Studies of the metabolism and permeation of monocarboxylic acids in *Escherichia coli* and *Aerobacter aerogenes*

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This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy in the University of Leicester, entitled "Studies of the metabolism and permeation of monocarboxylic acids in Escherichia coli and Aerobacter aerogenes" is the result of work done mainly by me during the period of registration for the above degree.

T.D.K. Brown

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ABSTRACT

1. Mutants of E. coli K12 devoid of acetate kinase and phosphotransacetylase activities have been isolated by selection for resistance to fluoroacetate. The properties of the mutants have been studied and the role of the acetate kinase/phosphotransacetylase system clarified. It has a role in acetate excretion and its control properties are consistent with a role in fermentative metabolism. It also has a role in acetate activation at high concentrations of acetate.

2. Studies of acetate incorporation by acetate kinase and phosphotransacetylase-negative mutants of E. coli K12 have revealed the existence of a second system capable of acetate activation in this organism. This system has properties consistent with a role in the activation of acetate at low concentrations. It is saturated at 2 mM acetate and is also subject to repression by glucose. An acetate thiokinase activity could be detected in cell-free extracts and a number of its properties could be correlated with in vivo observations on the second system. The activity was induced by acetate and repressed by glucose. It had a low K_m for acetate. The thiokinase also shows propionate thiokinase activity. Attempts to isolate mutants modified in this activity proved unsuccessful.

3. Problems relating to the study of monocarboxylic acid permeation have been reviewed. The uptake of acetate and lactate by washed cell suspensions of E. coli K12 has been investigated. The data obtained have been considered in the light of criteria required to establish the existence of specific transport systems. Two acetate uptake

processes have been found. One shows saturation kinetics with a K_m of approx. 10^{-5} M and its activity is repressed by glucose. The other operating at high acetate concentrations is apparently non-saturable. The effects of a number of factors on acetate uptake were investigated. Preliminary evidence that, under certain conditions, a membrane transport process may be rate-limiting in acetate uptake was obtained. The existence of specific transport systems for D- and L-lactate was established. The properties of these systems were investigated. Lactate uptake was found to be induced by lactate and repressed by glucose. Lactate was not actively concentrated within the cell, but uncoupling agents prevented its transport. The properties of a mutant defective in catabolite repression of L-lactate utilization are described.

4. Mutants of A. aerogenes 1033 devoid of acetate kinase and phosphotransacetylase activities have been isolated by selection for resistance to fluoroacetate. Their properties have been compared with those of the E. coli mutants. The acetate kinase/phosphotransacetylase system has an amphibolic role in A. aerogenes. No evidence for the operation of an alternative mechanism of acetate activation was obtained. The coarse control properties of the acetate kinase/phosphotransacetylase system were studied under a variety of conditions. The role of acetic acid as the inducer of the enzymes of the butanediol-forming system of A. aerogenes was established. Possible relationships between the operation of the acetate excreting system and the operation of the butanediol-forming system are discussed.

FIG. 1

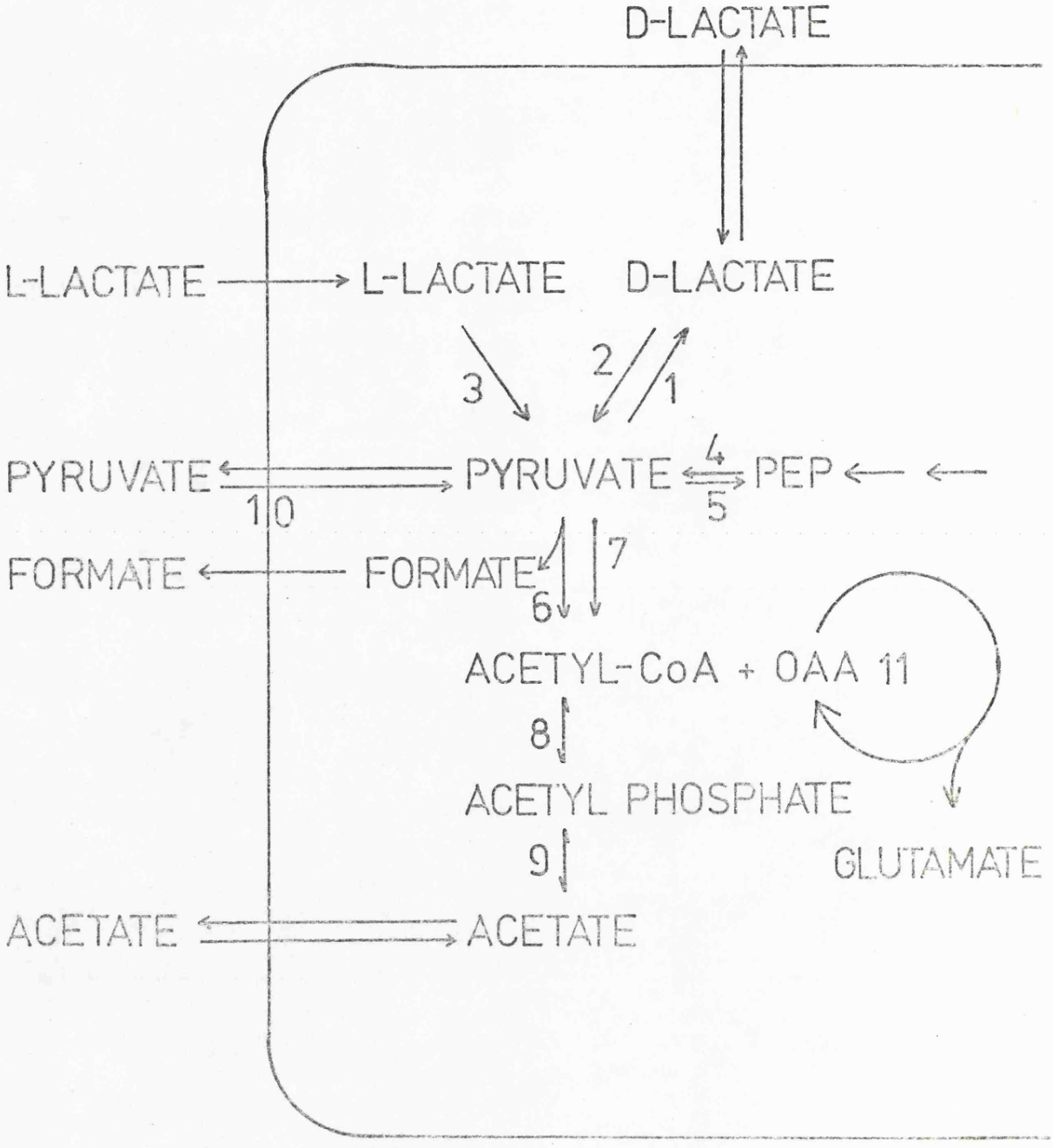

D-LACTATE
L-LACTATE
L-LACTATE
D-LACTATE
3
2
1
PYRUVATE
PYRUVATE
4
5
PEP
10
FORMATE
FORMATE
6
7
ACETYL-CoA + OAA 11
8
ACETYL PHOSPHATE
9
GLUTAMATE
ACETATE
ACETATE

Figure 1

Pathways of monocarboxylic acid metabolism in *E. coli*

1. lactate dehydrogenase; 2. D-lactate oxidase; 3. L-lactate oxidase; 4. pyruvate kinase; 5. PEP-synthase; 6. pyruvate formate-lyase; 7. pyruvate dehydrogenase; 8. phosphotransacetylase; 9. acetate kinase; 10. uptake system for pyruvate; 11. tricarboxylic acid cycle.

Table 1

Organism *Escherichia coli* *	Mating type	Growth requirements	Metabolic lesions	Origin
K12 strains:				
K1	Hfr	met,thy	-	lab. strain
K1HFAc1	Hfr	met,thy	ACK^-	this study
K1HFAc2	Hfr	met,thy	ACK^-	this study
K1HFAc3	Hfr	met,thy	PTA^-	this study
K2	F^-	his,thr,leu,arg, trp	-	lab. strain
K2FAc1	F^-	his,thr,leu,arg, trp	ACK^-	this study
K2FAc7	F^-	his,thr,leu,arg, trp	PTA^-	this study
K2HFAc3	F^-	his,thr,leu,arg, trp	PTA^-	this study
K2.3	F^-	his,arg,trp,ace	PDH^-	lab. strain
K2.1t	F^-	his,thr,leu,arg	PPS^-	lab.strain
K2.1.4	F^-	arg,thr,leu,glt	PPS^-,CS^-	lab.strain
AB1621	-	-	-	R.A. Cooper (Leicester)
AB1621 LLO	-	-	LLO^-	R.A. Cooper (Leicester)
1100	-	-	-	I. Pastan (Bethesda) [I]
5336	-	-	AC^-	I. Pastan (Bethesda) [I]
5336 L-lact$^+$	-	-	AC^-,LCR^-	this study
K12	Hfr	-	-	L.N. Ornston (New Haven) [II]

Table 1 (continued)

Organisms	Mating type	Growth requirements	Metabolic lesions	Origin
K12Glc102	Hfr	-	USG$^-$	L.N. Ornston (New Haven) [II]
Aerobacter aerogenes strains				
1033 †	-	-	-	H.E. Umbarger (Lafayette) [III]
1033 FAc6	-	-	PTA$^-$	this study
1033 FAc7	-	-	ACK/PTA$^-$	this study
1033 FAc8	-	-	ACK$^-$	this study
Pseudomonas aeruginosa strains				
D17	-	-	-	D. Jones (Leicester)
AT16	-	-	ACT$^-$	A.J. Skinner (Leicester)

* all strains of *E. coli* K12 listed have a requirement for thiamin (vit. B_1)

The following abbreviations are used to describe metabolic lesions: ACK = acetate kinase; PTA = phosphotransacetylase; PDH = pyruvate dehydrogenase; PPS = PEP-synthase; CS = citrate synthase; LLO = L-lactate oxidase; AC = adenyl cyclase; LCR = catabolite repression of L-lactate utilization; USG = uptake system for glycollate; ACT = acetate thiokinase.

Table 1 (continued)

The following abbreviations are used to describe growth requirements: his = histidine; thr = threonine; leu = leucine; arg = arginine; trp = tryptophan; ace = acetate; glt = glutamate; met = methionine; thy = thymine. Amino acids were routinely added to media at 100 µg/ml and thymine at 40 µg/ml.

The procedures used to select strains derived in the course of this study are described in the text.

[I] Perlman & Pastan, 1969

[II] Ornston & Ornston, 1970

[III] Størmer, 1967.

† Aerobacter aerogenes strain 1033 has been characterised as non-motile and urease positive and may therefore be classified as a strain of Klebsiella aerogenes (see International J. Systematic Bacteriology (1970) 20 221.).

INTRODUCTION

Two classes of control mechanism have been distinguished in living organisms. "Fine" control operates to regulate the rate at which a given enzyme molecule can catalyse a reaction, while "coarse" control operates to regulate the quantity of a given enzyme molecule in the cell by modifying its rate of synthesis or degradation (Kornberg, 1965a). The Enterobacteriaceae have the ability to use a wide range of compounds as sources of carbon and energy. This ability is expressed selectively and the selectivity depends largely on the operation of highly sophisticated "coarse" control mechanisms. A number of groups of coarse control systems operating on metabolic pathways can be distinguished in the Enterobacteriaceae. The selective use of carbon sources is normally managed through the operation of inducible systems. Superimposed on the inductive mechanism is the phenomenon of catabolite repression (Paigen & Williams, 1970). This is thought to be mediated in a unitary fashion by the control of 3',5'-cyclic AMP levels in the cell. These mechanisms serve to ensure that the proteins specified by a regulon under inducible control are synthesised at high rates only when two conditions are fulfilled:

1. the compound degraded by the pathway is present in the medium,
2. that no "better" carbon source, such as glucose, is present in the medium.

Control of biosynthetic pathways is normally achieved by repression. Thus, when amino acids are supplied in the growth medium, production of the enzymes required for their synthesis is reduced (Umbarger, 1969). A third type of control which might be distinguished from induction

and repression control as outlined above is that which operates to control functions whose expression is dependent on the supply of oxygen available to the bacterium. A range of systems appears to be controlled in this way, e.g. lactate dehydrogenase, acetate kinase/phosphotransacetylase, ethanol dehydrogenase, fumarate reductase, and enzymes of the tricarboxylic acid cycle, and it seems unlikely that a unitary mechanism can explain the available observations.

The Enterobacteriaceae can use a range of monocarboxylic acids as carbon and energy sources, such as acetate, lactate, pyruvate, glycollate and long chain fatty acids. A number of these are normally excreted when the bacteria are growing on a "rich" carbon source such as glucose. They are reused diauxically when the glucose level in the medium becomes growth-limiting. Amarasingham and Davis (1965) have suggested that this mode of growth gives a selective advantage to the Enterobacteriaceae in competition with other microbes. Thus, the competitive organisms may not have the capacity to adapt rapidly to the range of compounds excreted by the Enterobacteriaceae during the rapid initial dissimilation of glucose and these products are thus available for re-utilization by them at their leisure.

There are two possible methods for achieving this excretion followed by re-utilization. In the case of lactate utilization, two separate "pathways" are employed, one for the excretion of lactate and the other for its utilization. Production of D-lactate during fermentation of glucose is achieved by a soluble NAD-linked lactate dehydrogenase, while utilization of DL-lactate is dependent on the operation of two flavoprotein-linked, membrane-bound lactate oxidases.

It is thought that the situation is significantly different in the case of acetate in that a single system involving acetate kinase and phosphotransacetylase is responsible both for acetate production from acetyl CoA and for activation of exogenous acetate to acetyl CoA.

Thus the fine control properties of phosphotransacetylase have been discussed by Sanwal in terms of this dual role (Sanwal, 1970).

The utilization of acetate as a sole carbon source is dependent on the operation of the anaplerotic glyoxylate cycle. The enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, have been extensively investigated and shown to be closely linked and subject to co-ordinate induction and catabolite repression control (Kornberg, 1966). This system thus presents a contrasting picture of the control of acetate utilization. The coarse control properties of the acetate kinase/phosphotransacetylase system have not been critically examined, nor has its metabolic role been adequately defined. The isolation and study of mutants devoid of these enzymes has enabled this situation to be clarified.

While there exist many broad similarities between the metabolic processes of the enterobacteria Escherichia coli and Aerobacter aerogenes, there also exist a number of significant differences. A number of these are found in the patterns of fermentative metabolism. The most obvious difference lies in the operation of the acetoin/2,3-butanediol pathway in A. aerogenes, but not in E. coli (this difference constitutes the metabolic basis of the Voges-Proskauer test for distinguishing the aeroform and coliform members of the Enterobacteriaceae).

The operation of this pathway results in the production of the neutral compounds acetoin and 2,3-butanediol from pyruvate during the fermentation of glucose. It might be suggested that diversion of carbon and reducing power into neutral products allows energy production to continue after the pH level of the medium has dropped to a level at which further acid production would prove lethal. Acetic acid, one of the major acidic products of fermentation, is thought to play an important role in the induction of the acetoin/2,3-butanediol pathway (Størmer, 1968a). It is perhaps significant that the pK of acetic acid is such that the quantity of free acid will vary significantly as the pH of the medium falls during fermentation (Mickelson & Werkman, 1938). Acetate is also an activator of the pH 6 acetolactate-forming enzyme, the first enzyme in the pathway (Størmer, 1968b). It is therefore pertinent to examine the interaction of the acetoin/2,3-butanediol-forming pathway with the acetate excretory system. An attempt to identify acetate unequivocally as the inducer of the pathway has been made using mutants devoid of acetate activating ability. Studies of possible interactions between the pathways for acetate production and for acetoin/2,3-butanediol production have been carried out.

Studies such as these have revealed that the coarse control mechanisms operating in fermentative metabolism are complex. These control mechanisms are potentially interesting and may show features not seen in classical inductive and repressive control mechanisms. Thus there exists a group of fermentative enzymes in E. coli, including lactate dehydrogenase, alcohol dehydrogenase, acetate kinase and

phosphotransacetylase, whose levels are elevated during anaerobiosis. The mechanism of this "induction" is unknown, but it might be of a unitary type comparable to that which operates in catabolite repression. It is likely that the control of this group of enzymes can be distinguished from the control operating on, for example, the anaerobic "induction" of fumarate reductase (Wyn Jones & Lascelles, 1967). The repression of certain enzymes, notably those of the tricarboxylic acid cycle, by anaerobiosis presents another problem in anaerobic control. It seems likely that these mechanisms are distinct from catabolite repression. The problem presented by the control of the acetoin/2,3-butanediol pathway in A. aerogenes is particularly difficult. In this system, the induction is dependent on a number of factors:

1. the presence of acetate in the medium (probably as free acetic acid),
2. the metabolism of a glycolytic precursor, such as glucose,
3. low oxygen levels in the medium.

During batch cultures these factors will change and interact in a complex manner. Preliminary studies have been made of the control of enzymes induced by anaerobiosis using batch and fermenter culture.

Control of the utilization of compounds by the Enterobacteriaceae is frequently exerted at the level of membrane transport. Specific systems capable of catalysing the vectorial movement of compounds are present in the membrane and their synthesis is frequently under coarse control. Certain classes of compounds, however, are thought to enter cells by free diffusion. Monocarboxylic acid permeation is of interest because of conflicting evidence on the mechanism by which this class of compounds crosses biological membranes. A considerable

amount of work has been carried out on mitochondria and artificial membrane models which supports the idea that monocarboxylic acid transport is not carrier-linked. The observations on monocarboxylates are contrasted with those on di- and tricarboxylates (reviewed by Chappell, 1968).

In contrast to mitochondria, there is evidence that specific systems exist for the transport of monocarboxylates by bacteria. The existence of transport systems for pyruvate, glycollate and long chain fatty acids in E. coli has been reported. There have also been indications of membrane transport processes for acetate. This apparent difference between the behaviour of mitochondria and model membranes, and of bacteria, may reflect changes in intrinsic permeability due to differences in membrane structure. Thus mitochondria exist in an intracellular environment in which a high and non-specific permeability to metabolic precursors and end-products (frequently monocarboxylic acids) could be tolerated. The differences observed might, however, simply reflect differences in the methods and conditions used to study permeation.

Investigations in the broad areas outlined above (short chain fatty acid activation, fermentative metabolism and monocarboxylate permeation) have followed from initial attempts to study the mechanism of acetate permeation in E.coli. Many of the investigations are still in progress.

CHAPTER I

The Role of Acetate Kinase and Phosphotransacetylase in E. coli K12

Introduction

Since the detection of acetate kinase (ATP : acetate phosphotransferase, E.C. 2.7.4.3) (reaction 1) and phosphotransacetylase (acetyl CoA : orthophosphate acetyl transferase, E.C. 2.3.1.8) (reaction 2) in E. coli (Kaplan & Lipman, 1948; Rose et al., 1954; Gilvarg & Davis, 1956), it has been assumed that these two enzymes are involved in the activation of acetate to acetyl CoA.

1. acetate + ATP $\rightleftharpoons$ acetyl phosphate + ADP

2. acetyl phosphate + CoA $\rightleftharpoons$ acetyl CoA + P_i

Since E. coli can use acetate as a sole source of carbon and energy this would imply their ability to supply the acetyl CoA required for biosynthetic and energy metabolism during growth on acetate. The absence of any reports of acetate thiokinase (acetate : CoA ligase (AMP), E.C. 6.2.1.1) (reaction 3) activity in E. coli reinforces this view.

3. acetate + ATP + CoA $\rightleftharpoons$ acetyl CoA + PP_i + AMP

($PP_i + H_2O \longrightarrow 2P_i$)

Other enzymes capable of catalysing the formation of acetyl CoA from acetate, for example CoA transferases, have not been reported. The

role of the acetate kinase/phosphotransacetylase system in providing acetyl CoA from exogenous acetate must be reconciled with its role in the excretion of acetate by E. coli growing on glucose (Wood, 1961). Thus, on the basis of a failure to detect any alternative activation system and the ready reversibility of the two enzymes in E. coli, the pathway has been considered to be amphibolic (Sanwal, 1970).

The enzymes acetate kinase and phosphotransacetylase are examples of a group of acylate kinases and phosphotransacylases which are co-ordinately distributed in many anaerobic and facultative anaerobic bacterial species. The process catalysed by the acylate kinases is the most important energy-yielding reaction in anaerobes. It occurs in a wide range of Clostridia and in sulphate-reducing bacteria and has been considered to be a reaction characteristic of anaerobic life (Decker et al., 1970). The acyl phosphate substrates are formed mainly from acyl CoA's by phosphotransacylases. The free energy relationships of these two reactions contrast with those of an acylate thiokinase in which (in the presence of inorganic pyrophosphatase) the hydrolysis of two pyrophosphate bonds is involved. Systems of the acylate kinase/phosphotransacylase type have never been identified in strictly aerobic organisms.

The basic kinetic parameters of acetate kinase from E. coli ATCC 4157 have been established by Rose and his co-workers (Rose, 1962). At pH 7.4 in the direction of acetate phosphorylation, the K_m for ATP was 2×10^{-3}M, while the K_m for acetate was 0.3 M and the K_m for propionate was 0.47 M. In the reverse direction, the K_m for acetyl phosphate was 5×10^{-3}M and that for ADP, 1.5×10^{-3}M. More recent

mechanistic studies of acetate kinase from E. coli have been carried out by Satchell and White (1970) and by Anthony and Spector (1970). There have been no reports of effectors for E. coli acetate kinase. In a recent paper, Pelroy and Whitely (1971) have described an acetate kinase from Veillonella alcalescens which shows the allosteric properties of activation by acetyl phosphate and inhibition by ATP. These properties are consistent with a role in energy generation. The extremely high K_m for acetate shown by acetate kinase from E. coli might suggest that its primary role was to operate in the direction of acetate excretion rather than acetate activation. Thus the concentrations of acetate produced in the medium by growth on glucose are low compared with the K_m for acetate of acetate kinase (Holms & Bennett, 1971).

The kinetic properties of phosphotransactylase from E. coli B have been studied in some detail. The enzyme has been purified to homogeneity by Shimizu et al. (1969). A K_m value of 3×10^{-3} to 4×10^{-3}M for acetyl phosphate and a K_m value of 7.2×10^{-4}M for CoA were determined. Kinetic analysis has also been carried out by Satchell and his co-workers (Hibbert et al., 1971; Kyrtopoulos & Satchell, 1972). Effector studies on phosphotransacetylase from E. coli B reported by Suzuki have revealed interesting allosteric control properties (Suzuki, 1969). The enzyme is inhibited by nucleotides. NADH was the most powerful inhibitor tested. ADP and ATP also inhibited the enzyme, but their inhibitory strengths were respectively approximately one quarter and one tenth of that of NADH. The inhibitions were non-competitive with respect of CoA and sigmoidal

with respect to acetyl phosphate. Pyruvate activated the enzyme and reversed the inhibitory effect of nucleotides. The activation observed in the presence of NADH was considerably greater than that observed in the non-inhibited state. The possible significance of these allosteric properties in an amphibolic role for the enzyme has been discussed by Sanwal (Sanwal, 1970). The patterns can, however, be rationalised in terms of a role in fermentative metabolism with NADH and pyruvate seen as the key antagonistic effectors. These two compounds are of crucial importance for fermentative metabolism. Under conditions where the NADH level rises, the production of lactate from pyruvate will be required to reduce it and enable the maintenance of a high flux through the glycolytic system. The metabolism of pyruvate to acetate via phosphotransactylase is therefore inhibited. Under conditions where supply of pyruvate for lactate production is not limiting, the NADH inhibition is reversed and the energy-yielding metabolism of pyruvate to acetate can proceed. Thus the control properties of phosphotransacetylase can be used teleologically to support a role for the enzyme in fermentative metabolism.

Physiological studies of the role of acetate kinase and phosphotransacetylase in E. coli have been extremely limited. Halpern et al. (1964) compared the levels of acetate kinase in E. coli H grown on glucose and on succinate and found higher levels in the succinate-grown cells. Although the difference reported was small, Paigen and Williams (1970) cited these data in their review of catabolite repression and related control mechanisms, and indicated that the enzyme may be under catabolite repression control.

The work described here was carried out in order to clarify the role of the acetate kinase/phosphotransacetylase system in E. coli K12. The major part of the work involved studies of mutants devoid of, or containing low activities of, acetate kinase and phosphotransacetylase.

The properties of mutants devoid of acetate kinase and phosphotransacetylase isolated from Brevibacterium flavum have previously been reported by Shiio et al. (1969). The mutants were obtained by selection for glucose positive/acetate negative organisms. A large number of phosphotransacetylase negative mutants were isolated and one acetate kinase negative mutant. Two double mutants were obtained: one was deficient in both acetate kinase and phosphotransacetylase and the other in phosphotransacetylase and isocitrate lyase (L_S-isocitrate glyoxylate-lyase, E.C. 4.1.3.1). The mutants were all acetate negative. It is of particular interest that a mutant deficient in both phosphotransacetylase and isocitrate lyase was isolated. Given that the lesion was the result of a single mutation, it would suggest the genes might be under common control and thus confirm the role of the acetate kinase/phosphotransacetylase system in acetate utilization for which isocitrate lyase is essential in B. flavum (Shiio et al., 1969).

The mutants of E. coli K12 deficient in acetate kinase and phosphotransacetylase discussed in this thesis were obtained by selection for resistance to fluoroacetate. The classical studies of Peters and his co-workers have shown that, in many systems, fluoroacetate exerts its major toxic effect at the level of fluorocitrate inhibition

of aconitase (citrate [isocitrate] hydro-lyase, E.C. 4.2.1.3) (Peters, 1952; Peters et al., 1953; Morrison & Peters, 1954; Peters, 1957). Reports of the effects of fluoroacetate on the growth of E. coli have been published by Mager et al. (1955). Their work supports the idea that fluoroacetate blocks some steps in the tricarboxylic acid cycle in E. coli. Rose (1962) observed that fluoroacetate is not a substrate for acetate kinase, but the reactivity of fluoroacetyl phosphate in the E. coli acetate kinase/phosphotransacetylase system has been demonstrated by Marcus and Elliott (1959). The isolation of fluoroacetate-resistant mutants in Aspergillus nidulans has been reported by Apirion (1965) and in Thiobacillus neapolitanus by Kelly (1968).

The use of fluoroacetate-resistant mutants isolated in strains of E. coli K12 has clarified the amphibolic role of acetate kinase and phosphotransacetylase and revealed some novel features of acetate activation and excretion in this organism.

Materials and Methods

Reagents Acetyl phosphate lithium salt, ATP disodium salt, CoA and NAD were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium fluoroacetate was obtained from Sigma Chemical Co., or from British Drug Houses Ltd., Poole, Dorset, England. Sodium pyruvate was obtained from Boehringer Corporation (London) Ltd., England. [2-^{14}C]acetate sodium salt and [2-^{14}C]propionate sodium salt were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Citrate synthase and malate dehydrogenase were obtained from Boehringer Corporation (London) Ltd.

Other chemicals were A.R. grade where available.

Organisms The characteristics of organisms used in this Chapter are listed in Table 1.

Growth of Organisms Cells were grown at 37° in defined salts medium (Ashworth & Kornberg, 1966). Carbon sources were added at 25 mM unless otherwise stated. Cells were grown aerobically in either a Gallenkamp incubator shaker or in a Grant shaking water bath. Cells were grown anaerobically in two ways:

1. for the production of cells for enzyme assays, a small aerobically-grown inoculum was transferred to a Monax flask completely filled with freshly autoclaved culture medium. A Subaseal stopper was used to seal the flask. The maintenance of anaerobiosis was monitored by the addition of a small quantity of methylene blue.
2. for growth studies, the cells were grown in 250 ml Erlenmeyer flasks fitted with glass spargers and were sparged continuously with 95% N_2 : 5% CO_2. Samples were removed at intervals for growth measurements. In all anaerobic experiments, 25 mM sodium bicarbonate was added to the normal medium.

Growth was measured as absorbance at 680 nm; an absorbance of 1 corresponded to 0.68 mg dry weight.ml^{-1}.

Deviations from the above procedures are noted in the experimental section.

Assay of enzymes For experiments on aerobic enzyme levels, cells were grown in 50 ml volumes to an A_{680} of approximately 1 (corresponding to approx. 0.7 mg dry weight of cells.ml^{-1}). For experiments on anaerobic levels, cells were grown in 100 ml volumes to an A_{680} of approximately 0.6. Cultures were harvested by centrifuging the cells at 3000g for 10 minutes at 4^{o}; the cells were washed twice with 10 mM sodium phosphate buffer pH 7.5 containing 10 mM $MgCl_2$ and 1 mM sodium EDTA, were resuspended in 5 ml of this buffer and were sonicated for 1.5 minutes in a M.S.E. 100 watt sonicator at maximum output. The beaker containing the cell suspension was held in an ice bath. The cell extract was centrifuged for 1 h at 25,000g in a M.S.E. 18 centrifuge in order to remove cell debris and reduce the NADH oxidase levels for the phosphotransacetylase assays.

Acetate kinase was assayed at 30^{o} in the direction of acetate phosphorylation by the method of Rose (1955). Linearity of acetyl hydroxamate formation with time over the period of the assay and stoichiometric dependence of formation on the volume of cell-free extract added was checked. A standard curve for the assay was prepared using lithium acetyl phosphate.

Phosphotransacetylase was measured using a modification of the procedure used by Ochoa to assay citrate synthase (Ochoa, 1955). The formation of acetyl CoA from acetyl phosphate and CoA was measured as the reduction of NAD in the presence of L-malate, malate dehydrogenase (L-malate: NAD oxidoreductase E.C. 1.1.1.37) and citrate synthase (citrate oxaloacetatelyase (CoA-acetylating) E.C. 4.1.3.7). The reaction mixture consisted of 100 μmoles Tris-Cl pH 8.0,

5 µmoles $MgCl_2$; 0.5 µmoles NAD; 0.5 µmoles CoA; 5 µmoles L-malate; 12.5 µg crystalline malate dehydrogenase; 25 µg crystalline citrate synthase; 10 µmoles acetyl phosphate, and a quantity of cell-free extract in a final volume of 1 ml. The assays were carried out at a room temperature of 22-25°C. Care was taken to keep the rates of NADH appearance within stoichiometric limits by carrying out assays at at least two concentrations of cell-free extract. The assay is capable of detecting approximately a rate of approximately 0.03 µmoles $NADH.min^{-1}$.

Acetyl phosphate phosphatase activity was measured by following the disappearance of acetyl hydroxamate-forming material using the method described by Lipmann and Tuttle (1945). The phosphatase assays were carried out at either pH 5.65 in 0.09 M sodium acetate buffer or in 0.1 M Tris-Cl buffer pH 7.4. In both cases the assay mixture contained 5 mM $MgCl_2$ and 5 mM lithium acetyl phosphate. Samples were withdrawn over a period of 60 min and the residual acetyl phosphate was measured.

The protein content of the cell-free extracts was measured by the method of Lowry et al. (1951). (BSA was used as a standard).

Measurement of the incorporation of labelled compounds by cells growing on unlabelled carbon sources: Washed cell suspensions were inoculated at low cell densities into media containing an unlabelled carbon source and a [^{14}C]labelled substrate. The incorporation of label into the cells during logarithmic growth was followed by filtering 0.5 ml portions of cell suspension through nitro cellulose filters (0.45 µm pore size, Sartorius Membran Filter GMBH, 34 Gottingen, W. Germany) and washing with 5 ml of carbon-free salts medium. The

filters were then placed in 10 ml of toluene/methanol scintillant (70% toluene, 30% methanol containing 0.4% PPO (2,5-diphenyl-oxazole) and 0.02% POPOP (1,4-di-(2(5-phenyl-oxazoyl))benzene). Samples (0.05 ml) of the culture were taken at the beginning and end of each experiment to allow assessment of the oxidation of the added label to CO_2. These samples were also placed in 10 ml of toluene/methanol scintillant along with a dry nitrocellulose filter. All samples were counted in a Packard 4000 liquid scintillation spectrometer.

Estimations of acetate concentrations in the medium Samples for analysis were obtained by centrifuging 5 ml portions of culture for 10 min in a M.S.E. bench centrifuge to remove the cells. The cell-free supernatants were stored at -20°C until analysed for acetate. Samples were taken at various times during logarithmic growth.

Acetate was estimated in the samples of culture medium using a Pye Series 104 gas chromatograph (Pye Instruments Ltd., Cambridge, England), fitted with a DEGA + 2% H_3PO_4 column (10% diethylene glycol adipate on 100/200 mesh phosphoric acid-treated Diatomite C) run at 110°C. A flame ionisation detector was fitted to the instrument. Argon at 50 $ml.min^{-1}$ was used as carrier gas. Hydrogen was also supplied at 50 $ml.min^{-1}$. Air was supplied at 7 $lb.inch^{-2}$. The detector oven was set at 50°C above the column temperature. 0.3 ml samples of culture medium were treated with 0.06 ml of 50% ortho-phosphoric acid prior to analysis and stored at 0°C. Standard acetate solutions were run before and after each series of estimations. The areas under the peaks were estimated planimetrically.

Isolation of mutants Preliminary tests of fluoroacetate toxicity in E. coli K12 strains K1 and K2 were carried out both on plates and in liquid media. Toxicity was relatively low on glucose, glycerol and malate with considerably greater growth inhibition observed when the carbon source was pyruvate. Selections were thus carried out on plates containing 25 mM pyruvate with either 10 or 50 mM sodium fluoroacetate. The greater toxicity of fluoroacetate on pyruvate might be related to the activation of phosphotransacetylase observed by Suzuki (1969). Cultures of K1 and K2 were grown on Oxoid nutrient broth No.1, harvested in a bench centrifuge, washed with carbon-free salts medium and resuspended in salts medium. Approximately 10^8 cells were plated on the selective media. After 48-72 h of incubation at 37°C, resistant colonies appeared on the plates. A number of these colonies were picked from the plates and purified. Single colony isolates from these purifications were grown up on glucose medium and tested for the presence of acetate kinase and phosphotransacetylase.

The results of the assays showed interesting strain differences in the mutants isolated. K2 mutants resistant to 10 mM fluoroacetate were found in many cases to be acetate kinase-negative with a small number of phosphotransacetylase-negative organisms also appearing. A class of mutant in which no lesion could be detected in either acetate kinase or phosphotransacetylase under the conditions of assay used was also found. These organisms were acetate-negative. All the K2 mutants resistant to 50 mM fluoroacetate tested proved to be phosphotransacetylase-negative.

When K1 organisms resistant to fluoroacetate at the two

Table 2 Acetate kinase and phosphotransacetylase levels in *E. coli* K12 strains K2 and K2.3 (PDH^-) grown under various conditions

carbon source	specific activity acetate kinase	specific activity phosphotransacetylase
K2 aerobic		
glucose	1.5	1.2
glycerol	1.3	1.3
ribose	1.3	1.2
L-malate	1.7	1.5
acetate	1.8	1.3
pyruvate	4.1	2.6
DL-lactate	3.7	2.8
gluconate	3.6	2.5
glucose/acetate	1.6	2.0
K2.3 aerobic		
glucose/acetate	3.9	3.3
K2 anaerobic		
glucose	4.2	5.4

Cells were grown, cell-free extracts prepared and enzyme assays carried out as described in Materials and Methods. K2.3 was grown on 50 mM glucose/10 mM acetate medium. K2 was grown anaerobically on 25 mM glucose/25 mM sodium bicarbonate medium. Specific activities are expressed as μmol/min/mg protein.

different concentrations used were tested none of the low fluoroacetate-resistant organisms tested was found to be acetate kinase-or phosphotransacetylase-negative under the conditions of assay used. This group was able to grow rapidly on acetate. The high fluoro-acetate-resistant group consisted exclusively of acetate kinase-less or phosphotransacetylase-less organisms with the former type predominating. The residual levels of acetate kinase in these K1 high fluoroacetate-resistant acetate kinase-less strains appeared to be substantially lower than in the acetate kinase-negative strains isolated at 10 mM fluoroacetate in K2.

No organisms devoid of both acetate kinase and phospho-transacetylase were detected in the course of this selection for fluoroacetate resistance.

Results and Discussion

Levels of acetate kinase and phosphotransacetylase in *E. coli* K12 strain K2 grown under various conditions As a basis for the examination of the role of acetate kinase and phosphotransacetylase in *E. coli* K12, the levels of these enzymes in cultures of strain K2, grown aerobically and anaerobically on a variety of carbon sources, was measured (Table 2). The data indicate that there is a degree of co-ordinate expression of the enzymes under the conditions tested. The enzyme levels found in aerobically grown cells fall into two groups. Cells grown on carbon sources which can give rise to pyruvate without

FIG. 2

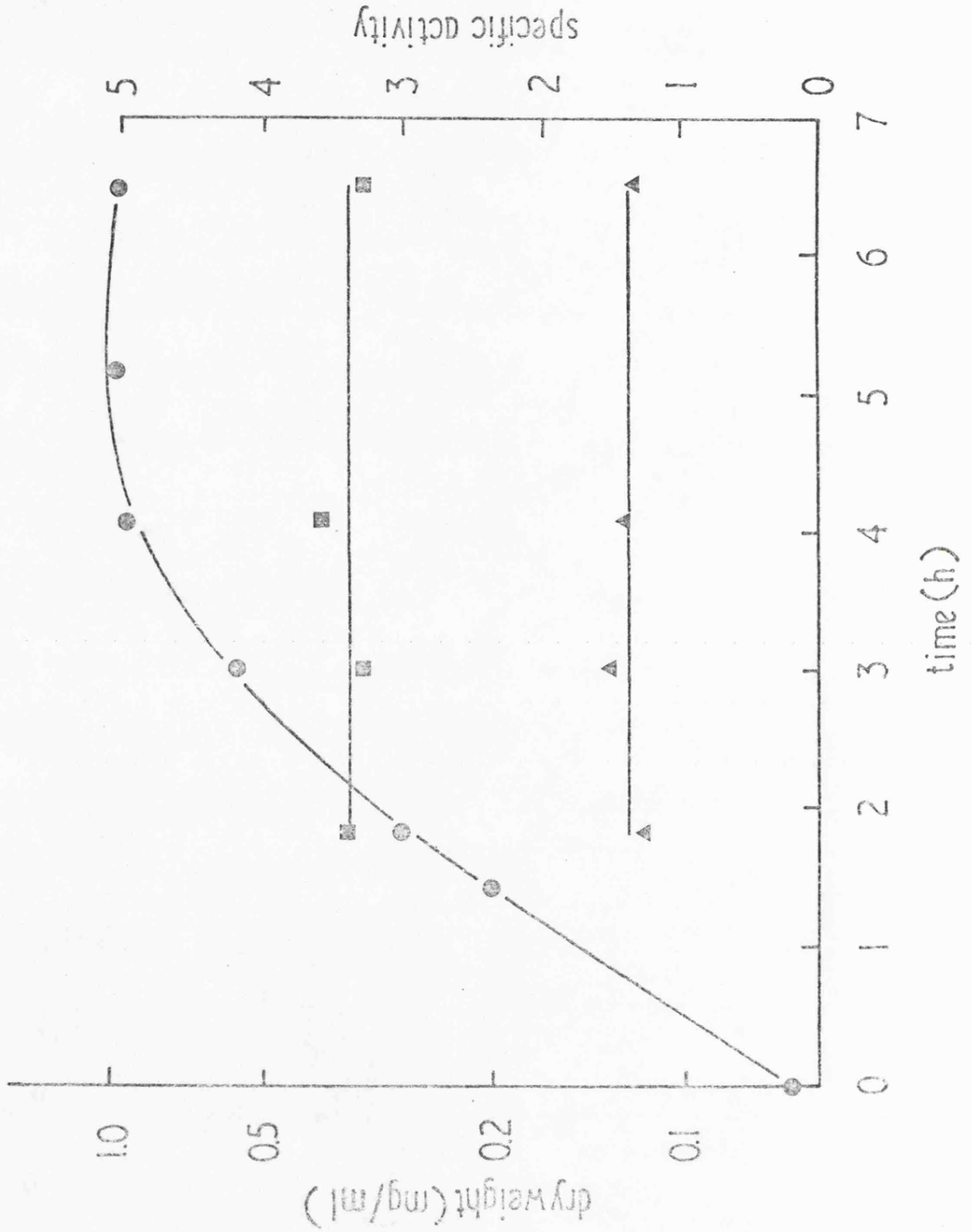

specific activity
5
4
3
2
1
0
7
6
5
4
3
2
1
0
time (h)
dry weight (mg/ml)
1.0
0.5
0.2
0.1

Figure 2

Variation in acetate kinase and phosphotransacetylase levels in *E. coli* K12 strain K2 during growth on 50 mM glucose with vigorous aeration. 500 ml cultures were grown in 2 litre Erlenmeyer flasks shaken at approximately 300 revolutions per min in a New Brunswick rotary shaker. Samples for enzyme assay were removed at intervals. Chloramphenicol was added to give a concentration of 100 μg/ml and the sample kept at 0°C prior to sonication. Cell-free extracts were prepared and enzyme assays carried out as described in Materials and Methods. Growth ● ; specific activity of phosphotransacetylase (μmol NADH/min/mg protein) ■ ; specific activity of acetate kinase (μmol acetyl hydroxamate/min/mg protein) ▲ .

the involvement of the glycolytic system (pyruvate itself, DL-lactate and gluconate) contain higher levels of acetate kinase and phosphotransacetylase than those grown on carbon sources obligatorily metabolised via at least a part of the glycolytic pathway (glucose, ribose and glycerol) or from which pyruvate is produced via PEP (malate and acetate).

In order to test the idea that pyruvate might be an important metabolite in the control of these enzymes under aerobic conditions, the levels of the enzymes in K2-3, a pyruvate dehydrogenase-negative derivative of K2, grown on glucose/acetate medium, were measured. Higher levels of acetate kinase and phosphotransacetylase than in the parent strain were observed. Pyruvate dehydrogenase-less mutants excrete pyruvate into the medium and might be expected to contain an elevated pyruvate pool.

As a batch culture of E. coli grows on glucose, the oxygen concentration in the medium, the pH of the medium, and the levels of compounds excreted into the medium, will change. The possibility that these changes cause induction or repression of acetate kinase and phosphotransacetylase was investigated. Samples were taken at various stages of growth from a culture of K2 growing on 50 mM glucose with vigorous aeration and the specific activities of acetate kinase and phosphotransacetylase measured. The results are presented in Figure 2. They show that the levels of the enzymes do not vary during growth under these conditions.

These data do not suggest that the acetate kinase/phosphotransacetylase system is subject to what is normally understood as

Table 3 Acetate kinase and phosphotransacetylase levels in acetate kinase-less and phosphotransacetylase-less mutants of *E. coli* K12 growing aerobically and anaerobically on glucose

		specific activity	
	organism	acetate kinase	phosphotrans-acetylase
aerobic	K2	1.8	2.0
	K2FAc1 (ACK^-)	0.1	1.9
	K2HFAc3 (PTA^-)	1.7	less than 0.03
anaerobic	K2	4.2	5.4
	K2FAc1 (ACK^-)	0.4	-
	K2HFAc3 (PTA^-)	-	less than 0.1

Cells were grown, cell-free extracts prepared, and enzyme assays carried out as described in Materials and Methods. Anaerobic growth was on 25 mM glucose/20 mM sodium bicarbonate medium. Specific activities are expressed as μmol/min/mg protein.

catabolite repression. A possible role for pyruvate in coarse control is interesting in view of the position of this compound at the major branch point in fermentative metabolism. The elevated levels of the enzymes in anaerobically grown cells are consistent with the expected role of this enzyme system in acetate excretion and energy production under anaerobic conditions.

Acetate kinase and phosphotransacetylase levels in strain K2 and its mutants K2FAc1 and K2HFAc3 The residual acetate kinase and phosphotransacetylase levels of the fluoroacetate-resistant mutants of strain K2 were measured in order to test the effect of a lesion in one enzyme on the level of the other. The possibility that anaerobic isoenzymes might exist was tested by measuring the levels of acetate kinase and phosphotransacetylase in the mutants after anaerobic growth on glucose. No pleiotropic effects on enzyme levels or indication of anaerobic isoenzymes were obtained (Table 3).

The resistance of K2FAc1 and K2HFAc3 to fluoroacetate in liquid culture The observation that in the selection of K2 mutants resistant to fluoroacetate the range of lesions obtained depended upon the selective level of fluoroacetate used prompted a comparison of the effects of fluoroacetate, at 10 and 50 mM, on the growth in 25 mM pyruvate media, of the mutants K2FAc1 (ACK^-) isolated at 10 mM fluoroacetate and K2HFAc3 (PTA^-) isolated at 50 mM fluoroacetate. Both the resistant organisms showed wild-type growth rates at the two fluoroacetate concentrations tested whereas growth of the parent

FIG. 3

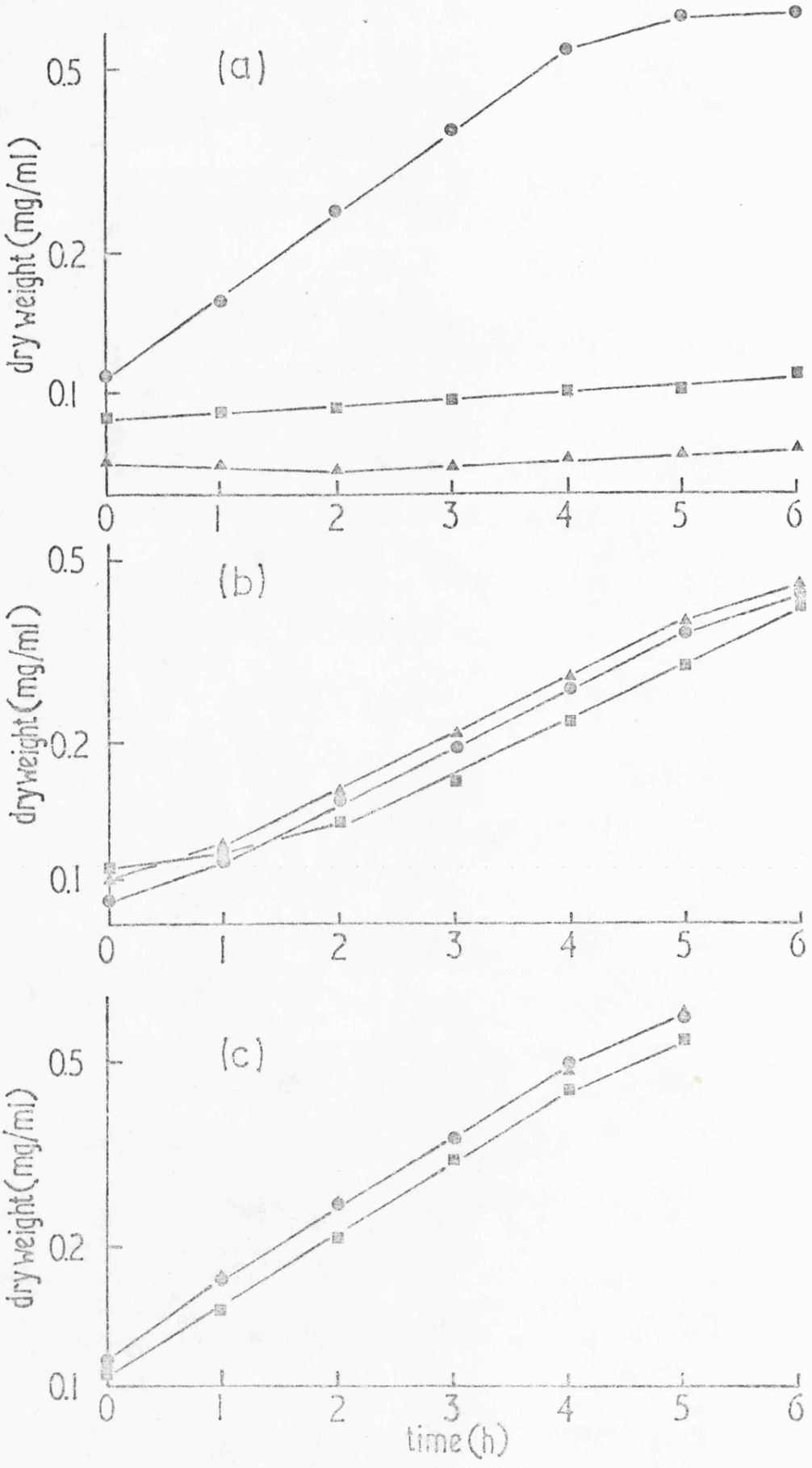


Figure 3

Effect of 10 mM and 50 mM sodium fluoroacetate on the growth of *E. coli* K12 strains K2, K2FAc1 (ACK^-) and K2HFAc3 (PTA^-) on 25 mM sodium pyruvate. The organisms were pregrown on pyruvate. Growth was measured as described in Materials and Methods. (a) K2 - no addition ● ; 10 mM fluoroacetate ▲ ; 50 mM fluoroacetate ■ . (b) K2FAc1 - no addition ● ; 10 mM fluoroacetate ▲ ; 50 mM fluoroacetate ■ . (c) K2HFAc3 - no addition ● ; 10 mM fluoroacetate ▲ ; 50 mM fluoroacetate ■ .

