i> ⁺ / <i>met</i> A, <i>icl</i> R-11, <i>pro</i> A, <i>pps</i> , <i>arg</i> H, <i>thi</i> , <i>rps</i> L | mating MLD 22 X MLD 34 → met + |

| <u>Strain</u> | <u>Genotype</u> | <u>Source</u> |
|--------------------|---|--|
| MLD 40 | F ⁺ met A ⁺ , icl R ⁺ , pgi ⁺ /met A, iclR-7, pro A, pps, arg H, thi, rps L | mating MLD 22 X MLD 32 → met + |
| PL 2 | Hfr H gal K | S. Adhya |
| MLD 41 | Hfr H suc A | P ₁ ·CGSC 4456 X PL2 → gal + |
| H 2 | Hfr H glt A | B.D. Sanwal |
| 604-30S | F ⁻ sdh, frd | T.C-Y Lo |
| DF 1651 | F ⁻ pyr D, pps, edd, his, tyr A, thi, gal K, rps L | D. Fraenkel |
| HG20 | F ⁻ pyr D, edd, his, tyr A, thi, gal K, rps L, pck, dme | H. Goldie |
| HG38 | Hfr Δ(lac, pro) mdh | H. Goldie et al (1978) |
| CGSC 4823 (UH-Ac2) | F ⁺ (?)ace E, trp, sup E, tyr T, mel | Henning et al (1964) |
| CGSC5476 (Ac-10) | F ⁺ (?)ace F, mel, sup E, tyr T, | Henning et al (1964) |
| MLD 42 | Hfr icd | EMS mutagenesis of CSH 78 |
| MLD 43 | Hfr glt A | ICR 191 mutagenesis of CSH 78 |

Enzyme Induction Experiments

In these experiments, the changes in the levels of enzymes (usually isocitrate lyase or isocitrate dehydrogenase) were followed after the removal of cells from one carbon source (usually glucose) into medium containing another carbon source. The following is a general outline of the procedure.

Cells were grown through at least three transfers on glucose before the start of the experiment. An overnight culture in 100 ml of glucose medium A was inoculated into 500 ml of fresh glucose medium A to a final density of approximately 100 Klett units. Cells were allowed to grow with vigorous shaking for approximately two hours by which time they were in log phase.

The cells were then spun down at 5000 g in a Sorvall RC-5 refrigerated centrifuge at 10°C, washed once in medium A with no carbon source, and resuspended in 500 ml of prewarmed medium A with 1% of the appropriate carbon source and any other supplements which might be required.

A 30 ml aliquot was taken immediately on resuspension, and cells were returned to 37°C where they were vigorously shaken over the course of the experiment. Further aliquots were taken, usually once an hour, over six to ten hours.

The aliquots were centrifuged in a Sorvall centrifuge at 12,000 g for ten minutes at 4°C, washed once in chilled medium A with no carbon source, and stored as pellets at -15°C until analyzed. The delay before analysis was usually no more than a few days.

Enzyme Assays

Cells to be extracted for enzyme assays, other than those from the induction experiments, were always grown on a given carbon source for at least three transfers before being collected.

All cells to be extracted for enzyme assays were resuspended in 0.1 M Tris-HCl (pH 7.5) containing 2 mM MgCl₂ and 20% glycerol. The glycerol was added to protect the isocitrate dehydrogenase which is cold labile (Reeves *et al* 1972).

Extracts were prepared by sonication using an Insonator Model 1000 sonicator with a standard microtip probe (Ultrasonic Systems Inc.). The cell suspensions, which were kept on ice, were sonicated 1 min per ml of suspension. The probe was cooled on ice for 30 sec after every minute of sonication.

Extracts were centrifuged at 4^oC in a Sorvall RC-5 centrifuge for 10 minutes at 27,000 g.

Protein determinations were done by the method of Lowry *et al* (1951). Specific activities are expressed in units per mg protein.

All spectrophotometric assays were done using a Gilford Recording Spectrophotometer Model 2400.

Isocitrate Lyase (Dixon and Kornberg 1959)

The assay mixture contained in 1 ml final volume:

80 μmoles KH_2PO_4 , pH 7.0

6 μmoles MgCl_2

2 μmoles DTT

4 μmoles DL- Na isocitrate

4 μmoles phenylhydrazine adjusted to approximately pH 7.0

with NaOH

The reaction was started by the addition of extract and followed for at least three minutes. There is an initial lag. The formation of glyoxylate phenylhydrazone was followed at 324 nm ($\epsilon_M = 1.7 \times 10^4$). One unit is the amount of enzyme which produces 1 μmole of glyoxylate phenylhydrazone per minute.

Isocitrate Dehydrogenase (Cribbs and Englesberg, 1964)

The assay mixture contained in 1 ml final volume:

100 μmoles Tris-HCl, pH 7.4

0.33 μmoles MnCl_2

0.033 μmoles NADP^+

4.0 μmoles DL- Na isocitrate

The reaction was started by the addition of extract, and the formation of NADPH was followed at 340 nm ($\epsilon_M = 6220$). One unit is the amount of enzyme which reduces 1 μmole of NADP^+ per minute.

Phosphoglucose Isomerase (Fraenkel and Levisohn 1967)

The assay mixture contained in 1 ml final volume:

50 μmoles Tris-HCl plus 10 μmoles MgCl_2 , pH 7.6

0.2 $\mu\text{moles NADP}^+$

0.4 $\mu\text{moles fructose-6-phosphate}$

0.3 units of glucose-6-phosphate dehydrogenase

The reaction was started by adding extract and the formation of NADPH was followed at 340 nm. One unit is the amount of enzyme which reduces 1 $\mu\text{mole of NADP}^+$ per minute.

α -Ketoglutarate Dehydrogenase and Pyruvate Dehydrogenase (Guest and Creaghan 1973)

The assay mixture contained in 1 ml final volume:

120 $\mu\text{moles Tris-HCl, pH 8.5}$

3 $\mu\text{moles DTT}$

0.08 $\mu\text{moles CoA}$

0.2 $\mu\text{moles thiamine pyrophosphate}$

0.8 $\mu\text{moles 3-acetyl NAD}^+$

Extract, containing up to 1 mg of protein, was incubated with the above mixture for 2-3 minutes. The reaction was started by adding 7.5 $\mu\text{moles of either Na } \alpha\text{-ketoglutarate or Na pyruvate to one cuvette}$. A second cuvette with no keto-acid was run as a control. The reduction of 3-acetyl NAD^+ was followed at 366 nm ($\epsilon_M = 9100$). One unit is the amount of enzyme which reduces 1 $\mu\text{mole of 3 acetyl NAD}^+$ per minute.

Malate Oxidase (Goldie *et al* 1978)

The assay mixture contained in 1 ml final volume:

100 μ moles Tris-HCl, pH 7.5

100 μ moles KCl

10 μ moles KCN

1 μ mole K ferricyanide

5 μ moles Na malate

The reaction was started by adding extract, and the reduction of ferricyanide was followed at 420 nm ($\epsilon_M = 1.0 \times 10^3$). One unit is the amount of enzyme which reduces 1 μ mole of ferricyanide per minute.

Malate Dehydrogenase (Ochoa 1955)

The assay mixture contained in 3 ml final volume:

75 μ moles potassium phosphate buffer, pH 7.4

0.15 μ moles NADH

The reaction was started by adding 0.76 μ moles of freshly prepared oxaloacetic acid.

A control with no oxaloacetic acid was also done.

The oxidation of NADH was followed at 340 nm ($\epsilon_M = 6220$).

One unit is the amount of enzyme which oxidizes 1 μ mole of NADH per minute.

PEP Carboxylase (Cánovas and Kornberg 1969)

The assay mixture contained in 1 ml final volume:

100 μ moles Tris-HCl, pH 8.5

5 μ moles $MgCl_2$

0.1 μ moles NADH

10 μ moles fresh $KHCO_3$

2.5 μ moles acetyl CoA

5 μ moles potassium PEP

2 units malate dehydrogenase

The crude extract was made 40% saturated by ammonium sulfate, and the supernatant was used. This removed NADH oxidase activity.

The reaction was started by adding PEP. The oxidation of NADH was followed at 340 nm. A blank reaction was run first before adding PEP to determine amount of oxidase remaining. One unit is the amount of enzyme which oxidizes 1 μ mole of NADH per minute.

PEP Synthase (Cooper and Kornberg 1969)

This assay was chosen because only crude extracts were examined.

The assay contained in 0.5 ml final volume:

50 μ moles Tris-HCl, pH 8.4

5 μ moles $MgCl_2$

5 μ moles ATP, pH 6.8

0.075 μ moles Na pyruvate

A control was also run which contained no ATP.

Tubes were equilibrated for 10 minutes at 30°C and the reaction was started by adding extract. The reaction was continued at 30°C. An 0.1 ml sample was removed immediately, and others at 5 minutes and 10 minutes from the test and control tubes.

The samples were placed in test tubes containing 0.9 ml of water and 0.33 ml of 0.1% 2,4-dinitrophenylhydrazine made up in 2N HCl.

After 10 minutes at 30°C 1.67 ml of 10% Na OH were added.

This reaction measures pyruvate remaining in the reaction mixture.

Tubes were read on the Gilford spectrophotometer at 445 nm. Under these conditions 1 μ mole of pyruvate has an absorbance of 6 absorbance units.

The Extraction of CoA and Acetyl CoA From Cells

The method used was basically that of Setlow and Setlow (1977). Cells were inoculated into fresh medium from an overnight culture and allowed to grow until they reached log phase, approximately 200 Klett units measured with a blue filter. The cultures were always adapted to a given carbon source by at least three transfers in it.

Total CoA

Two ml of growing cells were added directly to 8 ml of 95% ethanol

at approximately 80°C. The mixture was immediately made 5 mM in EDTA and 25 mM in potassium phosphate buffer pH 7.0, and incubated at 75°C for 15 minutes. It was then chilled on ice and centrifuged at 4°C for 10 minutes at 28,000 g.

The supernatant was removed very carefully with a Pasteur pipette into a 25 ml pear-shaped rotary evaporator flask. The mixture was made 2 mM in DTT and evaporated to dryness at 30°C on a rotary evaporator. Residues were stored dry and made up to 0.5 ml with distilled water at the time of the assay. The samples were usually analyzed within 24 hours.

Acetyl CoA

To obtain only acyl CoAs, 2 mM DTT was also present in the first extraction; after 15 minutes at 75°C the suspension was made 5 mM in NEM and incubated a further 10 minutes. The suspension was chilled and centrifuged as for the total CoA, and the supernatant fluid was made 5 mM in DTT before evaporation.

Samples were stored and redissolved as for the total CoA.

The first addition of DTT will reduce any CoA dimers that might be present. The NEM will then react with the reduced CoA, leaving only the acyl CoAs of which the major one is acetyl CoA. The further addition of 5 mM DTT reacts with excess NEM.

Determination of CoA and Acetyl CoA

The method of Allred and Guy (1969) was used. In this method, the CoA is recycled through a coupled enzyme system in which the rate of formation of NADH is proportional to CoA concentrations when the necessary enzymes and substrates are present in excess.

The assay mixture (premix) was made up as follows:

| <u>Component</u> | <u>Amount</u> |
|---------------------------|---------------|
| Tris buffer (1M, pH 7.2) | 0.5 ml |
| Potassium chloride (1M) | 0.1 ml |
| L-Malate (0.2 M) | 0.1 ml |
| Acetyl phosphate (0.08 M) | 0.1 ml |
| NAD ⁺ (0.02 M) | 0.1 ml |
| Malate dehydrogenase | ~1.6 units |
| Citrate synthase | ~3.5 units |

Solutions which were stable in the refrigerator for several weeks were combined in the above proportions just prior to use.

CoA standards or cell extracts were added to a fluorescence cuvette (1 cm X 1 cm) containing 0.9 ml of the premix, 0.1 ml of DTT (2 μ moles) and sufficient water to make the total volume up to 1.7 ml. After a 2 minute incubation at approximately 25^oC, 0.1 ml of phosphotrans-acetylase (30 units) was added to start the reaction. The reaction was carried out at room temperature, approximately 23^oC.

The change in NADH concentration was measured on an Aminco-Bowman spectrofluorophotometer with a J10-222A photomultiplier which

is equipped with a damping device. This helps to cut down electronic noise at the high sensitivity setting (setting = 1) which had to be used for these experiments.

Rates were followed on a Houston Instrument Omnigraphic 2000 X-Y recorder.

Conditions could not be reproduced identically each day, so a standard curve of rate of change in fluorescence versus CoA concentration was run for each set of experiments. All extract concentrations measured on the same day were compared to this standard curve.

Method for Collecting and Extracting Cells For Pyruvate Determination

The method was based closely on that of Lowry *et al* (1971) but with certain modifications. In this method, a known volume of medium containing log phase cells at a known density, is rapidly collected on a Millipore filter. The filter, with the cells, is then quick-frozen and treated at once with aqueous perchloric acid to extract pyruvate under conditions in which metabolic conversion is minimal.

Lowry *et al* were able to collect 30 to 50 ml of cells which corresponded to 0.1 to 0.15 mg/ml dry weight in 30 to 60 seconds. This was not possible with the vacuum pump (Millipore vacuum pressure pump) used for these experiments. It was, however, possible to collect 10 - 15 ml of cells with a density of 0.3 - 0.35 mg/ml dry weight in

this time. Since it is very important that the cells be collected as quickly as possible, this latter procedure was used for the pyruvate determination experiments.

Cells were grown through at least three transfers on glucose minimal medium. A fresh overnight culture was used as an inoculum for 500 ml of glucose medium in a 2 litre flask. This was added to a final density of approximately 80 Klett units (Blue No. 42 filter). Cells were allowed to grow to a density of approximately 160 Klett units by which time they had reached early log phase.

A sample of glucose-grown cells was taken as described below, then cells were collected, washed and resuspended in fresh medium with a new carbon source, as described for the enzyme induction experiments.

Samples for analysis were collected from 10 to 15 ml of medium on a 47 mm diameter 0.45 μ Millipore filter with suction using a Millipore vacuum pump. The cells were not washed but as soon as all the liquid had been removed (total filtration time not more than 60 sec) the filter was frozen in liquid nitrogen. The brittle filter was then broken into a 30 ml Corex centrifuge tube previously cooled in the liquid nitrogen.

To this tube was added 1 ml of 0.3 N HClO_4 containing 1 mM EDTA and the tube was thoroughly mixed with a Vortex mixer, and centrifuged for 10 minutes at 28,000 g.

A measured aliquot was removed into an Eppendorf centrifuge tube and neutralized with 0.2 volumes of 1.5 M K_2CO_3 . The resulting KClO_4

was removed by centrifugation in an Eppendorf Microcentrifuge.

The supernatant fluid was stored at -78°C until it could be analyzed.

Determination of Pyruvate

This assay was based on that of Lowry *et al* (1971) in which the change in the level of NADH produced by LDH was a measure of pyruvate concentration.

The assay mix contained 200 mM imidazole-acetate buffer (pH 7.0), 0.5 - 2 μM NADH and 12.5 units of beef heart lactate dehydrogenase.

Imidazole was used because it appears to reduce the natural fluorescence of *E. coli* extracts attributed to FMN (Lowry *et al* 1971). Special low fluorescence imidazole was used (Sigma).

Analyses were conducted with 1 ml of reagents in fluorescence cuvettes plus a measured aliquot of the neutralized HClO_4 extracts. Changes in NADH fluorescence were followed on an Aminco-Bowman spectrofluorophotometer. The amount of NADH used in the reaction varied with the amount of pyruvate present in the extracts. In a few cases, there was a change in NADH fluorescence even in the absence of lactate dehydrogenase. This seemed to be worse with some extracts than others. For this reason, a blank reaction without lactate dehydrogenase was run for all samples in addition to the reaction in the presence of lactate dehydrogenase. This blank reaction was taken into account when calculating the amount of pyruvate in a given sample.

A standard curve of NADH versus fluorescence was used to determine the change in NADH concentration and thus pyruvate concentration. The concentrations of the standard NADH solutions were determined by their absorbance at 340 nm, using $\epsilon_M = 6220$.

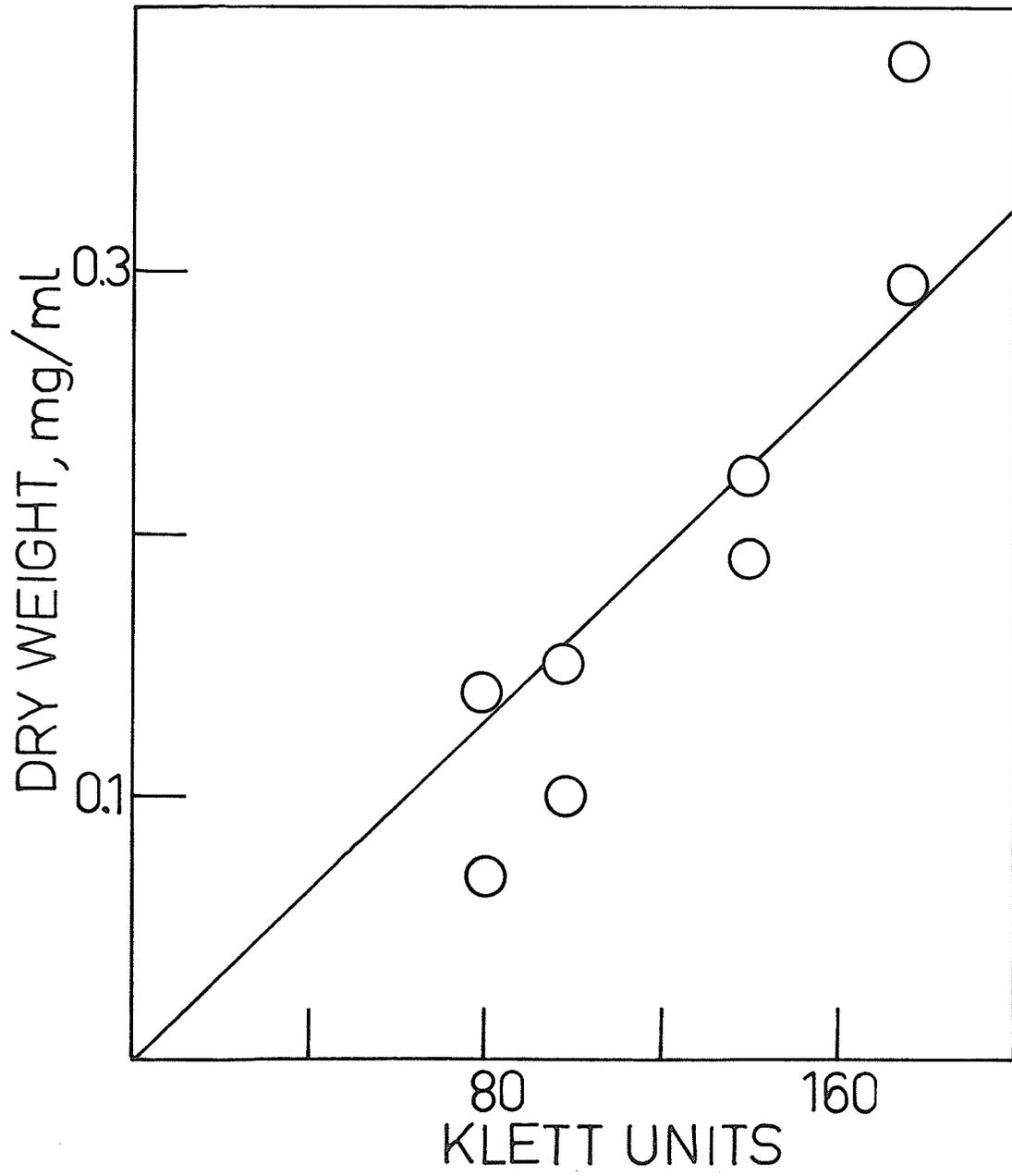
Dry Weight Determinations

CSH 78 was grown up on glucose medium A into late log phase. Cells were spun down in a Sorvall refrigerated centrifuge and resuspended in one-fifth the volume of medium A with no carbon source. This suspension was further diluted with medium A to various cell densities as measured on the Klett with a blue filter (approximately 420 nm).

Two 30 ml aliquots for each cell density were added to dried, weighted 50 ml polypropylene centrifuge tubes. The cells were spun down at 4°C in a Sorvall centrifuge at 28,000 g. The pellets were washed in 5 ml of ice cold deionized water very briefly and spun down again at 28,000 g for five minutes. The tubes were dried overnight in a drying oven at 100°C, weighed again, and the dry weights calculated by differences.

The dry weight standard curve is seen in Figure 4.

Figure 4. Standard curve of dry weights versus Klett units (blue filter). Cells were collected and dried as described in the text.



Genetic Methods

The general methods for the genetic experiments are those described by Miller (1972). Centrifuging for mutagenesis, P₁ phage lysate preparation, transductions, and matings was done in sterile five inch glass screw cap tubes in a table-top centrifuge.

Ethylmethane Sulfonate (EMS) Mutagenesis

Cells were grown to a density of 2-3 X 10⁸ cells/ml in 5 ml of glucose medium A. They were then spun down, washed in medium A with no carbon source, and resuspended in 2 ml of medium A containing 0.2 M Tris-HCl pH 7.5 (and no carbon source). To this was added 0.03 ml of EMS and the mixture was vortexed to dissolve the EMS. The culture was shaken vigorously for 2 hours at 37°C, then spun down, washed once with medium A and resuspended in 1 ml of medium A. This medium contained no carbon source. This suspension was diluted 10-fold into several different tubes containing 5 ml of selective medium and allowed to grow to saturation at 37°C.

ICR 191 Mutagenesis

ICR 191 is an acridine-based compound which causes frame-shift mutations in *E. coli*. It is unstable in solutions at room temperature and in the light. Solutions were stored wrapped in aluminum foil at -15°C and were thawed just before use.

A preliminary experiment was carried out to determine the best

concentration of ICR 191 to use. This is the highest concentration which still permits adequate growth.

A 10^{-4} dilution of a fresh overnight LB culture of the strain to be tested was prepared, and inoculated into several tubes containing 3 ml of enriched medium A with varying concentrations of ICR 191, as well as a control tube with no ICR 191. The tubes were covered with aluminum foil and grown with aeration overnight at 37°C . Tubes with an intermediate amount of growth compared to the control contained the best concentrations of ICR 191 for mutagenesis.

For the strains tested and the preparation of ICR 191 used in this work, the best concentration was $45.5\ \mu\text{g/ml}$. This is somewhat high compared to concentrations generally used ($10\ \mu\text{g/ml}$), but may reflect the purity of the ICR 191 used in these experiments.

The actual mutagenesis experiments were done as above, using $45.5\ \mu\text{g/ml}$ ICR 191. Several tubes were usually run, because mutants arising from the same tube might not be independent. Only one mutant from each tube was saved for further analysis.

After growth overnight, cells were washed in medium A with no carbon source and plated on selective medium.

Penicillin Selection

After mutagenesis, cells were grown up overnight on non-selective medium (for non-selective and selective media, see the description of the preparation of *pps* mutants, below). Usually several tubes from

the original mutagenesis were carried through this procedure to ensure independent mutants. Five ml of culture was spun down, washed twice in medium A with no carbon source, and resuspended in 1 ml of medium A. This was used to inoculate 5 ml of prewarmed selective medium to a density of $1-2 \times 10^7$ cells/ml. Cells were grown with aeration at 37°C until they reached log phase, approximately 2 hours.

At this point, 10,000 units/ml of penicillin were added and cells were shaken for a further 60 to 90 minutes until lysis, as seen by loss of turbidity, was complete. Cells were then spun down, washed once in medium A and resuspended in 5 ml of non-selective medium.

Cells were allowed to grow to saturation and then plated out for further testing. Only one mutant from each tube was saved.

Preparation of P₁ Phage Lysates

One drop of overnight culture was inoculated into 5 ml of LB broth containing $5 \times 10^{-3}\text{M}$ CaCl_2 . Cells were aerated until they reached a density of approximately 2×10^8 cells/ml. Coliphage P₁vir was preabsorbed by adding 10^7 phage to 1 ml of this culture and incubating at 37°C for 20 minutes without shaking. Then 2.5 ml of LB top agar at 45°C was added, and the entire contents was poured onto a fresh LB plate. The plates were incubated overnight, face up, at 37°C .

The agar layer was collected by adding 2 ml to each plate, and scraping the agar layer into a centrifuge tube. A few drops of chloroform were added to the tube and the mixture was vortexed

vigorously, then allowed to stand for 10 minutes before centrifuging.

The supernatant containing the P₁ lysate was collected in a sterile screw cap tube, and a few drops of chloroform were added. Lysates were stored at 4°C.

Transduction with P₁ Phage Lysates

Transductions with coliphage P₁ were used both in strain construction and genetic mapping experiments.

Five ml of a fresh overnight LB culture of the strain to be transduced were centrifuged, and the cells resuspended in 5 ml of MC buffer (0.1 M MgSO₄ plus 0.005 M CaCl₂) and shaken vigorously for 15 minutes. The P₁ lysate (made on the appropriate donor strain) was added at a multiplicity of infection (M.O.I.) of 1:1. A control, containing no phage was also run. Preadsorption was carried out by shaking gently at 37°C for 20 minutes.

To prevent excess killing by P₁vir (a virulent strain of P₁), an equal volume of 1 M sodium citrate was added after preadsorption to remove calcium ions which are required for adsorption of P₁. Cells were spun down, resuspended in 1 ml of 0.1 M sodium citrate, and plated, either directly or after dilution, onto selective medium.

Interrupted Mating

Donor cells (Hfr or F') were grown overnight at 37°C, without shaking, in 5 ml of LB medium. Recipient cells (F⁻) were grown

overnight in 5 ml of LB with shaking.

Five ml of fresh LB broth was added to the overnight donor cultures, and they were incubated for a further 3-4 hours at 37°C without shaking.

Two drops of the overnight recipient culture were inoculated into fresh LB medium and cells were grown with shaking at 37°C to a density of approximately 2×10^8 cells/ml.

A mating mixture was made to contain 10 recipient cells to 1 donor cell. One drop of 20% glucose was added to the mixture, which was then incubated with gentle shaking at 37°C for the time required for transfer of the given marker.

Mating pairs were broken apart by very vigorous vortexing for one minute, followed by chilling on ice. Cells were spun down, washed once in chilled medium A, and resuspended in 1-2 ml of medium A, then either diluted or plated directly onto selective medium.

Isolation of Mutants

PEP Synthase Mutants (Cooper and Kornberg 1967)

Strains CSH 78 and CSH 60 were mutagenized with EMS and allowed to recover overnight in glucose minimal medium. The cells were subjected to penicillin selection in the presence of lactate minimal medium, then allowed to recover in glucose minimal medium. Mutants were plated on glucose medium A and checked by replica plating for

the ability to grow on lactate, pyruvate and acetate.

icl R Constitutive Mutants

I. Different *pps* isolates of CSH 78 and CSH 60 were mutagenized with ICR 191. After growth in the presence of ICR 191 cells were washed and plated onto lactate medium A.

Colonies which grew on lactate were checked by replica plating for ability to grow on pyruvate. Cells which were pyruvate negative but lactate positive were grown up on glucose medium A and assayed for constitutive levels of isocitrate lyase (Brice and Kornberg 1968).

II. MLD 12 which was *ppc* was mutagenized with EMS, and allowed to recover in glucose medium A with required supplements. Cells were plated on glucose medium. Glucose positive cells could be either *ppc* revertants or *icl R*. A large number of colonies which grew up were assayed for constitutive levels of isocitrate lyase on glucose medium.

Isocitrate Dehydrogenase Mutants (*icd*)

Isocitrate dehydrogenase mutants were selected according to the method of Helling and Kukora (1971). Cells from an overnight LB culture were plated, directly or after EMS mutagenesis, onto LB plates containing 0.4% glucose and 10 µg/ml nalidixic acid. The nalidixic acid was made up as a 10 mg/ml stock in sterile 1 N NaOH.

Mutants which grew up on these plates were checked by replica

plating for a proline or glutamate requirement on glucose medium A. Colonies showing such a requirement were assayed for isocitrate dehydrogenase.

Thymidylate Synthase Mutants (*thy A*) (Miller 1972)

Overnight cultures were inoculated into 5 ml of glucose medium A plus supplements as required, and containing 200 µg/ml of thymidine and 200 µg/ml aminopterin. Cells were allowed to grow to saturation, and a drop was subcultured into identical medium. When the cells reached saturation for a second time they were plated onto glucose medium A plus required supplements and 200 µg/ml thymidine.

Colonies were checked by replica plating for their ability to grow with and without thymidine.

Recombination Deficient Mutants (*rec A*)

Strain CGSC 4206 is a *rec A* Hfr which donates both *thy A* and *rec A* within 5 minutes. This strain was mated with F⁻ *thy A* mutants for 30 minutes and recombinants were selected on glucose medium A lacking thymidine but with streptomycin to select against C6SC 4206. These cells were further tested for UV sensitivity which indicates the presence of the *rec A* gene.

Cells which no longer required thymidine were replica plated onto two LB plates. One was exposed to UV light for 20 seconds, the other served as a control. The UV source was a Sylvania germicidal

UV lamp set 25 cm from the plates. Plates were wrapped in aluminum foil to prevent photoreactivation and incubated at 37°C for approximately 6 hours.

Colonies which grew on the control plate but not on the irradiated plate were *rec A*.

F' Construction

The procedure for construction of F' strains which are diploid for the region of the *E. coli* chromosome (89-90 minutes) where *icl R*, *ace A* (isocitrate lyase), and *ace B* (malate synthase A) are located, was that of Low (1968).

Specific details for the construction of these F's are given in the results. The general procedure was as follows:

A recipient strain (F⁻) of the required genotype was made *rec A*. This was accomplished, by the methods previously described, by first making the strain *thy A*, and then mating with an Hfr *rec A* and selecting for *thy A*, *rec A* recombinants.

This strain was then mated with an Hfr which donates the relevant region of the chromosome early. After mating for approximately 30 minutes the pairs were broken apart and plated on medium which selected for the desired genotype.

The fact the recipient is *rec A* should prevent recombination from occurring, and merodiploids are created.

These strains were checked for UV sensitivity and the ability to transfer the relevant genes to a second F^- strain.

F' Curing

The following three methods for curing F' 's were used:

I. Acridine Orange (Hirota 1960)

Approximately 1000-2000 cells (0.1 ml of a 10^{-5} dilution of a fresh overnight LB culture) were inoculated into 5 ml of LB broth pH 7.6 containing 75 $\mu\text{g/ml}$ acridine orange. The pH of the broth is critical. Cells were grown overnight in the dark with shaking at 37°C . The cultures were then streaked onto selective medium. For the F' 's used in this work, the selective medium was TTC glucose which can differentiate between pgi^- and pgi^+ colonies.

II. Acridine Orange Plate Method (Fan 1969)

In this method, acridine orange is added directly to the indicator plate. For this work, TTC glucose plates were used. After autoclaving, 2.5 ml of 10 mg/ml acridine orange solution made up in sterile water and 3 ml of sterile 2N NaOH were added to 1 litre, final volume of medium.

The TTC glucose plates for this experiment were made with Difco Antibiotic Medium No. 2.

Cultures were streaked directly onto these plates, and incubated in the dark at 37°C.

The plating efficiency on this medium is 1/3 to 1/2 normal efficiency.

III. SDS Treatment (Inuzuka *et al* 1968)

This method was originally devised to eliminate the sex factor from Hfrs. It can also be used to cure F's.

Approximately 10^3 cells/ml were inoculated into 5 ml of Difco Antibiotic medium No. 3 adjusted to pH 7.6 and containing 10% (w/v) SDS. Cells were grown with shaking at 37°C until growth appeared, and then were streaked onto selective TTC glucose plates.

Chemicals

All chemicals except the ICR 191 were obtained from commercial sources and were of the highest purity available.

ICR 191 was synthesized by K. LaRue of the Chemistry Department, University of Manitoba by a procedure similar to that of Peck *et al* (1961).

CHAPTER 3

PHYSIOLOGICAL STUDIES

Steady-state Levels of Isocitrate Lyase on
Different Carbon Sources

The levels of the enzymes of the glyoxylate cycle in wild-type *E. coli* K12 vary according to the carbon source on which the cells are grown. Table 1 gives the specific activities of isocitrate lyase in two wild-type strains CSH78 and CGSC259 grown on different carbon sources.

Enzyme activity does vary from preparation to preparation on any given carbon source, usually by no more than a factor of two. This variability is small compared to the differences in specific activity of isocitrate lyase seen on going from one carbon source to another.

For these wild-type strains, glucose has the lowest specific activity for isocitrate lyase, acetate the highest, although it should be noted that the two strains do not show the same magnitude of difference. There appears to be no relationship between isocitrate lyase activity and whether the sugar is transported by the phosphotransferase system. Glucose, which is transported by this system produces very low levels of isocitrate lyase, but so does lactose which is not.

Cells grown on lactose and glycerol, in which the levels of cyclic AMP are elevated compared to glucose-grown cells, show very little difference in the activity of isocitrate lyase.

The levels of isocitrate lyase appear to reflect whether the

Table 1

The steady-state levels of isocitrate lyase in two wild-type *E. coli* K12 strains grown on different carbon sources.

| Carbon Source | Isocitrate Lyase (units/mg) | | | |
|-------------------------|-----------------------------|-------|---------|-------|
| | CSH78 | | CGSC259 | |
| glucose | 0.003 | (1) | 0.002 | (1) |
| glucose + acetate | 0.003 | (1) | 0.003 | (1.5) |
| lactose | 0.007 | (2.3) | N.D. | |
| glycerol | 0.014 | (4.7) | N.D. | |
| lactate | 0.025 | (8.1) | N.D. | |
| succinate | 0.09 | (33) | 0.047 | (24) |
| succinate + acetate | 0.065 | (22) | 0.066 | (33) |
| L-malate | 0.095 | (32) | 0.072 | (36) |
| fumarate | 0.089 | (30) | N.D. | |
| glycollate | 0.032 | (11) | N.D. | |
| L-proline | 0.17 | (56) | N.D. | |
| acetate | 0.29 | (96) | 0.099 | (50) |
| α -ketoglutarate | 0.32 | (107) | N.D. | |
| LB | 0.043 | (14) | 0.018 | (9) |

All cells were grown through at least three transfers on medium A with a given carbon source before being collected in log phase. All carbon sources were 0.4% except acetate which was 1%. Isocitrate lyase was assayed as described in Methods. The number in parentheses gives the enzyme level relative to that in glucose-grown cells.

carbon source is metabolized via glycolysis or the TCA cycle. The specific activity seen on proline, as compared to those on other compounds metabolized through the TCA cycle (succinate, malate, fumarate), is an interesting anomaly. This higher level of isocitrate lyase is reproducible; the point is interesting because proline is presumably metabolized through succinate. Kornberg (1966) had noted that proline, glutamate or γ -aminobutyrate were the only carbon and/or nitrogen sources on which acetate had a stimulatory effect on isocitrate lyase synthesis in *E. coli* W. *E. coli* K12 does not grow on glutamate as sole carbon source because of difficulty in transporting it into the cell, and therefore was not used in these steady-state studies.

Acetate has no inducing effect on isocitrate lyase synthesis in these strains when they are growing on glucose or succinate.

Table 2 gives the specific activities of isocitrate lyase during growth on different carbon sources in three strains which are constitutive (*icl* R) for the enzyme.

These constitutive strains, although they have higher levels on all carbon sources compared to the wild-type strains, still show a very similar hierarchy to that seen for the wild-type. Growth on glucose yields the lowest levels of isocitrate lyase, and growth on acetate the highest, although the difference between them is not as marked. Cyclic AMP has little effect on the level of isocitrate lyase in these *icl* R strains growing on glucose.

Table 2

The steady-state levels of isocitrate lyase in three strains constitutive for the glyoxylate cycle operon during growth on different carbon sources.

| Carbon Source | Isocitrate lyase (units/mg) | | |
|--|-----------------------------|------------|------------|
| | CSH60 | MLD 5 | MLD 8 |
| glucose | 0.07 (1) | 0.20 (1) | 0.22 (1) |
| glucose + 5 X 10 ⁻⁴ cyclic AMP | 0.12 (1.6) | 0.20 (1) | 0.28 (1.3) |
| glycerol | 0.19 (2.7) | N.D. | 0.43 (1.9) |
| lactate | 0.22 (3.1) | 0.46 (2.2) | 0.58 (2.5) |
| succinate | 0.30 (4.3) | 0.34 (1.7) | 0.42 (1.9) |
| L-proline | 0.39 (5.6) | 0.44 (2.1) | 0.64 (2.9) |
| acetate | 0.5 (7.1) | 0.5 (2.5) | 0.71 (1.3) |

Cultures were grown through at least three transfers on medium A with a given carbon source and collected in log phase. All carbon sources were 0.4% except acetate which was 1%. Numbers in parentheses give the enzyme level relative to that in glucose.

The Effect of the Nitrogen Source on Isocitrate Lyase Levels

The observation that proline produced levels of isocitrate lyase that were almost as high as those on acetate suggested that the nitrogen source has some regulatory effect on the synthesis of this enzyme. To test this possibility, CSH78 was grown on 0.4% glucose in medium A to which different nitrogen sources had been added. The results are seen in Table 3.

These results indicate quite clearly that if glucose is present as the carbon source, the nitrogen source has no effect on the level of isocitrate lyase. This is true even for proline which, as was shown above, gives elevated levels of that enzyme when the proline is being used as sole carbon source.

The Induction of Isocitrate Lyase in Wild-type

E. coli K12 Growing on Different Carbon Sources

The induction experiments were carried out with CSH78 as described in the Methods section. Cultures were grown up on glucose, which produced the lowest levels of isocitrate lyase, washed, and resuspended in the inducing medium containing 1% of the appropriate carbon source. Samples were taken at least every hour and assayed for isocitrate lyase activity. The initial enzyme levels vary somewhat between experiments, but the time of onset and rate of synthesis of isocitrate lyase is quite characteristic of a given carbon source.

