LIPOIC ACID TRANSPORT THROUGH THE BACTERIAL CELL MEMBRANE

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Submitted to the faculty of the Graduate College
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in partial fulfillment of the requirements
for the degree of
DOCTOR OF PHILOSOPHY
May, 1969

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ACKNOWLEDGEMENTS

The author wishes to express his deep appreciation to his major professor, Dr. F. R. Leach for continual guidance and encouragement throughout his graduate work.

Sincere appreciation is extended to Dr. K. E. Ebner, Dr. G.V.Odell, Dr. R. K. Gholson, and Dr. E. C. Noller for their suggestions and contributions as members of advisory committee.

Acknowledgement is due Mrs. D. L. Gimlin for her helpful suggestions of thesis preparation.

A very special thank is expressed to his wife, Myung Jae and his son, Michael for their encouragement, understanding and patience during the entire study.

Sincere gratitude is expressed to his parents for their spiritual encouragements and thoughts for the study.

Finally he is thankful for the support from National Science Foundation (Grant No. GB-3274) and Oklahoma Agricultural Experimental Station (Project No.1109) and for the facilities and equipments of Department of Biochemistry of Oklahoma State University.

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CHAPTER I

INTRODUCTION

The growth and maintenance of living cells depend upon transport of nutrients from the extracellular medium to the interior of the cells through a semipermeable membrane which regulates the process of penetration of the substances. Most transport studies use microbial cells, because of their relatively simple structure, their well controlled nutrition and their amenability to genetic analysis. Although both active and specific passive transport are recognized, their molecular mechanisms remain unknown. Recent advances in studying transport mechanisms have been made by isolating specific proteins from the cell membrane rather than by characterization of the phospholipids of the membrane.

Definite proof of the existence in bacteria of stereospecific permeation system for β-galactosides and amino acids was established by Cohen and Monod (1). For these systems they suggested the generic names "permease" which implies the presence of a protein, functional in transport and distinct from metabolic enzymes. The entry of organic substrates into bacterial cells through a selective permeation system is based on two observations: 1) the capacity of certain cells to accumulate internally certain nutrients against a concentration gradient and 2) the state of "crypticity" of certain cells toward particular substrates, i.e., their incapacity to metabolize a given substrate, even

though they possess the relevant enzyme system. Selective crypticity studied by Doudorff, Hassid, Putnam, Potter, and Lederberg (2) involved a mutant of Escherichia coli incapable of metabolizing glucose, although it possessed hexokinase and metabolized maltose via the enzyme amylomaltase (3) which catalyes the reversible reaction of maltose into amylose and glucose. Although free glucose is liberated in this reaction, the organisms metabolized quantitatively both moieties of the maltose molecule. Thus glucose could be used when liberated intracellularly by amylomaltase, while free glucose from the external medium could not be used by those cells. A cell membrane impermeable to glucose could not possibly be permeable to maltose, except via a stereospecific permeation system.

Cohen and Monod showed that the accumulation of galactosides was induced E. coli by the presence of a galactoside and was reversible. At saturating concentrations, the initial rate of entry is independent of galactoside concentration and the maximal amount of galactoside which the cells take up at saturating concentration vary widely from one compound to another compound, the ratio being, 5 to 1 for thiomethyl-galactoside and thiophenyl-galactoside. The accumulation of galactoside catalyzed by the permease is linked to the metabolic activity of the cells. The inhibition of accumulation by typical uncoupling agents such as 2,4-dinitrophenol or azide is not due to an action on the permease, but the energy coupling allows the permease reaction to function against a concentration gradient. The permease activity for the accumulation of galactosides into the cell is genetically controlled. Galactosides are not only substrates of the permease but also induce the permease and the induction is inhibited by chloramphenicol,

fluorophenylalanine or amino acid deprivation in an amino acid-requiring strain.