Table 4 Growth of acetate kinase-less and phosphotransacetylase-less strains of *E. coli* K12 on 25 mM acetate minimal medium

organism	mean generation time (h)
K2	2.8
K2FAc1 (ACK^-)	6.0
K2HFAc3 (PTA^-)	16
K1	2.6
K1HFAc1 (ACK^-)	5.5
K1HFAc3 (PTA^-)	10

The organisms were grown as described in Materials and Methods. K2 derivatives were pregrown on glycerol medium. K1 derivatives were transferred from 25 mM glycerol/10 mM acetate medium. Pregrowth on this medium did not cause reversion of the lesions. The growth of the cultures was measured as described in Materials and Methods.

Table 5 Growth of acetate kinase-less and phosphotransacetylase-less strains of *E. coli* K12 on glucose/bicarbonate medium under anaerobic conditions

organism	mean generation time (h)
K2	1.8
K2FAc1 (ACK^-)	1.8
K2HFAc3 (PTA^-)	3.8
K1	1.5
K1HFAc1 (ACK^-)	2.3
K1HFAc2 (ACK^-)	2.3
K1HFAc3 (PTA^-)	4.3

The organisms were grown as described in Materials and Methods. Cells were pregrown anaerobically on glucose/bicarbonate medium as described for enzyme assays, harvested, washed and resuspended in 25 mM glucose/ 20 mM sodium bicarbonate minimal medium. Growth of the cultures was measured as described in Materials and Methods.

strain was almost completely abolished under these conditions (Figure 3).

Growth studies of acetate kinase-negative and phosphotransacetylase-negative mutants

1. Aerobic growth on acetate The growth characteristics of K2FAc1, K2HFAc3, K1HFAc1 and K1HFAc3 on 25 mM acetate medium were compared with those of the parent strains. The mean generation times are shown in Table 4. K2FAc1 (ACK^-) maintained its growth on acetate after three cycles from a low cell density in acetate medium; the cells remain acetate kinase-negative. The maximum cell density obtained was lower than that of the parent strain. These data indicate that growth on acetate is impaired but not completely abolished in acetate kinase-negative organisms, but that phosphotransacetylase-negative strains are essentially acetate-negative. This difference in growth pattern is also observed with cells plated on acetate medium.

2. Anaerobic growth on glucose Acetate kinase-negative and phosphotransacetylase-negative mutants grow with wild-type mean generation times aerobically on glucose. However, when anaerobic growth is measured, a marked increase in the mean generation time of the phosphotransacetylase-negative mutants is observed. An increase in the mean generation time of the acetate kinase-negative mutants is seen in the K1 derivatives, but not in the K2 derivatives. The data are shown in Table 5. The slow growth of the phosphotrans-acetylase-less strains under these anaerobic conditions may reflect

FIG. 4

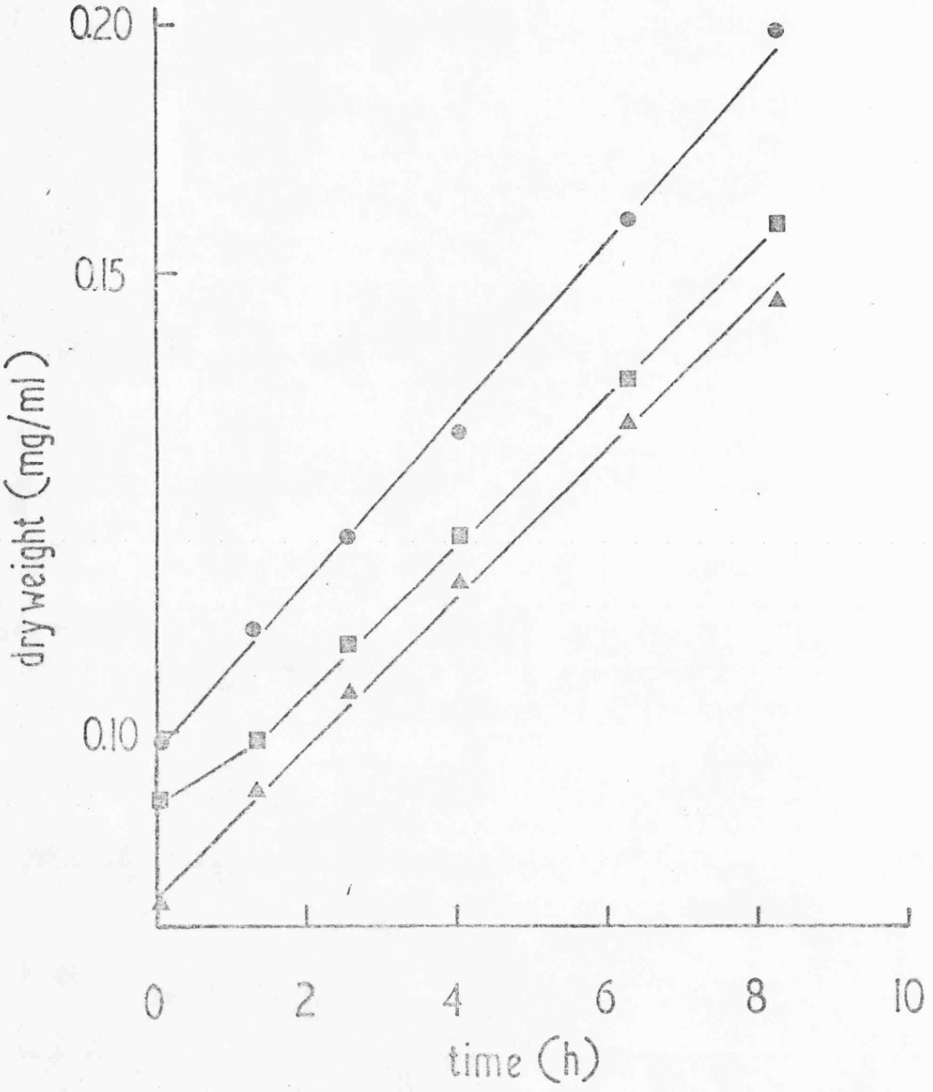


Figure 4

Growth of *E. coli* K12 strains K2, K2FAc1 (ACK^-) and K2HFAc3 (PTA^-) on 20 mM dilithium acetyl phosphate. The strains were pregrown on glycerol. Growth of the cultures was measured as described in Materials and Methods. K2 ● ; K2FAc1 ▲ ; K2HFAc3 ■ .

an accumulation of a toxic metabolite, possibly acetyl CoA. The failure to observe the same degree of inhibition in acetate kinase-less strains is interesting.

3. Aerobic growth on acetyl phosphate E. coli can use certain hexose phosphates as carbon sources and it was therefore of interest to test the possibility of growth on acetyl phosphate. Permeability to phosphorylated compounds is low unless specific transport systems are elaborated. The ability of wild-type, acetate kinase-negative and phosphotransacetylase-negative strains to grow on 20 mM acetyl phosphate was tested, (Figure 4). Slow growth was observed in the parental and mutant strains. The slow growth of the phosphotrans-acetylase-less organism suggests that it is unlikely that acetyl phosphate per se is being used. Acetyl phosphate is susceptible to hydrolysis in aqueous solution at neutral pH (Stadtman, 1955). The possibility that growth of a phosphotransacetylase-less strain may be possible on the low concentrations of acetate maintained by hydrolysis of acetyl phosphate is of interest in the light of incorporation experiments described below.

Acetate excretion by K2, K2FAc1 and K2FAc7 growing aerobically on glucose and by K1, K1HFAc1, K1HFAc2 and K1HFAc3 growing anaerobically on glucose The effect of lesions in acetate kinase and phosphotrans-acetylase on acetate excretion by cells growing aerobically and anaerobically on glucose was tested in order to investigate their role in excretory metabolism. The particular importance of

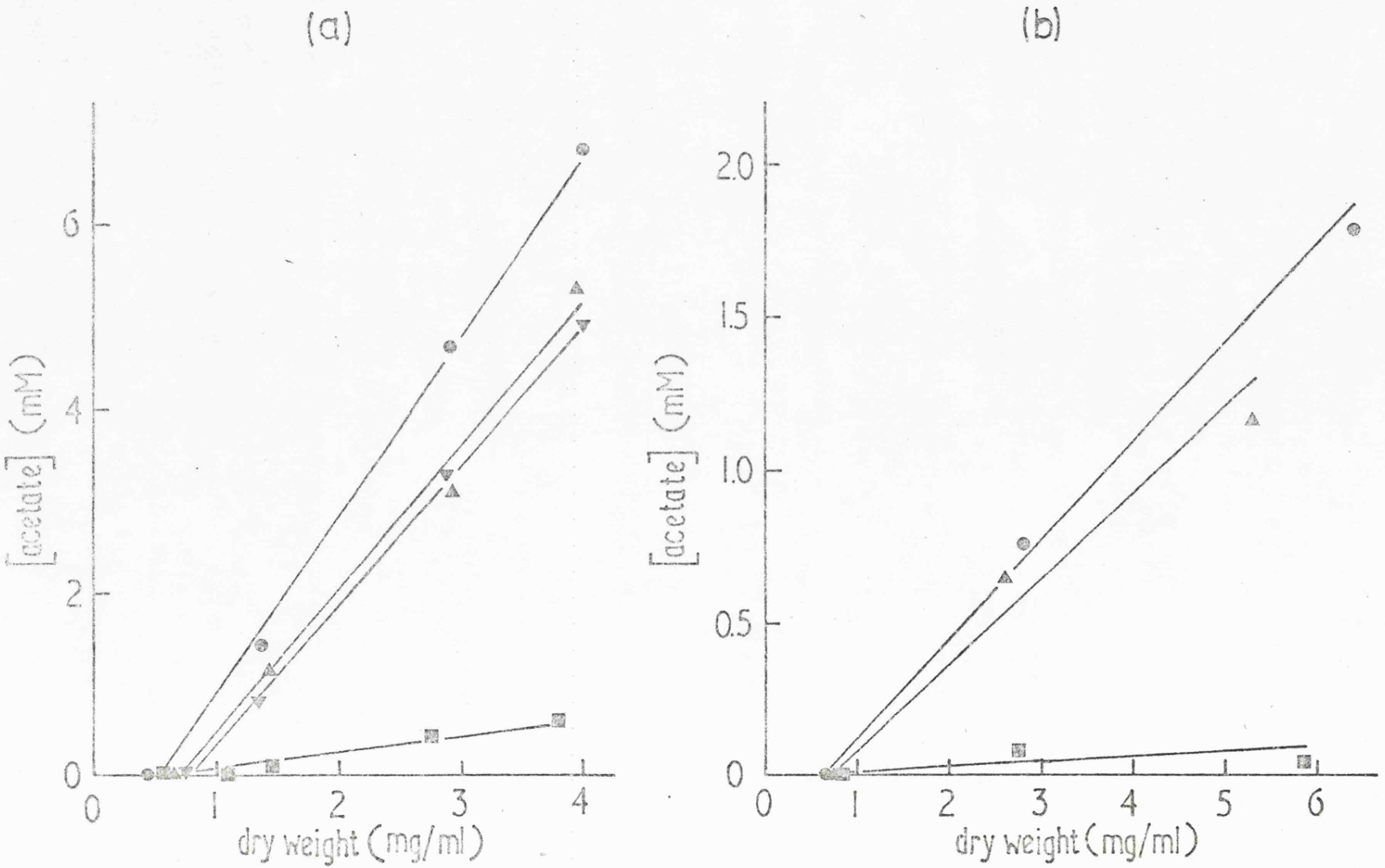


FIG. 5

Figure 5

Acetate excretion by acetate kinase-negative and phosphotransacetylase-negative mutants of K1 and K2 growing anaerobically and aerobically on glucose. Acetate concentrations in the culture medium were measured as described in Materials and Methods. (a) Anaerobic growth on 25 mM glucose/25 mM sodium bicarbonate medium. Cultures were grown and growth measured as described in Materials and Methods. K1 ● ; K1HFAc1 (ACK^-) ▲ ; K1HFAc2 (ACK^-) ▼ ; K1HFAc3 (PTA^-) ■ . (b) Aerobic growth on 25 mM glucose. Culture growth was measured as described in Materials and Methods. K2 ● ; K2FAc1 (ACK^-) ▲ ; K2FAc7 (PTA^-) ■ .

phosphotransacetylase during anaerobic growth on glucose is suggested by the decreased growth rates observed in the phosphotransacetylase mutants (Table 5). The effect of the lesions in acetate kinase is less clear cut. Studies of acetate excretion in the mutants growing anaerobically on glucose suggest possible reasons for the growth observations. As shown in Figure 5, the phosphotransacetylase-negative mutants excrete essentially no acetate either aerobically or anaerobically, but the three acetate kinase-negative strains tested (two of which contained extremely low residual levels of enzyme) showed a rate of acetate excretion close to that found in the wild-type. Two possible explanations for this observation were tested:

1. The organisms excrete acetyl phosphate. The presence of acetyl phosphate in the medium was tested for by the method of Lipmann and Tuttle (1945). None could be detected in the culture medium at any time during the growth of the cultures.

2. There is a specific acetyl phosphate phosphatase (acetyl phosphate phosphohydrolase, E.C. 3.6.1.7). Cell-free extracts from anaerobically grown cells were tested for acetyl phosphatase activity. At pH 7.4 no activity could be detected and the acid phosphatase activity tested at pH 5.65 could scarcely be detected. The growth conditions used are known powerfully to repress the periplasmic acid and alkaline phosphatases (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1 and 2) (Torriani, 1960; Hsie & Rickenberg, 1967). High levels of spontaneous acetyl phosphate hydrolysis were observed at 37°C and it is possible that this hydrolysis might account for the observed

excretion in the acetate kinase-negative mutants (Stadtman, 1955).

An attempt was also made to test the possibility that a primary phosphate acceptor other than ADP might be involved in the breakdown of acetyl phosphate. Anderson and Kamel (1966) have reported the purification of an enzyme from A. aerogenes which catalyses the transfer of the phosphate group from acetyl phosphate to D-glucose to form D-glucose 6-phosphate (acetyl phosphate : D-glucose 6-phosphotransferase). This activity could not be detected in E. coli.

In view of the rapid spontaneous hydrolysis of acetyl phosphate, and the finding that lesions in acetate kinase have only a limited effect on acetate excretion by cells growing on glucose, an attempt was made to test the role of acetate kinase in energy metabolism. If E. coli could be grown anaerobically on pyruvate, the sole energy yielding process available to the cell would be the acetate kinase reaction. Acetate kinase-less mutants would be expected to be unable to grow anaerobically on pyruvate. It did not, however, prove possible to get reasonable growth of the wild-type under the conditions tested.

The incorporation of [2-^{14}C]acetate by acetate kinase-negative and phosphotransacetylase-negative mutants growing on an unlabelled carbon source Preliminary observations (described in Chapter III) had shown that washed cell suspensions of acetate kinase-negative and phosphotransacetylase-negative mutants were able to take up labelled acetate as readily as the wild-type at low concentrations and that this

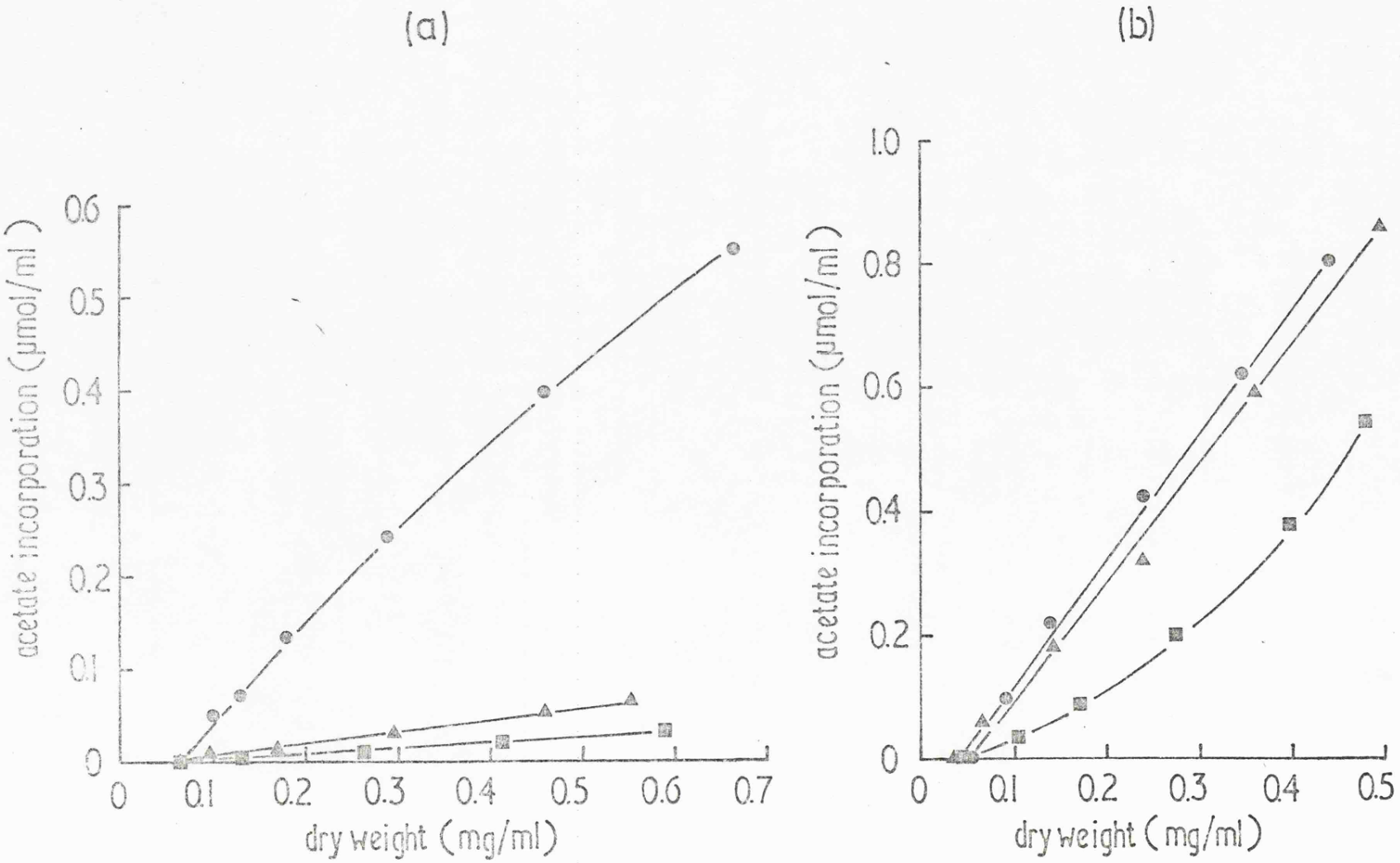

(a)
(b)
acetate incorporation (μmol/ml)
dry weight (mg/ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
1.0

FIG. 6

Figure 6

Comparison of 2 mM sodium [2^{14}C]acetate incorporation by (a) glucose-grown strains K2 ● ; K2FAc1 (ACK$^-$) ▲ ; K2HFAc3 (PTA$^-$) ■ , growing on 25 mM glucose, and (b) glycerol-grown strains K2 ● ; K2FAc1 ▲ ; K2HFAc3 ■ , growing on 25 mM glycerol. Incorporation of radioactive label into cell material was measured as described in Materials and Methods.

FIG. 7 (a)

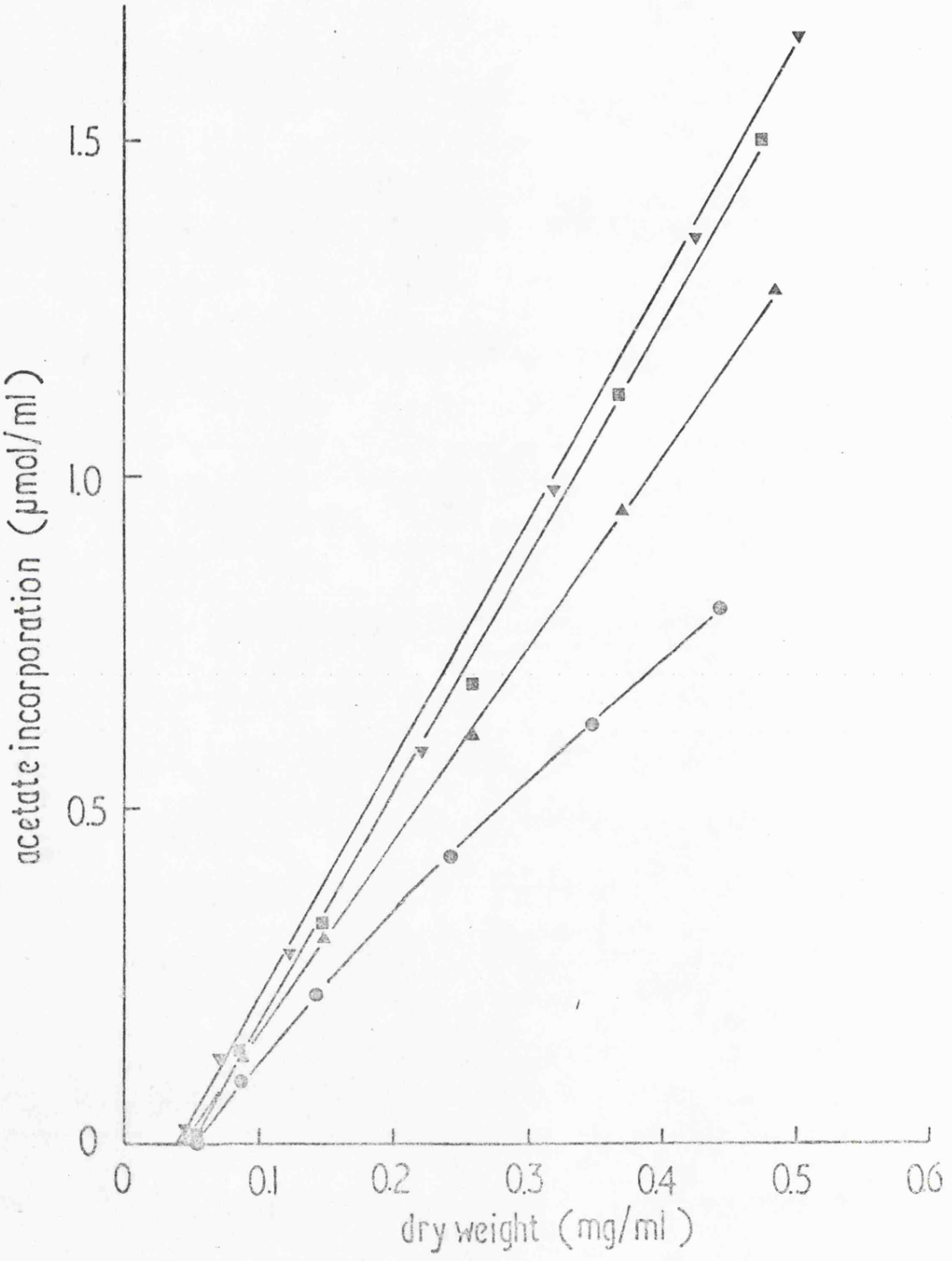

acetate incorporation (μmol/ml)
1.5
1.0
0.5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
dry weight (mg/ml)

FIG. 7 (b)

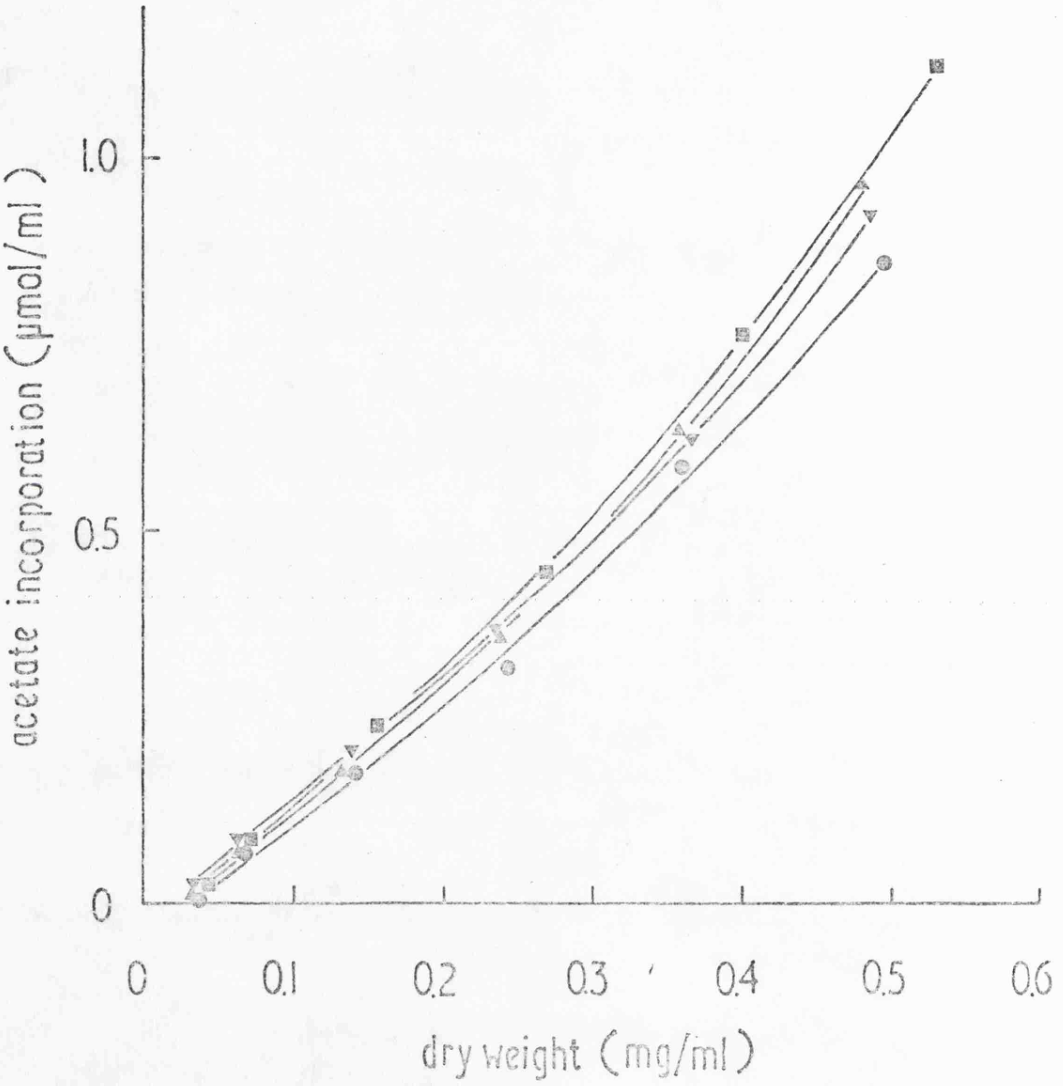

acetate incorporation (μmol/ml)
1.0
0.5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
dry weight (mg/ml)

FIG. 7 (c)

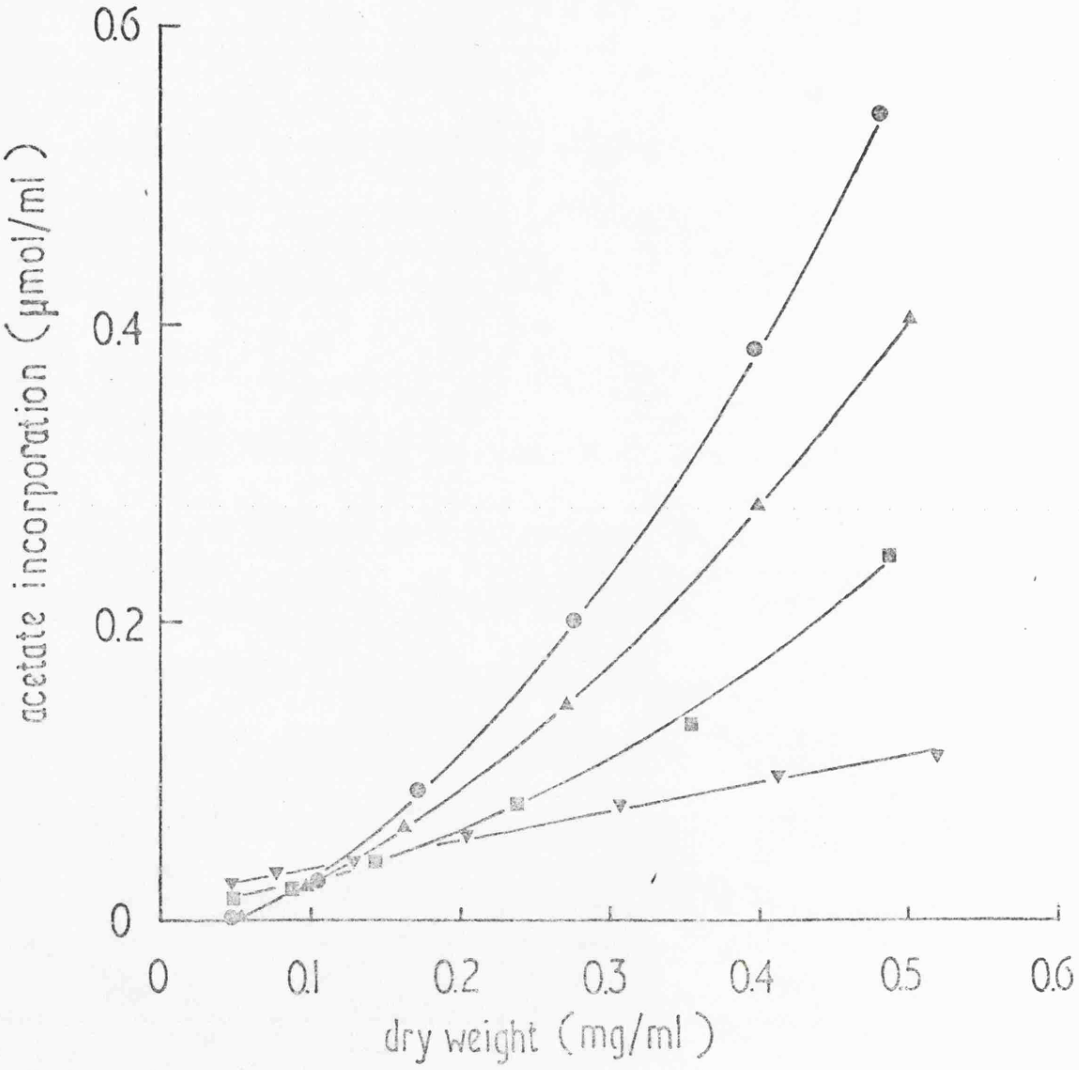

0.6
0.4
0.2
0
acetate incorporation (μmol/ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
dry weight (mg/ml)

Figure 7

Incorporation of sodium [2^{14}C]acetate by E. coli K12 strain K2, K2FAc1 (ACK^-) and K2HFAc3 (PTA^-) at 2, 5, 10 and 20 mM. All strains were pregrown on glycerol, harvested, washed and transferred to glycerol/acetate medium. Incorporation of radioactive label into cell material was measured as described in Materials and Methods.

(a) K2 - 2 mM ● ; 5 mM ▲ ; 10 mM ■ ; 20 mM ▼ ;

(b) K2FAc1 - 2 mM ● ; 5 mM ▲ ; 10 mM ■ ; 20 mM ▼ ;

(c) K2HFAc3 - 2 mM ● ; 5 mM ▲ ; 10 mM ■ ; 20 mM ▼ .

uptake process was repressed by glucose. The possibility of a second activation system for acetate was also indicated by the slow, but logarithmic, growth of acetate kinase-less mutants on acetate. In order to study the effect of lesions in acetate kinase and phosphotransacetylase upon the utilization of acetate under more "physiological" conditions than were used in the short term washed cell suspension experiments, a series of studies of acetate incorporation into cultures growing logarithmically on an unlabelled carbon source was carried out. Comparisons of the incorporation of labelled acetate at 2 mM by strains K2, K2FAc1 (ACK^-) and K2HFAc3 (PTA^-) growing on glucose and glycerol were carried out (Figure 6). Lesions in acetate kinase and phosphotransacetylase almost completely abolish the incorporation of acetate when the cells are pregrown on glucose and glucose is used as the unlabelled carbon source. A different pattern is observed when glycerol-grown cells growing on glycerol are exposed to 2 mM acetate. The acetate kinase-negative mutant retains the ability to incorporate acetate at close to wild-type levels and a considerable residual ability to incorporate acetate is also observed in the phosphotransacetylase-less organism.

The effect of increasing the acetate concentration from 2 to 20 mM is shown in Figure 7 for glycerol-grown cells growing on [^{14}C]acetate plus glycerol. In the wild-type, incorporation increases as the acetate concentration is increased (Figure 7a) whereas in the acetate kinase-less mutant there is essentially no increase in acetate incorporation as the acetate concentration is increased (Figure 7b). The ability of the phosphotransacetylase-negative mutant to incorporate

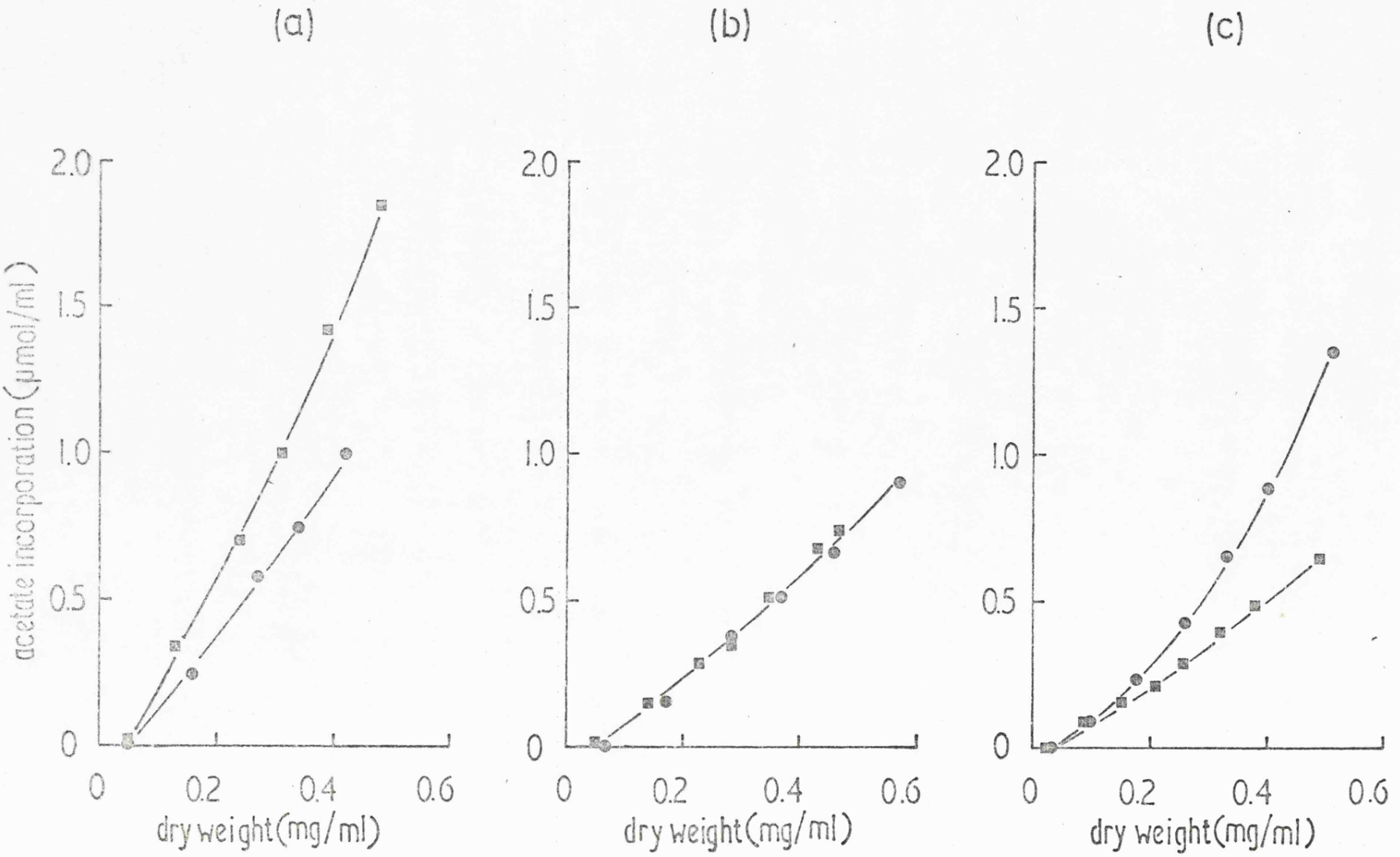

(a)
(b)
(c)
acetate incorporation (μmol/ml)
2.0
1.5
1.0
0.5
0
0
0.2
0.4
0.6
dry weight (mg/ml)

FIG. 8

Figure 8

Incorporation of sodium [2-^{14}C]acetate by *E. coli* K12 strain K1, K1HFAc1 (ACK^-) and K1HFAc3 (PTA^-) growing on 25 mM glycerol. All strains were pregrown on glycerol, harvested, washed and transferred to fresh glycerol/acetate medium. Incorporation of radioactive label into cells was measured as described in Materials and Methods. (a) K1 - 2 mM ● ; 20 mM ■ . (b) K1HFAc1 - 2 mM ● ; 20 mM ■ . (c) K1HFAc3 - 2 mM ● ; 20 mM ■ .

FIG. 9

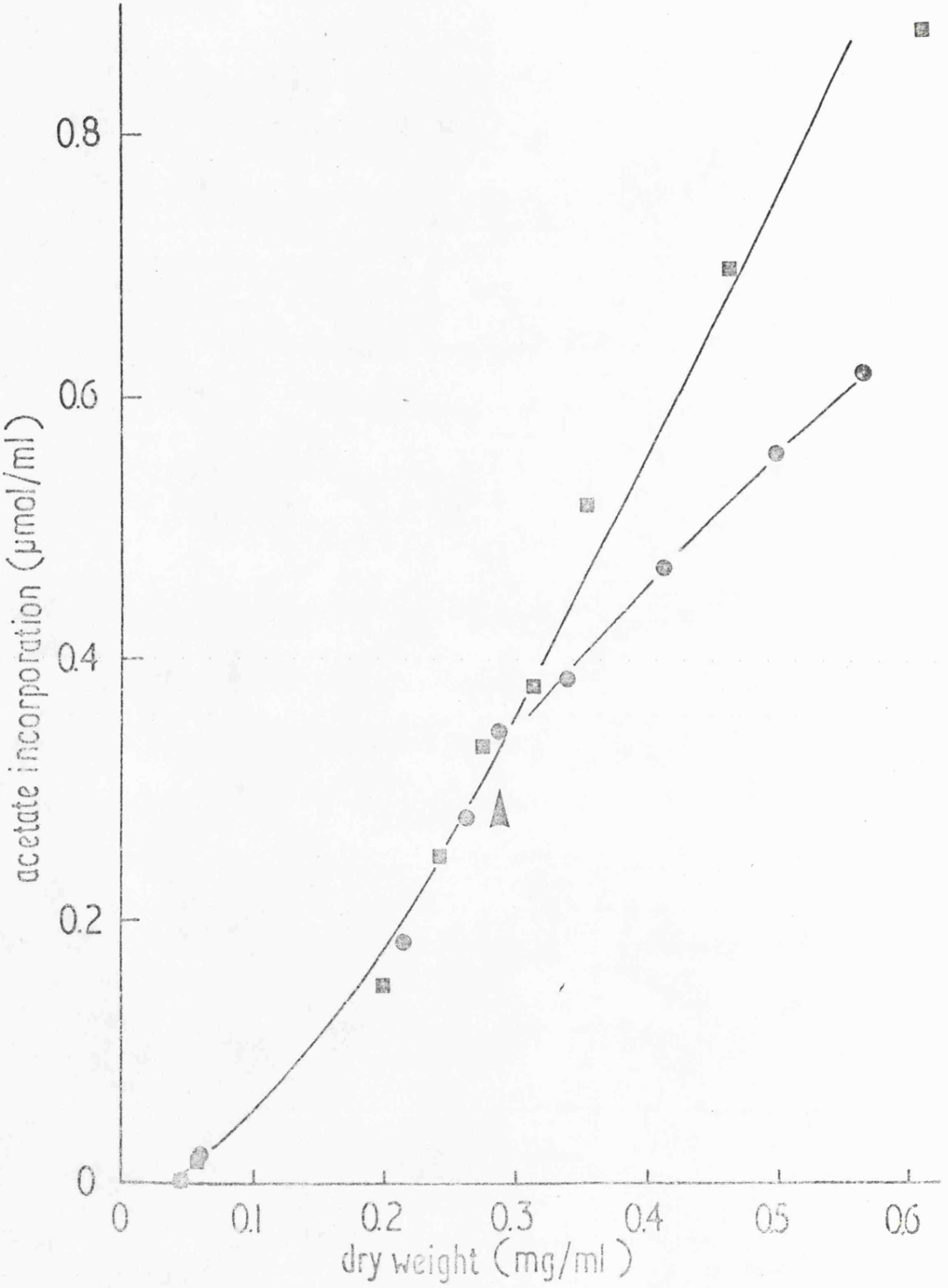

0.8
0.6
0.4
0.2
0
acetate incorporation (μmol/ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
dry weight (mg/ml)

Figure 9

The effect of 25 mM glucose addition upon the incorporation of 2 mM sodium [2^{14}C]acetate by strain K2FAc1 (ACK$^-$) growing on 25 mM glycerol. K2FAc1 was pregrown on glycerol, harvested, washed and transferred to fresh glycerol/acetate medium. Incorporation of radioactive label into cells was measured as described in Materials and Methods. The glucose addition was made at the point indicated by the arrow. Incorporation by control culture (no glucose addition) ■ ; incorporation by culture to which 25 mM glucose was added at the point indicated ● .

acetate actually decreases dramatically as the acetate concentration is raised from 2 to 20 mM (Figure 7c). This observation is consistent with the difference observed in the growth rates of acetate kinase-less and phosphotransacetylase-less mutants on 25 mM acetate medium. These observations were confirmed with acetate kinase-negative and phosphotransacetylase-negative mutants isolated from K1 (Figure 8). The relative degree of inhibition of incorporation at 20 mM acetate compared with the incorporation at 2 mM in the phosphotransacetylase-negative K1 derivative tested (Figure 8c) was less than observed with the similar K2 derivative (Figure 7c), but a qualitatively similar pattern of incorporation was observed with the wild-type, acetate kinase-less and phosphotransacetylase-less strains of K1 (Figures 8a, b and c).

The possibility that the low rates of acetate incorporation at 2 mM observed when strains lacking acetate kinase or phosphotransacetylase are growing on glucose (after pregrowth on glucose) could be due to an inhibitory effect of glucose (or some metabolite derived from glucose) on a second activating system, rather than to a repressive effect, was tested. The effect of the addition of 25 mM glucose to a culture of K2FAc1 (ACK^-) pregrown on glycerol and growing on 25 mM glycerol + 2 mM [2-^{14}C]acetate upon incorporation of the labelled acetate was measured (Figure 9). Upon addition of the glucose there is an immediate reduction in the rate of acetate incorporation, but the rate was not reduced to the level observed in K2FAc1 pregrown on glucose and growing on 25 mM glucose + 2 mM labelled acetate (Figure 6). As growth proceeds in the presence of the added glucose

there is evidence of a further decrease in the ability to incorporate acetate which may reflect repression of the synthesis of the second system and its dilution as the culture grows. This experiment suggests that there may be both repression and inhibition by glucose of the alternative activating system with the former effect being quantitatively more significant.

Effect of exogenous acetyl phosphate upon growth of K2 upon acetate

The effect of increasing acetate concentration upon acetate incorporation in phosphotransacetylase-negative strains suggests the possibility that elevated acetyl phosphate levels caused by an inability to metabolise acetyl phosphate to acetyl CoA may inhibit acetate utilization perhaps at the level of the second acetate activation system. The addition of 10 mM lithium acetyl phosphate to a culture of K2 growing on acetate fails to inhibit growth. It is likely that the lack of effect of exogenous acetyl phosphate is due to its failure to get into the cell in spite of the concentration used.

Dependence of growth rate upon medium acetate concentration in K1

The dependence of growth rate of strain K1 upon acetate concentration in the medium was measured in order to determine whether it would reflect the contrasting properties of the two activating systems revealed by incorporation studies with wild-type and mutant strains. The growth rate was measured over a range of acetate concentrations from 1 to 50 mM. This presented considerable technical problems. Thus in order to observe a reasonable percentage change in cell mass

Table 6 Dependence of growth rate of *E. coli* K12 strain K1 upon acetate concentrations

concentration of acetate in medium (mM)	mean generation time (h)
50	2.0
20	2.9
10	4.1
5	5.5
3	4.3
2	4.7
1	5.2

Cultures were grown as described in Materials and Methods. Pregrowth was on glycerol/acetate medium. Growth of the cultures was measured as described in Materials and Methods.

at low concentrations of acetate, it was necessary to initiate the growth at a low A_{680} with consequent loss of accuracy in the absorbance data. The growth rates at low acetate concentrations were therefore difficult to measure accurately. Strain K1 was pregrown on glycerol/acetate medium, washed twice with carbon-free basal medium and resuspended in media containing acetate at different concentrations. The growth rates measured at various acetate concentrations are shown in Table 6. The growth rate appears to be concentration dependent at high acetate concentrations, but to have a constant value at acetate concentrations below 5 mM. The rate observed at low acetate concentrations is similar to that observed for acetate kinase-less mutants. It is thus possible that the growth pattern reflects the high and low concentration activation systems suggested by the incorporation experiments described above. The possibility that acetate concentration may affect the operation of other systems required for acetate growth, for example induction of the glyoxylate by-pass, cannot, of course, be eliminated in this experiment.