Table 3

The levels of isocitrate lyase in the wild-type strain CSH78 grown on different carbon and nitrogen sources.

| Carbon and Nitrogen Source | Isocitrate Lyase (units/mg) |
|-----------------------------------|-----------------------------|
| glucose, NH_4^+ > 1 mM | 0.05 |
| acetate, NH_4^+ > 1 mM | 0.25 |
| glucose, 0.075 mM NH_4^+ | 0.006 |
| glucose, 10 mM glycine | 0.005 |
| glucose, 10 mM L-glutamate | 0.004 |
| glucose, 10 mM L-proline | 0.004 |
| glucose+acetate, 10 mM L-proline | 0.007 |

Medium A without $(\text{NH}_4)_2\text{SO}_4$ was used for these experiments and supplemented with the specified nitrogen source. All carbon sources were 0.4%.

Acetate Induction

Since acetate is the carbon source which produces the highest steady-state levels of isocitrate lyase, it is the standard against which the other inducing carbon sources can be compared. Figure 5 shows the induction of isocitrate lyase in CSH78 transferred from glucose to 1% acetate. Although acetate produces the highest steady-state levels of isocitrate lyase, the initial rate of induction is slow compared to several other carbon sources as seen below.

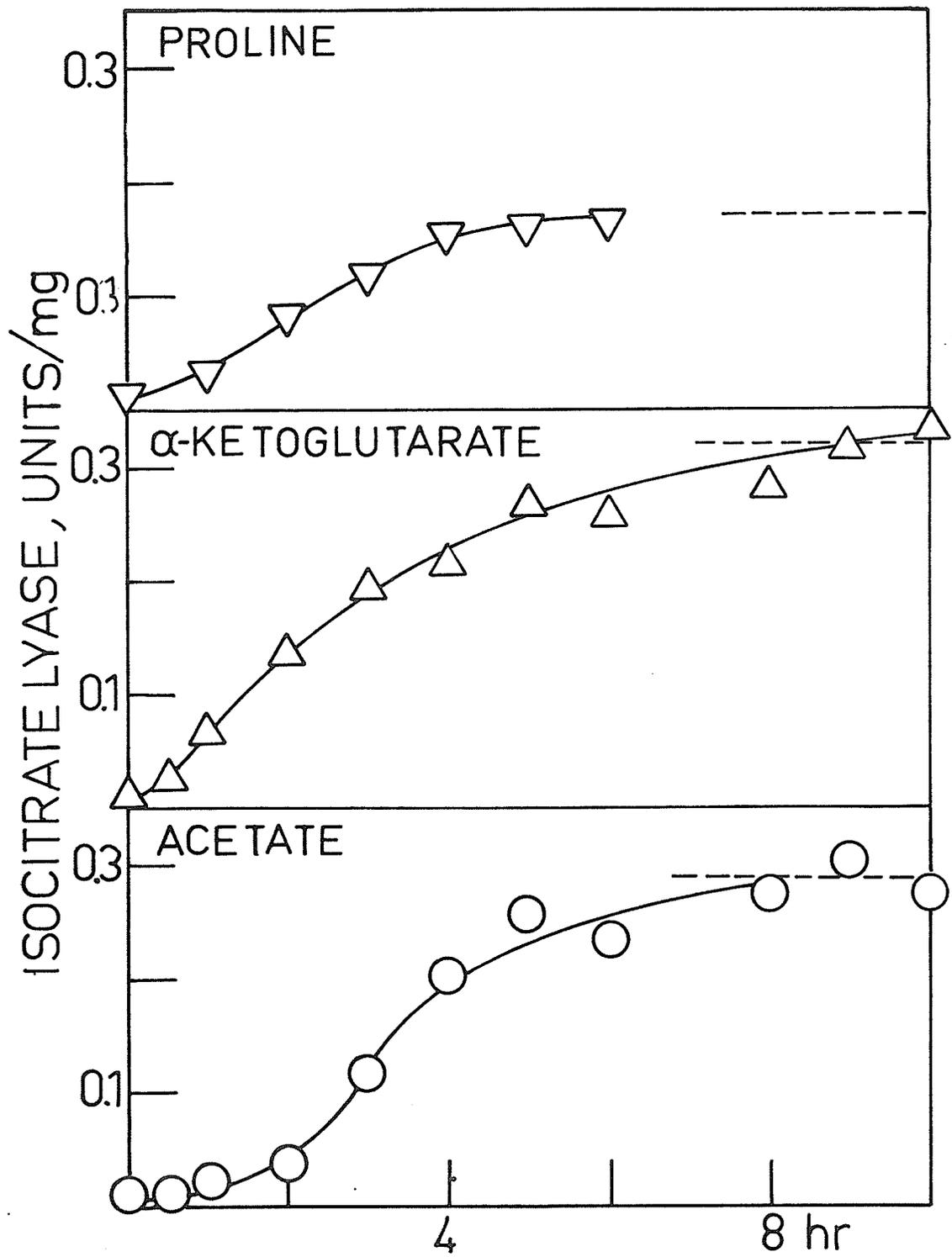
Induction on Proline

Figure 5 also shows the induction of isocitrate lyase in CSH78 on 1% proline. The initial rate of induction of the enzyme appears faster on this carbon source, although it begins to level off at a lower specific activity than in the acetate induced cells. This specific activity corresponds quite well to that seen for steady-state levels of isocitrate lyase in cells growing on proline as sole carbon source.

Induction on α -Ketoglutarate

As stated earlier, Kornberg (1966) made the observation that if glutamate, proline or γ -aminobutyrate was being used as a carbon and/or nitrogen source by *E. coli* W, acetate could induce higher levels of isocitrate lyase. Proline and γ -aminobutyrate are metabolized via glutamate, and all three compounds are metabolized via α -ketoglutarate. This suggested to me that either glutamate or α -ketoglutarate might be the compound which was having the actual effect on the levels of isocitrate lyase. *E. coli* K12 is not very

Figure 5. The induction of isocitrate lyase in CSH78 on 1% acetate (○), 1% α -ketoglutarate (Δ), and 1% proline (∇). Induction experiments were carried out as described in Methods. The zero-time sample was taken after resuspension in the new carbon source. The dotted line represents the approximate steady-state level of isocitrate lyase on the given carbon source, from Table 1.



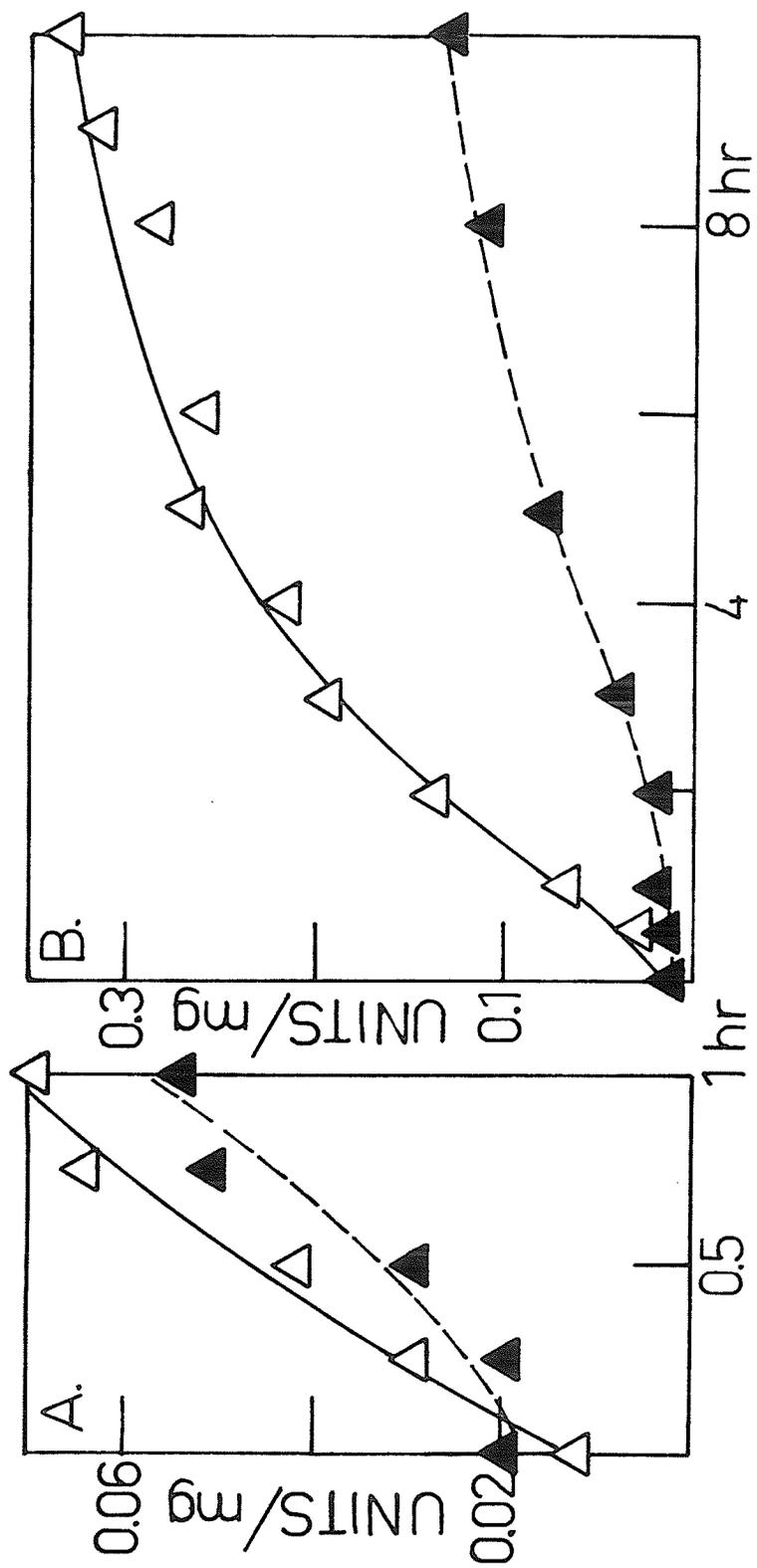
permeable to glutamate, and so α -ketoglutarate was chosen for an induction experiment. Figure 5 shows the induction of isocitrate lyase in CSH78 on 1% α -ketoglutarate. The rate of induction on this carbon source is faster than on either acetate or proline, and the specific activity of isocitrate lyase rises to levels comparable to those seen on fully induced acetate-grown cells. This result strongly suggests that proline does have its inducing effect on isocitrate lyase because it is metabolized to α -ketoglutarate.

Induction of α -Ketoglutarate Dehydrogenase by Acetate and α -Ketoglutarate

The experiments of Amarsingham and Davis (1965) showed that acetate and α -ketoglutarate could induce an increase in the level of α -ketoglutarate dehydrogenase in *E. coli*. They suggested that acetate caused an accumulation of α -ketoglutarate dehydrogenase. Since both of these carbon sources can also induce increases in the level of isocitrate lyase, it was important to find out whether these compounds have to be metabolized before they have their inducing effect on isocitrate lyase, or whether the induction is due to α -ketoglutarate.

Figure 6A shows the induction of α -ketoglutarate dehydrogenase and isocitrate lyase on α -ketoglutarate in strain CSH78 over an hour. This Figure indicates that there is a parallel induction of the two enzymes over this time. Figure 6B shows a similar induction over a longer time. This Figure shows a lag in the induction of α -ketoglutarate dehydrogenase compared to that of isocitrate lyase. These experiments were not repeated several times, so it is difficult

Figure 6. The induction of isocitrate lyase (\triangle) and α -ketoglutarate dehydrogenase (\blacktriangle) in CSH78 on 1% α -ketoglutarate. A. Induction of the two enzymes over 1 hour. B. Induction over 10 hours. Enzymes were assayed as described in Methods. α -Ketoglutarate dehydrogenase activities were multiplied by 10 before being plotted on the graph.



to decide on the basis of these results whether in fact α -ketoglutarate induces both enzymes at the same rate. There is, however, a low basal level of α -ketoglutarate dehydrogenase in these cells which could still allow some metabolism of α -ketoglutarate beyond the dehydrogenase reaction.

Figure 7A shows a similar experiment with CSH78 in which acetate is the inducing carbon source. These results indicate that on acetate, α -ketoglutarate dehydrogenase and isocitrate lyase are induced at similar rates. Figure 7B shows the induction of the two enzymes on acetate in another wild-type strain MP180. Although the induction of both enzymes is not as great in this strain, the isocitrate lyase and α -ketoglutarate dehydrogenase again appear to be induced at similar rates.

In Figure 8 the α -ketoglutarate dehydrogenase specific activities are plotted versus the isocitrate lyase specific activities to show the relationship between the two more clearly. The induction of the two enzymes by acetate may well be co-ordinate, and this finding raises the possibility that acetate can induce isocitrate lyase only after being metabolized through the α -ketoglutarate dehydrogenase reaction.

To investigate this possibility further, induction experiments were carried out using succinate and L-malate as the inducing carbon source.

Induction of Isocitrate Lyase on Succinate and L-Malate

Figure 9 gives the induction of isocitrate lyase on 1% succinate.

Figure 7. A. The induction of isocitrate lyase (○) and α -ketoglutarate dehydrogenase (●) in CSH78 on 1% acetate. The dotted line represents steady-state levels of α -ketoglutarate dehydrogenase in acetate-grown CSH78. B. The induction of isocitrate lyase (○) and α -ketoglutarate dehydrogenase (●) in the wild-type strain MP 180 on acetate. α -Ketoglutarate dehydrogenase activities were multiplied by 10 before being plotted on the graphs.

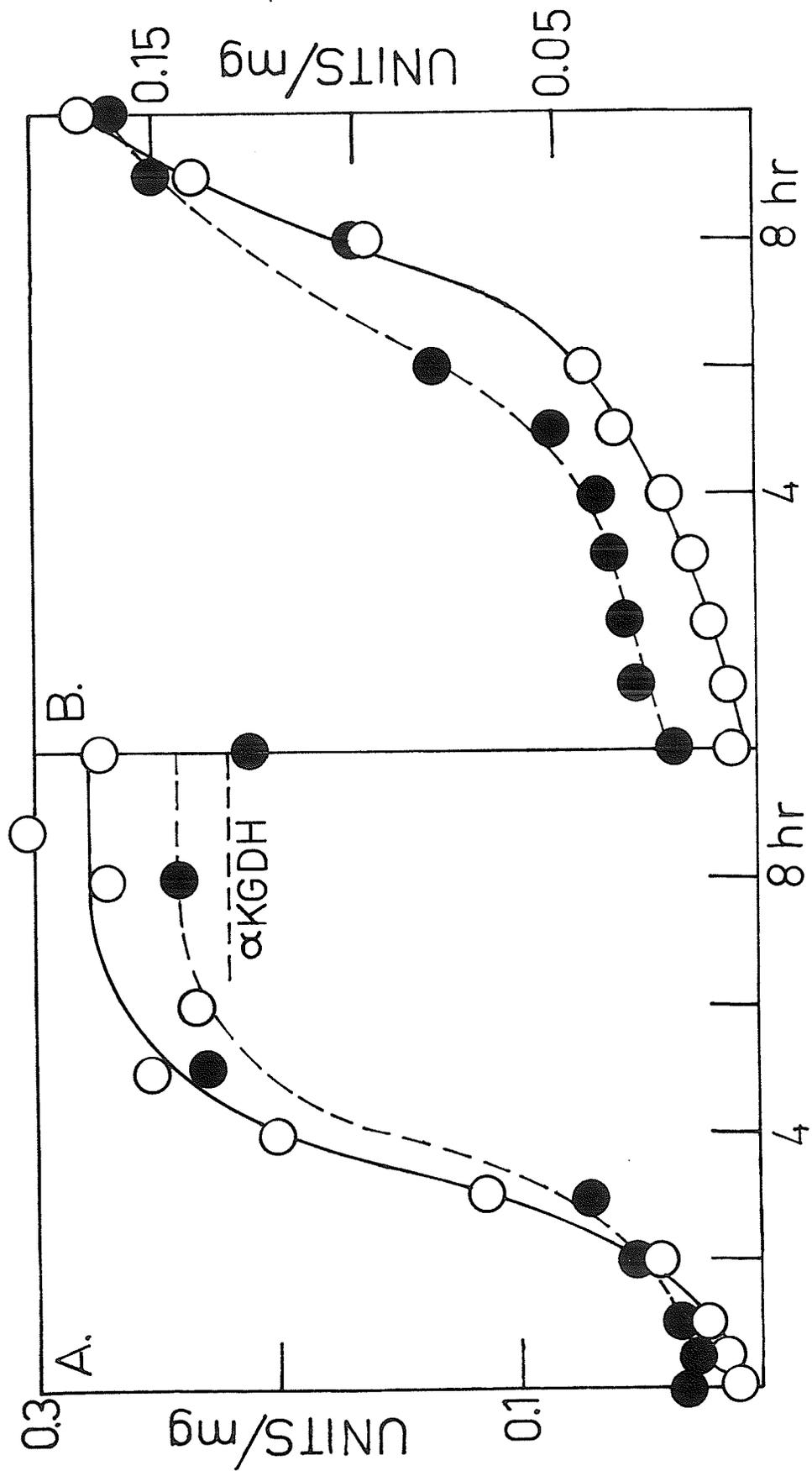


Figure 8. A plot of isocitrate lyase activity versus α -ketoglutarate dehydrogenase activity in CSH78 (●) and MP180 (○) on acetate and CSH78 on α -ketoglutarate (Δ). This is a replot of data in Figures 6B, 7A and 7B.

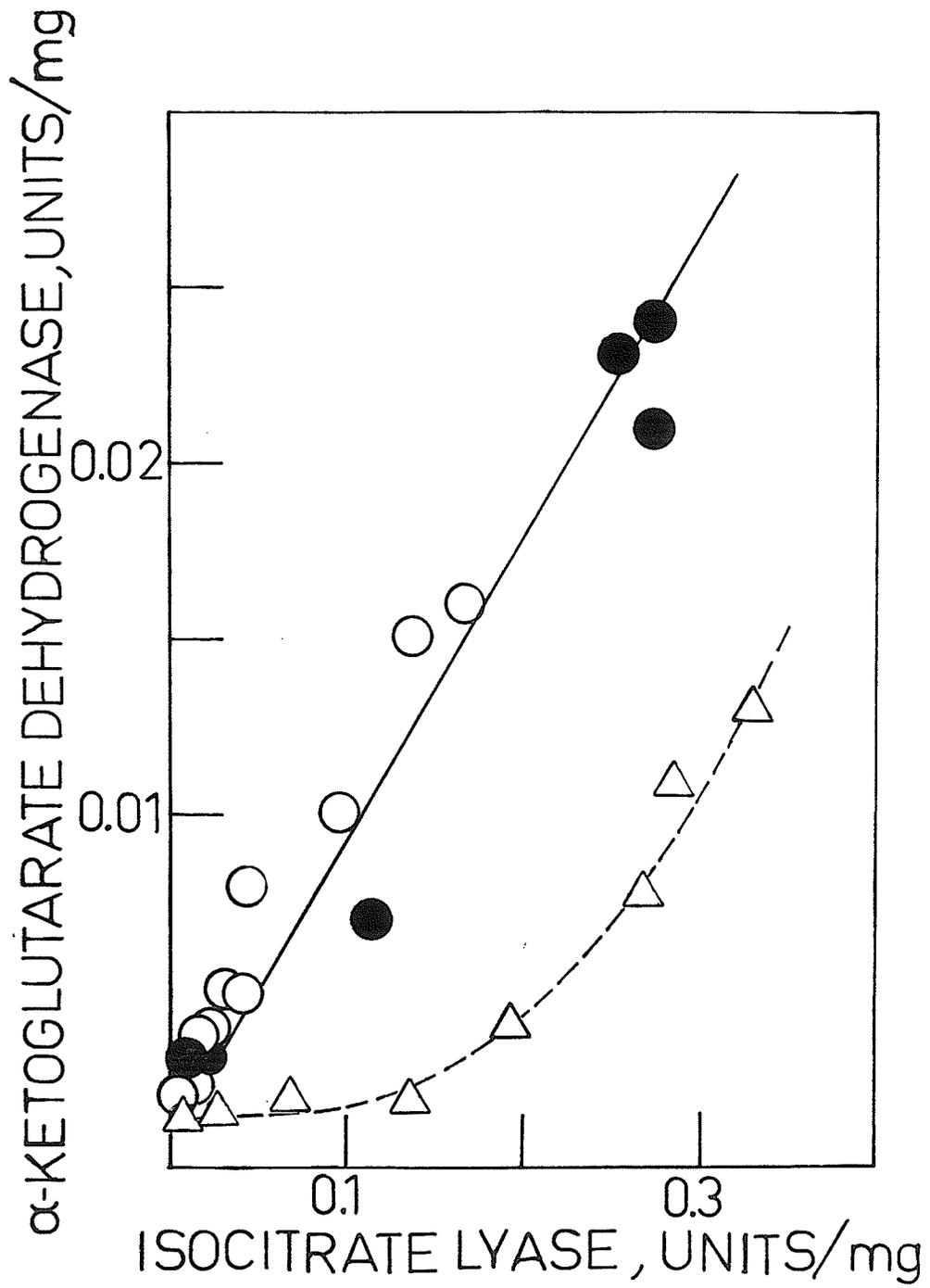
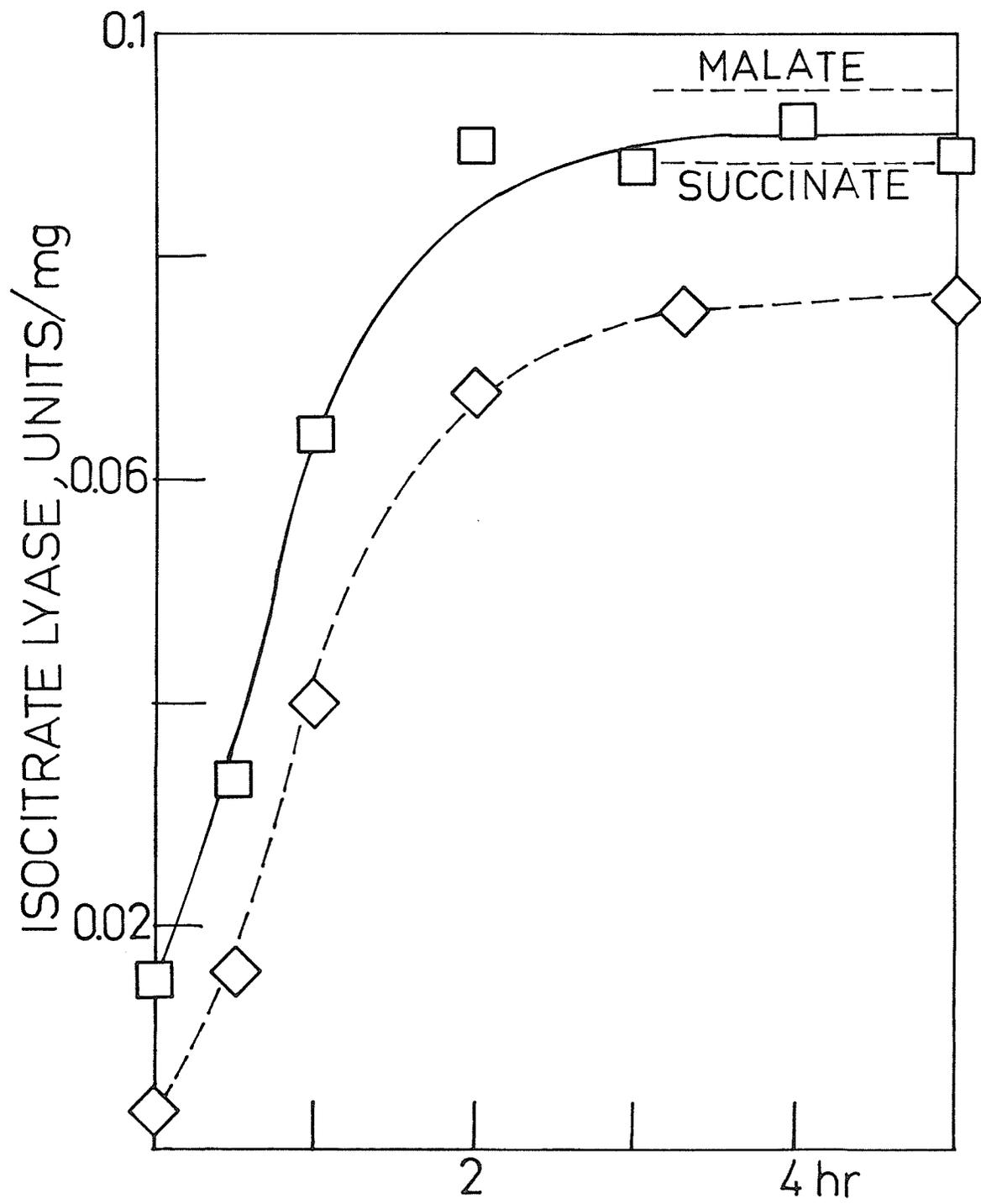


Figure 9. The induction of isocitrate lyase in CSH78 on 1% succinate (□) and 1% L-malate (◇). Other details as in Figure 5.



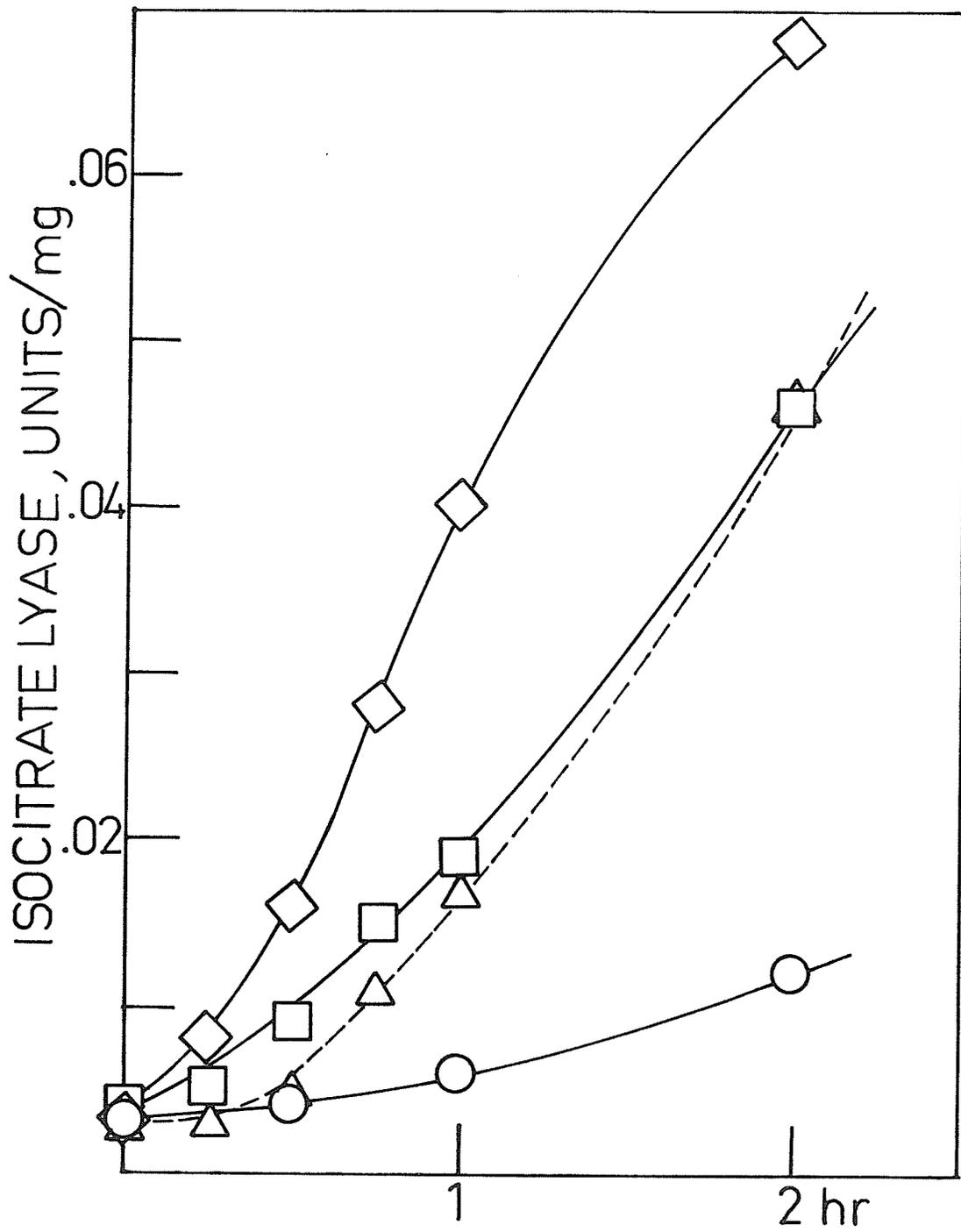
As was seen with the α -ketoglutarate, there is a very rapid rise in the specific activity of the isocitrate lyase, but unlike the result with α -ketoglutarate or acetate, the increase levels off after two hours. The specific activity after two hours is very similar to the steady-state specific activity of isocitrate lyase in CSH78 succinate-grown cells. Figure 9 also shows the induction of isocitrate lyase by 1% L-malate. Again, the rise in specific activity is very rapid but levels off after two hours.

These experiments indicate that the C_4 -dicarboxylic acids of the TCA cycle are also able to bring about very rapid increases in the specific activity of isocitrate lyase. This implies that acetate and α -ketoglutarate are perhaps having their inducing effect on this enzyme only after they are metabolized at least to the level of the C_4 dicarboxylic acids. The effects of succinate and L-malate are not identical to those of acetate and α -ketoglutarate, however. Although they are able to bring about a very rapid increase in the specific activity of isocitrate lyase, this increase stops at a lower level than for either α -ketoglutarate or acetate.

Short-term Induction of Isocitrate Lyase

Since important differences between the various carbon sources were seen in times of onset and initial rates of induction of isocitrate lyase, short term induction experiments were carried out to compare these parameters in more detail. Figure 10 shows the results of several of these experiments. It is very interesting that acetate, the carbon

Figure 10. The short-term induction of isocitrate lyase in CSH78 on acetate (○), α -ketoglutarate (Δ), succinate (\square) and malate (\diamond). All carbon sources were present at 1%. Other details as in Figure 5.



source which is able to produce the highest levels of isocitrate lyase, has its effect only very slowly compared to α -ketoglutarate, succinate and L-malate. The fact that α -ketoglutarate is somewhat slower than succinate or L-malate may reflect the time required to induce α -ketoglutarate dehydrogenase.

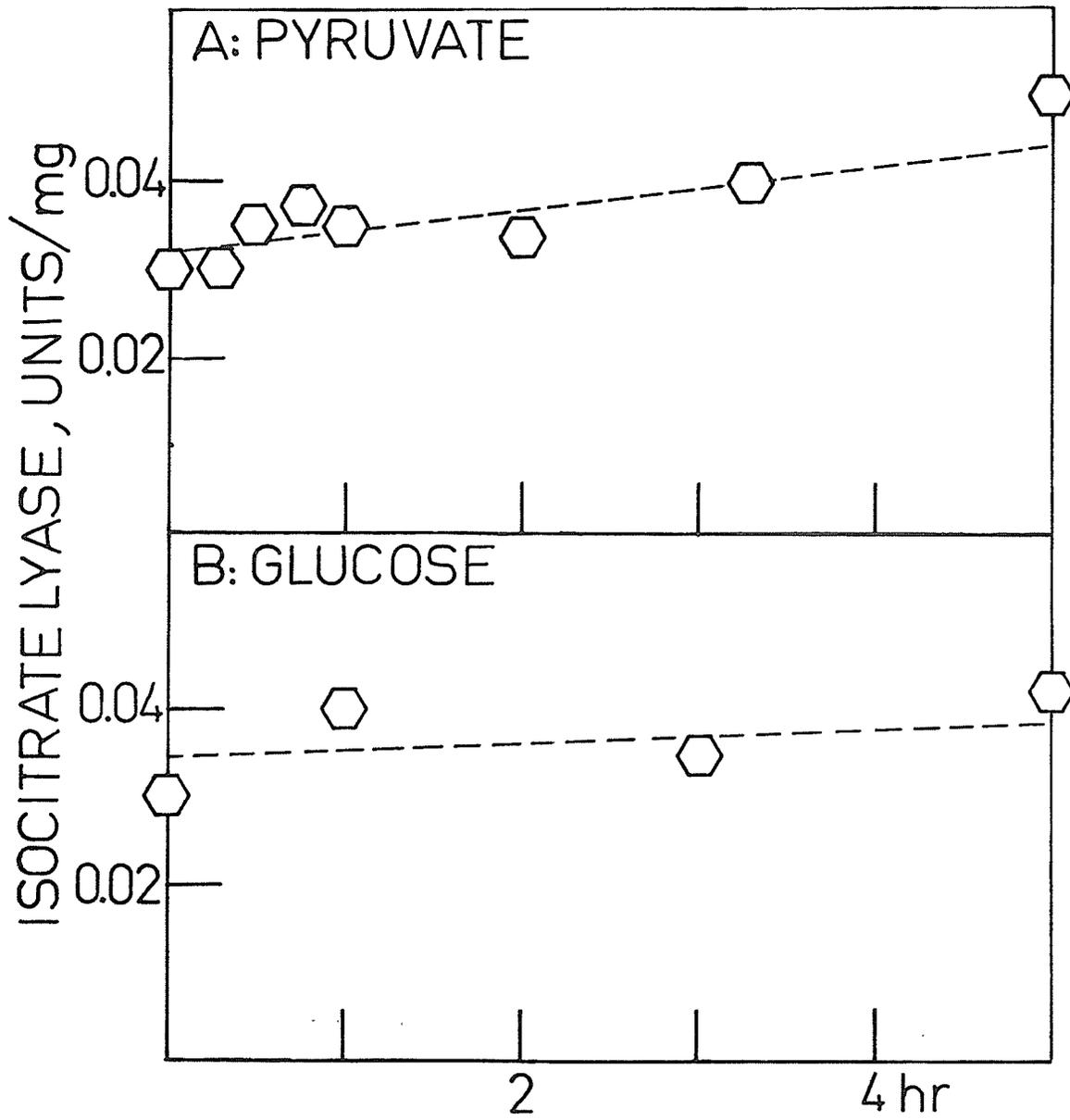
Induction on Pyruvate

One tenable theory for the regulation of isocitrate lyase synthesis, as we have seen, is that pyruvate is a co-repressor of the glyoxylate cycle operon. Figure 11A shows that over a period of five hours, there is essentially no induction of isocitrate lyase on 1% pyruvate in CSH78. This is as might be expected if pyruvate were a co-repressor. It is more difficult to visualize, however, how succinate, malate and α -ketoglutarate are able to bring about a very rapid decline in pyruvate levels whereas acetate can do so only much more slowly.

Figure 11B also shows a control induction experiment in which the glucose-grown cells were transferred to fresh glucose medium. As expected, there is no induction of isocitrate lyase.

These induction experiments show the interesting finding that the time of onset and the initial rate of induction of isocitrate lyase, for the carbon sources studied, do not follow the same hierarchy as that seen for the steady-state levels on these carbon sources. Malate and succinate, which give only intermediate levels of isocitrate lyase, reach these levels very rapidly. Acetate, which produces the highest steady-state levels of the glyoxylate cycle enzymes, induces

Figure 11A. The induction of isocitrate lyase in CSH78 on 1% pyruvate. B. A control experiment showing the effect of glucose on isocitrate lyase induction in CSH78. Cells were treated as described in Methods, resuspended in fresh glucose medium and sampled as for the other carbon sources.



isocitrate lyase rather slowly. This may imply that a certain amount of metabolism of acetate must occur before it can have its inducing effect. The effect of α -ketoglutarate is intermediate: it is able to begin the induction of isocitrate lyase almost as quickly as succinate and L-malate can, and yet it produces steady-state levels of the enzyme which are almost as high as those found on acetate. Table 4 summarizes the time required for the initial doubling of isocitrate lyase on the different carbon sources.

The Effect of Cyclic AMP on the Induction of Isocitrate Lyase

The observation of Makman and Sutherland (1965) that glucose lowers cyclic AMP levels in *E. coli*, coupled with the finding that glucose represses the synthesis of β -galactosidase and other catabolic enzymes, led to the discovery by Perlman and Pastan (1968) that added cyclic AMP can overcome the repressive effect of glucose, not only on β -galactosidase, but on a variety of catabolic enzymes.

Two classes of mutants have been isolated in which the synthesis of enzymes under the control of cyclic AMP is greatly diminished. One class is defective in adenylate cyclase (*cya*) and the second class is missing a cyclic AMP binding protein (*crp*). The defect in a *cya* mutant but not a *crp* mutant can be overcome by adding cyclic AMP to the medium.