Cohen and Rickenberg (4,5) found that when E. coli K12 cells were shaken at 37° in the presence of radioactive valine under condition where protein synthesis was blocked, radioactivity was rapidly accumulated in the cells up to 500-fold. The accumulation was inhibited by 2,4-dinitrophenol and sodium azide and is dependent upon an external energy source. The accumulation of valine was reversible; intracellular ^{14}C-valine was displaced by nonradioactive valine and also by the structurally related amino acids, leucine or isoleucine, in a competitive manner. Only the L-isomers are effective competitors. Similarly, peptides containing valine, leucine or isoleucine have little or no affinity for the valine accumulating system. Such a stereospecific system for accumulation of peptides has been studied in Lactobacillus casei by Leach and Snell (6). Glycine and L-alanine are accumulated from either the free amino acids or their peptides by an energy-dependent process which can be inhibited by 2,4-dinitrophenol. Competition experiments revealed an uptake system for glycine, another uptake system for both D- or L-alanine and an uptake system for the peptide.

Mitchell hypothesized that normal metabolic enzymes located on the membrane are the conductors of transport (7) and that metabolic energy is converted to osmotic work during the forming and opening of covalent links between the enzymes on the membrane and they carried molecules exactly as in enzyme-catalyzed group transfer reactions (8), whereas Cohen and Monod assume the existence of an osmotic barrier enclosing the entire cell, which is impermeable to most substrates, and the existence of stereospecific permeases which are functionally

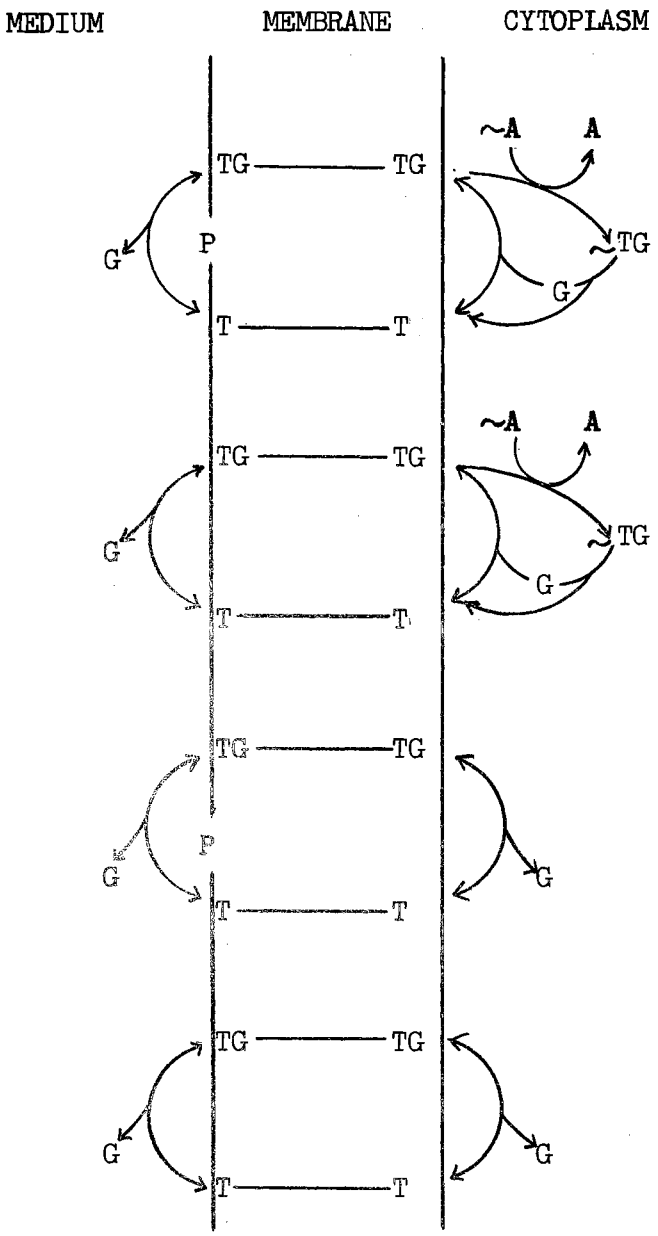
specialized for the transport of specific substrates through the osmotic barrier and which are distinct from the normal metabolic enzymes (1).