The incorporation of [2-^{14}C]propionate by acetate kinase-negative and phosphotransacetylase-negative mutants growing on glycerol

Propionate is a poor sole carbon source for many strains of E. coli, but it can be utilized under some conditions (Wegener et al., 1968). This utilization is thought to involve activation to propionyl CoA. In view of Rose's observation that propionate is a substrate for acetate kinase, albeit a poor one, and the observation that the acetate kinase/phosphotransacetylase system is involved in acetate

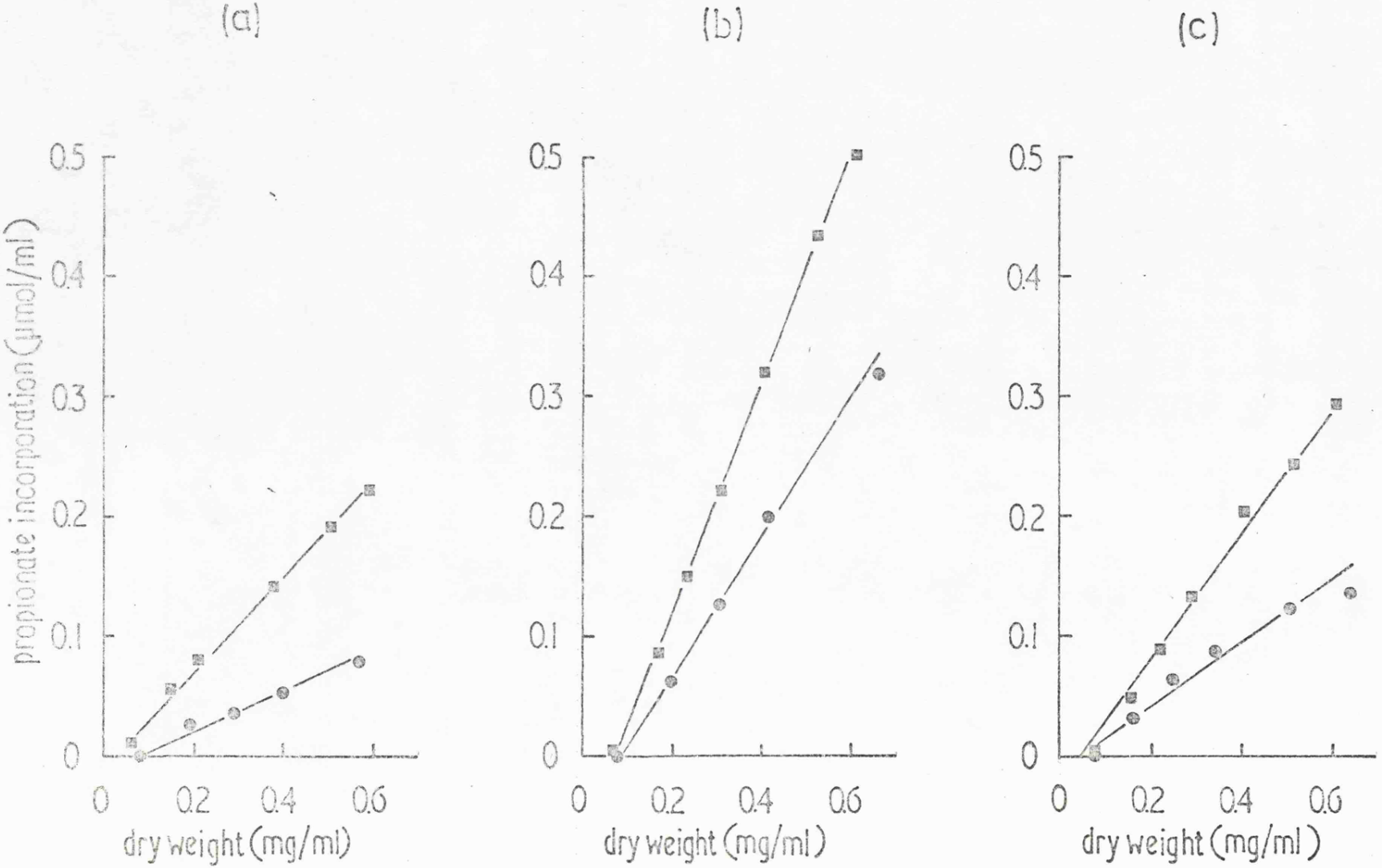

(a)
(b)
(c)
propionate incorporation (μmol/ml)
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
dry weight (mg/ml)

FIG.10

Figure 10

The incorporation of 2 and 20 mM sodium [2-^{14}C]propionate by cultures of *E. coli* K12 strains K1, K1HFAc1 (ACK^-) and K1HFAc3 (PTA^-) growing on 25 mM glycerol after pregrowth on 25 mM glycerol/20 mM sodium propionate medium. Incorporation of radioactive label into cells was measured as described in Materials and Methods. (a) K1 - 2 mM ● ; 20 mM ■ . (b) K1HFAc1 - 2 mM ● ; 20 mM ■ . (c) K1HFAc3 - 2 mM ● ; 20 mM ■ .

FIG. 11

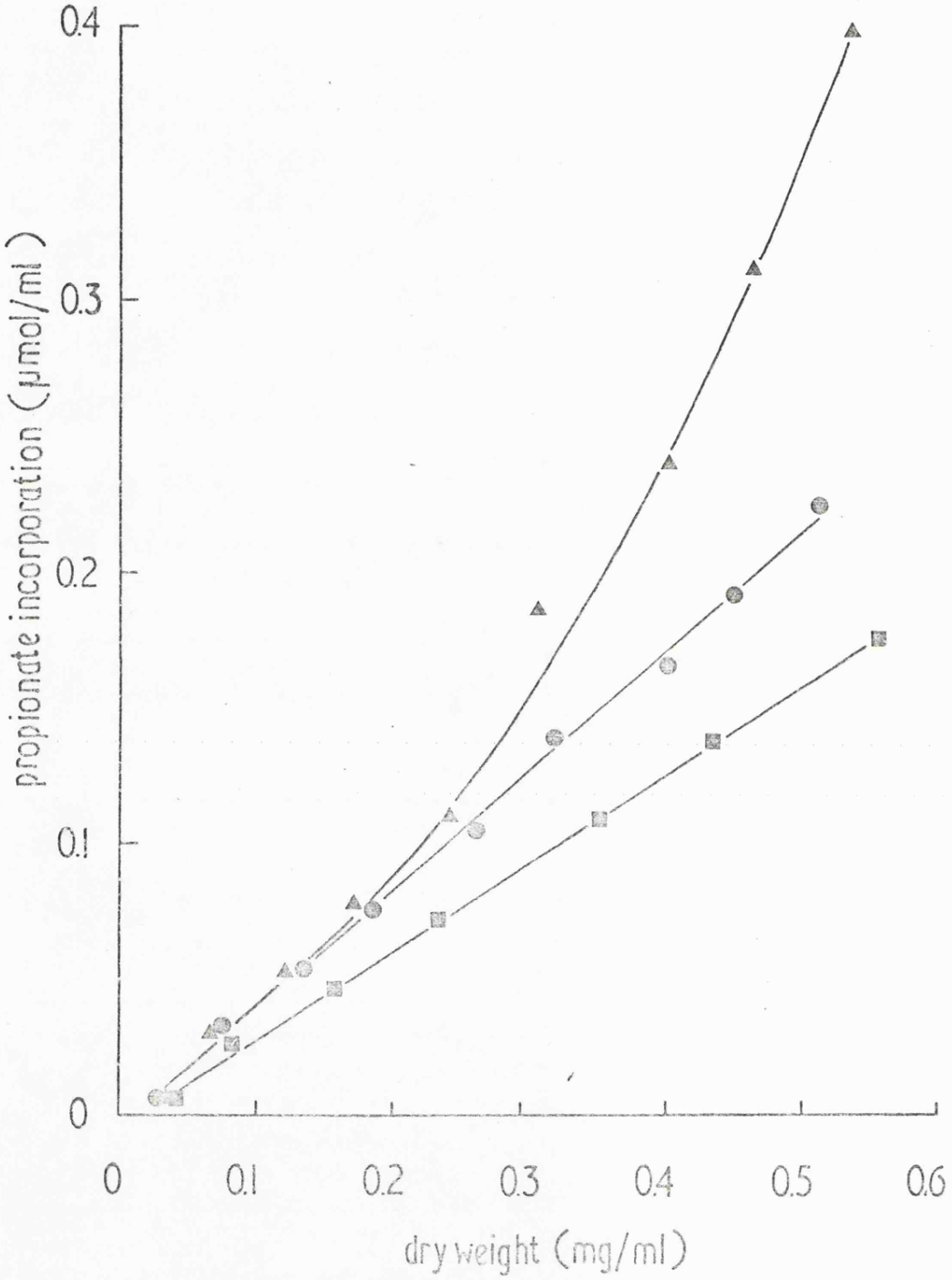

0.4
0.3
0.2
0.1
0
propionate incorporation (μmol/ml)
0
0.1
0.2
0.3
0.4
0.5
0.6
dry weight (mg/ml)

Figure 11

Incorporation of 2 mM sodium [2^{14}C]propionate by strains K1, K1HFAc1 (ACK$^-$) and K1HFAc3 (PTA$^-$) growing on 25 mM glycerol after growth on 25 mM glycerol. Cells were harvested, washed and resuspended in fresh glycerol/propionate medium. Incorporation of radioactive label into cells was measured as described in Materials and Methods. K1 ● ; K1HFAc1 ▲ ; K1HFAc3 ■ .

FIG.12

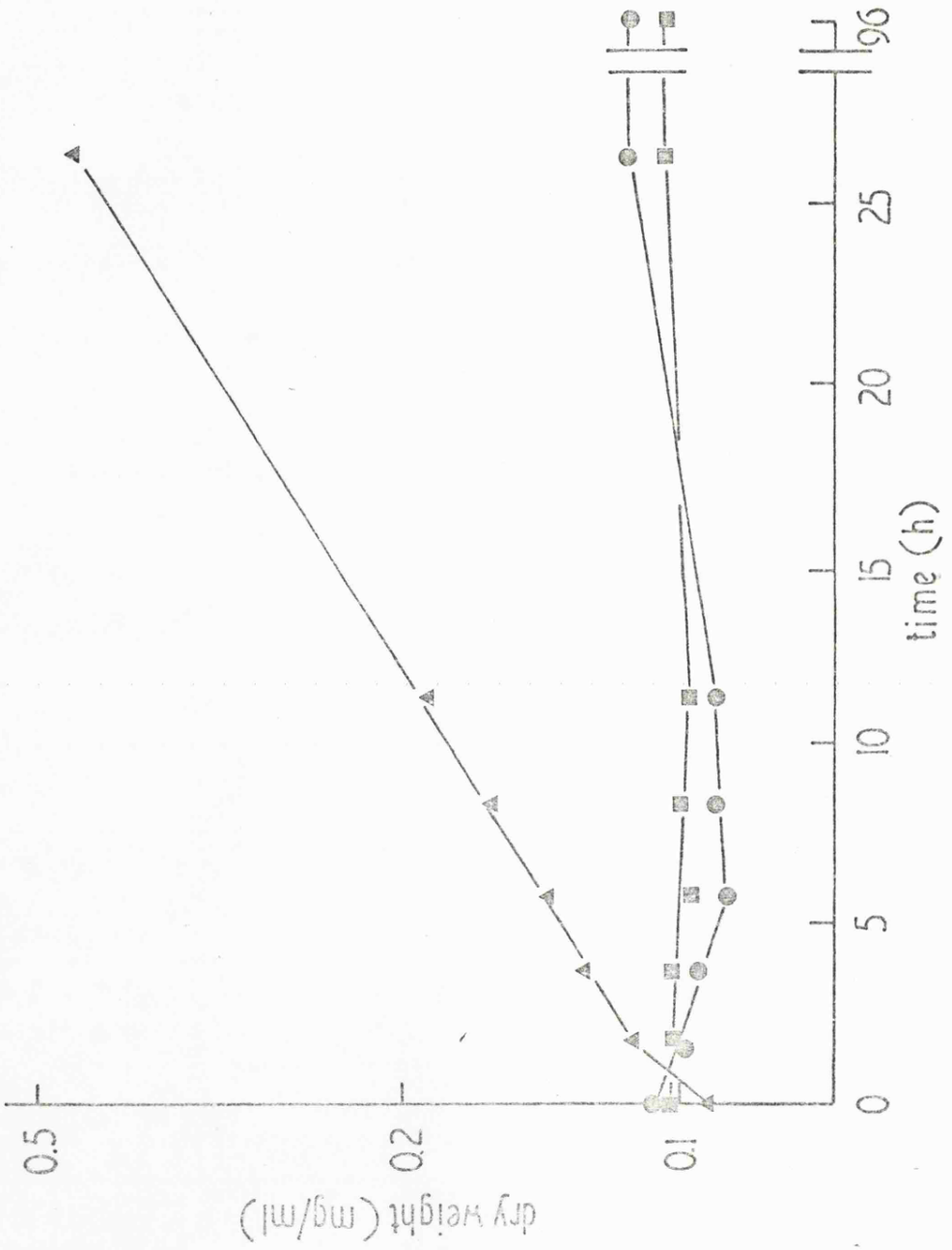


Figure 12

Growth of *E. coli* K12 strains K1, K1HFAc1 (ACK^-) and K1HFAc3 (PTA^-) on 25 mM sodium propionate after transfer from glycerol medium. Growth of the cultures was measured as described in Materials and Methods. K1 ● ; K1HFAc1 ▲ ; K1HFAc3 ■ .

activation at high acetate concentrations (Figures 6, 7 and 8), it was decided to determine whether the enzyme system played any role in propionate activation in E. coli K12.

The incorporation of labelled propionate at 2 and 20 mM by cells growing on glycerol after pregrowth on either glycerol alone or glycerol + propionate was measured and the results are presented in Figures 10 and 11. These suggest that the acetate kinase/phosphotransacetylase system does not play any role in propionate activation in K1 except perhaps a negative one. Induction by propionate of a system apparently required for propionate incorporation after transfer from glycerol medium is observed only in the acetate kinase-less derivative of K1, and higher levels of incorporation are observed in the acetate kinase-less derivative after pregrowth in the presence of propionate than in the wild-type or the phosphotransacetylase strain. These observations are in agreement with the finding that, after transfer from glycerol minimal medium to propionate minimal medium, K1HFAc1 (ACK^-) enters log phase within 2-3 hours whereas K1 and K2HFAc3 (PTA^-) fail to adapt to growth on propionate minimal medium within 96 hours (Figure 12). It is possible that propionyl phosphate levels are elevated in both the wild-type and phosphotransacetylase-less strain to the extent that propionyl phosphate inhibits the induction of a system required for propionate utilization. Propionyl phosphate is a poor substrate for phosphotransacetylase in E. coli (Hibbert et al., 1971).

CHAPTER II

Studies of a second acetate activation system in E. coli and its relation to other fatty acid activating systems

Introduction

The growth and incorporation experiments described in the previous chapter suggest the existence of a second acetate activation system in E. coli K12. The data from the incorporation experiments would suggest that this system must have a number of properties:

1. It would have a low K_m for acetate. Acetate activation by the second system as evidenced by incorporation of labelled acetate is saturated at 2 mM. The system thus contrasts with the high K_m for acetate shown by acetate kinase (Rose, 1962).
2. It would be subject to glucose repression. Acetate incorporation is higher in glycerol-grown acetate kinase-less mutants growing on glycerol than in glucose-grown cells growing on glucose and this is not due simply to an inhibitory effect of the glucose.
3. It would be expected to be essentially irreversible as a lesion in phosphotransacetylase almost totally abolishes acetate excretion.

These data prompted and guided a search for an alternative to the acetate kinase/phosphotransacetylase system for the activation of acetate. The most likely candidate for an alternative acetate activating system would be an acetate thiokinase. There have been no reports of acetate thiokinase activity in E. coli. Acetate thiokinase is found in certain bacteria and in animals and plants

(Beinert et al., 1953; Millerd & Bonner, 1954; Eisenberg, 1955, 1957; Fuller et al., 1961; Benedict & Rinne, 1964; Hoare & Gibson, 1964; Pearce & Carr, 1967; Flavell & Fincham, 1968; Skinner & Clarke, 1968; Trust & Millis, 1971). Thiokinases are essentially irreversible in the presence of inorganic pyrophosphatase:

$$\text{acetate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + PP_i$$

$$PP_i + H_2O \longrightarrow 2\ P_i$$

An examination of the literature on systems implicated in the activation of fatty acids by E. coli has clarified their possible relationships to the second acetate activation whose existence is suggested by the data described in Chapter I.

E. coli is capable of activating a range of short, medium and long-chain fatty acids. The best characterized activating enzyme is the acyl-CoA synthetase acting on medium and long chain fatty acids (acid : CoA ligase (AMP) E.C. 6.2.1.3). Its regulation has been the subject of intensive study by Overath and his colleagues and by Wakil's group (Overath et al., 1969; Klein et al., 1971; Weeks et al., 1969). Their work is in broad agreement. The enzyme is active against saturated, mono- and polyunsaturated acids from C_8-C_{20} and is induced by fatty acids with more than twelve carbon atoms. Glucose represses the induction. The ability of wild-type strains of E. coli to use fatty acids as sole carbon sources is determined in part by the inducer requirements of the activation and β-oxidation systems. The specific activity of the enzyme is low under the conditions of measurement used by Overath et al. (1969).

Samuel, Estroumza and Ailhaud (1970) have reported a partial purification of an acyl-CoA synthetase from an E. coli K12. It is

active on C_4-C_{18} fatty acids. They presented evidence for the existence of two enzymes, one acting on short and medium chain fatty acids and the other one on long chain fatty acids. This finding that the acyl-CoA synthetase is active against short and medium chain acids contrasts with Overath's findings and the work of Wakil's group. All the organisms studied were K12 strains. It is unlikely that the acyl-CoA synthetase described by these workers could be involved in acetate activation in E. coli K12.

The mechanisms of utilization of short and medium chain fatty acids as sole carbon sources are more complex than those of long chain acids. Genetic manipulations have been used to construct a variety of strains able to grow rapidly on short and medium chain fatty acids. The genetic plasticity of E. coli is strikingly illustrated by the ease with which such strains can be selected. The mechanisms of fatty acid activation have not been fully defined in any of these strains.

Genetic evidence for the existence of a short chain fatty acid activating system comes from the work of Salanitro and Wegener (1971a), who have selected a mutant capable of growth on butyrate and valerate by prolonged cycling in nonanoate medium. In view of the growth properties it seems likely that the organism has been modified in both short chain fatty acid activation and in β-oxidation. Thus specific elimination of either the observed derepressed β-oxidation activity or the uptake/activation system can be achieved. Selection for oleate negativity eliminates the derepressed β-oxidation system, while selection for 4-pentenoate resistance during growth

on acetate eliminates the uptake/activation process. The failure to identify the activation process by direct enzyme assay limits the utility of the data. The observation that modification of activating activity as evidenced by growth data has no effect on acetate and propionate growth is interesting in relation to the second acetate activation system. The 4-pentenoate-resistant mutants which are probably defective in activation also seem to be unaffected in their growth on acetate or propionate at 20 mM.

Overath has isolated strains (dec 16 but 1 and dec 16 but 12) that are capable of using butyrate as sole carbon source (Overath, 1969). A parent organism derepressed for the synthesis of the enzymes of long chain fatty acid degradation (acyl-CoA synthetase and β-oxidation) selected for growth on decanoate, was used for the selection of butyrate^{+} revertants. No butyrate activation system was identified in these organisms. The two mutants differ in that one (dec 16 but 1) possesses a very high level of thiolase and grows well on butyrate, but poorly on long chain fatty acids while the other (dec 16 but 12) grows well on butyrate and on fatty acids with more than eight carbon atoms. The relationship of the high thiolase (acyl-CoA : acetyl-CoA C-acyltransferase E.C. 2.3.1.16) activity to the growth properties of dec 16 but 1 is not known. Overath suggests that the butyrate activating system might involve a thiophorase system (butyryl : acetate-CoA transferase E.C. 2.8.3) or a butyrate kinase/phosphotransbutyrylase system. Both these systems were first identified in Clostridia (Stadtman, 1953; Valentine & Wolfe, 1960).

Two reports by Vanderwinkel and co-workers have described

the properties of mutants capable of growth on butyrate and valerate (Vanderwinkel et al., 1968; Vanderwinkel et al., 1971). If organisms capable of growth on butyrate are selected from a wild-type strain, KL16, they show slow growth on butyrate or valerate with a doubling time of 5-10 hours. If a strain constitutive for the glyoxylate cycle, D5H3G7, is used for the selection, the doubling time of the mutants obtained falls to 3-5 hours. If these mutants are further mutagenized to produce a lesion giving constitutive β-oxidation system activity, the doubling time falls to less than 3 hours. The common property of these butyrate$^+$ mutants is the presence of a CoA transferase activity capable of catalysing the reaction:

$$\text{acetyl CoA} + \text{butyrate} \rightleftharpoons \text{acetate} + \text{butyryl CoA}$$

$$\text{acetyl CoA} + \text{valerate} \rightleftharpoons \text{acetate} + \text{valeryl CoA}$$

The elevated levels of this activity are associated with elevated levels of thiolase. The thiolase levels also seem to be determined in some way by the state of control of the β-oxidation system. While the potential role of the CoA transferase in the utilization of butyrate and valerate is evident, the role of the thiolase activity is less obvious (Overath, 1969). It is likely that the glyoxylate-short chain fatty acid acyl CoA ester condensing activities postulated by Furmanski et al., (1967) to have a role in growth on medium chain fatty acids were artefactual being the result of operation of the CoA transferase and malate synthase (L-malate glyoxylate-lyase E.C. 4.1.3.2). The available data on short-chain fatty acid activation in E. coli suggest that none of the systems described is involved in acetate or propionate activity.

It is not known how propionate is activated in E. coli. Rose found that acetate kinase will catalyse the formation of propionyl phosphate from propionate and ATP. The K_m for propionate under the assay conditions used was, however, extremely high (Rose, 1962). The propionyl phosphate formed is a poor substrate for phosphotransacetylase (Hibbert et al., 1971). The incorporation experiments described in chapter I suggest that the acetate kinase/phosphotransacetylase system is unlikely to be involved in propionate activation in E. coli K12. The mechanism of propionate metabolism in E. coli remains largely obscure in spite of considerable effort by Ajl's group (Wegener et al., 1967; 1968a; 1968b; Kolodziej et al., 1968; Wegener et al., 1969). None of this work identified a propionate activation system.

The work described below was carried out in order broadly to characterize the second acetate activation system and to distinguish it from other known systems, e.g. the acetate kinase/phosphotransacetylase system and the butyrate/valerate activating system. Basic kinetic parameters were measured using crude extracts and the repression/induction behaviour briefly investigated. The relationship of the activity measured in vitro to the physiological processes measured in incorporation and uptake experiments was considered.

It seems likely that previous attempts to detect an acetate thiokinase in E. coli may have been unsuccessful for a number of reasons:

1. the low specific activity of the enzyme (cf. specific activity of long chain acyl-CoA synthetase),

2. repression of enzyme activity by glucose,

3. the high levels of acetate kinase found in E. coli grown under all conditions interfere with the commonly used hydroxylamine assay. Acetyl phosphate reacts much more readily than acetyl-CoA with hydroxylamine at the pH values used in the assays.

In these studies, an acetate kinase-less mutant, K1HFAc1, with extremely low residual acetate kinase levels was employed. Cells were grown on a glycerol/acetate medium and low acetate concentrations were used in the thiokinase assays. The use of this procedure allowed the detection of a second activating system for acetate in E. coli K12.

Materials and Methods

Ethane methane sulphonate (EMS) was obtained from Kodak Ltd., Kirby, Liverpool, England. β-fluoropropionate was obtained from K & K Chemicals, Hollywood, California, U.S.A.

Organisms The organisms used in this chapter are listed in Table 1.

Enzyme Assays For preparation of cell-free extracts, 600 ml cultures of K1HFAc1 (ACK^-) were grown on 50 mM acetate/50 mM glycerol medium in 2 litre Erlenmeyer flasks. A glycerol-grown inoculum was used and the cultures were shaken overnight in a New Brunswick rotary shaker at 37°C. Stationary phase cells were harvested by centrifugation at 12,000g for 10 minutes in a M.S.E. 18 preparative centrifuge, washed

once with ice-cold, carbon-free basal medium and once with ice-cold sonication buffer (0.1 M Tris-Cl; 10 mM $MgCl_2$; 1 mM sodium EDTA pH 8.0). The cells were finally resuspended in 10 ml of sonication buffer. The cells were sonicated for 2 x 30 seconds with a 30 second interval between each sonication in a beaker suspended in a mixture of ice and water. A M.S.E. 150 watt sonicator was used at an output of 9-10 μ peak to peak. The sonic extract was centrifuged for 1.5 hours at 150,000g in a M.S.E. 50 Superspeed centrifuge to sediment NADH oxidase and remove cell debris.

For studies of enzyme induction and repression, cells were grown in 1 litre Erlenmeyer flasks inoculated after pregrowth on the appropriate carbon source. Carbon sources were added at 50 mM. The washed cells were resuspended in 5 ml of buffer for sonication.

The thiokinase activity recovered with various sonication buffers was tested using the hydroxylamine assay (described below). 0.1 M sodium phosphate buffer pH 6.8 gave a considerably lower activity than 0.1 M Tris-Cl/0.01 $MgCl_2$/0.001 M Na EDTA buffer at pH 7.5 or 8.0. The pH 8.0 buffer gave optimum activity. Studies of the stability of this activity demonstrated that it was more stable at -20°C than at 0°C and that pH 8.0 gave better protection against inactivation than pH 7.5 or 6.8. Cell-free extracts could be stored deep frozen for up to a week in pH 8.0 Tris-Cl without loss of activity.

Three types of assay were used to detect acetate thiokinase in cell-free extracts of K1HFAc1:

1. Measurement of acetyl-hydroxamate formed by reaction of acetyl CoA with hydroxylamine. The absorbance of a ferric-hydroxamate complex

is measured at 520 nm in acid solution. This is a stop assay based on the procedure of Jones and Lipmann (Jones & Lipmann, 1955).

2. Measurement of acetyl-CoA production by a continuous assay with malate dehydrogenase and citrate synthase. In this modification of the Ochoa (1955) method for determining citrate synthase, the reduction of NAD is followed at 340 nm.

3. Polarographic determination of CoA disappearance. The decay in amplitude of the sulfhydryl wave of CoA occurring as acetyl-CoA is formed is followed (Weitzman, 1969).

Attempts to develop convenient radioactive assays were unsuccessful. The measurement of the appearance of absorption at 233 nm due to thiol-ester bond formation was impossible in the presence of the concentrations of ATP required in the assay. This assay was, however, useful in the measurement of phosphotransacetylase activity (Bergmeyer, 1963).

All the successful assays did, however, have disadvantages. The hydroxamate assay is insensitive and thus requires prolonged incubation and a large utilization of the substrates, acetate and ATP, to detect activity. This presents problems if the K_m for a substrate is high. The coupled assay is not stoichiometric at low rates. It suffers from the usual problems of coupled assays (for example, see Bergmeyer, 1965) and residual NADH oxidase presents problems in the measurement of low rates. The polarographic assay is continuous and sensitive and provided a direct measurement of substrate disappearance. The utility of the polarographic measurement of CoA disappearance is, however, limited by the dependence of the

reaction rate on CoA concentration. In order to observe the disappearance of CoA a reasonable proportion of the added CoA must be consumed in the reaction. This presents problems in attempting kinetic work.

Hydroxamate assay The routine assay was carried out as follows. The reaction mixture contained in a volume of 3 ml, 150 µmoles Tris-Cl pH 8.5; approximately 300 µmoles NH_2OH ($NH_2OH.HCl$ neutralised with NH_3 to pH 8.5); 15 µmoles $MgCl_2$; 15 µmoles sodium acetate; 1 µmole CoA; 10 µmoles ATP disodium salt and a quantity of cell-free extract. The reaction was initiated by the addition of ATP. The assay was incubated for 1 h at 37°C. The reaction was terminated by the addition of 3 ml of 2.5% Fe Cl_3 in 10% perchloric acid/2N HCl. Precipitated protein was removed by centrifugation in a bench centrifuge and the absorbance was measured at 520 nm with a Unicam SP600 spectrophotometer within 30 min of completion of the assay. The limits of stoichiometric dependence on enzyme addition and linearity with time were determined. The assay was found to be linearly dependent upon enzyme addition up to a rate of approximately 2 µmoles/h and linear with time over at least 1 h. Lithium acetyl phosphate was used to prepare a standard curve for the assay. Blanks without CoA and ATP were prepared for each set of determinations. The CoA-independent rate is probably a reflection of residual acetate kinase activity and the acetate thiokinase activity dependent on endogenous CoA. For assay of propionate activation, 300 µmoles of sodium propionate were substituted for the sodium acetate. The extinction of propionyl-hydroxamate was assumed to be the same as that for acetyl-hydroxamate.

Spectrophotometric coupled assay This was carried out in essentially the same way as the phosphotransacetylase assay described previously except that acetate and ATP were substituted for acetyl phosphate. 10 µmoles of ATP were used per 1 ml assay.

Polarographic assay Acetate thiokinase was assayed polarographically by following the acetate-dependent CoA disappearance (at -0.3 V versus a saturated calomel electrode measured at a dropping mercury electrode) in a Radiometer PO4 Polariter (Radiometer Ltd., Copenhagen, Denmark). The assays contained in 1 ml, 100 µmoles Tris-Cl pH 8.5; 5 µmoles $MgCl_2$; 10 µmoles ATP disodium salt and varying quantities of acetate and CoA.

EMS Mutagenesis EMS mutagenesis was carried out as follows. Log phase cells growing on nutrient broth were harvested, washed and resuspended in carbon-free basal medium. 2.5 ml of the cell suspension was added to 2.45 ml of basal medium in which 0.05 ml of EMS had been dissolved. The suspension was shaken in the presence of EMS for 1.5 h at 37°C. The cells were then harvested and plated on selective media.

Results and Discussion

Detection of acetate thiokinase activity in cell-free extracts of K1HFAc1 Acetate thiokinase was detected in cell-free extracts prepared from K1HFAc1 (ACK^-) grown on glycerol/acetate medium by all three assays described in the Methods section. The requirements for

Table 7 (a) Requirements for acetate thiokinase in cell-free extracts of glycerol/acetate grown *E. coli* K12 strain K1HFAc1 (ACK^-)

	specific activity
complete	54.3
-acetate	0.8
-CoA	0.8
-ATP	0
$-MgCl_2$	32.1
$-MgCl_2$ + 3.3 mM EDTA	0.8
+ 2-mercaptoethanol 3.3 mM	44.4
-ATP + 3.3 mM GTP	5.7
+ 10 mM potassium chloride	56.2
+ 1.6 mg/ml Triton X-100	53.3

(b) Distribution of acetate thiokinase activity between membranes and supernatant in a cell-free extract prepared from glycerol/acetate-grown strain K1HFAc1 (ACK^-)

	% total activity
membrane fraction	4
supernatant	96

Cell-free extracts were prepared and acetate thiokinase assays carried out as described in Materials and Methods with the exception that 5 mM sodium acetate was used in the assays of membrane and supernatant activity. Specific activity is expressed as nmol acetyl hydroxamate formed/min/mg protein. Membrane and supernatant fractions were prepared as described in the text.

activity were tested using the hydroxamate stop assay. Table 7 shows the results of experiments to determine the dependence of activity on acetate, ATP, CoA and Mg^{2+} and the effect of a number of compounds which might be expected to influence the acetate thiokinase activity. Thus Overath's (1969) work suggested an examination of the effects of 2-mercaptoethanol and Triton X-100 and results obtained with mammalian acetate thiokinase (Webster, 1966) suggested that the effect of K^{+} ions be also tested; similarly, the ability of GTP to replace ATP was also tested. The results indicate a reaction dependence characteristic of an acylate thiokinase. Omission of $MgCl_2$ from the assay ($MgCl_2$ is, however, present in the sonic extract) results in an approximately 40% inhibition of activity. The addition of 3.3 mM EDTA drastically reduces activity to approximately 3% of that found in the control. Thus a strong dependence on divalent metal ions for activity is observed. 2-mercaptoethanol exerts a small inhibitory effect at 3.3 mM whereas Overath found 2-mercaptoethanol to stimulate the activity of acyl-CoA synthetase. Triton X-100 is without effect on either membrane-free or membrane-containing sonic extracts. The acetate activating activity observed in these crude extracts is thus clearly distinguished from the acetate kinase/phosphotransacetylase system by the requirement for CoA to observe formation of acetyl-hydroxamate. Acetate kinase catalyses the CoA independent formation of acetyl phosphate (CoA is without effect on acetate kinase activity under the conditions tested). Acetyl phosphate is considerably more reactive towards hydroxylamine than is acetyl-CoA at the pH used in the assay. Thus no CoA-dependent role for phosphotransacetylase can be envisaged. The saturation curve

Table 8 Effect of pH on acetate thiokinase activity in a cell-free extract prepared from glycerol/acetate-grown strain K1HFAc1 (ACK^-)

pH	specific activity
7.5	17
8.0	35
8.5	59
9.0	36

Cell-free extracts and enzyme assays were carried out as described in Materials and Methods with the exception that Tris-HCl buffers of the above pH's were used and the hydroxylamine-hydrochloride was titrated to the appropriate pH with concentrated ammonia solution. Specific activities are expressed as nmol acetyl hydroxamate/min/mg protein.

of acetyl hydroxamate formation versus enzyme addition for the acetate thiokinase assay reflects the low reactivity of acetyl-CoA towards hydroxylamine. The slight inhibitory effect of 2-mercaptoethanol contrasts with Overath's observation of stimulation of long-chain acyl-CoA synthetase activity as does the lack of effect of Triton X-100.

Effect of pH on acetate thiokinase activity The difference between acetate kinase and the acetate thiokinase activity was further established by a study of the dependence of activity on pH (Table 8). For the hydroxamate assay used here, samples of hydroxylamine hydrochloride were neutralised with concentrated ammonia to the appropriate pH. The measurements were carried out in Tris-Cl buffer. Care was taken to check the stability of the activity under the different pH conditions and to check the assay saturation since the reactivity of acetyl-CoA with hydroxylamine is pH-dependent. The pH optimum of 8.5 differs from that of acetate kinase (7.4) and succinate thiokinase (7.2) (succinate : CoA ligase (ADP) E.C. 6.2.1.5). The value is similar to that obtained by Overath for the long chain fatty acyl-CoA synthetase (Overath, 1969).

Location of acetate thiokinase activity The location of the activity in soluble and membrane fractions was examined. A cell-free extract was prepared by the standard sonication procedure. Cell debris and unbroken cells were removed by centrifugation at 5,000g for 5 minutes in a M.S.E. 18 centrifuge. The membrane fraction was then isolated by a 1 hour spin at 18,000g and resuspended in the initial volume of buffer.

Table 9 Dependence of the rate of acetate thiokinase activity in a cell-free extract prepared from glycerol/acetate-grown strain K1HFAc1 (ACK^-) upon acetate concentration over the range 2.5 to 50 mM

mM sodium acetate	specific activity
2.5	28
5.0	31
10.0	32
25.0	32
50.0	31

Cell-free extract was prepared and acetate thiokinase activity assayed as described in Materials and Methods. Specific activity is expressed as nmol acetyl hydroxamate formed/min/mg protein.

FIG. 13

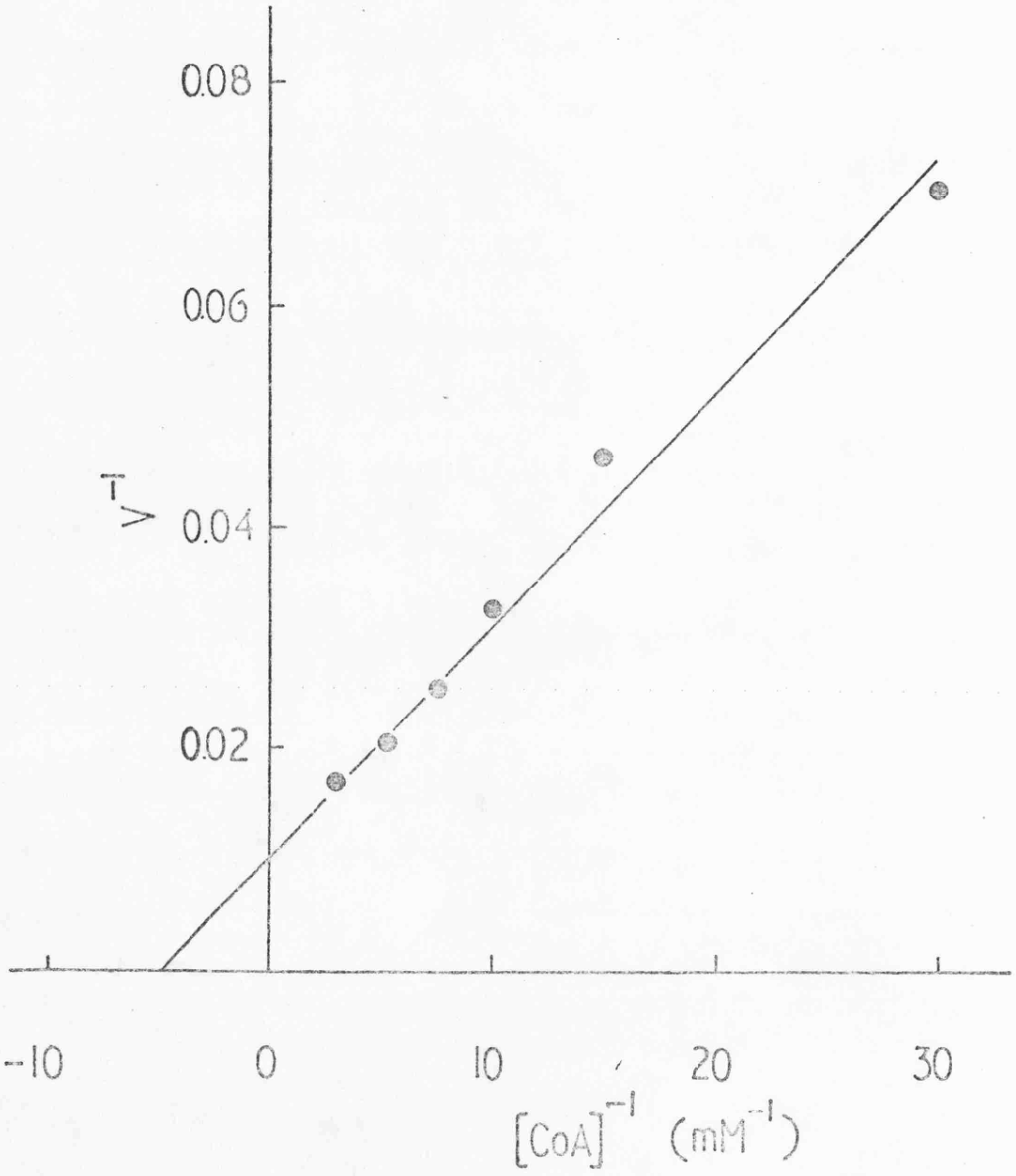


Figure 13

Dependence of rate of acetate thiokinase activity in a cell-free extract prepared from cells of *E. coli* K12 strain K1HFAc1 (ACK^-) grown on glycerol/acetate medium (see Materials and Methods) on CoA concentration. The data are presented in the form of a Lineweaver-Burk plot. The rate of acetyl hydroxamate formation was determined as described in Materials and Methods at various CoA concentrations in the presence of fixed concentrations of sodium acetate (20 mM) and ATP (3.3 mM). v is expressed as nmol acetyl hydroxamate formed/min/mg protein. The assays were carried out at 37°C.

FIG. 14

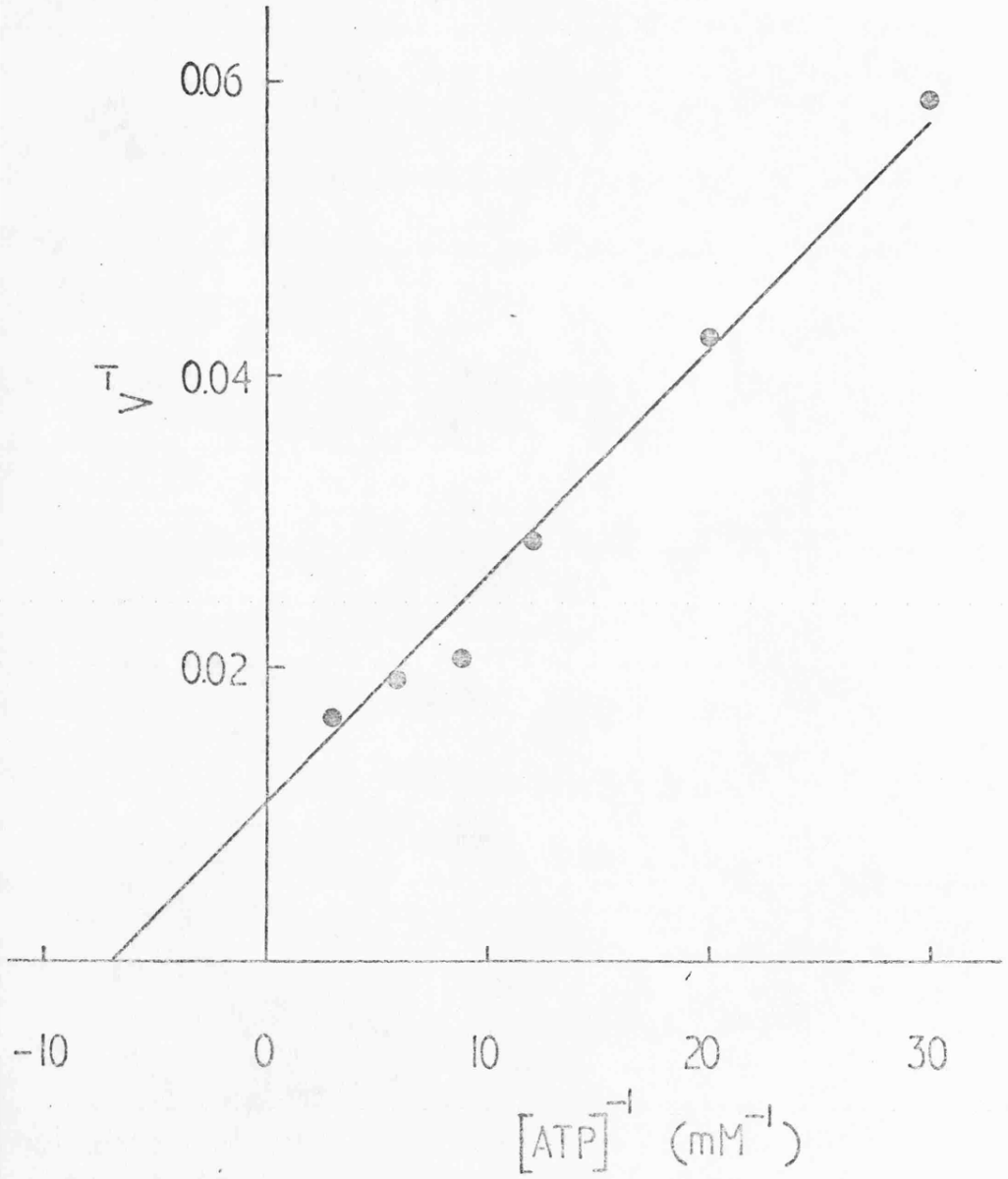


Figure 14

Dependence of rate of acetate thiokinase activity in a cell-free extract prepared from cells of E. coli K12 strain K1HFAc1 (ACK^-) grown on glycerol/acetate medium (see Materials and Methods) on ATP concentration. The data are presented in the form of a Lineweaver-Burk plot. The rate of acetyl hydroxamate formation was determined as described in Materials and Methods at various ATP concentrations in the presence of fixed concentrations of sodium acetate (20 mM) and CoA (0.33 mM). v is expressed as nmol acetyl hydroxamate formed/min/mg protein. The assays were carried out at 37°C.

The percentage of the total activity recovered in the supernatant and membrane fractions is shown in Table 7. The membrane fraction was not washed in order to minimize any loss of possibly loosely-bound enzymes and the residual activity in the membrane fraction may represent contamination with supernatant. Sonication is a vigorous procedure for disrupting cells and it remains possible that the activity might be loosely bound (cf. long chain acyl-CoA synthetase).

Kinetic parameters of acetate thiokinase Apparent K_m values for acetate, CoA and ATP were measured at fixed concentrations of the other two substrates. The relative insensitivity of the hydroxylamine assay presented problems in that a considerable quantity of substrate must be consumed in order to detect activity. This consideration is unimportant in the case of CoA which acts catalytically under these assay conditions, but poses technical problems in the cases of ATP and of acetate. The utility of the assay in estimating kinetic parameters is thus dependent on the values of these parameters since the minimum quantity of substrate usable is of the order of 1 μmole. This restriction allowed estimation of the K_m for ATP, but not for acetate by this method. Thus, within the range of concentrations useable in the hydroxylamine assay, the dependence of rate upon acetate concentration was extremely low (Table 9). Apparent K_m values were, however, obtained for CoA and ATP. The following values were determined. The K_m for CoA at 3.3 mM ATP and 20 mM sodium acetate was 2×10^{-4} M and the V_{max} 100 nmoles.min^{-1}.mg protein^{-1} and the K_m for ATP at 0.3 mM CoA and 20 mM sodium acetate was 1 mM with a V_{max} of 100 nmoles.min^{-1}.mg protein^{-1} (Figures 13 and 14).

FIG. 15

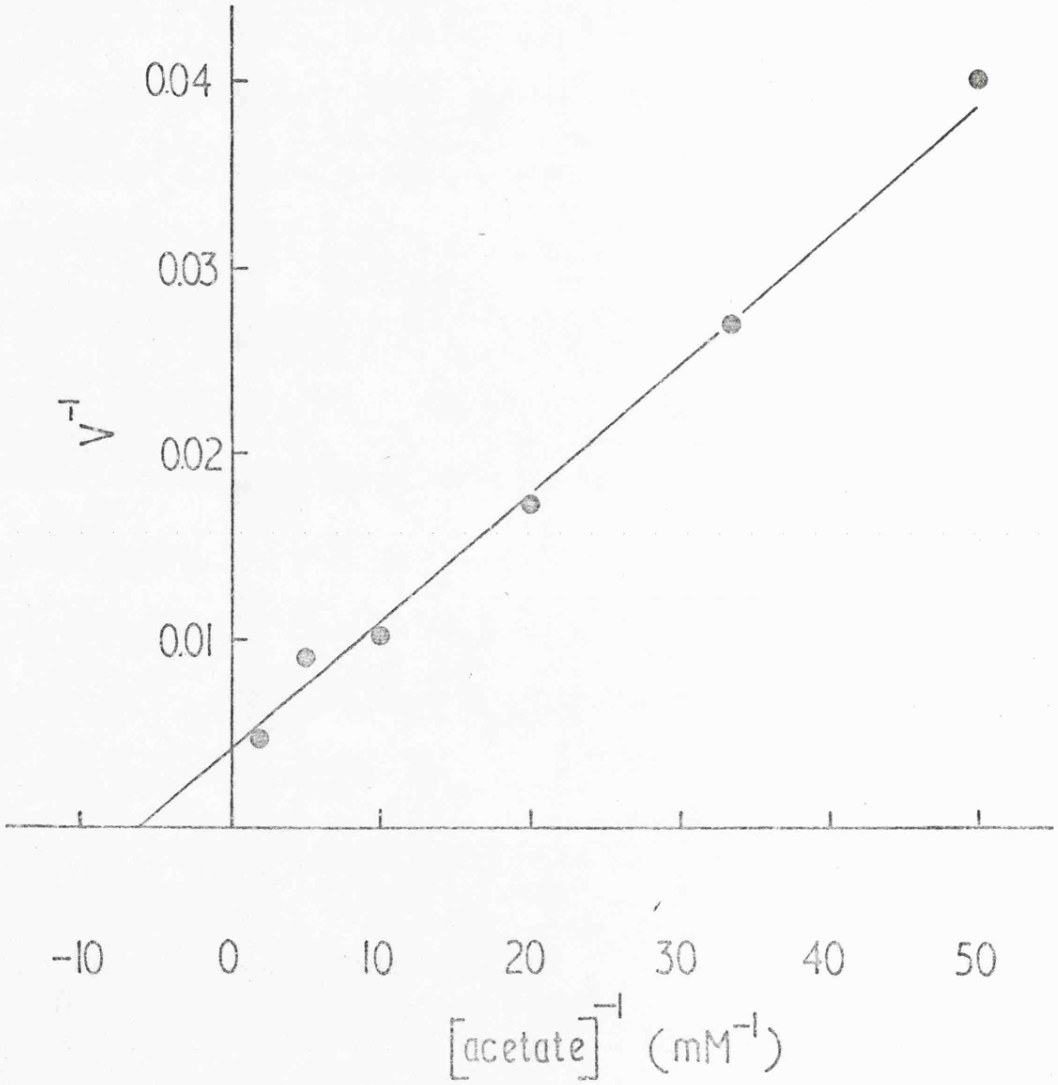


Figure 15

Dependence of rate of acetate thiokinase activity assayed in a cell-free extract prepared from glycerol/acetate-grown cells of strain K1HFAc1 (ACK^-) (see Materials and Methods) on sodium acetate concentration. The data are expressed in the form of a Lineweaver-Burk plot. The rates were measured using the spectrophotometric coupled assay described in Materials and Methods. Various concentrations of sodium acetate were tested at fixed concentrations of ATP (5 mM) and CoA (0.5 mM). v is expressed as nmol NADH formed/min/mg protein. The assays were carried out at 25°C.