As was seen in Table 1, the levels of isocitrate lyase in *E. coli*

Table 4

A summary of the initial rates of increase of isocitrate lyase in CSH78 on different carbon sources.

| Carbon Source | Time Needed For Doubling of Initial Specific Activity (min) | Time Needed To Reach 4 Times Initial Specific Activity (min) |
|-------------------------|---|--|
| malate | 12 | 25 |
| succinate | 25 | 54 |
| α -ketoglutarate | 33 | 48 |
| acetate | 60 | 120 |
| pyruvate | >300 | - |

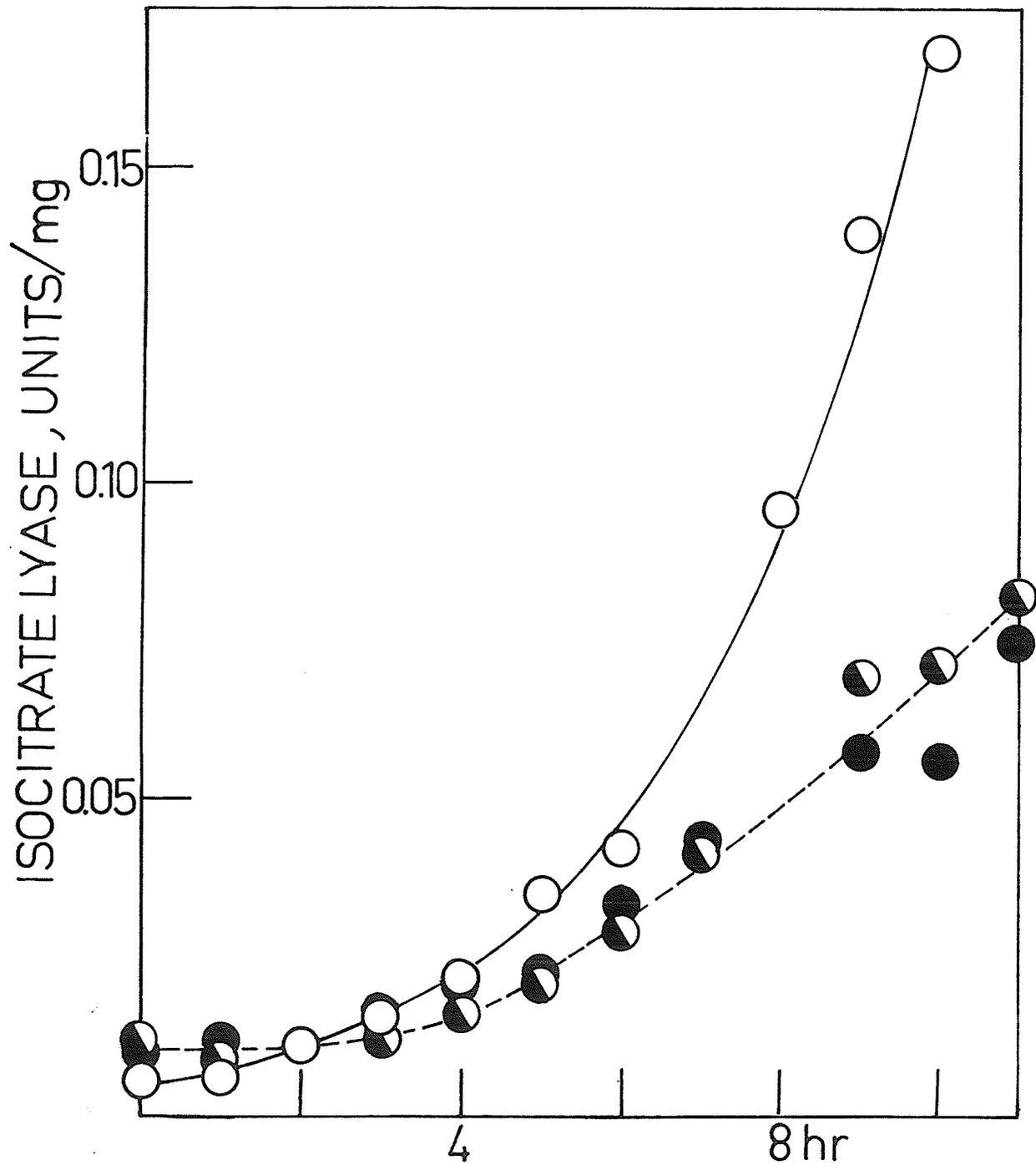
The times required to reach 2X and 4X the initial levels of isocitrate lyase are calculated from the data shown in Figures 10 and 11.

K12 vary greatly depending on the carbon source on which the cells are grown. The lowest levels are found in glucose-grown cells, the highest in acetate-grown cells. This hierarchy of carbon sources suggested the possibility that cyclic AMP might be involved in the regulation of the glyoxylate cycle enzymes. This idea was further reinforced when it was found that strains MP258 (*crp*) and MP259 (*cya*) could not grow on acetate, but that if 1 mM cyclic AMP was added to the *cya* strain, growth on acetate could occur. It is known, however, that cyclic AMP is required for the syntheses of the oxidative phosphorylation system (Hempfling and Beeman 1971), cytochromes (Broman *et al* 1974) and succinate dehydrogenase (Takahaski 1975). It was therefore a possibility that *cya* and *crp* mutants do not grow on acetate because of a lack of cytochromes or succinate dehydrogenase, rather than because they are unable to induce the glyoxylate cycle enzymes.

In order to test whether there is a direct effect of cyclic AMP on isocitrate lyase synthesis, a series of induction experiments was carried out using MP258 (*crp*), MP259 (*cya*) and the parent strain MP180. The *crp* strain was not used extensively because it reverts at a rather high frequency. The *cya* strain is quite stable.

Figure 12 shows the induction of MP180, MP258, and MP259 on 1% acetate. All three strains are able to induce isocitrate lyase on acetate although the *cya* and *crp* mutants seem to initiate induction on this carbon source somewhat more slowly than the parent MP180 does. The mutants have a higher initial level of isocitrate lyase than does the wild-type parent. This seems to be characteristic of the

Figure 12. The induction of isocitrate lyase on 1% acetate in the wild-type parent MP180 (○) and in MP258 (*crp*) (◐) and MP259 (*cya*) (●). Details as for Figure 5.



cya and *crp* mutants and was seen repeatedly during induction experiments on different carbon sources. The reason for this somewhat elevated level (3-4X) is not known.

Figure 13 shows the induction of isocitrate lyase in MP259 (*cya*) on 1% acetate with 1 mM cyclic AMP added. This Figure also shows another induction experiment of MP259 on acetate alone as a control. The *cya* mutant shows a greater induction of isocitrate lyase on acetate with cyclic AMP than without cyclic AMP, but the time of onset of induction is similar in both cases. The mutant in the presence of cyclic AMP induces isocitrate lyase to a slightly greater extent than the parent MP180 does over the time of the experiment (compare Figure 12) but this may simply reflect an increase in metabolism in the presence of cyclic AMP.

Unless cyclic AMP is added, a *cya* mutant is also unable to grow on proline, α -ketoglutarate, or succinate - all compounds which in the wild-type *E. coli* can induce isocitrate lyase to varying extents.

Figure 14 shows the induction of isocitrate lyase in the *cya* mutant MP259 on α -ketoglutarate and succinate. α -Ketoglutarate does not induce as well in this mutant as it does in the parent MP180 (not shown), nor does it induce as well as acetate. Succinate shows only a very slight effect on the level of isocitrate lyase in this strain. The minimal effect of succinate in this mutant is probably a reflection of the fact that cyclic AMP is required for the induction of the succinate transport system in *E. coli* (Lo *et al* 1972).

Figure 13. The induction of the isocitrate lyase in the *cya* mutant MP259 on 1% acetate alone (○), and 1% acetate plus 1 mM cyclic AMP (●). Cyclic AMP was added to the cells only at the time of resuspension in the acetate medium.

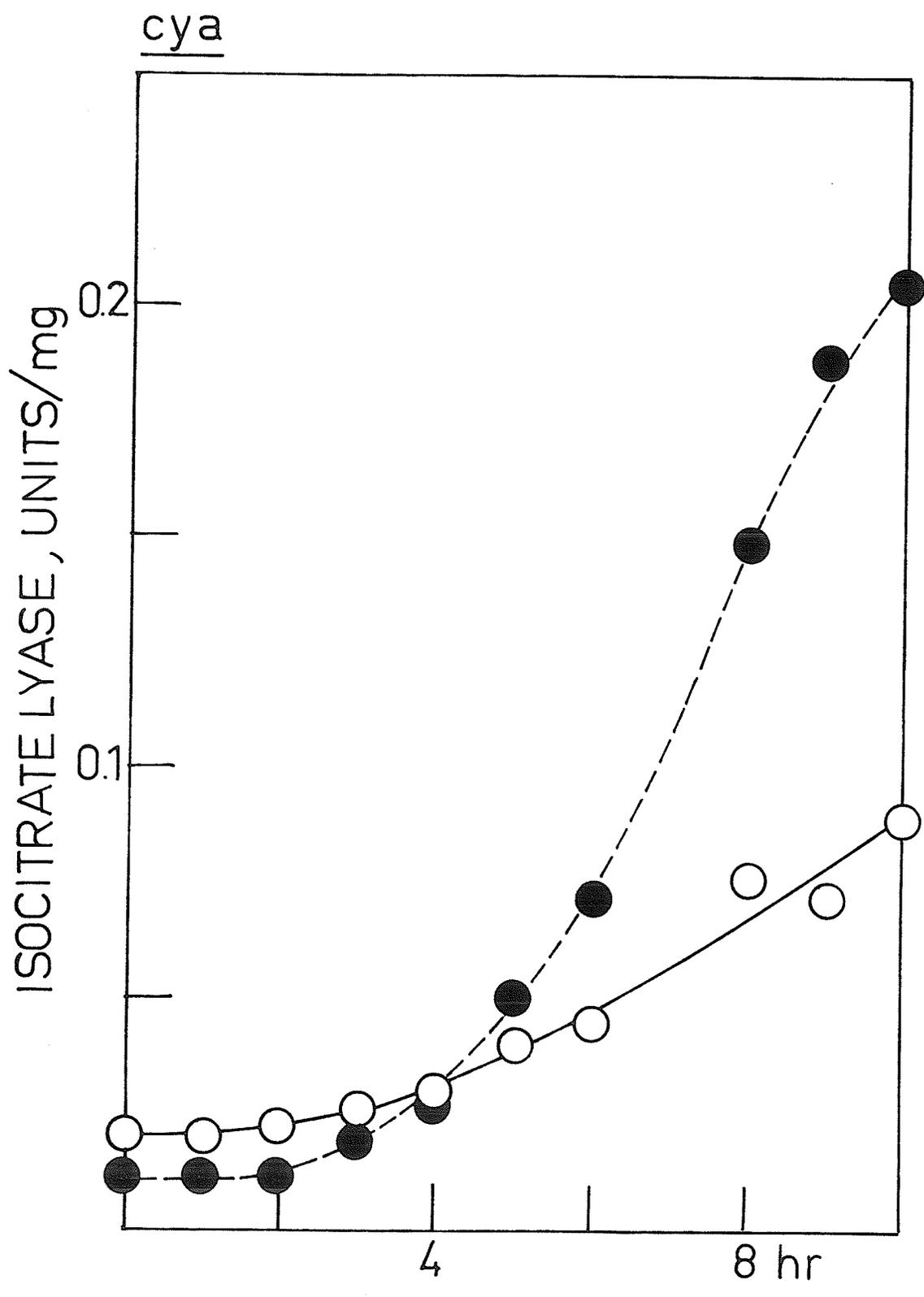
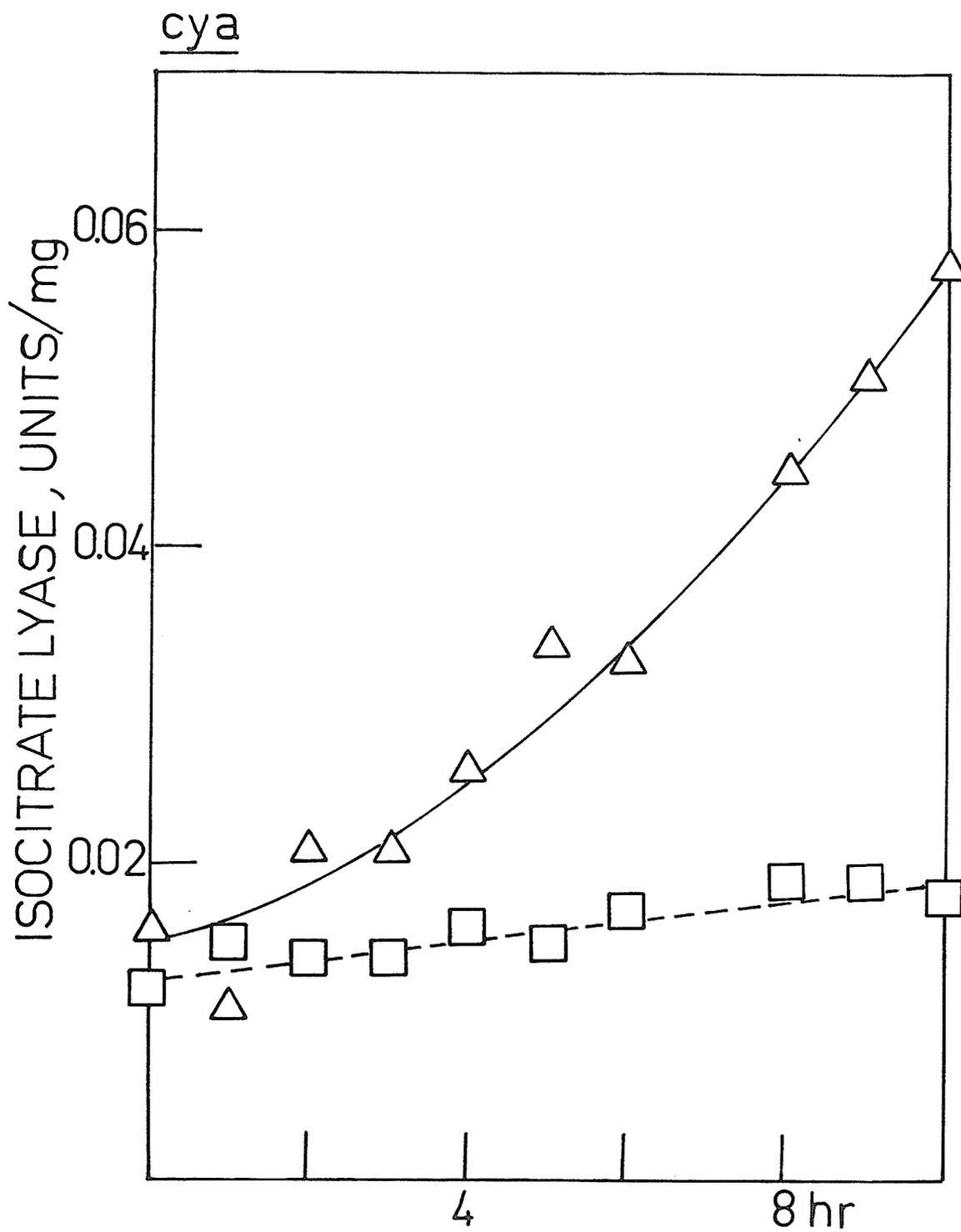


Figure 14. The induction of isocitrate lyase in MP259 (*cya*)
on 1% α -ketoglutarate (Δ) and 1% succinate (\square).



Whether cyclic AMP also has an effect on the transport of α -ketoglutarate is not known. The somewhat poorer inducing effect of α -ketoglutarate might be due to reduced metabolism through the TCA cycle because of low levels of succinate dehydrogenase and cytochromes. These factors as stated earlier might be expected to influence acetate metabolism as well, and to some extent they do. It is quite clear, however, that the actual induction of isocitrate lyase, and by inference that of malate synthase A, does not require the presence of cyclic AMP. Glucose must therefore be having a repressive effect on this operon by a different mechanism than that seen for many catabolic enzymes.

The Induction of Isocitrate Lyase in Mutants of the TCA Cycle

The use of mutants of the TCA cycle enzymes as a means of trying to determine the regulatory compound of the glyoxylate cycle was suggested by two observations:

1. Carbon sources other than acetate are rapid and effective inducers of isocitrate lyase.
2. It appears at least for acetate and α -ketoglutarate that some metabolism occurs before induction.

Mutants have been used in previous work on the glyoxylate cycle, although not mutants of the TCA cycle except for one lacking citrate synthase (Kornberg 1966). The approach taken for the experiments

described in this work was not to grow mutants on particular carbon sources, but rather to carry out induction experiments as had been done for the wild-type. Such experiments using the adenylate cyclase (*cyd*) mutant MP259 indicated that enzyme induction can occur even under conditions in which the strain is unable to grow. Thus, by using different combinations of mutants and carbon sources it should be possible to gain further insights into the control of isocitrate lyase synthesis.

Induction in Citrate Synthase (*glt A*) Mutants

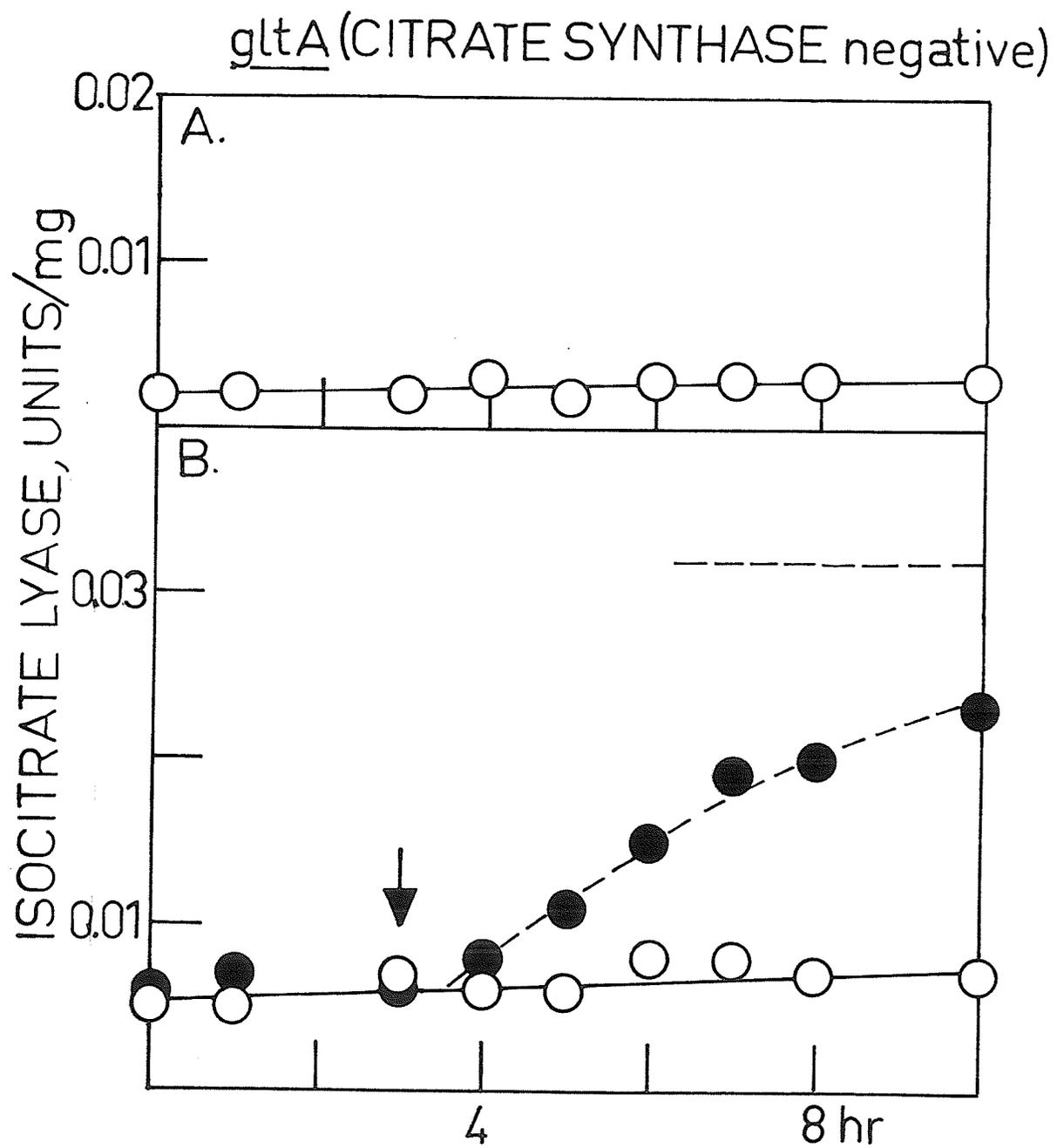
Two different *glt A* mutants were used for these experiments, H2 and MLD 43. Neither is able to grow on acetate, or glucose. If 0.01% proline or glutamate is added as a supplement, both are able to grow on glucose, but not acetate.

Figure 15 shows the induction on 1% acetate of both H2 (Figure 15A) and MLD 43 (Figure 15B). Casamino acids (0.1%) were also added to try to overcome any glutamate starvation. Neither strain shows any induction of isocitrate lyase on acetate.

Figure 15B also shows the induction of MLD 43 on 1% acetate to which after 3 hours, 0.5% glycollate was added. This is the reverse experiment to one that Kornberg (1966) did in which he added acetate to cells growing on glycollate plus glutamate. The induction experiment shows that after the addition of glycollate, isocitrate lyase levels rise, and after eight hours approach those seen for steady-state levels on glycollate. Glycollate can therefore induce isocitrate lyase in

Figure 15. A. The induction of isocitrate lyase in the *glt A* mutant H2 on 1% acetate plus 0.1% Casamino acids (○).

B. The induction of isocitrate lyase in the *glt A* mutant MLD 43 on 1% acetate plus 0.1% Casamino acids (○) and on the same carbon source to which 0.4% glycollate was added (↓) (○). The dotted line shows the steady-state level of isocitrate lyase in the wild-type parent CSH78 on glycollate. Both mutants were grown on 0.4% glucose plus 0.01% proline prior to the induction experiment.



this strain even in the presence of acetate. Kornberg found that in a *glt A* mutant of *E. coli* W growing on glycollate and glutamate, acetate caused a decrease in isocitrate lyase levels and argued from this that a co-repressor was being formed in these cells. The experiment presented here is not in agreement with this proposal.

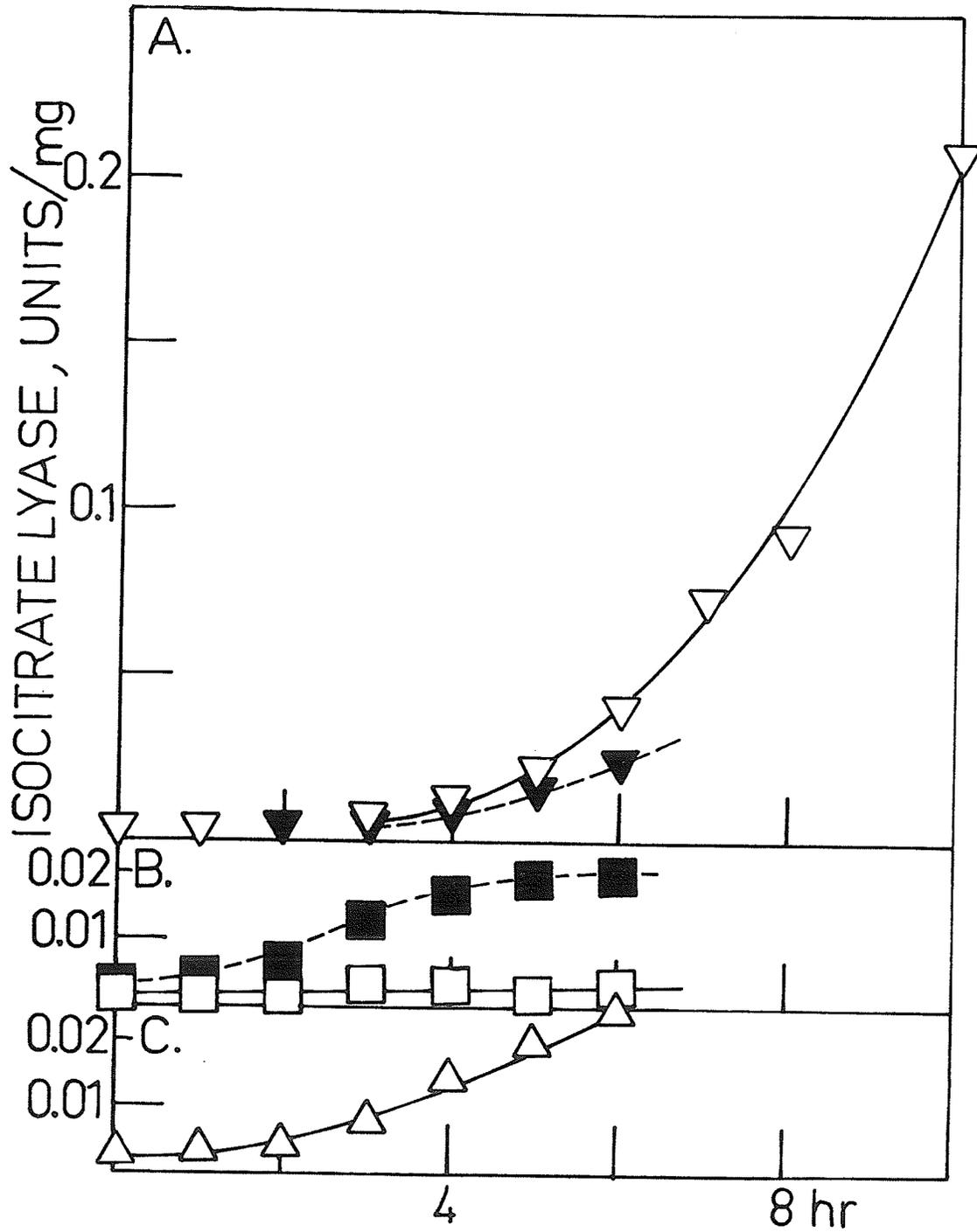
Proline is an extremely good inducer of isocitrate lyase in the *glt A* strain H2 (Figure 16A). The induction curve is very similar to that seen for the wild-type strain (Figure 5), although the onset of induction does appear to be somewhat slower.

This finding is confirmed by a similar induction pattern for 1% α -ketoglutarate (Figure 16C). This experiment was conducted over a shorter time, but the amount of induction over five hours is similar for proline and α -ketoglutarate.

Figure 16B shows the induction of strain H2 on 1% succinate, plus or minus 0.01% proline which was added as a supplement. Succinate, with no proline added, has no inducing effect on isocitrate lyase. When the small amount of proline is present, there is an induction although the rate of increase of the enzyme is slower than the wild-type and the level reached after six hours is lower. The rate, nonetheless is faster than that seen for proline or α -ketoglutarate alone. The control experiment, in which induction was carried out in 0.01% proline alone (Figure 16A) also produces an increase in isocitrate lyase. The onset of the induction is considerably slower, however, than that seen for succinate plus proline.

Figure 16. A. The induction of isocitrate lyase in the *glt A* mutant H2 on 1% proline (∇) and 0.01% proline (\blacktriangledown). B. The induction of isocitrate lyase in H2 on 1% succinate (\square) and 1% succinate plus 0.01% proline (\blacksquare). C. The induction of isocitrate lyase in H2 on 1% α -ketoglutarate (\triangle).

gltA (CITRATE SYNTHASE negative)



Induction in an Isocitrate Dehydrogenase (*icd*) Mutant

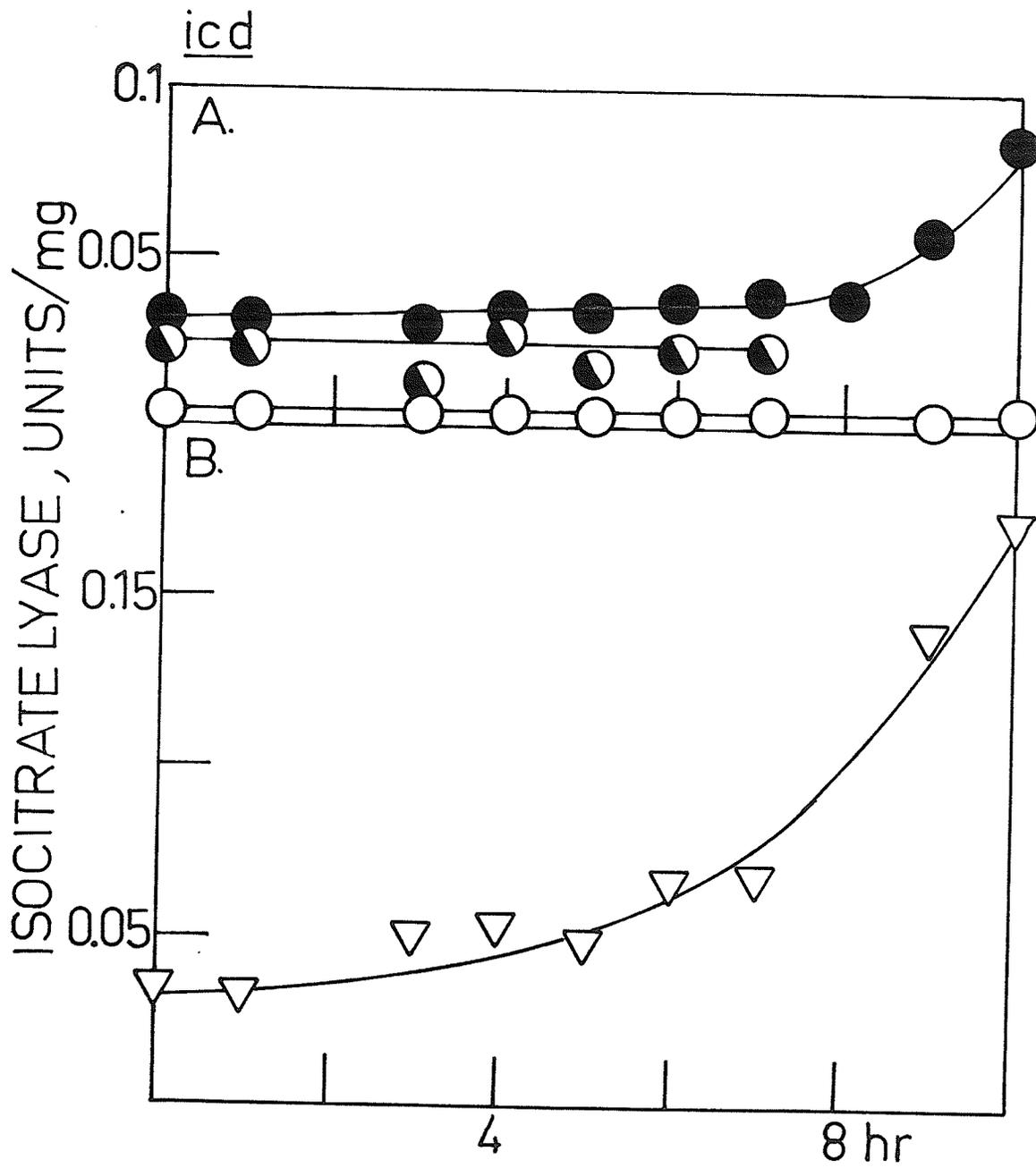
The strain MLD 42 was used for these experiments. It was isolated as a nalidixic acid resistant (10 µg/ml) mutant which is unable to grow on acetate or glucose (Helling and Kukora 1971). This strain will grow on glucose if supplemented with proline or glutamate.

Figure 17A shows three induction experiments with this mutant on 1% acetate with or without proline or Casamino acids as a supplement. In none of these experiments is there any induction by acetate at early times. After ten hours, the acetate plus proline experiment does show a two-fold increase in isocitrate lyase specific activity. This may be due to the late induction by the small amount (0.01%) of proline present. Figure 17B shows that 1% proline is able to stimulate induction of isocitrate lyase in this mutant. The induction is again rather slower than that seen for a wild-type strain, but similar to that for a *glt A* strain.

The initial levels of isocitrate lyase from glucose-grown *icd* cells are consistently higher than those in the wild-type or the *glt A* strain. It could be argued that the small amount of proline added as a supplement to the glucose medium was having an inducing effect even in this carbon source, but a similar supplement is added to the glucose medium of the *glt A* mutants, and they do not show these elevated levels.

These induction experiments with the *icd* mutant confirm the results from the *glt A* mutant. Since acetate does not induce

Figure 17. A. The induction of isocitrate lyase
icd mutant MLD 42 on 1% acetate (○), 1% acetate plus 0.01% proline
(●) and 1% acetate plus 0.1% Casamino acids (◐). B. The
induction of isocitrate lyase in MLD 42 on 1% proline (▽). The
cells were grown on 0.4% glucose plus 0.01% proline prior to the
induction experiment.



isocitrate lyase, but proline does, isocitrate does not appear to be involved in the induction or derepression of the glyoxylate cycle operon.

Induction in an α -Ketoglutarate Dehydrogenase (*suc A*) Mutant

The mutant originally used in these induction experiments was CGSC4456 (*suc A*). This strain lacks the decarboxylase component of α -ketoglutarate dehydrogenase and requires either succinate, or lysine plus methionine (Herbert and Guest 1968) as a supplement while growing on glucose. It does not grow on acetate or proline as sole carbon source, and only very poorly on succinate.

When the steady-state levels of isocitrate lyase on glucose were examined in this strain, however, it was found to have elevated levels compared to the wild-type strain CSH78 (Table 5).

To test whether the elevated levels were characteristic of a *suc A* mutation, or whether they were due to a fortuitous *icl R* mutation, CGSC4456 was transduced to *suc A*⁺ by selecting glucose positive transductants. Two such transductants were tested for levels of isocitrate lyase on glucose, and these were found still to be elevated (Table 5). The two-fold higher levels in the transductants as compared to the *suc A* strain probably do not reflect a significant difference, but rather simply the better growth on glucose of these wild-type cells.

It appears therefore that *suc A* mutants do not have elevated

Table 5

The specific activity of isocitrate lyase in wild-type CSH78, and in CGSC4456 (*suc A*) and a *suc A*⁺ transductant of CGSC4456 on glucose.

| Strain | Carbon Source | Isocitrate lyase (units/mg) |
|------------------------------------|---------------|--------------------------------|
| CSH78 | glucose | 0.003 |
| CSH78 | acetate | 0.29 |
| CGSC4456 | glucose | 0.12 |
| CGSC4456 <i>suc A</i> ⁺ | glucose | 0.20, 0.21 |

levels of isocitrate lyase because of a mutation in α -ketoglutarate dehydrogenase. Whether there is a selective pressure in these mutants to produce *icl R* mutations is not clear. Strain CGSC4456 still cannot grow on acetate even with the constitutive mutation. Genetic experiments (see Genetic Studies) have shown this constitutive mutation to map at *icl R*.

For the induction experiments presented here, the *suc A* mutation was transduced into strain PL2 which is *gal K*, and galactose positive transductants were selected on medium containing lysine plus methionine. One of these, MLD 41, which could not grow on glucose unless supplemented with succinate or lysine plus methionine, was used for further experiments.

This α -ketoglutarate dehydrogenase mutant does not induce isocitrate lyase on 1% acetate (Figure 18A). The level of enzyme actually appears to decline slightly. The other carbon sources tried in induction experiments, proline, α -ketoglutarate and succinate also give no induction of isocitrate lyase (Figure 18 B+D).

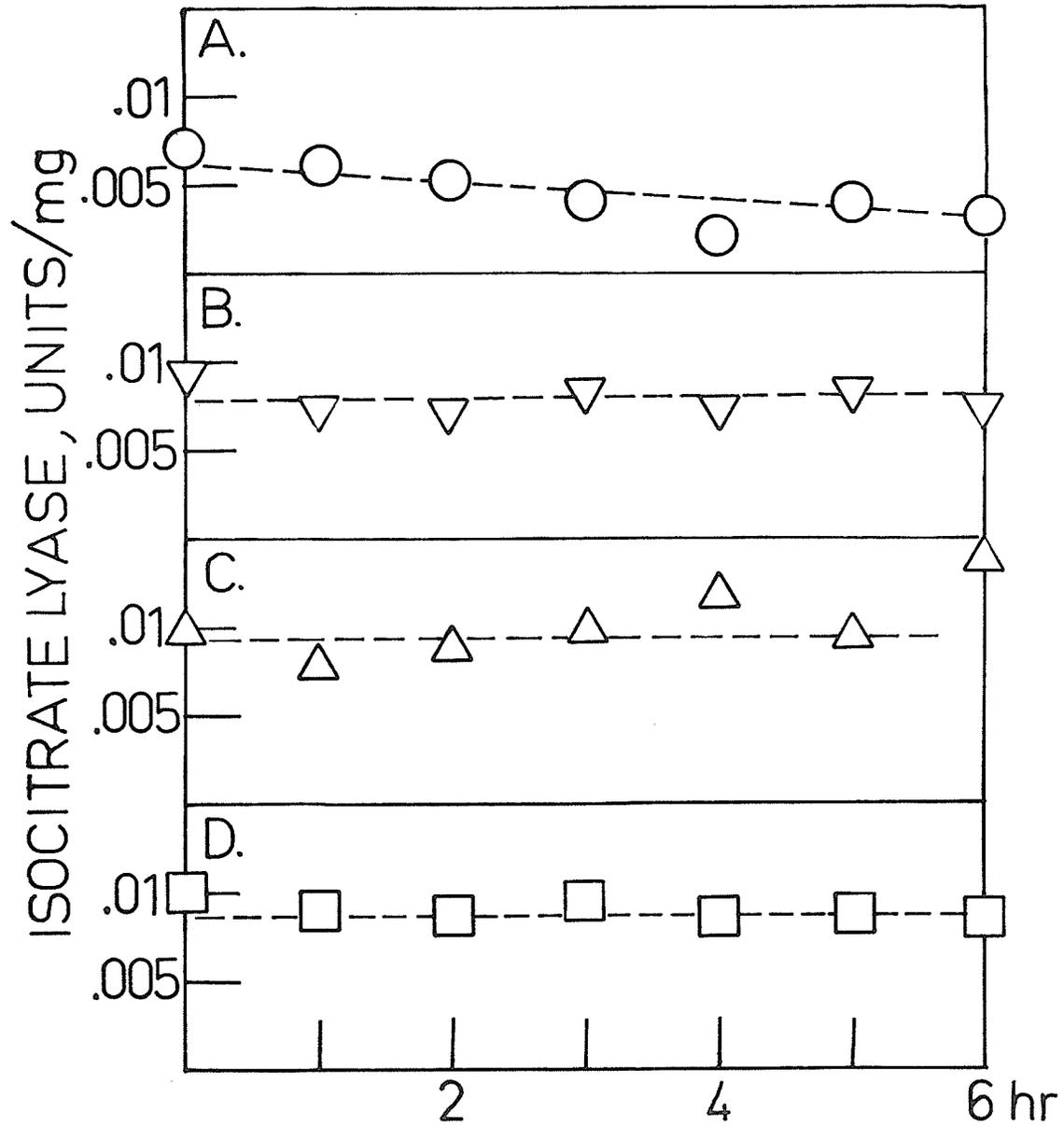
It is perhaps not surprising that α -ketoglutarate and proline cannot induce in a *suc A* mutant. Further metabolism of these compounds cannot occur in this mutant, and unless either of them is an inducer itself, no induction could occur.