Mitchell showed that the plasma membrane of Staphylococcus aureus is a complex lipo-protein sheet about 50 Å-thick containing 20% lipid, 40% protein exhibiting many oxidative and hydrolytic enzyme activities, and including some 90% of the total succinic, lactic, and formic dehydrogenases and the bulk of the cytochromes of the cell (9). Mitchell proposed the concept of the anisotropy for the enzymes and catalytic carriers. According to this theory the part of the system causing transport represents a metabolic system for which substrate accessibility is spatially anisotropic, chemical anisotropy being determined by the substrate selectivities of the membranes, that is, anisotropic arrangement of enzyme and catalytic carriers relative to structural elements of membranes and each other would require the enzyme and carrier molecule to carry both locational and catalytic substrate specificities (10). Concerning the possible asymmetry of the oxidation reduction process, he found that the succinate and malonate accepting region of the succinic oxidase system of Micrococcus lysodeikticus is situated at the inner side of the plasma membrane (11). At neutral pH the membrane is impermeable to hydrophilic solutes carrying more than four water molecules, such as ATP, NAD^+, glucose, succinate, malonate and ferricyanide but is freely permeable to lipid-soluble solutes such as ethanol. The intact cells rapidly oxidize externally added succinate, using molecular oxygen, but this oxidation is not inhibited by externally added malonate. However, if the pH of the cell suspension is briefly changed to 5 to allow malonate to pass in without damaging

the cells, the oxidation of succinate is almost completely inhibited.

Mitchell used glucose-6-phosphatase to illustrate the crypticity of certain cells to specific substrates. Only about 6 per cent of this enzyme in E. coli is associated with the cell membrane, and it is therefore classified as a "soluble" enzyme (7). Mitchell found that although glucose-6-phosphate did not penetrate the plasma membrane of intact cells, the cells fermented it at a rate equivalent to the total glucose-6-phosphatase of the cell. An intact cell suspension liberated the inorganic phosphate from glucose-6-phosphate into the mediu m, and not within the area confined by the membrane.

Kepes (12) proposed the formation of a rate-limiting Michaelis enzyme-substrate complex between the galactoside and its permease and then a spontaneous association-dissociation reaction of the complex to provide the carrier mechanism. The current theory for the galactoside permease proposed by Koch (13) consists of four different types where the energy couplings are placed inside the permeability membrane as shown in Fig 1. The most rapid process is the reaction in which the permease or carrier mediates the substrate transport with the expenditure of energy, against the concentration gradient (a). Cryptic mutants which lack the permease slowly take up the substrates via the carrier process (b). Some transport can occur when the energy coupling system is inhibited by azide or 2,4-dinitrophenol (c). Under these conditions no concentration of the substrate occurs within the cell, and the amount which had been previously concentrated greater than the equilibrium value is rapidly lost. The facilitated diffusion process is the reaction in which substrates in the extracellular medium binds the carrier on membrane to form a complex which then diffuses across



G = substrate, P = permease, T = carrier, A = energy source.

Figure 1. Proposed transport mechanisms

the membrane (d). Thus the prime mover of chemical transformation and transport is diffusion, and the spatial pathway of this process is channelled by permease and carrier. The complete process is active transport where substrate specifically binds permease and then carrier by the expenditure of energy whereas facilitated diffusion is carried out only by carrier.

The enzymatic and transport function carried out by the bacterial cell membrane is extremely difficult to study at the molecular level in relation to the membrane structures. The unit membrane concept of Robertson (14) suggests that the proteins and enzymes are localized at the membrane surface rather than constituting an intrinsic part of barrier between cytoplasm and external medium. Benson (15) suggested that certain proteins because of their primary structure may assume conformations with a high specific affinity for membrane lipids and thus may be intercalated directly into the membrane. The cell envelopes of gram-negative bacteria appears to be complex multilayered structures. The chemical and cytological evidence for the existence of layers indicated that the lipoprotein is the outmost layer of the cell wall overlying the lipopolysaccharide layer. The lipopolysaccharide represents a double layer with channels which provide communication between the cytoplasmic membrane and the outside environment. This lipopolysaccharide layer is on a rigid glucosaminopeptide layer that is responsible for most of the cell wall's rigidity and retains the shape of the bacterial wall. The rigid layer is partially covered by a layer of material that can be removed by proteolytic enzymes like trypsin, pepsin or pronase. It contains some round units which range in diameter from 75 $\mathring{A}$ to 250 $\mathring{A}$ (16,17). The periplasmic space between protoplasmic

membrane and rigid cell wall has been observed under electron microscope (7).

