Comparative Studies of the Regulation of Some Enzymes in Bacteria

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ABSTRACT

Reduced diphosphopyridine nucleotide (DPNH) has been found to be a potent inhibitor of malate dehydrogenase and malic enzyme throughout a selection of physiologically distinctive bacteria. Phosphoenolpyruvate carboxykinase is also inhibited by DPNH, but apparently only in bacteria which contain malate dehydrogenase. The previously reported inhibition of citrate synthetase by DPNH was also confirmed. In view of these results, it would seem likely that allosteric regulation by DPNH is a general property of bacterial enzyme systems.

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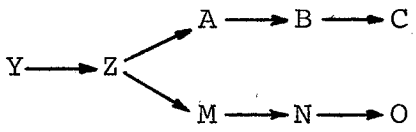
ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CS	citrate synthetase
CoA	coenzyme A
DNA	deoxyribonucleic acid
DPN	diphosphopyridine nucleotide
DPNH	reduced diphosphopyridine nucleotide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EMP	Embden-Meyerhof-Parnas
ED	Entner-Doudoroff
EDTA	ethylenediaminetetraacetic acid
F-6-P	fructose-6-phosphate
FDP	fructose 1,6-diphosphate
GSH	reduced glutathione
G-C	guanine-cytosine
GTP	guanosine triphosphate
HMP	hexose monophosphate pathway
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid
IDH	isocitrate dehydrogenase
MDH	malic dehydrogenase
ME	malic enzyme

NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
OAA	oxalacetate
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PS	protamine sulphate
TCA	tricarboxylic acid
TPN	triphosphopyridine nucleotide
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

During the past decade the understanding of cellular control systems has advanced rapidly. A particularly important regulatory system in the bacteria is constituted of allosteric proteins - enzymes located at key (branching) points in the metabolic scheme which are controlled by certain metabolites acting as signals (44). These metabolites, collectively called effectors, bind to the enzyme molecule and either inhibit or activate the enzyme involved, thus favouring one or the other pathway in a branched scheme (44). The effector molecule is often the end-product of one of the pathways (44). A typical example



would have metabolite C inhibiting the enzyme which catalyzes the conversion of Z to A. This study has been involved with the inhibition of allosteric enzymes by DPNH, an 'end-product' of the TCA cycle.

Regulatory enzymes have a quaternary or subunit structure, i.e., the enzyme molecule is composed of associated, identical units, usually two or four in number (44). An enzyme is considered to be a true 'allosteric' protein when it has been established that an effector, such as metabolite C,

binds to the subunit at a site physically separate from the binding area ('active site') of substrate Z. The binding of the effector usually changes the affinity of the enzyme for its substrate (45). The possible nature of this change in the enzyme molecule has been discussed at length in papers by Monod (45) and Koshland (32).

Enzymes other than those located at metabolic branching points may also be allosterically controlled. For example, the interconversion of F-6-P and FDP is catalyzed by two irreversible enzymes. The glycolytic function (F-6-P to FDP) is performed by phosphofructokinase which is allosterically activated by AMP (3, 33), while the reverse reaction, catalyzed by fructose diphosphatase, is inhibited by AMP in an allosteric manner (3, 33). Thus, the concentration of AMP in the cell can effect a sensitive control of carbohydrate metabolism.

The use of AMP in the illustrative example above is not unimportant. Many workers have assumed that the adenylates AMP, ADP and ATP are the only important allosteric effectors in the control of the cell's energy metabolism (3, 33). Recently, four regulatory enzymes of the TCA cycle in *E. coli* were found to be inhibited by DPNH (55, 56, 72, 80). The work presented here was undertaken to examine whether the function accorded the adenylate compounds falls to DPNH in the bacteria.

HISTORICAL

Citrate synthetase (citrate oxalacetate lyase(CoA-acetylating) EC 4.1.3.7) is probably the most important enzyme of the citric acid cycle. In catalyzing reaction (I), CS facilitates

$$\text{acetyl-CoA} + \text{OAA} + H_2O \longrightarrow \text{citric acid} + \text{CoA} \qquad \text{(I)}$$

the entry of the products of catabolism into the TCA cycle. CS is known to be ". . . the rate-limiting step of the tricarboxylic acid cycle" (74), thus, the regulation of this essentially irreversible enzyme (12) will greatly affect the supply of biosynthetic intermediates and energy available to the aerobically growing cell.

In 1966, Weitzman published his discovery that DPNH was a potent inhibitor of CS in E. coli (71). He soon complemented this success by revealing the allosteric nature of this regulation (72). Weitzman subsequently initiated a survey of CS regulation throughout a large and diverse selection of bacterial genera (74). He found that the citrate synthetases of gram (-) bacteria were invariably inhibited by DPNH (74). Furthermore, the citrate synthetases of the gram (+) bacteria, yeast, and pig heart were not controlled (71, 74). It has been discovered that these enzymes are much smaller molecules (75). Weitzman has postulated that they are not composed of subunits, and hence are not susceptible to inhibition by DPNH (75). A further difference in the

susceptibility of CS to DPNH inhibition was also noted among the aerobic and facultatively anaerobic gram (-) organisms. In some cases, CS inhibited by DPNH could be reactivated by adding AMP to the reaction mixture. Reactivation occurred only with the citrate synthetases from the gram (-) aerobes, however (74). Wright et al (79) found a second allosteric inhibitor of CS in E. coli, α-ketoglutarate. Another survey by Weitzman and Dunmore (76) revealed a pattern of CS inhibition interestingly similar to that earlier published by the same authors. Inhibition of CS by α-ketoglutarate occurred in the gram (-) enterics, which are facultatively anaerobic bacteria. The CS from gram (-) aerobic bacteria, gram (+) bacteria, yeast, wheat germ, and pig heart were not affected by this compound (76).

Before describing the other three enzymes detailed in the following study, it is of interest to note how it was discovered that they too were inhibited by DPNH in E. coli. In the Enterobacteriaceae (4), PEP may be metabolized directly to OAA by PEP carboxylase or catabolism may proceed by the usual route through pyruvate and acetyl-CoA, into the TCA cycle. Since the intermediate compounds of the citric acid cycle are also utilized as precursors in other biosynthetic reactions, there is a constant need for their replacement. This is accomplished by enzymes such as PEP carboxylase. Kornberg has termed their function 'anaplerotic', meaning

replenishing (30). Pyruvate carboxylase fulfils the anaplerotic function in animals, yeast and some bacteria, notably the pseudomonads and probably the Bacilli (10, 60, 78). It is now thought that the availability of OAA to the TCA cycle is controlled through these two enzymes. Acetyl-CoA is a powerful activator of both PEP carboxylase and the pyruvate carboxylase in animals (78), and has recently been shown to be involved in a novel regulation of pyruvate carboxylase synthesis in both mesophilic and thermophilic Bacilli (10, 60). The mechanism for regulating OAA supply has been represented as follows; assume that no acetyl-CoA or OAA is present in the cell, then the anaplerotic enzymes will be inactive (i.e., acetyl-CoA absent) and PEP will be catabolized to pyruvate and acetyl-CoA by pyruvate kinase and the pyruvate dehydrogenase complex respectively. Since no OAA is present, acetyl-CoA cannot enter the TCA cycle and the concentration of acetyl-CoA will continue to rise until activation of one of the anaplerotic carboxylases occurs. The OAA thus produced, and the acetyl-CoA already present, will then be condensed by the CS enzyme to produce citrate and final oxidation can proceed (30).