One of the primary interests in determining kinetic parameters for this activity was to estimate the apparent K_m and V_{max} values for acetate and compare them with the K_m and V_{max} values obtained for acetate uptake by washed cell suspensions. Accurate determination of values proved impossible largely because of the low K_m for acetate. Attempts were, however, made to use both the spectrophotometric coupled assay and the polarographic assay. The residual NADH oxidase levels in the cell-free extract proved particularly troublesome in attempts to measure rates at low acetate concentrations. The results obtained indicated that the K_m for acetate was low. The data are presented in Figure 15 although they are quantitatively unreliable. Polarographic estimation of CoA disappearance provides a direct measure of reaction rate and thus avoids the problems of the coupled assay. The measurement of substrate disappearance does, however, limit the usefulness of the assay. At low acetate concentrations, since the amount of CoA consumed in the reaction must be a substantial part of the total present, the amount of CoA which can be added is low. Since the K_m for CoA is relatively high, the reaction rate will be linear for only a short period. Initial instability in the polarograph makes it difficult to obtain accurate values for these initial rates. The polarographic results did, however, confirm the low K_m for acetate (this was estimated to be below 10^{-4} M). Thus this acetate thiokinase activity appears to have a low K_m for acetate which would allow it to fulfill the postulated role of acetate activation at low acetate concentrations.

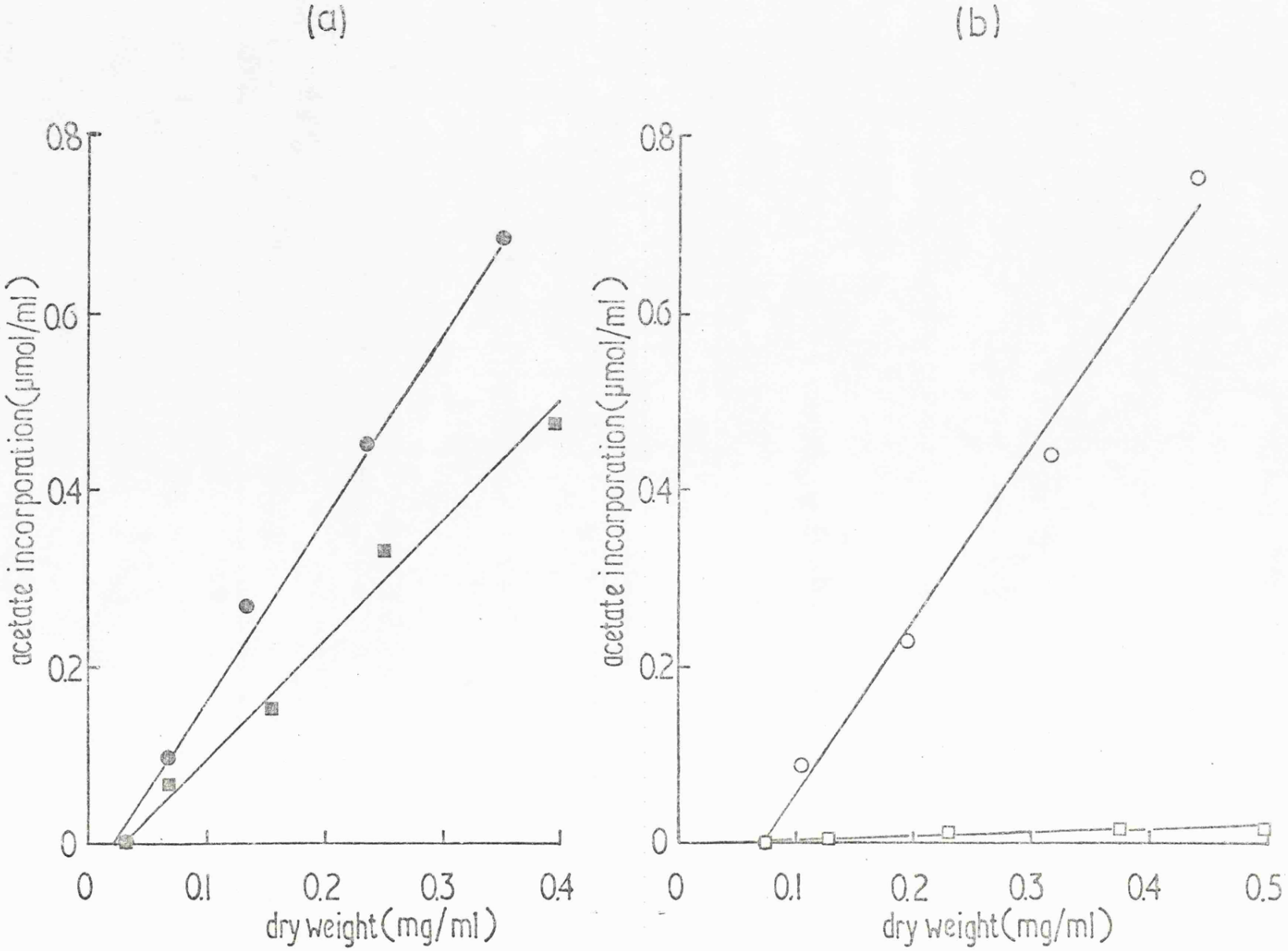

(a)
(b)
acetate incorporation(μmol/ml)
0.8
0.6
0.4
0.2
0
0
0.1
0.2
0.3
0.4
0.5
dry weight(mg/ml)

FIG. 16

Figure 16

The effect of 10 mM sodium pyruvate upon the incorporation of 2 mM sodium [2-^{14}C]acetate by *E. coli* K12 strains K2 and K2HFAc3 (PTA^-) growing on 25 mM glycerol. The strains were pregrown on 25 mM glycerol, harvested, washed and transferred to fresh medium. Incorporation of radioactive label into cell material was measured as described in Materials and Methods (Chapter I). K2 - no addition ● ; + 10 mM sodium pyruvate ■ .
K2HFAc3 - no addition ○ ; + 10 mM sodium pyruvate □ .

Studies of potential effectors of acetate thiokinase Incorporation experiments using growing cultures, and uptake experiments using washed cell suspensions (see Chapter III), suggest that at least two effectors might be involved in controlling acetate activation by the second system. Pyruvate, at concentrations down to 10^{-5} M, is a potent inhibitor of acetate uptake and acetate incorporation is also totally blocked in K2HFAc3 (PTA^-) by 10 mM pyruvate (higher concentrations must be used as pyruvate is consumed during the incorporation experiment) (Figure 16b). The control of acetate utilization by pyruvate (or compounds close to pyruvate) through modulation of the glyoxylate cycle, has been extensively studied (Ashworth & Kornberg, 1963; Kornberg, 1965b). It is therefore interesting to see another potential role for pyruvate in the control of acetate utilization.

Evidence for a possible inhibitory effect of acetyl phosphate upon the second activation system comes from observations of the inhibition of acetate incorporation observed in phosphotransacetylase-less organisms as the acetate concentration is increased (see chapter I). It might be suggested that the inhibition results from production of an elevated level of acetyl phosphate within the cell (this compound cannot be metabolised in the mutant). Exogenous acetyl phosphate is not toxic to E. coli (see chapter I), but this is not surprising since phosphorylated compounds will penetrate the cell membrane only poorly in the absence of a specific transport systems.

In an effort to localise further the site of inhibition, the effects of pyruvate and of acetyl phosphate on acetate thiokinase activity were tested. Pyruvate failed to inhibit activation at

Table 10 Action of potential effectors on acetate thiokinase activity in a cell-free extract prepared from glycerol/acetate-grown strain K1HFAc1 (ACK^-)

	addition	% control activity
pH 8.5	none	100
	10 mM sodium pyruvate	95
	10 mM PEP	110
	10 mM sodium pyrophosphate	66
	10 mM sodium pyrophosphate + 10 mM $MgCl_2$	101
	10 mM sodium phosphate	99
	1 mM ADP	100
	1 mM AMP	83
	1 mM NADH	81
	10 mM glucose 6-phosphate	25
pH 7.0	10 mM sodium pyruvate	90
	10 mM PEP	100

Cell-free extract was prepared and acetate thiokinase activity assayed as described in Materials and Methods with the exception that 50 mM sodium phosphate buffer was substituted for Tris-HCl buffer in the assays carried out at pH 7.0. "% control activity" is the % of the activity observed in the absence of any addition (100%).

10 mM when tested using the hydroxamate assay both at pH 8.5 and pH 7.0. PEP also failed to inhibit under the conditions used. Evidence that pyruvate itself is the inhibitor of the second acetate activation system is presented in chapter III. Acetyl phosphate cannot, of course, be included in the hydroxamate assay and an attempt was made to obtain a qualitative idea of its effect on acetate thiokinase activity by means of the coupled assay; a cell-free extract prepared from a phosphotransacetylase-less strain, K2HFAc3, was used as source of thiokinase. No inhibition of acetate thiokinase activity by acetyl phosphate was observed. The action of a number of other potential effectors was also tested (Table 10).

Glucose 6-phosphate was the only compound to produce a major effect on the acetate thiokinase activity. This inhibition is interesting in view of the observation that glucose and 2-deoxyglucose produce inhibition of acetate uptake in short-term experiments (described in chapter III). None of the other minor inhibitory effects observed is likely to be of significance in the control of the activity. The failure to observe acetyl phosphate inhibition of acetate thiokinase activity suggests that it may produce an effect at some other point in the pathway of acetate metabolism.

In these experiments, pyruvate did not inhibit acetate thiokinase activity; this may have a number of explanations:

1. the acetate thiokinase activity detected *in vitro* may not be involved in acetate activation *in vivo*,
2. its *in vivo* state differs from that *in vitro*, for example, in membrane attachment,

Table 11 (a) Monocarboxylic acid activation by a cell-free extract prepared from glycerol/acetate-grown strain K2HFAc1 (ACK^-)

	specific activity
5 mM sodium acetate	15.8
100 mM sodium formate	0
100 mM sodium glycollate	0.7
100 mM sodium propionate	7.0
100 mM sodium butyrate	0

(b) Ability of monocarboxylic acids to inhibit activation of acetate by a cell-free extract prepared from glycerol/acetate-grown strain K1HFAc1 (ACK^-)

	specific activity
5 mM sodium acetate	15.8
5 mM sodium acetate + 100 mM sodium formate	14.2
5 mM sodium acetate + 100 mM sodium glycollate	14.4
5 mM sodium acetate + 100 mM sodium propionate	12.0
5 mM sodium acetate + 100 mM sodium butyrate	12.5

Cell-free extract was prepared and enzyme assays carried out as described in Materials and Methods except for the modifications described above and the use of 0.22 mM CoA. Specific activity is expressed as nmol acetyl hydroxamate formed/min/mg protein.

FIG.17

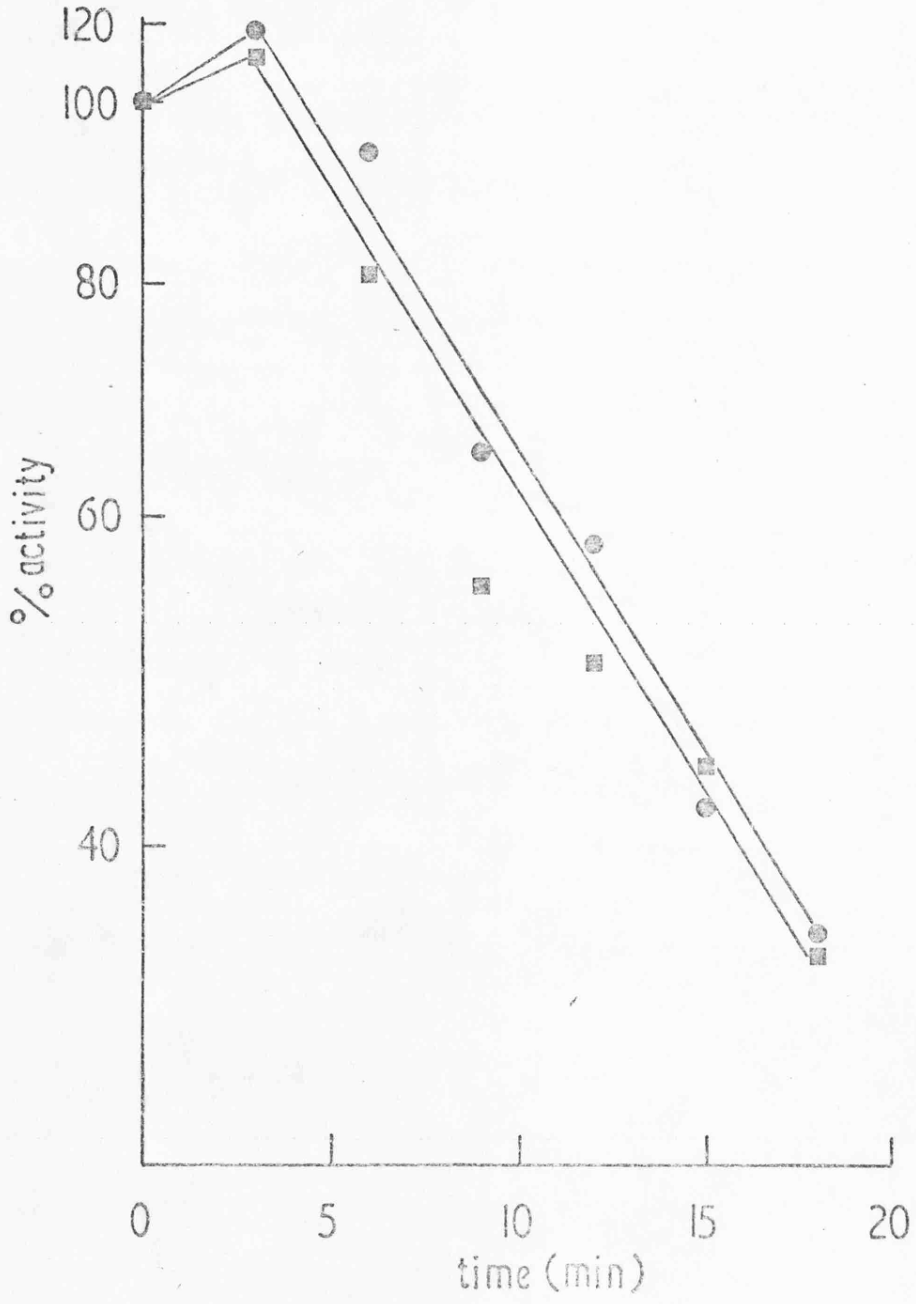


Figure 17

Thermal denaturation at 50°C of acetate and propionate thiokinase activities in a cell-free extract prepared from glycerol/acetate-grown cells of strain K1HFAc1 (ACK^-) (see Materials and Methods). Activities were measured using the hydroxamate assay described in Materials and Methods and are expressed as percentages of the initial activity which remained after a given time. %-acetate thiokinase activity ● ; %-propionate thiokinase activity ■ .

3. the site of the pyruvate inhibition may not be at the level of acetate activation.

This last point can, however, be dealt with. The total inhibition of acetate incorporation caused by pyruvate in K2HFAc3 contrasts with the partial inhibition observed in the parent strain which possesses an intact acetate kinase/phosphotransacetylase system (Figure 16a). The major problem raised by the first possibility can only readily be resolved by the isolation of mutants defective in the second activation system.

The ability of cell-free extracts containing acetate thiokinase activity to activate monocarboxylic acids and the inhibition of acetate activation by potential analogues The ability of an extract of K2HFAc1 (ACK^-) prepared from glycerol/acetate-grown cells to activate a number of short chain monocarboxylic acids was tested (Table 11). A considerable ability to activate propionate was observed and evidence for a limited activation of glycollate was obtained. Butyrate was not significantly activated by this system. The possibility that the propionate activation was not carried out by the same enzyme that carried the acetate activation was tested by a study of the effects of thermal denaturation. Coordinate loss of propionate and acetate activating ability was observed (Figure 17). This suggests that both activations are catalysed by the same enzyme. A role for this enzyme in propionate activation might provide a means of selecting mutants modified in the second acetate activating system.

The ability of a monocarboxylic acid, added at 100 mM, to inhibit

Table 12 Levels of acetate thiokinase activity in cell-free extracts of strain K1HFAc1 (ACK^-) grown on various carbon sources

carbon source	specific activity
25 mM sodium acetate/25 mM glycerol	22
50 mM sodium succinate	7
50 mM glycerol	4
50 mM glucose	1

Cultures were grown, cell-free extracts prepared, and acetate thiokinase activity assayed as described in Materials and Methods with the exceptions that 5 mM sodium acetate and 0.22 mM CoA were used in the assays. Specific activity is expressed as nmol acetyl hydroxamate formed/min/mg protein.

the activation of 5 mM acetate by cell-free extract was also tested (Table 11). There is some evidence of inhibition by all the acids tested, but this might represent an effect of increased ionic strength. It seems likely that propionate inhibits activation since the total hydroxamate formed is considerably less than the sum of the independently measured propionate and acetate activation. These data may be compared with the results of uptake experiments in which inhibition by potential analogues was studied (presented in chapter III).

Coarse control of acetate thiokinase activity The levels of acetate thiokinase in cultures grown on different carbon sources were measured. The results indicated that the activity was induced by acetate and repressed by glucose (Table 12). Succinate failed to induce the activity,which confirms that the activity is not simply a reflection of succinate thiokinase activity which is known to be induced in E. coli by growth on succinate. These coarse control properties suggest a role in acetate activation and contrast with those observed for the acetate kinase/phosphotransacetylase system.

Attempts to select mutants in the second system The data presented above provide only indirect evidence that the acetate activating activity detected in cell-free extracts is involved in the activation of acetate in vivo. While the observations on K_m and V_{max} values, the pattern of inhibition by analogues, and the coarse control properties are in line with in vivo observations, definitive evidence

of the physiological role of the thiokinase requires the isolation of mutants deficient or modified in this activity. A variety of approaches to this problem have been tried but none has proved successful as yet.

The most obvious approach was to vary the conditions of fluoroacetate resistance selection in order to obtain conditions under which toxicity was still observed in mutant deficient in the acetate kinase/phosphotransacetylase system. The original selection used pyruvate as a carbon source to obtain maximum toxicity, but pyruvate appears to inhibit the second system in vivo and it is unlikely that its use would allow selection of mutants in it. It thus seemed possible that if an alternative carbon source to pyruvate on which fluoroacetate was also toxic could be found, then reselection for fluoroacetate resistance of acetate kinase-negative mutants might yield organisms deficient in the second system. It was found that reasonable fluoroacetate toxicity (growth inhibition at 50-100 mM) could be obtained with the wild-type using 25 mM DL-lactate as carbon source, but that acetate kinase-less and phosphotransacetylase-less organisms were resistant to fluoroacetate under these conditions.

The observation that propionate is a substrate for the acetate thiokinase activity in vitro and that lesions in acetate kinase and phosphotransacetylase did not affect ability to incorporate labelled propionate, suggested that selection of propionate-negative strains might result in the isolation of organisms defective in the second acetate activating system. If organisms devoid of acetate kinase or phosphotransacetylase were used, the second system would be the sole

means of acetate activation. The low growth rates of K12 strains of E. coli on propionate make conventional selection with mutagenesis followed by penicillin counter-selection difficult. An alternative approach uses selection for resistance to β-fluoropropionate. This compound was found to be toxic to K1HFAc1 (ACK^-) and K1HFAc3 (PTA^-) at 50 mM on glucose or glycerol. The effect of β-fluoropropionate on Thiobacillus neapolitanus has been reported by Kelly (1970). Organisms resistant to fluoropropionate were isolated after prolonged incubation on glucose and glycerol plates containing 50 mM β-fluoropropionate with both K1HFAc1 and K1HFAc3. These organisms have not yet been adequately characterized, but may well provide information on the poorly understood mechanism of propionate utilization. Preliminary data suggest that they remain capable of acetate and propionate activation.

Information on the physiological role of an enzyme system may be obtained not only by the selection of mutants lacking a function, but also by the selection of mutants derepressed in that function. Two approaches to this end have been employed. The attempt to select a mutant able to grow more rapidly upon acetate than the parent strain K2FAc1 (ACK^-), which grows slowly on acetate, was unsuccessful. No fast growers on acetate were obtained after a number of cycles through acetate minimal medium. Similarly, no fast-growing colonies were detected above the background when acetate kinase-less strains were plated on acetate medium even when the cultures were mutagenized with EMS. $Acetate^+$ revertants of PTA^- strains could be selected: these were found to be PTA^+. This is not surprising since the second acetate

activation system is inhibited at the high acetate concentrations used for the selection.

The possibility that the inability of K2 strains to grow on propionate was due to a failure to activate propionate at a sufficiently rapid rate was investigated. K2 derivatives capable of propionate growth on plates were selected and tested for elevated levels of acetate thiokinase activity. No increase in level was detected in these organisms.

The role of the second acetate activation system in propionate activation by E. coli K12 The metabolism of propionate in E. coli is imperfectly understood. Little work on its mechanism of activation has been carried out and its relationship to acetate activation is unknown. In some organisms, for example, Rhodospirillum rubrum (Eisenberg, 1955) and Prototheca zopfii (Callely & Lloyd, 1964), propionate and acetate are thought to be activated by the same thiokinase. Skinner and Clarke (1968) reported isolation of mutants of Pseudomonas aeruginosa which lacked acetate thiokinase activity. Out of eight mutants isolated, five were propionate-negative, but three grew normally on propionate. The authors concluded that propionate was activated by acetate thiokinase in this organism.

The common identity of acetate and propionate thiokinase activity is suggested by the thermal inactivation data presented in Figure 17. The K_m for propionate was determined using a cell-free extract of K2HFAc1 grown on glycerol/acetate medium. It was found to to relatively high (10^{-2} M) compared to the K_m for acetate (less than

FIG. 18

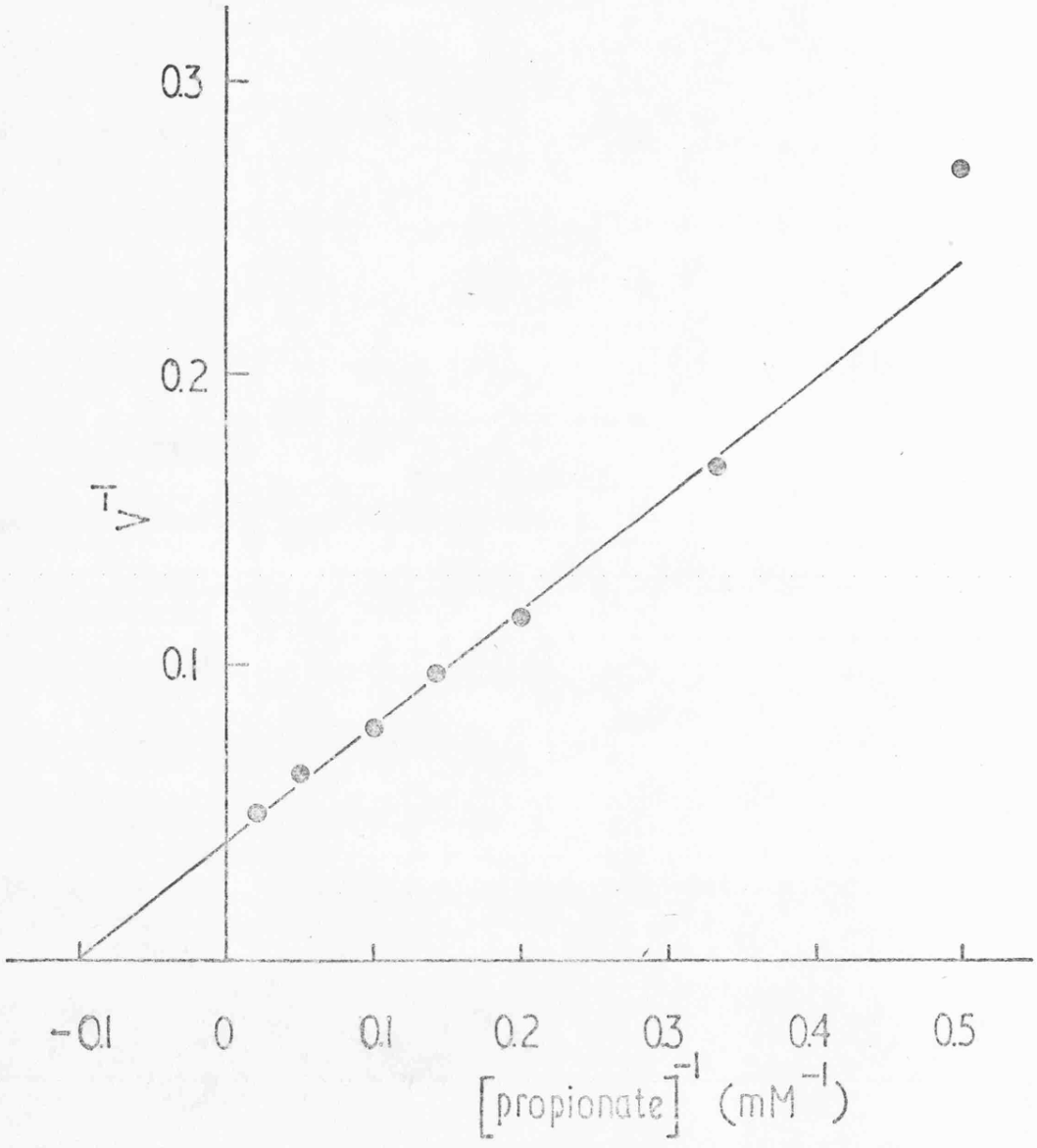


Figure 18

Dependence of rate of propionate thiokinase activity assayed in a cell-free extract prepared from glycerol/acetate-grown cells of strain K1HFAc1 (ACK^-) (see Materials and Methods) on sodium propionate concentration. The rates were determined using the hydroxamate assay described in Materials and Methods. Various concentrations of sodium propionate were tested at fixed concentrations of CoA (0.33 mM) and ATP (3.3 mM). v is expressed as nmol propionyl hydroxamate formed/min/mg protein. The assays were carried out at $37^{\circ}C$.

10^{-4} M) (Figure 18). Studies with acetate kinase-less and phosphotransacetylase-less mutants failed to demonstrate a role for the acetate kinase/phosphotransacetylase system in propionate utilization. The lack of activation by this system is probably due to the ineffectiveness of phosphotransacetylase in metabolising propionyl phosphate (Hibbert et al., 1971), since significant CoA-independent formation of propionyl-hydroxamate was observed in standard propionate thiokinase assays with wild-type cells.

The incorporation data presented in chapter I suggested that propionate was capable of inducing a system required for propionate incorporation in K1HFAc1, an acetate kinase-less strain. The possibility that propionate might be able to induce an activating enzyme was tested by comparing the levels of acetate and propionate thiokinase in K1HFAc1 grown on glycerol or glycerol + 50 mM sodium propionate. No differences in activity were observed. Cells grown on propionate as sole carbon source similarly did not show elevated levels of the activating ability. It seems likely that the propionate thiokinase activity is a reflection of a system which normally activates acetate. This is indicated by the high K_m for propionate compared to acetate and the failure of propionate to induce the activity to any degree.

The data presented in chapter I demonstrate a role for the acetate kinase/phosphotransacetylase system in acetate excretion and support the existence of a second acetate activation system in E. coli K12. The data presented in this chapter were gathered in an attempt to illuminate the properties of the second system with in vitro studies. The results may be summed up as follows. An acetate activating system

with the properties of an acylate thiokinase which differs from previously reported fatty acid activating systems in E. coli has been demonstrated in cell-free extracts of E. coli K12 strains. Its primary specificity appears to be for acetate and a number of the properties of this activity are consistent with a role in acetate activation under certain conditions (it might be of particular importance when the acetate concentration in the medium was low). In the absence of mutants devoid of the activity its physiological role cannot be firmly established. The failure to observe inhibition of the activity by pyruvate in vitro clearly raises questions about the relationship of the activity measured in vitro to that suggested by the in vivo experiments. Control of the system by pyruvate would be teleologically consistent with observations on the control of the glyoxylate shunt.

It is likely that the second acetate activating system is involved in propionate activation. Taken together, the findings that the acetate kinase/phosphotransacetylase system and the butyrate system have no role in propionate activation, the case for another system capable of short-chain fatty acid activation is strong. While the acetate activating system described above has properties consistent with observations on acetate and propionate metabolism in E. coli, final proof of its role will probably await the isolation of mutants defective in propionate activation.

CHAPTER III

Carboxylic Acid Permeation in E. coli

Introduction

In the course of my work on the mechanism of acetate permeation in E. coli K12 it became apparent to me that, in certain studies of monocarboxylic acid permeation, too little attention has been paid to meeting the experimental criteria required to define the existence of specific transport systems: the possibility exists that some monocarboxylic acids may enter the cell in the absence of specific transport systems. In order to clarify this situation, I have attempted to review this field in the introduction to this chapter, and, particularly, to evaluate the experimental criteria required for establishing the existence of bacterial transport systems. The possibility that simple diffusion might be involved in monocarboxylate permeation is considered against the background of data obtained from studies of mitochondrial permeation.

Theoretical considérations Biological membranes have many functions, but perhaps the most obvious is to provide a permeability barrier which separates living cells from their external environment. Their selective permeability properties thus control the composition of the internal environment. Since the formulation of the membrane theory of control of cell permeability, much work has been carried out to elucidate the relationship of structure to function in membranes.

The basic concept of membrane structure advanced by Danielli and Davson in 1935 has remained central to thinking about membrane structure up to the present time (Danielli & Davson, 1935). They pictured biological membranes as consisting of bimolecular phospholipid layers with protein adsorbed on their surfaces. While many of the basic properties of a wide range of biological membranes can be explained in terms of refinements of such a model, there are large quantitative variations in certain membrane properties, for example, permeability. Large differences in protein/lipid ratios, phospholipid and sterol composition have been observed in studies on myelin, erythrocyte membranes, inner and outer mitochondrial membranes and bacterial membrane, but the relationship of these differences to functional differences is imperfectly understood (Korn, 1969).

In general, three classes of mechanism by which substances can cross membranes are distinguished:

1. simple diffusion,
2. facilitated diffusion,
3. active transport.

Simple diffusion occurs in the absence of any specific transport system. In the two extreme models of diffusion across biological membranes, molecules are considered either to dissolve in the bimolecular lipid layer or to move through water-filled "pores". Facilitated diffusion involves the operation of a specific transport system in the membrane; movement of the transported compound will be to dissipate an existing electrochemical gradient. Active transport involves the specific transport of a compound against an electrochemical gradient, which requires an input of free energy. Both facilitated

and active transport are generally considered to involve highly specific interactions of the permeant with membrane proteins (Stein, 1967).

The intrinsic permeability, (i.e. the permeability in the absence of specific transport systems), of a number of membrane systems has been studied in some detail. Important differences have been revealed. Thus the red cell membrane is intrinsically very permeable to small anions and lipid-soluble monocarboxylic acids and is less permeable to larger anions and dicarboxylic acids (Whittam, 1964). These general features are also found to hold for skeletal muscle, nerve and certain plant cells. The inner mitochondrial membrane on the other hand is intrinsically impermeable to all anions and cations (Chappell, 1968; Mitchell & Moyle, 1969). An even more dramatic difference is found between the permeability properties of the inner and outer mitochondrial membranes. Under the conditions normally used for study the outer membrane does not act as a permeability barrier to low molecular weight compounds (Klingenberg, 1970). There is no reason to believe that all membranes have closely related structures and it seems likely that the structural variations observed are reflected in differences in permeability properties.

A number of factors will be important in determining whether a given acid can cross a membrane by simple diffusion. Firstly, the structure of the membrane would seem to be important as indicated by the observed variations in intrinsic permeability of different membranes. Stein (1967) has summarised the structural features of a permeant molecule which are likely to control its diffusion across a

classical Danielli-Davson membrane. Three factors are considered to be important:

1. size,
2. hydrogen bonding capacity,
3. unshielded $-CH_2-$ groups.

Both increased size and hydrogen bonding ability will reduce the rate of diffusion across a membrane while the presence of unscreened aliphatic chains will increase lipid solubility and enhance the rate of diffusion across the hydrophobic region of the membrane. In cases where only the free acid can cross the membrane, the pH of the medium and the pK of the acid will have crucial roles in controlling the rate of permeation. Thus the requirement for specific anion transporters for di- and tricarboxylic acids, but not monocarboxylic acids in mitochondria has been ascribed to the difference between the ratio of anion to free acid at neutral pH. Thus between the homologues acetate (pK 4.86) and succinate (pKs 4.19 and 5.48) there is a difference in this ratio of 10^3. It is, however, likely that other factors must contribute. Thus the permeation of model membranes by trichloroacetate, which has an extremely low pK (0.7), is rapid (comparable with that of monochloroacetate, pK 2.86), even though the ratio of free acid to anion will differ by at least a factor of 10^2. In physiological situations, a large difference between the intrinsic permeability of membranes to acetate (pK 4.86) and pyruvate (pK 2.48) might be expected on the basis of the free acid permeation model. A difference is observed in model studies, but this is not as large as might be predicted (McGivan, 1968). Considerations of the kind

outlined above are important in a preliminary assessment of likely permeation mechanisms.

Carboxylic acid permeation in mitochondrial and model systems In a discussion of likely mechanisms of carboxylate permeation in E. coli, the most useful available comparison is with the properties of the inner mitochondrial membrane. This membrane has a number of similarities with bacterial membranes. The presence of cardiolipin as a major phospholipid may be important in determining the intrinsic permeability properties of the membrane. Thus smectic mesophases of lecithin and cardiolipin are relatively impermeable to small anions when compared with smectic mesophases of lecithin and diacetyl phosphate (McGivan, 1968). Extremely low intrinsic permeability to small ions seems to be a feature of the inner mitochondrial membrane. A requirement for extremely low permeability is presumably associated with the role of the membrane in energy conservation. Many bacterial membranes have similar roles to that of the inner mitochondrial membrane in energy transduction.

The permeation of mitochondria by carboxylic acids has been extensively studied by Chappell's group (Chappell, 1968). The most widely used technique has been that of isotonic swelling in which a semi-quantitative measure of permeability is obtained by following the rate of light scattering changes due to swelling of the mitochondria in ammonium salts of the acids (Chappell et al., 1968). This suffers from the disadvantage that the concentration of permeant to which the mitochondrion is exposed is necessarily high. Studies of permeation

at low concentrations to obtain accurate kinetic data present technical problems and have been used only to provide confirmation of results obtained using isotonic swelling or other indirect techniques in a limited number of cases. There thus exists the possibility that,in some cases, the mechanisms operating at high concentrations might differ from those operating at lower, more "physiological", concentrations. Kinetic data obtained for dicarboxylate permeation using the inhibitor stop technique has, however, amply confirmed the earlier semi-quantitative analysis (Palmieri et al., 1971).

Considerable success has been achieved in defining the anion permeation systems operating in mitochondria. Monocarboxylates appear to cross the membrane as the free acids by simple diffusion under the conditions studied. There remains some question about the existence of a specific transporter for pyruvate. Thus Papa et al. (1971) have presented evidence for a saturable pyruvate permeation mechanism in rat liver mitochondria and Hansford (1972) has commented upon the anomalous nature of pyruvate transport in blowfly flight-muscle mitochondria. Lardy's group has, however, challenged the interpretation of Papa's data and suggest that they can be explained in terms of adsorption rather than transport (Zahlten et al., 1972). Di- and tricarboxylates are transported by exchange diffusion carriers (antiporters) (reviewed by Chappell, 1968). Thus, the dicarboxylate carrier exchanges dicarboxylate anion for dicarboxylate anion or phosphate and the tricarboxylate carrier exchanges tricarboxylate anion for dicarboxylate anion.

Experiments with model membranes also support the idea that

monocarboxylates can cross membranes by simple diffusion as free acids, whereas di- and tricarboxylates cannot (McGivan, 1968). In the case of both mitochondria and model systems, there is a relationship between the rate of swelling in isotonic solutions of ammonium salts and the pK of the monocarboxylate. To summarize, in the case of mitochondria there is much evidence to support the idea that, at least at high concentrations, most monocarboxylates cross the membrane as free acids by a non-specific diffusion process, whereas di- and tricarboxylates are transported by specific systems. Some doubt remains about the mechanism of pyruvate permeation. This situation must by contrasted with that described for E. coli and other bacteria.

Carboxylic acid permeation in bacteria E. coli is capable of growth on a range of carboxylic acids and there is evidence that the movement of at least some of these acids across the cell membrane requires the operation of specific transport systems. The criteria used by workers studying bacterial permeation merit detailed attention. The studies can be placed in two broad groups:

1. those in which direct evidence for the existence of a specific transport system was presented,
2. those in which its existence was postulated on the basis of less direct evidence.

Establishing direct evidence for the existence of a transport system involves studying the uptake of a compound isolated from further metabolism. The separation of movement across the membrane from subsequent metabolism is frequently achieved in bacterial studies by the isolation of mutants with appropriate metabolic lesions. In

certain cases in which a compound has many possible metabolic fates, e.g. pyruvate, this may not be possible. The use of specific inhibitors to inhibit further metabolism is also possible under certain circumstances. This approach was used in studies of citrate transport by Lawford and Williams (1971). Another approach to the isolation of transport from metabolism depends on the use of analogues. Successful use will depend on the transport step having a different specificity from that of subsequent enzymic steps.

Having succeeded in isolating transport from metabolic utilization certain properties of the transport process can be examined. The most commonly examined properties are:

1. dependence of rate of penetration on concentration. The rate is not expected to be directly proportional to concentration, but to reach a limiting (saturation) value as the concentration is increased in the case of specifically-mediated transport,
2. effect of structural analogues on permeation rates. Analogues may be expected to compete with permeants if specific transport systems operate,
3. effect of protein reagents on the rate of permeation,
4. study of counter-transport, in which movement of a structurally related compound is driven against an electrochemical gradient by facilitated movement down an electrochemical gradient of the permeant.

An important criterion available principally in investigations of bacterial and fungal studies is the isolation of mutants specifically defective in the transport of a compound. The isolated mutant must be shown to be modified only in permeation. Isolation of mutants which

are simply defective in the uptake of a labelled compound can be misleading. Thus, in diffusion or facilitated diffusion systems in which there is no accumulation of transported material within the cell, certain metabolic lesions will result in the appearance of defective transport. Isolation of transport mutants does not in itself establish that the rate-limiting process measured under certain conditions of uptake is occurring at the membrane level. Other criteria may be required to establish this.

A number of indirect criteria used in the study of carboxylic acid permeation will be discussed in detail in view of the doubtful utility of some of them. The observation of saturation kinetics for overall uptake of radioactively labelled permeant into cell material by washed cell suspensions has been extensively used as evidence to support the existence of specific transport systems (e.g. Klein *et al.*, 1971; Salanitro & Wegener, 1971b; Vanderwinkel *et al.*, 1971; Wagner *et al.*, 1972). The possibility that the process being measured in this type of experiment does not represent a rate-limiting membrane step must be considered very seriously, particularly in the case of compounds which might be suspected to cross membranes by simple diffusion. At a more sophisticated level, the kinetic parameters for the uptake process have been compared with those of the initial enzyme of the pathway for utilization of the compound (Klein *et al.*, 1971). Overath's group has used evidence of this type to support the existence of a permeation system for long chain fatty acids in *E. coli* K12. The possibility that the *in vivo* properties of the enzymes will differ from those which can be measured *in vitro* remains a problem. This difficulty is particularly acute in the case of

membrane-bound enzymes (cf. Overath et al., 1969). The use of competition data is also inadequate unless the target of the inhibition is clearly defined. In this case, a distinction between the properties of competition at the permeation step and at the initial metabolic step is likely to be justified as only these two processes will have closely related specificity properties. However, in the absence of an attempt to study both processes the use of competition data to support the existence of transport systems is not justified. A number of other approaches to the study of transport systems in which the fate of added label is inadequately defined may be cited. The observation of coarse control of uptake may reflect changes in the levels of metabolic enzymes rather than in a transport system. Similarly, the action of inhibitors on uptake may reflect effects on requirements for energy to catalyse flux through a metabolic pathway or interfere with the pooling of a compound remote from that with which the cells are challenged rather than a direct effect on membrane permeation. With adequate controls this approach can yield useful data. Knowles and Smith (1971) have used inhibitors and uncouplers of energy metabolism in studies of lactate uptake by Azotobacter vinelandii. The effect of these compounds on the oxidation of DL-lactate by whole cells was compared with the effect in cell-free extracts. Uncouplers had a powerful inhibitory effect on oxidation in whole cells, but not in cell-free extracts. This suggests that the uncouplers can affect a membrane transport process, although alternative explanations might be entertained. The use of osmotic shock effects on uptake as a criterion for the existence of a membrane

transport process can be criticised in cases where the fate of the label is not adequately defined (Salanitro & Wegener, 1971b). Thus osmotic shock is known to have a number of effects besides release of binding proteins (Neu & Heppel, 1965; Heppel, 1969). Release of soluble components, such as nucleotide pools, from the cell might be expected to interfere with metabolic activity. Thus in a facilitated diffusion system, the removal of labelled compound from the medium might be inhibited indirectly and this would be reflected in the observed rate of uptake.

A more successful criterion for establishing the existence of a transport system was used in many early studies (Campbell & Stokes, 1951; Kogut & Podoski, 1953; Shilo & Stanier, 1957; Clarke & Meadow, 1959). It depends on establishing that the failure of an organism to utilize a given compound is caused by a lack of permeability towards it rather than the lack of a capacity to metabolize it. Many early studies of the transport of TCA cycle intermediates depended on this method. Recently, methods have become available for specifically modifying membrane properties and this approach may be used to test the possibility that the process being measured in simple uptake experiments detecting total label appearance in the cell is rate-limited by a membrane process. A number of approaches may be used (reviewed by Cronan & Vagelos, 1972). The effect of phospholipid phase changes on transport activity has been studied. Arrhenius plots for the transport of glucosides and galactosides are biphasic with the break in the curve corresponding to <u>in vitro</u> X-ray scattering observations of changes in the order of phospholipid bilayers. This finding has

many applications particularly if the lipid composition of the membrane is manipulated by using mutants defective in the synthesis and degradation of fatty acids. Thus Overath et al. (1970) have tested the effect of temperature on the uptake of glycerol and found no break in the temperature profile. This indicates that permeation is not the rate-limiting step in glycerol uptake under the conditions tested. The existence of a specific transport system has been suggested, but the equilibration of glycerol across the membrane is known to be extremely rapid (Sanno et al., 1968). Another approach to the detection of membrane effects on transport is to study the effect of pleiotropic membrane lesions on uptake. Von Meyenburg (1971) has isolated mutants of E. coli B/r which require increased nutrient concentrations for half-maximal growth rate. This requirement is associated with a pleiotropic effect on transport affinities. It is thus likely that compounds whose uptake is modified by lesions of this type will be dependent upon specific membrane transport for their utilization.

When publications on carboxylic acid transport in bacteria are considered it is obvious that most of the direct evidence for carboxylic acid transport systems comes from work on di- and tricarboxylic acid utilization. There are, however, two reports which directly confirm the existence of monocarboxylate transport systems in E. coli. A problem with studies of carboxylate transport in E. coli is that the acids do not appear to accumulate to any great extent within the cell. Thus the isolation of mutants devoid of specific transport activity

provides the most satisfactory method of defining the existence of a transport system. Monocarboxylic acids may well be capable of non-specific penetration of the cell membrane at the high concentrations normally used for growth studies and mutant selection. Thus the isolation of mutants by direct methods of mutagenesis, enrichment and plating on selective media may not work as satisfactorily as in many other cases. Although there are a relatively large number of reports of transport systems for di- and tricarboxylic acids in bacteria, there are few of monocarboxylic acid transport systems. This is due to the difficulties in defining the parameters of monocarboxylate transport. It is interesting that this situation contrasts with that found with studies of carboxylate permeation in mitochondria.

Evidence has been provided for the existence of transport systems for TCA cycle intermediates in a variety of bacterial species. Many early studies of Pseudomonas spp. using crypticity method demonstrated the existence of di- and tricarboxylic acid transport systems (Campbell & Stokes, 1951; Kogut & Podoski, 1953; Clarke & Meadow, 1959). Shilo and Stanier (1957) demonstrated tartrate permeases in Pseudomonas spp. More recently, studies of citrate transport in two Pseudomonas species by Lawford and Williams (1971) who used fluorocitrate to reduce oxidation of citrate and thus isolate entry from further metabolism. They obtained evidence for accumulation of label which was shown to be present entirely as citrate. As well as characterising citrate transport in Pseudomonas fluorescens, Lawford and Williams also isolated a Pseudomonas species capable of growth on tricarballylic acid. They

examined the exchange properties of tricarboxylate transport in this organism. No evidence of an antiporter mechanism related to that found in the inner mitochondrial membrane was obtained. Studies of citrate transport in Aerobacter aerogenes have been reported (Villareal-Moguel & Ruiz-Herrera, 1969; Wilkerson & Eagon, 1971). Specific transport systems were identified, but there is a disagreement in the data on pooling of citrate. Uncouplers inhibited uptake. Willecke and Pardee (1971) characterized citrate transport in the Gram-negative bacterium Bacillus subtilis. Aconitase-negative mutants were used to isolate transport from metabolism. The system was inducible and subject to glucose repression. The system was capable of concentrating citrate 100 times and could maintain an internal citrate concentration of 86 mM.

Dicarboxylate accumulation by B. subtilis is similarly characterized by high pool levels. 132-fold accumulation was found in a succinate dehydrogenase-negative strain (Ghei & Kay, 1972). Kay and Kornberg (1969; 1971) examined the dicarboxylic acid transport system in E. coli K12. It was found to be highly specific, C_4-acids other than succinate, malate, fumarate, aspartate and maleate were taken up poorly. Mutants devoid of the ability to transport C_4-acids were isolated and mapped. The study of mutants with lesions in TCA cycle enzymes revealed that there is very little accumulation of labelled material in the absence of further metabolism. Studies of dicarboxylate transport have also been reported for Azotobacter vinelandii and a Streptococcus sp. (Repaske et al., 1960; London & Meyer, 1970).