Neu and Heppel (18) found that a number of degradative enzymes and other protein which are localized in periplasmic space are released from bacteria by osmotic shock. The cells are exposed by ethylenediaminetetraacetate (EDTA) in a hypertonic solution of sucrose, followed by immediate suspension of the cells into ice cold hypotonic solution containing magnesium ions. The treatment with EDTA release the acid-soluble nucleotide pool and renders the cells permeable to actinomycin (19), puromycin (20) and nucleotides (21). The change is probably related to the release of lipopolysaccharide components of the cell wall (22). However, the alteration of permeability is separate from the change occurring in osmotic shock, since there is no enzyme release.

Schatzmann (23) found that the red blood cell adenosine triphosphatase (ATPase) is of a lipoprotein nature and the cell ghosts treated with clostridial lecithinase lost both their ouabain-sensitive and insensitive ATPase. Skou (24) found that the transfer of the energy-rich phosphate from ATP to ATPase required magnesium and the hydrolysis of the phosphorylated enzyme and then required Na^+ and K^+. Both processes were inhibited by sulfhydryl inhibitors but at different rates.

Studies on amino acid transport (25), glycoside transport (26), galactose transport (27), galactosides transport (28) all in E. coli and sulfate transport (29) in Salmonella typhimurium have been conducted using positive and negative mutants.

Kundig, Ghosh and Roseman (30) isolated a bacterial

phosphotransferase system which catalyzes the transfer of phosphate from phosphoenolpyruvate to various carbohydrates according to the following reaction scheme.

$$\text{P-enolpyruvate} + \text{HPr} \xrightarrow{E_I,\ Mg^{++}} \text{pyruvate} + \text{P-HPr}$$

$$\text{P-HPr} + \text{sugar} \xrightarrow{E_{II},\ Mg^{++}} \text{sugar-P} + \text{HPr}$$

$$\text{P-enolpyruvate} + \text{sugar} \xrightarrow[Mg^{++}]{\text{HPr},\ E_I,\ E_{II},} \text{sugar-P} + \text{pyruvate}$$

the system is composed of two enzymes, E_I (a soluble fraction) and E_{II} (a membrane-bound particulate fraction) and HPr (a soluble low molecular weight, 10,000, heat-stable protein). The carbohydrate specificity of the reaction is determined by E_{II} which contains a number of components, each specific for a different carbohydrate. Kundig, Kundig, Anderson and Roseman (26) demonstrated that whole cells of E. coli W 2244 subjected to the osmotic shocking procedure of Neu and Heppel (18) lost the ability to concentrate α-methylglucoside and methyl-β-thio-D-galactopyranoside. Furthermore the ability to concentrate these compounds was completely restored by the addition of HPr to the reaction mixture. This study represents the first evidence that the P-enolpyruvate-P-transferase system is involved in carbohydrate transport. Genetic evidence for the transport system came from the findings of pleiotropic mutants of Aerobacter aerogenes (31) and E. coli (32) which lost simultaneously the ability to grow on several sugars. These mutations involve the loss of E_I or HPr. A similar pleiotropic effect of a mutation of E_I occurs in Samonella typhimurium (33). The mutant was unable to grow on nine simple

carbohydrates.

Rickenberg, Cohen, Buttin and Monod (34) observed that labeled thiogalactosides added to resting cells of E. coli grown in the presence of lactose or thiogalactosides, were rapidly taken up by the cells to a concentration far higher than that in the medium. The specificity, kinetics and energy requirements of this active transport system led Cohen and Monod to the idea of galactoside permease that was well controlled genetically. In 1965, Fox and Kennedy (28) used nonradioactive N-ethylmaleimide in the presence of thiogalactoside to block reactive sites not protected by the substrate. The protected sites are subsequently labeled with radioactive N-ethylmaleimide. Labeling associated with transport appears in the particulate membrane fraction of sonicated cells and the protein component, called M protein is released only by treatment with detergents. The protein has been purified and genetic evidence strongly indicates that it is related to the product of the y gene located between β-galactosidase gene and β-galactoside transacetylase gene of the lactose operon (35). Kennedy proposed a model for the transport and accumulation of -galactosides medicated by the lactose system where two processes are sharply distinguished, (a) a facilitated entry, and (b) the accumulation of the β-galactosides against a concentration gradient, dependent upon metabolic energy as shown in Fig. 2. A molecule of β-galactoside in the medium binds to the M protein in the membrane by Michaelis-Menten type interaction. The β-galactoside binding site may alternately be exposed to the medium and to the cytoplasm by a process of rotational diffusion. The complex on the inner surface of the membrane may dissociate and if the substrate is attacked by β-galactosidase, the continuous breakdown of