This delicate regulation of OAA supply is complicated by the presence of two other enzymes which compete strongly with CS for the use of OAA as a substrate. Sanwal, working with E. coli, has stated that " . . . in the absence of

compartmentation controls in bacteria, such as are present in higher organisms (due to the presence of mitochondria) excess oxalacetate can very easily be diverted via malate dehydrogenase and malic enzyme (converging pathway) to pyruvate and thence probably to acetyl-CoA. To prevent this unnecessary and wasteful recycling . . . mechanisms should be available which regulate the activity of the enzymes of the converging pathway." (56). Sanwal and his group found " . . . that the levels of DPNH in Escherichia coli (and perhaps also in the other enteric bacteria) are the central control signals for co-ordinating the activity of a variety of enzymes which utilize oxalacetate or malate as substrates." (80). It is now known that in addition to CS (72), TPN-specific malic enzyme (55), DPN-specific malate dehydrogenase (56) and phosphoenolpyruvate carboxykinase (80) are allosterically inhibited by DPNH in E. coli. The pyruvate dehydrogenase complex of E. coli has also been reported to be inhibited by DPNH (20), but it is not known for certain whether this represents an inhibition of an allosteric nature.

The in vivo level of DPNH in E. coli is 1.5 to 2.0 times higher when grown on glucose rather than succinate (80). Sufficient ATP is generated by anaerobic oxidation of glucose to satisfy the cell's energy needs. The operation of the TCA cycle and the electron-transport system for energy production purposes thus become largely superfluous.

Repression of these two enzyme systems during aerobic growth on glucose is well established (80). Due to a repressed cytochrome system, little re-oxidation of DPNH can occur and its concentration therefore remains high during active glycolysis (80). Since optimal operation of the citric acid cycle is not required, (Amarashingham (2) has proposed that under these conditions its primary function is biosynthetic) inhibition of the pyruvate dehydrogenase complex (20), CS and MDH by DPNH is not illogical.

Our comparative study of enzyme regulation involved four enzymes, all of which compete for OAA as a substrate. The controls on one of these, CS, and its importance in terminal oxidation and biosynthesis, has been reviewed (page 3). Two gluconeogenic systems can also utilize OAA. PEPCK is an important gluconeogenic enzyme in both animals and microorganisms, converting OAA to PEP directly (33). ME, in conjunction with MDH, constitutes a second possibility for gluconeogenesis in bacteria. During glycolysis the gluconeogenic enzymes should not be essential. This reasoning led to the discovery that DPNH, whose concentration is high during glycolysis, inhibited both gluconeogenic systems in E. coli (55, 56, 80). Due to the wide acceptance of the adenylate control hypothesis, (discussed later, page 13) it became important to determine if all bacteria or just perhaps the enterics (whose aforementioned physiological characteristics might be termed atypical of the bacteria in general) were

affected by this novel inhibitor. A brief review of the history and physiological importance of these enzymes is included to better understand the significance of their regulation by DPNH.

Although malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) was discovered very early in this century, relatively little work has been done with it. The molecular

$$\text{L-malic acid} + \text{NAD} \rightleftharpoons \text{OAA} + \text{NADH}_2 \qquad \text{(II)}$$

properties of MDH were first determined as recently as 1956 (77) and it has been studied in the bacteria only within the last five years (1, 46). A bacterial MDH has been obtained in crystalline form from B. subtilis (81, 82).

Sanwal has found that the MDH of E. coli is allosterically inhibited by DPNH in both the forward (oxidation) and reverse directions (56). As mentioned before, inhibition of the enzyme in the forward direction is a check on the operation of the TCA cycle. MDH has an equilibrium constant such that OAA reduction is greatly favoured over malate oxidation (12). During glycolysis, the CS of E. coli will be inhibited, thus leaving OAA susceptible to reduction by MDH and oxidative decarboxylation by ME to form pyruvate. Since a gluconeogenic system is not needed by the cell during this period, an effective control is necessary. DPNH was found to be the controlling effector, inhibiting both enzymes in the appropriate direction (55, 56).

Malic enzyme (L-malate:NADP oxidoreductase(decarboxylating) EC 1.1.1.40) is unique among enzymes. While widespread in

$$\text{L-malic acid} + \text{NADP} \xrightleftharpoons{Mn^{++}} \text{pyruvic acid} + CO_2 + NADPH_2 \quad \text{(III)}$$

nature, it has not yet been proven to perform a function of great value to the cell. ME was first discovered in pigeon liver by Ochoa et al (47) and the TPN-specific enzyme was soon observed in bacteria by a number of workers (35). Because of its relative unimportance, little work has been done with ME until recently. Since the enzyme is reversible (12), it has been postulated to perform both an anaplerotic and/or a gluconeogenic function in a system involving MDH and PEPCK.

$$\text{Pyruvate} \xrightarrow{\text{ME}} \text{Malate} \xrightarrow{\text{MDH}} \text{OAA} \xrightarrow{\text{PEPCK}} \text{PEP}$$

Much evidence has accumulated against the postulated role of ME in the above pathway. For example, ME is predominantly a soluble enzyme and is located outside the mitochondria of the cells in higher organisms (78), yet such mitochondria are able to phosphorylate pyruvate (35). In effect, then, ME is denied access to the metabolites and enzymes of the TCA cycle in all organisms except the bacteria. An anaplerotic role for ME in bacteria has also been disproved since Kornberg

has shown that PEP carboxylase is the anaplerotic enzyme in E. coli when it is grown on glucose (30). Even in media containing pyruvate as sole carbon source, E. coli mutants devoid of PEP synthase (it should be noted that E. coli sp. do not contain pyruvate carboxylase) do not grow (30). A possible gluconeogenic function for ME has been proved to be unlikely, at least in bacteria which normally contain PEPCK·PEP synthase is synthesized only when lactate or pyruvate are present as the sole carbon-containing compounds, thus there seems to be no possibility that malate is converted to PEP via ME and PEP synthase during growth on intermediates of the TCA cycle (30). E. coli mutants of PEPCK do not grow on C_4 acids of the citric acid cycle, thus providing additional evidence against ME functioning in gluconeogenesis (24).

To further complicate matters, Katsuki et al (29) have reported the existence of an allosteric DPN-specific malic enzyme in E. coli which is activated by aspartate (29, 61). The authors noted that ME was 5-10 times more active than its DPN-linked counterpart when grown on a minimal malate medium (29). "In micro-organisms, the general consensus of opinion seems to be that it (ME) has a role in the generation of pyruvate when the supply of C_4 compounds is abundant . . ." (53), and " . . . malic enzyme (TPN-specific) in both bacterial and mammalian systems, is concerned with the generation of

pyruvate and reducing power for lipogenesis." (55). Takeo et al (61) have postulated a similar function for the DPN-linked malic enzyme, and to date it is only under the above conditions that ME is known to perform any physiological function.

In accord with this known function, ME has recently been found to be an allosteric protein subject to end-product inhibition by acetyl-CoA (53). Sanwal has hypothesized that the recycling of OAA and malate to pyruvate during active glycolysis (DPNH concentration is high) would be illogical, and results published recently (55) show that ME is allosterically inhibited by DPNH and OAA.

It has also been observed that ME is allosterically inhibited by cAMP (54). In mammalian tissues, the actions of a number of hormones are mediated through cAMP (49), i.e., cAMP is a known effector of some mammalian enzymes. The level of cAMP in E. coli has been observed to rise sharply during periods of glucose starvation and it was subsequently found that ME was inhibited by this compound, the first known case in bacteria (54). It would seem, then, that cAMP is another indicator of the state of glycolysis in E. coli and for this reason inhibits ME.

Phosphoenolpyruvate carboxykinase (ATP: oxalacetate carboxy-lyase (transphosphorylating) EC 4.1.1.32) was discovered by Utter and Kurahashi (67)

$$OAA + ATP \xrightleftharpoons{Mg^{++}} PEP + ADP + CO_2 \qquad (IV)$$

in 1954. The original enzyme, isolated from chicken liver, was named oxalacetate carboxylase. The enzyme from mammalian sources requires GTP as a coenzyme, while ATP is generally necessary in microorganisms. Early investigations of the enzyme mechanism revealed that it was reversible. PEP was found to be the product of oxalacetate decarboxylation and it, in turn, was the acceptor molecule involved in the CO_2 fixation reaction (66). Subsequent to this finding, the enzyme was renamed PEPCK (18). The ATP requirement of PEPCK in microorganisms was first established for yeast (8). A later paper suggested that PEPCK functioned primarily as a gluconeogenic enzyme in yeast (65), and an abundance of evidence now supports this view for both procaryotic and eucaryotic cells (24, 30, 57, 62, 78). PEPCK does not serve an anaplerotic role in bacteria and, as has been mentioned, seems to be necessary for growth in minimal media using intermediates of the TCA cycle as carbon sources (24, 30). Mammalian liver mitochondria phosphorylate pyruvate at a high rate (35), and both pyruvate carboxylase and PEPCK are present in these mitochondria at a concentration sufficient to account for the observed production of PEP (34, 78). There seems little doubt that PEPCK is important mainly in gluconeogenesis.