Direct evidence for the existence of specific systems for

monocarboxylate permeation is much more limited. Only two definitive reports are available at the present time. Kornberg and Smith (1967) have reported the isolation of mutants of E. coli K12 specifically defective in pyruvate transport (USP^-). No effect of the lesion on the uptake of other α-oxoacids was observed. The mutants were isolated by a resistance technique. A PEP-synthase-less strain was used as the parent organism and mutants capable of rapid growth on acetate/pyruvate medium were selected. A number of these were found to be defective in pyruvate transport. Selection for resistance to pyruvate rather than the inability to grow on pyruvate is probably important in ensuring isolation of the mutants. It is perhaps significant that the pK of pyruvate is low (2.48). No further data on the properties of pyruvate transport were presented. Ornston and Ornston (1970) have reported the properties of glycollate uptake by mutant strains of E. coli K12. They isolated one mutant which had lost the ability to oxidise glycollate, one which was impermeable to the compound and a third which had undergone a loss in permeability and which was in addition defective in either glyoxylate reductase or malate synthase G. Results from the mutant blocked in glycollate oxidation suggested a six-fold accumulation of intracellular glycollate, but the nature of the pool material was not determined. Glycollate transport seems not to be induced by internal glycollate since the initial rate of glycollate uptake in the glycollate oxidation mutant is only about 2.5% of the induced rate in the wild-type. It is interesting that glycollate is the inducer of malate synthase G, but not of its own transport system. All studies of labelled glycollate uptake were carried out in the presence

of 10 mM unlabelled acetate. Acetate is a potential analogue of glycollate.

The only other data which provide direct evidence for transport systems for monocarboxylates come from studies of phenyl-substituted monocarboxylates. Cook and Fewson (1972) have presented preliminary data upon the transport of benzoate and mandelate by a bacterial strain, N.C.I.B. 8250. Various degrees of permeability barrier to mandelate were observed after growth under conditions which did not induce the enzymes for mandelate oxidation. No mandelate pool was identified in wild-type organisms induced for mandelate utilization, but, under non-inducing conditions, or in mutants lacking L-mandelate dehydrogenase, the label in the cells could be identified as mandelate. With the mutant lacking L-mandelate dehydrogenase grown in the presence of phenylglyoxylate the equilibration of mandelate across the membrane was rapid. No permeability barrier to benzoate could be detected even in uninduced cells but evidence for the operation of a facilitated diffusion system was obtained from exit counterflow experiments. Thus addition of benzoate to cells containing salicylate caused an efflux of salicylate against a concentration gradient. Benzoate also competitively prevented the appearance of 4-fluorobenzoate inside the cell. Thus, in spite of indications that permeation is extremely rapid, it is suggested that benzoic acid (as the free acid a rather hydrophobic molecule) penetrates the cell membrane by facilitated rather than simple diffusion. This case might be analogous to that suggested by Lin for glycerol permeation in E. coli (Sanno et al., 1967). Conflicting evidence on the nature of mandelate permeation in Pseudomonas putida

has been presented. Hegeman (1966) found no evidence for a mandelate transport system in Ps. putida in a study of rates of equilibration in induced and uninduced cells. The $T_{\frac{1}{2}}$ for equilibration was less than five seconds at 30°C. Recently, Higgins and Mandelstam (1972) have described some strange features of mandelate transport in the same organism. They also found equilibration irrespective of the state of induction of the systems for mandelate utilization, but found that the six-fold accumulation of free mandelate was observed only in induced cells.

Many more studies which claim to have examined transport of monocarboxylates indirectly have been reported. Frequently, these have used the terms uptake and transport interchangeably and a similar confusion has arisen in the interpretation of the data presented, but with suitable controls indirect methods will provide satisfactory evidence for the existence of a transport system.

Clarke and Meadow (1959) studied the ability of washed suspensions and cell-free extracts of Pseudomonas aeruginosa to oxidise acetate after growth on malate. A lag in oxidative ability was observed in the case of washed suspensions but not in the crude extracts. The lag observed was short (approximately 10 minutes). The observation of a lag is interpreted as indicating that the cell already contains a small amount of the transport system but the amount is so low as to make the entry rate-limiting. The possibility that oxidation in whole cells may be controlled by the presence of the glyoxylate shunt and that its induction may modify oxidation rates is not considered. Kogut and Podoski (1953) found similar indications of a specific acetate transport system in Pseudomonas spp. using this crypticity technique.

Knowles and Smith (1971) also used a crypticity approach which provided evidence for a lactate transport system in Azotobacter vinelandii.

Two general studies of carboxylic acid permeation in E. coli which touch on the problem of monocarboxylate permeation have been reported. Both have used the plasmolysis technique which necessitates the use of high concentrations of permeant. Bovell, Packer and Helgerson (1963) used light scattering techniques to follow equilibration of organic compounds across the cell membrane of E. coli B. Sodium formate equilibrated across the membrane rapidly. No evidence on whether this equilibration was mediated by a specific system was presented. Plasmolysis has also been used by Mitchell and Moyle (1956a; 1956b) to follow permeation. They examined the effect of high concentrations of the salts of monocarboxylic acids on E. coli. Sodium acetate was found to penetrate the cells only slowly, while ammonium acetate penetrated rapidly particularly at lower pH. The ability of ammonium acetate to plasmolyse the cells varied from culture to culture.

A number of studies on the uptake/transport of aliphatic monocarboxylic acids has recently been carried out and in some cases the authors' interpretation of the data presented in their papers is open to question. Wagner et al. (1972) have studied labelled acetate uptake by washed cell suspensions of E. coli W: indeed, theirs are the only data reported on acetate uptake in E. coli at the present time. A number of properties of the uptake system were reported. Biphasic saturation kinetics for labelled acetate uptake were observed. The K_m for the first phase of uptake was found to be 6.0 μM and that for the

second phase found to be 0.74 mM. Competitive inhibition of uptake by propionate, but not butyrate, was observed. Prior growth on glucose repressed the ability of washed cell suspensions to take up labelled acetate. No pooling of acetate within the cell was detected. Although these data thus describe some of the factors governing the entry of acetate into metabolic pathways, they fail to provide evidence about the nature of the acetate permeation process *per se*. The major problem is that in none of the experiments reported can the transport of acetate be dissociated from its subsequent metabolic fate.

Two recent studies have described the properties of uptake systems for short chain fatty acids in *E. coli* K12. Salanitro and Wegener (1971b) have presented some evidence for the existence of specific transport systems. They have isolated mutants capable of growth on butyrate and valerate (see Chapter II). They suggest that the ability to grow on these acids is dependent on the introduction of a mutation which permits entry of short chain acids. Two classes of mutant were obtained; one takes up short chain fatty acids constitutively while the other shows inducible behaviour. The nature of these lesions is not adequately clarified. A hypothesis is proposed in which the uptake system is a complex, synthesis of which is controlled by a regulatory gene and which consists of two components: (1) a diffusion component and (2) an activation component or components. The diffusion component is required for the permeation of the fatty acid anion at pH 7, but not for penetration of the undissociated acid at pH 5. It therefore seems likely that a specific or facilitated diffusion component is being invoked but the bulk of Salanitro and Wegener's evidence fails to illuminate their hypothesis. Demonstration of saturation kinetics, effect of osmotic shock and competition data

are all capable of equivocal interpretation. Evidence of specific permeation mechanisms is provided by the pH-dependence of equilibration across the membrane. There is apparent rapid equilibration at pH 5 but not at pH 7. This is interpreted in terms of a high intrinsic permeability to the free acid, but not the anion. Permeation of the anion thus requires the presence of a specific system. It is unfortunate that the labelled material present inside the cell in these equilibration experiments is not characterized. Similar evidence comes from the pH-dependence of pentenoate growth inhibition in the wild-type organism. An interesting observation is that non-specific uptake at pH 7 increases with chain length when medium chain fatty acid uptake is examined. Thus, while some evidence for an uptake system is presented, claims that the acids are concentrated by the cell are not justified and many observations areequivocal. The work of Vanderwinkel *et al.* (1971) similarly demonstrates a saturable system for the uptake of short chain fatty acids in *E. coli* K12. The existence of a specific entry system is assumed.

Overath's group has reported the properties of a transport system for long chain fatty acids in *E. coli* K12. The system is reported to be inducible under the same conditions required for the induction of the enzymes of fatty acid degradation and to have a chain length specificity similar to that of the acyl-CoA synthetase. Various lesions in fatty acid degradation were found to reduce the rate of uptake. Thus mutants lacking acyl-CoA synthetase were unable to take up fatty acids to any significant extent. The evidence for the existence of a specific fatty acid transport system is that fatty

acid uptake shows saturation kinetics with kinetic parameters differing from those of the acyl-CoA synthetase. It is suggested by Klein et al. (1971) that the acyl-CoA synthetase may be vectorially involved in fatty acid transport. The tight coupling between metabolism and uptake is viewed in terms of feedback inhibition, perhaps to prevent the accumulation of long chain acyl-CoA derivatives which have detergent properties. The more simple minded view that this is precisely the behaviour expected of a non-specific or facilitated diffusion system is not considered. The justification for invoking the existence of a specific transport system without further evidence is limited.

One of the most interesting pieces of indirect evidence on the mechanism of monocarboxylate permeation comes from the work of von Meyenburg (1971). Using a qualitative technique based on the growth rate obtained over a concentration gradient of a nutrient established on a plate, it was possible to observe differences in the concentration dependence of growth on pyruvate and acetate between the wild-type and the mutant pleiotropically affected in membrane properties. The amplification of this type of study would provide convincing evidence that a membrane transport process in involved in the utilization of aliphatic monocarboxylic acids.

The evidence for the existence of carboxylate transport systems may be summarised as follows. In a range of bacterial species there is strong evidence for the presence of specific transport systems for di- and tricarboxylic acids. The evidence for such systems acting on monocarboxylates is more equivocal and the bulk of the work has been carried out on E. coli. The evidence from mutant and direct transport

studies along with considerations of the relation of structure to likely mode of permeation would suggest pyruvate, glycollate and mandelate are transported by specific systems. Considerations of structure and pK would suggest that lactate, glycerate and glyoxylate would fall in the same group. (There is little doubt that longer chain polysubstituted monocarboxylates such as gluconate would require specific transport systems). The case of the aliphatic monocarboxylates is more open to question. There is no mutant evidence to suggest the existence of specific systems, but this may result from the inherent difficulties in isolating such mutants. It is possible that at high concentrations simple diffusion will play an important role in permeation and slow growth will still be possible in the absence of a transport system. At the lower concentrations which might be encountered in a natural environment, the scavenging function of a transport system might be very significant. Thus the evidence for their existence is built on indirect studies none of which can be said to settle the problem decisively. It might be suggested that the weight of the evidence favours their existence. This contrast with the mitochondrial situation may be a reflection of a number of factors:

1. requirement for a higher rate of flux across the membrane in bacteria than in mitochondria. This does not seem likely in view of the rates measured for dicarboxylate flux in mitochondria (Palmieri et al., 1971).

2. Requirement for rapid flux at lower concentrations in bacteria which normally live outside the cell.

3. Differences in intrinsic permeability due to differences in membrane structure. This area remains largely unexplored at the present time.

The ionic relationships of anion transport in bacteria are not well understood. Much work has been carried out on anion transport in mitochondria where transporters for phosphate, sulphate, di- and tricarboxylates, glutamate and aspartate have been identified (Mitchell, 1970). Theoretical aspects of the problem have been defined and discussed in great depth by Mitchell (1967). Two types of ionic relationships are possible:

1. anion-cation symport,
2. anion-anion antiport.

Both types of system are observed in eucaryotic cells. Thus mitochondrial phosphate permeation is thought to involve proton symport, while di- and tricarboxylate permeation involves anion-anion antiport (reviewed by Mitchell, 1970). Recent work has demonstrated proton fluxes associated with β-galactoside transport in E. coli but much of the evidence for the role of proton symporters is indirect coming from studies of the effects of uncouplers (proton conductors) and ionophoric antibiotics on transport (Mitchell, 1970; Harold, 1970; West, 1971; Mitchell & West, 1972). The complexity of anion transporting systems may well be considerable as extensive linkage between symporters and antiporters is possible as is found in mitochondria. Thus, phosphate might be antiported in exchange for another anion and itself recovered by a proton symport system. The possibility that endogenous anion production might play a role in anion transport has not been extensively

considered. Our knowledge of these processes thus remains primitive at the present time.

A considerable part of the work to be described below was carried out in order to clarify the mechanism of acetate permeation in E. coli. Previous studies of aliphatic monocarboxylic acid permeation in bacteria have failed to resolve this problem, while the excellent studies of mitochondrial transport deal with a very different physiological situation. The technical problems of studying acetate permeation have proved difficult to overcome and it has been necessary to resort to indirect methods. Some other studies of carboxylate transport in less refractory systems are also described.

Materials and Methods

Lithium D-lactate and carbonylcyanide m-chlorophenylhydrazone (ClCCP) were obtained from Calbiochem Corp., London. Lithium L-lactate, lithium DL-lactate, free D- and L-lactic acids and gramicidin D were obtained from Sigma Corp.

[2-^{14}C]chloroacetate sodium salt, [1-^{14}C]glycollate sodium salt, [1-^{14}C]DL-lactate sodium salt and [1,4-^{14}C]DL-tartaric acid were purchased from the Radiochemical Centre.

Organisms The organisms used in this chapter are listed in Table 1.

Measurement of uptake of labelled compounds by washed cell suspensions Cells for use in uptake experiments were routinely grown at 37°C in a shaking water bath. They were harvested in late logarithmic phase using an M.S.E. bench centrifuge and washed twice with the buffer to be used in the uptake experiment (in most cases this was phosphate buffered basal medium without a carbon source, pH 7.0). Cells were resuspended to give an A_{680} of approximately 0.5. Cell suspensions were shaken for 10 minutes at the temperature to be used for the uptake (37°C unless otherwise stated) prior to the addition of the labelled material. At intervals after the addition of label, samples were withdrawn and rapidly filtered through 0.45 μm pore size Sartorius filters. The filters were washed with a 5 ml of the uptake buffer which was kept at the temperature used for the uptake. Filters were placed in Bray's fluid (Bray, 1960) or toluene/methanol scintillant (see above) and counted in a Packard Model 4000 liquid scintillation spectrometer. For determination of trichloroacetic acid (TCA)-insoluble material, portions of cell suspension were added to an equal volume of 10% TCA left for 15 minutes and then filtered through a Sartorius filter. The filter was washed with 5% TCA and counted as described above.

Extraction and analysis of pool materials For extraction and analysis of soluble labelled material present in cells after exposure to a labelled compound, cells were rapidly filtered through a 47 mm diameter Millipore filter of 0.8 μm pore size and washed with 10 volumes of buffer. The filter was then placed in 2 ml of an ice-cold toluene/

water mixture (5 : 95) and deep frozen overnight. The mixture was subsequently thawed and the cells detached from the filter by vortex mixing. The cells were removed by centrifugation and the supernatant solution evaporated to dryness at room temperature under a stream of air. The dried material was redissolved in water and a portion applied to a 300 μm silica gel G thin layer plate. Chromatography was carried out in either ethanol/ammonia/water (80 : 16 : 4) for monocarboxylic acids or ethanol/water/ammonia (100 : 12 : 16) for dicarboxylic acids (Randerath, 1963). Authentic labelled materials were used as standards. Radioactivity was detected either by radioautography on Kodak "Blue Brand" X-ray film or by scanning with a Panax TLC plate scanner.

Preparation of spheroplasts Cells in mid log phase were harvested by centrifugation at 12,000 g for 5 minutes in a M.S.E. 18 centrifuge. The cells were washed twice with 0.01 M Tris-Cl buffer pH 8.0 at 5°C. The washed cells were suspended in 0.03 M Tris-Cl buffer pH 8.0 containing 20% sucrose at a cell concentration of 1 g wet weight per 80 ml of buffer. Sodium EDTA was added to give a concentration of 0.01 M, lysozyme to 0.5 $mg.ml^{-1}$, and deoxyribonuclease to 10 $\mu g.ml^{-1}$. The cell suspension was stirred gently at room temperature for 30 minutes and the spheroplasts collected by centrifugation at 16,000 g for 10 minutes. The spheroplasts were washed in 20 mM potassium PIPES (piperazine-N,N'-bis[2-ethane-sulphonic acid])/20% sucrose buffer pH 7.0. The cell density was adjusted to an A_{680} of approximately 15.

Iso-osmotic swelling Swelling of spheroplasts in iso-osmotic solutions of the salts of carboxylic acids buffered at pH 7.0 with potassium PIPES was followed at 680 nm in a Hilger-Gilford recording spectrophotometer. 0.1 ml of spheroplast suspension was added to 2.4 ml of the iso-osmotic salt solution in a 3 ml glass cuvette at room temperature and changes in absorbance measured. Decrease in A_{680} was measured of the swelling of the spheroplasts. Swelling is dependent on the spheroplasts being permeable to the suspending salt. The effectiveness of the procedure for preparing the spheroplasts was tested by following their swelling in distilled water.

Oxygen electrode measurements of substrate oxidation Cells for oxygen electrode studies were grown as described for uptake experiments. Washed cell suspensions were prepared as described. Endogenous substrate oxidation was reduced by leaving the cells suspended in buffer at room temperature for 4-5 hours prior to use. Oxygen consumption was measured at 37°C in a Clark oxygen electrode fitted with a water jacket and a magnetic stirrer.

FIG. 19

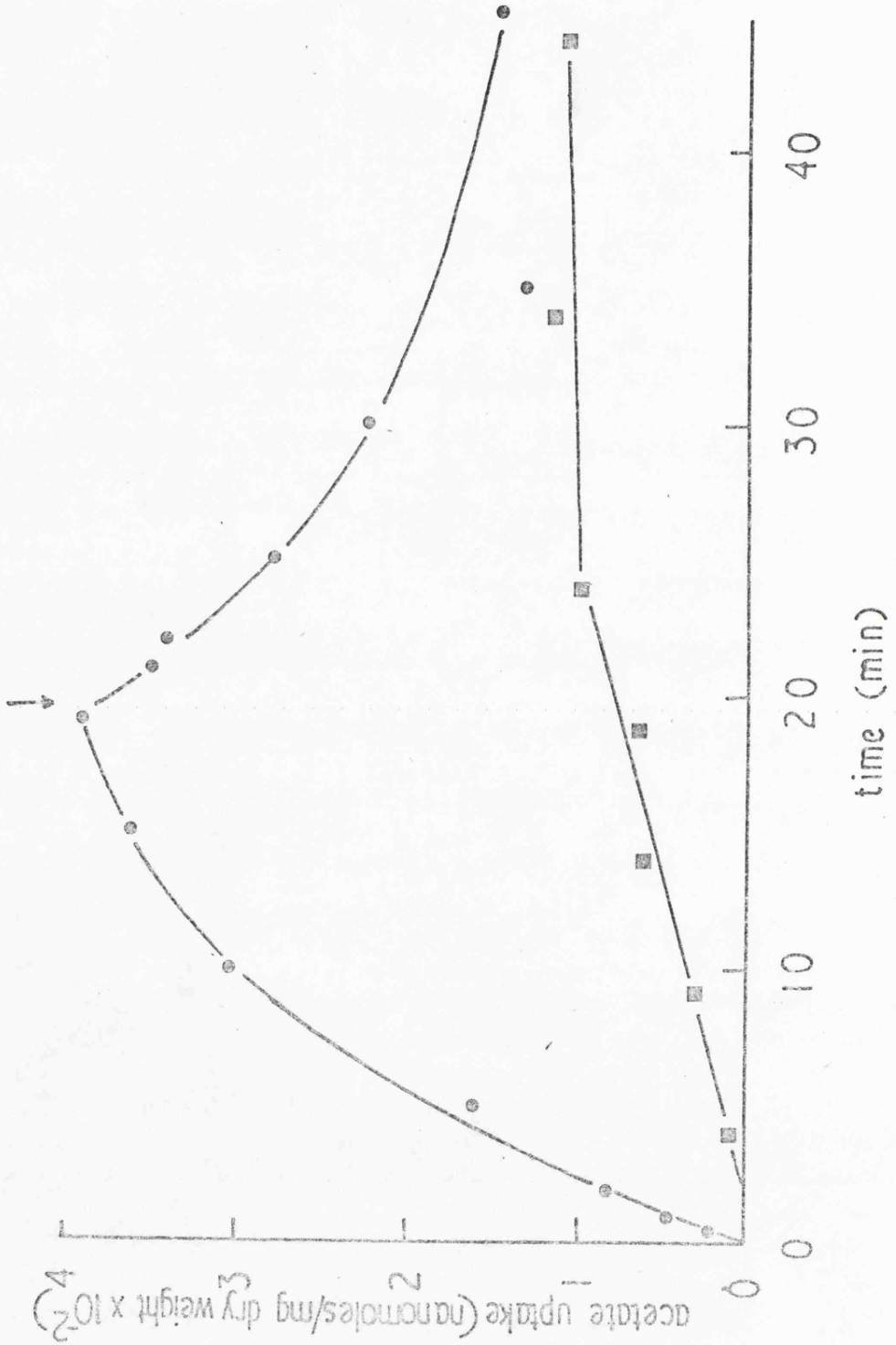

acetate uptake (nanomoles/mg dry weight x 10^2)
0
1
2
0
10
20
30
40
time (min)

Figure 19

Uptake of 0.1 mM sodium [2^{14}C]acetate by glycerol-grown *E. coli* K12 strain K2. Measurement of the uptake of labelled material was carried out as described in Materials and Methods. 10 mM [^{12}C]acetate was added at the point indicated by the arrow (20 min).

● total uptake of labelled acetate, ■ incorporation of label into TCA-insoluble form.

Results and Discussion

Acetate Uptake in E. coli K12

General Properties The work carried out on acetate uptake and permeation can be divided into two main parts. The first deals with the properties of acetate uptake in wild-type cells and a variety of mutant strains. The second deals with attempts to relate the observed properties to the mechanism of acetate permeation in E. coli.

Washed cell suspensions of wild-type E. coli, grown on glycerol, take up radioactively labelled acetate from the medium rapidly. Linear initial rates can be obtained and incorporation of label into TCA-insoluble material is low. The rate of uptake of label decreases after a time and at this point there is a considerably higher concentration of label inside the cells than in the surrounding medium (Figure 19). This label can be "chased" from the cells by excess cold acetate (Figure 19) or the pool discharged by the addition of sodium azide. The rapid efflux of label from the cells upon the addition of excess unlabelled acetate to the medium is considered to represent equilibration of the intracellular pool with the unlabelled acetate. These properties might suggest that acetate is accumulated within the cell; it was thus essential to check the intracellular fate of the labelled acetate.

Two approaches were used. In the first, the ability of a citrate synthase-less strain (which cannot metabolise acetate via the tricarboxylic acid cycle) to take up acetate was compared with that of the parent strain. The citrate synthase-less strain was found to

FIG. 20

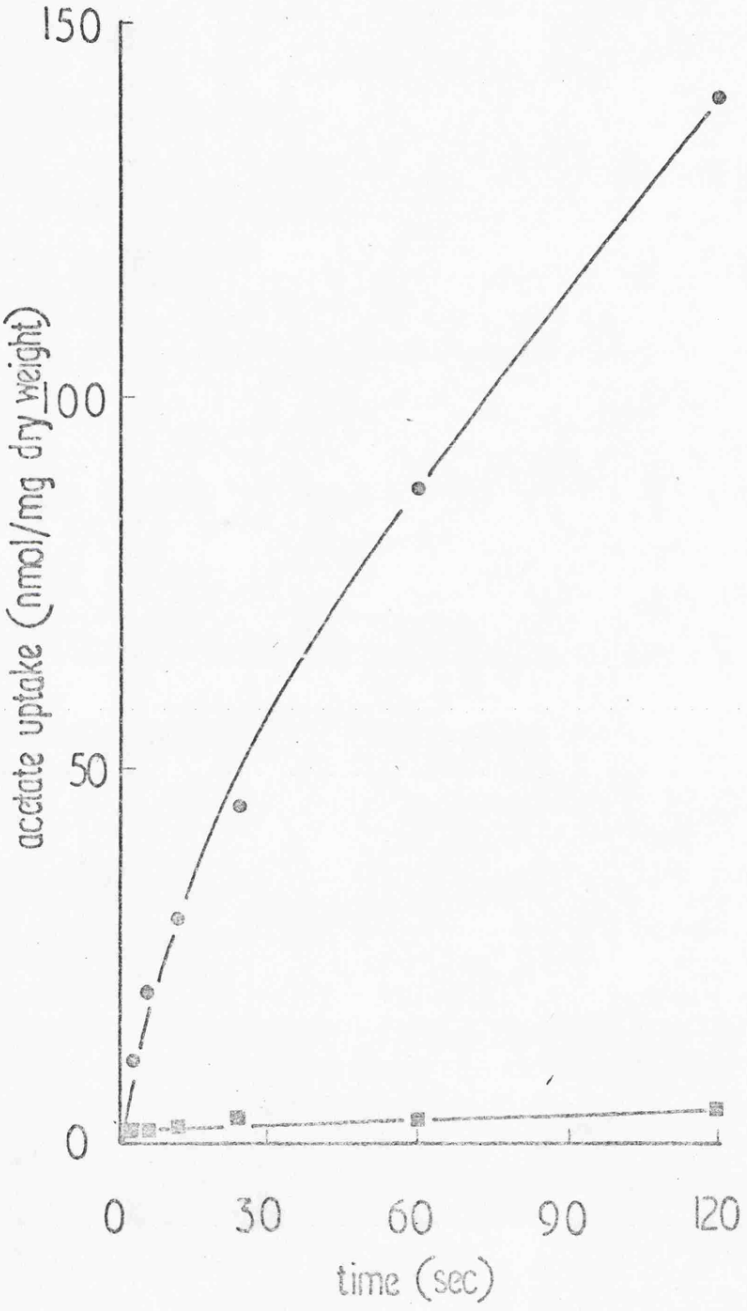

150
100
50
0
acetate uptake (nmol/mg dry weight)
0
30
60
90
120
time (sec)

Figure 20

Effect of a lesion in citrate synthase upon the uptake of 0.1 mM [2^{14}C]acetate. Strains K2.1t (PPS^-) and K2.1.4 (PPS^-, CS^-) were grown on nutrient broth containing 50 mM acetate. Measurement of uptake of labelled material was carried out as described in Materials and Methods. ● acetate uptake by K2.1t; ■ acetate uptake by K2.1.4.

Table 13

Effect of carbon source used for growth on the initial rate of 0.01 mM sodium [2-^{14}C]acetate uptake by washed suspensions of strain K2

Carbon source	Initial rate of uptake*
glycerol	100
L-malate	100
glucose	11
gluconate	22
pyruvate	64

Uptake of radioactive label was measured as described in Materials and Methods. Cells were grown on 25 mM carbon sources and washed cell suspensions prepared as described in Materials and Methods.

* The initial rate of uptake is expressed as a percentage of the initial rate of uptake by glycerol-grown cells.

take up acetate at a low rate compared to the parent strain (Figure 20). In order to confirm that this observation was not the result of a failure in the mutant of an energy generating process required for uptake, the fate of label in a wild-type strain was examined as described in Materials and Methods. Thin layer chromatography of the pool extracted after E. coli K12 strain K2 had been allowed to take up labelled acetate for 10 minutes revealed that the major product of acetate uptake under the conditions employed was glutamate. Traces of succinate were also found, but there was no evidence of the presence of acetate either from the radioautography or from acid treatment of a sample of the pool material. It may be concluded that E. coli does not actively concentrate acetate within the cell under the conditions tested. It is thus apparent that the process being examined in acetate uptake could be a multi-enzyme process involving six steps and that interpretation of this process in terms of permeation requires careful consideration.

The effect of carbon sources and growth conditions upon the ability to take up acetate The effect of carbon source used for the growth of the organisms on their ability to take up labelled acetate from the medium was examined using washed cell suspensions of strain K2. It can be seen from Table 13 that the carbon source used for growth has a considerable effect on the acetate uptake. Uptake is low in glucose-grown cells and high in glycerol-grown cells with other compounds giving intermediate levels of uptake. Thus glucose appears to have a repressive effect upon acetate uptake. This is in agreement

FIG. 21

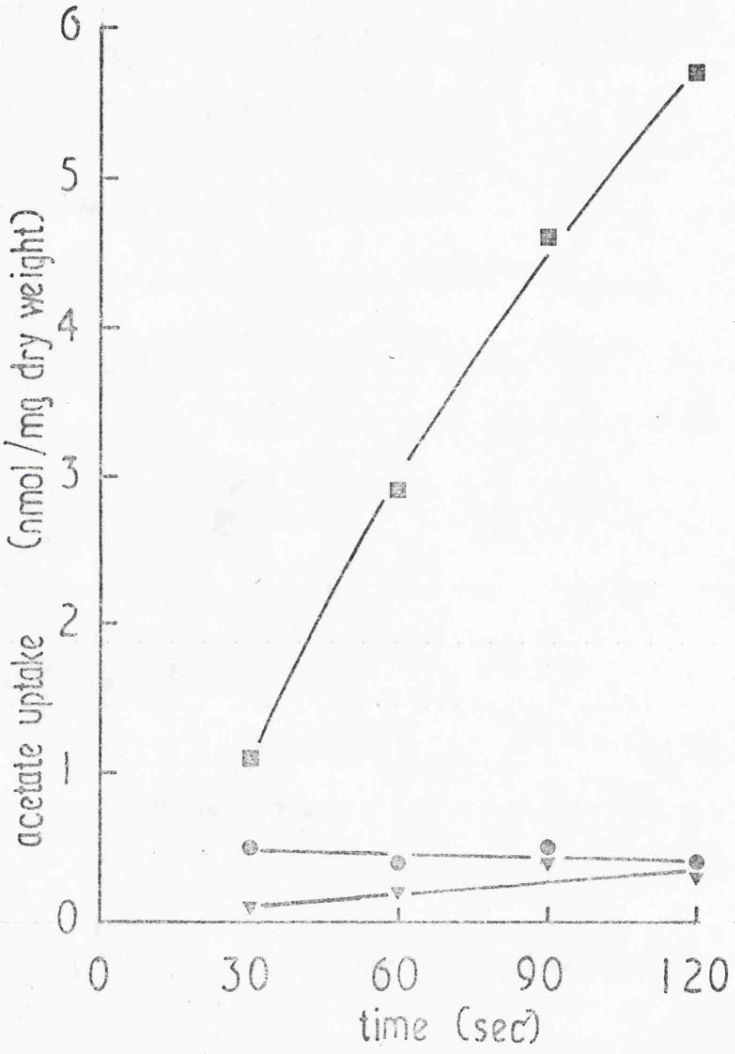

acetate uptake (nmol/mg dry weight)
0
1
2
3
4
5
6
0
30
60
90
120
time (sec)

Figure 21

Effect of growth under anaerobic conditions upon the uptake of 0.1 mM [2^{14}C]acetate by strains K2, K2FAc1 (ACK^-) and K2HFAc3 (PTA^-). The organisms were grown on 25 mM glucose/25 mM sodium bicarbonate medium. Measurement of uptake of labelled material was carried out as described in Materials and Methods. ● acetate uptake by K2; ▼ K2FAc1; ■ K2HFAc3.

with the finding of Wagner et al. (1972). The site of the effect is not, however, clarified by these observations. The synthesis of a number of enzymes required for the formation of glutamate from acetate is known to be repressed by glucose in E. coli (Gray et al., 1966b) and the acetate thiokinase activity described in the previous chapter is also subject to glucose repression.

The ability of wild-type, acetate kinase-less and phosphotransacetylase-less strain to take up labelled acetate after growth on glucose/bicarbonate medium under anaerobic conditions was also tested. The rate of uptake was found to be low in all cases (Figure 21). The activities of a number of tricarboxylic acid cycle enzymes are low after anaerobic growth on glucose (Gray et al., 1966b) and the site of this effect of anaerobic growth on acetate uptake cannot be readily determined.

The effect of lesions in acetate kinase and phosphotransacetylase on acetate uptake The roles of acetate kinase and of phosphotransacetylase and of acetate thiokinase in acetate activation were described in the two previous chapters. These results indicate that the acetate kinase/phosphotransacetylase system has a role in acetate activation at high external acetate concentrations while the thiokinase may have a role at lower acetate concentrations. The effect of these lesions on the ability of cells to take up acetate at high, intermediate and low concentrations was examined in cells that had been grown on glucose or glycerol. The acetate kinase-less and phosphotransacetylase-less strains are able to incorporate acetate only poorly

FIG. 22 (a)

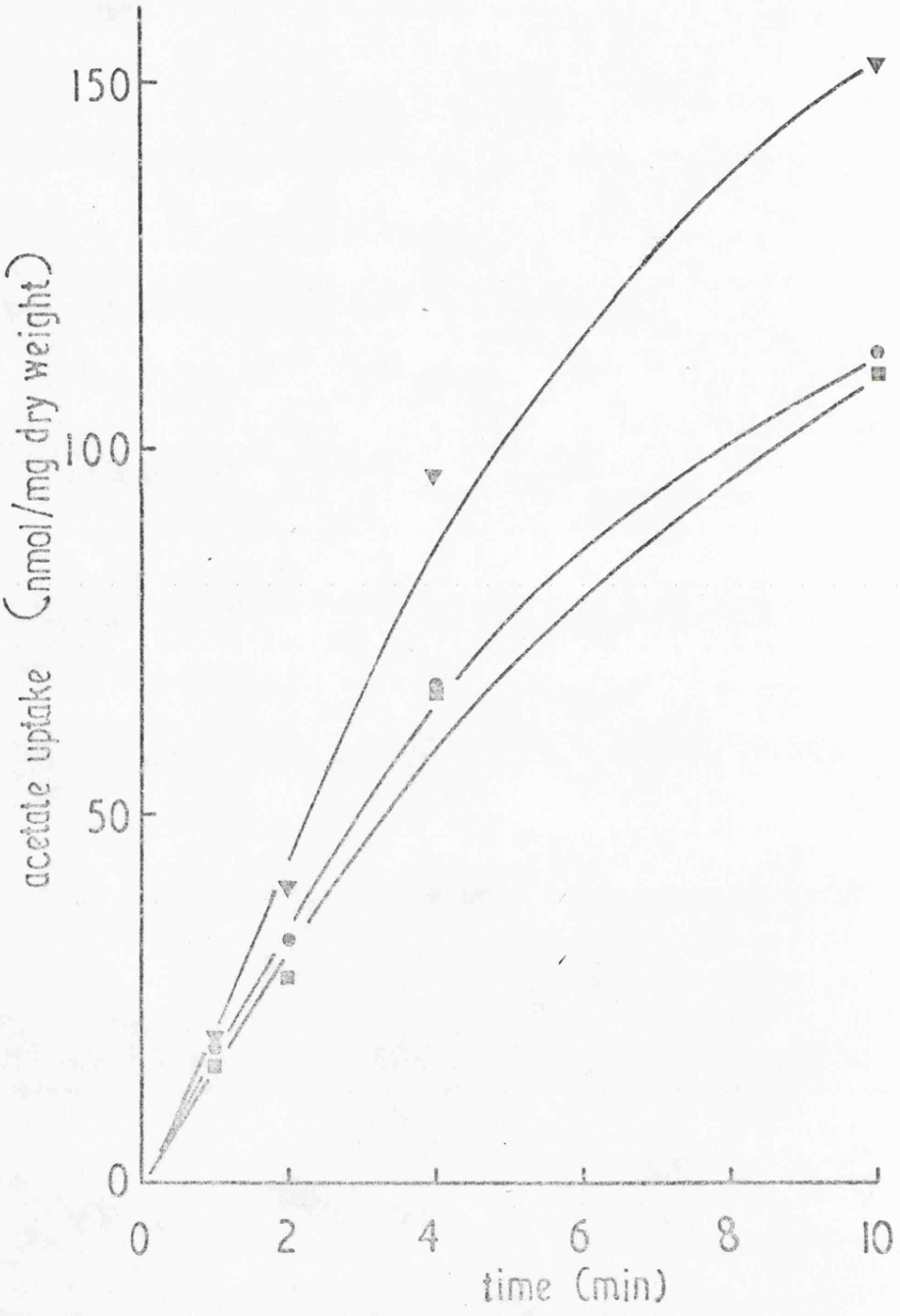

150
100
50
0
acetate uptake (nmol/mg dry weight)
0
2
4
6
8
10
time (min)

FIG. 22 (b)

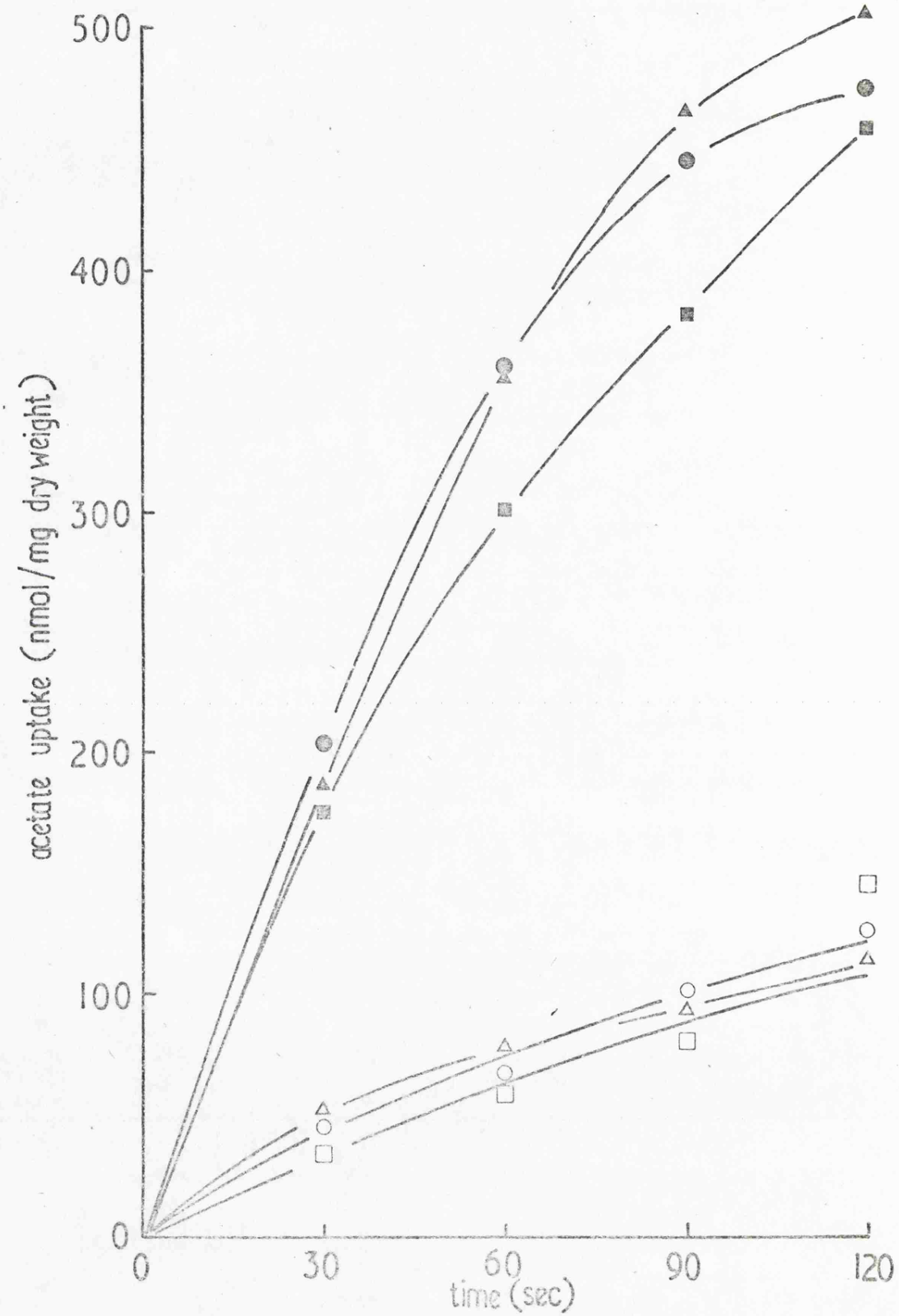

500
400
300
200
100
0
acetate uptake (nmol/mg dry weight)
0
30
60
90
120
time (sec)

FIG. 22(c)

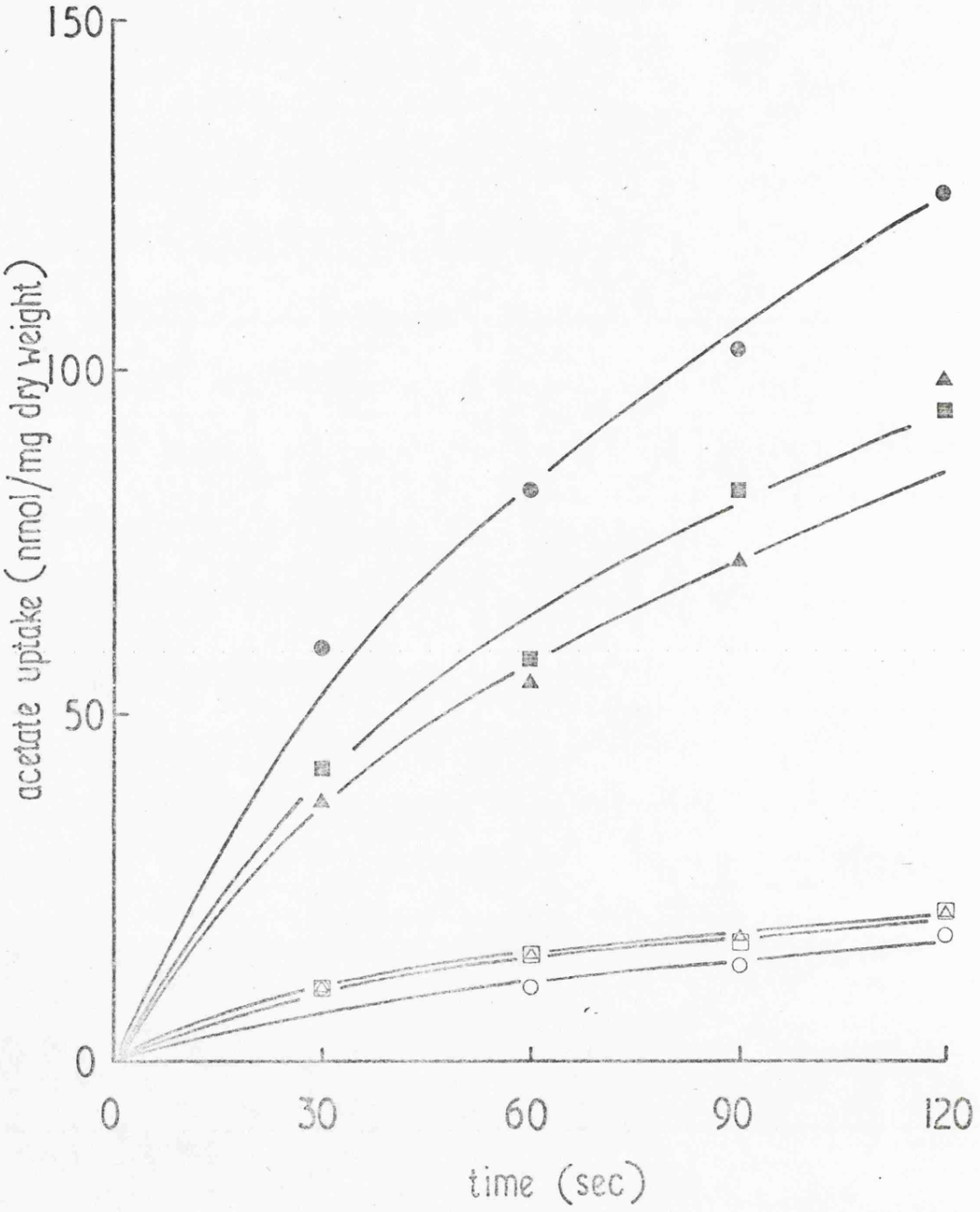

150
100
50
0
acetate uptake (nmol/mg dry weight)
0
30
60
90
120
time (sec)

Figure 22

Effect of lesions in acetate kinase and phosphotransacetylase upon acetate uptake in K2 strains. Uptake of labelled material was measured as described in Materials and Methods.

(a) Uptake of 0.1 mM [2^{14}C]acetate by glycerol-grown K2 ● ; K2FAc1 (ACK^-) ▼ ; and K2HFAc3 (PTA^-) ■ .

(b) Uptake of [2^{14}C]acetate at 2 and 20 mM by glycerol-grown K2 ○ (2 mM); ● (20 mM); K2FAc1 (ACK^-) △ (2 mM); ▲ (20 mM); and K2HFAc3 (PTA^-) □ (2 mM), ■ (20 mM).

(c) Uptake of [2^{14}C]acetate at 2 and 20 mM by glucose-grown K2 ○ (2 mM), ● (20 mM); K2FAc1 (ACK^-) △ (2 mM), ▲ (20 mM); and K2HFAc3 (PTA^-) □ (2 mM), ■ (20 mM).

FIG. 23

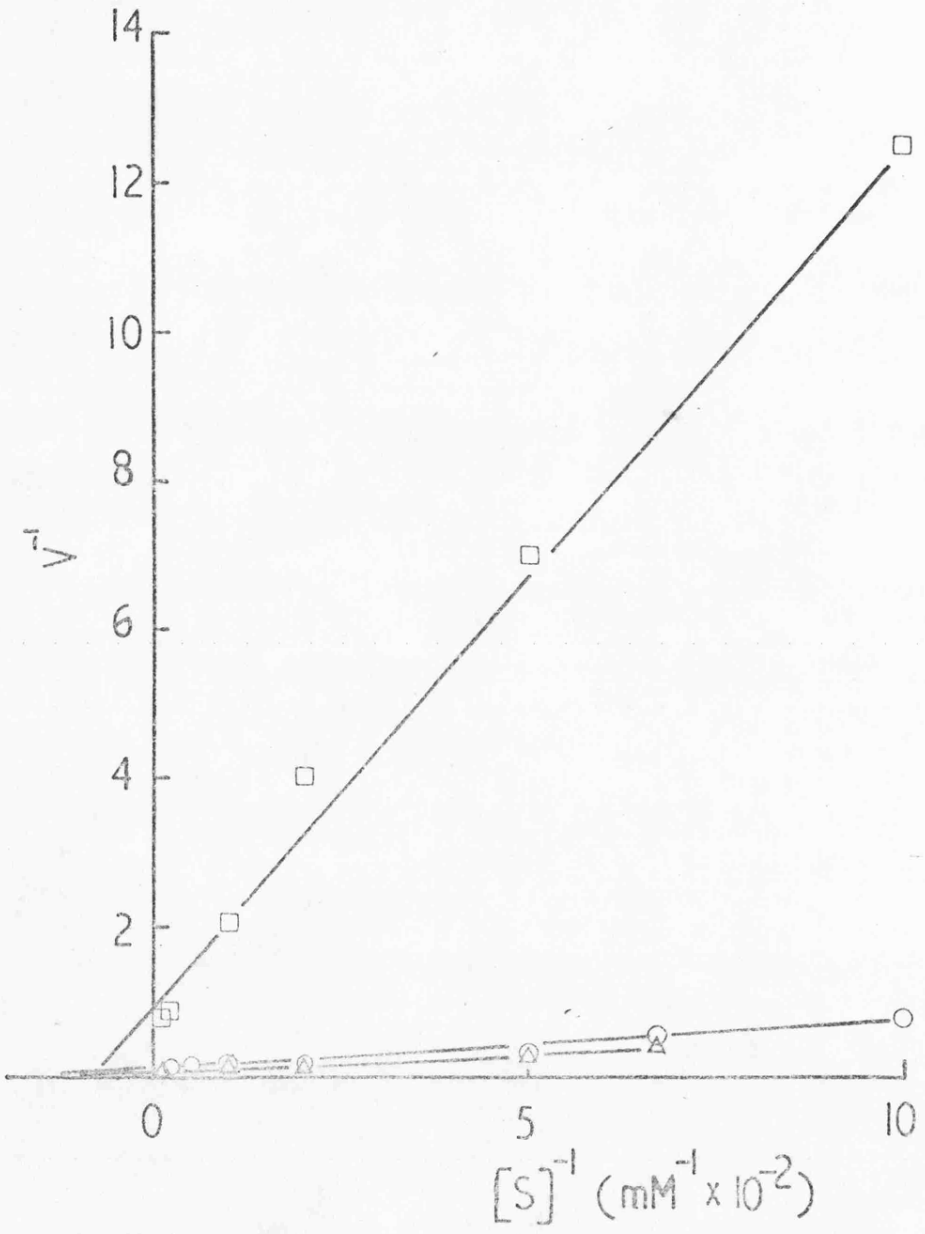


Figure 23

Concentration dependence of initial rates of sodium [2^{14}C]acetate uptake by E. coli K12 strain K2 grown on glucose, glycerol, and acetate. Uptake of labelled material was measured as described in Materials and Methods. The data are expressed in the form of a Lineweaver-Burk plot. V is expressed in nmol acetate uptake.min^{-1}.mg dry weight of cells^{-1}. □ glucose-grown; ○ glycerol-grown; △ acetate-grown.