Induction in a Succinate Dehydrogenase Mutant (*sdh*)

The strain used for these experiments was 604-30S which is lacking succinate dehydrogenase (*sdh*) and fumarate reductase (*frd*). This

Figure 18. The induction of isocitrate lyase in the *suc A* mutant MLD 41 on A: 1% acetate (○); B: 1% proline (▽); C: 1% α-ketoglutarate (△); and D: 1% succinate (□). All cells were grown on 0.4% glucose plus 0.1% succinate prior to the induction experiment.

sucA (α -KGDH negative)



latter enzyme is important in *E. coli* only during growth on glucose, or under anaerobic conditions. This mutant can grow on glucose, but not on succinate or acetate. Transductants which were selected for growth on succinate became acetate positive as well.

Figure 19A shows the induction of 604-30S on 1% acetate with and without 0.1% Casamino acids. In neither case is there any induction of isocitrate lyase. The Casamino acids were added to try to overcome any amino acid starvation that might occur. A similar experiment (data not shown) in which 0.05% L-malate was added with 1% acetate showed identical results to 1% acetate alone. Malate was added to try to eliminate the possibility that acetate could not induce because there was not sufficient oxaloacetic acid in the cell to react with any acetyl CoA formed.

Proline shows a very slight (3X) induction of isocitrate lyase with and without Casamino acids (Figure 19B), but this is far below the amount of induction seen on a wild-type or *glt A* strain over the same time. Figure 19C also shows that α -ketoglutarate is unable to induce isocitrate lyase.

These results would appear to indicate that if a carbon source cannot be metabolized beyond succinate it is unable to have an inducing effect on the synthesis of isocitrate lyase. However, when fumarate and malate are used as the inducing carbon sources (Figure 20 A,B) they also have only very small effects on levels of the enzyme. Fumarate shows about a three-fold increase in activity after six hours, and malate a similar increase. Since malate and presumably

Figure 19. The induction of isocitrate lyase in the *sdh* mutant 604-30S on A: 1% acetate with (●) and without 0.1% Casamino acids (○); B: 1% proline with (▼) and without (▽) Casamino acids; and C; 1% α -ketoglutarate (Δ).

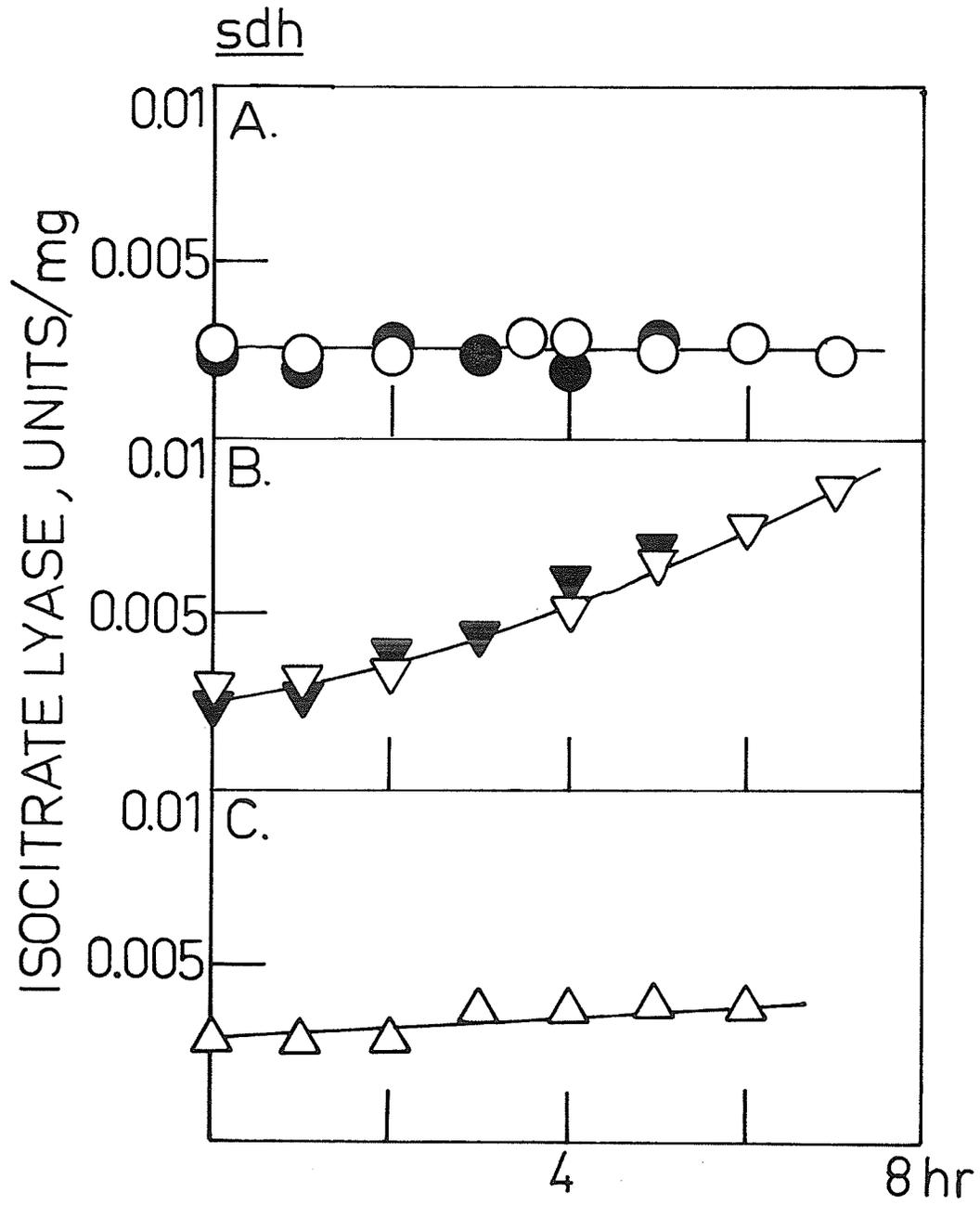
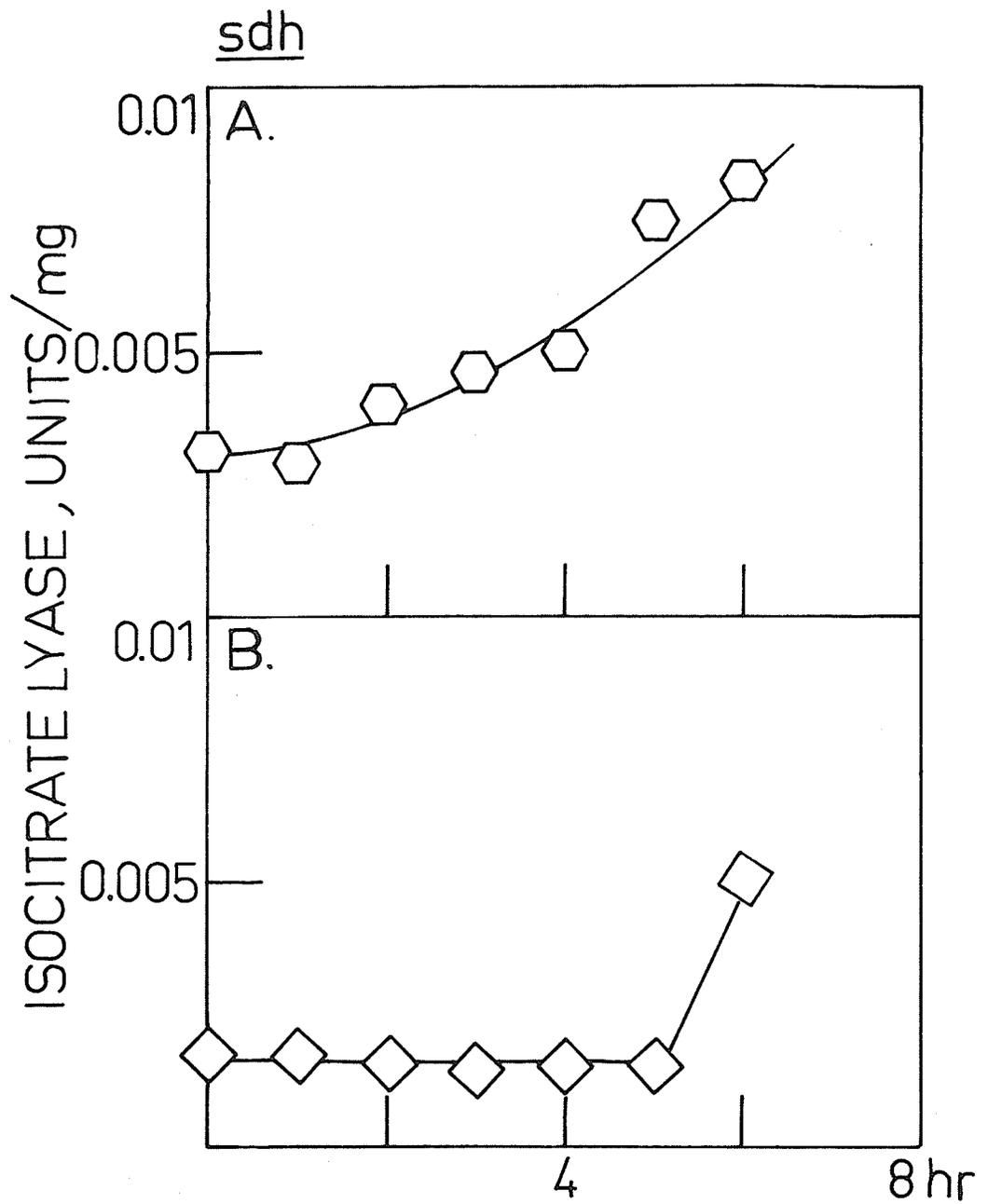


Figure 20. A. The induction of isocitrate lyase in the *sdh* mutant 604-30S on 1% fumarate (\circ). B. The induction on 1% DL-malate (\diamond).



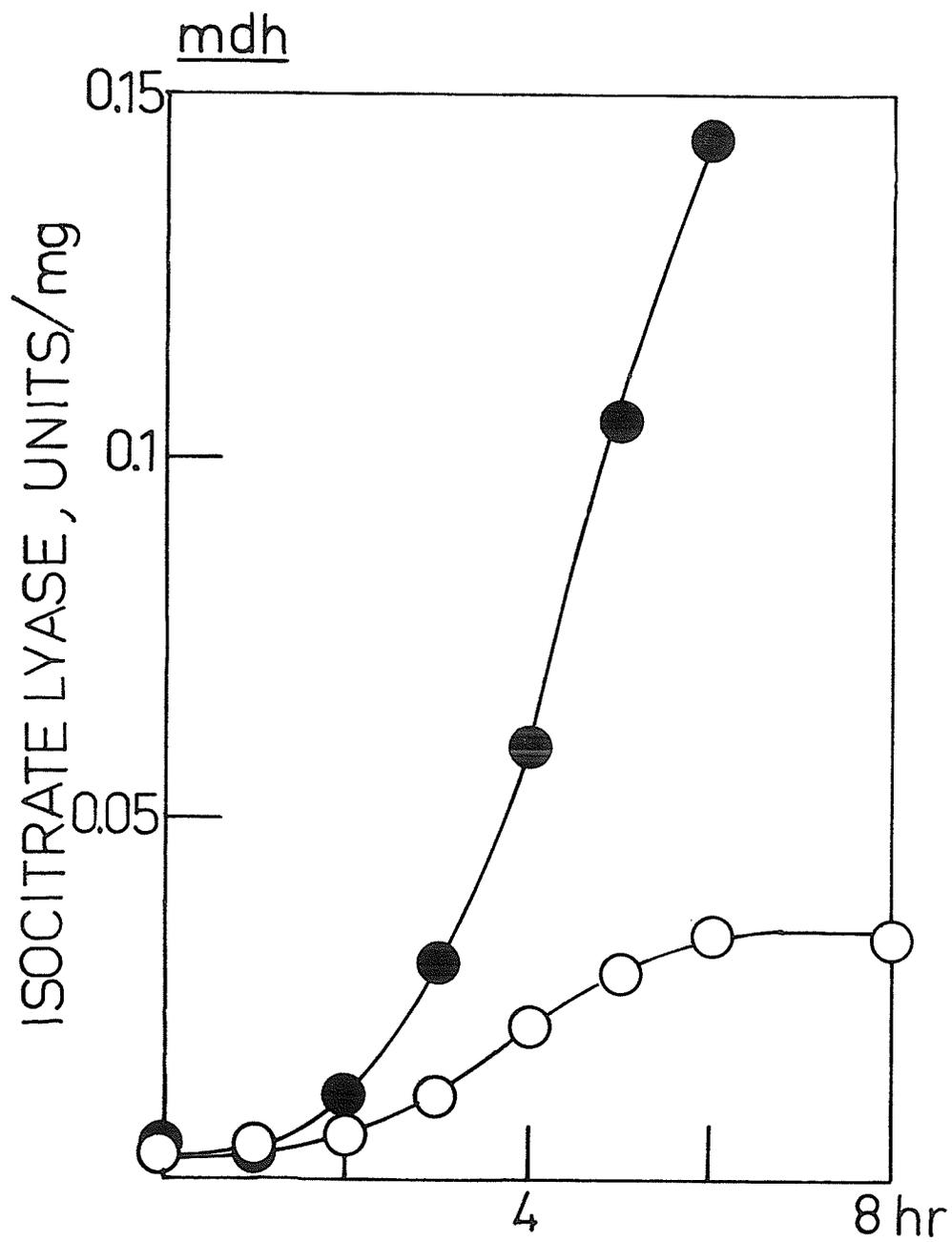
fumarate can induce isocitrate lyase in a wild-type and since they occur beyond the succinate dehydrogenase block, it is somewhat unexpected that these carbon sources should not have a greater effect on the synthesis of isocitrate lyase.

Induction in a Malate Dehydrogenase Mutant (*mdh*)

The malate dehydrogenase mutant (*mdh*) used for these induction experiments was HG38. This strain is also deleted for the lactose-proline region (Δ *lac pro*) and requires proline as a supplement. It is unable to grow on L-malate or acetate, but it can grow on glucose and to some extent succinate.

Figure 21 shows the induction of isocitrate lyase in HG38 on 1% acetate with a proline supplement of 0.01%. This strain shows good induction on acetate, and the time of onset of induction, although perhaps not the rate, is similar to that of the wild-type strain. The presence of 0.01% proline in the growth medium might be complicating the results, however, since with the *glt A* mutant it was found that this concentration of proline had an inducing effect on isocitrate lyase (Figure 16A). Figure 21 also shows an induction experiment on acetate in which a proline supplement of only 0.002% was added. Again, there is induction, although the final level of isocitrate lyase is quite low. This low level probably reflects the extremely small supply of proline added to satisfy the amino acid requirement in this experiment. The time of onset of isocitrate lyase induction, and the initial rate of increase, are nonetheless similar for both proline concentrations.

Figure 21. The induction of isocitrate lyase in the *mdh* mutant HG38 on 1% acetate supplemented with 0.01% proline (●), and with 0.002% proline (○).

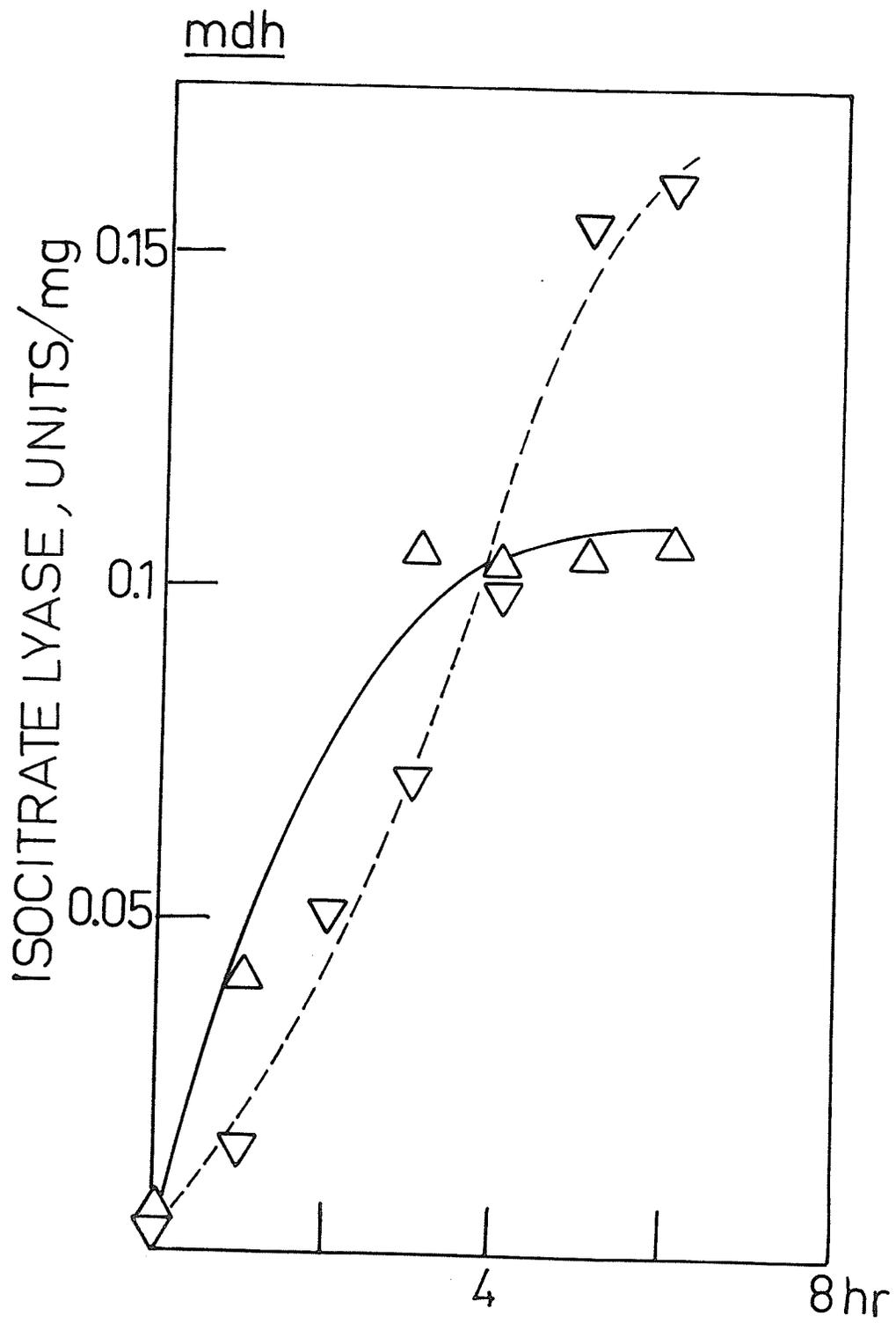


Proline is a very good inducer of isocitrate lyase in this strain, although α -ketoglutarate is not as effective (Figure 22). In the wild-type strain, it will be recalled, these two compounds show very similar induction levels. As in the wild-type, α -ketoglutarate induces isocitrate lyase more rapidly than proline does, but unlike the wild-type this induction levels off at a lower specific activity in the *mdh* mutant.

This *mdh* mutant is the first mutation in an enzyme in the TCA cycle beyond α -ketoglutarate dehydrogenase which shows induction of isocitrate lyase on acetate and proline. It is the only mutant of a TCA cycle enzyme of those tested in these induction experiments, which induces isocitrate lyase on acetate. This presents the intriguing question of how this mutant is able to metabolize the acetate in order to bring about this induction since the mutant should have difficulty producing oxaloacetate, and it has been shown above that a *glt A* mutant, which cannot condense acetyl CoA and oxaloacetate, cannot induce on acetate.

HG38 contains no malate dehydrogenase, and it is not a leaky mutant. Goldie *et al* (1978) have demonstrated the presence of a malate oxidase in mutants such as HG38 which totally lack malate dehydrogenase. To test whether this mutant was inducing a malate oxidase, this enzyme was assayed in the same samples as were assayed for isocitrate lyase after induction on proline and on acetate. Levels of the malate oxidase were extremely low (0.016 ± 0.003 units/mg)

Figure 22. The induction of isocitrate lyase in the *mdh* mutant HG38 on 1% proline (∇) and 1% α -ketoglutarate (Δ). The α -ketoglutarate medium was supplemented with 0.01% proline since this strain is (Δ *lac pro*).



and did not increase during the induction experiment. It therefore seems highly unlikely that this mutant is able to produce oxaloacetic acid via a malate oxidase under the conditions of these experiments.

Another alternative is that malate is metabolized via the NAD-malic enzyme to pyruvate which is then metabolized to PEP via PEP synthase. Oxaloacetic acid could then be formed from PEP by PEP carboxylase. Whatever route is used to make oxaloacetic acid, it does not appear to be efficient enough to allow these mutants to grow on acetate as sole carbon source.

The Induction of Isocitrate Lyase in a Malate Synthase Mutant

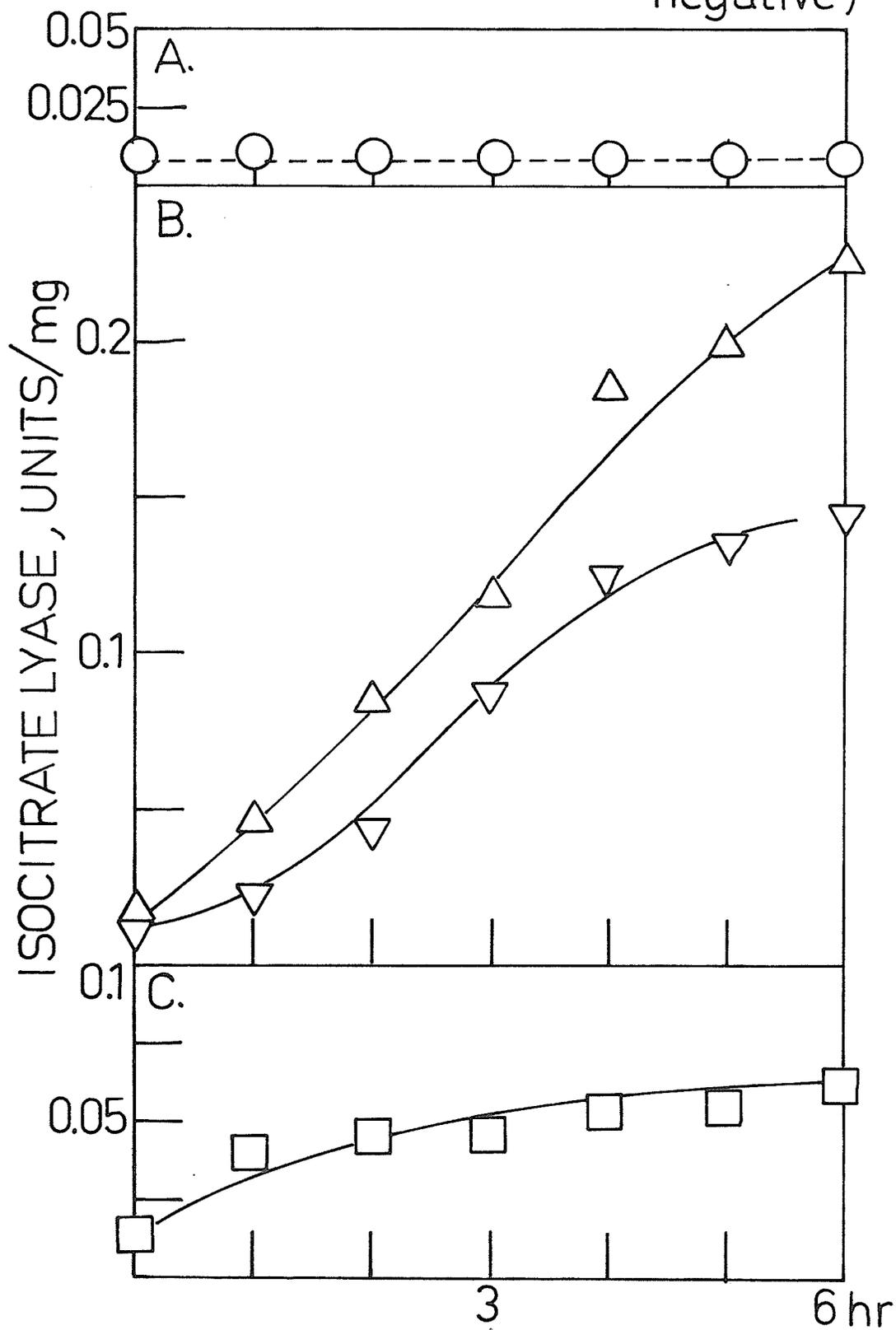
The strain MLD 44, which is a double mutant for malate synthase A (*ace B*) and malate synthase G (*gla*), was used in these induction experiments. The work of Vanderwinkel and DeVlieghere (1968) has clearly established the presence of two malate synthases in *E. coli*, and the fact that even though malate synthase G is induced by glyoxylate, both enzymes have to be mutated before a strain becomes unable to grow on acetate.

MLD 44 cannot grow on glyoxylate or acetate, although it can grow on most other carbon sources, including proline. A mutant which lacks isocitrate lyase (*ace A*) is also able to grow on proline, although again, not on acetate.

Figure 23A shows that there is no induction of isocitrate lyase in MLD 44 on 1% acetate. This indicates that acetate must

Figure 23. A. The induction of isocitrate lyase in the *ace B*, *glc* mutant MLD 44 on 1% acetate (○). B. Induction on 1% proline (▽) and on 1% α -ketoglutarate (Δ). C. Induction on 1% succinate (□).

aceB glc (MALATE SYNTHASE
negative)

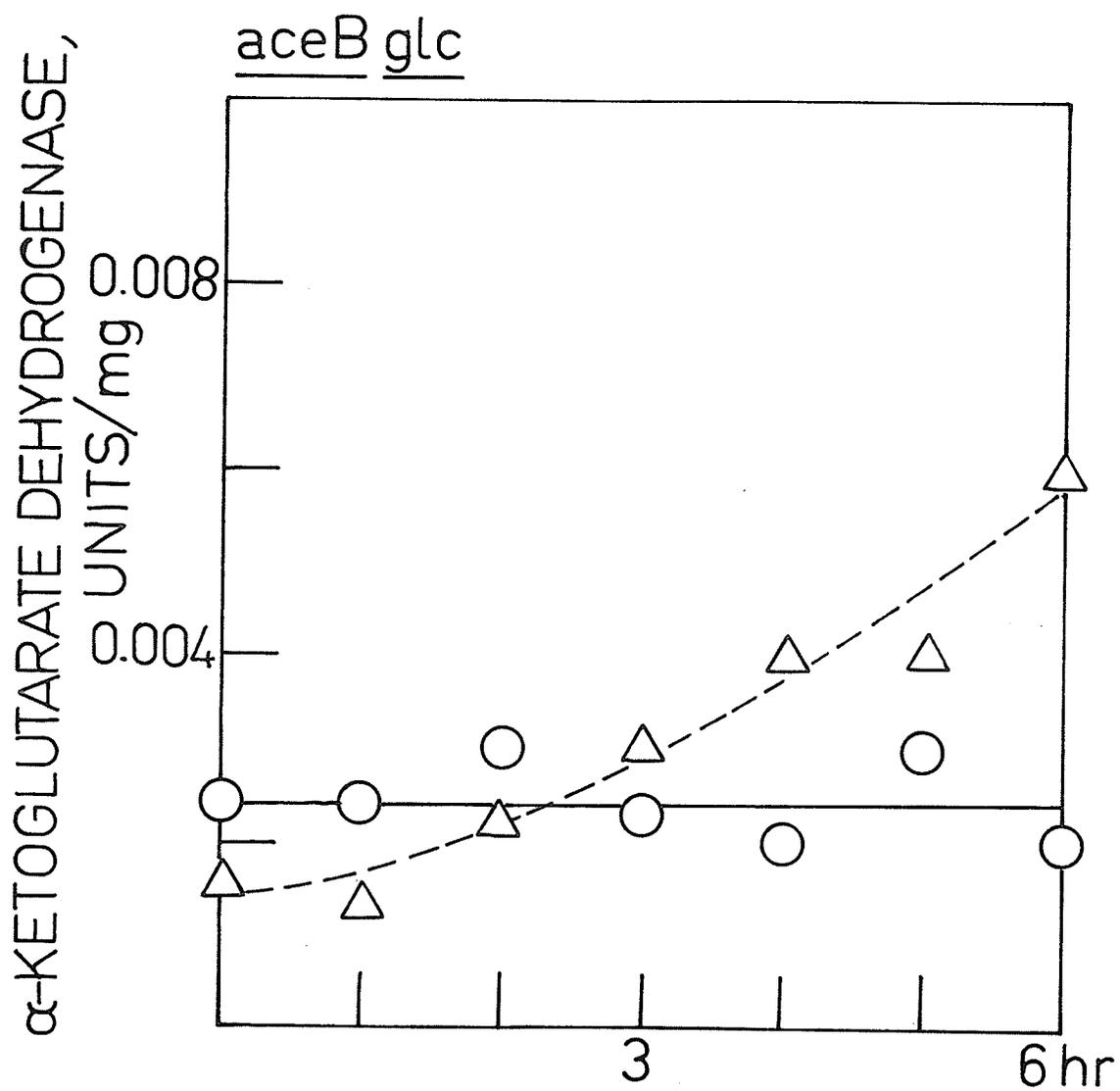


be metabolized through the glyoxylate cycle, at least to some extent, before it can have its inducing effect. It might be thought that at least during the initial stages, it would be sufficient for the acetate to be metabolized through the TCA cycle via α -ketoglutarate dehydrogenase. Induction experiments with the wild-type, as we have seen, show a rise in α -ketoglutarate dehydrogenase activity on acetate, which closely parallels the rise in isocitrate lyase activity. In Figure 24 it can be seen, however, that in this *ace B, glc* mutant induced on acetate there is no increase in α -ketoglutarate dehydrogenase activity.

Unlike acetate, proline is a very effective inducer of isocitrate lyase in this mutant, as is α -ketoglutarate (Figure 23B). The patterns of induction of isocitrate lyase on these carbon sources are similar to those seen with a wild-type strain, with α -ketoglutarate inducing somewhat more rapidly than proline (Figure 5). Figure 24 shows that α -ketoglutarate dehydrogenase is induced on α -ketoglutarate in this *ace B, glc* mutant.

Succinate was also tried as an inducer of isocitrate lyase in this strain (Figure 23C) and although it produces a rapid four-fold rise in activity in the first hour, the isocitrate lyase levels increase only slightly after that time. The amount of induction is not as great as on the wild-type, but the initial level of isocitrate lyase in this induction experiment is already about three- to four-fold higher than that seen with the wild-type CSH78 (Figure 10).

Figure 24. The induction of α -ketoglutarate dehydrogenase in the *ace B*, *glc* mutant MLD 44 on 1% acetate (○) and 1% α -ketoglutarate (△).



Thus, although the amount of induction is not as great, the final level of isocitrate lyase is similar to the steady-state level seen with the wild-type on succinate (Table 1).

Induction of Isocitrate Lyase in a PEP Carboxykinase (*pek*),

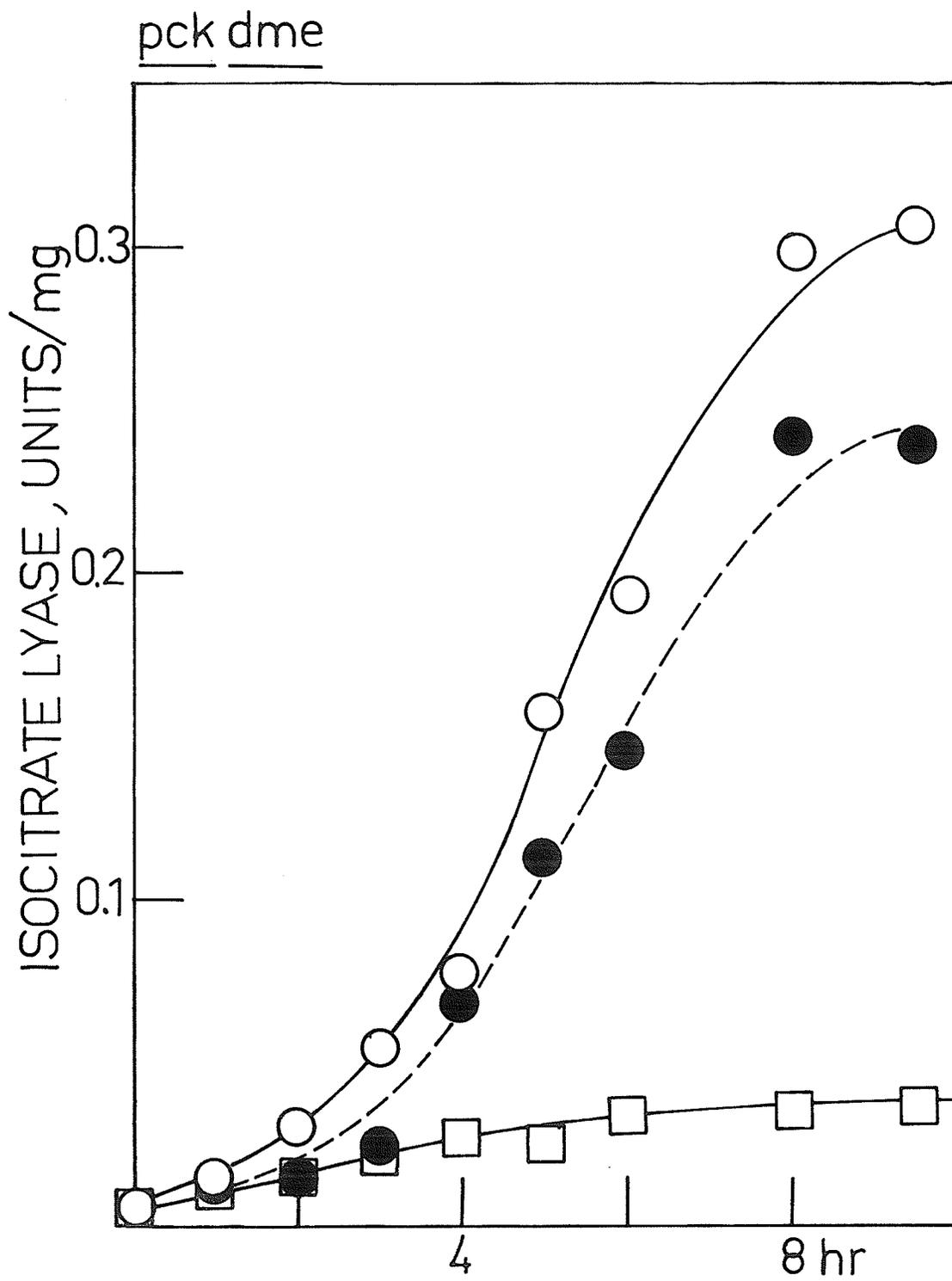
NAD - Malic Enzyme (*dne*) Mutant

The two routes to gluconeogenesis from intermediates of the TCA cycle are via PEP carboxykinase, or via NAD-malic enzyme to pyruvate which can then be metabolized to PEP using PEP synthase (Hansen and Juni 1974). The mutant used in these induction experiments, HG20, lacks both these enzymes. It is unable to grow on succinate or acetate, but it grows on glucose.

The theory for the control of the glyoxylate cycle operon suggested by Dietrich and Henning (1970) is that pyruvate is a co-repressor of the operon. The *pek*, *dne* mutant should be unable to produce pyruvate from succinate to any great extent. Figure 25 shows the induction of HG20 on succinate. The initial rate of increase of isocitrate lyase is slower than that seen for the wild-type, and the specific activity does not increase to the steady-state levels seen on succinate with a wild-type strain (Figure 9).

Figure 25 also shows that acetate is able to induce very well in this mutant. The induction pattern is very similar to that of a wild-type on acetate. An even more interesting result, also seen in Figure 25, is the induction of isocitrate lyase in this mutant on 1% acetate plus 1% succinate. It is evident that the induction pattern

Figure 25. The induction of isocitrate lyase in the *pep*, *dme* mutant HG20 on 1% acetate (○), 1% succinate (□) and 1% succinate plus 1% acetate (●). HG20 is also *his*, *pyrD*, *tyr A*, so all media were supplemented with 0.01% each of histidine, uridine and tyrosine.



on this mixture resembles that of acetate, rather than of succinate as in the wild-type. The level of isocitrate lyase reached is similar to that seen on acetate alone.

Figure 26 shows the induction of this mutant on 1% proline. Although there is a fifteen-fold increase in the specific activity of isocitrate lyase, the rate of induction is somewhat slower than is seen for the wild-type (compare Figure 5), and the level of activity reached after seven hours is also somewhat low compared to those seen on *glt A* and *icd* mutants, however (see Figures 16A, 17B).