The efficient control of CO_2 fixation and the distribution of PEP is of vital importance to the cell. A complicated

regulatory system exists in the enteric bacteria since PEPCK, PEP carboxylase, and pyruvate kinase compete for PEP. Several allosteric effectors have been detailed for PEP carboxylase and pyruvate kinase in E. coli and Salmonella (9, 38, 39, 40, 51, 52). Recently, Wright and Sanwal (80) have reported the allosteric regulation of PEPCK by DPNH, the first known effector of this enzyme.

In bacteria, the allosteric regulation of enzymes in the TCA cycle is a relatively new discovery. Before Weitzman's work with bacterial citrate synthetases, it was generally considered that the only indicators of the energy level in the cell were the adenylates. Publications by Krebs (33) and Atkinson (3) strongly support this hypothesis. There is an impressive amount of evidence to support this theory in eucaryotic cells. For example, the most important enzymes of glycolysis and gluconeogenesis are those which are essentially physiologically irreversible. These enzymes (several are listed below) are regulated by the adenylates and their control is vital to the cell, both procaryotic and eucaryotic. Very briefly, Atkinson's theory supposes that in the energy starved cell (i.e., AMP level high, ATP level low) IDH is activated, CS is not inhibited, and energy production proceeds via the TCA cycle. If a high energy condition should occur, IDH and CS would be relatively inactive and citrate would accumulate, thus activating acetyl-CoA carboxylase. Excess energy would then be stored

as fat (3). While it seems a very tidy theory, all the above controls have not been demonstrated in any single eucaryotic cell (3).

Only two enzymes of the TCA cycle are under allosteric control in eucaryotes. One of these is DPN-dependent IDH. Bacteria contain only the TPN-dependent IDH for which no effectors have yet been found (40). There is also no conclusive proof to date that ATP is an important effector of CS in bacteria.

Process	Activator	Inhibitor
(a) Eucaryotes:Glycolysis phosphofructokinase F-6-P ⟶ FDP	F-6-P,ADP AMP(3,33)	ATP,citrate (3,33)
(b) Eucaryotes:Gluconeogenesis fructose diphosphatase FDP ⟶ F-6-P		AMP,FDP (3,33)
(c) Eucaryotes:Terminal Oxidation CS acetyl-CoA + OAA ⟶ citrate		ATP (3)
Isocitrate dehydrogenase isocitrate ⟶ α-ketoglutarate	AMP (3)	
(d) Procaryotes:Glycolysis phosphofructokinase F-6-P ⟶ FDP	ADP,GDP(5)	PEP(5)
pyruvate kinase PEP ⟶ pyruvate	AMP,FDP(39)	

Jangaard et al (27) have reported the inhibition of E. coli CS by ATP, but the effect is greatly altered by changes in pH, so much so that at pH 7.5, a pH at which the enzyme is optimally active, no inhibition is observed. Recently, it has been shown that CS activity from B. subtilis is reduced

75% in the presence of 5 mM ATP over a wide pH range (16). While this is quite a high ATP concentration, (Kreb's calculations (33) assume that the sum of the concentrations of AMP, ADP, and ATP in the cell is 5 mM) this report may be of greater significance than that of Jangaard (27) since no control of CS has yet been demonstrated in the gram (+) bacteria. These are the only reported instances of adenylate control in the citric acid cycle of procaryotic cells.

MATERIALS AND METHODS

Cultures

Fourteen different bacteria were used in this study (Table I). Four members of the Family Enterobacteriaceae were included to test the universality of the regulatory patterns established in another enteric, Escherichia coli (55, 56, 72, 80). The other ten organisms were selected on the basis of gram-reaction, taxonomic diversity, and ease of cultivation in chemically-defined media.

Water Supply and Sterilization

Glass-distilled-water was used in all operations. Unless otherwise noted, all solutions and media were sterilized by autoclaving at 121° for 15 minutes.

Media

Whenever possible, cultures were grown in Difco nutrient broth plus an added carbon source (Table III). In some instances, however, the TCA cycle enzymes were repressed in this complex medium (see Results, page 26) and variations of the chemically-defined-medium given below were employed in these cases (Table III). Medium A was usually kept as a stock solution prepared at 10X final concentration and diluted as required. Selected carbon sources were added to either the mineral salts medium or to nutrient broth in

order to induce the desired enzyme. Unless otherwise stated, all carbon sources in Table III were prepared and sterilized separately at 10X their final concentration, then added to an appropriate volume of medium using aseptic technique.

Mineral Salts Medium A

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$Na_3citrate.2H_2O$	0.47 g
$MgSO_4$(anhydrous)	0.05 g
$(NH_4)_2SO_4$	1.5 g
$MnCl_2.4H_2O$	2.0 mg
$FeSO_4.7H_2O$	4.0 mg

The above compounds were dissolved in water to a final volume of one liter. In preparation, magnesium sulphate was dissolved separately and added after the other salts had solubilized. Final pH was 7.0.

Glutamate was prepared at 50X final concentration. Both succinic and malic acids were neutralized to pH 7.0 with NaOH before sterilization. Thiamine hydrochloride was dissolved in water at 100X final concentration (pH was 4.2) and sterilized by autoclaving at 110° for 25 minutes.

Cultural Techniques

All cultures were grown aerobically (rotary shaker) in 2000 ml Erlenmeyer flasks. Final volume per flask was 900 ml including inoculum and added carbon sources. Micrococcus and Nocardia were incubated at room temperature (22-24°) while the remaining cultures were grown at 28°. In all cases, 50 ml of the rapidly growing culture was used to inoculate the larger flasks. Organisms to be grown in a mineral salts medium were first adapted to that medium. This was accomplished by sequential transfer of a 10% volume of inoculum from nutrient broth to mineral salts medium until good growth occurred. Contents of the two-liter flasks were harvested by centrifugation at 16,000 g for 15 minutes. This and all subsequent operations to be described were carried out at 4°. Nutrient broth cultures were harvested 24 hours after inoculation and those grown in mineral salts at 48-72 hours. Each flask was tested for cultural purity by microscopic observation of the gram stain and cultural characteristics on nutrient agar. The entire contents of a single culture flask were used for the analysis of each of the four enzymes, i.e., 3600 ml of media was harvested per organism. After the initial centrifugation, the cells were washed once in the appropriate harvest buffer (Table II) and were then resuspended in this buffer (50% w/v) and stored at -20° until needed (generally within two weeks).

This preparation was then thawed at 22-24° and the cells disrupted by sonic oscillation at 8-10° for 15 minutes. The resulting crude enzyme was then treated as in Table IV.

Chemicals

The following chemicals and coenzymes were purchased from Sigma Chemical Co. Diphosphopyridine nucleotide (β-DPN^+), diphosphopyridine nucleotide, reduced form (β-DPNH), monosodium triphosphopyridine nucleotide (TPN^+), s-acetyl coenzyme A, sodium salt, 71% assay (acetyl CoA), crystalline adenosine-3',5'-cyclic phosphoric acid (cAMP), tetrasodium salt of ethylenediamine-tetraacetic acid (EDTA), tri-hydroxymethylaminomethane buffer (Tris), protamine sulphate, from salmon, (PS) and the sodium salt of adenosine-5'-triphosphate (ATP). Other special chemicals were as follows; Aldrich Chemical Co. - 5,5'-dithiobis-(2-nitrobenzoic acid) i.e., DTNB; Nutritional Biochemicals Corp. - oxalacetic acid (OAA) and D-biotin; Calbiochem - crystalline reduced glutathione (GSH), dithiotheitol (Cleland's Reagent) and N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES); Nuclear Chicago - the radioisotope C^{14} in $NaHCO_3$. All other chemicals used were of reagent grade and had been purchased from established chemical companies.