FIG. 24

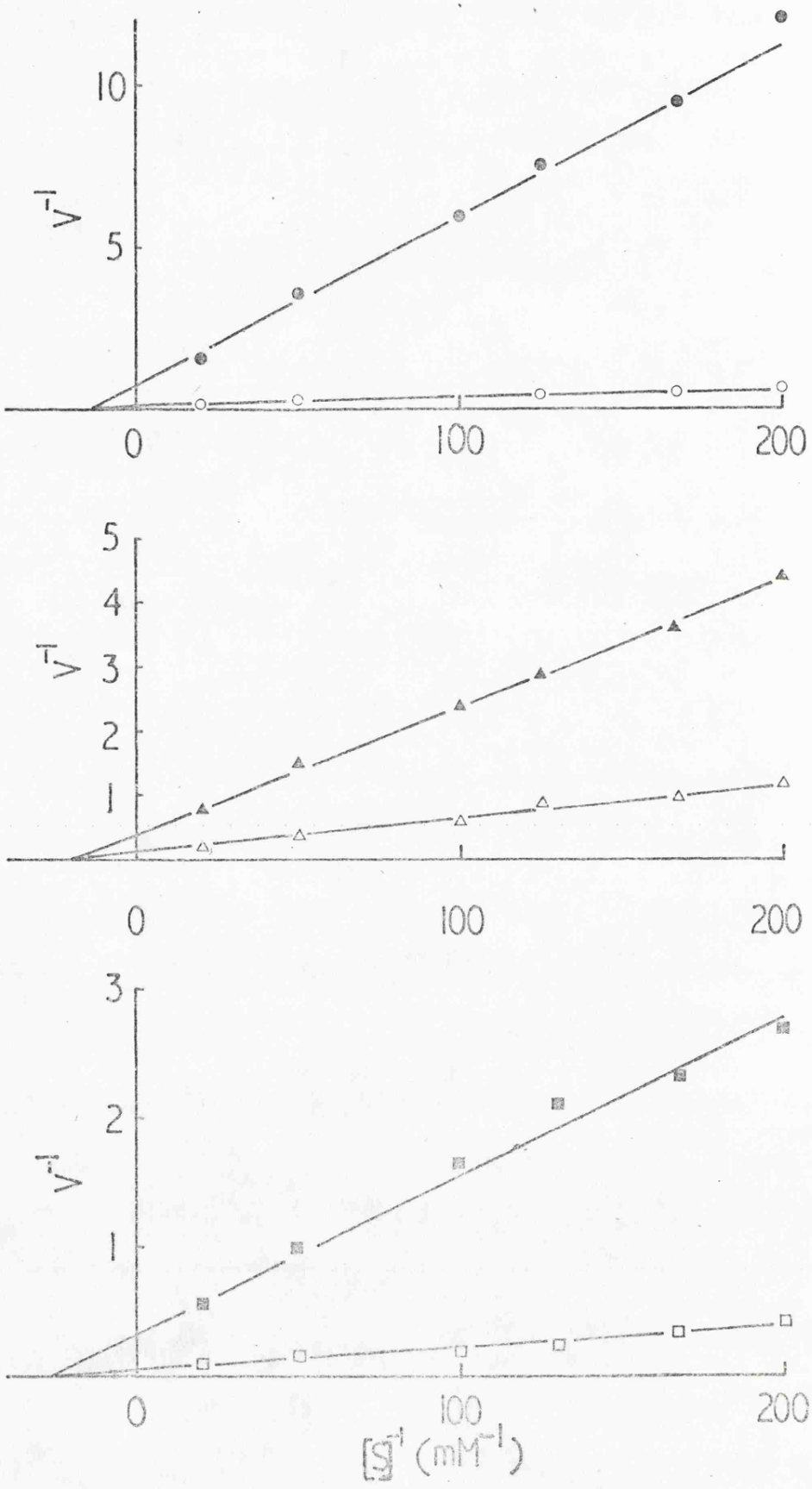

10
5
$\overline{v}^{-1}$
0
100
200
5
4
3
2
1
$\overline{\tau}$
0
100
200
3
2
1
$\overline{v}^{-1}$
0
100
200
$[S]^{-1}$ (mM^{-1})

Figure 24

Concentration dependence of initial rates of sodium [2^{14}C]acetate uptake by strains K1, K1HFAc1 (ACK$^-$) and K1HFAc3 (PTA$^-$) grown on glucose and glycerol. Uptake of labelled material was measured as described in Materials and Methods. The data are presented in the form of a Lineweaver-Burk plot. V is expressed as nmol acetate taken up.min^{-1}. mg dry weight of cells^{-1}. K1 ● glucose-grown, ○ glycerol-grown; K1HFAc1 (ACK$^-$) ▲ glucose-grown, △ glycerol-grown; K1HFAc3 (PTA$^-$) ■ glucose-grown, □ glycerol-grown.

when grown on glucose, but can incorporate it well during growth on glycerol (see Chapter I) although their ability to incorporate it is impaired at high concentrations of acetate. It was thus of interest to investigate whether the uptake process reflected modifications in activating ability. No changes in pattern of uptake comparable with changes in incorporation were observed (Figure 22). Lesions in acetate kinase and phosphotransacetylase have only limited effects on the uptake of 20 mM labelled acetate by washed cell suspensions compared with their effects on the ability of cultures growing on glucose or glycerol to incorporate 20 mM labelled acetate. The processes measured in the two types of experiment are obviously different. It is also seen that glucose repression of uptake occurs at high acetate concentrations as well as at low concentrations.

The determination of kinetic parameters for acetate uptake Further information on the effect of the carbon source used for growth and of lesions in acetate kinase and phosphotransacetylase on acetate uptake was obtained from studies of the variation in initial rate of uptake with acetate concentration. It was of interest to find out whether the uptake process showed saturation kinetics. Apparent K_m and V_{max} values for the process were determined by plotting the data according to the method of Lineweaver and Burk (1934). The effect of carbon source used for growth is illustrated by the values for K_m and V_{max} obtained for strain K2 grown on glucose, glycerol or acetate (Figure 23). The K_m and V_{max} values for strains K1, K1HFAc1 (ACK^-) and K1HFAc3 (PTA^-) grown on glucose and glycerol were also compared (Figure 24). These determinations were carried out using a

FIG. 25

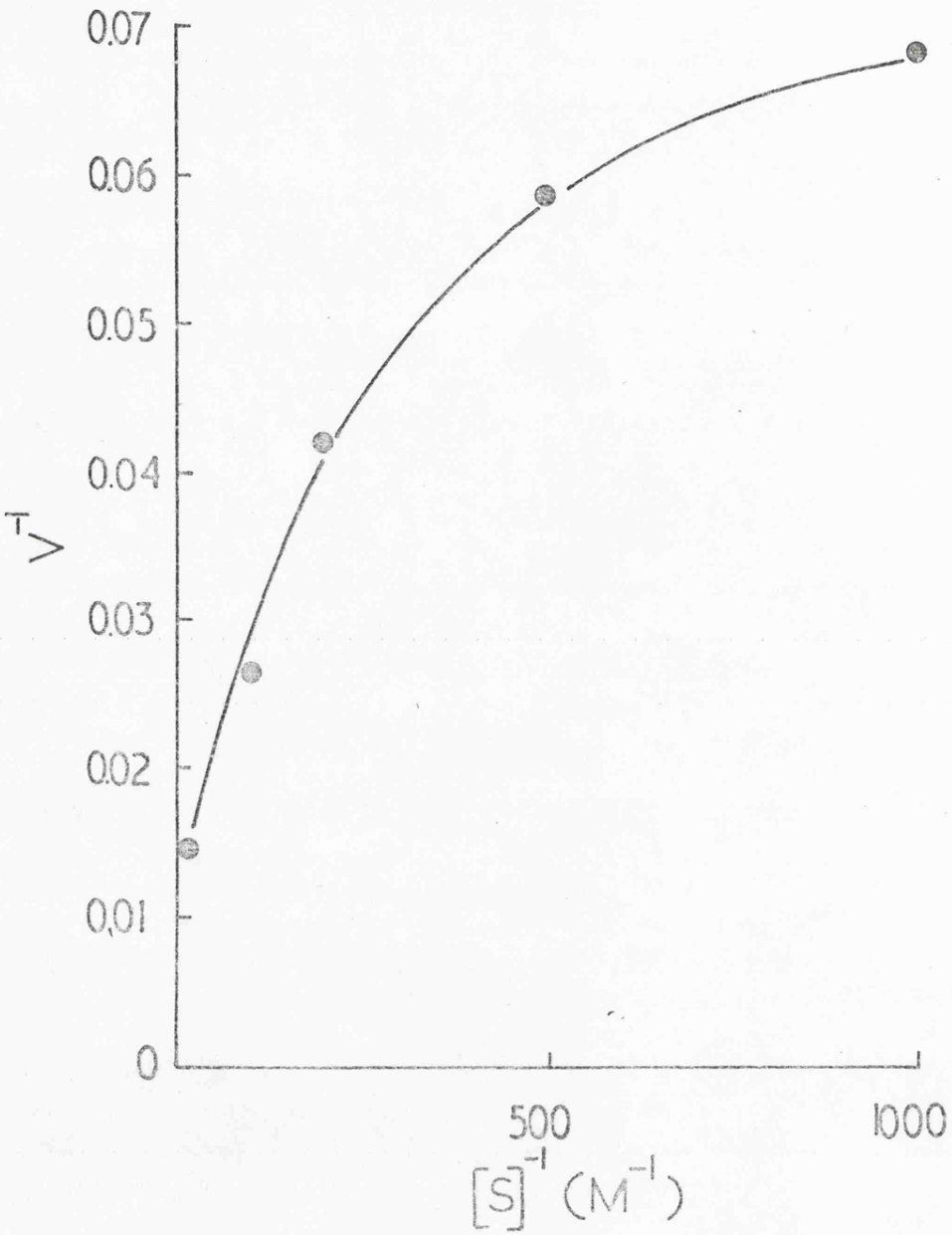


Figure 25

Concentration dependence of the initial rate of sodium [2-^{14}C]acetate uptake by washed suspensions of glycerol-grown strain K2 at high acetate concentrations. Uptake of labelled material was measured as described in Materials and Methods. The data are presented in the form of Lineweaver-Burk plot. V is expressed as nmole acetate taken up.min^{-1}.mg dry weight of cells^{-1}.

FIG. 26

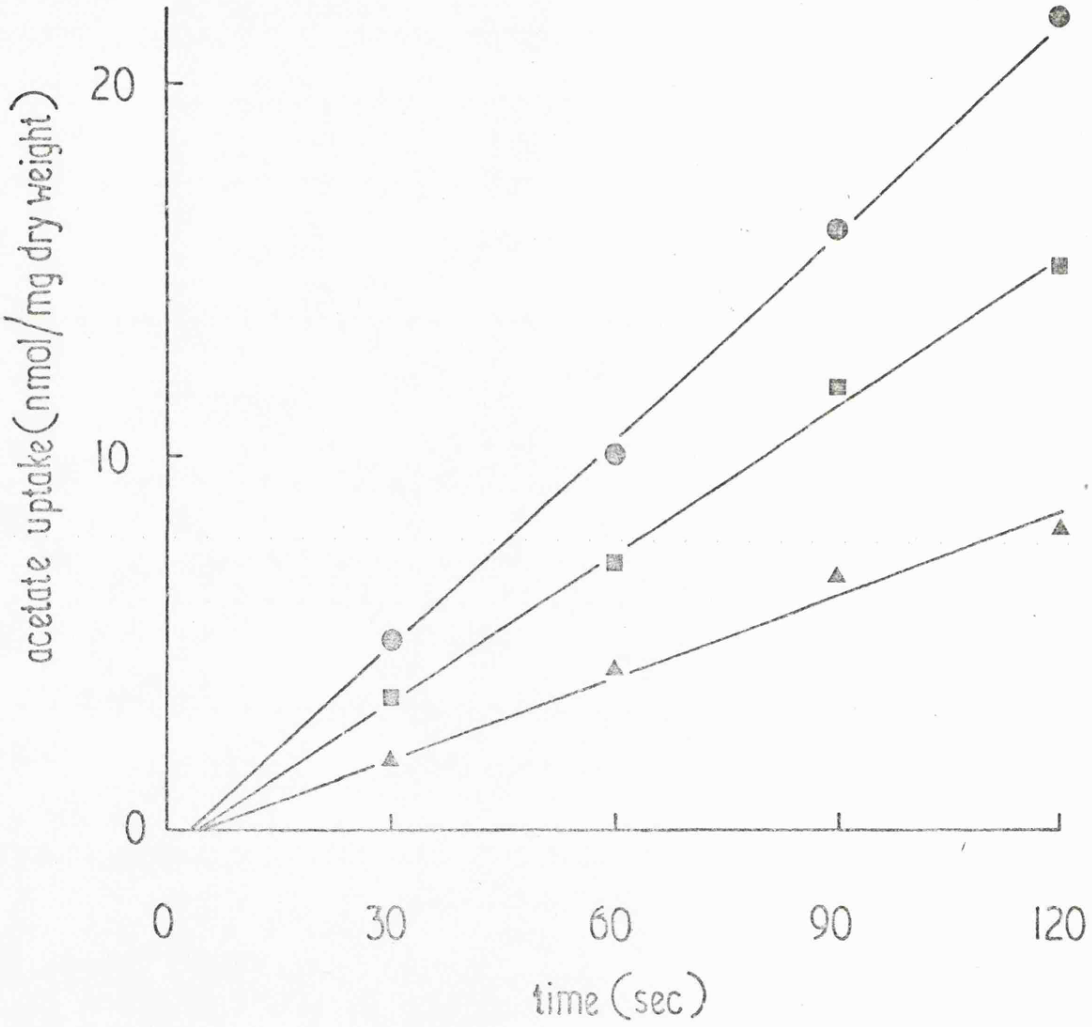

acetate uptake (nmol/mg dry weight)
20
10
0
0
30
60
90
120
time (sec)

Figure 26

The effect of monocarboxylic acid addition upon [2^{14}C]acetate uptake by washed suspensions of glycerol-grown strain K2. Labelled acetate was added at 0.01 mM and unlabelled monocarboxylates at 1 mM. All additions were made at zero time. No addition ● ; sodium propionate ▲ ; sodium glycollate ■ . The following compounds produced no significant effect when tested at 1 mM; sodium butyrate, sodium formate, sodium chloroacetate, sodium cyanoacetate, sodium hydroxypyruvate, lithium D-lactate, sodium glyoxylate, sodium succinate and acetamide. Uptake of labelled material was measured as described in Materials and Methods.

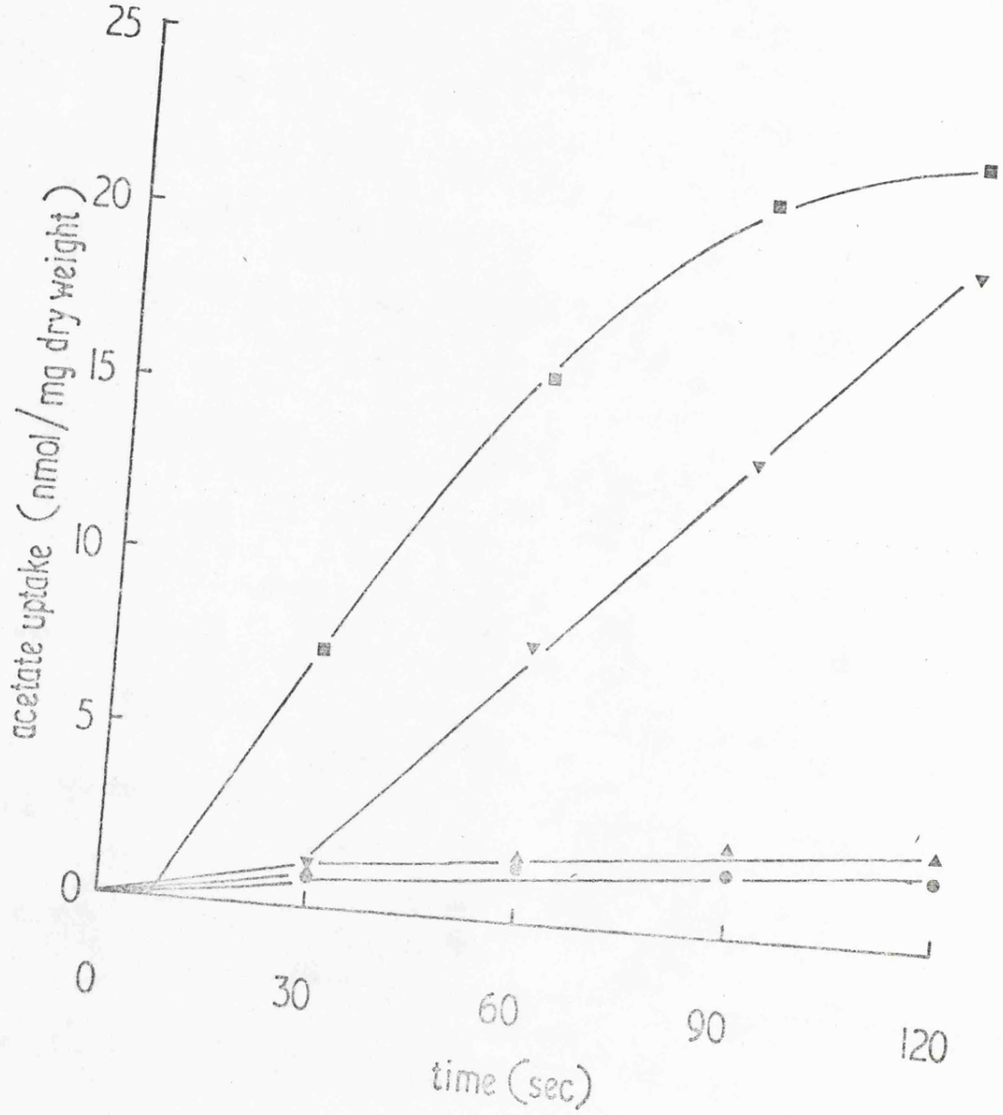
concentration range from 10^{-4} M to 10^{-6} M. The kinetics of acetate uptake at higher concentrations was examined with strain K2 grown on glycerol (Figure 25).

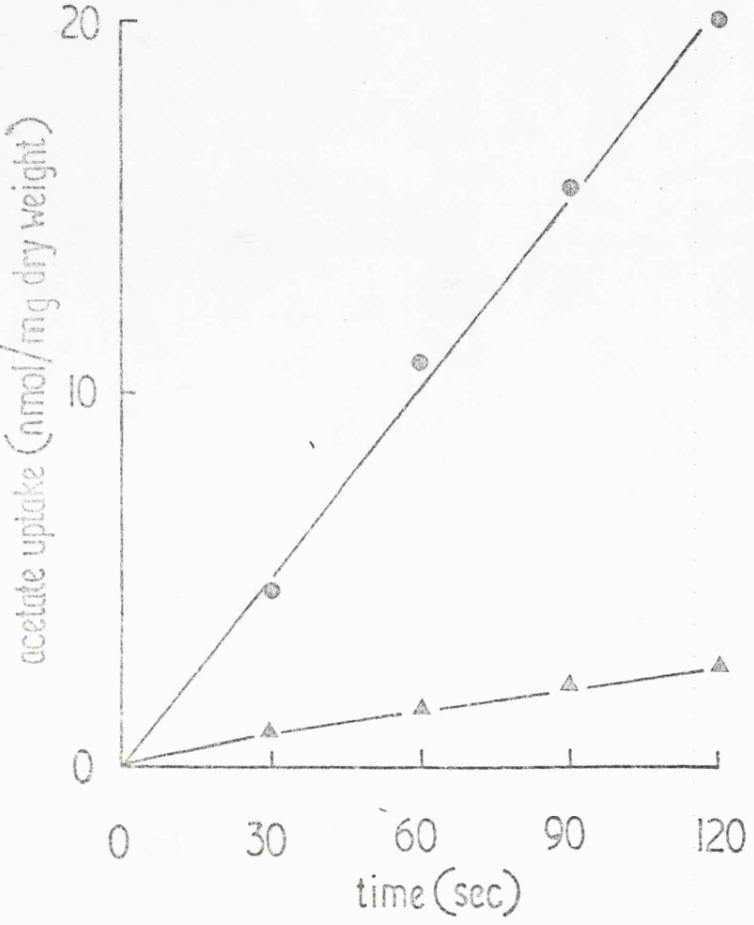
Thesedata suggest that there are two acetate uptake processes. One, operating at low concentrations of acetate, shows saturation behaviour, while the other process, operating at higher acetate concentrations, may not. The K_m determined for the system operating at low acetate concentrations did not vary significantly with the carbon source upon which the cells had been grown. The V_{max} did, however, depend upon the carbon source used for growth (acetate$>$ glycerol$>$ glucose). K_m and V_{max} values for the uptake process were similarly not significantly affected in acetate kinase-less and phosphotransacetylase-less organisms. The accuracy with which the kinetic parameters for the saturable system can be determined is adversely affected by the intervention of the apparently non-saturable system at acetate concentrations only about ten times greater than the K_m for the saturable system.

The effects of potential analogues and inhibitors on acetate uptake

A number of compounds were screened for their effects on acetate uptake. Most of these were monocarboxylic acids which were considered to be potential analogues of acetate and therefore possibly useful in these studies of permeation. Of the monocarboxylates tested, only propionate, glycollate and pyruvate had any effect on uptake under the conditions used (Figure 26). Propionate and glycollate had only weakly inhibitory effects even when used in considerable excess. Pyruvate,on the other hand, proved to be an extremely powerful inhibitor.

FIG. 27(a)


25
20
15
10
5
0
acetate uptake (nmol/mg dry weight)
0
30
60
90
120
time (sec)


20
10
0
acetate uptake (nmol/mg dry weight)
0
30
60
90
120
time (sec)

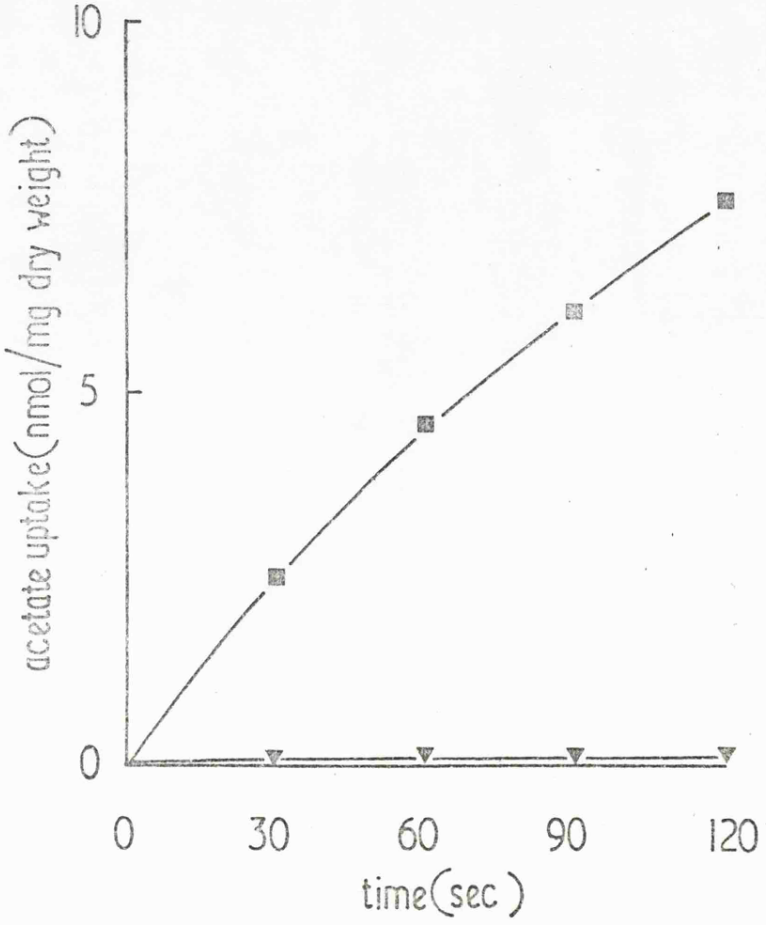

10
5
0
acetate uptake (nmol/mg dry weight)
0
30
60
90
120
time (sec)

FIG. 27(b)

FIG. 27 (c)

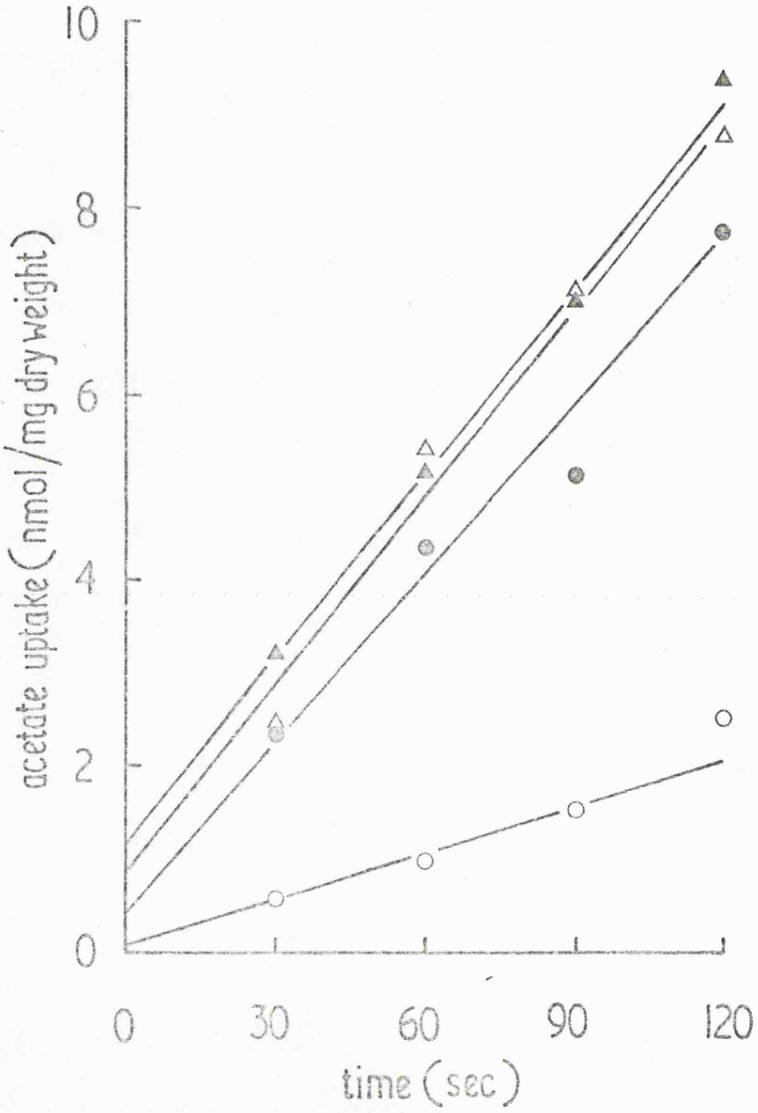

10
8
6
4
2
0
acetate uptake (nmol/mg dry weight)
0
30
60
90
120
time (sec)

Figure 27

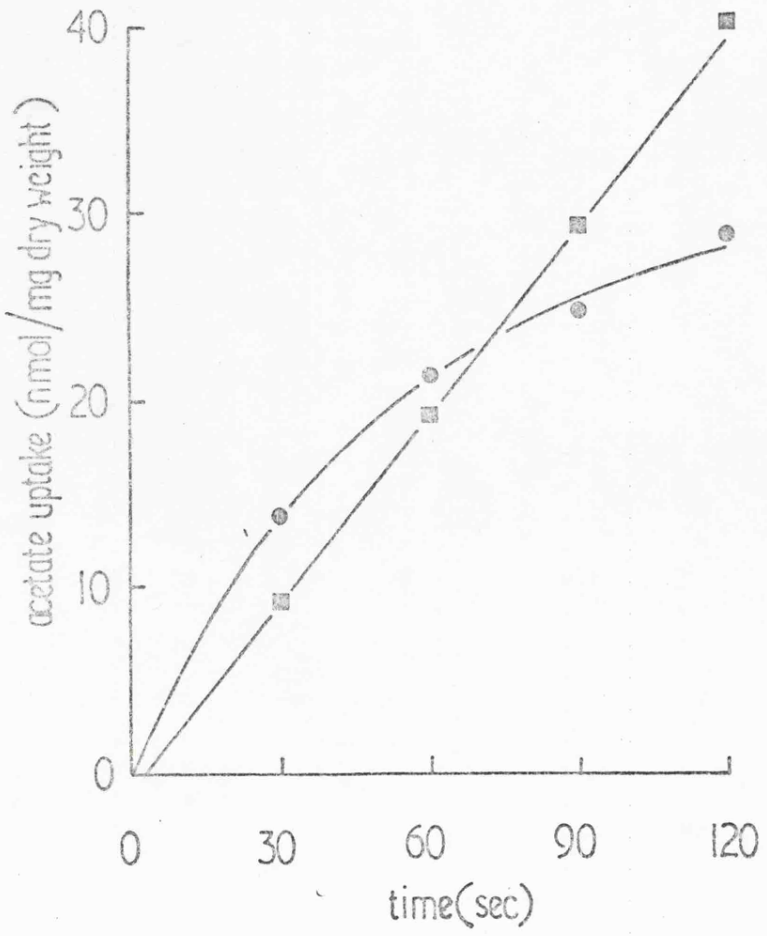
(a) The effect of pyruvate addition upon 0.1 mM [2^{14}C]acetate uptake by washed suspensions of glycerol-grown strain K2. Uptake of labelled material was measured as described in Materials and Methods. Pyruvate was added at zero time. No addition ■ ; 0.01 mM sodium pyruvate ▼ ; 0.1 mM sodium pyruvate ▲ ; 1 mM sodium pyruvate ● .

(b) The effect of pyruvate addition upon [2^{14}C]acetate uptake by suspensions of glycerol/acetate-grown strain K2.3 (PDH$^-$) and glycerol-grown strain K2.1t (PPS$^-$). Uptake of labelled material was measured as described in Materials and Methods.
K2.3, 0.1 mM sodium [2^{14}C]acetate. No addition ● ; 1 mM sodium pyruvate ▲ .
K2.1t, 0.01 mM sodium [2^{14}C]acetate. No addition ■ ; 1 mM sodium pyruvate ▼ .

(c) The effect of 0.01 mM sodium pyruvate addition upon the uptake of 0.01 mM sodium [2^{14}C]acetate by suspension of glycerol-grown strains K2 and K2.6 (USP$^-$). Uptake of labelled material was measured as described in Materials and Methods. Pyruvate was added at zero time.
K2 no addition ● ; pyruvate added O . K2.6 no addition ▲ ; pyruvate added △ .

The effect of propionate and glycollate on acetate uptake was paralleled by the effect of acetate on propionate and glycollate uptake. Acetate was also found to inhibit formate uptake to a limited extent.

The well documented role of compounds related to pyruvate in the control of acetate metabolism (Kornberg, 1965b) and the powerful nature of the inhibition of acetate uptake prompted further investigation of the effect of pyruvate upon acetate uptake. The concentration dependence of inhibition was studied and pyruvate was found to be inhibitory at concentrations down to 10^{-5} M (Figure 27a). The rapid removal and metabolism of pyruvate under the conditions used was demonstrated in control experiments on pyruvate uptake and this rapid removal prevented study of the kinetic properties of the inhibition; it also suggested the possibility that pyruvate *per se* may not be the inhibitor of acetate uptake. Thus acetyl CoA produced by the action of the pyruvate dehydrogenase complex might be considered to be a potential inhibitor of acetate activation. An attempt to clarify this possibility was made by studying the pyruvate inhibition of acetate uptake in two mutants in which two major metabolic routes from pyruvate are blocked. A PEP-synthase-negative and a pyruvate dehydrogenase-negative mutant were studied. With both mutants, pyruvate inhibition of acetate uptake was still observed, (Figure 27b). Glyoxylate at 1 mM failed to reverse the inhibitory effect of 1 mM pyruvate (cf. Ashworth & Kornberg, 1963). It is thus unlikely that the pyruvate inhibition of acetate is related to the phenomenon studied by Ashworth and Kornberg. An attempt to locate the site of pyruvate inhibition was also made. Two sites of


acetate uptake (n mol/mg dry weight)
40
30
20
10
0
0
30
60
90
120
time(sec)

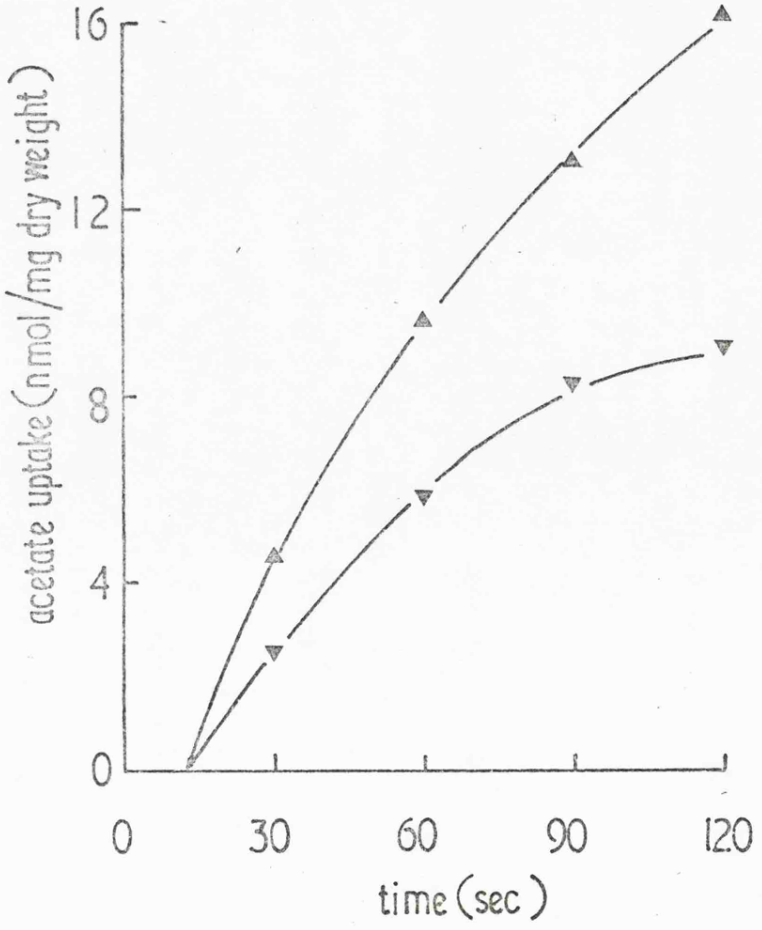

acetate uptake (n mol/mg dry weight)
16
12
8
4
0
0
30
60
90
120
time (sec)

FIG. 28

Figure 28

The effect of glucose and 2-deoxyglucose addition upon [2^{14}C]acetate uptake by suspensions of glycerol-grown strain K2. Uptake of labelled material was measured as described in Materials and Methods. 0.1 mM sodium [2^{14}C]acetate ● ; 0.1 mM sodium [2^{14}C]acetate + 1 mM glucose added 5 min prior to the acetate ■ ; 0.01 mM sodium [2^{14}C]acetate ▲ ; 0.01 mM sodium [2^{14}C]acetate + 1 mM 2-deoxyglucose added 5 min prior to acetate ▼ .

action are possible. It might act competitively at the cell surface interacting with an acetate carrier or intracellularly on some subsequent metabolic step. Evidence for an extracellular site of action would provide support for the existence of a specific transport system for acetate. The ability of pyruvate to inhibit acetate uptake in strain K2.6, a pyruvate transport-negative strain, was tested: it was found that the ability of pyruvate to inhibit uptake in this mutant was drastically reduced, but that acetate uptake was normal, demonstrating that acetate is not transported by the pyruvate system (Figure 27c). This suggests that the site of pyruvate inhibition is intracellular. The data thus agree with the incorporation data presented in Chapter II of this thesis. 10 mM pyruvate was found to inhibit acetate incorporation completely in a phosphotransacetylase-negative strain, but not in a wild-type strain. Inhibition at the transport level would be expected to be reflected in equal inhibition in both strains.

The effect of glucose on acetate uptake was also studied. Acetate is excreted by E. coli during growth on glucose and it is possible that glucose might exert a controlling effect on acetate uptake (Wood, 1961). Glucose was in fact found to cause a limited inhibition of uptake. That this inhibition of uptake was not simply caused by metabolic production of acetate from glucose is suggested by the finding that 2-deoxyglucose, a non-metabolisable analogue of glucose, caused a similar inhibition (Figure 28).

Succinate was without effect on acetate uptake and lesions in the dicarboxylic acid transport (dct) system did not modify the

FIG. 29

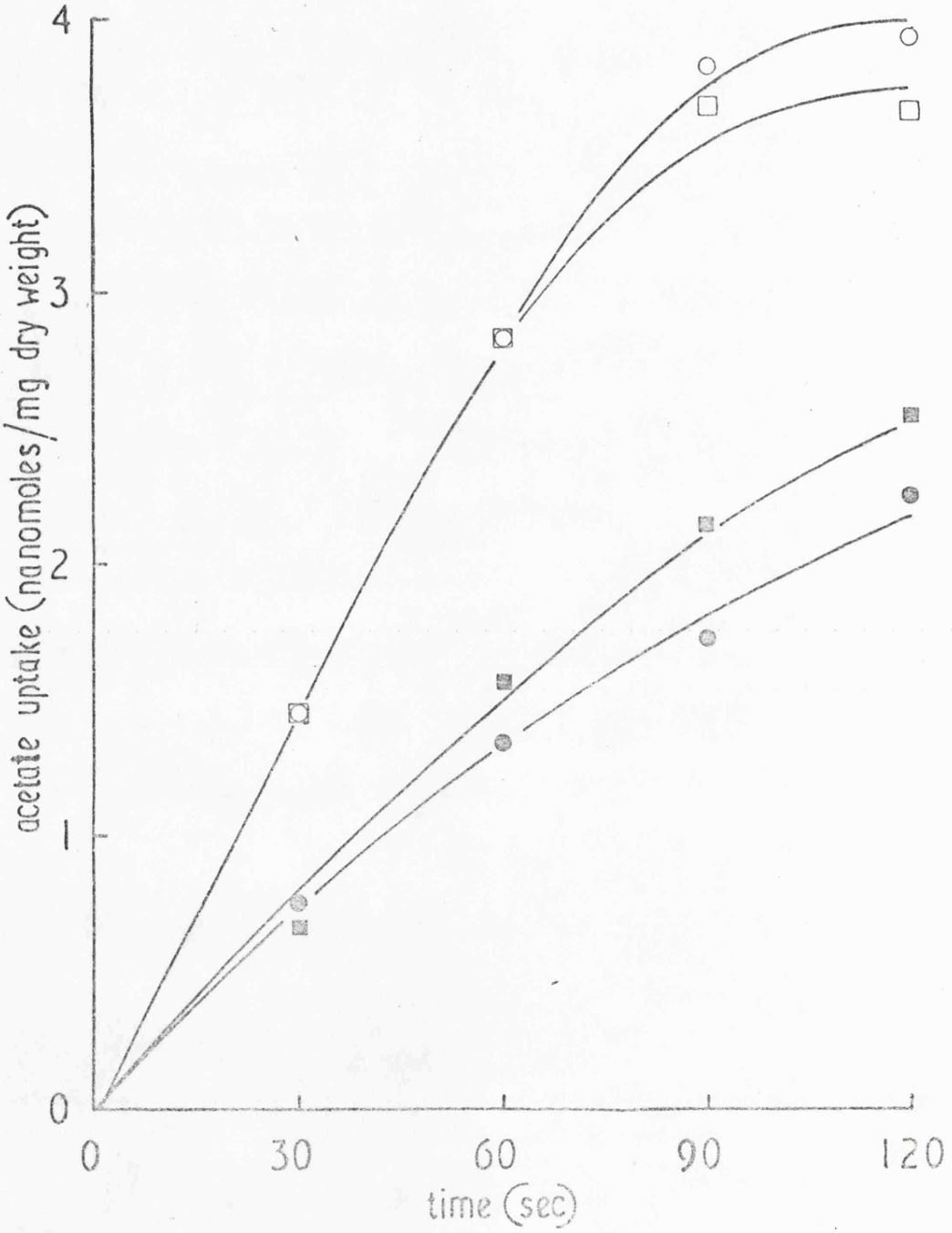

acetate uptake (nanomoles/mg dry weight)
4
3
2
1
0
0
30
60
90
120
time (sec)

Figure 29

Effect of 1 mM sodium glycollate addition upon 0.001 mM sodium [2-^{14}C]acetate uptake by K12 and K12Glc102 (USG^-). The strains were grown on glycerol. Glycollate was added at zero time. Uptake of labelled material was measured as described in Materials and Methods. K12 - no addition □ ; glycollate ■ .
K12Glc102 - no addition ○ ; glycollate ● .

ability to take up acetate (W.W. Kay, personal communication). Thus acetate is not transported by the dct system.

In an attempt to gain positive indications of the existence of an acetate transport system from competition experiments with acetate analogues, the experimental design used in the experiment on the site of pyruvate inhibition was employed. A glycollate transport-negative mutant (Ornston & Ornston, 1970) was used to test the effect of glycollate on acetate uptake. The observation of a competitive effect on uptake in the presence of a non-permeant analogue provided evidence for the existence of a specific transport system (Figure 29).

Studies of acetate transport using a citrate synthase-less strain of E. coli K12 A number of experiments were carried out in an attempt to examine permeation isolated from subsequent metabolic steps in the tricarboxylic acid cycle. The experiments described above had shown that uptake is considerably reduced in a citrate synthase-less organism (K2.1.4). The ability of the citrate-synthase-less strain to accumulate acetate is not enhanced by the addition of an energy source, such as glucose: E. coli does not accumulate acetate within the cell. It might be expected that the equilibration of acetate across the membrane could be detected in citrate synthase-less strains. However, consideration of the initial rate of acetate uptake in a wild-type strain suggests that equilibration at low concentrations of acetate (10^{-4} M) would be complete within 5 seconds at 37°C, and this estimate of the equilibration time assumes that the rate-limiting step measured in uptake experiments with the wild-type strain is

permeation. If the rate of permeation were of the order found for the permeation of mitochondria by aliphatic monocarboxylic acids ($T_{\frac{1}{2}}$ approx. 1 millisecond) then measurement of equilibration would prove impossible since the time during which the cells are exposed to the washing buffer after filtration would be long (5 seconds) compared with the equilibration time. The ability to detect equilibration of acetate across the cell membrane would in fact be an indication of a much lower rate of permeation than is found in mitochondria.

The metabolism of acetate is not, of course, completely abolished in a citrate synthase-less strain. Acetyl CoA can still be produced, but it cannot be oxidised via the tricarboxylic acid cycle. The pool level of CoA in E. coli is rather high (approx. 0.5 mM) (Alberts & Vagelos, 1966; Hansen & Henning, 1966) and acetyl CoA might be a major product of acetate uptake in a citrate synthase-less strain at low acetate concentrations, but not at higher concentrations.

A group of experiments were carried out at low acetate concentrations using phosphate buffered medium pH 7.0. The rate of uptake of labelled acetate from the medium in these experiments was extremely low compared with the rate expected from studies with wild-type strains. Control experiments with wild-type cells had shown that the glutamate required for growth of the citrate synthase-less strain did not cause a massive repression of ability to take up acetate. The rate of uptake in K2.1.4 varied with the carbon source used for growth of the cells, being more rapid in glycerol-grown than in

glucose-grown cells. Preincubation of cells in carbon-free buffer for 3 hours resulted in a change of pattern of uptake with a much more rapid movement to the calculated equilibrium level of the label now being observed. In the phosphtate buffered medium, using low concentrations of labelled acetate, DNP and azide were found to inhibit uptake after 30 minutes preincubation. The medium contained ammonium ions in molar excess of the added acetate in these experiments. High concentrations of propionate were found to inhibit acetate uptake under these conditions.

A series of experiments were also carried out to study acetate uptake at high concentrations. In most of these the cells were suspended in 50 mM sodium dimethylglutarate buffer at pH 5.0 or 7.0. The effect of pH on sodium acetate uptake by citrate synthase-less cells was tested at 1 and 50 mM. The initial rate of uptake could not be measured under the conditions employed and only the final level of label within the cell could be estimated. At both 1 and 50 mM acetate the level of label within the cells was much lower at pH 5.0 than at pH 7.0. The nature of the label present within the cells at pH 7.0 was tested after acetate uptake at both 1 and 50 mM. In both cases free acetate could be detected within the cells using thin layer chromatography of the pool material, but there was evidence of glutamate in the case of uptake carried out at 1 mM. Labelled material which did not move from the origin was also observed in the chromatographic system used (see Materials and Methods). Preincubation for 30 minutes with a mixture of ClCCP, azide and arsenate considerably reduced the level of label appearing in the cells in uptakes with 1 and 50 mM sodium acetate.

The substitution of ammonium acetate for sodium acetate stimulated the level of label inside the cells at pH 7.0 but not at pH 5.0. It is interesting that, in contrast, the level of propionate uptake at 50 mM was greater at pH 5.0 than at pH 7.0.

These experiments demonstrate some of the problems of studying acetate permeation. The lack of mutants in which acetate metabolism is abolished is a major hindrance. The inability to measure rates of equilibration across the membrane (cf. Salanitro & Wegener, 1971b) means that one is reduced to studying the effect of, for example, pH on the final level of label inside the cell which may be determined in the case of weak acids by factors such as pH gradients. The fact that inhibitors of energy metabolism affect the uptake process at all acetate concentrations tested suggests that there must be some involvment of energy in the equilibration of acetate across the membrane, but it is not clear that this is a direct effect. The possibility that specifically mediated ion fluxes, e.g. H^+, might control the movement of acetate by simple diffusion must be considered, but the ammonium ion effect suggests that this is not likely. The ability to detect free acetate within the cell suggests that the rate of equilibration across the E. coli membrane cannot be as rapid as that observed in mitochondria.

Attempts to study monocarboxylate permeation using [2-^{14}C]chloroacetate

Although competition experiments indicated that chloroacetate is a poor analogue of acetate, an attempt was made to use it to study monocarboxylate permeation as the labelled material is cheaply and readily available. The commercially available material contains traces of acetate and in the wild-type strains apparent chloroacetate

uptake was found to be due to the scavenging of traces of acetate. When a citrate synthase-less strain which cannot scavenge acetate in this way was used, the rate of uptake of label was extremely slow. Some indication that acetate would compete with chloroacetate in this system was obtained. With high carrier concentrations a slow movement to apparent equilibration of label was observed. This may represent a largely diffusional process. It might be possible to estimate the rate of diffusion of monocarboxylate across the membrane using, for example, labelled benzoic acid and thus compare non-mediated rates of equilibration with possibly mediated rates determined from simple uptake experiments.

Attempts to study acetate permeation in E. coli using the iso-osmotic swelling techniques The swelling of E. coli spheroplasts (prepared as described in Materials and Methods) in 400 mM solutions of acetate salts buffered with 20 mM potassium PIPES at pH 7.0 was studied. In potassium acetate solution, swelling was observed and this was stimulated by the addition of gramicidin D at 2.4 µg/ml. The rate of swelling observed varied from spheroplast preparation to spheroplast preparation (cf. Mitchell, 1956a). When suspended in ammonium acetate, no swelling was observed even after the addition of gramicidin D or ClCCP. This contrasts with Mitchell's (1956a) finding that E. coli was plasmolysed in concentrated ammonium acetate solution. These experiments threwno light on the mechanism of acetate permeation.