Induction in Pyruvate Dehydrogenase Mutants

Two strains, each mutated in a different component of the pyruvate dehydrogenase complex were used for these induction experiments. CGSC4823 is mutated in the pyruvate dehydrogenase component of the complex (*ace E*) and strain CGSC5476 is mutated in the dihydrolipoamide transacetylase component (*ace F*). Both mutants require the addition of acetate as a supplement when growing on glucose. Mutants of pyruvate dehydrogenase are able to grow on acetate, although CGSC5476 does so only poorly, perhaps because of another unknown mutation. CGSC 4823, however, grows well on acetate.

Figure 27A shows the induction of isocitrate lyase in these strains on 1% acetate. The *ace E* strain shows a pattern of induction which is very similar to that of the wild-type. The *ace F* mutant, which grows only poorly on acetate, induces the isocitrate lyase much more slowly, but induction does occur to a small extent over the course

Figure 26. The induction of isocitrate lyase in the *pck*, *dme* mutant HG20 on 1% proline. The medium also contained histidine, uridine and tyrosine as in Figure 25.

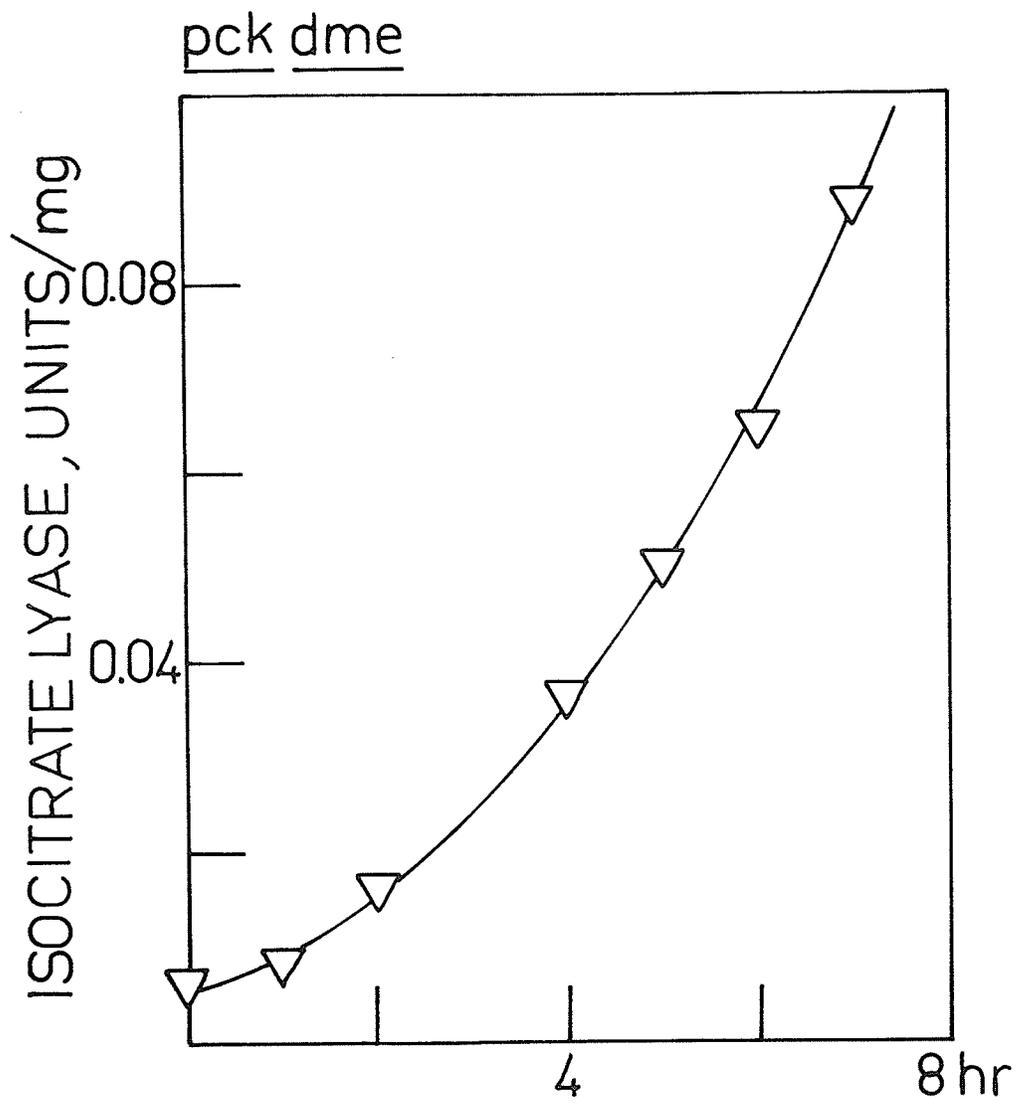
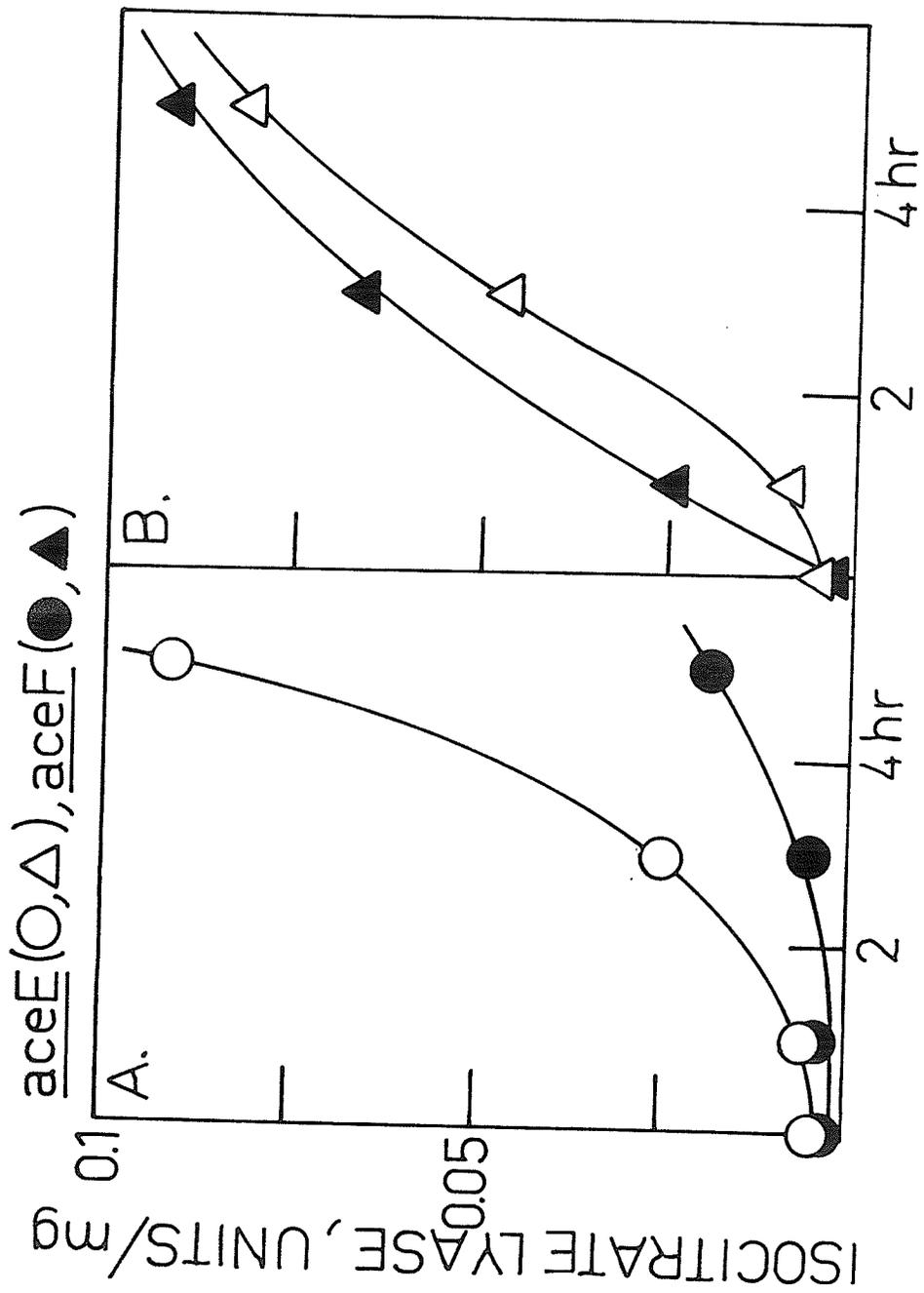


Figure 27A. The induction of isocitrate lyase in the *ace E* mutant CGSC4823 (○) and the *ace F* mutant CGSC5476 (●) on 1% acetate.

B. The induction of isocitrate lyase on 1% α -ketoglutarate plus 0.2% acetate in CGSC4823 (\triangle) and CGSC5476 (\blacktriangle). Both strains were grown up on 0.4% glucose plus 0.2% acetate prior to the induction experiment.



of the experiment.

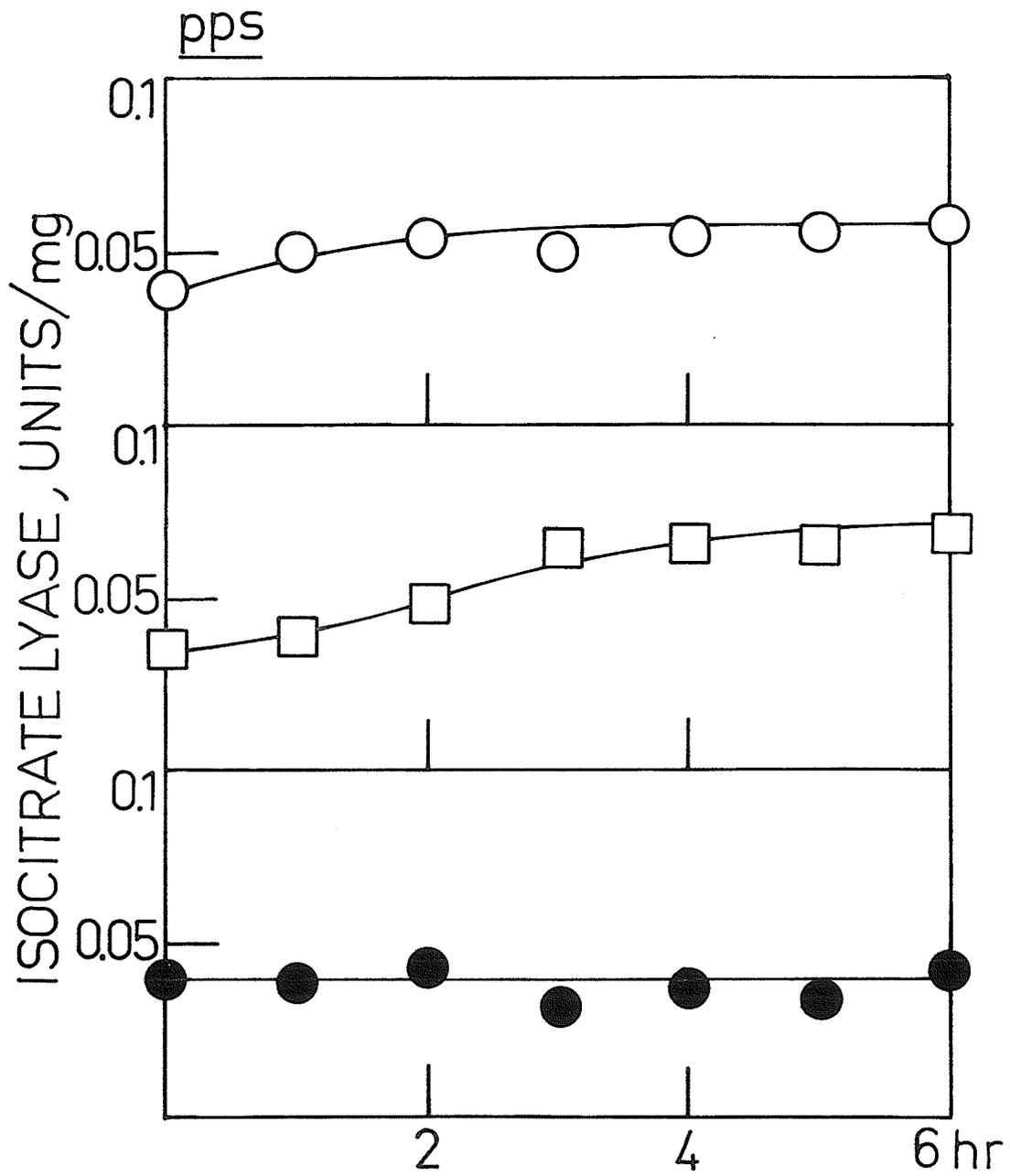
The induction patterns on 1% α -ketoglutarate, (Figure 27B) to which 0.2% acetate was added as a supplement, show that both mutants are able to induce isocitrate lyase very well on this combination of carbon sources. Acetate was added to try to prevent any starvation for acetyl CoA which could occur on α -ketoglutarate. The initial rate of induction seen for both mutants, as in the wild-type, is faster on α -ketoglutarate than on acetate. The fact that CGSC5476 (*ace F*) can induce isocitrate lyase on α -ketoglutarate shows that the reason it does not grow well on acetate is not simply the mutation in this enzyme.

Induction in a PEP Synthase (*pps*) Mutant

The PEP synthase mutant (*pps*) used for these induction experiments was DF1651. This strain grows, with appropriate supplements, on glucose, succinate and acetate but does not grow on lactate or pyruvate.

Figure 28 shows the results of three induction experiments with this strain on acetate, succinate, and succinate plus acetate. The initial level of isocitrate lyase is about ten-fold higher for all these experiments, than is normally seen for a wild-type strain. The reason for this high specific activity on glucose is unknown. It is probably not a result of the growth supplements which are required by this strain, because strain HG20 (*pck*, *dme*) is derived from DF1651, and although it requires the same supplements, it does not show elevated levels after growth on glucose.

Figure 28. The induction of isocitrate lyase in the *pps* mutant DF1651 on 1% acetate (○), 1% succinate (□) and 1% acetate plus 1% succinate (●). All media contained 0.01% supplements of histidine, uridine and tyrosine since this strain is *his*, *pyr D* and *tyr A*.



Although the initial level of isocitrate lyase is high, there is little further induction on any of the carbon sources. Induction by acetate is barely detectable, even though DF1651 is able to grow on acetate. Succinate shows a limited two-fold induction over the initial level, although the final level of isocitrate lyase approaches that seen in the wild-type steady state. In this strain, the combination of succinate plus acetate produces levels of isocitrate lyase which are not even as high as those seen with succinate or acetate alone (Figure 28).

Acetyl CoA and CoA in *E. coli*

Cells were extracted and assayed for total CoA and acetyl CoA as described in Methods.

Table 6 shows the concentrations of total CoA and acetyl CoA in the wild-type strain CSH78 and a glyoxylate cycle constitutive strain CSH60, grown on different carbon sources. Table 6 also includes the ratio of acetyl CoA to total CoA concentrations for each carbon source, and the specific activity of isocitrate lyase determined in the same cells.

Table 7 and Figure 29 show the results of an isocitrate lyase induction experiment using the wild-type strain CSH78 on 1% acetate. Samples taken during the course of the induction were used to measure total CoA and acetyl CoA, and also to measure the specific activity

Table 6

The levels of total CoA, acetyl CoA and isocitrate lyase in CSH78 (wild-type) and CSH60 (*icl R*) on different carbon sources.

| Strain | Carbon Source | Total CoA μ moles/gram dry weight | Acetyl CoA μ moles/gram dry weight | Acetyl CoA/ Total CoA | Isocitrate Lyase units/mg |
|--------|---------------|---|--|--------------------------|---------------------------------|
| CSH78 | glucose | 4.06 | 1.31 | 0.25 | 0.005 |
| | acetate | 7.76 | 1.21 | 0.15 | 0.22 |
| | pyruvate | 6.62 | 1.15 | 0.17 | 0.02 |
| | succinate | 4.34 | 1.21 | 0.27 | 0.04 |
| | L-proline | 12.7 | 2.17 | 0.17 | 0.13 |
| CSH60 | glucose | 13.2 | 2.33 | 0.18 | 0.15 |
| | acetate | 15.6 | 1.71 | 0.11 | 0.48 |

All cells were grown through at least three transfers on a given carbon source. Cells were sampled and assayed for total CoA and acetyl CoA as described in Methods. Isocitrate lyase assays were done on the same cultures that were used for the CoA and acetyl CoA determinations.

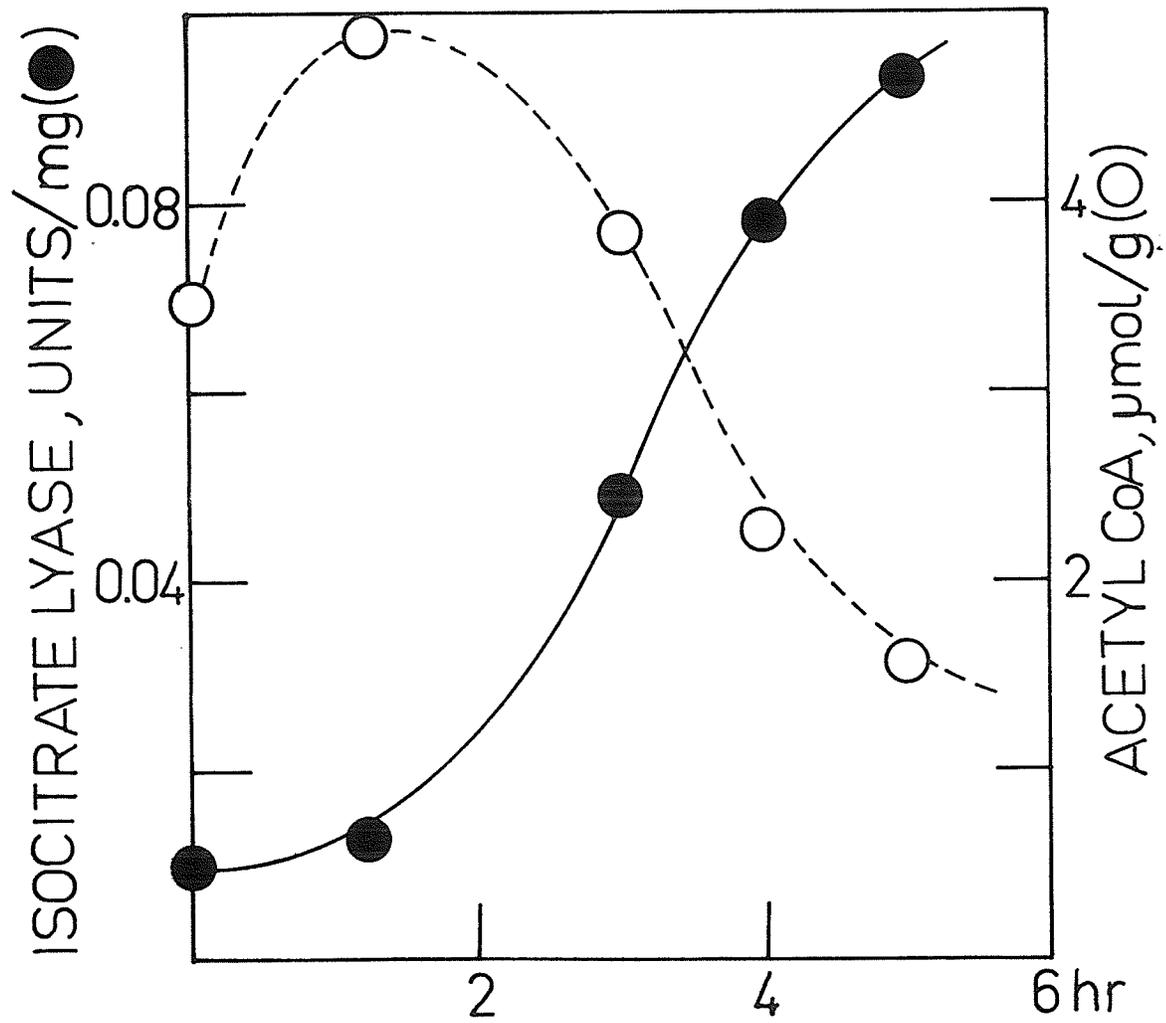
Table 7

The levels of total CoA, acetyl CoA and isocitrate lyase in CSH78 during induction on acetate.

| Time (hr) | Total CoA μ moles/gram dry weight | Acetyl CoA μ moles/gram dry weight | Acetyl CoA/ Total CoA | Isocitrate Lyase (units/mg) |
|-----------|---|--|--------------------------|-----------------------------------|
| 0 | 9.9 | 3.48 | 0.38 | 0.010 |
| 1.25 | 7.40 | 4.87 | 0.66 | 0.013 |
| 3 | 6.83 | 3.83 | 0.56 | 0.049 |
| 5 | 7.95 | 1.46 | 0.18 | 0.093 |

Cells were grown to stationary phase in glucose, washed and re-suspended in 1% acetate. The zero time point for all assays was taken as soon as possible after resuspension in acetate. Cells were extracted and analyzed as described in Methods.

Figure 29. The levels of acetyl CoA (○) and isocitrate lyase (●) in CSH78 during induction on 1% acetate. The zero time point was taken as soon as possible after cells were resuspended in 1% acetate medium. Cells were sampled, extracted and assayed for acetyl CoA as described in Methods. These data are also shown in Table 7.



of isocitrate lyase. Figure 29 shows that before isocitrate lyase levels begin to rise in the cell, acetyl CoA levels increase. These levels fall very quickly, however, as soon as isocitrate lyase is induced. After five hours in the presence of acetate, the acetyl CoA: total CoA ratio resembles that seen for acetate-grown cells rather than glucose-grown cells.

Table 8 and Figure 30 show the results of a similar experiment with CSH78 in which 1% proline was used as the inducing carbon source. As is typical of isocitrate lyase induction on proline, the enzyme levels rise rapidly. No increase is seen in acetyl CoA levels however. Instead, they fall steadily during the experiment and again the ratio of acetyl CoA: total CoA reaches that seen in cells growing on proline as sole carbon source.

Table 9 shows the levels of total CoA and acetyl CoA in the citrate synthase mutant H2 during an induction experiment on 1% acetate. This strain shows a very high level of total CoA at the beginning of the experiment, most of which appears to be acetyl CoA. As might be expected with this strain, there is little change in the levels of total CoA and acetyl CoA and the ratio of the two never approaches that seen in a wild-type strain (Table 6). As has been noted earlier for citrate synthase mutants, there is no induction of isocitrate lyase.

Table 8

The levels of total CoA, acetyl CoA and isocitrate lyase in CSH78 during induction on proline.

| Time (hr) | Total CoA μ moles/gram dry weight | Acetyl CoA μ moles/gram dry weight | Acetyl CoA/ Total CoA | Isocitrate Lyase (units/mg) |
|-----------|---|--|--------------------------|-----------------------------------|
| 0 | 7.55 | 2.13 | 0.28 | 0.0070 |
| 1 | 8.12 | 1.81 | 0.22 | 0.018 |
| 2 | 8.12 | 1.40 | 0.17 | 0.044 |
| 3 | 7.55 | 1.74 | 0.23 | 0.084 |
| 4 | 7.48 | 1.25 | 0.17 | 0.12 |
| 5 | 7.61 | 1.32 | 0.17 | 0.13 |

Cells were grown to log phase on 0.4% glucose, washed, and resuspended in 1% proline medium. The zero time samples were taken as soon as possible after resuspension in proline and extracted and assayed as described in Methods.

Figure 30. The induction of isocitrate lyase (●) and the change in the levels of acetyl CoA (○) in CSH78 on 1% proline. The zero time point for both determinations was taken as soon as possible after resuspension in proline.

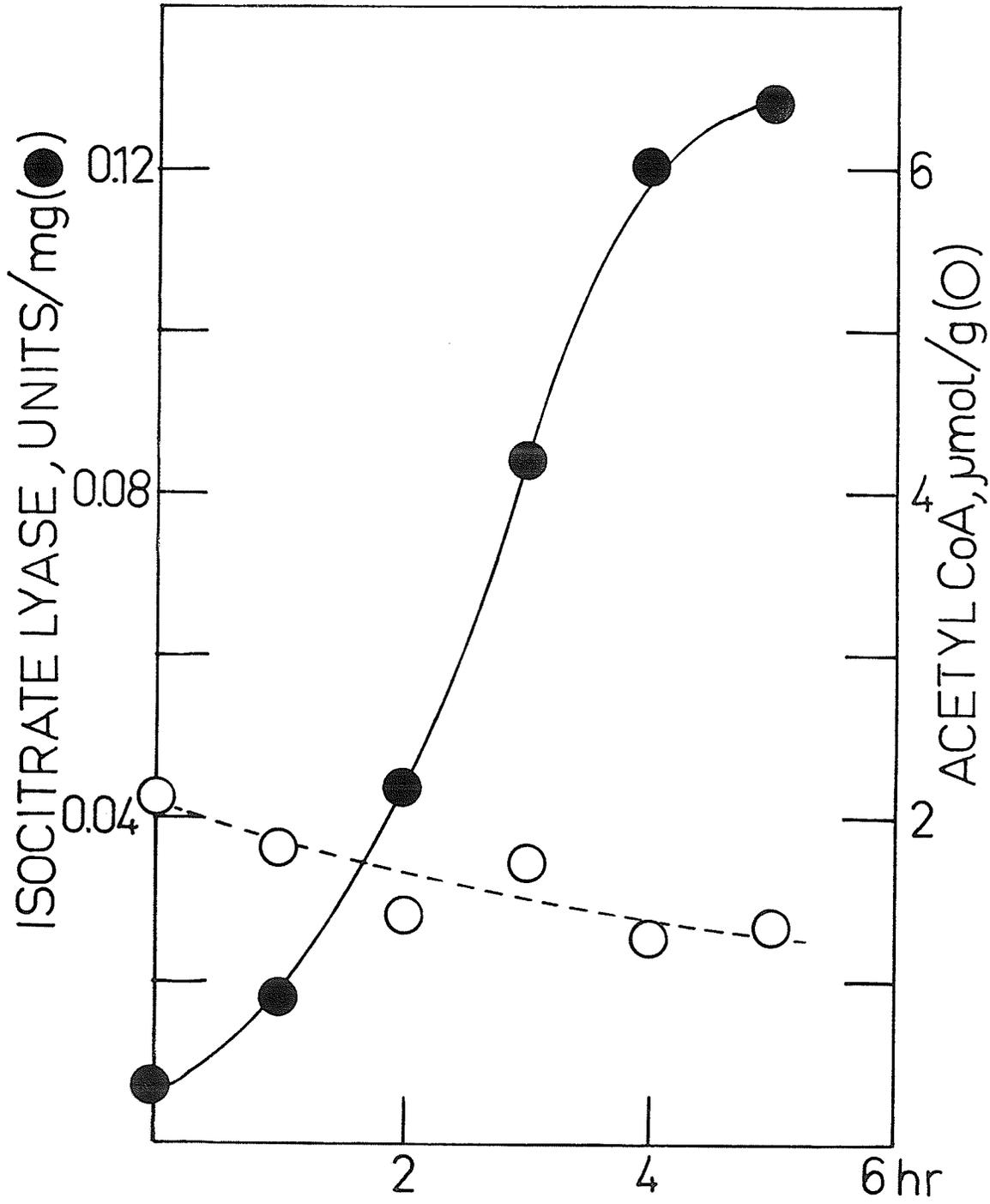


Table 9

The levels of total CoA, acetyl CoA and isocitrate lyase in the *glt A* mutant H2 during induction on acetate.

| Time (hr) | Total CoA μ moles/gram dry weight | Acetyl CoA μ moles/gram dry weight | Acetyl CoA/ Total CoA | Isocitrate Lyase (units/mg) |
|-----------|---|--|--------------------------|-----------------------------------|
| 0 | 19.2 | 15.3 | 0.80 | 0.002 |
| 1 | 14.9 | 10.6 | 0.71 | 0.002 |
| 2 | 17.9 | 10.0 | 0.56 | 0.002 |
| 3 | 17.9 | 7.71 | 0.43 | 0.002 |
| 4 | 18.1 | 10.8 | 0.60 | 0.003 |

Cells were grown on 0.4% glucose plus 0.01% proline prior to the induction experiment. The zero time samples were taken as soon as possible after resuspension in 1% acetate medium. Cells were sampled, extracted and assayed as described in Methods.

The Determination of Pyruvate Levels During the Induction
of Isocitrate Lyase

The levels of pyruvate were determined during the course of a series of experiments identical to those which studied the induction of isocitrate lyase on different carbon sources. The changes in the levels of pyruvate in the wild-type strain CSH78 during incubation in acetate, α -ketoglutarate, L-malate and succinate were followed.

The cells were sampled, extracted and assayed as described in Methods. Table 10 and Figures 31 and 32 show the levels of pyruvate in the cells at the different times after resuspension in the new carbon source.

There is a large drop in the pyruvate levels during the centrifugation and washing procedure. In all experiments, the initial levels of pyruvate were much lower than those for the glucose-grown cells. The levels do rise again quite quickly after resuspension in L-malate, succinate and α -ketoglutarate. They remain low, however, on acetate.

There appears to be no correlation between the levels of pyruvate in these cells, and the rate at which isocitrate lyase is induced. Acetate which induces the enzyme more slowly does not produce as high levels of pyruvate as do succinate, malate or α -ketoglutarate which induce isocitrate lyase very rapidly. Lowry *et al* (1971) showed that pyruvate levels are very low on acetate-grown *E. coli*, while glucose and succinate cells have higher levels. The experiments

Table 10

The levels of pyruvate in CSH78 during induction experiments on acetate, L-malate, succinate and α -ketoglutarate.

| Sample Time(min) | Acetate | | Pyruvate μ moles/g dry weight | | | | | |
|------------------|---------|---------|-----------------------------------|---------|-----------|-------------------------|------|---------|
| | | | L-Malate | | Succinate | α -Ketoglutarate | | |
| 0 | 0.55 | (1.6) | 0.84 | (2.8) | 0.16 | (3.0) | 1.1 | (4.3) |
| 5 | 0.17 | (6.8) | 0.85 | (7.8) | 0.37 | (10) | 1.29 | (7.8) |
| 15 | 0.48 | (14.8) | 1.89 | (13.8) | 0.44 | (12.3) | 1.83 | (13.8) |
| 30 | 0.19 | (29.8) | 1.5 | (18.8) | 1.4 | (16.3) | 0.96 | (31.3) |
| 60 | 0.17 | (71.3) | 1.01 | (58.8) | 2.04 | (72.3) | 1.26 | (18.8) |
| 120 | 0.17 | (71.3) | 1.01 | (58.8) | 2.04 | (72.3) | 1.11 | (58.8) |
| 120 | 0.66 | (119.8) | 2.54 | (118.8) | 1.03 | (122.3) | 1.26 | (118.8) |
| 180 | 0.35 | (184.3) | 1.39 | (186.3) | 1.05 | (187.3) | 0.97 | (181.3) |

Pyruvate levels on glucose-grown cells are 3.18 ± 1.1 μ moles/g dry weight.

Cells were grown on glucose medium A washed and resuspended in fresh medium containing 1% of the carbon source indicated. Samples were collected, extracted and analyzed as described in the Methods. The number in parentheses indicates the actual time of sampling in minutes, after the cells were resuspended in the new carbon source.

Figure 31. The changes in the levels of pyruvate (○,△) and isocitrate lyase (●,▲) in CSH78 during induction on 1% acetate and 1% α-ketoglutarate. The pyruvate value in the hatched area is the level in these cells growing on glucose (3.18 ± 1.1 μmoles/g dry weight). The hatched area represents the period required for centrifugation and washing. Cells were collected, extracted and assayed as described in Methods. These data are also shown in Table 10.

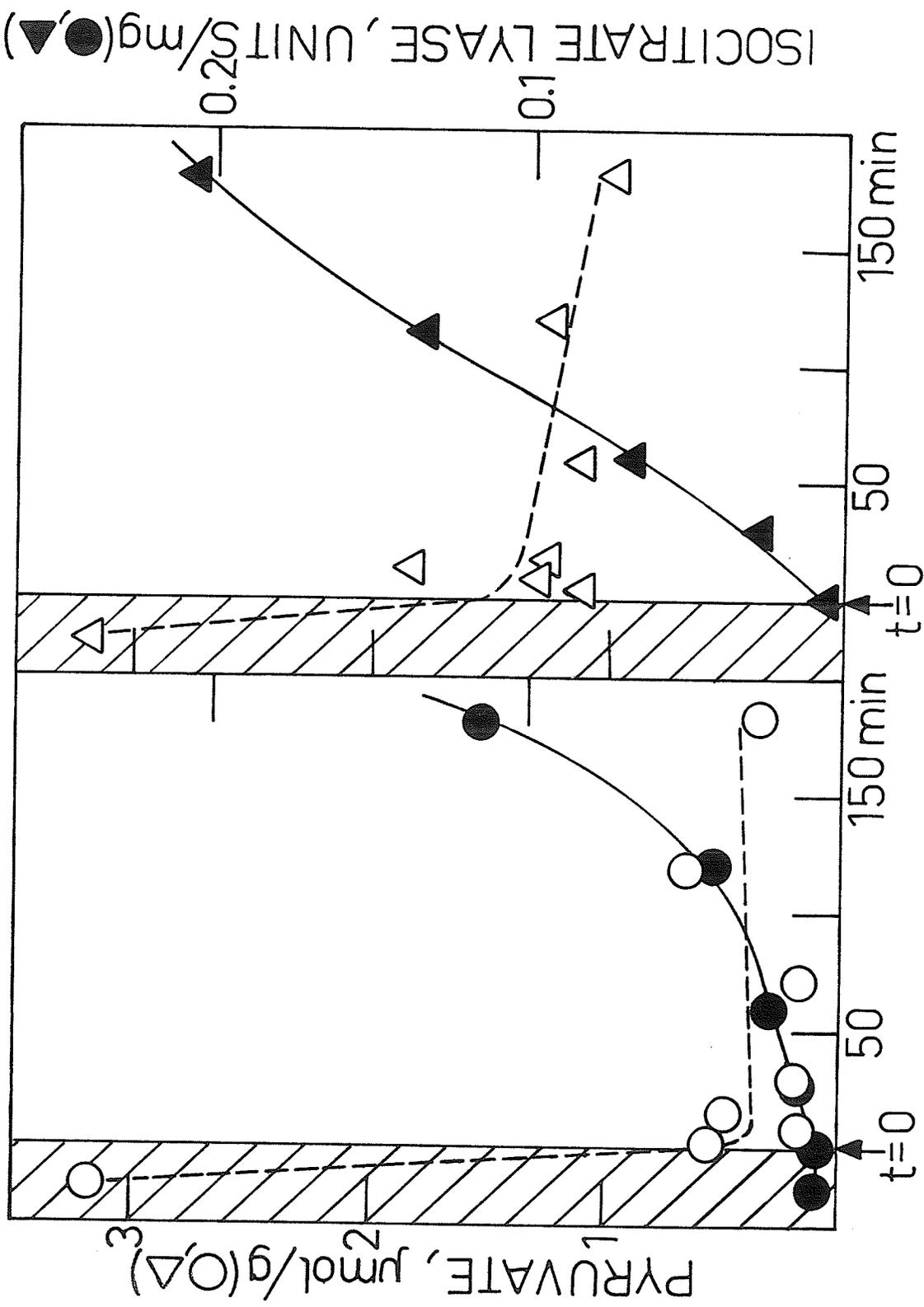
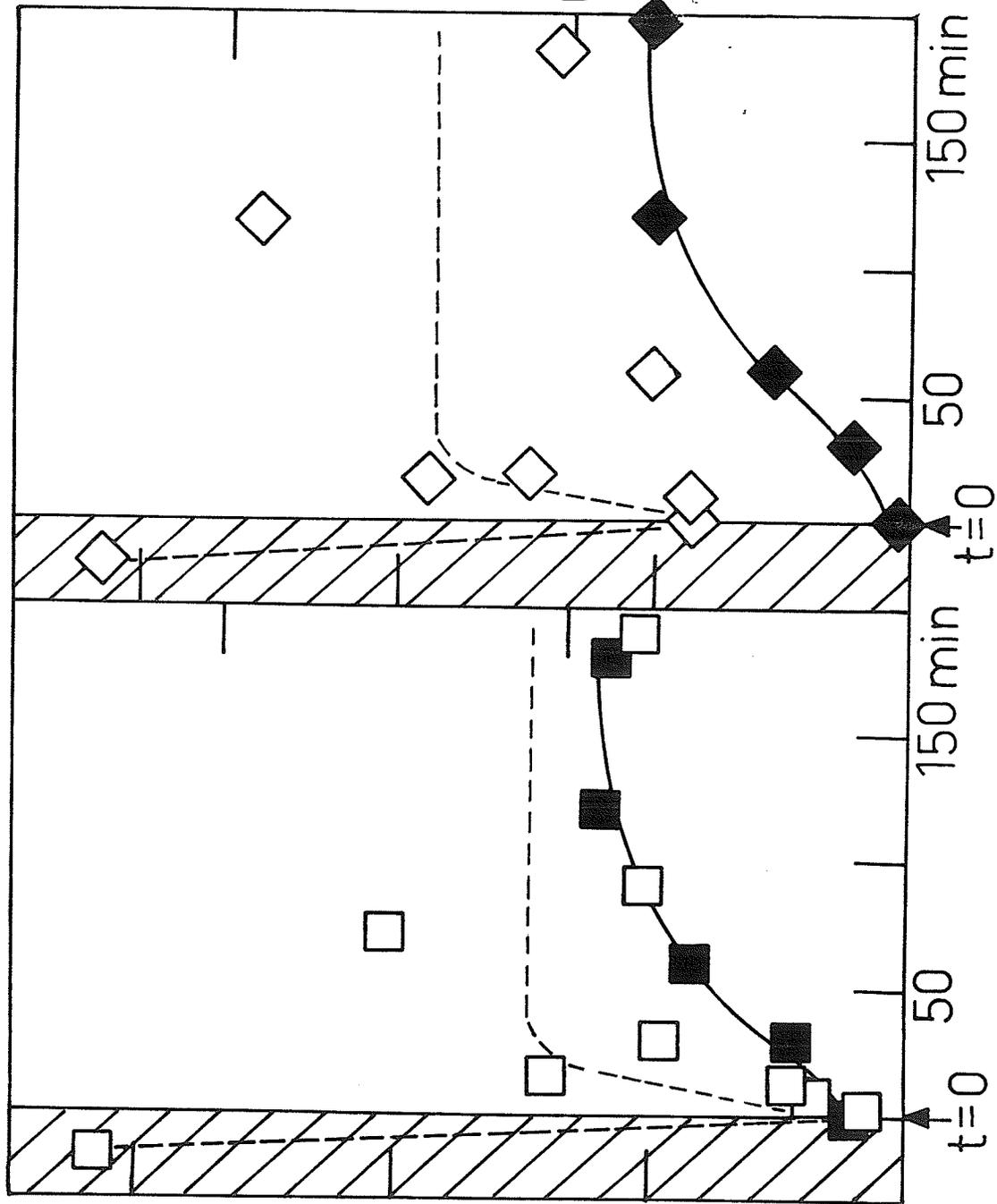


Figure 32. The changes in levels of pyruvate (\square, \diamond) and isocitrate lyase ($\blacksquare, \blacklozenge$) in CSH78 during induction on 1% succinate and 1% L-malate. The pyruvate value in the hatched area is the level in these cells growing on glucose ($3.18 \pm 1.1 \mu\text{moles/g dry weight}$). The hatched area represents the period required for centrifugation and washing. Cells were collected, extracted and assayed as described in Methods. These data are also shown in Table 10.