Partial Purification of Enzymes

The procedures listed in Table IV were employed mainly to eliminate DPNH-oxidizing activity from the crude extracts

(DPNH oxidase activity is associated with the plasma membrane in many bacteria) (42). Also, a partial purification of the citrate synthetase enzyme was necessary to prevent non-specific colour development of the indicator DTNB used in this assay. Aside from Table IV, one other purification scheme was used. The MDH of Staphylococcus aureus was found to be bound to the cell membrane (43) and a modification of the procedure published by Phizackerley and Francis (48) was used to isolate the enzyme. Staphylococcus was harvested and the cells sonicated as previously described (page 18).

The enzyme crude was then centrifuged at 12,000 g for 10 minutes. The pellet from this step was discarded and the supernate tested for DPN-dependent MDH activity. None was found. The supernate was then ultracentrifuged at 100,000 g for one hour. The supernate was discarded and the pellet was resuspended in the MDH harvest buffer (Table II) pH 7.5. The ultracentrifugation was repeated as above and the supernate discarded. The pellet was resuspended in 10 ml of the MDH assay buffer, pH 9.0 (Table II) and subjected to sonic oscillation for 10 minutes. This preparation was tested for DPN-dependent MDH activity and it was found to be present (Table V).

The protein concentration in all enzyme preparations was determined by the method of Lowry (36) using crystalline bovine serum albumin as a standard.

Enzyme Assay Systems

Spectrophotometric assays were done using a Beckman DU monochromator connected to a Gilford 2000 recorder. Silica cuvettes of one cm light path were employed. The buffers referred to in the following assays have been detailed in Table II. All assays were performed at room temperature (22-24°).

(a) Malic dehydrogenase

MDH activity was assayed by measuring the increase in absorption at 340 mμ using a reaction mixture containing 5 mM L-malate, 0.46 mM DPN^+, MDH assay buffer and a suitable volume of the enzyme. Final volume was 3.0 ml. L-malic acid was prepared in water at 30X the concentration above and neutralized to pH 7.4 using NaOH. DPN^+ was freshly prepared in water at 30X concentration (56).

(b) Malic Enzyme

ME was determined spectrophotometrically in the same way as MDH. The reaction mixture contained 1 mM $MnCl_2$, 10 mM L-malate and 0.077 mM TPN^+ (55). Unless otherwise stated, Tris buffer was used in the assay for ME. Final volume, including enzyme, was 3.0 ml. $MnCl_2$ was prepared in water at 30X the above concentration. Malic acid was prepared in water at 30X concentration and neutralized to pH 7.4 with

NaOH before use. TPN^+ was freshly prepared in Tris assay buffer at 30X final concentration.

In the assay for ME in the presence of cAMP, slightly different conditions were employed. L-malate was added to a final concentration of 2.0 mM instead of as above. Due to the lesser amount of malate, a new and more concentrated enzyme preparation was required (cells were resuspended in as little buffer as possible, approximately 100% w/v). Upon the addition of cAMP to these preparations, precipitation problems were often encountered. In these cases, HEPES buffer replaced Tris in the assay (Table IV). It was observed that the ME of some bacteria showed substantially greater activity in HEPES buffer.

(c) Citrate Synthetase

CS activity was assayed by measuring the increase in absorption at 412 mμ. The reaction mixture consisted of 0.1 mM OAA, 0.04 mM acetyl CoA, 0.025 mM DTNB, CS assay buffer and a suitable volume of the enzyme (79). Final volume was 1.0 ml. Acetyl CoA was freshly prepared in Tris buffer at 10X the above concentration. DTNB was also prepared at 10X concentration in Tris. OAA was freshly prepared in water at 100X concentration and neutralized to pH 6.5 with NaOH before use. The reaction was started by adding OAA.

(d) Phosphoenolpyruvate carboxykinase

The assay for PEPCK involves an ATP-dependent exchange of C^{14} between $NaH^{14}CO_3$ and OAA (80). The reaction mixture contained 5 mM OAA, 20 mM $NaHCO_3$ (0.05 microcurries), 3.5 mM ATP, 2.5 mM $MgCl_2$, Tris assay buffer and 0.5 ml of the enzyme preparation. Final volume was 2.0 ml. $NaHCO_3$ and ATP were freshly prepared in Tris buffer at 20X the above concentration. $MgCl_2$ was prepared in water at 20X concentration. OAA was freshly prepared in water at 20X concentration and neutralized to pH 6.5 with NaOH before use.

The reaction was carried out at room temperature (22-24°) and ended after five minutes by adding H_2SO_4 to a final concentration of 0.4 N. Carbon dioxide was bubbled through the acidified mixture for five minutes and aliquots (each scintillation vial contained 0.8 ml reacted enzyme mixture plus 10 ml scintillation fluid) were counted in a Packard Tri-Carb liquid scintillation counter for two minutes. Bray's (6) scintillation fluid was used and all counts were corrected for quenching and efficiency of counting errors by utilizing the Packard's automatic external standard (AES) system. Appropriate control experiments were conducted and activities reported (Table V) are dependent on the presence of both enzyme and ATP.

DPNH Oxidizing Activity

The presence of DPNH oxidizing activity was assayed for before testing the effect of DPNH on any enzyme. This was done by measuring the decrease in absorbance at 340 mμ. In the MDH, ME and CS assays, the reaction mixture for the assay included either 0.1 mM or 0.5 mM DPNH, the amount of enzyme to be assayed for inhibition, and all cofactors and substrates other than DPN^+, TPN^+ and OAA respectively. The CS preparations containing DPNH were also tested at 412 mμ before the final substrate (OAA) was added. PEPCK preparations were examined for DPNH oxidizing activity by measuring the decrease in absorbance at 340 mμ in a reaction mixture containing 0.5 ml enzyme, 0.1 mM DPNH and 2.4 ml assay buffer. In each instance, any significant oxidation of DPNH was removed by further purification of the enzyme preparation.

Preparation of Inhibitors

Three potential inhibitors were examined for their effects on various enzymes. OAA and cAMP were tested solely on ME. OAA was freshly prepared in water at a concentration of 3.0 mM and neutralized with NaOH before use. This stock solution was added to the reaction mixture in the cuvette to give a final concentration of either 0.05 mM, 0.1 mM or 0.2 mM OAA. Cyclic AMP was freshly prepared in Tris ME assay buffer at a concentration of 30 mM and diluted

in the cuvette to give a final concentration of 2 mM. DPNH was always freshly prepared in Tris ME assay buffer, pH 7.5. A number of suitable concentrations were used as stock solutions. In the PEPCK assay, DPNH was added in solid form to give a final concentration of 5 mM in the reaction mixture. The K_i for DPNH inhibition of MDH, referred to in Table V, was determined by plotting velocity vs. mM DPNH for at least three different concentrations of DPNH. K_i is defined as the concentration of DPNH which reduces the maximum velocity of the enzyme reaction by 50%.