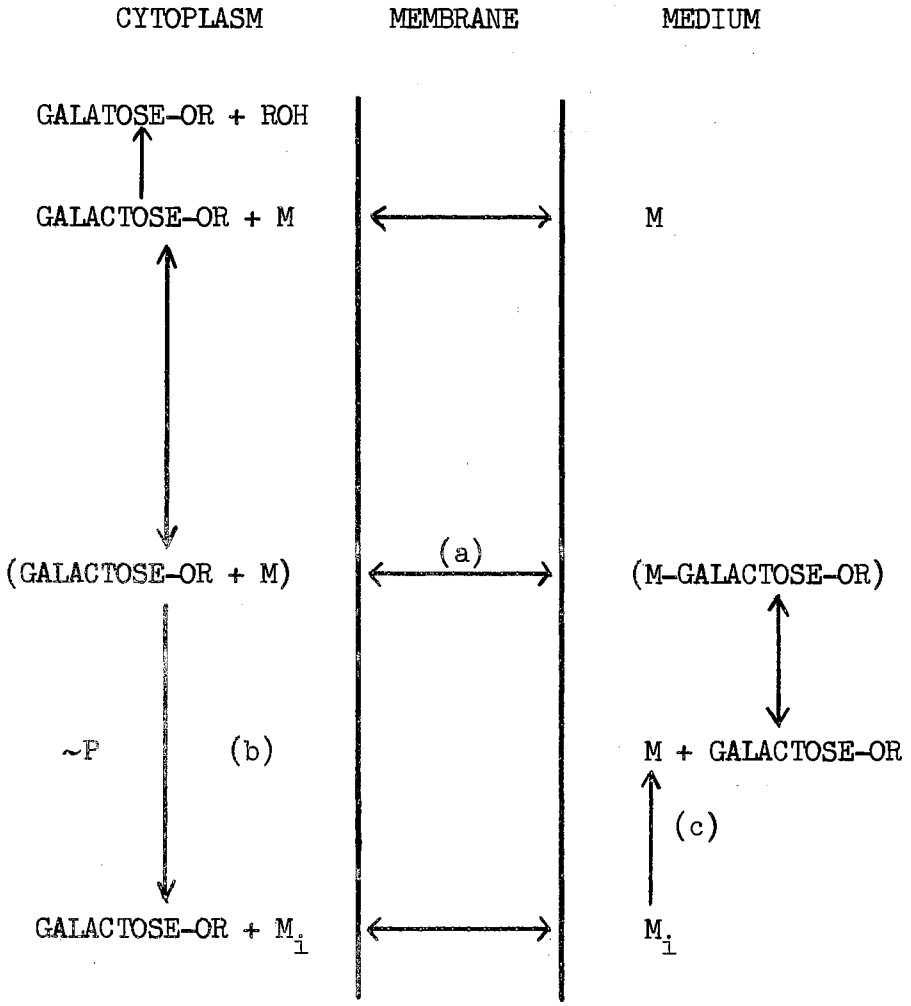


Figure 2. A model of function of M protein in the transport of β-galactosides.

galactoside may lead to a substantial flow of galactoside from the medium into the cells. This process is independent of metabolic energy. On the other hand the galactoside M protein complex may undergo an energy-requiring transformation in which the protein is converted to a form Mi with a greatly reduced affinity for galactosides and then Mi protein may be converted back M protein with high affinity for substrate. The process of accumulation is then dependent upon the asymmetry caused by interaction of the M protein with the energy yielding system.

Pardee (36) crystallized a sulfate binding protein from S. typhimurium. The binding protein was released by osmotic shock. The sulfate transport function has been identified with the cys A region of the bacterial chromosome which contains at least three cistrons, a defect in one of which leads to impaired transport. Some transport negative mutants however were still able to bind inorganic sulfate.