ISOCITRATE LYASE, UNITS/mg (■, ◆)



PYRUVATE, $\mu\text{mol/g}$ (□, ◇)

presented here indicate that these new levels are reached quite rapidly when cells are transferred from one carbon source to another.

Isocitrate Dehydrogenase in *E. coli* K12

Levels of Isocitrate Lyase and Isocitrate Dehydrogenase on Different Carbon Sources

A comparison of the levels of isocitrate lyase and isocitrate dehydrogenase in the wild-type strain CSH78, grown on different carbon sources, is shown in Table 11. Although the isocitrate dehydrogenase levels do not show the same very marked differences in specific activities that are seen for isocitrate lyase, there are differences nonetheless. Glycerol, succinate and pyruvate show the highest specific activities of isocitrate dehydrogenase among the carbon sources tested, with glucose showing a slightly lower level. For these experiments, acetate gave the lowest specific activity, and proline and α -ketoglutarate showed intermediate levels of isocitrate dehydrogenase. These results are typical, although as will be seen in the induction experiments below, the levels of isocitrate dehydrogenase on proline often fall below those seen on acetate.

Table 12 shows a comparison of isocitrate lyase and isocitrate dehydrogenase levels on different carbon sources in two strains, MLD 8 and CSH60, which are constitutive for isocitrate lyase. The differences in the specific activities of the two enzymes on the various carbon

Table 11

The specific activities of isocitrate dehydrogenase and isocitrate lyase in CSH78 on different carbon sources.

| Carbon Source | Isocitrate Lyase (units/mg) | Isocitrate Dehydrogenase (units/mg) |
|-------------------------|--------------------------------|--|
| glucose | 0.0040 | 0.89 |
| glycerol | 0.020 | 1.21 |
| succinate | 0.070 | 1.29 |
| pyruvate | 0.0090 | 1.34 |
| L-proline | 0.16 | 0.59 |
| α -ketoglutarate | 0.15 | 0.63 |
| acetate | 0.29 | 0.34 |

Cells were grown through three transfers on a given carbon source before being collected in log phase and assayed as described in Methods. All carbon sources were present at 0.4% except acetate which was 1%.

Table 12

The specific activities of isocitrate dehydrogenase and isocitrate lyase in the *icl R* mutants MLD 8 and CSH60 on different carbon sources.

| Carbon Source | MLD 8 | | CSH 60 | |
|---|------------------------------|--------------------------------------|------------------------------|--------------------------------------|
| | Isocitrate Lyase units/mg | Isocitrate Dehydrogenase units/mg | Isocitrate Lyase units/mg | Isocitrate Dehydrogenase units/mg |
| glucose | 0.22 | 0.83 | 0.070 | 0.67 |
| glucose + 5×10^{-4} cyclic AMP | 0.28 | 0.69 | 0.12 | 0.71 |
| glycerol | 0.43 | 0.80 | 0.19 | 0.73 |
| lactate | 0.58 | 0.67 | 0.22 | 0.79 |
| succinate | 0.42 | 0.73 | 0.30 | 0.88 |
| L-proline | 0.64 | 0.088 | 0.39 | 0.35 |
| acetate | 0.71 | 0.046 | 0.50 | 0.52 |

Cells were grown through three transfers on a given carbon source before being collected in log phase and assayed as described in Methods. All carbon sources were present at 0.4% except acetate which was 1%.

sources are less marked than was seen in the wild-type strain except in the cases of acetate and proline. The levels of isocitrate dehydrogenase are very much lower in MLD 8 on these two substrates, and are also lower in CSH60, although less markedly so.

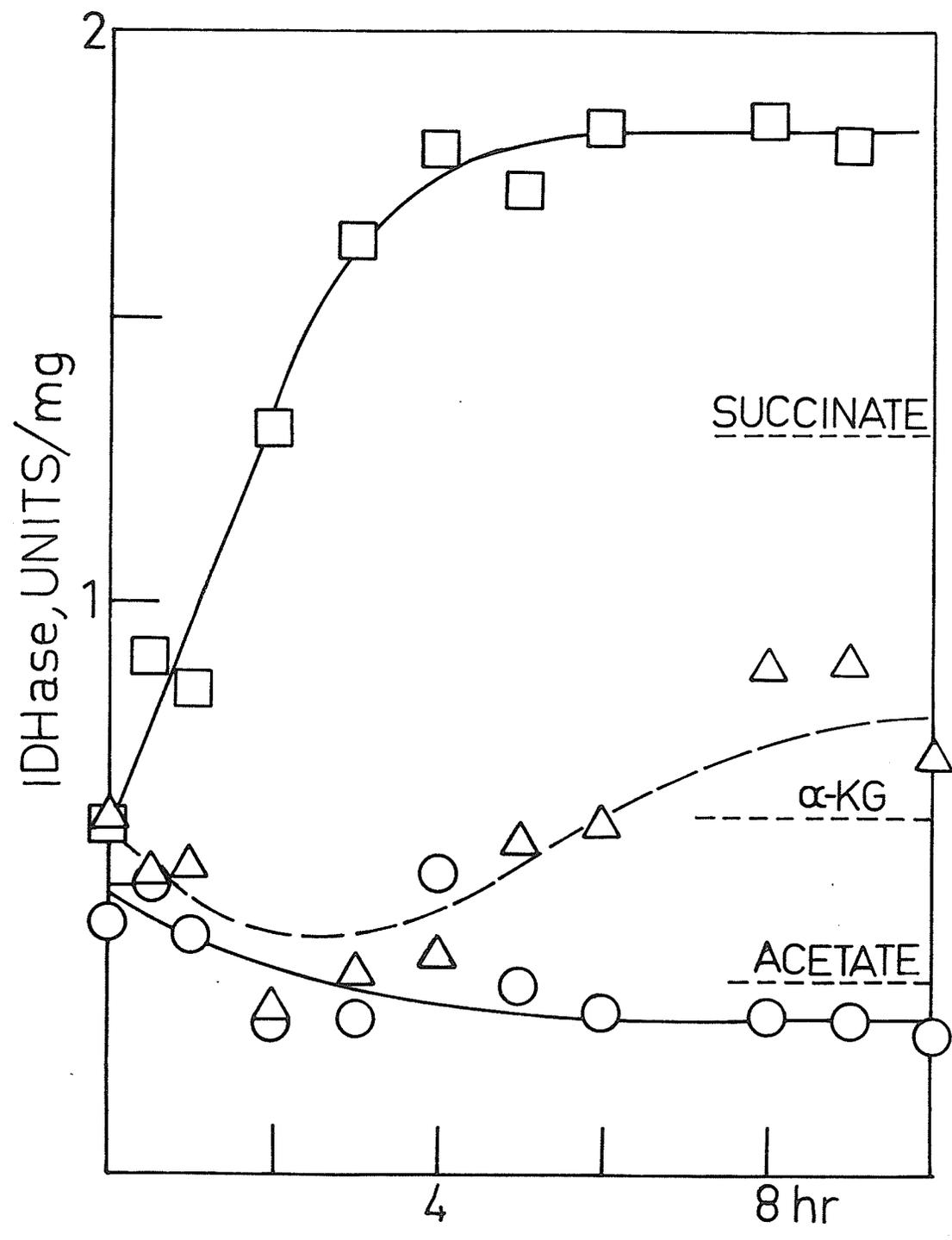
The isocitrate dehydrogenase levels in both these strains are lower for all carbon sources (except perhaps glucose) than in the wild-type. Whether this is a consequence of the *icl R* mutation is not clear. Like the wild-type, however, these mutants have a roughly constant amount of isocitrate dehydrogenase on glucose, glycerol, lactate and succinate, and a significantly lower amount on proline and acetate.

Changes in the Levels of Isocitrate Dehydrogenase in a Wild-Type *E. coli* K12

Induction experiments, identical to those already described to follow changes in isocitrate lyase levels, were carried out to follow changes in isocitrate dehydrogenase specific activity. In many experiments, the same sample was assayed for both enzymes.

Figure 33 shows the changes in specific activity of isocitrate dehydrogenase in CSH78 on going from glucose to acetate, α -ketoglutarate and succinate. Both acetate and α -ketoglutarate produce within one hour a drop in the specific activity of isocitrate dehydrogenase. In the case of acetate, a steady-state level is reached after two hours which is thereafter maintained. The α -ketoglutarate produces a similar initial drop in the isocitrate dehydrogenase activity but in this experiment, the activity again rises to a level greater than

Figure 33. The changes in the specific activity of isocitrate dehydrogenase in CSH 78 on the transfer of cells from glucose medium to medium containing 1% acetate (○), 1% α -ketoglutarate (Δ), or 1% succinate (□). The dotted lines represent the steady-state levels of this enzyme in CSH 78 on the given carbon source (Table 11). The zero time samples were taken as soon as possible after resuspension in the new carbon source.



that seen for the steady-state on this carbon source.

Succinate produces a rapid increase in the specific activity of isocitrate dehydrogenase. During the time of the experiment, the activity of the enzyme rises to levels greater than that seen for the steady-state on this carbon source.

The initial levels of isocitrate dehydrogenase in these induction experiments often differ by a factor of two, even for replicate experiments with the same strain. The reason for this is not known. Changes in the activity of the enzyme appear to be very rapid. The zero time sample for these experiments was taken as soon as possible after resuspension in the new carbon source, but it is possible that changes in the enzyme activity can occur during the centrifugation and washing. Alternatively, the enzyme is known to be cold sensitive (Reeves *et al* 1972) and although glycerol is added to the extraction buffer, and assays are done immediately, storage of the cell pellet at -15°C for varying lengths of time before the enzyme is extracted, may affect the reproducibility of the assays.

Figure 34 shows an experiment which is the reverse of that in Figure 33. CSH 78 was grown on acetate, then resuspended in medium containing glucose, pyruvate, succinate, α -ketoglutarate or proline. Pyruvate and succinate produced rapid increases in the specific activity of isocitrate dehydrogenase. Glucose also produced an increase in the activity of this enzyme, but rather more slowly. There was little change in the specific activity of isocitrate dehydrogenase on transfer from acetate to α -ketoglutarate and about a two-fold reduction in activity when cells were resuspended in proline.

Figure 34. The changes in the specific activity of isocitrate dehydrogenase in CSH 78 on the transfer of cells from acetate medium to medium containing pyruvate (\odot), glucose (\ominus), succinate (\square), α -ketoglutarate (\triangle) or proline (∇) all present at 1%. Cells were grown on medium A containing 1% acetate, centrifuged, washed and resuspended in the new carbon source as described in Methods. The zero time activity is the specific activity of the culture on acetate before resuspension in the new carbon source.

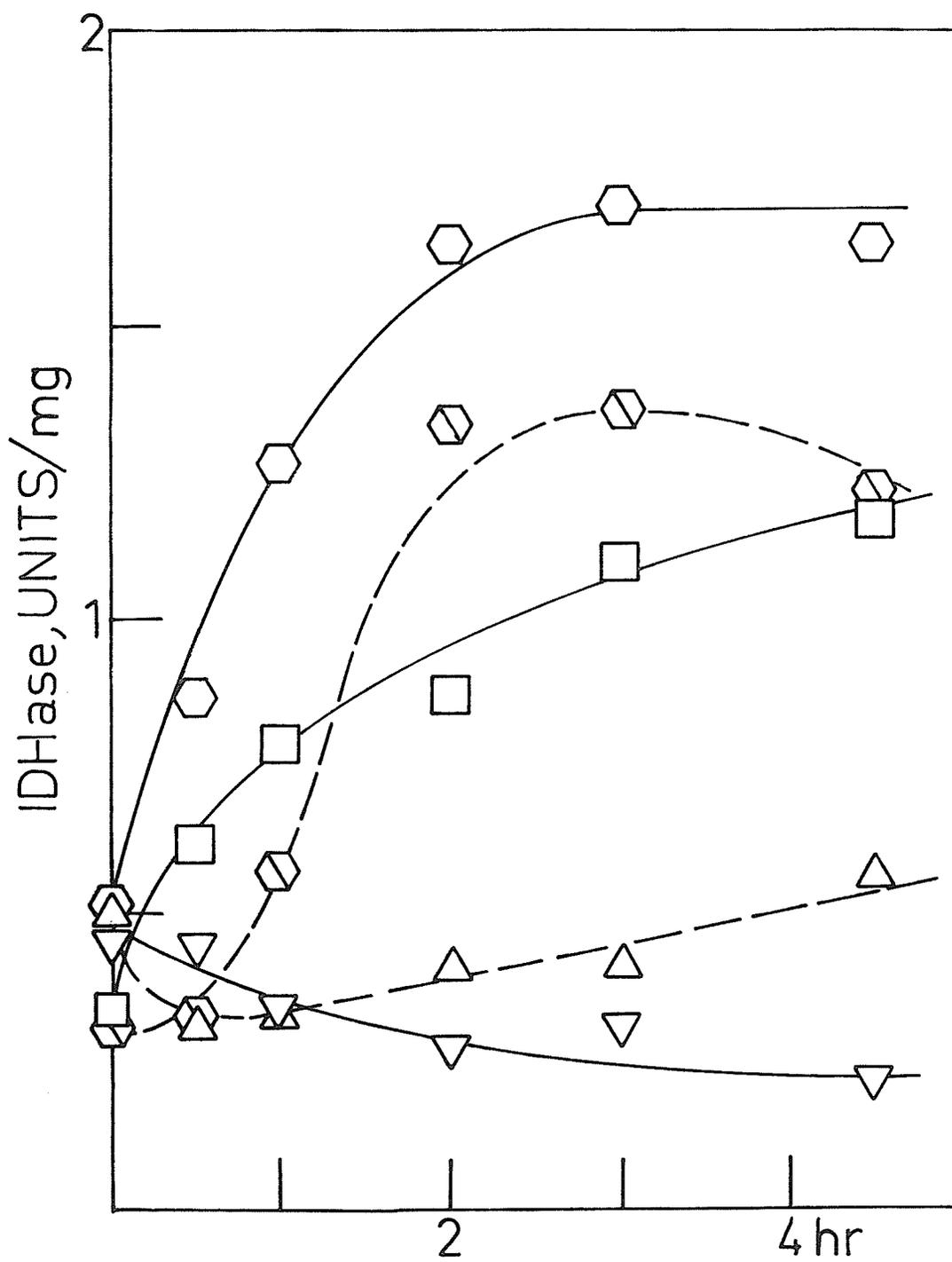


Figure 35. The changes in the specific activity of isocitrate lyase in CSH 78 on transfer of cells from acetate medium to medium containing pyruvate (\circ), glucose (\diamond), succinate (\square), α -keto-glutarate (\triangle), or proline (∇) all present at 1%. These are the same extracts as were assayed for isocitrate dehydrogenase activity in Figure 34. The broken line is calculated from the known protein concentrations of the extracts (which increased with time, because the cells were growing), on the assumption that no further synthesis of isocitrate lyase occurred after resuspension in the new carbon source. This may be called the "dilution model" for decline in specific activity of this enzyme.

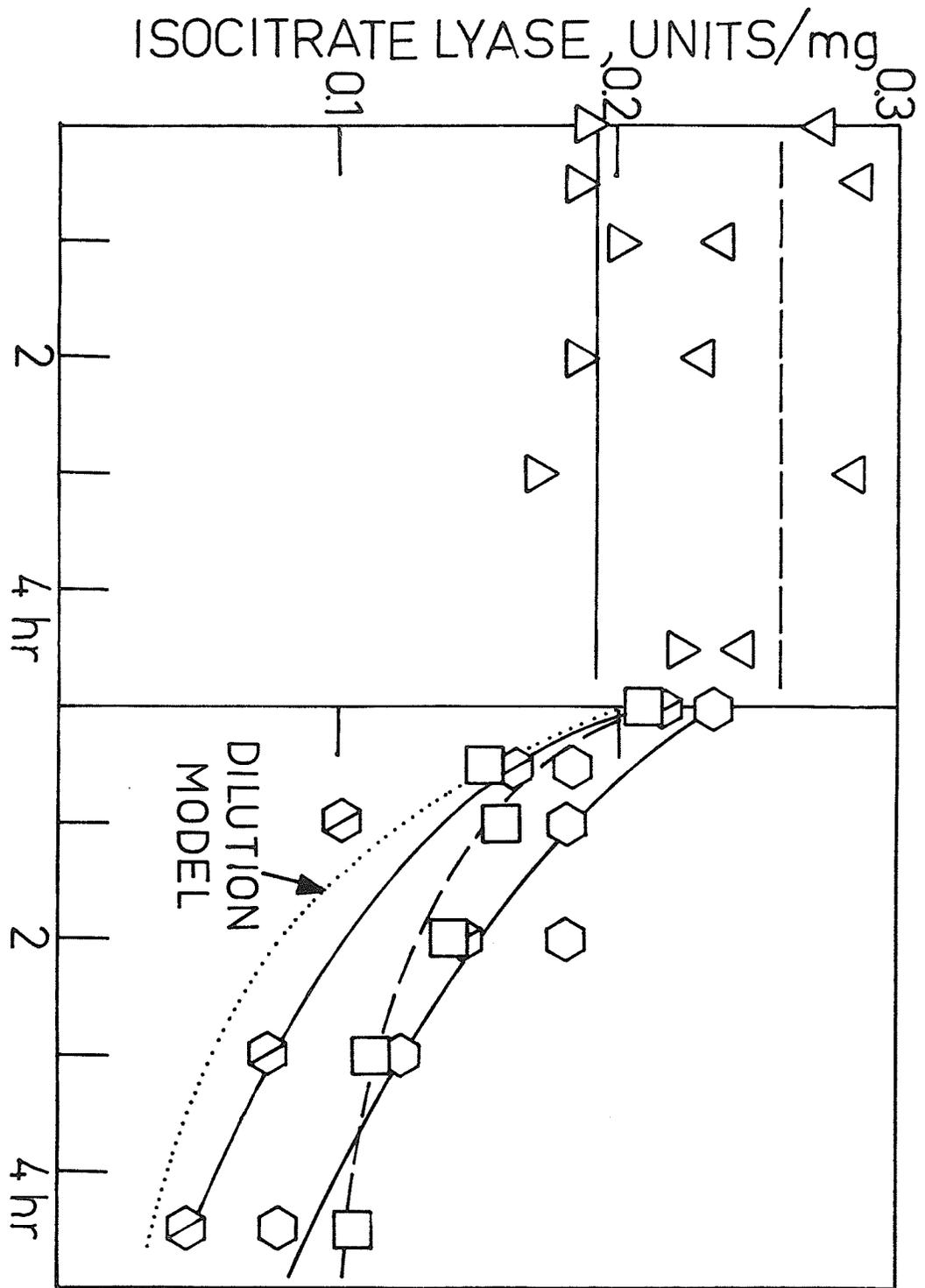


Figure 35 shows the changes in levels of isocitrate lyase measured in the same extracts. There is little change in the specific activity of isocitrate lyase on transfer from acetate medium to medium containing either proline or α -ketoglutarate. Levels of this enzyme do decrease however, when cells are resuspended in glucose, pyruvate or succinate.

Although the changes which occur in the specific activity of isocitrate dehydrogenase are quite rapid, the changes in the levels of isocitrate lyase are slower and appear to reflect simply a dilution of the specific activity of this enzyme during growth on the new carbon source (see broken line in Figure 35).

Changes in Isocitrate Dehydrogenase Activity in a Malate Synthase

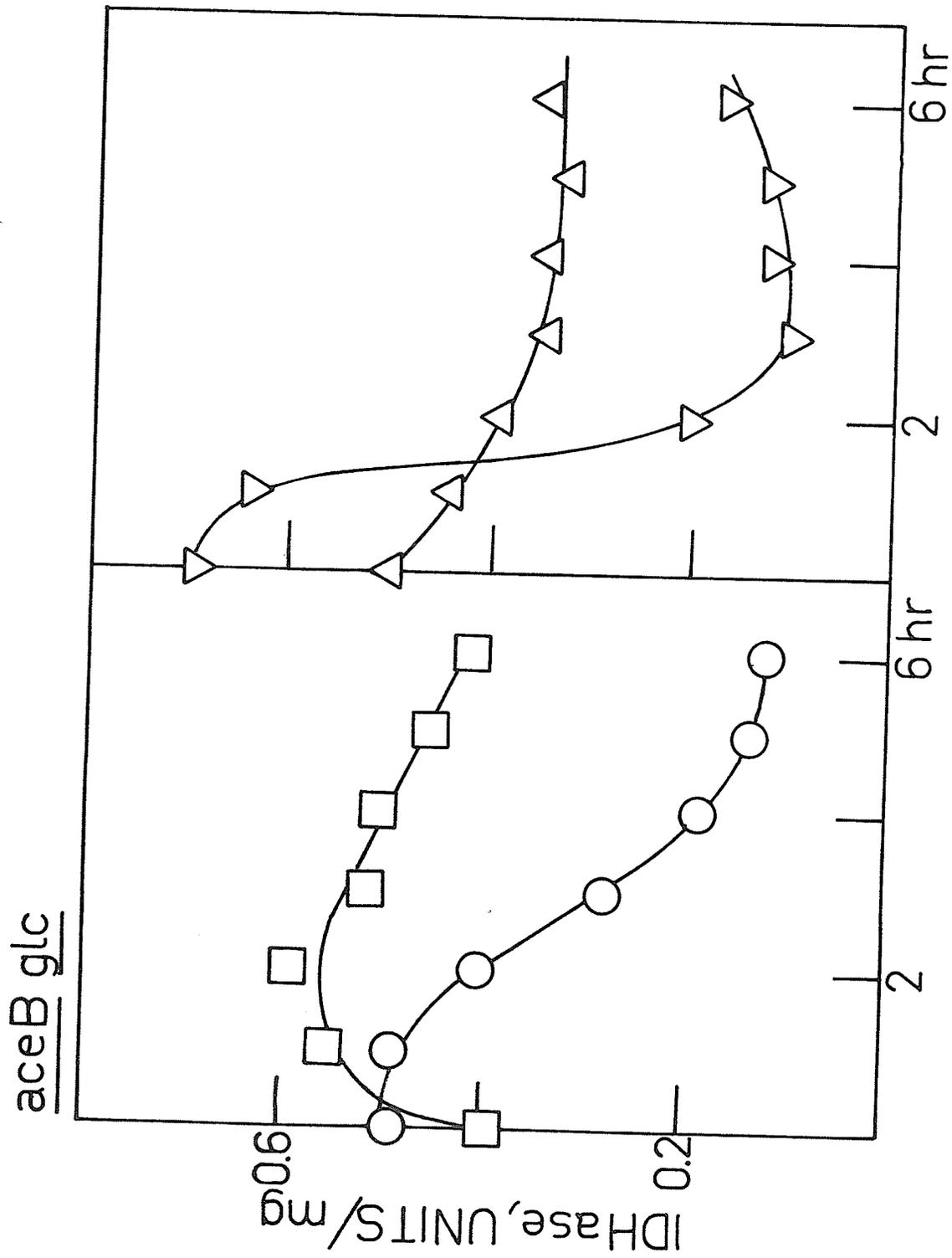
Mutant

The mutant used for these experiments, MLD 44, is mutated in both malate synthases (*ace B*, *glc*) and is the same strain that was used for the isocitrate lyase induction experiments described earlier. As in those experiments, glucose-grown cultures were resuspended in different carbon sources, and the changes in the activity of isocitrate dehydrogenase were measured.

Figure 36 shows the changes which occur in the specific activity of isocitrate dehydrogenase in MLD 44 on going from glucose to acetate, proline, α -ketoglutarate and succinate.

The enzyme activity falls very sharply on proline, with only 15% of the original activity remaining after three hours. This level recovers slightly over the next three hours, but only to 25% of the initial level. Acetate produces a drop in isocitrate dehydro-

Figure 36. The changes in the specific activity of isocitrate dehydrogenase in the *ace B*, *glc* mutant MLD 44 on transfer from glucose to acetate (○), succinate (□), α -ketoglutarate (Δ), and proline (∇) all at 1%. The zero time samples were taken after resuspension in the new carbon sources.



genase activity also, but this occurs more slowly than was seen with proline. Although the initial activity of isocitrate dehydrogenase in these acetate cells is not as great as that seen for the proline cells, the final enzyme activities reached in the two cases are similar. In this *ace B, glc* strain, α -ketoglutarate produces only a slight drop in isocitrate dehydrogenase activity, and succinate, which in a wild-type produces a considerable increase in specific activity, shows only a small and temporary rise.

Isocitrate Dehydrogenase Activity in Mutants of the TCA Cycle

α -Ketoglutarate Dehydrogenase Mutant Figure 37 shows isocitrate dehydrogenase activity in the *suc A* mutant MLD 41 incubated in succinate and in α -ketoglutarate. As was seen in this strain with isocitrate levels, there is essentially no change in the specific activity of isocitrate dehydrogenase on either carbon source.

Succinate Dehydrogenase Mutant Figure 38 shows the changes in isocitrate dehydrogenase activity in the *sdh* strain 604-30S, resuspended in acetate, malate and fumarate. There is essentially no change in the enzyme activity, which is initially quite low, on either acetate or malate. There is only a very slight increase in isocitrate dehydrogenase on fumarate.

Malate Dehydrogenase Mutant The changes in the activity of isocitrate dehydrogenase in the *mdh* mutant HG38 on acetate and on proline are shown in Figure 39. Both carbon sources produce a marked drop in activity of isocitrate dehydrogenase, but proline seems to act

Figure 37. The changes in the specific activity of isocitrate dehydrogenase in the *suc A* mutant MLD 41 after transfer from glucose to 1% α -ketoglutarate (Δ) and 1% succinate (\square).

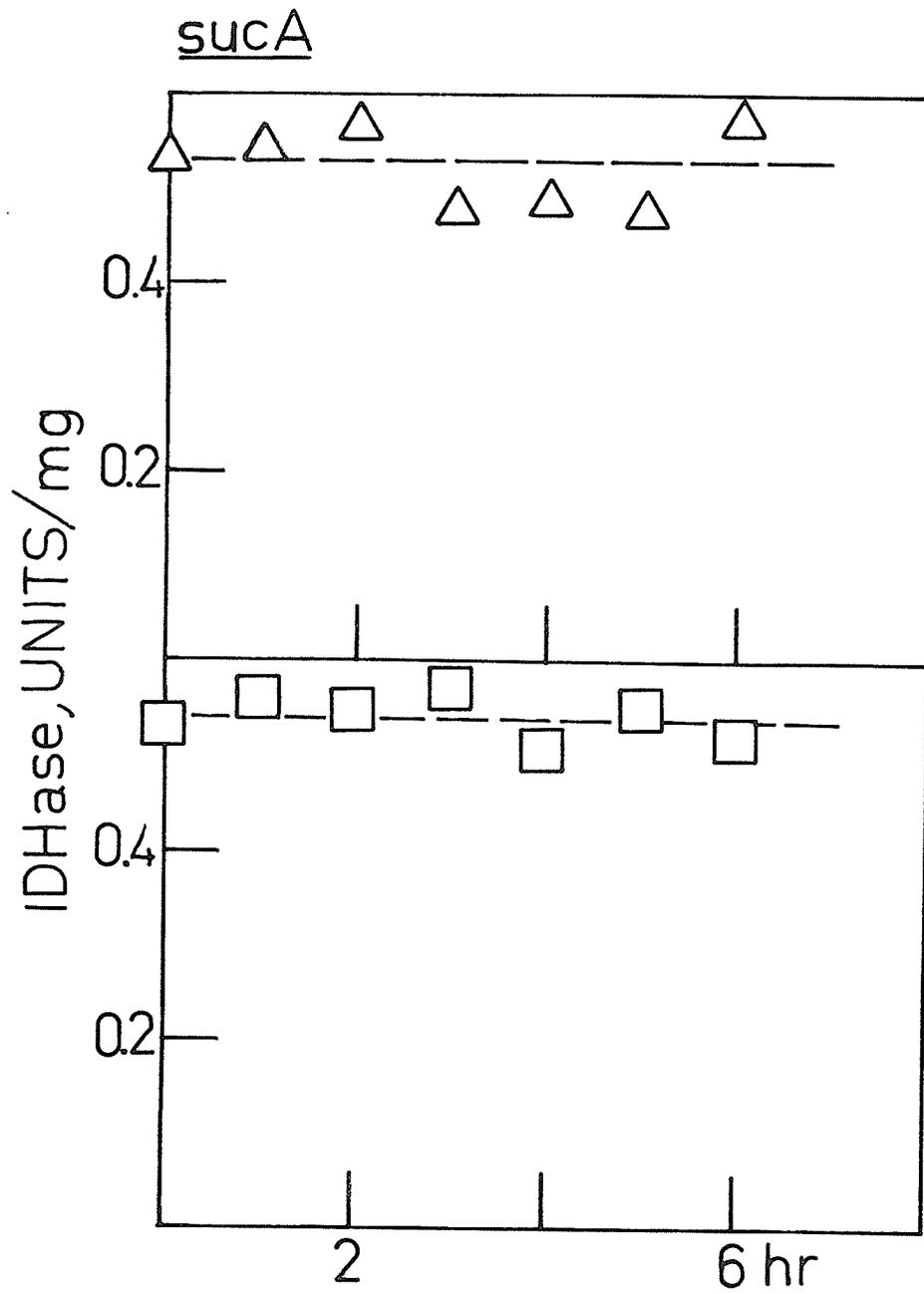


Figure 38. The changes in the specific activity of isocitrate dehydrogenase in the *sdh* mutant 604-30S after transfer from glucose to 1% acetate (○), 1% DL-malate (◊) and 1% fumarate (◻).

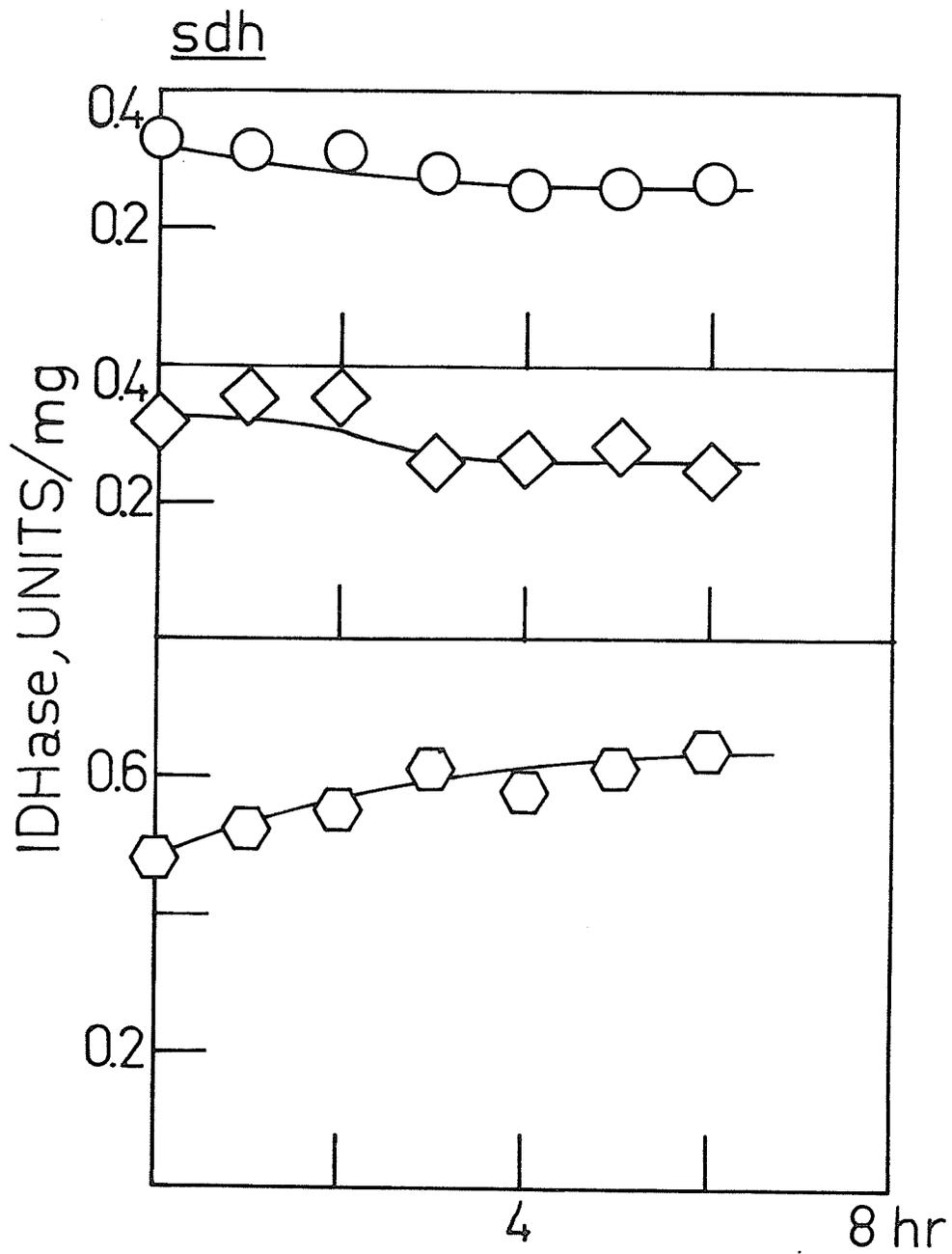
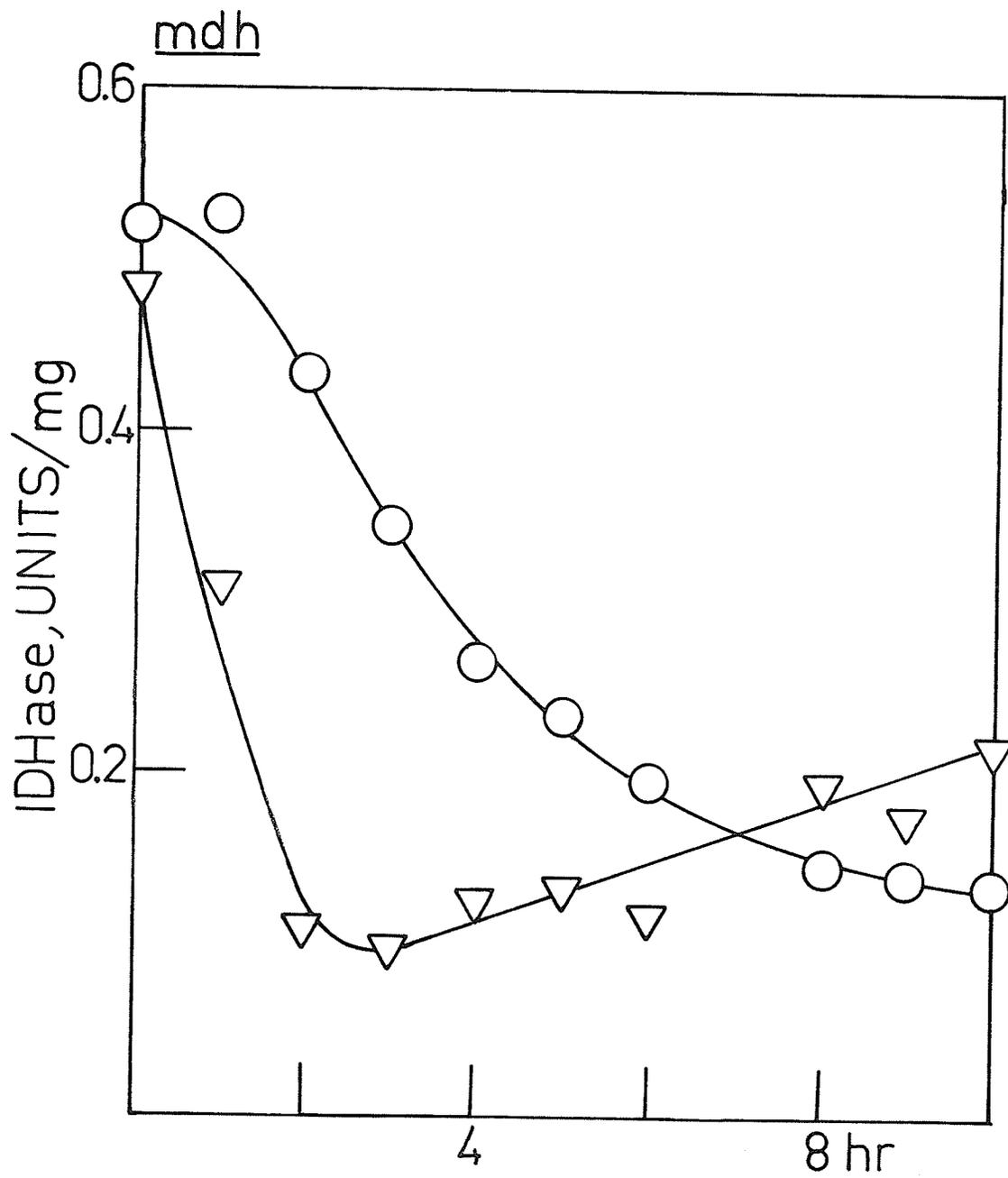


Figure 39. The changes in the specific activity of isocitrate dehydrogenase in the *mdh* mutant HG 38 after transfer from glucose to 1% acetate (○), and 1% proline (▽).



more quickly than acetate, a phenomenon which was also seen in the malate synthase mutant.