RESULTS

Repression of enzymes in the TCA cycle presented problems in the case of Bacillus cereus and Micrococcus lysodeikticus. Both organisms were originally grown in nutrient broth plus succinate, but neither MDH nor ME could be detected in either bacterium. An appropriate mineral salts medium was decided upon based on the literature (31). MDH and CS were subsequently found in Micrococcus although the activities were weak (Tables V, VI). In Bacillus, however, the maximal activity of the TCA cycle coincides with sporulation (21) and is subject to both catabolite and feedback repression (16, 41). Hanson and Cox (23) have reported that the citric acid cycle in B. subtilis may be thought of as having anabolic (citrate to α-ketoglutarate and glutamate) plus catabolic (α-ketoglutarate to OAA) portions. The B. cereus culture used in this study was originally grown in nutrient broth and harvested after 24 hours, but apparently sporulation had not begun since neither anabolic (CS) nor catabolic (MDH, ME) enzymes were detected. The anabolic portion of the cycle (in particular CS and aconitase) is thought to be most severely repressed by the products of glucose catabolism in the presence of glutamate or α-ketoglutarate (23). During repression of these enzymes, sporulation will not occur (21). Thus the anabolic portion seemed the key to maximal enzyme production. An earlier report showed that high levels of

aconitase were formed by B. subtilis in a mineral salts medium containing acetate and glutamate as sole carbon sources (22). Since glutamate was implicated as a co-repressor of aconitase, it seemed sensible to reduce its concentration to a level just sufficient for good growth (B. cereus requires an organic nitrogen source when growing on acetate) and success was achieved with a mineral salts medium containing 0.5% acetate and 5 mM glutamate (Table III). Presumably sporulation of B. cereus occurred during growth in this medium, for high levels of CS were produced (Table VI). The same medium was used to induce MDH and ME, but succinate, rather than acetate, was included as the primary carbon source (Table III).

Citrate Synthetase

The experiments conducted with CS (Table VI) confirmed the results of Weitzman (74). The enteric bacteria were uniformly sensitive to DPNH inhibition as evidenced by the low concentration which completely inactivated these enzymes. The citrate synthetases of aerobic bacteria were also strongly controlled, although both the Azotobacter and Pseudomonas enzymes were only partially inhibited at a level of DPNH which produced 100% inhibition in the enterics. With certain reservations, we agree that CS is inhibited by DPNH only in the gram (-) bacteria (74). It seems very unlikely that citrate synthetases from Bacillus sp. are affected (16, 74

and Table VI). In view of the extremely low activity observed, the study of the enzyme from Micrococcus is open to question. We are thus in no position to confirm or deny Weitzman's results for the other citrate synthetases from gram (+) species.

The citrate synthetases of Erwinia, Nocardia and Staphylococcus could not be located. There are several possible explanations for this. Weitzman stated that some of the enzymes were very low in activity and resorted to polarography for their measurement (70, 74). The link between pyruvate and the TCA cycle is known to be weak in S. aureus (59). Alternately, the citrate synthetases in question may have been inactivated by DTNB in the assay system as experienced by Weitzman (74), or the enzymes may have been lost in purification. In any event, these three citrate synthetases were found by Weitzman (i.e., they are soluble in the cytoplasm) and their regulation fits the pattern discussed previously (74).

Malic Enzyme

Unlike CS, DPNH was a potent inhibitor of all the malic enzymes tested, whether from gram (-) aerobes, the enterics or gram (+) bacteria (Table VII). OAA also inhibited ME in every bacterium tested.

It is important to note the low concentration of OAA required to cause inhibition. Because OAA is an allosteric

inhibitor, small increases (e.g., from 0.05 to 0.10 mM OAA) in concentration were observed to cause large increases in percentage inhibition. As an example, Azotobacter ME showed no inhibition at 0.05 mM OAA, but was inhibited 50% by 0.10 mM OAA. Unfortunately, the allosteric effect is not illustrated by Table VII. The results using cAMP were not conclusive. Only three of the ME tested were inhibited by cAMP. Surprisingly, the ME of two of the enterics were not inhibited at a concentration of 2 mM cAMP and thus no definite pattern of regulation is discernible using this compound. The enzymes in P. aeruginosa, Erwinia and Salmonella, which showed some degree of control, were far less susceptible than the published result for the enzyme from E. coli (54). The use of HEPES rather than a phosphate buffer in the assay may have contributed to the rather large differences observed. Also, the enzyme from E. coli was quite highly purified as compared to our crudes (54). Nevertheless, from the results it would seem unlikely that cAMP is important for the fine control of ME in the bacteria.

No TPN-specific ME was found in Micrococcus, but the levels of MDH and CS detected in this bacterium were noticeably low. Because of this, there would seem a possibility that Micrococcal ME is membrane-bound and hence insoluble. The ME of S. aureus is known to be so located (43), but was probably solubilized (in this survey ME was found in the supernatant fraction) by prolonged sonication. The relationship between Micrococcus and Staphylococcus is discussed in greater detail later (page 36).

Malic Dehydrogenase

In this survey, ten of the original twelve bacteria (Ps. convexa and C. lividum were added later) possessed an active MDH (Table V). Each of the enzymes was strongly inhibited by DPNH, assaying in the direction malate to OAA. Thus, both the ME and MDH of all bacteria tested were inhibited by DPNH, regardless of their gram-reaction, carbohydrate metabolism or oxygen requirements.

An interesting development in the search for MDH controls was the finding that certain bacteria did not have a soluble, nucleotide-dependent MDH. It has already been mentioned that an insoluble DPN-dependent MDH is bound to the cell membrane in Staphylococcus (43). Two organisms used in this survey, Micrococcus lysodeikticus and Azotobacter vinelandii, are known to have both a flavoprotein linked L-malate oxidase or oxidoreductase (EC 1.1.3.3) and a soluble MDH (17, 28). With this in mind, it was found that Ps. aeruginosa had only minimal MDH activity (Table V). Under the same growth conditions, the activity of ME in this organism was nearly ten times higher. Another experiment was conducted with Ps. aeruginosa cells grown as before (Table III), but treated as Francis et al (17) did their extract of Pseudomonas B_2aba. Once again there was little reduction of DPN^+, and it was concluded that Ps. aeruginosa did not have

" . . . a readily solubilized NAD-dependent malate dehydrogenase which requires a particulate system to provide the link to oxygen" (17), as does Ps. B_2aba. These results indicated that the main enzyme responsible for malate oxidation in Ps. aeruginosa might be malate oxidase. A recent publication by Tiwari and Campbell (63) confirmed this hypothesis. Two other pseudomonads, Ps. fluorescens and Ps. ovalis, Chester, are also known to possess the malate oxidase enzyme in preference to the more common MDH (17).

MDH was similarly absent from extracts of Nocardia corallina (Table V) although, once again, ME was very active. It would seem probable that Nocardia, too, has the L-malate oxidase enzyme since a membrane preparation (as in S. aureus) for both Nocardia and Ps. aeruginosa showed no DPN-linked MDH to be present in either case. Unfortunately, one cannot definitely state that Nocardia does not have a MDH since negative results are open to question when working with such crude techniques. There is, however, some indirect supporting evidence for this hypothesis since at least two species of Mycobacterium, a genus closely related to Nocardia, contain malate oxidase (4, 15, 28).

Phosphoenolpyruvate carboxykinase

Wright and Sanwal (80) have shown that the PEPCK of E. coli is allosterically inhibited by DPNH. This inhibition

was characteristic of all the enterics tested as well as C. violaceum, (Weitzman (74) refers to this organism as an aerobe) another facultative anaerobe (13). For the sake of comparison, we tested C. lividum, which is oxidative in its metabolism of carbohydrates (13), and found that it contained both a soluble MDH and PEPCK. The presence of both enzymes, and the fact that PEPCK was inhibited by DPNH in this aerobe, is an important result, particularly since only one other PEPCK (Ps. aeruginosa, Table V) is available for comparison in the gram (-) aerobes. A single PEPCK was found among the gram (+) bacteria tested, and that in N. corallina, an obligate aerobe.

The PEPCK enzymes in Pseudomonas and Nocardia were not inhibited by DPNH. In view of the result from C. lividum, the absence of inhibition of PEPCK is probably not related to the oxidative metabolism of these organisms. Also, the absence of a DPN-linked MDH in both of the bacteria having no control of PEPCK, would seem more than coincidental. This point will be discussed in greater detail later (page 41).

It should be noted that the high concentration (5 mM) of DPNH employed in the PEPCK inhibition studies was necessary only to obviate interference by MDH in the crude extracts. The purified PEPCK enzyme in E. coli is inhibited in both directions at concentrations of DPNH below 1 mM (80).