Acetate uptake in Pseudomonas aeruginosa The availability of mutants

of Ps. aeruginosa deficient in acetate thiokinase prompted the examination of acetate uptake in order to determine whether or not an active transport system for acetate exists in this organism (Skinner & Clarke, 1968). The ability of wild-type and mutant strains to take up acetate was compared. An acetate thiokinase-negative mutant AT16 was considerably reduced in its ability to take up acetate when compared with the wild-type. The level of label accumulated in the mutant did, however, represent a considerable concentration of label. The nature of this accumulated material was therefore checked by thin layer chromatography as described in Materials and Methods. Some acetate could be detected within the cells, but the major spot on the chromatogram corresponded to glutamate. The residual ability to synthesize glutamate from acetate may reflect leakiness in the acetate thiokinase lesion or the operation of an alternative activation system present at low levels. The failure to observe accumulation of acetate is interesting because of the observation that acetamide, which is less polar and therefore probably diffuses more rapidly through biological membranes than acetic acid, is actively transported by Ps. aeruginosa (Brammar et al., 1966; Stein, 1967).

These studies of acetate uptake in E. coli K12 have outlined some of the factors controlling its entry into metabolic routes, but have failed adequately to define its mechanism of permeation. No direct criteria for the existence of a specific transport system could be successfully employed and evidence that the uptake process measured at low acetate concentrations is rate-limited by a membrane permeation step rests on indirect evidence. Thus, under conditions where glycollate is a non-permeant, it was found to compete with acetate uptake. The idea

that membrane permeation is a rate-limiting step in acetate metabolism under certain conditions is supported by the observation of von Meyenburg (1971). It is, however, possible that the uptake process observed at high acetate concentrations is not rate-limited by a permeation step. The data presented demonstrate that factors affecting acetate metabolism can have powerful effects on the rates of acetate uptake by washed cell suspensions. Thus great caution must be exercised in the interpretation of data obtained from simple uptake experiments.

Lactate uptake in E. coli K12 The metabolism of D- and L-lactate by E. coli presents interesting problems (Kline and Mahler, 1965). D-lactate is a major product of glucose fermentation in E. coli under certain conditions and synthesis of D-lactate from pyruvate is catalysed by a soluble NAD-linked lactate dehydrogenase (D-lactate : NAD oxidoreductase E.C. 1.1.1.28). The oxidations of D- and L-lactate to pyruvate during growth on these compounds are catalysed by two distinct membrane-bound, flavoprotein-linked oxidases. The control of these oxidases is somewhat anomalous. Thus the D-lactate oxidase is constitutive, while the L-lactate oxidase is inducible by D- or L-lactate. The role of the D-lactate oxidase in providing energy for the active transport of certain compounds has been extensively investigated by Kaback's group (Barnes & Kaback, 1971; Kaback & Barnes, 1971). It has been suggested that its constitutivity might relate to this role. Work with mutants in the D- and L-lactate oxidases suggests that the enzymes are required for growth on their respective isomers and that metabolism therefore proceeds via the specific oxidase (Pascal et al., 1969;

C. Wenz, personal communication) rather than being funnelled through one oxidase by a racemase system.

There have been no reports on the mechanism of lactate transport in E. coli; it was therefore of interest to investigate the nature of this process. Knowledge of the relationship of its control to the role of the oxidase systems wouldbe particularly interesting. A number of problems relating to lactate transport are apparent:

1. is there a specific transport system or does lactate enter by simple diffusion?
2. given that entry is mediated by a specific system, are there separate systems for the transport of D- and L-lactate?
3. if there are two systems, what controls operate upon them? Is D-lactate transport constitutive like D-lactate oxidase,and L-lactate transport inducible like the L-lactate oxidase? Does growth on one isomer induce transport of the other?
4. given the existence of a transport system, what ionic interactions are involved in its operation?

Attempts to obtain answers to these questions have been made and some preliminary answers obtained.

A number of experimental approaches were used in attempts to demonstrate the existence of a lactate transport system and investigate its properties. Much of the evidence comes from indirect technique.

Attempts to isolate mutants defective in lactate transport An attempt was made to select mutants deficient in D- or L-lactate transport

by selection for resistance of a PEP-synthase-less strain to 10 mM D- or L-lactate while growing on acetate. Previous experience had shown that this selection procedure could yield mutants defective in pyruvate transport (Kornberg & Smith, 1967). An advantage of this selection might be that a complete abolition of uptake will not be required to obtain resistance. The lesions in lactate transport might be expected to be leaky at high lactate concentration.

E. coli K12 strain K2.1t (PPS^-) was grown on acetate medium, harvested and plated on 25 mM acetate/10 mM lithium D- or L-lactate plates. Lithium had previously been shown to be non-toxic under these conditions. Spontaneous appearance of resistant colonies was observed after 48-72 hours. A large number of colonies were picked and screened for resistance to 10 mM pyruvate while growing on 25 mM acetate. Many of the colonies were found to have remained sensitive to inhibition by pyruvate. This suggested that the lesions giving rise to resistance to lactate in these organisms involved modification of lactate utilization rather than reversion to PPS^+ or selection of isocitratelyase constitutive organisms. Growth tests of D- and L-lactate-resistant colonies on 10 mM D- and L-lactate plates to which 2 mM D-glucosamine had been added to supply gluconeogenic requirements suggested that the colonies were still able to grow slowly on D- or L-lactate. This approach is still being pursued. Alternative screening methods may have to be used as it is possible that lactate transport mutants might show slow plate growth on lactate.

The possibility of using an L-lactate specific revertant of an adenyl cyclase-negative strain (described below) for selection of

organisms resistant to DL-β-fluorolactate is also being investigated. A strain capable of using only one of the optical isomers is required for this selection.

No evidence for the existence of specific lactate transport system has come as yet from the isolation of lactate mutants, but it is hoped to follow this approach further.

Studies of lactate uptake in a mutant of E. coli K12 strain AB1621 defective in lactate metabolism Use of a mutant in which metabolism of a compound whose transport is being investigated is blocked can yield information about the presence of specific transport systems. This approach has been used to obtain evidence for the existence of a lactate transport system. Given that permeation of the cell by the compound under investigation can be measured a number of criteria for a specific system as opposed to free diffusion can be applied. In this case, evidence of saturation, competition by analogues and modification of rate of entry by growth conditions were sought. Evidence that the initial rate of uptake obtained in the blocked mutant could be correlated with initial rates observed in the wild-type was also sought.

In order to carry out these studies a mutant unable to grow on DL-lactate, AB1621 (LLO^-), was used. This strain is deficient in L-lactate oxidase and shows no growth on either D- or L-lactate. Revertants capable of growth on D-lactate are readily isolated. The most likely explanation of the inability to use both L- and D-lactate is that the organism is also impermeable to D-lactate. This cannot,

however, be readily confirmed without the use of specifically labelled isomers of lactate. Certain competition data support this explanation. Construction of a double mutant lacking both D- and L-lactate oxidase is being attempted.

[1-^{14}C]DL-lactate sodium salt was used for the uptake experiments. The lithium salts of D- and L-lactate were used as carrier. The presence of lithium ions was found not to affect lactate uptake. The use of carrier lactate prepared from the free acids resulted in inhibition of uptake which occurred only to the level normally observed in the blocked mutant. Solutions of free lactic acids are known to contain considerable quantities of lactoyl lactate and lactide (Lockwood et al., 1965) and it seems likely that one or both these compounds may effectively inhibit the further metabolism, but not entry, of lactate. It might be possible to use these compounds to study the role of the D-lactate oxidase in active transport systems.

In order to obtain a more accurate estimate of the initial rate of lactate uptake in the blocked mutant, many of the experiments to be described were carried out at 22°C. Experiments with the wild-type indicated that the rapid rate of uptake in the mutant could not be measured accurately in fully induced cells. Thus glycerol-grown cells containing uninduced, but not catabolite repressed, levels of lactate utilizing systems were frequently used. The accuracy with which initial rates of lactate uptake can be measured in AB1621 (LLO^-) precludes estimation of a K_m for lactate uptake.

As a preliminary precaution before carrying out studies of uptake with the blocked mutant, the nature of the labelled material

FIG. 30

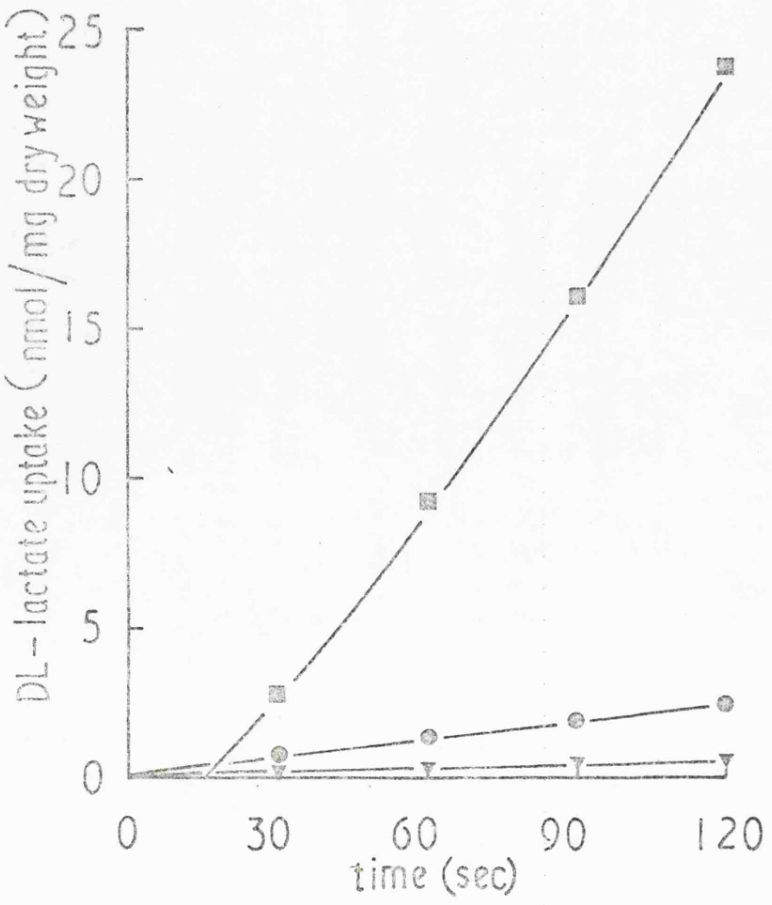
^{14}C DL-lactate uptake (nmol/mg dry weight)
0.5
1.0
0
30
60
90
120
time (sec)

^{14}C DL-lactate uptake (nmol/mg dry weight)
0.5
1.0
0
30
60
90
120
time (sec)

Figure 30

Effect of D-, L- and DL-lactate and glycollate addition upon 0.1 mM sodium [1^{14}C]DL-lactate uptake by glycerol-grown AB1621LLO (LLO$^-$). Lithium salts of lactate were used and glycollate was added as the sodium salt. All additions were made at zero time. The uptake of labelled material was measured as described in Materials and Methods with the exception that a temperature of 22^{o}C was employed. The addition of unlabelled lactate is considered to modify a rate of uptake calculated from the initial specific activity of the labelled lactate rather than from the final specific activity obtaining after the addition of unlabelled lactate, i.e. the unlabelled material is considered to compete with the labelled material.

0.1 mM sodium [1^{14}C]DL-lactate ■ ; 0.1 mM sodium [1^{14}C]DL-lactate + 2 mM L-lactate ● ; 0.1 mM sodium [1^{14}C]DL-lactate + 1 mM D-lactate ▲ ; 0.1 mM sodium [1^{14}C]DL-lactate + 2 mM DL-lactate ▽ ; 0.1 mM sodium [1^{14}C]DL-lactate + 10 mM glycollate O .


DL-lactate uptake (nmol/mg dry weight)
25
20
15
10
5
0
0
30
60
90
120
time (sec)

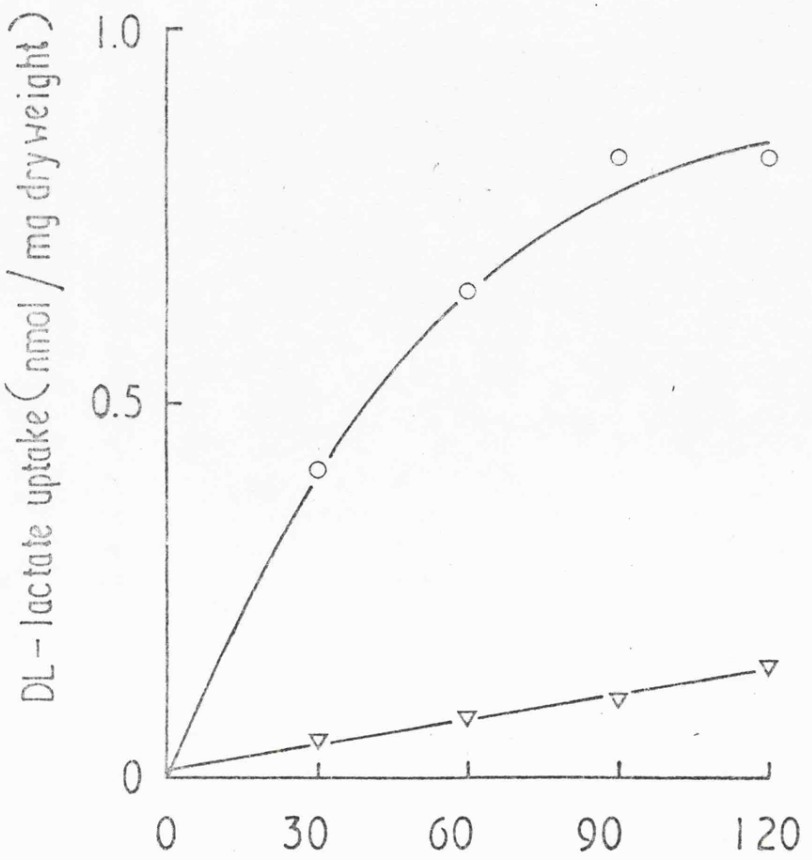

DL-lactate uptake (nmol/mg dry weight)
1.0
0.5
0
0
30
60
90
120

FIG. 31

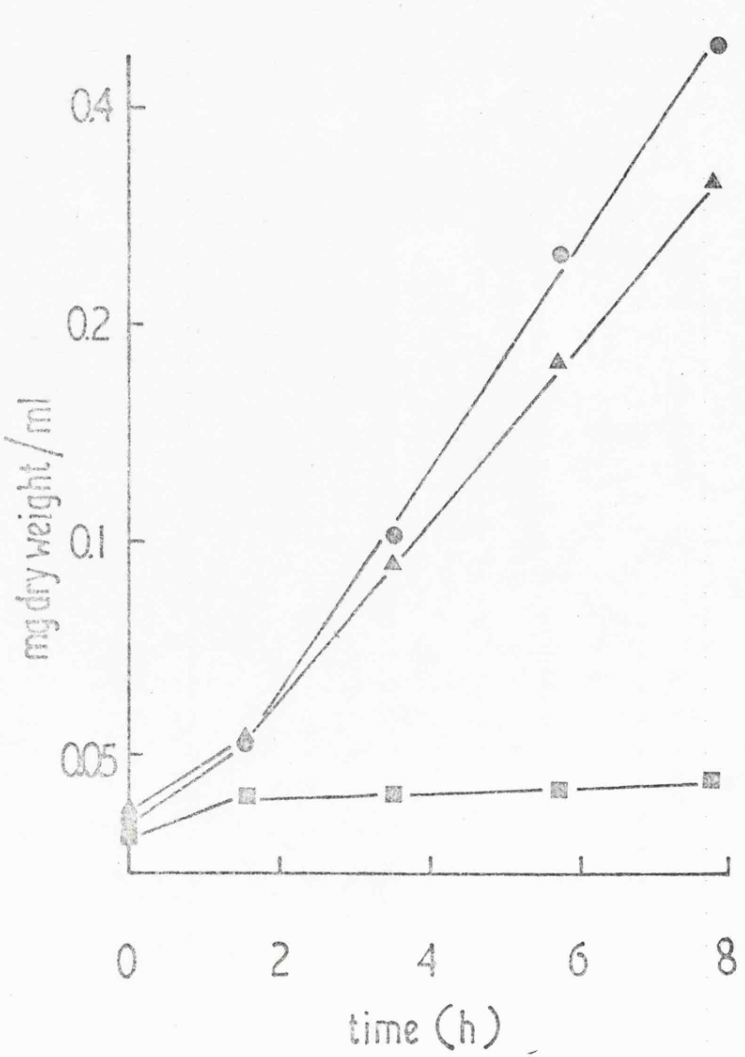
Figure 31

Effect of carbon source used for growth on the ability of washed suspensions of strains AB1621 and AB1621LLO (LLO^-) to take up 0.1 mM sodium [1-^{14}C]DL-lactate. Uptake of labelled material was measured as described in Materials and Methods with the exception that a temperature of 22° was employed. AB1621 - glucose-grown ▼ ; glycerol-grown ● ; sodium DL-lactate-grown ■ ; AB1621LLO - glucose-grown ▽ ; glycerol-grown ○ .

appearing inside the cell when the mutant was exposed to labelled DL-lactate was checked. Extraction of the labelled pool material after allowing uptake of 10^{-4} M sodium [1-^{14}C]DL-lactate to proceed for 2 minutes was carried out. Analysis by thin layer chromatography demonstrated the presence of free lactate within the cell. Binding of lactate at the origin presented problems in this chromatographic system. It thus seems likely that the process being measured in uptake experiments with the mutant blocked in lactate metabolism represents the permeation of lactate into the cell and that experiments can be carried out to test the existence of a specific transport system.

Uptake of sodium [1-^{14}C]DL-lactate by glycerol-grown AB1621 (LLO^-) showed saturation. Thus addition of 2 mM lithium DL-lactate to 0.1 mM labelled DL-lactate decreased the rate of label appearance within the cell by approximately 50%. Glycollate also acted as a weak competitor of lactate uptake at 100-fold molar excess. When 2 mM lithium D-lactate and 2 mM L-lactate were substituted for 2 mM lithium DL-lactate, more powerful inhibition was observed with D- than with L-lactate. This suggests the possibility that D-lactate may be acting as a non-permeant competitor (Figure 30).

The effect of the carbon source used for growth on DL-lactate uptake in AB1621 (LLO^-) is shown in Figure 31. Glucose has a strong repressive effect on uptake. The uptake of DL-lactate by the wild-type strain is shown for comparison. The observed rates for glucose-and glycerol-grown cells show reasonable agreement between the wild-type and the mutant. It is thus likely that the rate-limiting step in


0.4
0.2
0.1
0.05
mg dry weight / ml
0
2
4
6
8
time (h)

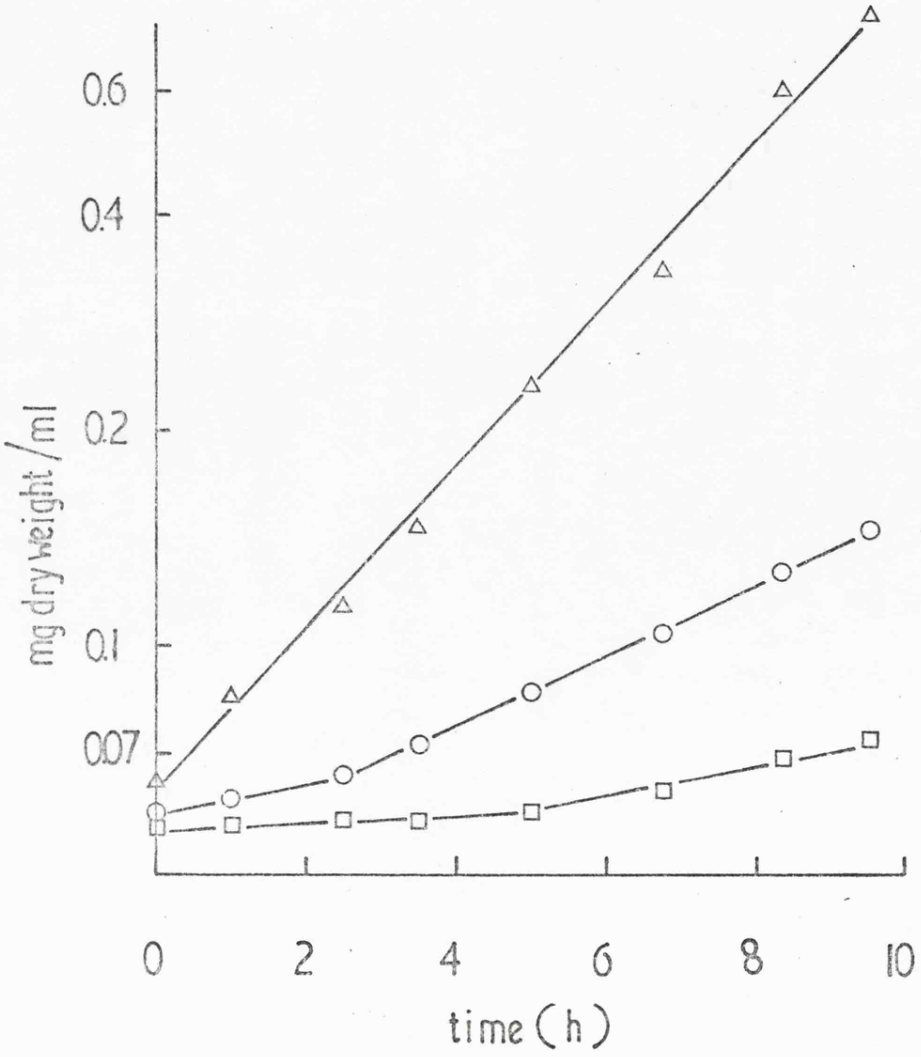

0.6
0.4
0.2
0.1
0.07
mg dry weight / ml
0
2
4
6
8
10
time (h)

FIG. 32

Figure 32

Growth properties of an adenyl cyclase-deficient mutant, 5336 (AC^-) and a mutant derived from it which is specifically resistant to catabolite repression of L-lactate utilization, 5336 L-$lact^+$ (AC^-,LCR^-). Growth was measured as described in Materials and Methods (Chapter I). Cultures of 5336 and 5336 L-$lact^+$ were pregrown on glucose, harvested, washed and transferred to fresh medium containing carbon sources at 25 mM. 5336-$lact^+$ - sodium DL-lactate ● ; glucose ▲ ; mannitol ■ . 5336 - sodium DL-lactate ○ ; glucose △ ; mannitol □ .

lactate uptake experiments under these conditions is membrane permeation and that experiments with wild-type cells could be used to study features of the membrane transport of lactate in E. coli.

Studies of lactate metabolism in an adenyl cyclase-negative mutant of E. coli K12 and in an L-lactate$^+$ revertant derived from it Further evidence for the existence of specific transport systems for D- and L-lactate in E. coli was obtained from studies of lactate growth and uptake in an adenyl cyclase-negative mutant, 5336, and an L-lactate$^+$ revertant, 5336 L-lact$^+$, derived from it. The adenyl cyclase-negative mutant was described by Perlman and Pastan (1969). They found that the mutant grew more slowly than the parent strain, 1100, on a wide range of carbon sources. When the ability of strain 5336 to grow on DL-lactate was tested, a mean generation time of approximately 6 hours was found. This compares with a value of approximately 1.5 hours for the parent strain. The slow growth of the mutant on DL-lactate was confirmed in plate tests and it proved possible to isolate a number of L-lactate$^+$ revertants of 5336. 5336 grown on glucose/nutrient broth medium was harvested and spread on 10 mM lithium L-lactate plates and incubated at 37°C for 48 hours. Spontaneous revertants to growth on L-lactate were picked and purified. Most of these had pleiotropically regained the ability to grow on a wide range of carbon sources. One isolate, however, remained negative on all other carbon sources tested including D-lactate (Figure 32). This strain was designated 5336 L-lact*. Its properties were consistent with a specific abolition of catabolite repression in the utilisation of L-lactate. Thus, upon transfer from growth on glucose, adaptation to the use of L-lactate was

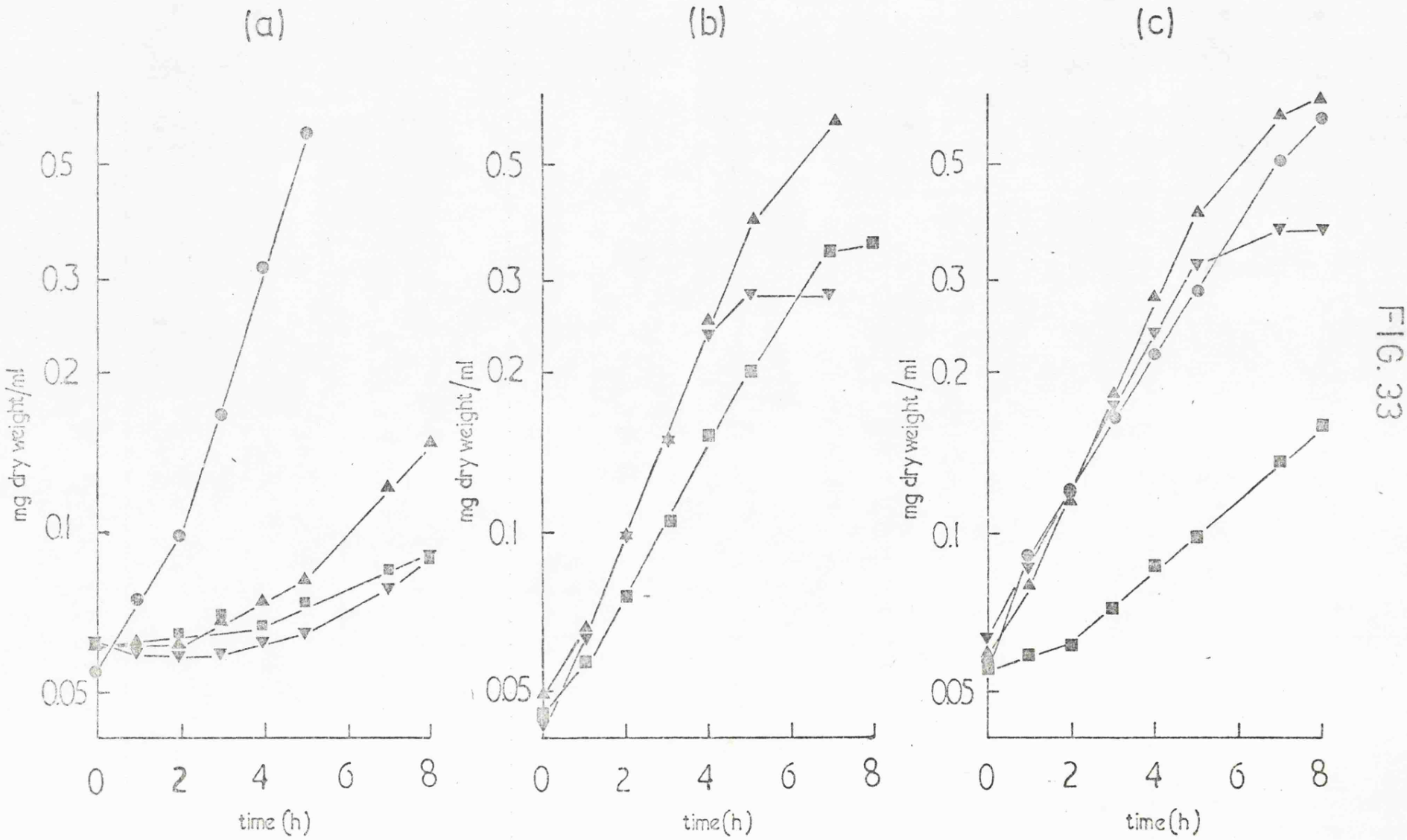


FIG. 33

Figure 33

Growth of 1100 (parent strain of 5336) and 5336 L-lact$^+$ (AC$^-$,LCR$^-$) on glucose and lactate after transfer from glucose and DL-lactate growth. 1100 was pregrown on glucose and DL-lactate and 5336 L-lact$^+$ on glucose. Cells were harvested, washed and resuspended in fresh media containing one of a number of carbon sources. Growth was measured as described in Materials and Methods (Chapter I).

(a) 1100 pregrown on 25 mM glucose and transferred to: 20 mM glucose ● ; 20 mM sodium DL-lactate ▲ ; 10 mM lithium L-lactate ▼ ; 10 mM lithium D-lactate ■ .

(b) 1100 pregrown on 25 mM sodium DL-lactate transferred to: 20 mM sodium DL-lactate ▲ ; 10 mM lithium L-lactate ▼ ; 10 mM lithium D-lactate ■ .

(c) 5336 L-lact$^+$ pregrown on 25 mM glucose transferred to: 20 mM glucose ● ; 20 mM sodium DL-lactate ▲ ; 10 mM lithium L-lactate ▼ ; 10 mM lithium D-lactate ■ .

FIG. 34

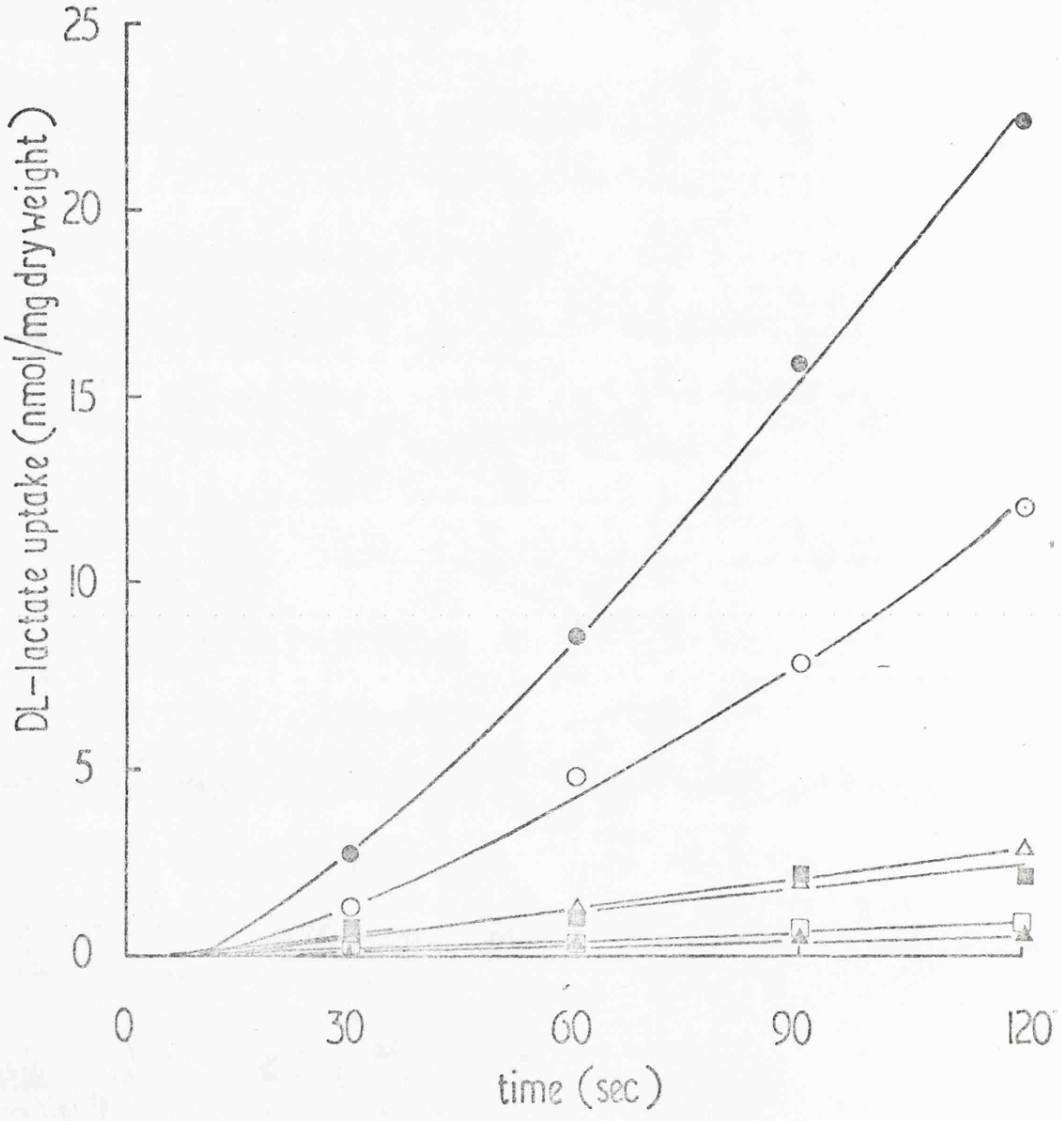

DL-lactate uptake (nmol/mg dry weight)
25
20
15
10
5
0
0
30
60
90
120
time (sec)

Figure 34

Uptake of 0.1 mM sodium [1-^{14}C]DL-lactate by suspensions of strains 1100, 5336 (AC^-) and 5336 L-$lact^+$ (AC^-,LCR^-). Uptake of labelled material was measured as described in Materials and Methods.
1100 grown on sodium DL-lactate ● ; 5336 L-$lact^+$ grown on sodium DL-lactate ○ ; 1100 grown on glycerol ■ ; 5336 L-$lact^+$ grown on glucose △ ; 1100 grown on glucose ▲ ; 5336 grown on glucose □ .

rapid in contrast to the situation observed with 1100 (Figure 33). Similarly, the uptake of DL-lactate was found to be inducible, but not subject to catabolite repression. Thus the level of uptake observed in glucose-grown 5336 L-lact$^{\pm}$ corresponded to the level of uptake observed in glycerol-grown, but not glucose-grown, strain 1100. The level of DL-lactate uptake observed in glucose-grown 5336 was similar to that observed in glucose-grown 1100. DL-lactate uptake in 5336 L-lact$^{\pm}$ grown on DL-lactate was rapid and occurred at approximately 50% of the rate found for 1100 grown on DL-lactate (Figure 34). This is in agreement with the observation that the relief of catabolite repression is observed only for L-lactate utilization. Studies of mannitol uptake in 5336 L-lact^{+} confirm the non pleiotropic nature of the L-lactate^{+} lesion. The fact that D-lactate utilization is severely impaired in 5336 even though E. coli possesses a constitutive D-lactate oxidase (Kline & Mahler, 1965) suggests that another inducible/catabolite repressible system must be involved in D-lactate utilization. Since 5336 L-lact^{+} can utilize L-lactate at wild-type rates, but cannot use D-lactate rapidly, the dysfunction in D-lactate utilization in 5336 must involve lactate permeation. These observations support the existence of specific transport systems for both D- and L-lactate in E. coli.

Oxygen electrode studies of the ability of E. coli K12 AB1621 to oxidise D- and L-lactate Another source of indirect evidence for the existence of lactate transport systems is the study of ability to oxidise D- and L-lactate after growth on different carbon sources. The

Table 14

Lactate oxidation by washed cell suspensions of strain AB1621

Carbon source used for growth	Substrate	µg atoms $O.min^{-1}.mg\ dry\ wt^{-1}$
glycerol	0.05 mM D-lactate	0.032
	0.05 mM L-lactate	0.035
	25 mM D-lactate	0.063
	25 mM L-lactate	0.063
L-lactate	0.05 mM D-lactate	0.28
	0.05 mM L-lactate	0.34
D-lactate	0.05 mM D-lactate	0.37
	0.05 mM L-lactate	0.42
DL-lactate	0.05 mM D-lactate	0.27
	0.05 mM L-lactate	0.31

Substrate-dependent oxygen consumption was measured as described in Materials and Methods. Cells were grown on 25 mM carbon sources. Lithium salts of lactate were used. Substrates were lithium salts of lactate.

ability of cells pregrown on glycerol to oxidise D- and L-lactate was compared with ability of cells pregrown on D-, L- or DL-lactate to oxidise D- and L-lactate (Table 14). It was found that glycerol-grown cells oxidised D- and L-lactate at approximately the same rate and that this rate was approximately 10% of the rate of oxidation after pregrowth on lactate. Thus, in spite of the presence of a constitutive D-lactate oxidase, the cells grown in the absence of lactate were unable to oxidise D-lactate at a rapid rate. Lactate was able to induce a system required for the oxidation of D-lactate. In glycerol-grown cells, the final common path for oxidation was not limiting since the rate of oxidation observed upon addition of both D- and L-lactate was equal to the sum of the individual rates. Pregrowth on L-lactate induced the ability to oxidise both L- and D-lactate and pregrowth on D-lactate also induced the ability to oxidise both isomers. This suggests that the transport systems for D- and L-lactate uptake can be induced by either D- or L-lactate (cf. Kline & Mahler, 1965). It is not possible to eliminate the possibility that traces of contamination with the other isomer might be responsible for the induction. Attempts to decryptify lactate oxidation in uninduced cells by the addition of toluene were unsuccessful (cf. Jackson & DeMoss, 1965).

Studies of the effects of agents modifying energy transduction upon uptake in E. coli K12 AB1621 The intracellular levels of lactate label observed in AB1621 (LLO^-) do not rise above approximately 2-3 times the level in the surrounding medium. The uptake process does not therefore correspond to the typical active transport system and it does

not seem likely that the small concentration gradient observed is maintained by the same type of mechanisms operating to maintain the large concentration gradients observed in, for example, amino acid transport. The ability of cellular constituents to bind anions and a relatively small error in the estimation of the intracellular volume used to calculate the intracellular concentration could explain the small apparent concentration gradient observed and it seems likely that the system can be considered as a facilitated diffusion (cf. Kay & Kornberg, 1971) system.

It seems likely that, at pH 7.0, the pH of the medium routinely used for uptake studies, the species most likely to be transported across the cell membrane by a specific system would be the lactate anion. This raises the question of how lactate permeation might be linked to other cation and anion fluxes across the membrane.

As the lactate transport system appears to involve facilitated diffusion rather than active transport, it might a priori seem unlikely that metabolic inhibitors known to act directly on energy metabolism would have any effect on lactate permeation. In view of the possibility that linkage between lactate permeation and other ionic fluxes might occur preliminary studies of the effect of inhibitors and uncouplers of electron transport, agents inhibiting membrane ATPase and ionophores were undertaken. Claims have been made that the facilitated diffusion of neutral species in energy poisoned cells can be made energy dependent if the energy depletion process is taken far enough (Koch, 1971). Many reports have indicated that, under 'normal' conditions of energy-poisoning, the transporters are capable of operating at high rates (Pavlasova & Harold, 1969). Preliminary attempts have

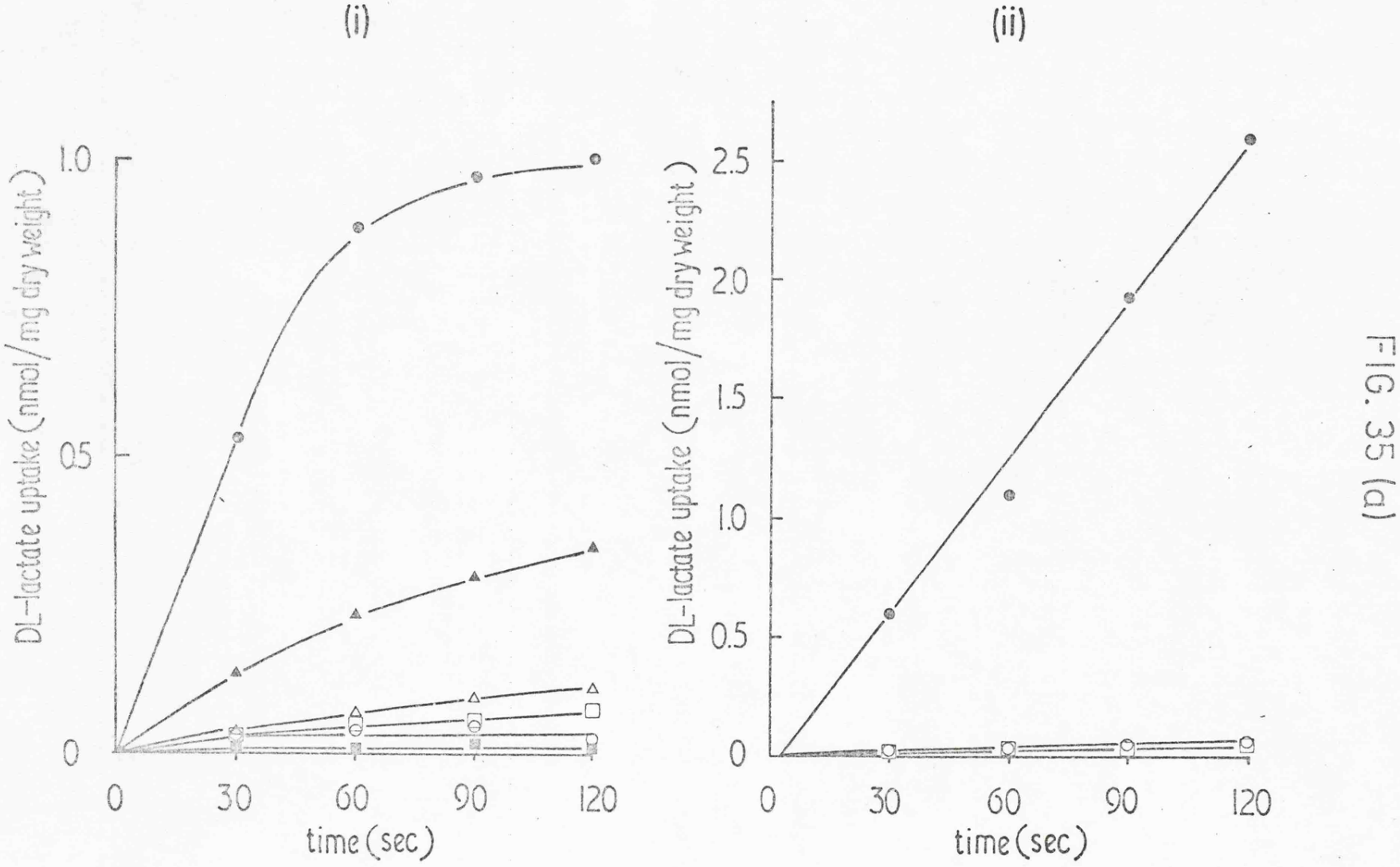
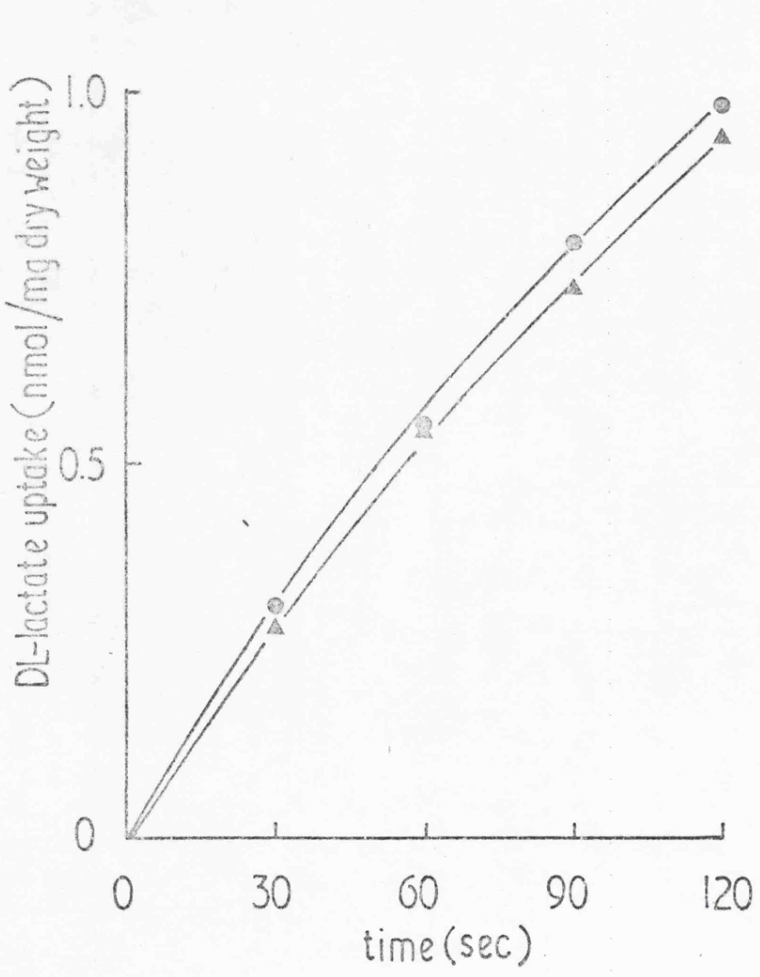

(i)
(ii)
DL-lactate uptake (nmol/mg dry weight)
1.0
0.5
0
0
30
60
90
120
time (sec)
2.5
2.0
1.5
1.0
0.5
0
0
30
60
90
120
time (sec)

FIG. 35 (a)


DL-lactate uptake (nmol/mg dry weight)
1.0
0.5
0
0
30
60
90
120
time (sec)

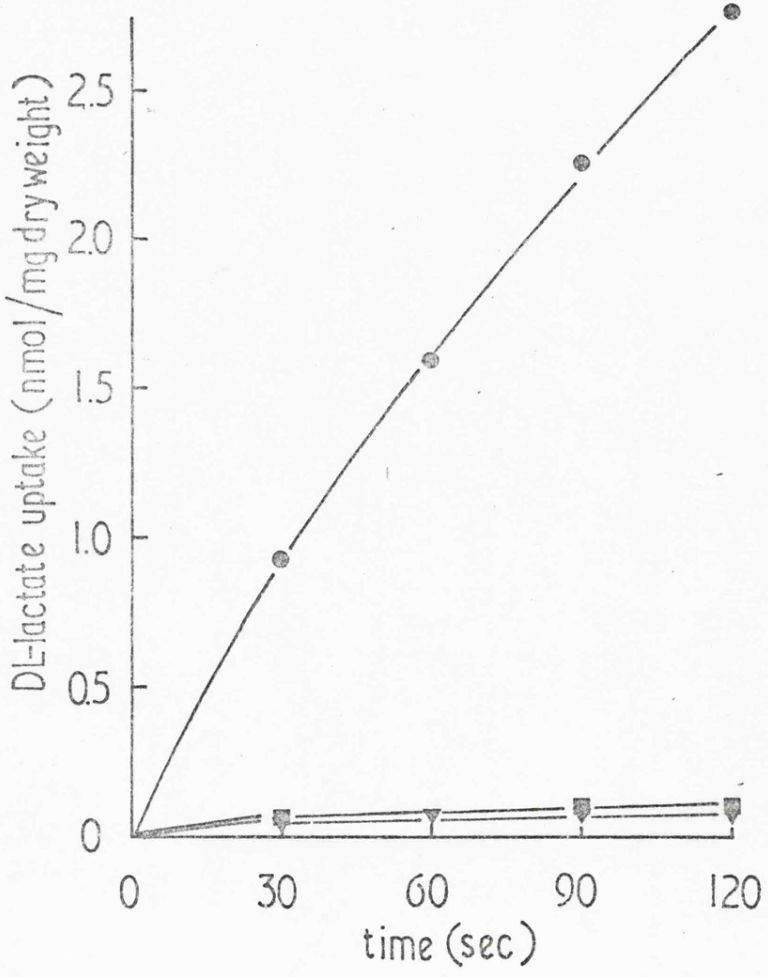

DL-lactate uptake (nmol/mg dry weight)
2.5
2.0
1.5
1.0
0.5
0
0
30
60
90
120
time (sec)

FIG. 35 (b)

Figure 35

(a) Effect of inhibitors and uncouplers of energy metabolism on the uptake of 0.1 mM sodium [1-^{14}C]DL-lactate by strains AB1621LLO (LLO$^-$) and AB1621. Both strains were grown on glycerol. Uptake of labelled material was measured as described in Materials and Methods with the exception that a temperature of 22°C was employed.