Isocitrate Dehydrogenase Activity in a PEP Carboxykinase, NAD-Malic Enzyme Mutant

The *pck*, *dme* mutant, HG20, was used for these experiments and Figure 40 shows the changes in specific activity of isocitrate dehydrogenase in this mutant after transfer to acetate, to succinate and to acetate plus succinate. Acetate produces an increase in enzyme specific activity over the first two hours, but the activity then falls. It rises again slightly, but the final activity is only 60% of the initial level. This *pck*, *dme* mutant is the only strain among those tested which shows an initial rise in isocitrate dehydrogenase activity on acetate.

Succinate produces an increase in enzyme activity, as well, but there is little or no subsequent drop. Succinate plus acetate shows the initial increase in activity seen with acetate and succinate, and also the later decline in activity seen on acetate alone. This decline is not nearly as marked as that seen with acetate alone, and the level finally reached is very similar to the initial activity.

Isocitrate Dehydrogenase Activity in a PEP Synthase Mutant

Figure 41 shows the changes in the levels of isocitrate dehydrogenase in the *pps* mutant DF1651, on acetate, succinate and acetate plus succinate. As with HG20, which is derived from this strain, the

Figure 40. The changes in the specific activity of isocitrate dehydrogenase in the *pc̄k*, *dme* mutant HG20 after transfer from glucose to 1% acetate (○), 1% succinate (□), and 1% acetate plus 1% succinate (■).

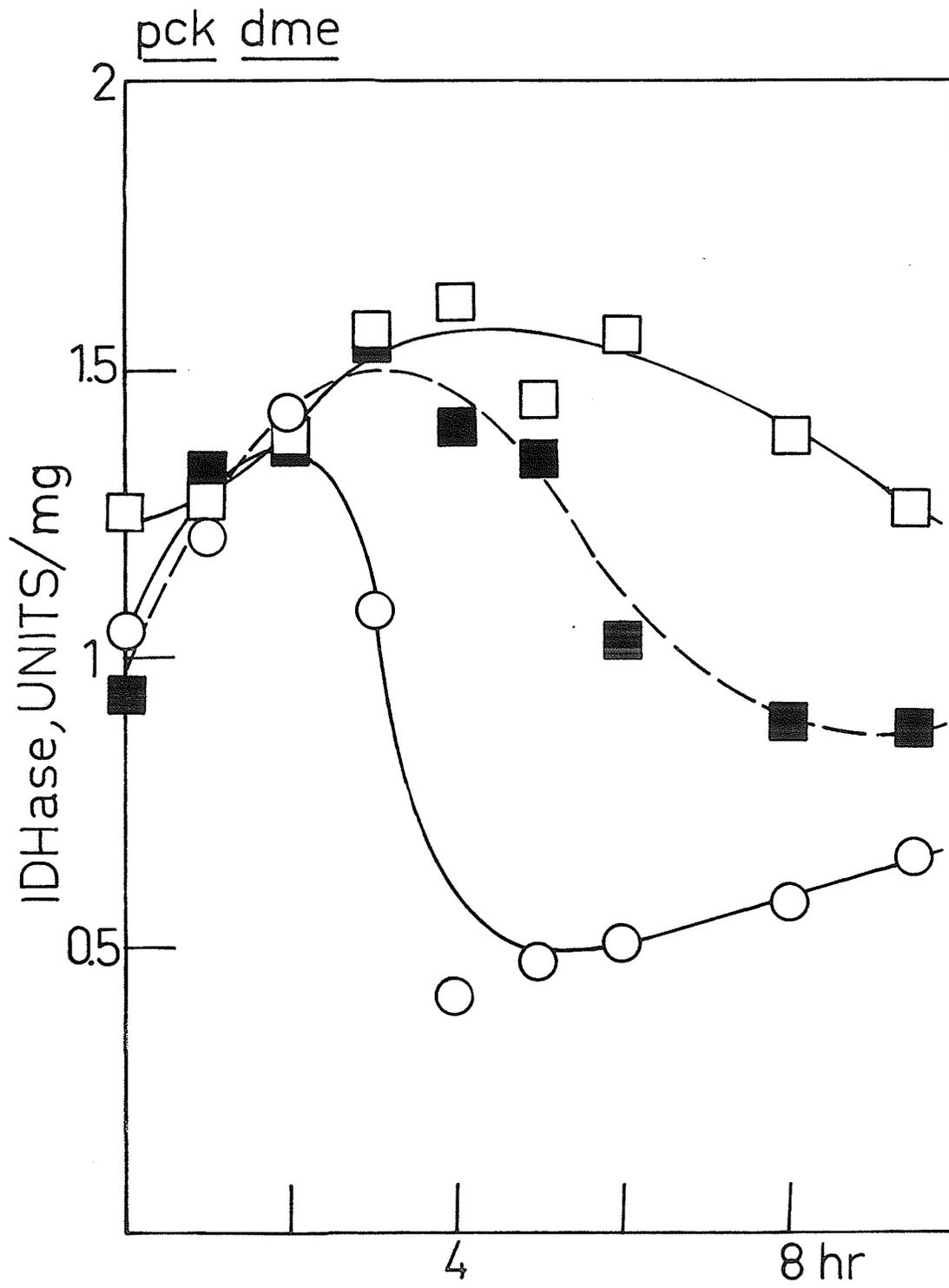
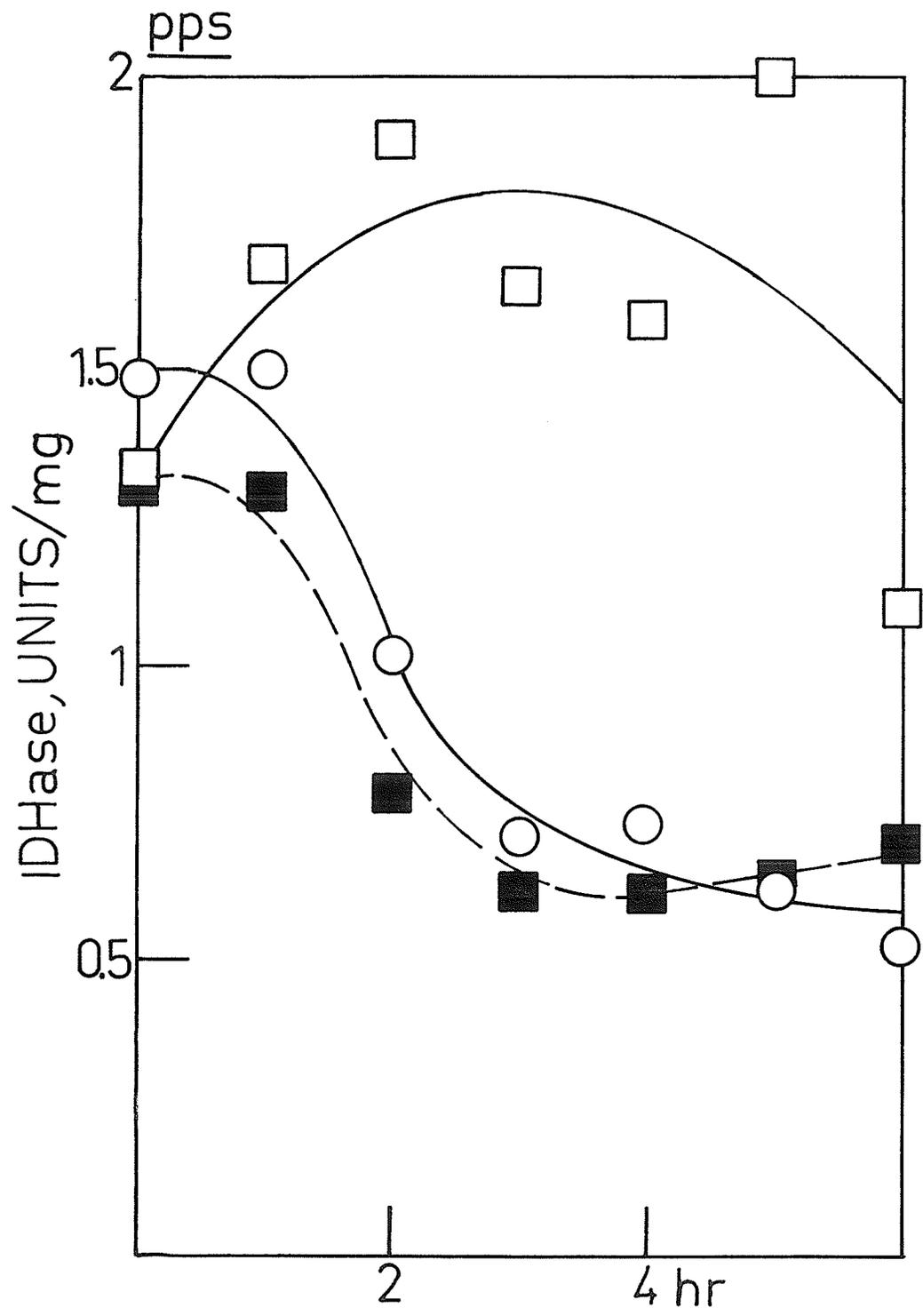


Figure 41. The changes in the specific activity of isocitrate dehydrogenase in the *pps* mutant DF1651 after transfer from glucose to 1% acetate (○), 1% succinate (□), and 1% acetate plus 1% succinate (■).



initial specific activity of isocitrate dehydrogenase in this mutant is high. There is a decline in activity on acetate after one hour, with activity falling to about 35% of the initial value after six hours. Succinate produces a rapid rise in specific activity in this mutant and the combination of succinate plus acetate leads to isocitrate dehydrogenase levels that are essentially the same as those produced on acetate alone.

The Effect of Cyclic AMP on Isocitrate Dehydrogenase Activity in
E. coli K12

The adenylate cyclase (*cya*) mutant MP259 was studied to test the effect of cyclic AMP on the loss of activity of isocitrate dehydrogenase. Figure 42 shows the effects of acetate, α -ketoglutarate and succinate on the activity of the enzyme in the *cya* mutant. There is a marked drop in activity on both acetate and α -ketoglutarate. The decrease in activity looks much greater for the acetate culture, but this is because this particular culture initially had a much higher activity. The final specific activities on both these carbon sources are the same. Succinate has very little effect on the activity of isocitrate dehydrogenase in this strain. There is a slow decline in the levels over the course of the experiment, but this may not be due to any active process. There is also very little effect of succinate on the levels of isocitrate lyase in this strain (Figure 14). Both these results are probably due to the poor transport of succinate in a *cya* mutant (Lo *et al* 1972).

Figure 42. The changes in the specific activity of isocitrate dehydrogenase in the *cya* mutant MP 259 after transfer from glucose to 1% acetate (○), 1% α -ketoglutarate (Δ) and 1% succinate (□).

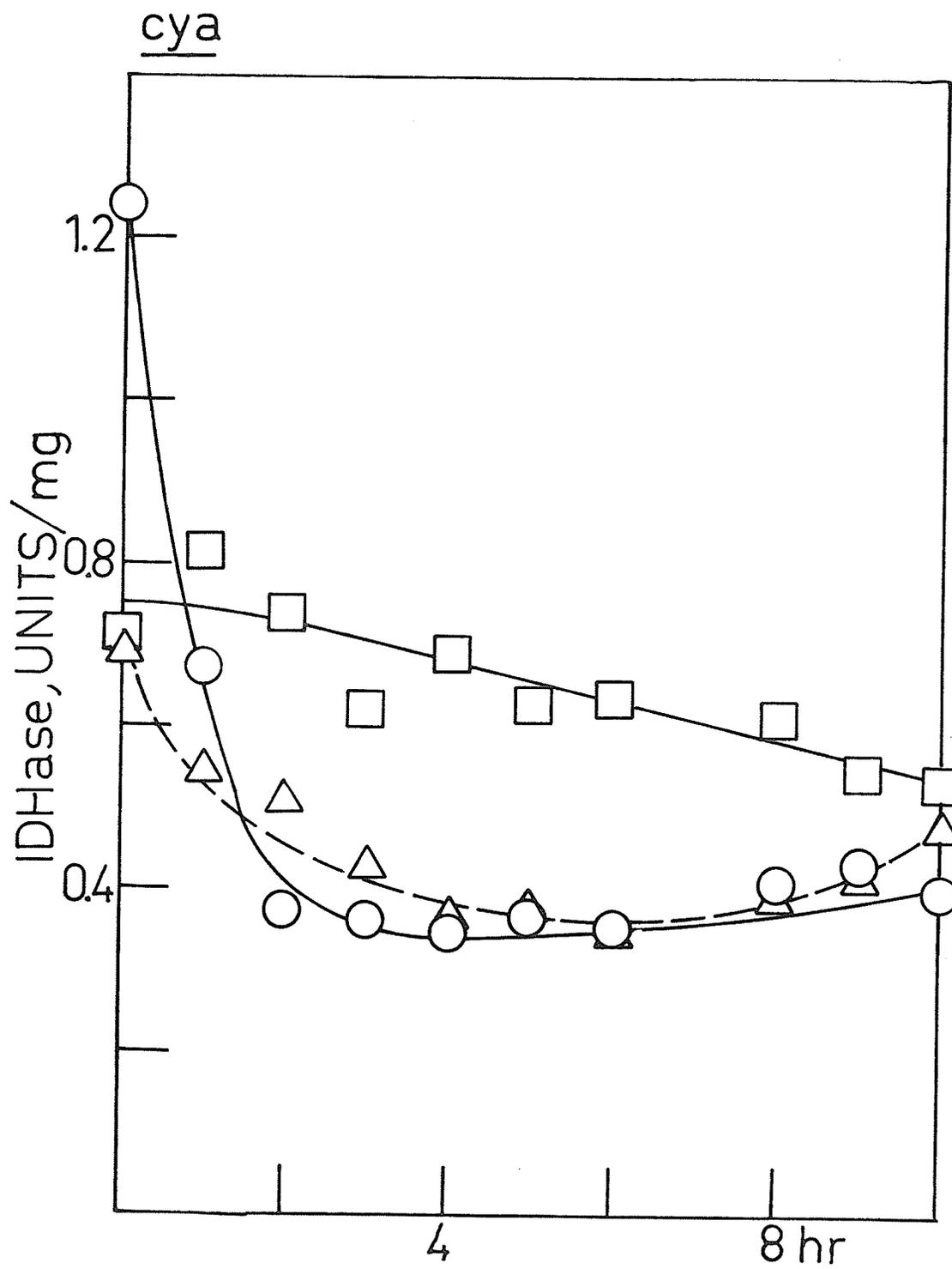
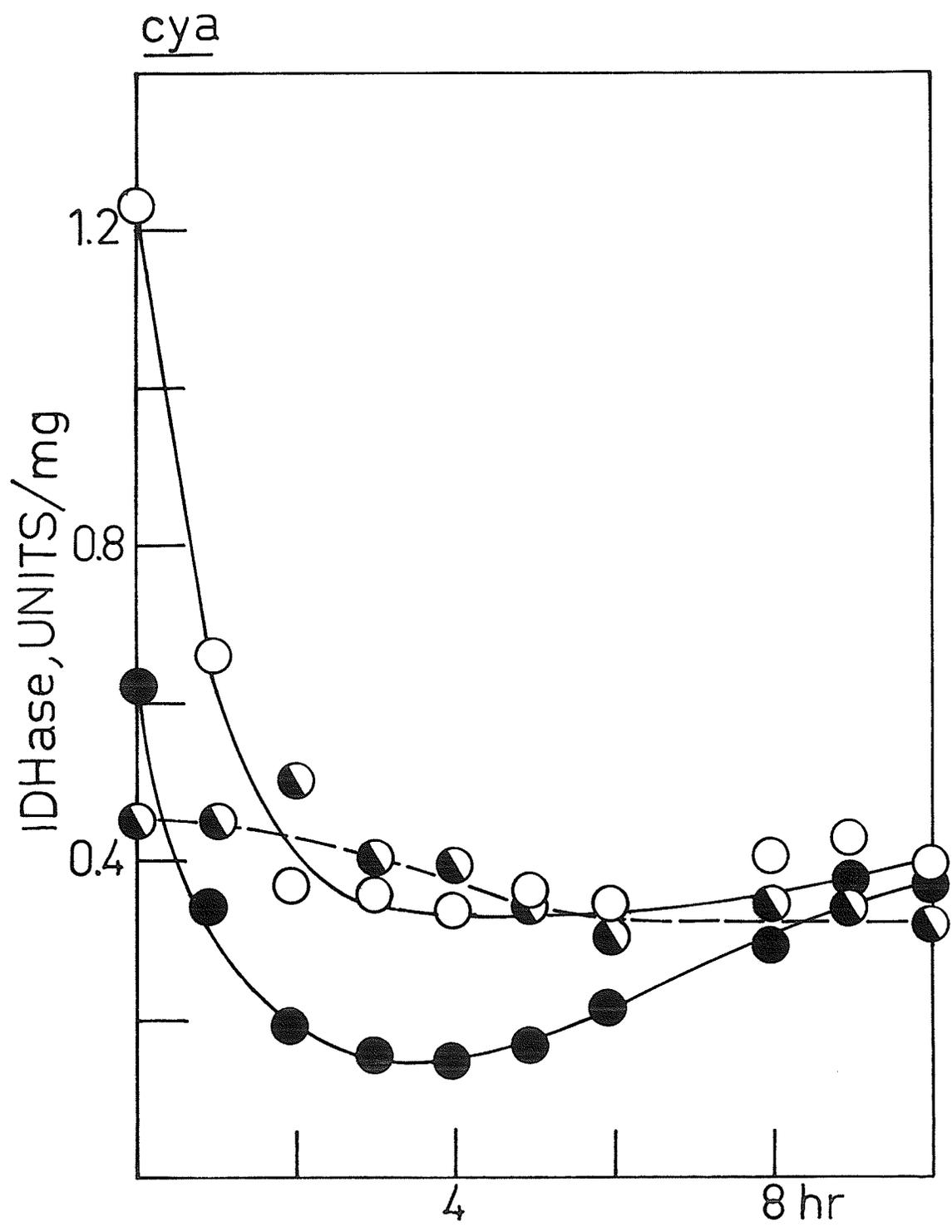


Figure 43 shows the effect of acetate with and without 1 mM cyclic AMP on isocitrate dehydrogenase activity in this *cya* strain. The activity of the enzyme in the acetate plus cyclic AMP culture starts at a lower level than the culture on acetate alone, and falls to a lower value. The ultimate activity reached after ten hours is the same, however, with or without cyclic AMP. Figure 43 also shows the activity of isocitrate dehydrogenase in the parent MP180 on acetate. The level in these cells starts at a low specific activity and does not change significantly over the course of the experiment. This value is comparable to the final specific activity seen with the *cya* strain, with and without cyclic AMP.

Figure 43. The changes in the specific activity of isocitrate dehydrogenase in the *cya* mutant MP 259 after transfer from glucose to 1% acetate (○), and 1% acetate plus 1 mM cyclic AMP (●) and its parent MP 180 after transfer to 1% acetate (◐). The *cya* mutant was grown on glucose in the absence of cyclic AMP.



DISCUSSION

DISCUSSION

The Glyoxylate Cycle

Experiments presented in this thesis support the view that acetyl CoA cannot be an inducer of the glyoxylate cycle operon. The levels of acetyl CoA appear to remain fairly constant on the different carbon sources although the fraction of total CoA which is present as acetyl CoA does vary. The fraction of CoA present as acetyl CoA is lower in cells with elevated levels of glyoxylate cycle enzymes. This can be seen for the wild-type and an *icl* *R* mutant growing on acetate, and for the wild-type growing on proline (Table 6).

When acetyl CoA levels are followed during adaptation of cells to growth on acetate, these levels initially rise, but fall as soon as isocitrate lyase is induced and the acetyl CoA is therefore more readily metabolized (Figure 29). The acetyl CoA levels during adaptation to growth on proline show no such rise, but also begin to decline as isocitrate lyase is induced (Figure 30).

The fact that *glt* *A* mutants are not able to induce isocitrate lyase on acetate further confirms that acetyl CoA cannot be the inducer. Levels of acetyl CoA are high in these cells even during growth on glucose, although isocitrate lyase specific activities are comparable to wild-type, and there is very little change in these levels when these cells are transferred to acetate medium, a fact which indicates that there is probably no difficulty in taking up acetate in these

mutants (Table 9).

The data of Lowry *et al* (1971) on metabolite levels in *E. coli* growing on different carbon sources have essentially eliminated the possibility that PEP is a co-repressor of the glyoxylate cycle operon. The levels of this metabolite are actually higher on acetate than they are on glucose, and the levels on the few other carbon sources tested by this group bear no relationship to the levels of isocitrate lyase. The same cannot be said for their data on pyruvate, however. The levels of this metabolite vary inversely with the levels of isocitrate lyase, for all the carbon sources they studied. This fact, taken in conjunction with the experiments of Kornberg (1966) and Dietrich and Henning (1970) makes pyruvate a rather likely candidate as a co-repressor of the glyoxylate cycle operon.

Experiments presented in this thesis do not support this hypothesis, however. The time of onset of isocitrate lyase induction, not only the level of this enzyme finally reached, depends markedly on the carbon source upon which the induction is taking place (Figures 5, 9). If pyruvate were the co-repressor, the rate at which its levels drop should correlate with the time of onset of induction - slowly with acetate, more quickly with α -ketoglutarate and succinate, and very quickly with malate. No such correlation was seen. With all four substrates, the drop in pyruvate was rapid and dramatic, and indeed was almost over by the time the cells had been centrifuged, washed, and resuspended (Figures 32, 33). This drop probably results from a decrease in the production of pyruvate from PEP, once glucose is removed

and the phosphotransferase system ceases to operate.

The observation of this rapid decrease in the amounts of pyruvate, during the rather simple manipulations needed to transfer the cells from one medium to another, emphasizes the importance of the precautions taken in the procedure of Lowry *et al* (1971) to freeze the cells as quickly as possible. Collecting the cells by centrifugation cannot be expected to give accurate results. Even with rapid freezing, the reproducibility of the measurements is not very good, and this may be a result of rapid metabolism of pyruvate during the collection of the cells on the filters.

Once the cells are resuspended in a new carbon source, there is a prompt readjustment to new pyruvate levels. Malate, succinate and α -ketoglutarate all produce rapid rises in the pyruvate concentration which levels off well below the amounts seen for glucose-grown cells, but above that found on acetate-grown cells. The concentration of pyruvate in the acetate cells is approximately 20 to 25% of that seen on the other three substrates.

These changes in pyruvate levels obviously do not correlate at all with changes in specific activity of isocitrate lyase seen during these induction experiments. If pyruvate were the controlling metabolite one would expect to find an immediate increase in isocitrate lyase levels on acetate rather than the slow onset which is actually seen. Succinate, malate, and α -ketoglutarate should not show such rapid increases in isocitrate lyase levels.

It is interesting to note that although α -ketoglutarate produces rather higher levels of isocitrate lyase than malate or succinate the levels of pyruvate finally reached on these three compounds are all about the same (Table 10; Figures 32, 33).

My pyruvate measurements confirm the findings of Lowry *et al* (1971) that glucose-grown cells have high pyruvate levels, succinate intermediate levels, and acetate low levels. They also indicate that the changes in the concentrations of metabolites can be very rapid when cells are transferred from one carbon source to another.

The absolute values for pyruvate levels which I measured in these cells are higher, however, than the steady-state values given by Lowry *et al* (1971). This could be due to strain differences or possibly to the rather unusual conditions under which the experiments were carried out. Lowry *et al* (1971) reported pyruvate values for cells which were fully adapted to different carbon sources, and they do mention that they obtained values of pyruvate of 5 μ moles/g dry weight in cells growing on acetate to which glucose had been added shortly before the measurement was made. It may therefore not be unusual to find higher levels under experimental conditions like those in my experiments.

Experiments with some of the TCA cycle mutants also suggest that pyruvate is not the co-repressor of the glyoxylate cycle operon.

Although acetate is not able to induce isocitrate lyase in *glt A* mutant, proline and α -ketoglutarate (and probably succinate, although this is less clear-cut) certainly can (Figure 16). Pyruvate levels

cannot be reduced in this strain by the formation of acetyl CoA which then reacts with oxaloacetate, since this reaction is missing in a *glt A* mutant. It might rather be expected that pyruvate levels would increase on these carbon sources.

An *mdh* mutant can induce isocitrate lyase on proline and on acetate (Figures 21, 22). The malate oxidase noted by Goldie *et al* (1978) in this strain does not appear to be present in very high levels under the conditions of these induction experiments. The only other routes for the production of oxaloacetate in these cells would be via PEP carboxykinase and PEP carboxylase or via NAD -malic enzyme, PEP synthase and PEP carboxylase; both routes might be expected to produce increases in pyruvate as well as in oxaloacetate.

Mutants in enzymes of the pyruvate dehydrogenase complex are able to induce isocitrate lyase on α -ketoglutarate to which a small amount of acetate, a necessary growth supplement for these mutants, was added (Figure 27B). Under the conditions of the experiment it might be expected that pyruvate would accumulate, but there is no obvious repressive effect on isocitrate lyase synthesis. Induction in the mutants occurs about as quickly as in the wild-type. One of these mutants, CGSC4823 (*ace E*), also induces on acetate (Figure 27A). This is not unexpected since mutants in the pyruvate dehydrogenase complex can grow on acetate. The second mutant, CGSC5476 (*ace F*) does not grow well on acetate and induces isocitrate lyase only poorly on this carbon source (Figure 27A). Since isocitrate lyase

induces extremely well on α -ketoglutarate, I suspect that some other unknown mutation in this strain, in addition to *ace F*, explains the poor growth on acetate.

The PEP synthase (*pps*), DF1651, behaves somewhat strangely. It shows rather elevated levels of isocitrate lyase even during growth on glucose. These levels are changed only very slightly after transfer to acetate, succinate, or succinate plus acetate (Figure 28). Since a *pps* mutant should grow on acetate, these rather low levels of isocitrate lyase in that carbon source are difficult to explain. In the absence of an explanation, the results obtained with this strain probably should not be used to argue for or against pyruvate as a co-repressor.

These direct and indirect indications that pyruvate levels do not correlate with the isocitrate lyase levels leave the identity of the regulating metabolite of the glyoxylate cycle operon an open question. Some useful indications about the glyoxylate cycle regulation, however can be obtained from the results in this thesis. An hypothesis which would explain most of the results obtained with the TCA cycle mutants is that induction of the glyoxylate cycle operon will occur only if levels of C_4 TCA cycle intermediates close to malate are high, and there is also a good supply of energy from the oxidative steps of the cycle which are coupled to oxidative phosphorylation. The isocitrate dehydrogenase step does not have an energy-producing role in *E. coli* since it is an NADP- rather than an NAD-linked enzyme. This leaves α -ketoglutarate dehydrogenase and succinate dehydrogenase

as the important energy-producing steps in this organism.

Induction on acetate will occur according to this hypothesis, only if the glyoxylate cycle enzymes are present, thus providing a means of increasing the C_4 intermediates, and if the oxidative steps of the TCA cycle are available. The *ace B*, *glc* mutant (Figure 24), which is not able to induce isocitrate lyase on acetate, is unable to produce high levels of C_4 intermediates. The *gltA* mutant (Figure 15) is unable both to provide increased C_4 acids, and to metabolize acetate to the oxidative energy-producing steps. The *icd* mutant (Figure 17A) is unable to metabolize acetate as far as the first oxidative step of the TCA cycle, and the *suc A* mutant (Figure 18A) is blocked at that energy-producing step. The *sdh* mutant (Figure 19A) is unable to metabolize acetate completely through the glyoxylate cycle, and is also blocked at a major energy-producing step of the TCA cycle. None of these mutants is able to induce isocitrate lyase on acetate. The *mdh* mutant should be able both to produce C_4 intermediates and to metabolize acetate through the oxidative steps of the TCA cycle, because there are alternative routes around the *mdh* block. This mutant does in fact show some induction of isocitrate lyase on acetate (Figure 21).

When induction of the glyoxylate cycle is carried out on α -keto-glutarate or proline, all the energy-producing steps of the TCA cycle are beyond the point of entry of these compounds into the cycle. They can therefore cause a rise in C_4 intermediates and energy supply

simultaneously. As would be predicted, the *ace B*, *glc* mutant (Figure 23), the *glt A* mutant (Figure 16A,C), and the *icd* mutant (Figure 17B) are all able to induce isocitrate lyase on these carbon sources. Metabolism of these compounds is completely prevented in a *suc A* mutant, and as expected, there is no induction of isocitrate lyase in this mutant (Figure 18B,C). Very little metabolism can occur in a *sdh* mutant on proline or α -ketoglutarate, and this mutant shows only a very slight increase in isocitrate lyase levels on these carbon sources (Figure 19B,C). The *mdh* mutant, which should be unimpaired on these carbon sources, shows good induction on proline, and significant though somewhat less induction on α -ketoglutarate (Figure 22).

When considering induction on succinate and malate, it must be remembered that these compounds will not yield full amounts of energy unless they are metabolized, at least in part, to pyruvate and acetyl CoA which will allow them to be oxidized completely through the TCA cycle. Since the *ace B*, *glc* mutant is not impaired in any function of the TCA cycle, this mutant should be able to induce isocitrate lyase on succinate, and indeed, it does (Figure 23). No induction would be predicted in a *glt A* mutant, since it is unable to condense acetyl CoA and oxaloacetate and thus allow the continuation of the TCA cycle. Some induction on succinate is seen with this mutant, but only if a small amount of proline is present (Figure 16B). Since this amount of proline itself has an inducing effect, although somewhat delayed compared to that of succinate plus proline (Figures 16A,B), interpretation of these results is made more difficult. On the basis of the

hypothesis, however, no induction on succinate would be expected, because of energy starvation. There is no induction of a *suc A* mutant on succinate. This strain although it is able to convert succinate to other C₄ intermediates, is missing α -ketoglutarate dehydrogenase, a major energy-producing step in the cycle. No induction on C₄ intermediates might also be predicted for the *sdh* mutant which is missing another energy-producing enzyme. There is some limited induction of this strain on fumarate (Figure 20). Presumably the energy derived from the other energy-producing reactions of the TCA cycle is sufficient to allow this small effect.

The *pck*, *dme* mutations prevent the metabolism of TCA cycle intermediates out of the cycle. The hypothesis would predict that this mutation should still allow induction of the glyoxylate cycle on acetate, α -ketoglutarate and proline, but not on malate or succinate which must be metabolized to pyruvate and acetyl CoA in order to satisfy the energy requirement. Acetate is in fact an extremely good inducer of the glyoxylate cycle in this mutant (Figure 25), while succinate shows very poor induction (Figure 25). Proline can also induce isocitrate lyase in this strain, although not as well as acetate (Figure 26). Since succinate cannot prevent acetate levels of induction being reached in this mutant (Figure 25) as it can in the wild-type and in some other mutants, it would appear that succinate dominates acetate in the wild-type by a process which requires metabolism through PEP carboxykinase and NAD-malic enzyme.

Further predictions of the hypothesis are that the *pps* mutation would not affect induction of the glyoxylate cycle on any of the

carbon sources mentioned, and that the *ace E* and *ace F* mutations would prevent induction only on succinate or malate because there is no route by which they may be converted to acetyl CoA. These mutants do in fact show induction on α -ketoglutarate and acetate (Figures 27A, B).

Although the hypothesis explains why induction of the glyoxylate cycle enzymes occurs with particular mutants on particular carbon sources, it leaves unanswered the question of the metabolic regulator responsible for the steady-state levels of these enzymes on different carbon sources. This regulator is unlikely to be a C_4 intermediate itself. Malate levels do not correlate (Lowry *et al* 1971) with glyoxylate cycle enzymes. Oxaloacetate levels have not been measured accurately, but are probably low. None of the mutants studied were certainly unable to make oxaloacetate. The *mdh* mutant might be making it via a malate oxidase (Goldie *et al* 1978) or via the NAD-malic enzyme, PEP synthase, PEP carboxylase sequence. Also, if a C_4 intermediate were an inducer, succinate and malate might be expected to give higher levels of glyoxylate cycle enzymes than acetate at the steady-state. The opposite is the case.

While the kinetics of the changes in pyruvate levels, when cells are transferred from glucose to another carbon source, are too rapid for a drop in pyruvate concentration to be the trigger which induces isocitrate lyase, it is still possible that this compound is the regulator which maintains the steady-state levels. The pyruvate concentrations on different media do vary inversely with isocitrate lyase levels as has been noted, at least as far as the available

data show.

On malate and succinate, one would expect significant pyruvate levels, because these compounds must be metabolized to some extent through pyruvate to acetyl CoA for energy production. No pyruvate production is needed on acetate itself, of course, and on proline and α -ketoglutarate metabolism through the energy-producing steps of the TCA cycle does not require a source of acetyl CoA. It might be predicted that pyruvate levels on proline and α -ketoglutarate would also be low during steady-state growth on these carbon sources, but this is not true of cells adapting to growth on α -ketoglutarate (Table 10) where pyruvate levels are comparable to those on malate and succinate. The fact that the *pek*, *dme* mutant does not induce isocitrate lyase as well on proline as the wild-type does also seems to indicate that some metabolism through pyruvate and acetyl CoA must normally occur on this carbon source.

Although a hierarchy of pyruvate levels can be rationalized in light of the metabolic needs of the cell, it should be noted that the known regulation of the malic enzymes (Sanwal and Smando 1969, Sanwal 1970), which would yield pyruvate from malate and succinate, does not seem sufficient to explain why more pyruvate should be produced during growth on these substrates than on α -ketoglutarate, proline, or acetate itself. If pyruvate is a regulator of the glyoxylate cycle, then the metabolic forces which control its concentration are still to be understood.

The induction experiments with the *aya* and *crp* mutants show that although these mutants are not able to grow on acetate, or α -ketoglutarate, they are able to induce isocitrate lyase on these carbon sources (Figures 12, 13). The amount of induction in these mutants does not appear very great when compared to the wild-type or to the *aya* strain grown in the presence of cyclic AMP, but these mutants on glucose already have four- to five-times higher levels of isocitrate lyase than a wild-type cell (Figure 12). Therefore, cyclic AMP does not appear to be necessary for the expression of the glyoxylate cycle enzymes. It would seem more likely that the reason why these strains do not grow on acetate is that they have reduced levels of cytochromes (Broman *et al* 1974) and succinate dehydrogenase (Takahashi 1975) which are known to require cyclic AMP for their synthesis. Low levels of both of these would severely limit the amount of metabolism which could occur on acetate. Since acetate and to a lesser extent α -ketoglutarate are able to induce isocitrate lyase in these cells, there must be sufficient TCA cycle enzymes and cytochromes present to allow at least some limited metabolism.

Succinate fails to induce these strains probably because cyclic AMP is required for the induction of the succinate transport system (Lo *et al* 1972) and thus succinate does not enter the cells to any great extent.

Isocitrate Dehydrogenase

Like Bennett and Holms (1971, 1975), and Garnak and Reeves (1979) I have observed that there is a partial inactivation of isocitrate dehydrogenase when *E. coli* cells are transferred from glucose to acetate medium. Since this effect is the opposite of the effect of acetate upon isocitrate lyase levels, it was of interest to see the extent to which the two effects mirrored one another. One important new finding was that α -ketoglutarate and proline, which are strong inducers of isocitrate lyase, both cause isocitrate dehydrogenase inactivation (Figure 33).

There are striking differences between the effects on isocitrate lyase and isocitrate dehydrogenase, however. In the induction experiments, succinate, unlike acetate or α -ketoglutarate, produces an increase in activity of isocitrate dehydrogenase relative to that seen on the glucose-grown cells (Figure 33), whereas this compound resembles acetate and α -ketoglutarate in its effect on isocitrate lyase. Acetate is able to cause an inactivation of isocitrate dehydrogenase even in the absence of glyoxylate cycle enzymes. The *ace B*, *glc* mutant which is missing both malate synthases, and does not induce isocitrate lyase, still is able to produce a marked drop in isocitrate dehydrogenase activity on acetate (Figure 36). The rates of inactivation of isocitrate dehydrogenase on acetate, proline and α -ketoglutarate are similar (Figure 33), whereas isocitrate lyase production is much slower on acetate than α -ketoglutarate (Figure 5).

These differences indicate that there is probably not a common regulatory event that stimulates isocitrate lyase synthesis and inactivates isocitrate dehydrogenase.