It is difficult to comment on the failure to find PEPCK in several bacteria since very little information

is available about this enzyme in microorganisms. It is known that both Micrococcus lysodeikticus and Azotobacter vinelandii possess oxalacetate (OAA⟶pyruvate) decarboxylase, an enzyme which is not important in CO_2 fixation (28,66). In the experiments with Bacillus, Flavobacterium, and Staphylococcus, high ME activity was observed, but PEPCK was never found in the same extracts, thus repression of PEPCK is unlikely. It is very tempting to theorize that PEP synthase is present in these organisms, i.e., that PEPCK may not be the only possible gluconeogenic pathway leading to PEP in bacteria. Of course this is strictly hypothetical. It is equally probable that we have failed to isolate, or satisfy the cofactor requirements of, PEPCK enzymes present in any or all of the above.

DISCUSSION

Most bacteriologists would agree that classical bacterial taxonomy, as in the seventh edition of Bergey's Manual (4), is no longer adequate. A new taxonomy is developing based on the size of the bacterial genome, the base composition of bacterial DNA and the homology of these DNAs (15).

An average size bacterial genome might contain 1500-3500 cistrons (15). Classical methods rarely consider more than 30 characters, i.e., about 150 cistrons, and therefore approximately 10% of the genetic information available is used to classify most bacteria (15). The realization of this fact has led to increasing use of numerical (Adansonian) methods, in which 100-200 characters are considered. A much more significant percentage of the genome is exposed in such a classification. Also, the classical biochemical tests of bacterial taxonomy determine only the presence of a single product, and do not reveal the mechanisms of the reactions involved in the formation of that product. As a result, enzyme assays are utilized far more often in numerical taxonomy and comparative enzyme regulation (surveys by Weitzman (74), Cohen (11) and this study) is just now becoming a part of the new taxonomy.

DNA base composition ranges from 25-75 moles-percent guanine-cytosine (G-C) in the bacteria (15). This is a

conveniently wide range since it has been established that organisms 16-20 mole-percent apart in G-C base composition can share only four percent of their cistrons (15). Conversely, two organisms with identical DNA base composition are not necessarily similar. The number of possible arrangements of the G-C nucleotide pairs is enormous. In such a situation, the relatedness of the two bacteria can be determined by DNA homology experiments (15). Thus, while of little practical value in differentiating between bacteria at the species level, DNA base composition can determine whether it is possible for an unknown bacterial isolate to belong to any genus with a previously established 'type DNA'.

The enteric bacteria have been subjected to numerous taxonomic studies. We wish only to point out (see page 36) that P. vulgaris does not appear to be closely related to the majority of the enteric bacteria. E. coli, Salmonella, Serratia and Erwinia are clustered in a group at 50-60 mole-percent G-C. P. vulgaris is widely separated by this criterion. Despite this, P. vulgaris, and the other enterics tested, exhibited enzyme inhibition patterns (Table V, VI, VII) which were essentially identical to those published for E. coli (55, 56, 80). A possibility exists that the enterics might be separated according to the response of their ME to cAMP (Table VII), but further purification of these enzymes would be necessary to prove or disprove this point.

A further inspection of some other DNA base compositions reveals other anomalies in Bergey's classification. Micrococcus and Staphylococcus, two traditional members of the gram (+) cocci (4), possess genomes which must be almost entirely different. The results reflect several differences. S. aureus has a membrane-bound MDH and an active ME. This is supported by other authors (43). M. lysodeikticus possesses both MDH and a malate oxidase, but no trace was found of ME. Micrococcus sp. have a DNA base composition similar to that of Azotobacter and other obligate aerobes. These bacteria are known to possess many more membrane-bound enzyme systems than facultative anaerobes (19, 42). This, then, could account for the lack of success in isolating the TCA cycle enzymes from Micrococcus, and further serve to differentiate between Staphylococcus and Micrococcus.

DNA Base Composition of Some Bacteria

Organism	Mole-Percent G-C (15)
E. coli sp.	50-55
Salmonella	50-55
Serratia	53-60
Erwinia	50-57
Proteus vulgaris	35-42
Flavobacterium	30-42,64-70
Pseudomonas	43-48,56-70
Hydrogenomonas	67-68
Azotobacter	64-68
Chromobacterium lividum (aerobe)	65-72
C. violaceum sp. (facultative anaerobes)	64-68

DNA Base Composition of Some Bacteria (Cont'd)

Organism	Mole-Percent G-C (15)
Staphylococcus	32-38
Micrococcus	62-75
Nocardia	68-74
Mycobacterium	58-70
Bacillus subtilis (aerobic)	42-47
Bacillus cereus (fac. an.)	32-40

From the above, it is also obvious that the genus Flavobacterium needs revision and the actual taxonomic position of F. devorans (used in this survey, Table I) is in doubt. The pseudomonads, as they are now classified, are extremely heterogeneous in their DNA base composition. This massive genus is in a state of flux and has already been extensively reorganized by Stanier et al (58). Nocardial DNA is similar to that in the genus Mycobacterium (see page 31), and to the DNA of other actinomycetes (15). Some interesting differences can also be observed between the oxidative and fermentative Chromobacteria and Bacilli.

Bacterial taxonomy has traditionally relied heavily on comparative carbohydrate metabolism (14). In a recent survey (74), Weitzman suggested that AMP reactivates (i.e., after CS has been exposed to DPNH) only the citrate synthetases of the gram (-) bacteria which are oxidative in their metabolism of glucose. Since the obligately aerobic pseudomonads and Azotobacter used by Weitzman probably all utilize the Entner-Doudoroff (ED) pathway for glucose oxidation, these organisms will either not have a phosphofructokinase enzyme or it will be weak in activity (14, 74). AMP is a known activator

of phosphofructokinase, thus the author concluded that in its absence AMP controlled CS instead (74). There are at least two flaws in this argument however. For example, Weitzman included C. violaceum in the above group of AMP-reactivated citrate synthetases, but this organism has known fermentative abilities (13), and thus must possess an active phosphofructokinase. Also, Weitzman previously stated that E. coli CS was reactivated by AMP (73). In view of these contradictions, a taxonomic division of the gram (-) bacteria on the basis of carbohydrate metabolism would seem less than foolproof.

The results show that DPNH and OAA are important effectors of bacterial enzymes of the citric acid cycle (Tables V, VI, VII). It is particularly important that DPNH inhibits MDH and ME in all bacteria tested. Its effect cannot be correlated with the mode of carbohydrate metabolism or the gram-reaction of the organism involved. Below is a list of the different routes of glucose catabolism in some of the bacteria surveyed. Theoretically, the concentration of DPNH in vivo could vary widely according to the pathway used.

Comparative Glucose Catabolism in Some Bacteria

Organism	Principle Pathway of Glucose Catabolism	Oxygen Requirements	Reference
S. typhimurium	EMP	fac. an.	(14)
Ps. aeruginosa	ED	aerobe	(14)
A. vinelandii	cyclic ED	aerobe	(14)
N. corallina	HMP	aerobe	(14)
S. aureus	EMP + HMP	fac. an.	(59)

The Embden-Meyerhof-Parnas (EMP) pathway should produce four moles of DPNH per mole of glucose catabolized to acetyl-CoA. DPNH is formed from DPN^+ by glyceraldehyde-3-phosphate dehydrogenase and the pyruvate dehydrogenase complex enzymes. The ED pathway splits glucose into pyruvate and glyceraldehyde phosphate and therefore will produce only three moles of DPNH. A number of bacteria which use the ED scheme lack phospho-fructokinase and also have a very weak 'shunt' from glyceraldehyde phosphate to pyruvate (14,69). This relatively unknown pathway, referred to as the cyclic ED, is present in A. vinelandii and several other genera (14). One mole of glucose oxidized by this route would produce two moles of DPNH. The ED pathway has not been found in the gram (+) bacteria and it is, therefore, likely that obligate aerobes such as Nocardia corallina contain

the hexose monophosphate pathway (HMP). The HMP can be of two types, either a 'by-pass' mechanism or a truly cyclic pathway (69). In either case, the number of moles of DPNH formed is four.