Cohen and Rickenberg (4) described the competitive interactions among leucine, isoleucine and valine during their accumulation into E. coli. The isolation of mutants of E. coli which were defective in the transport of D-serine, glycine, and L-alanine indicated that these three amino acids probably migrate by a common transport system (37). Britten and McClure (38) proposed a carrier model to explain maintenance of the neutral amino acid pool, according to which an external amino acid diffuses into the cell and collides with an unoccupied carrier. The complex diffuses through the cell and collides with a site. In a reaction coupled to an energy donor, the amino acid is transferred from the carrier to the site. Studies on the uptake of the amino acids histidine, tryptophan, tyrosine and phenylalanine in S. typhimurium indicated that there is a general permease able to transport

all four amino acids as well as a wide variety of structural analogs(39).

Recently, Piperno and Oxender (40) reported the isolation from E. coli K12 cells of a protein that specifically binds the branched chain amino acids leucine, isoleucine and valine, and suggested a role for this protein in the transport of the branched amino acids in E. coli from the results of the similarity of the kinetic constants for cellular transport (0.2-0.4 M) and binding activity of the protein (dissociation constant K_D, 0.2-0.4 M), the simultaneous repression of leucine transport and the synthesis of binding protein when cells are grown in the presence of leucine, and the finding that the binding protein is localized in the cell envelop (41). The binding protein was crystallized and its molecular weight of 34,000 was determined (42).

Kundig, Kundig, Anderson and Roseman (26) found that the HPr component of the phosphoenolpyruvate kinase system enhanced the accumulation of certain galactosides in osmotically shocked cells. Anraku (27) has found restoration of galactose uptake in E. coli by the addition of the concentrated shock fluid to osmotically shocked cells. However the M protein of β-galactoside transport in bound firmly to the cell membrane. Kaback and Stadtman (43) found an active accumulation of proline in spheroplast membranes of E. coli. Most recently Pardee and Watanabe (44) studied the intracellular location of the sulfate-binding protein using diazo-7-amino-1,3-naphthalenedisulfonate. This reagent is incapable of penetrating the cell membrane so only proteins outside the membrane are inactivated. The sulfate binding protein appeared to be present on the outside surface of the membrane.

Oginsky (45) reported that cells of a vitamin B_{12}-deficient mutant strain of E. coli were capable of adsorbing radioactive vitamin B_{12}

labeled with ^{60}Co, in excess of amounts required of growth. Cells of a normal strain of E. coli also adsorbed vitamin B_{12} rapidly, whereas cells of the mutant strain grown with the vitamin exhibited a slower uptake. The adsorption rate of the mutant cells was decreased and desorption was increased by the addition of glucose.

Traub and Lichstein (46) demonstrated the inability of Lactobacillus arabinosus to grow in the complete absence of biotin. Lichstein and his collaborators (47) observed that biotin deficient cells of L. arabinosus accumulated large amounts of bound biotin in the presence of glucose and the accumulation was inhibited partially or wholly by iodoacetate or homobiotin.

Wood and Hitchings (48) studied the uptake of folic acid in various bacterial specieis. Glucose is essential for this function in all cases, but the capacity of uptake varied between cell types. Streptococcus faecalis took up and quantitatively released the adsorbed folic acid and the uptake of folic acid was dependent upon cell concentration, pH (6.5) and temperature (37°). Uptake of radioactive thiamine by Lactobacillus fermenti was studied by Neujahr (49). Evidence was that the uptake of thiamine was markedly stimulated by glucose and Mg ion whereas it was inhibited by iodoacetate.

Sanders and Leach (50,51) demonstrated an energy-requiring, temperature-dependent system capable of concentrating lipoic acid in the cell pool in S. faecalis and was also found in E. coli, S. aureus, and A. aerogenes. The system was easily saturated with lipoic acid and was constitutive. The rapid uptake of lipoic acid was followed by a slight decline and a plateau and the uptake at 0° was about 10% of that of uptake at 37°. Octanoic acid competed with lipoic acid while the