(i) AB1621LLO - no addition ● ; 4 mM DCCD 30 min preincubation ▲ ; 0.001 mM C1CCP 5 min preincubation △ ; 30 mM sodium azide 5 min preincubation □ ; 1 mM DNP 5 min preincubation ○ ; 1% toluene 5 min preincubation ■ .

(ii) AB1621 - no addition ● ; 30 mM sodium azide 5 min preincubation □ ; 1 mM DNP 5 min preincubation ○ .

(b) Effect of gramicidin D on 0.1 mM sodium [1-^{14}C]DL-lactate uptake in untreated and in DNP-inhibited cells of glycerol-grown AB1621LLO. Uptake of labelled material was measured as described in Materials and Methods with the exception that a temperature of 22°C was used. No addition ● ; 6 μg/ml gramicidin D 5 min preincubation ▲ ; 1 mM DNP 5 min preincubation ■ ; 1 mM DNP + 6 μg/ml gramicidin D 5 min preincubation ▼ .

also been made to study the effect of modifying the intra- and extracellular ionic composition upon lactate uptake.

The uncouplers DNP and ClCCP were extremely effective inhibitors of DL-lactate permeation in AB1621 (LLO^-) at concentrations of 10^{-3} and 10^{-6} M respectively. Azide was a powerful inhibitor at 30 mM. (Azide has an uncoupling effect at this concentration). These are concentrations typically effective against transport processes in E. coli. The effect of a 30 minute preincubation with 4 mM dicyclohexylcarbodiimide (DCCD) was also tested. Inhibition was observed although it was not as powerful as that given by azide and the uncouplers. DNP and azide were also effective against lactate uptake in wild-type cells (Figure 35a). These observations suggest that permeation of lactate might involve an energy-dependent process even though there does not appear to be a significant accumulation of lactate within the cell under the conditions tested. Some evidence has been put forward which suggests that uncouplers might have direct effects on membranes unrelated to their role as proton conductors (Weinbach & Garbus, 1968a, 1968b). The possibility that permeation might involve cation symport was investigated. If the inhibitory effect of an uncoupling agent was due to the inhibition of movement of alkali metal cations across the membrane, an agent capable of transporting such ions across the membrane might be expected to release the inhibition. Gramicidin D is an alkali metal and proton ionophore of relatively low specificity (Henderson, 1971). The effect of gramicidin D on lactate uptake in the presence and absence of an inhibitor concentration of DNP was therefore tested. Control experiments demonstrated an inhibitory effect of gramicidin D on L-leucine uptake in Tris-EDTA treated cells

(Leive, 1968). There was no effect on the uptake of 2-deoxyglucose. Tris-EDTA treatment is required to make E. coli and other gram-negative bacteria permeable to peptide ionophores such as gramicidin D and valinomycin (Harold, 1970). Gramicidin D was without effect on lactate uptake and failed to reverse the inhibition due to DNP (Figure 35b). This suggests that the inhibition by DNP is not simply due to an inhibition of cation symport. The proton-conducting role of uncouplers (Mitchell & Moyle, 1967 ; Harold & Baarda, 1968; Pavlasova & Harold, 1969) also suggests that the inhibition of proton symport cannot be important in the inhibition of lactate uptake by DNP and ClCCP. The effect of sodium and potassium ion levels in the medium was tested. No dramatic effects were observed. An attempt to examine the possible role of anion-anion antiport was made by substituting the poorly permeant maleate anion for the phosphate normally used in uptake experiments. In an attempt to ensure that endogenous permeant anions did not supply anions for antiport the cells were subjected to cold osmotic down shock from basal medium into distilled water. This removed a considerable quantity of nucleotide material from the cells as evidenced by measurements of the A_{260} and A_{280} of the medium after down shock. The ability of cold water shock to remove the pool material accumulated during acetate uptake was also checked. The shocked cells were resuspended in Tris-maleate buffer or phosphate buffer and uptake experiments carried out. Essentially, no difference between the uptake carried out in Tris-maleate buffer and that carried out in phosphate buffer was observed. A role for endogenously generated anions, for example, bicarbonate, cannot be eliminated by this experiment.

FIG. 36

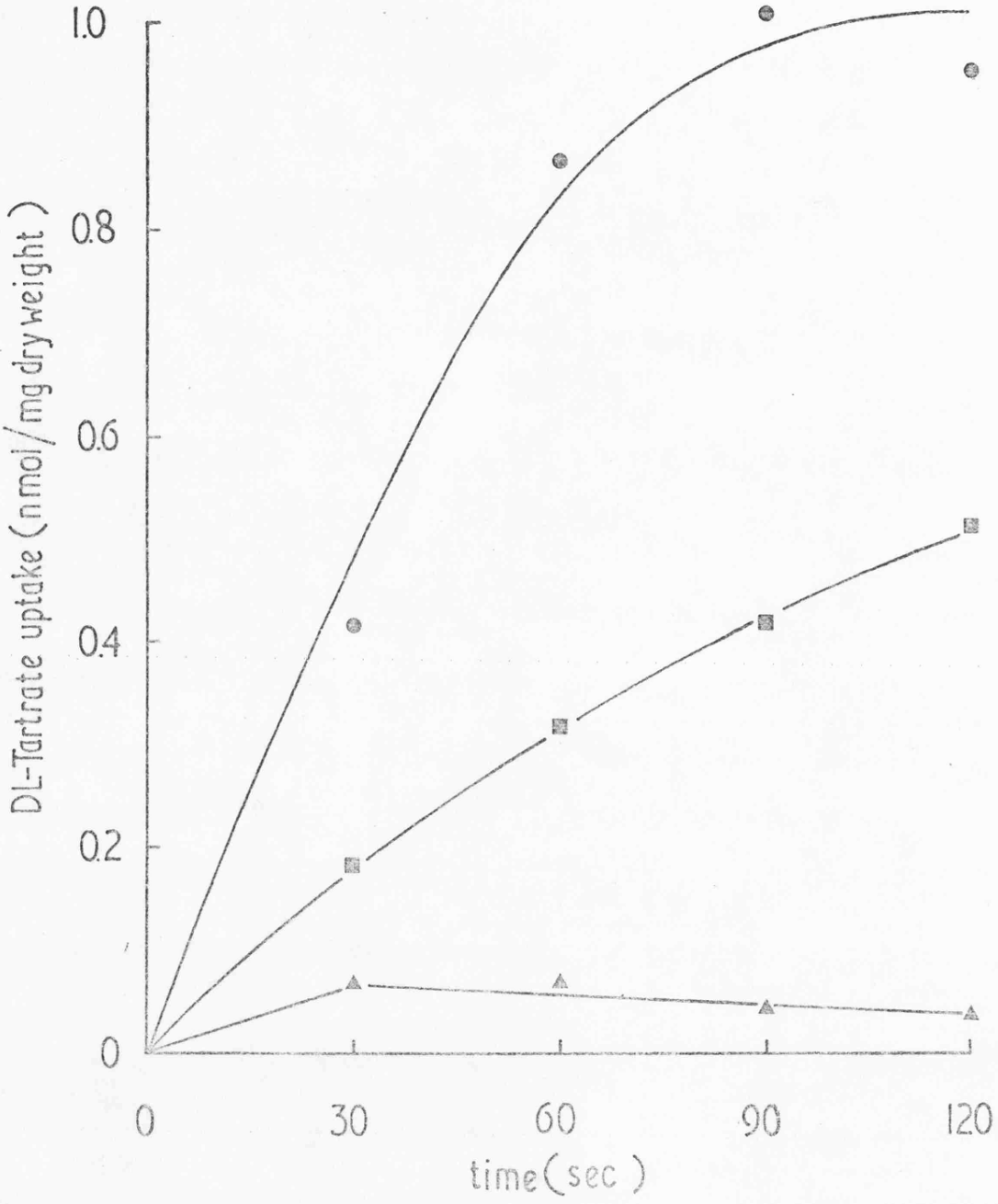

1.0
0.8
0.6
0.4
0.2
0
DL-Tartrate uptake (n mol/mg dry weight)
0
30
60
90
120
time (sec)

Figure 36

Effect of DNP and DCCD on the uptake of 0.1 mM [1,4 ^{14}C]DL-tartaric acid by strain K2.1.4 (PPS^-,CS^-) grown on nutrient broth containing 5 mM glutamate and 20 mM L-malate. Uptake of labelled material was measured as described in Materials and Methods with the exception that a temperature of 22°C was used. No addition ● ; 1 mM DCCD 5 min preincubation ■ ; 1 mM DNP 5 min preincubation ▲ .

In order to test whether or not the inhibition of lactate uptake by uncouplers and inhibitors of electron transport was a general feature of carboxylate anion permeation experiments on DL-tartrate uptake were carried out. DL-tartrate is a non-metabolisable analogue of the dicarboxylic acids normally taken up by the dct system (Kay & Kornberg, 1971). The extremely slow residual metabolism can be essentially eliminated if a citrate synthase-less strain is used. E. coli K12 strain K2.1.4 was therefore grown on nutrient broth/malate/glutamate medium, the C_4-acid being added to induce the dct system. The uptake of DL-tartrate was readily measured and was found to be powerfully inhibited by 1 mM DNP. DCCD also inhibited the uptake (Figure 36). Extraction of the pool and thin layer chromatography of it demonstrated that the label present inside the cell was tartrate. It thus seems likely that the effects observed on DL-lactate uptake are more generally observed with carboxylate permeation in E. coli.

A potentially interesting observation in relation to the possible role of electron transport processes in lactate uptake and to the role of lactate oxidases in the uptake of other compounds was made when an attempt to study the kinetics of lactate uptake at various concentrations was made using cells of AB1621 grown on DL-lactate. The initial rates obtained were found to be low compared to the rates observed after 2 minutes. An autocatalytic effect has been observed in lactate oxidation by Azotobacter vinelandii (Knowles & Smith, 1971).

FIG. 37

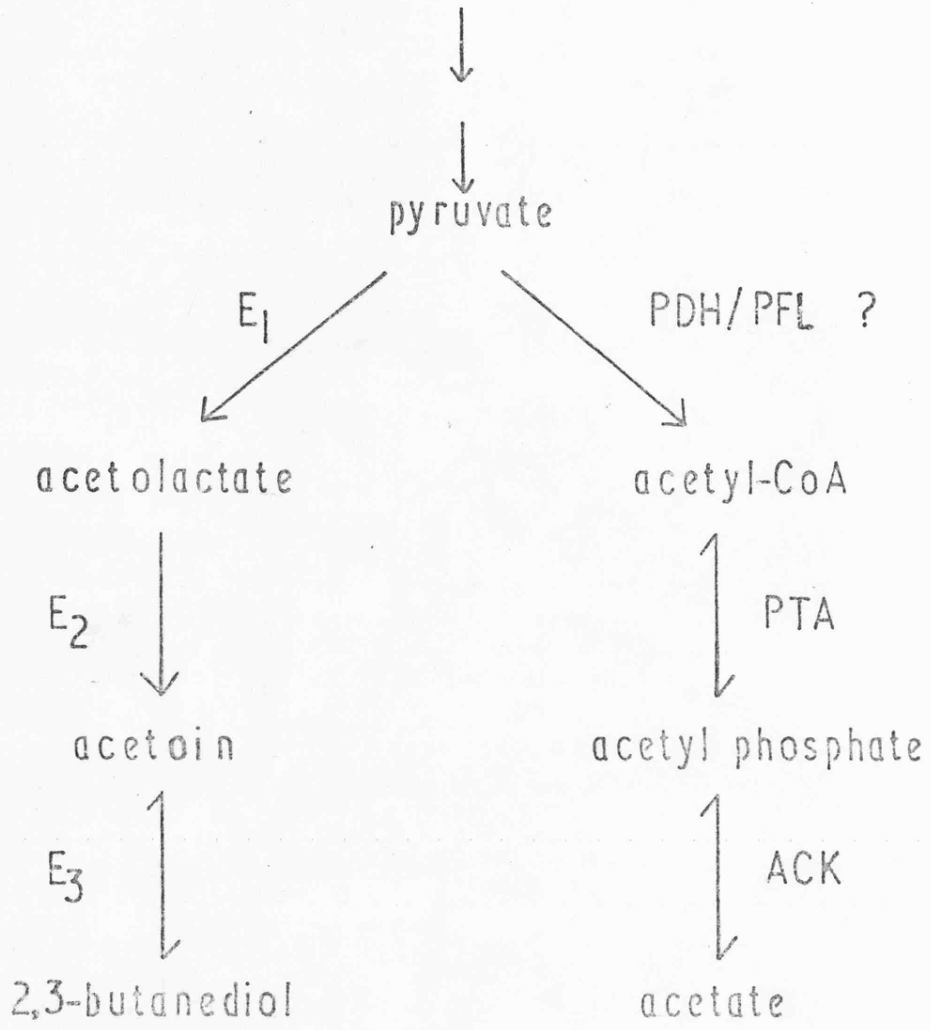

pyruvate
E1
PDH/PFL ?
acetolactate
acetyl-CoA
E2
PTA
acetoin
acetyl phosphate
E3
ACK
2,3-butanediol
acetate

Figure 37

Pathway of acetate and 2,3-butanediol formation in A. aerogenes. ACK = acetate kinase, PTA = phosphotransacetylase, E_1 = pH 6 acetolactate-forming enzyme, E_2 = acetolactate decarboxylase, E_3 = diacetyl (acetoin) reductase, PDH = pyruvate dehydrogenase complex and PFL = pyruvate formate lyase system.

CHAPTER IV

Studies of the acetate kinase/phosphotransacetylase and the 2,3-butanediol-forming systems in Aerobacter aerogenes 1033

Introduction

Many homologies have been found between metabolic processes in E. coli and A. aerogenes but certain broad differences distinguish the organisms. One of these differences, which is used as a taxonomic indicator for the separation of coliform and aeroform types, involves the fermentative properties of the groups. Thus A. aerogenes can ferment glucose to produce acetoin and 2,3-butanediol, but E. coli cannot. Other differences in the pattern of fermentation products are also observed (Wood, 1961).

The production of acetoin and 2,3-butanediol from pyruvate in A. aerogenes involves three enzymes, the pH 6 acetolactate-forming enzyme, acetolactate decarboxylase and 2,3-butanediol dehydrogenase (Figure 37) (Størmer, 1967; Løken & Størmer, 1970; Bryn, Hetland & Størmer, 1971). The synthesis of these enzymes is coordinately induced by acetate in the presence of a metabolisable carbon source, such as glucose (Størmer, 1968a). Besides its role in the coarse control of the 2,3-butanediol-forming pathway, acetate also appears to have a role in its fine control. Acetate has been shown to be an activator of the pH 6 acetolactate-forming enzyme (Størmer, 1968b). The saturation curve for pyruvate is hyperbolic in acetate buffer, but

sigmoidal kinetics are obtained in other buffers. The enzyme has a sharp pH optimum at pH 5.8 in acetate buffer. No effect of acetate on diacetyl (acetoin) reductase is observed if the reaction is examined in the direction of acetoin reduction. In the reverse direction, however, the K_m for 2,3-butanediol increases about 10-fold in acetate as compared with phosphate buffer at pH 5.8, but no effect of acetate is observed at pH 7.0 (unpublished).

These observations of the interaction between acetate and the enzymes of the acetoin/2,3-butanediol-forming system prompted an examination of certain properties of the acetate kinase/phosphotransacetylase system in A. aerogenes and their relationship to those of the butanediol pathway. The controls acting on the fermentative systems producing acetate, and acetoin and 2,3-butanediol, might be expected to show certain correlations in their properties. The study of the control of fermentative enzymes in the Enterobacteriaceae has been limited. Much work has been carried out on factors controlling the spectrum of fermentation products, but relatively little is known of how the production is controlled at the enzyme level. It seemed likely that useful information on the control of the synthesis of fermentation products could be obtained from integrated studies of the acetate kinase/phosphotransacetylase and of the acetoin/2,3-butanediol-forming system of A. aerogenes.

Studies of the products of glucose fermentation and the external factors modulating their synthesis reveal interesting differences between A. aerogenes and E. coli. Qualitative differences, such as the absence of the butanediol-forming enzymes from E. coli, exist alongside the quantitative differences in the amounts of products

formed. The response of the fermentative mechanisms to variations in external factors, such as oxygen concentration and pH, also differs in the two species.

One of the most important differences between A. aerogenes and E. coli fermenations of glucose lies in the response to the pH of the medium. E. coli shows relatively little difference in the products formed at acid or alkaline pH with the exception that formate is produced at alkaline pH, but replaced by hydrogen and carbon dioxide at acid pH (Blackwood, Neish & Ledingham, 1956; Gray et al., 1966a). A. aerogenes shows considerable differences (Mickelson & Werkman, 1938; Harrison & Pirt, 1967). The major difference lies in the virtual absence of butanediol production at pH 7.4 which contrasts with its role as the major fermentation product at pH 6.0. Harrison and Pirt (1967) used continuous culture techniques to investigate the effect of different forms of growth limitation on the products. Oxygen concentration and medium pH emerged as the most critical factors.

Preliminary investigations of the coarse control mechansism acting on the acetate and butanediol-forming systems of A. aerogenes 1033 have been carried out in collaboration with Dr. F.C. Størmer (Universitetet i Oslo). The role of the acetate kinase/phosphotransacetylase system was investigated using mutants devoid of these enzymes. Mutants in acetate activation were used to study the nature of the inducer of the enzymes of the butanediol-forming pathway.

Materials and Methods

Organisms The organisms used in this chapter are listed in Table 1.

Growth of organisms *A. aerogenes* 1033 and derivatives prepared from it were grown on minimal medium supplemented with trace elements (Ashworth & Kornberg, 1966). Carbon sources were added at 50 mM unless otherwise stated. Cultures for enzyme assays were grown either aerobically in a rotary shaker at 37°C or anaerobically in a fermenter sparged with 95% N_2/5% CO_2 of the purest grade available. During anaerobic growth, the pH of the medium was kept constant using a Titrator TTr2 (Radiometer Ltd., Copenhagen, Denmark) connected to an Autoburette ABU 13 obtained from the same Company. Additions of 0.1 M NaOH were therefore made automatically to compensate for the acid produced during fermentation. Studies of aerobic and anaerobic growth rates were carried out as described for *E. coli* in Chapter I.

Enzyme assays Cell-free extracts for the assay of acetate kinase and phosphotransacetylase were prepared in 0.1 M Tris-HCl, 10 mM $MgCl_2$ and 1 mM sodium EDTA buffer pH 7.5 as described in Chapter I. 50 mM potassium phosphate buffer was used to prepare the extracts for assay of diacetyl (acetoin) reductase. Acetate kinase and phosphotransacetylase were assayed as described in Chapter I. Phosphotransacetylase was also assayed by the method of Bergmeyer (1963). Diacetyl (acetoin) reductase was measured by following the formation from 2,3-butanediol and NAD. The incubation mixture contained in 1 ml: 1.5 μmoles NAD, 100 μmoles

2,3-butanediol and 50 μmoles potassium phosphate buffer pH 7.0. Protein was estimate by the method of Lowry et al. (1951).

Selection of mutants Mutants devoid of acetate kinase, phosphotransacetylase,or both activities, were selected by plating approximately 10^8 cells on 50 mM DL-lactate plates containing 50 mM fluoroacetate. Spontaneously resistant colonies whichappeared after approximately 48 h were picked and purified by repeated streaking for single colonies.

Results and Discussion

The role of the acetate kinase/phosphotransacetylase system in A. aerogenes. Studies with fluoroacetate-resistant mutants The action of acetate in controlling the production of acetoin and butanediol by A. aerogenes is dependent on the functioning of the acetate excretory system. The properties of mutants devoid of acetate kinase and phosphotransacetylase were therefore studied in order to confirm the amphibolic role of this system. It is also of interest to compare the properties of the mutants with those isolated in E. coli K12.

Assay of the levels of acetate kinase and phosphotransacetylase in fluoroacetate-resistant mutants of A. aerogenes revealed three classes of mutant: acetate kinase-negative, phosphotransacetylase-negative and acetate kinase/phosphotransacetylase-negative. The isolation of mutants devoid of both enzymes by spontaneous mutation to fluoroacetate resistance suggests that the genes specifying their synthesis may be closely linked or under coordinate control. No examples of this class

Table 15 Growth properties of fluoroacetate-resistant mutants of *A. aerogenes* 1033

Organism	Mean generation time on acetate	Mean generation time on glucose/bicarbonate
wild type	3 h	1 h
FAc8 (ACK^-)	c.16 h	2.5 h
FAc6 (PTA^-)	c.16 h	2.5 h
FAc7 (ACK/PTA^-)	c.16 h	2.5 h

Growth on acetate was measured in cultures shaken vigorously at 37°C. Sodium acetate was added at 50 mM. Growth was measured as described in Materials and Methods (Chapter I). Anaerobic growth was measured at 37°C in cultures sparged continuously with 95% N_2 ; 5% CO_2, as described in Materials and Methods (Chapter I). The medium contained 25 mM glucose and 25 mM sodium bicarbonate.

of mutant were isolated in E. coli K12. Only a limited number of transducing phages are available for A. aerogenes strains and these have a restricted host range. Transduction of Klebsiella pneumoniae by phage P1 has been reported (Streicher, Gurney & Valentine, 1971) but it was found to be ineffective against A. aerogenes 1033. It is thus impossible to carry out linkage studies by conventional techniques to determine whether the structural genes for acetate kinase and phosphotransacetylase are in fact closely linked in A. aerogenes.

The growth properties of the mutants were studied under conditions where one or other of the dual roles of the acetate kinase/phosphotransacetylase might be expected to be significant (Table 15). The growth of a representative of each class of mutant was tested on acetate. Under these conditions, the system might be expected to function in the activation of acetate. The mutants were essentially acetate-negative even after prolonged incubation in acetate medium. This would suggest that no acetate activating system other than the acetate kinase/phosphotransacetylase system operates at a significant level under these conditions. Acetate kinase and phosphotransacetylase-negative mutants of Brevibacterium flavum are also acetate-negative (Shiio et al., 1969) but acetate kinase-negative mutants of E. coli K12 show slow, but logarithmic, growth on acetate (see Chapter I). The growth of the mutant strains on glucose/sodium bicarbonate medium under anaerobic conditions was also studied (Table 15). During the fermentation of glucose the excretory function of the acetate kinase/phosphotransacetylase system might be expected to be important. There was a considerable increase in the mean generation time of all classes of mutant compared with the wild-type under these growth conditions and

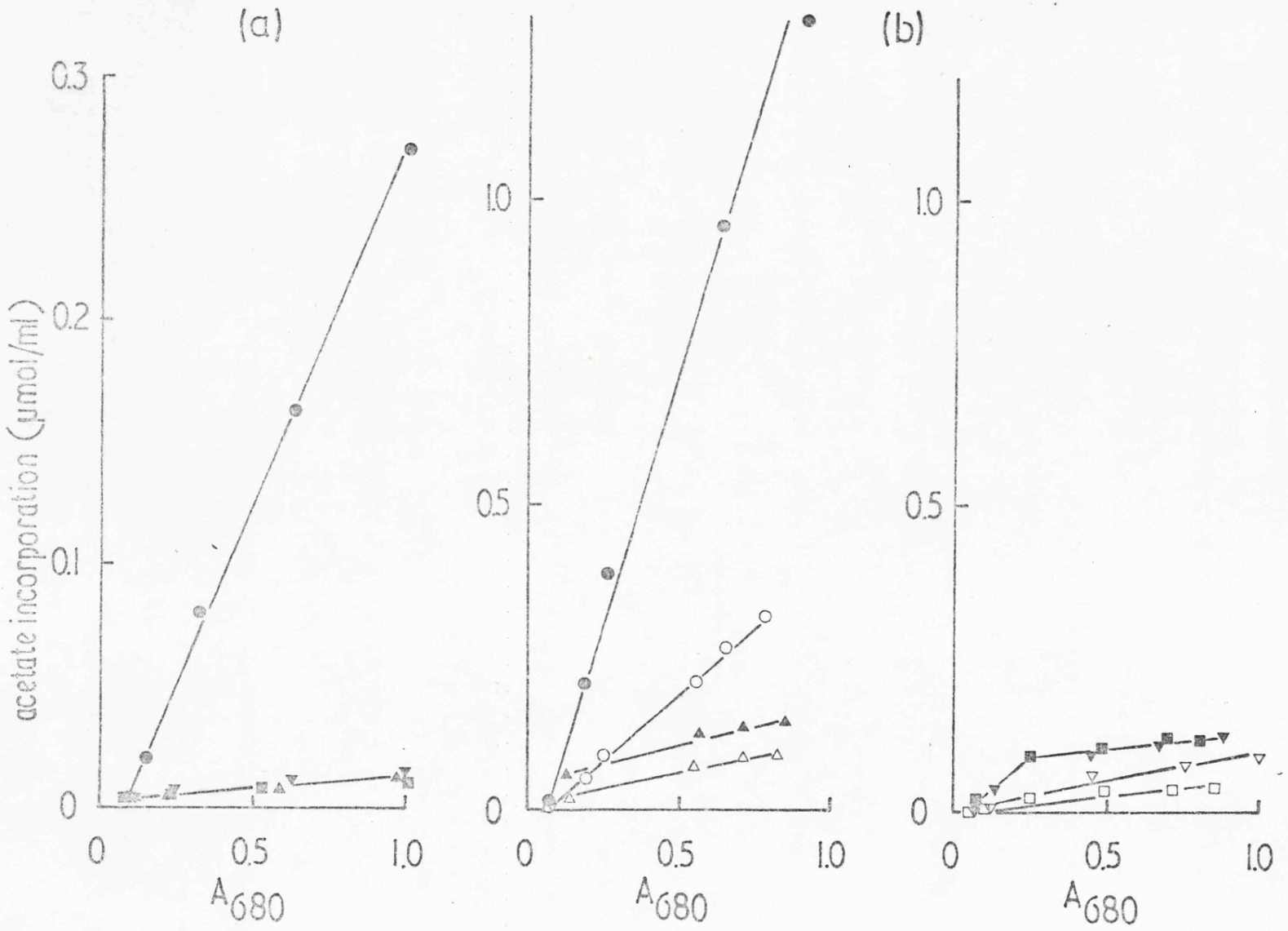


FIG. 38

Figure 38

Incorporation of [2^{14}C]acetate by mutants of A. aerogenes 1033 devoid of acetate kinase and phosphotransacetylase activity.

Incorporation of radioactive label into cell material was measured as described in Materials and Methods (Chapter I).

(a) Incorporation of 2 mM sodium [2^{14}C]acetate by A. aerogenes 1033 ● ; FAc6 (PTA$^-$) ▲ ; FAc7 (ACK/PTA$^-$) ▼ ; FAc8 (ACK$^-$) ■ ; during growth on 25 mM glucose after pregrowth on glucose.

(b) Incorporation of 2 mM and 20 mM sodium [2^{14}C]acetate by A. aerogenes 1033 ○ (2 mM); ● (20 mM) ; FAc6 (PTA$^-$) △ (2 mM); ▲ (20 mM); FAc7 (ACK/PTA$^-$) ▽ (2 mM); ▼ (20 mM); FAc8 (ACK$^-$) □ (2 mM); ■ (20 mM).

Table 16 Levels of acetate kinase and phosphotransacetylase in cultures of A. aerogenes 1033 grown on various carbon sources.

Carbon source	specific activity	
	acetate kinase	phosphotransacetylase
glucose	1.7	2.4
glycerol	1.3	2.1
pyruvate	5.8	8.4
acetate	1.0	1.7

Cultures were grown with vigorous aeration to an A_{680} of 1. Carbon sources were added at 50 mM. Cell-free extracts were prepared and enzyme levels estimated as described in Materials and Methods. Phosphotransacetylase activities were measured using the coupled assay. Specific activities are expressed as μmol acetyl hydroxamate formed/min/mg protein (acetate kinase) and μmol NADH formed/min/mg protein (phosphotransacetylase).

this confirms the role of the acetate kinase/phosphotransacetylase system in fermentative metabolism. These data suggest that the system has an amphibolic role in A. aerogenes.

Incorporation experiments in which cells were exposed to labelled acetate at 2 and 20 mM while growing on glucose or glycerol were carried out. They demonstrated the abolition of essentially all acetate activating ability in the mutants during growth on both glucose and glycerol (Figure 38). Pregrowth on glycerol/acetate medium failed to stimulate incorporation. The results of the incorporation experiments show a good correlation with the acetate growth properties. The properties of the mutants in A. aerogenes contrast with those of the mutants in E. coli K12. Incorporation data from acetate kinase-less strains of E. coli K12 provided evidence for a second acetate activation system. In contrast, only low levels of acetate thiokinase activity were detected in cell-free extracts prepared from an acetate kinase-less strain of A. aerogenes grown on glycerol/acetate medium.

Variation in acetate kinase and phosphotransacetylase levels with growth conditions The possibility that the coarse control properties of the acetate kinase/phosphotransacetylase system might reflect its role in fermentative metabolism and, more particularly, its relationship to the control of the acetoin/2,3-butanediol-forming pathway, was investigated. Preliminary studies of the levels of acetate kinase and phosphotransacetylase in cultures grown on various carbon sources were carried out (Table 16). Coordinate expression of the enzyme activities is observed. Elevated levels of the enzymes were found in cultures grown on pyruvate. Pyruvate lies at a key branch point in

FIG. 39

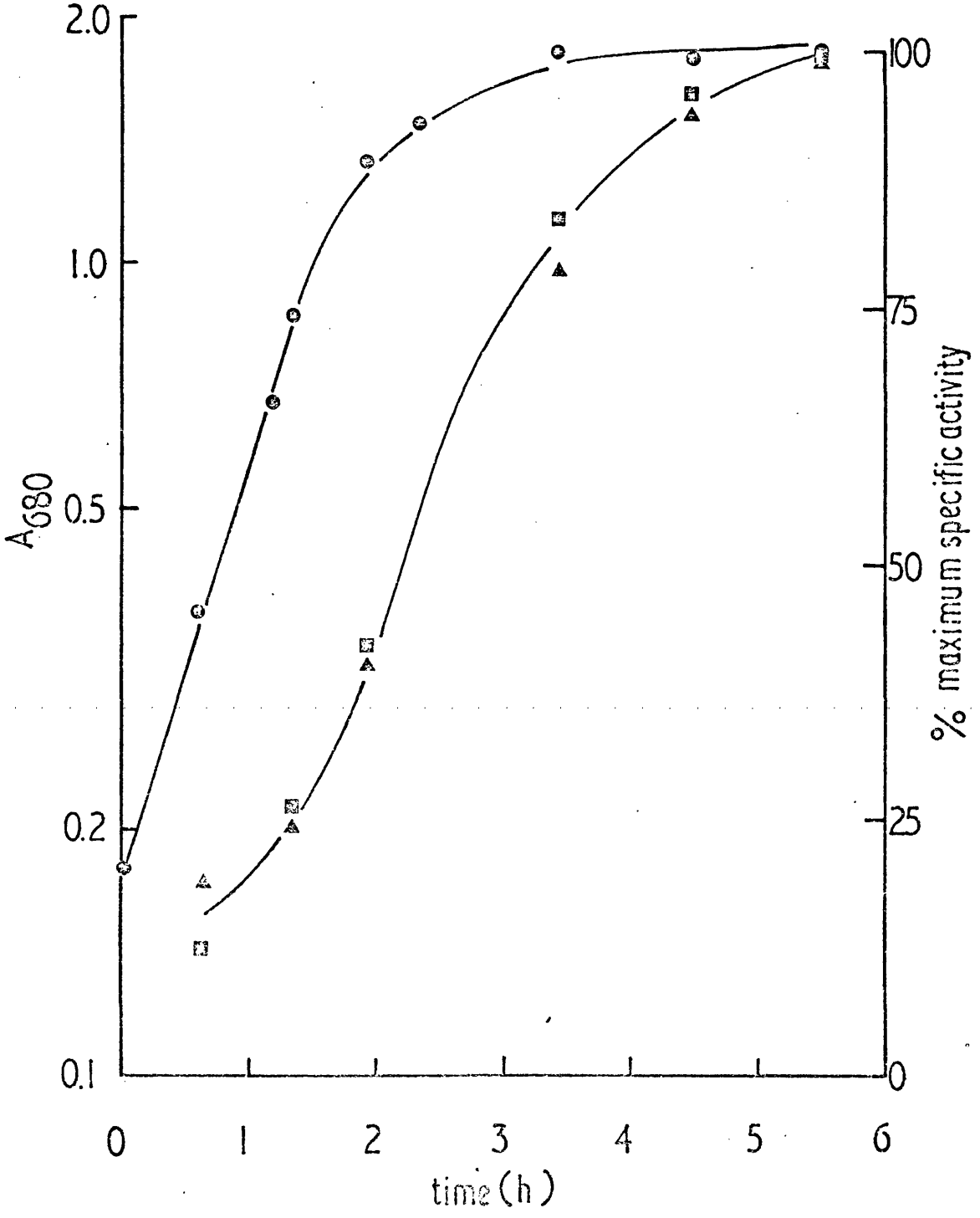

2.0
1.0
0.5
0.2
0.1
A_{680}
100
75
50
25
0
% maximum specific activity
0
1
2
3
4
5
6
time (h)

Figure 39

Variation in specific activities of acetate kinase and phosphotransacetylase in A. aerogenes 1033 during growth on 50 mM glucose with vigorous aeration. 500 ml cultures were grown in 2 litre Erlenmeyer flasks shaken at approximately 300 revolutions per min in a New Brunswick rotary shaker. Samples for enzyme assay were removed at intervals. Chloramphenicol was added to give a concentration of 100 µg/ml and the samples were cooled in ice. Cell-free extracts were prepared and enzymes assayed as described in Materials and Methods. The coupled assay was used to estimate phosphotransacetylase. The maximum specific activity of acetate kinase was 2.4 and that of phosphotransacetylase 4.0. Growth of the culture was measured as described in Materials and Methods. Growth (A_{680}) ● ; % maximum specific activity of acetate kinase ■ ; % maximum specific activity of phosphotransacetylase ▲ .

FIG.40

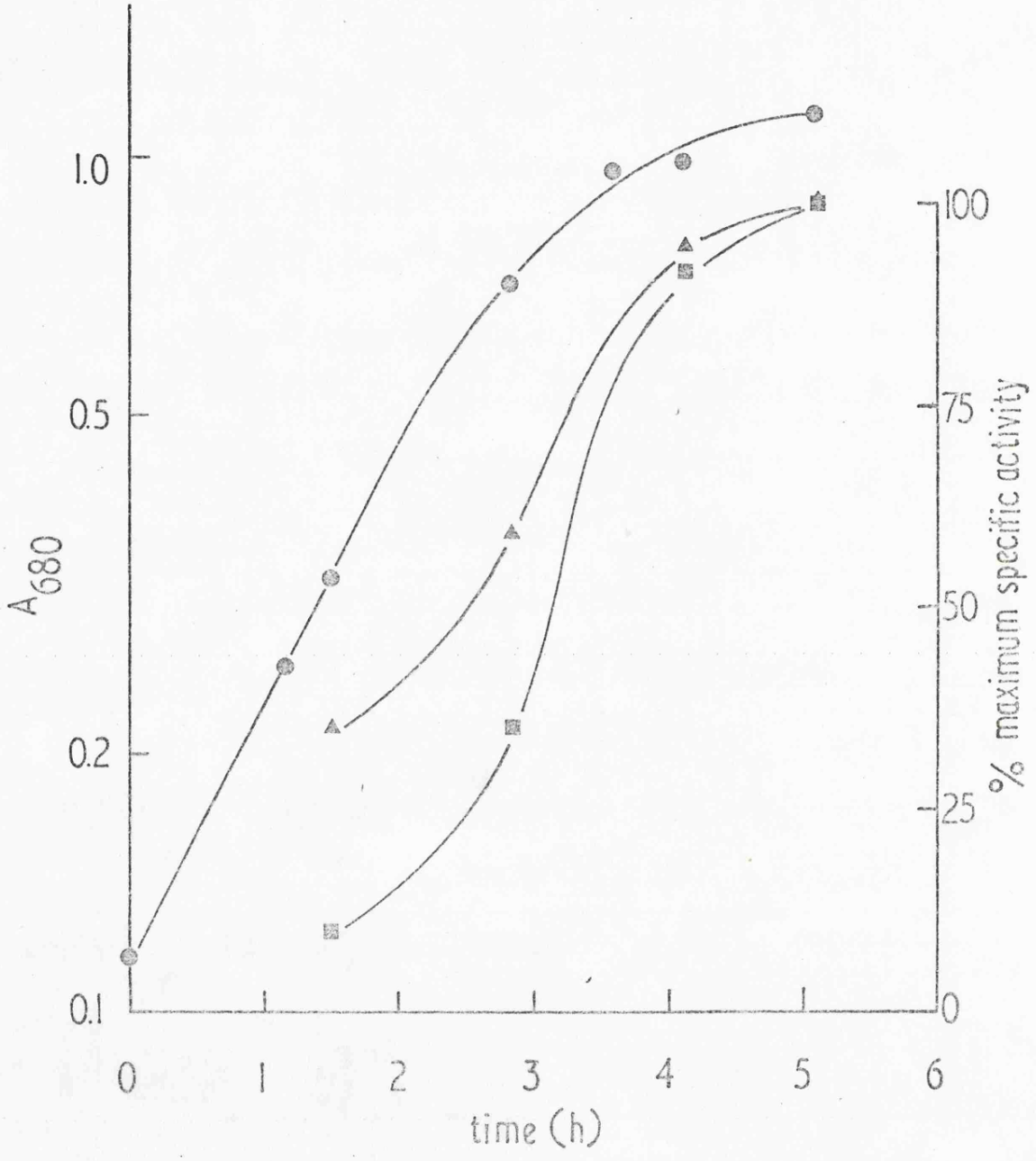

1.0
0.5
0.2
0.1
A_{680}
100
75
50
25
0
% maximum specific activity
0
1
2
3
4
5
6
time (h)

Figure 40

Variation in specific activities of acetate kinase and phosphotransacetylase in A. aerogenes 1033 during growth on 50 mM sodium pyruvate with vigorous aeration. 500 ml cultures were grown in 2 litre Erlenmeyer flasks shaken at approximately 300 revolutions per min in a New Brunswick rotary shaker. Samples for enzyme assay were removed at intervals. Chloramphenicol was added to give a concentration of 100 μg/ml and the samples were cooled in ice. Cell-free extracts were prepared and enzyme assays carried out as described in Materials and Methods. The coupled assay was used to estimate phosphotransacetylase. The maximum specific activity of acetate kinase was 5.6 and that of phosphotransacetylase 11.3. Growth of the culture was measured as described in Materials and Methods. Growth (A_{680}) ● ; % maximum specific activity of acetate kinase ■ ; % maximum specific activity of phosphotransacetylase ▲ .

fermentative metabolism and acetate is a major product of the fermentation of pyruvate (Krebs, 1937). Elevated levels of the acetate kinase/phosphotransacetylase system were also found in E. coli grown on pyruvate (see Chapter I, Table 2).

The variation in levels of acetate kinase and phosphotransacetylase in cultures growing aerobically on glucose was studied (Figure 39). There is a coordinate rise in the levels of the two enzymes as growth proceeds. During growth on glucose in batch culture, a number of factors which might be seen as potentially controlling the synthesis of the acetate kinase/phosphotransacetylase system will vary: these include the pH of the medium, the oxygen concentration in the medium, and the concentrations of low molecular weight metabolites excreted into the medium during fermentation. In view of the observation that elevated levels of the enzymes are found in pyruvate-grown cells and that pyruvate is excreted by A. aerogenes growing aerobically on glucose under some conditions (Dagley et al., 1951; Harrison & Pirt, 1967), the variation of acetate kinase and phosphotransacetylase levels during growth on pyruvate was tested. Variation of levels was observed (Figure 40). This suggests that accumulation of pyruvate in the medium during growth on glucose cannot be the sole cause of the increase in levels observed. In order to investigate the factors controlling enzyme levels during fermentative growth, further experiments were undertaken with a fermenter in which both the pH of the medium and the oxygen concentration in the medium can be controlled. The variation in medium composition cannot, of course, be controlled in these experiments. In these experiments, the variations in acetate kinase and phosphotransacetylase

Table 17 Acetate kinase, phosphotransacetylase and diacetyl (acetoin) reductase levels in cultures of A. aerogenes 1033 growing anaerobically on glucose at constant pH

A_{680}	specific activity		
	acetate kinase	phosphotransacetylase	diacetyl (acetoin) reductase
		pH 7.0	
0.17	1.1	0.7	-
0.63	2.7	0.8	0.03
0.95	3.0	1.6	0.05
1.26	3.0	2.2	0.09
1.32	2.6	2.2	0.10
		pH 5.8	
0.13	1.6	1.4	0.24
0.32	2.2	1.8	2.0
0.66	1.6	1.8	4.4
0.89	1.7	1.8	6.3
0.87	1.3	1.8	6.0

Cultures were grown on 50 mM glucose as described in Materials and Methods. Enzyme assays were carried out as described in Materials and Methods. The thiolester absorption assay was used to determine phosphotransacetylase. Specific activities are expressed as μmol/min/mg protein.

Table 18 The effect of acetate on the formation of diacetyl (acetoin) reductase in A. aerogenes 1033 and FAc7 (ACK/PTA$^-$)

mM acetate	wild-type	specific activity acetate kinase/phosphotransacetylase$^-$
0	0.03	0.04
25	0.17	0.21
50	0.24	0.37
150	0.43	0.54
200	0.41	0.67

The cells were pregrown on 50 mM glucose and harvested when they had reached an A_{680} of 0.5; they were washed and resuspended in minimal medium containing 50 mM glucose and various amounts of sodium acetate (pH 7.0) at a final A_{680} of 1.5 in a volume of 50 ml. The cell suspensions were shaken for 60 min at approximately 100 strokes per min in a water bath at 37°C. Cell-free extracts were prepared and enzyme assays carried out as described in Materials and Methods. Specific activity is expressed as μmol NADH formed/min/mg protein.

specific activities during growth on glucose with the pH of the medium maintained constant at pH 5.8 or pH 7.0 under anaerobic conditions were measured (Table 17). The increase in specific activities observed during anaerobic growth on glucose under these conditions were much smaller than those found in aerobic cultures growing on glucose. This would suggest that the variation in pH of the medium or its oxygen concentration might be significant factors in controlling the synthesis of acetate kinase and phosphotransacetylase. The observations that the enzyme levels are similar at both pH 5.8 and 7.0 suggest that the latter factor must be of primary importance.

Variation in diacetyl (acetoin) reductase levels with acetate concentration and growth conditions The effect of varying concentrations of acetate upon the levels of diacetyl (acetoin) reductase in wild-type and in the acetate kinase/phosphotransacetylase-negative mutant of A. aerogenes 1033 is shown in Table 18. The induction experiments were carried out under semi-anaerobic conditions in the presence of 50 mM glucose. The results strongly suggest that acetate per se is the inducer of the butanediol-forming enzyme: neither acetyl phosphate nor acetyl CoA can be formed from acetate in the mutant and hence neither of these compounds can be the inducer. The demonstration of the role of acetate as the inducer is significant in view of the large changes in concentration of free acetic acid which will occur as the pH of the medium falls during the fermentation of glucose. The importance of the concentration of free acetic acid in controlling the products of fermentation in Aerobacter was already recognized by Mickelson and Werkman (1938).

The formation of diacetyl (acetoin) reductase had also been examined during anaerobic growth on glucose at constant pH (Table 17). At pH 5.8 there is a rapid rise in diacetyl (acetoin) reductase activity in the culture during growth. At pH 7.0 there is only a small rise and the final level of the enzyme formed is only about 2% of that finally attained in the culture grown at pH 5.8. Negligible synthesis of diacetyl (acetoin) reductase is observed in cultures grown on glucose with vigorous aeration. The importance of oxygen concentration in controlling the production of acetate and 2,3-butanediol was demonstrated by Harrison and Pirt (1967). It is possible that oxygen concentration may affect the pH of the medium achieved in batch culture experiments.

CONCLUSION

The work described in this thesis has largely stemmed from attempts to study the permeation of *E. coli* K12 by aliphatic monocarboxylic acids, in particular, its permeation by acetate. Three broad areas of investigation arose from the consideration of this initial problem:

1. The nature of the acetate activation mechanism(s) in *E. coli* K12 and *A. aerogenes*.
2. The control of the enzymes responsible for acetate activation and excretion and the relationship of this control to possible cellular functions of these enzymes.
3. The mechanism of monocarboxylic acid permeation in *E. coli* K12, in particular, the role of specific permeation mechanisms in the utilization of acetate and lactate.

The studies with *A. aerogenes* carried out in collaboration with Dr. Størmer were initiated as a result of interest in the role of acetate in the control of fermentative metabolism in this organism.

The studies of acetate activation were a prerequisite for the investigation of acetate permeation, but proved interesting in their own right. They revealed the existence of a second system capable of activating acetate in *E. coli* K12. (The ability of the acetate kinase/phosphotransacetylase system to activate acetate *in vitro* has been known for many years). The properties of the second system were consistent with a role in acetate activation at low acetate concentrations

but lack of success in the isolation of mutants defective in the activating activity has prevented a definitive description of its role in the metabolism of acetate. The failure to observe pyruvate inhibition of the acetate thiokinase activity assayed *in vitro* remains a substantial problem. In contrast to the results obtained with *E. coli*, work with *A. aerogenes* suggests that the thiokinase is not important in acetate activation in this latter organism, at least under the conditions studied.

Investigation of the role of the acetate kinase/phosphotransacetylase system in acetate activation and excretion clearly revealed its amphibolic function. Studies with mutants devoid of the enzyme activities demonstrated that in *E. coli* K12 the system is necessary for the activation of acetate at high acetate concentrations (20 mM), but that acetate activation can continue at wild-type rates at lower acetate concentrations (2 mM) even in the absence of acetate kinase activity. This contrasts with the properties of similar mutants isolated in *A. aerogenes* where acetate activation was severely restricted at both 2 and 20 mM. It is likely that the acetate kinase/phosphotransacetylase system is capable of considerable activation of acetate at 2 mM even in *E. coli* K12 where the second system can be demonstrated. The coarse control properties of the acetate kinase/phosphotransacetylase system in both *E. coli* K12 and *A. aerogenes* are consistent with its postulated role in acetate excretion. Interesting differences emerged in the coarse control properties of the system in the two organisms and some success was achieved in relating its properties to reported patterns of fermentation products. The role of coarse control

of enzyme activity in determining the type and quantity of fermentation products in A. aerogenes has not been extensively investigated in contrast to the many reports of the role of environmental factors in the control of fermentation product excretion.

The role of specific transport systems in the metabolism of monocarboxylic acids remains a problem. The properties of acetate uptake by washed cell suspensions of E. coli K12 were examined and evidence obtained that membrane permeation is a rate-limiting step in acetate uptake under certain conditions, but that factors remote from membrane permeation may affect the observed rate of uptake. The results obtained suggest that the uptake process operating at high acetate concentrations (which are normally used to study growth) may differe from that operating at lower acetate concentrations. The role of the systems cannot be adequately defined in the absence of mutants modified in transport activity. Evidence for the existence of specific transport systems for D- and L-lactate in E. coli K12 was also obtained. It appears likely that monocarboxylic acid permeation requires specific transport systems at least under certain conditions. It still remains to be established whether or not passive diffusion plays a role in monocarboxylic acid permeation at high acid concentrations; this will require the isolation of mutants devoid of transport activities. The very existence of specific transport systems for monocarboxylates in E. coli K12 provides an interesting contrast with the situation found for mitochondrial permeation. It is to be hoped that permeability differences of this type will ultimately be explicable in terms of differences in membrane structure.

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