The inhibition of MDH and ME by DPNH would appear to be a universal trait of the aerobic and facultatively anaerobic bacteria. CS is controlled in all the gram (-) bacteria, regardless of their metabolism. The citrate synthetases of gram (+) bacteria are not inhibited by DPNH (74). This could be due to a single genetic character, i,e., the presence of a smaller or non-allosteric CS enzyme (75), whose regulatory function might be superfluous if, for example, the pyruvate dehydrogenase complex (20) is allosterically inhibited by DPNH in gram (+) organisms. The regulation of PEPCK by DPNH was variable. The reasons for this variation are unknown (further discussion page 41). The results imply that in all bacteria there exists a mechanism (analogous to the one in E. coli) which controls the concentration of DPNH in the cell (80). Repression of the cytochrome system during aerobic growth in complex media containing glucose has been observed in gram (+) bacteria, in particular B. subtilis (68) and S. aureus (59).

The most interesting results concern the inhibition of PEPCK (Table V) by DPNH. Six gram (-) bacteria (5 facultative anaerobes, 1 aerobe) were found which contained a DPNH-inhibited PEPCK enzyme. These organisms all possessed a soluble MDH. Two other bacteria, Nocardia and Ps. aeruginosa, had PEPCKs which were not inhibited by DPNH. Neither organism had a soluble MDH. An attempt was made to study the PEPCK of a pseudomonad known to have a soluble MDH (e.g., Kornberg's (17) Ps. B_2aba). Ps. convexa was found to have the MDH enzyme but, unfortunately, a preliminary experiment failed to detect PEPCK in the crude enzyme preparation. No further work was done with this culture. We believe that the relationship between MDH and PEPCK is significant. The experiments relevant to these results were repeated several times and no significant variation was observed. A rather attractive, if unproven, scheme can be hypothesized based on the collective results for Ps. aeruginosa. Assume that the DPNH concentration is high. Normally the oxidation of malate to OAA is blocked by DPNH inhibition of MDH. Malate oxidase is mainly responsible for malate oxidation in this organism (63), thus, in all probability OAA will be produced. The pathway leading from malate to pyruvate is blocked since ME is inhibited. CS and PEPCK will compete for OAA as a substrate. CS will be inhibited by DPNH while

PEPCK will be activated. Kinetic studies of Pseudomonas CS[1] and PEPCK would prove very interesting, particularly since the enzymes from E. coli (80) have already been done.

One can only guess under what circumstances the above system might function in the cell. Such a scheme could affect gluconeogenesis, the glyoxylate cycle or the dicarboxylic acid cycle operating in pseudomonads (30). Some pseudomonads effect gluconeogenesis by a reversal of the non-oxidative portion of the pentose phosphate pathway (34, 50, 64). This would seem to apply to the organism used in this survey (Ps. aeruginosa 7700) since it is known to lack FDP aldolase (34), making gluconeogenesis by way of the EMP pathway impossible. A recent report has shown that Ps. aeruginosa 9027 (64) has malate oxidase rather than MDH, but gluconeogenesis is through the reverse EMP pathway.

It is certain that regulatory enzyme patterns in the pseudomonads are quite different from those operating in the enteric bacteria, and PEPCK is further proof of this fact. The use of the enzyme effectors as taxonomic tools is becoming more widespread, and all the results so far gathered emphasize the differences between the enterics and the pseudomonads. Acetyl-CoA inhibits the ME of E. coli (53) but not the ME of Ps. fluorescens (26). Wright et al (79)

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[1] A detailed kinetic study of E. coli CS will be available soon - J. Wright, Doctoral Dissertation, this department.

have shown that the CS of E. coli is inhibited by α-ketoglutarate. Weitzman has since surveyed other genera and found that only citrate synthetases in the enteric bacteria are affected (76). No other procaryotic (including the pseudomonads) or eucaryotic citrate synthetases were inhibited by α-ketoglutarate. An interesting paper by Cohen et al (11) has described the regulatory patterns of aspartokinase and homoserine dehydrogenase in some pseudomonad and coliform bacteria. The two families were shown to have distinctly different control mechanisms (11). A subdivision of the pseudomonads on this basis was possible, and agreed with a previously proposed taxonomy for the genus.

The massive and authoritative study of the aerobic pseudomonads mentioned above (58) incorporates many of the best ideas of the new taxonomy. Unfortunately, none of the organisms in our study were classified therein, but several relevant points should be mentioned. Pseudomonas B_2aba (soluble MDH), used by Kornberg in numerous experiments (17), is included in this classification and is assigned to the group Ps. acidovorans (58). Stanier (58) remarked on the differences in malate oxidation among the pseudomonads and pointed out that Ps. ovalis, Chester (only malate oxidase) fell in a different taxonomic group (Ps. putida) in their survey. Previously it was stated that Ps. convexa (a genus classified as Ps. putida by Stanier) contained MDH. Although this result does not fit the above scheme, it is certainly an intriging idea and one which may be of practical use in taxonomy. Also of interest was the inclusion of the genus Hydrogenomonas in the pseudomonad family (58). Recently there

has been a report (37) that phosphoribulokinase is strongly activated by NADH in Hydrogenomonas facilis. Both phosphoribulokinase of Hydrogenomonas and PEPCK of Pseudomonas are ATP-dependent, CO_2-fixing enzymes activated by DPNH. It is a rather striking analogy, especially in view of the close taxonomic relationship between the two genera (58).

In any survey, liberties are taken with not too much regard to detailed facts. There has been no proof presented that any enzyme in this study is allosterically inhibited by DPNH, although it is repeatedly implied by referral to the allosteric enzymes in E. coli. Also, there is little evidence in the literature and none herein to support the supposition that DPNH is present in vivo at a concentration sufficient to cause inhibition. Nonetheless, it is hoped the reader has taken our work seriously enough to come this far.

SUMMARY

(1) Citrate synthetase was inhibited by reduced diphosphopyridine nucleotide (DPNH) in all gram-negative bacteria tested. Citrate synthetases from gram-positive organisms were not affected.

(2) Malic enzyme was inhibited by both DPNH and oxalacetic acid in every bacterium surveyed. Only three of the eleven malic enzymes studied were inhibited by cyclic adenosine monophosphoric acid (cAMP), and it seems unlikely that cAMP is important in the fine control of malic enzyme in bacteria.

(3) Malate dehydrogenase was inhibited by DPNH in all bacteria studied.

(4) Phosphoenolpyruvate carboxykinase was inhibited by DPNH in those bacteria which also possessed malate dehydrogenase. Both Pseudomonas aeruginosa and Nocardia corallina lack the malate dehydrogenase enzyme and no inhibition of phosphoenolpyruvate carboxykinase was observed. The possible significance of this relationship was discussed.

(5) The inhibition of malate dehydrogenase, malic enzyme, and phosphoenolpyruvate carboxykinase by DPNH cannot be successfully correlated with the gram-reaction and/or carbohydrate metabolism of the organisms surveyed. It is probable that allosteric regulation of enzyme activity by DPNH is indigenous to all bacteria with a functional TCA cycle.

TABLE I

Cultures

Culture	Carbohydrate[1] Metabolism	Gram Reaction	ATCC	MCC
Azotobacter vinelandii	oxidative	(-)	9104	120
Chromobacterium violaceum	fermentative	(-)		210
Chromobacterium lividum	oxidative	(-)	12473	123
Flavobacterium devorans	oxidative	(-)	10829	259
Pseudomonas aeruginosa	oxidative	(-)	7700	74
Pseudomonas convexa var. *hippuricum*	oxidative	(-)		75
Erwinia carotavora	fermentative	(-)	495	37
Proteus vulgaris	fermentative	(-)		73
Salmonella typhimurium LT-2	fermentative	(-)		118
Serratia marcescens	fermentative	(-)		92
Bacillus cereus	fermentative	(+)	10206	15
Micrococcus lysodeikticus	oxidative	(+)	4698	54
Nocardia corallina	oxidative	(+)		66
Staphylococcus aureus	fermentative	(+)		116

ATCC = American Type Culture Collection
MCC = culture collection of the Dept. Microbiology, Univ. Manitoba
1 = based on reaction in Hugh-Leifson medium (25) as given in (13).