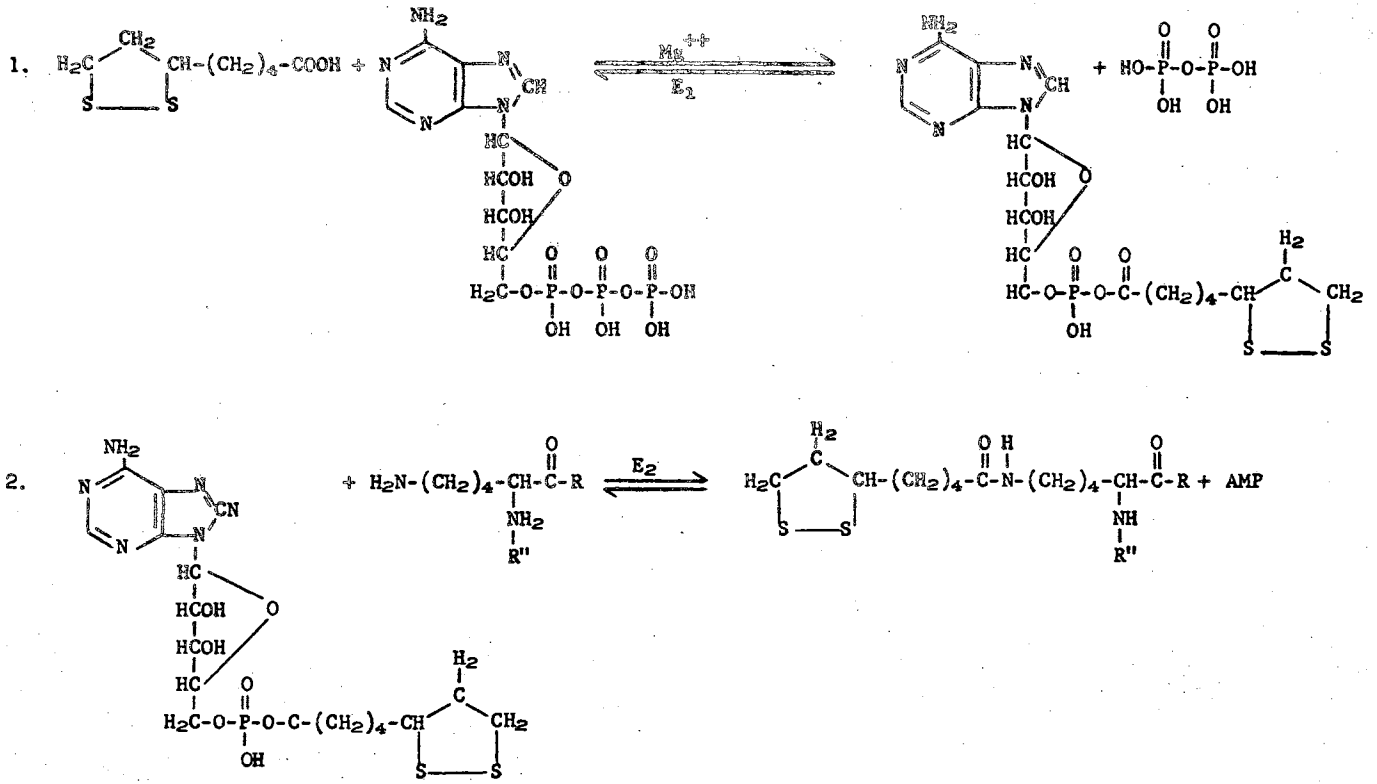
analogues 8-methylthiotic acid, 1,2-dithiolane-3-caproic acid, and 1,2-dithiolane-3-butyric acid were without effect, and the transport was inhibited by 2,4-dinitrophenol. Pyruvate induced metabolic swelling only in lipoic acid sufficient S. faecalis protoplasts and its swelling was proportional to the amount of lipoic acid present, indicating that metabolic swelling was related to oxidative metabolism (52).

Snell, Tatum, and Peterson (53) observed that extracts of several natural materials stimulated the growth of lactic acid bacteria which led to the isolation of lipoic acid. Within a period of about six years following the discovery of the vitamin, lipoic acid was isolated and characterized and the basic features of its site and mode of action were established. The well-defined role of lipoic acid is as a prosthetic group bound in amide linkage to the ε-amino group of a lysine residue in the multienzyme complexes which catalyze the oxidative decarboxylation of pyruvate and α-ketoglutarate to produce acetylcoenzyme A and succinyl coenzyme A. Pyruvic acid produces CO_2 and an enzyme decarboxylase-bound "aldehyde-thiamine pyrophosphate", and then the "active aldehyde" is transformed to a reductive acylation of protein-bound lipoic acid by an enzyme lipoic reductase transacetylase. Transacetylation of the lipoic acid bound acetylmoiety yield the enzyme bound dihydrolipoic acid and acetyl CoA. The reduced enzyme bound lipoic acid is the oxidized by an FAD-flavoprotein dihydrolipoyl dehydrogenase that is in turn oxidized through electron-transfer reaction by NAD (54).