Largely because of difficulties stabilizing isocitrate dehydrogenase in cell-free extracts during the early phases of these studies, I did not test many mutants for the inactivation of isocitrate dehydrogenase. Since the demonstration of inactivation in the malate synthase mutant shows that acetate need not be metabolized through the glyoxylate cycle, it would be interesting to see whether inactivation is found in a *glt A* mutant, where metabolism would be stopped at acetyl CoA. There is essentially no change in the activity of isocitrate dehydrogenase in *suc A* or *sdh* mutants on any of the carbon sources tested (Figures 37, 38). This may mean that, as was suggested for the induction of isocitrate lyase, energy is required for the inactivation-activation of isocitrate dehydrogenase - either for the phenomenon itself, or for transport of the carbon compounds.

Since isocitrate dehydrogenase regulation channels more carbon through the glyoxylate cycle, this regulation could contribute to the isocitrate lyase induction by increasing C₄ intermediate accumulation on acetate. This probably is not an important effect on proline, however, since this carbon source induces isocitrate lyase well in a *glt A* mutant (Figure 16A), which cannot produce isocitrate at all.

It seems likely that acetate, proline and α -ketoglutarate inactivate isocitrate dehydrogenase by a common mechanism. Perhaps acetate must be converted to α -ketoglutarate first, but experiments on

isocitrate dehydrogenase inactivation in a *glt A* mutant have so far given equivocal results. Alternatively, the production of pyruvate could trigger the activation of isocitrate dehydrogenase. Cells transferred from acetate to pyruvate, succinate or glucose (Figure 34) show rapid rises in isocitrate dehydrogenase activity on all these carbon sources, but especially on pyruvate. The *pep*, *dme* mutant however, still shows increased activity of isocitrate dehydrogenase on succinate, even though pyruvate presumably is not produced in this strain on succinate (Figure 40). This mutant also shows an initial increase in activity on acetate and acetate plus succinate, but these levels later fall (Figure 40).

Whatever the trigger, the mechanism of inactivation-activation appears to involve a covalent modification as demonstrated by Garnak and Reeves (1979). This may be a phosphorylation, but their evidence does not really rule out an adenylation like that established in glutamine synthase (Shapiro and Stadtman 1970) which in fact is subject to regulation by α -ketoglutarate, among other compounds.

The decline in the activity of isocitrate dehydrogenase on acetate and α -ketoglutarate can still occur in a *cya* mutant without added cyclic AMP (Figure 43). Garnak and Reeves (1979) have postulated the operation of a protein kinase which phosphorylates the isocitrate dehydrogenase and causes inactivation. If such a protein kinase is present in *E. coli*, it appears not to be dependent on cyclic AMP.

How the inactivation-activation of isocitrate dehydrogenase is sufficient to regulate the flow of isocitrate through the two cycles

is still unclear. Lowry *et al* (1971) saw a real shift of metabolism in the isocitrate dehydrogenase direction when glucose was added to cells growing on acetate, in spite of the fact that these cells were fully induced for the glyoxylate cycle. The published K_M values for isocitrate are 1.8×10^{-5} M for isocitrate lyase (Ashworth and Kornberg 1963) and 1.56×10^{-5} M for isocitrate dehydrogenase (Reeves *et al* 1972). Thus, the simple activation of isocitrate dehydrogenase seen in my studies is not enough to explain this shift. The effect would seem to require some inhibition, or inactivation of isocitrate lyase. Lowry *et al* (1971) showed that of Kornberg's suggested inhibitors of isocitrate lyase, PEP was present in higher concentrations on acetate than glucose, and pyruvate did not accumulate to high enough levels to show significant inhibition. I found that isocitrate dehydrogenase levels rise about two-fold over an hour (Figure 34), when cells are transferred from acetate to glucose. When cells are transferred from acetate to pyruvate, isocitrate dehydrogenase levels increase about four-fold in an hour (Figure 34). In both cases, isocitrate lyase levels fall no more rapidly than one would expect from simple cessation of new enzyme synthesis (Figure 35). This latter finding indicates that isocitrate lyase is not inactivated upon transfer to glucose, although an unidentified inhibitor may still be involved.

It is obvious that further experiments are required before there can be a complete understanding of the regulation of the glyoxylate cycle in *E. coli*. The difficulty with the mutant experiments is

that there are so many interrelated reactions going on simultaneously, that there is a very real danger of oversimplification and even misinterpretation. More extensive measurements of metabolites, in particular pyruvate, and if possible oxaloacetate, might be useful, especially in some of the mutants studied in this work.

With the isocitrate dehydrogenase, more mutant experiments might prove useful in determining the regulator of this system. A fuller characterization of the modification involved in the inactivation-activation of the enzyme also offers a fruitful area of study.

There are special problems involved in working with the glyoxylate cycle regulation. Since it is a cycle, integrated into an even larger cycle, the regulator cannot be at the beginning or the end of a pathway as it is in the classical operons (*lac*, *his*, *trp*, *ara*) which have been studied. The inducing or repressing compound is probably always present in the cells, although its level will vary. Acetate is present in the medium of cells growing on glucose, and all the compounds to which acetate can be metabolized are also present in glucose-grown cells, although in different proportions. Rather small fluctuations in the metabolites may be enough to induce the glyoxylate cycle operon, and the techniques needed to identify the critical fluctuations must be correspondingly precise.

CHAPTER 4

GENETIC STUDIES

GENETIC STUDIES

The genes of the glyoxylate cycle, specifying isocitrate lyase (*ace A*) and malate synthase A (*ace B*) have been shown to map together at what is now designated 90 minutes on the *E. coli* chromosome (Vanderwinkel and DeVlieghere 1968). Very close to these genes is the locus designated *icl R*, mutants at which can confer constitutivity upon the glyoxylate cycle enzymes (Vanderwinkel and DeVlieghere 1968, Brice and Kornberg 1968). Figure 44 shows the order and location of these genes on the *E. coli* genetic map.

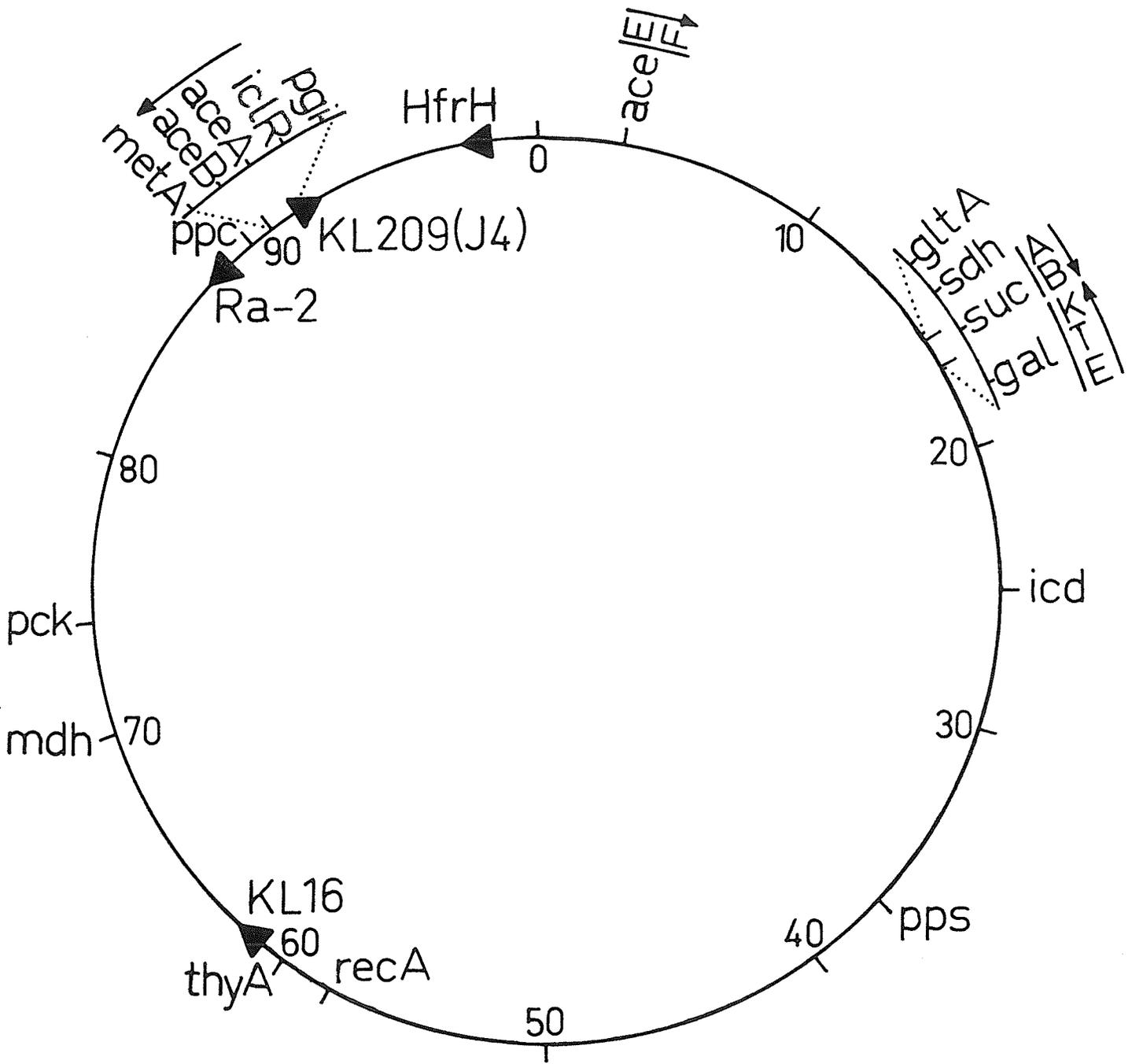
The co-ordinate induction of isocitrate lyase and malate synthase A, and the location of the two genes not only in close proximity to one another, but also to a locus which regulates this induction, implies that these genes are transcribed as an operon (Jacob and Monod 1961). The exact nature of the regulation at the genetic level, however is not known. This section will describe the isolation of a series of glyoxylate cycle constitutive mutants, their mapping, and the formation of F' merodiploids to study the dominant-recessive patterns of these *icl R* mutations.

The Isolation of Glyoxylate Cycle Constitutive Mutants (*icl R*)

Constitutive mutants of the glyoxylate cycle were obtained as:

1. Lactate positive, but pyruvate negative isolates of PEP synthase (*pps*) mutants (Brice and Kornberg 1968).

Figure 44. The chromosome of *Escherichia coli* K12 showing locations of relevant genetic markers. Numbers refer to the time scale on the genetic map. The arrows designate the location of Hfr's. The point of the arrowhead for any Hfr indicates the point of origin. During conjugation, transfer of the point of origin is immediately followed by transfer of the base of the arrowhead. The Hfr's KL209 (J₄) and Ra2 were used in the formation of F's covering the glyoxylate cycle operon (*icl R*, *ace A*, *ace B*). The Hfr KL16 was used in the construction of *rec A* strains.



2. Glucose positive isolates of a PEP carboxylase (*ppc*) mutant (Vanderwinkel *et al* 1963).
3. Spontaneous mutants.

Table 13 shows the growth characteristics, and PEP synthase and isocitrate lyase levels in four PEP synthase (*pps*) mutants isolated by EMS mutagenesis as described in Methods.

Table 14 shows the growth characteristics and isocitrate lyase levels in a series of *icl R* constitutive strains isolated from these *pps* mutants by ICR 191 mutagenesis, and one isolated by EMS mutagenesis.

One constitutive mutant was isolated after EMS mutagenesis as a glucose positive PEP carboxylase (*ppc*) mutant. The levels of isocitrate lyase in this mutant, and other mutants which were found fortuitously to have elevated levels of isocitrate lyase, are shown in Table 15. One such fortuitous mutation was found in an isolate of CSH60 which otherwise appears to be a wild-type cell. Another *icl R* mutation was found in strain CGSC4456 which is also *suc A*. It is not clear whether this *icl R* mutation would have arisen because of a selective pressure from the *suc A* mutation.

Strain CGSC5859, which is known to carry an *icl R* mutation (Creaghan and Guest 1972), is also shown in Table 15.

Mapping of *icl R* Mutants

The *icl R* mutations were mapped by P₁ transduction. Table 16 shows the data obtained from three point transductions. P₁ lysates were prepared on the various *icl R* mutants and were used to transduce

Table 13

Growth characteristics and enzyme levels in PEP synthase mutants.

| Strain | Growth Characteristics | | | Isocitrate Lyase units/mg | PEP Synthase units/mg |
|---------------|------------------------|---------|---------|---------------------------------|--------------------------|
| | Glucose | Lactate | Acetate | | |
| CSH78 | + | + | + | 0.015 | 0.17 |
| CSH60 | + | + | + | 0.016 | N.D. |
| MLD 1 (CSH60) | + | - | + | 0.019 | <.001 |
| MLD 2 (CSH78) | + | - | + | 0.016 | <.001 |
| MLD 3 (CSH78) | + | - | + | 0.023 | 0.01 |
| MLD 4 (CSH78) | + | - | + | 0.017 | <.001 |

Mutants were selected by EMS mutagenesis and penicillin enrichment procedure on lactate as described in Genetics Methods. The strain number in parentheses indicates the parent strain. Cells were grown on LB for isocitrate lyase determinations, and LB plus 0.4% lactate for the PEP synthase determinations.

Table 14

Growth characteristics and isocitrate lyase levels of *icl R* mutants.

| Strain | Mutant Isolation Method | Growth Characteristics | | | | Isocitrate Lyase units/mg |
|--------|------------------------------------|------------------------|---------|----------|---------|---------------------------|
| | | Glucose | Lactate | Pyruvate | Acetate | |
| MLD 5 | ICR191-MLD 1 (<i>icl R</i> -2) | + | + | ± | + | 0.18 |
| MLD 6 | ICR191-MLD 1 (<i>icl R</i> -3) | + | + | ± | + | 0.13 |
| MLD 7 | ICR191-MLD 2 (<i>icl R</i> -4) | + | + | ± | + | 0.13 |
| MLD 8 | ICR191-MLD 3 (<i>icl R</i> -5) | + | + | - | + | 0.13 |
| MLD 9 | ICR191-MLD 3 (<i>icl R</i> -6) | + | + | ± | + | 0.15 |
| MLD 10 | ICR191-MLD 3 (<i>icl R</i> -8) | + | + | ± | + | 0.19 |
| MLD 11 | EMS-MLD 4 (<i>icl R</i> -9) | + | + | ± | + | 0.22 |

Mutants were isolated by ICR191 mutagenesis or EMS mutagenesis as described in Genetic Methods. Cells were plated on glucose minimal medium and lactate positive, pyruvate negative colonies were selected by replica plating. Cells were grown on minimal medium with 0.4% glucose for the isocitrate lyase determinations. This Table also shows the parent of each mutant and the *icl R* designation number.

Table 15

Derivations of *icl R* mutants and their isocitrate lyase levels.

| Strain | Source | Isocitrate Lyase units/mg |
|----------|------------------------------------|------------------------------|
| MLD 12 | parent | 0.022 |
| MLD 13 | EMS MLD 12 (<i>icl R</i> -10) | 0.23 |
| CGSC4456 | spontaneous (<i>icl R</i> -11) | 0.19 |
| CSH60 | spontaneous (<i>icl R</i> -1) | 0.17 |
| CGSC5859 | <i>icl R</i> -7 | 0.12 |

MLD 13 was isolated by EMS mutagenesis as a glucose positive *ppc* mutant. CGSC4456 and CSH60 have spontaneous *icl R* mutations, and CGSC5859 is JRG548 of J.R. Guest. Each mutant is given an *icl R* designation number. Cells for isocitrate lyase assays were grown on LB.

Table 16

Genetic mapping of the different *icl* R mutations by P₁ transduction.

| Donor | Selected Marker | Number Analyzed | Frequencies of co-transduction (%) ¹ of selected marker with | | Frequencies of co-transduction (%) ¹ of | | |
|------------------------------------|---------------------------|-----------------|---|---------------------------|--|--|--|
| | | | <i>ace</i> A ⁺ | <i>met</i> A ⁺ | <i>icl</i> R | <i>ace</i> A ⁺ , <i>icl</i> R | <i>met</i> A ⁺ , <i>icl</i> R |
| CSH60(<i>icl</i> R-1) | <i>ace</i> A ⁺ | 109 | | 98 (107) | 100 (109) | | 98 (107) |
| | <i>met</i> A ⁺ | 300 | 93 (279) | | 81 (242) | 87 (242) | |
| MLD 5(<i>icl</i> R-2) | <i>met</i> A ⁺ | 50 | 90 (45) | | 90 (45) | 100 (45) | |
| MLD 6(<i>icl</i> R-3) | <i>met</i> A ⁺ | 400 | 95 (378) | | 86 (342) | 91 (342) | |
| MLD 7(<i>icl</i> R-4) | <i>ace</i> A ⁺ | 157 | | 90 (142) | 100 (157) | | 90 (142) |
| | <i>met</i> A ⁺ | 385 | 88 (338) | | 82 (314) | 93 (214) | |
| MLD 8(<i>icl</i> R-5) | <i>met</i> A ⁺ | 540 | 92 (494) | | 84 (454) | 92 (454) | |
| MLD 9(<i>icl</i> R-6) | <i>met</i> A ⁺ | 540 | 91 (491) | | 79 (426) | 87 (426) | |
| MLD 10(<i>icl</i> R-8) | <i>met</i> A ⁺ | 540 | 89 (478) | | 79 (424) | 91 (424) | |
| MLD 11(<i>icl</i> R-9) | <i>met</i> A ⁺ | 540 | 87 (470) | | 79 (428) | 91 (428) | |
| MLD 13(<i>icl</i> R-10) | <i>met</i> A ⁺ | 684 | 92 (632) | | 92 (631) | 100 (631) | |
| CGSC4456(<i>icl</i> R-11) | <i>met</i> A ⁺ | 412 | 70 (290) | | 54 (221) | 76 (221) | |
| CSH78(<i>icl</i> R ⁺) | <i>met</i> A ⁺ | 539 | 92 (496) | | | | |
| | <i>ace</i> A ⁺ | 338 | | 92 (319) | | | |

¹Actual numbers of transductants scored are given in parentheses.

P₁ lysates were prepared on the various *icl* R mutants, and transductions were done as described in Genetic Methods. The recipient strain in all transductions was CGSC4869 (*met* A, *ace* A, *icl* R⁺, *pps*, *pro* A, *arg* H, *thi*). Either *ace* A⁺ or *met* A⁺ was used as the selective marker. The *icl* R locus was scored as lactate positive, pyruvate negative colonies.

the multiple auxotrophic strain CGSC4869. Either *ace A*⁺ or *met A*⁺ was used as the selective marker. The *icl R* locus was scored as lactate positive, pyruvate negative colonies in this *pps* strain.

All the *icl R* mutants co-transduce the glyoxylate cycle constitutive mutation at a high frequency with both *met A*, and *ace A*. The frequency of co-transduction is greater between the *ace A* and the *icl R* mutation than between the *met A* and the *icl R* mutation for all the mutants tested. The co-transduction frequencies are similar to those obtained for *icl R* constitutive mutants by Brice and Kornberg (1968) and Vinopal and Fraenkel (1974) and are compatible with the gene order:

met A ace A icl R

Production of F's

Once it was established that all the constitutive mutants mapped at the *icl R* locus as part of the glyoxylate cycle operon it was possible to proceed to make F's which would cover this region of the chromosome.

Several of the mutants were derived from the parent strains CSH78 and CSH60. These strains are both Hfr's whose F factors are integrated into the chromosome near, and donate early, the genes of the glyoxylate cycle operon (Figure 44). Most of the *icl R* mutants derived from these strains still are Hfr's and retain the ability

to transfer these genes.

These strains were used to make F's according to the method of Low (1968) which is described in a general way in Genetic Methods. This method involves mating an Hfr, which donates the genes of interest early, into a female strain which is *rec A*. This mating then gives rise to merodiploids rather than recombinants.

To ensure that the progeny of a mating between the *icl R* Hfr's and the recipient strain would cover the entire region of the glyoxylate cycle operon, the recipient used was MLD 18. This strain is derived from CGSC5134, and was made *rec A* as described in Genetic Methods. It has the genotype *met A*, *pgi*, *his*, *rps L*, *rec A* and is wild-type for all the loci of the glyoxylate cycle operon. The loci *pgi* and *met A* map on either side of the operon (Figure 44).

Matings were carried out, as described in Genetic Methods, between several of the *icl R* Hfr's and MLD 18. Progeny were selected on glucose minimal medium supplemented with histidine and streptomycin, to select against the Hfr's. Isolates were tested for UV sensitivity, for the ability to transfer the *ace A*, *met A* genes, and on TTC glucose for the presence of a *pgi*⁺ gene. The precise lengths of the F's were not determined. Table 17 lists the strains obtained by this method.

Not all the *icl R* mutants could be used to produce F's by this method. For these strains, a second procedure was used. P₁ lysates were prepared on these *icl R* strains and used to transduce MLD 16 (*met A*, *ace A*, *pps*, *pro A*, *arg H*, *thy A*), selecting for acetate positive,

Table 17

F' strains obtained by mating Hfrs with MLD 18.

| Strain | <i>icl R</i> |
|--------|------------------------------|
| MLD 19 | F' <i>icl R</i> -4 |
| MLD 20 | F' <i>icl R</i> -1 |
| MLD 21 | F' <i>icl R</i> ⁺ |
| MLD 22 | F' <i>icl R</i> ⁺ |
| MLD 23 | F' <i>icl R</i> -5 |
| MLD 24 | F' <i>icl R</i> -6 |
| MLD 25 | F' <i>icl R</i> -8 |
| MLD 26 | F' <i>icl R</i> -1 |
| MLD 27 | F' <i>icl R</i> -3 |
| MLD 28 | F' <i>icl R</i> -2 |

All F's grew on glucose minimal medium supplemented with histidine, were glucose positive on TTC glucose and UV sensitive, and could transfer *met A*⁺ and *ace A*⁺ to strain CGSC4869 (*ace A*, *met A*, *pro A*, *arg H*).

methionine-requiring colonies. These isolates were then tested for their ability to grow on lactate but not pyruvate, an indication that they had received the *icl R* gene. Isolates were also tested for the presence of constitutive levels of isocitrate lyase. The *icl R* isolates were then made *rec A* as described in Genetic Methods.

Each of this series of *icl R* strains was then mated with MLD 22, which carries an F' with a wild-type *icl R⁺* gene. Colonies were selected which were *met A⁺*. These isolates were tested for UV sensitivity and the ability to transfer *met A⁺* and *pgi⁺*. Table 18 lists the strains obtained by this method.

Curing the F' Strains

All of the F' strains derived by mating MLD 22, which carries only wild-type genes on the F', with *icl R* recipients cured spontaneously if grown in medium which contained methionine. MLD 22 itself also cured spontaneously at a high frequency if not grown in medium in which there was a selective pressure to retain the F'.

Strains MLD 20 and MLD 26, both of which are derived from CSH60, also cured at a high frequency.

None of the other strains (MLD-19,-21, -23 to -25, -27 and -28) could be cured by any of the standard procedures with acridine orange or SDS as described in Genetic Methods.

Levels of Isocitrate Lyase in the F' Strains

Each of the F' strains produced in this work carried both a wild-

Table 18

F' strains obtained by mating MLD 22 (F' *icl R*⁺) with F⁻ *icl R* *rec A* mutants.

| Strain | <i>icl R</i> |
|--------|---|
| MLD 37 | F' <i>icl R</i> ⁺ / <i>icl R</i> -9 |
| MLD 38 | F' <i>icl R</i> ⁺ / <i>icl R</i> -10 |
| MLD 39 | F' <i>icl R</i> ⁺ / <i>icl R</i> -11 |
| MLD 40 | F' <i>icl R</i> ⁺ / <i>icl R</i> -7 |

All F's could grow on glucose plus proline and histidine, were UV sensitive, and could transfer *pgi*⁺ and *met A*⁺ to strain CGSC5134 (*met A*, *pgi*, *his*).

type *icl R* gene and a mutant *icl R* gene which produced constitutive levels of isocitrate lyase in a haploid cell. All other genes of the glyoxylate cycle enzymes, isocitrate lyase and malate synthase A, were wild-type on both the F' and the chromosome.

Table 19 gives the specific activities of isocitrate lyase in these F' strains and where available, their cured derivatives. It also includes the specific activity for phosphoglucose isomerase in these strains.

Strains MLD 19 - MLD 28 all show the presence of phosphoglucose isomerase which is missing in the parent strain CGSC5134, and in those F' strains which have been cured. Strains MLD 37 - MLD 40 show approximately double the levels of phosphoglucose isomerase that is seen in their cured derivatives. The levels of this enzyme provide an internal check for the presence of an F' in these strains.

The *icl R* mutations fall into two categories: those which are dominant to the wild-type and those which are recessive.

Table 20 shows the specific activities, on four standard carbon sources, of four *icl R* strains, two of which are dominant (*icl R*-5, *icl R*-2) in these complementation studies, and two of which are recessive (*icl R*-1, *icl R*-9). Neither type of *icl R* is completely constitutive: both show lower levels on glucose than on acetate, and intermediate levels on succinate and proline. The dominant mutations show a narrower range of activities than the recessive mutation.

Table 19

The levels of isocitrate lyase and phosphoglucose isomerase in F' strains and cured derivatives.

| Strain | Isocitrate Lyase units/mg | Phosphoglucose Isomerase units/mg |
|---|---------------------------------|---|
| CGSC5134 | 0.021 | 0.001 |
| MLD 19 F' <i>icl</i> R-4/ <i>icl</i> R ⁺ | 0.16 | 0.48 |
| MLD 20 F' <i>icl</i> R-1/ <i>icl</i> R ⁺ | 0.016 | 0.49 |
| MLD 20 cured (<i>icl</i> R ⁺) | 0.019 | 0.002 |
| MLD 21 F' <i>icl</i> R ⁺ / <i>icl</i> R ⁺ | 0.007 | 0.60 |
| MLD 22 F' <i>icl</i> R ⁺ / <i>icl</i> R ⁺ | 0.001 | 0.84 |
| MLD 22 cured | 0.005 | 0.003 |
| MLD 23 F' <i>icl</i> R-5/ <i>icl</i> R ⁺ | 0.16 | 0.65 |
| MLD 24 F' <i>icl</i> R-6/ <i>icl</i> R ⁺ | 0.19 | 0.58 |
| MLD 25 F' <i>icl</i> R-7/ <i>icl</i> R ⁺ | 0.17 | 0.70 |
| MLD 26 F' <i>icl</i> R-1/ <i>icl</i> R ⁺ | 0.017 | 0.64 |
| MLD 26 cured (<i>icl</i> R ⁺) | 0.017 | 0.004 |
| MLD 27 F' <i>icl</i> R-8/ <i>icl</i> R ⁺ | 0.20 | 0.70 |
| MLD 28 F' <i>icl</i> R-2/ <i>icl</i> R ⁺ | 0.15 | 0.93 |
| MLD 37 F' <i>icl</i> R ⁺ / <i>icl</i> R-9 | 0.007 | 1.33 |
| MLD 37 cured (<i>icl</i> R-9) | 0.22 | 0.81 |
| MLD 38 F' <i>icl</i> R ⁺ / <i>icl</i> R-10 | 0.007 | 1.54 |
| MLD 38 cured (<i>icl</i> R-10) | 0.25 | 0.66 |
| MLD 39 F' <i>icl</i> R ⁺ / <i>icl</i> R-11 | 0.007 | 1.42 |
| MLD 39 F' cured (<i>icl</i> R-11) | 0.11 | 0.67 |
| MLD 40 F' <i>icl</i> R ⁺ / <i>icl</i> R-7 | 0.005 | 1.42 |
| MLD 40 cured (<i>icl</i> R-7) | 0.12 | 0.73 |

Strains MLD 19 - MLD 28 were grown on glucose medium A plus histidine. CGSC5134 and the cured derivatives of MLD 20, 22 and 26 were grown on glycerol medium A plus methionine and histidine. Strains MLD 37 - MLD 40 were grown on glucose medium A plus arginine, and proline. Their cured derivatives were grown on the same medium plus methionine.

Table 20

The levels of isocitrate lyase in dominant and recessive *icl R* mutants on different carbon sources.

| Carbon Source | Isocitrate lyase, units/mg | | | |
|----------------------|------------------------------|------------------------------|-----------------------------|-----------------------------|
| | CSH 60 (<i>icl R</i> -1) | MLD 11 (<i>icl R</i> -9) | MLD 8 (<i>icl R</i> -5) | MLD 5 (<i>icl R</i> -2) |
| glucose | 0.070 | 0.080 | 0.22 | 0.20 |
| glucose + cyclic AMP | 0.12 | 0.10 | 0.28 | 0.21 |
| succinate | 0.30 | N.D. | 0.42 | 0.34 |
| proline | 0.38 | 0.49 | 0.64 | 0.44 |
| acetate | 0.50 | 0.55 | 0.71 | 0.50 |

Cells were grown on the given carbon source through three transfers before being assayed for isocitrate lyase. CSH60 and MLD 11 have recessive *icl R* mutations in complementation studies, and MLD 5 and MLD 8 have dominant *icl R* mutations. All carbon sources were 0.4% except acetate which was 1%. When present, cyclic AMP was 5×10^{-4} M.

DISCUSSION

DISCUSSION

The use of merodiploids to study regulatory mechanisms in bacteria originated with the famous Pa Ja Ma experiment of Pardee *et al* (1959) in which temporary merozygotes formed during conjugation were used to examine the regulation of synthesis of the enzymes of the *lac* operon. The discovery of the stable F' *lac* by Jacob and Adelberg (1959) made it possible to carry out the complementation studies which led to the concept of the repressor and the operator.

In the inducible lactose system i^- constitutive repressor mutations are recessive to the wild-type both in *cis* and in *trans*. The operator mutation is dominant to the wild-type, but only in the *cis* configuration (Jacob and Monod 1961). Another type of repressor mutation has also been identified, i^{-d} , which is partially dominant to the wild-type in both *cis* and *trans*. This phenotype is due to the formation of mixed multimers of wild-type and mutant repressor subunits, which produce much less efficient repression than the wild-type (Davies and Jacob 1968).

The *icl R* mutations studied in this work also fall into two classes: one which is dominant to the wild-type allele, and one which is recessive. It is tempting to speculate that the recessive type of *icl R* is due to a mutation in a repressor-like molecule, while the dominant type *icl R* is due to an operator mutation. It is not possible, however, from these experiments to distinguish between the

operator-type of mutation and the dominant repressor type of mutation. The F's produced for this study carry wild-type genes for the other enzymes of the glyoxylate cycle operon on both the episome and the chromosome. Experiments would have to be carried out with the *icl R* mutation in *cis* to an *ace A* mutation, before one could distinguish between the two types.

An interesting difference between all of the *icl R* mutations studied here, and the constitutive mutants of the *lac* operon is that those repressor-type *icl R* mutations which are recessive to the wild-type, do not show fully constitutive levels on all carbon sources. At least two of these mutants continue to show different levels of isocitrate lyase on glucose and acetate (Table 20). The repressor mutations of the lactose system (i^-) show elevated levels of β -galactosidase in the absence of inducer, levels which are often higher than those seen in induced wild-type cells (Jacob and Monad 1961). The recessive *icl R* mutants in this study, at least in some cases, show rather lower levels of isocitrate lyase under all growth conditions, compared to the dominant *icl R* mutants under similar conditions (Table 20).

There is no reason why the regulation of the glyoxylate operon need conform to the lactose operon pattern. The accepted view of the regulation of the glyoxylate cycle operon, in the literature, has been that the system is repressible rather than inducible, and that PEP or pyruvate acts as the co-repressor (Kornberg 1966, Vanderwinkel and DeVlieghere 1968). Although the work presented

in this thesis now makes it doubtful that either of these compounds is responsible for the repression of the operon, it could still be a repressible rather than an inducible system.

The repressible systems which have been studied are all ones for which an exogenous supply of the product of the pathway, or the functional integrity of the pathway, is required at all times. These systems include several which are responsible for the biosynthesis of amino acids: tryptophan (Platt 1978), histidine and arginine (for both, see Gots and Benson 1974).

Although the glyoxylate cycle need function only when the cells are growing on acetate, the products of the cycle, succinate and malate, are vital to the cell at all times and thus one might expect a regulatory mechanism which would respond to high levels of a product of the glyoxylate cycle by shutting down the synthesis of these enzymes when further synthesis of this product was no longer required.

A third possibility for the regulation of the glyoxylate cycle operon is that it is a positively controlled system. The classic example of such a regulatory mechanism is the arabinose operon (Lee 1978). In this system the *ara C* gene product acts as a repressor of the operon in the absence of arabinose, and as a positive regulator in the presence of arabinose, the inducer.

It is not possible to decide, on the basis of the complementation experiments done in this work, whether this is a possible regulatory mechanism for the glyoxylate cycle operon. The recessive *icl R* mutations could be mutated in a protein which no longer binds to the

DNA to prevent transcription. Any wild-type product would function normally in a diploid cell, and prevent the expression of the operon. The dominant mutation would then be operator mutations which do not bind the repressor protein.

Without the isolation and study of *icl R* mutants which are non-inducible, however, it is not possible to distinguish between these various mechanisms. Uninducible *lac I* mutations have been shown to produce 'super-repressors' which bind to the operator more efficiently than the wild-type repressor and are therefore *trans* dominant in diploid studies (Davies and Jacob 1968). Uninducible mutants of the *ara* operon have been shown to be recessive to the wild-type allele and to the constitutive mutant allele because they produce a gene product which does not bind the inducer (Lee 1978).

Although this work is not able to distinguish the type of genetic mechanism which is regulating the glyoxylate cycle operon, it does show that a variety of different *icl R* constitutive mutants, obtained using different selective pressures, all map in the same region of the chromosome and in very close proximity to the genes that the *icl R* locus controls. Since so few constitutive mutants of the glyoxylate cycle have been mapped (Brice and Kornberg 1968, Vinopal and Fraenkel 1974) it was always possible that more than one locus might be involved in the regulation of the operon. The data presented here show that this is probably not the case. The mapping data provide no indication, however that the two types of constitutive mutations map at different distances from the *ace A*

locus (Table 21). If the regulation were similar to that of the *lac* operon the recessive (repressor) mutations might be expected to map somewhat farther from the *ace A* locus than the dominant (operator) mutations do.

There was some difficulty in scoring *icl R* colonies in the mapping experiments. They were identified, as has been explained, as lactate positive, pyruvate negative colonies of a *pps* mutant. When *ace A* was used as the selective marker, essentially all of the colonies were also lactate positive and thus were scored as *icl R*. It is not clear whether there is a bias towards constitutive mutants when acetate is used as the selective medium. Vinopal and Fraenkel (1974) noticed a similar phenomenon in their mapping experiments when *ace A* was the selective marker. A difficulty also arose, however, when *met A* was used as the selective marker. All colonies which were scored as acetate positive also showed a tendency to grow on lactate. A difference could be detected between *icl R* and wild-type colonies if the colonies were scored early enough, but if they were left too long, it became difficult to distinguish between them. This phenomenon was first noticed when a wild-type *icl R* produced lactate positive colonies. This scoring difficulty may have led to linkages which are higher than they should be, and thus it would be difficult to distinguish between two loci which might be closely linked. Only CGSC4456 showed an *icl R* mutation which was not as closely linked as the others to *met A*. This is in keeping with the fact that this *icl R* is a recessive, repressor-

Table 21

The frequency of co-transduction of dominant and recessive *icl R* mutations with *met A* and *ace A*.

| Strain | Type of Mutation | Frequency of Cotransduction % | |
|------------------------------|------------------|-------------------------------|----------------------|
| | | <i>ace A - icl R</i> | <i>met A - icl R</i> |
| MLD 6 (<i>icl R</i> -3) | D | 91 | 86 |
| MLD 7 (<i>icl R</i> -4) | D | 93 | 82 |
| MLD 8 (<i>icl R</i> -5) | D | 92 | 84 |
| MLD 9 (<i>icl R</i> -6) | D | 87 | 79 |
| MLD 10 (<i>icl R</i> -8) | D | 87 | 79 |
| CSH60 (<i>icl R</i> -1) | R | 87 | 81 |
| MLD 11 (<i>icl R</i> -9) | R | 93 | 82 |
| MLD 13 (<i>icl R</i> -10) | R | 100 | 92 |
| CGSC4456 (<i>icl R</i> -11) | R | 76 | 54 |

D is a dominant *icl R* mutation, R is a recessive mutation. Data were obtained from Table 16.

type mutation.

The fact that several of the F' strains cannot be cured is somewhat worrying. Other F's produced from these *icl R* strains proved too unstable for use, so that the F's used in the complementation experiments were chosen because of their stability. Except for the fact that they cannot be cured, the strains show all the properties of F's: they transfer the *met A* and *ace A* genes at a high frequency and they are UV sensitive and thus still *rec A*. Low (1972) has reported that there are some F's which are resistant to curing. All of the F' strains which do not cure contain *icl R* mutations derived by ICR 191 mutagenesis and appear as dominant. The possibility must be considered that these strains are not in fact F's, but because they are all *met A*⁺, *icl R*, *pgi*⁺ and *rec A*, it seems rather unlikely that they have arisen by recombination.

Much work still must be done to determine the regulation of the glyoxylate cycle operon, both at the genetic level, and in the identification of the regulating metabolite. Physiological experiments have probably told us as much as they are able. The search for the regulating metabolite must involve the study of the actual levels of the possible regulators under a variety of conditions. Only then can the physiological observations be placed in a secure context.

The simple genetic experiments have now also been completed. Further complementation experiments, with uninducible mutants

if they can be isolated, might give further insights into the genetic regulation, and deletion mapping would give a more clear-cut idea about the organization of *icl R* locus. As Beckwith and Rossow (1974) have pointed out, however, one must exercise caution in the interpretation of the *in vivo* complementation data. It is likely that an *in vitro* protein synthesizing system will eventually prove useful in the final unravelling of the regulation of the *E. coli* glyoxylate cycle operon. Almost thirty years after its discovery, the glyoxylate cycle is still proving a challenge.

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