TABLE II
Buffers

	MDH	ME	PEPCK	CS
HARVEST BUFFERS	0.05 M Tris-HCl pH 7.5 1 mM EDTA	0.05 M sodium phosphate pH 7.0 10 mM EDTA 10 mM Cleland	0.1 M Tris-HCl pH 7.5 1 mM EDTA 1 mM GSH	0.02 M Tris-HCl pH 8.0 1 mM EDTA 10 mM $MgCl_2$
ASSAY BUFFERS	0.15 M Tris-CH_3COOH pH 9.0 15 mM EDTA	0.1 M Tris-HCl pH 7.5 or 0.1 M HEPES-NaOH pH 7.5	0.1 M Tris-HCl pH 7.5	0.02 M Tris-HCl pH 8.0

TABLE III

Media used for enzyme production

CULTURE	MDH	ME	PEPCK	CS
Azotobacter	BR,s	BR,s	BR,s	BR,a
Chromobacterium lividum	NB,m	-	NB,m	-
Chromobacterium violaceum	NB,s	NB,s	NB,s	NB,a
Flavobacterium	NB,s	NB,s	A,g,s	A,g,a
Pseudomonas aeruginosa	NB,s	NB,s	NB,s	NB,a
Pseudomonas convexa	NB,m	NB,m	NB,m	-
Erwinia	NB,s	NB,s	NB,s	A,g,a
Proteus	NB,s	NB,s	NB,s	NB,a
Salmonella	NB,s	NB,s	NB,s	NB,a
Serratia	NB,s	NB,s	NB,s	NB,a
Bacillus	B,g,s	B,g,s	B,g,s	B,g,a
Micrococcus	pA,g,s,bt	pA,g,s,bt	pA,g,s,bt	pA,g,a,bt
Nocardia	B,m,t	NB,m	NB,m	B,a,t
Staphylococcus	NB,s	NB,s	NB,s	NB,a

A= medium A (page 17)
pA= medium A adjusted to pH 8.0 with NaOH
B= medium A minus citrate
NB= Difco nutrient broth
BR= Brown's nitrogen-free medium (7)
a= 0.5% sodium acetate
s= 0.5% sodium succinate
m= 1.0% sodium DL-malate
g= 5 mM sodium-L-glutamate
t= 0.2 ug/ml thiamine hydrochloride
bt= 10 ug/ml D-biotin

LEGEND - TABLE IV

C = centrifugation at 12,000 g for 10 minutes. Pellet discarded.

U = enzyme crude centrifuged as in C followed by ultra-centrifugation at 100,000 g for 120 minutes. Pellet discarded.

PS = dropwise addition of one volume of 2% w/v protamine sulphate (previously adjusted to pH 7.0 with NaOH) to one volume of enzyme crude. Stirred at 4° for 30 minutes followed by step C. Pellet discarded.

AS = PS supernate adjusted to 30% of saturation with solid $(NH_4)_2SO_4$. Stirred at 4° for 30 minutes followed by step C. Pellet discarded.

D = dialysis at 4° in 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 50 mM KCl. Samples were dialyzed for 3-10 hours. Ratio of buffer to sample volume was 50-100:1.

H = HEPES buffer used in enzyme assay.

HT = step C supernate held at 55° for 5 minutes and cooled by plunging into ice-water bath. Step C repeated. Pellet discarded.

M = enzyme bound to membrane. Details of purification on page 18.

AD = air dialysis of the PS treated extract at 22-24° until original sample volume regained.

TABLE IV

Procedures used to partially purify enzymes.

CULTURES	MDH	ME	ME cAMP	PEPCK	CS
Azotobacter	PS	PS	C,H	C	PS,D
Chromobacterium lividum	PS	-	-	C	-
Chromobacterium violaceum	PS	PS	C,H	PS	PS,D
Flavobacterium	PS	U	U	C	PS,D
Pseudomonas aeruginosa	PS	PS	C,H	PS,AD	PS,AS,D
Pseudomonas convexa	C	C	-	C	-
Erwinia	PS	U	U,H	PS	PS,D
Proteus	PS	U	C,H	PS	PS,AS,D
Salmonella	PS	U	U,H	PS	PS,AS,D
Serratia	PS	U	C,H	PS	PS,D
Bacillus	C,HT	C,HT	U	C	PS,AS,D
Micrococcus	PS	C	-	C	PS,D
Nocardia	C	U	U,H	U	PS,D
Staphylococcus	M	U	U,H	C	PS,D

TABLE V

Inhibition of MDH and PEPCK by DPNH

CULTURE	Specific[1] Activity of MDH	K_i (mM DPNH)	Specific[2] Activity PEPCK	Percent Inhibition (5mM DPNH)
Azotobacter	0.03	0.20	-	-
Chromobacterium lividum	5.7	0.15	1.0	45%
Chromobacterium violaceum	3.0	0.10	1.2	50%
Flaovbacterium	0.14	0.10	-	-
Pseudomonas aeruginosa	0.02	nd	0.18	60%[3]
Pseudomonas convexa	p	-	-	-
Erwinia	36.0	0.10	1.1	65%
Proteus	7.2	0.15	1.4	80%
Salmonella	11.8	0.25	2.2	80%
Serratia	6.1	0.10	1.4	75%
Bacillus	0.43	0.25	-	-
Micrococcus	0.05	0.10	-	-
Nocardia	-	-	1.1	0%
Staphylococcus	0.12	0.05	-	-

[1]One unit is a change in absorbance of 1.0/min/mg protein

[2]One unit is the picomoles $^{14}CO_2$ fixed/5 min/mg protein

[3]This is activation, not inhibition.

nd= K_i not determined due to low activity. Enzyme was inhibited 100% at 1 mM DPNH however.

p= strong MDH and ME were present, but activities were not determined.

TABLE VI

Inhibition of CS by DPNH

CULTURE	Specific[1] Activity of CS	DPNH (mM)	Percent Inhibition by DPNH
Azotobacter	0.09	0.5	30%
Chromobacterium violaceum	0.14	0.5	100%
Flavobacterium	0.06	0.5	100%
Pseudomonas aeruginosa	0.89	0.5	50%
Erwinia	-	-	-
Proteus	0.24	0.5	100%
Salmonella	0.28	0.5	100%
Serratia	0.13	0.5	100%
Bacillus	0.40	1.0	0%
Micrococcus	0.02	1.0	0%
Nocardia	-	-	-
Staphylococcus	-	-	-

[1]One unit is a change in absorbance of 1.0/min/mg protein.

TABLE VII

Inhibition of ME by DPNH, OAA and cAMP

CULTURE	Specific[1] Activity	Inhibition by DPNH (0.5 mM)	Inhibition by OAA (mM)	Percent Inhibition	Specific[2] Activity of MEcAMP	Inhibition by cAMP (2 mM)
Azotobacter	0.04	75%	0.10	50%	0.05	nsi
Chromobacterium violaceum	0.11	50%	0.05	15%	0.04	nsi
Flavobacterium	0.06	70%	0.05	10%	0.03	nsi
Pseudomonas aeruginosa	0.17	75%	0.05	20%	0.07	20%
Erwinia	0.38	70%	0.05	20%	0.11	20%
Proteus	0.04	55%	0.05	15%	0.02	nsi
Salmonella	0.18	75%	0.07	15%	0.09	15%
Serratia	0.06	80%	0.05	20%	0.03	nsi
Bacillus	0.43	60%	0.10	10%	0.04	nsi
Micrococcus	-	-	-	-	-	-
Nocardia	0.35	60%	0.20	30%	0.08	nsi
Staphylococcus	0.07	100%	0.10	20%	0.03	nsi

[1]One unit is a change in absorbance of 1.0/min/mg protein

[2]Specific activity is as defined above but different assay system was used (page 22).

nsi = no significant inhibition. Assay accuracy was taken to be $\pm$ 5%, therefore any inhibition between 0 and 10% was denoted as nsi.

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