Reed, Leach and Koike (55) observed that incubation of extracts of lipoic acid-deficient S. faecalis cells with lipoic acid gave preparations which were capable of catalyzing the oxidation of pyruvate

or α-ketobutyrate. Lipoic acid labeled with ^{35}S was converted to a protein-bound form. Fractionation of the lipoic acid-deficient extracts revealed requirements for ATP, Mg^{++} and two activating enzymes (E_1 and E_2) for the incorporation of lipoic acid into the apopyruvate dehydrogenation system. The reaction scheme is shown in Fig. 3.

The activation reaction by the enzymes resemble what would be expected in the energy-dependent transport system. Therefore, a study of the intracellular distribution of the lipoic acid activating enzymes was the first objective of this thesis. The second objective is to determine if a binding protein exists for lipoic acid and to characterize its function in transport.



E_1= fraction PS-2A.

E_2= fraction PS-2B.

$H_2N-(CH_2)_4-\underset{\underset{R''}{|}}{\overset{\overset{NH}{|}}{CH}}-\overset{\overset{O}{||}}{C}-R$= apopyruvate dehydrogenase complex

Figure 3. Reaction Scheme of Lipoic Acid Activating System

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

Unlabeled D,L-α-lipoic acid was generously supplied by Dr. D.S. Acker of E.I. Du Pont De Nemours and Co. and Dr. L.J. Reed of University of Texas. Radioactive D,L-α-lipoic acid-^{35}S was prepared by Dr. F.R. Leach (20.1 μc/mg). Lysozyme (E.C. No.3.2.1.17), lactate dehydrogenase (E.C. No.1.1.1.27), deoxyribonuclease (E.C. 3.1.4.6) and dry cultures of Clostridium kluyveri were obtained from Worthington Biochemical Co. Thiamine pyrophosphate was purchased from California Biochemical Research. Adenosine triphosphate, coenzyme A and NAD^+ were Pabst Laboratories products. NADH and glucose-6-phosphate were purchased from C.F. boenhringer and Soehne GmbH Mannheim, West Germany. ^{14}C-L-Valine (0.19 mc/mmole)and ^{14}C-L-proline (0.2 mc/mmole) were obtained from New England Nuclear. p-chloromercuribenzoate and N-ethylmaleimide are Mann Research Laboratories products. Brij 58 was a product of Atlas Chemical Industries, Inc. The fine glass beads were Prismo 200, of Prismo Safety Corp. Bactotryptone and Bacto-agar were Difco Laboratories products.

Methods

Growth of Cells

Cells of S. faecalis (strain 10C1) were grown for 10-12 hours in

the lipoic acid-free synthetic medium at 37° described by Gunsalus and Razzell (56). The cells were harvested in late log phase. Cells of E. coli (Crookes strain) were grown with vigorous aeration for 6-8 hours at 37° in a synthetic medium (57). The cells were harvested in a refrigerated sharples supercentrifuge and washed twice with cold distilled water.

Preparation of Cell Membranes

The S. faecalis 10C1 cells were suspended to an A_{630} of 0.4 in 0.6 M sucrose-0.75 M potassium phosphate buffer of pH 6.3. The cell suspension was incubated with 50 μg/ml of lysozyme for approximately five hours at 37°. The protoplasts were obtained by centrifugation of the cell suspension in a Sorvall RC-2 Centrifuge at 14,000 x g for 10 minutes at 2°. Cells of E. coli were treated rapidly at 4° with 42 μg/ml of lysozyme in 0.6 M sucrose-0.03 M Tris-HCl buffer containing 4 mM EDTA of pH 7.8 to obtain the protoplasts (58). Both types of protoplasts were lysed with a 0.5% Brij 58 solution and 50 μg of deoxyribonuclease in the presence of 0.01 M of $MgSO_4$ at 4° unless otherwise indicated.

The lytic mixtures were centrifuged at 4,300 x g for 5 minutes and then at 24,000 x g for 15 minutes at 2°. The supernatant solutions containing soluble enzymes were retained for further experiments (MS) and the membrane fraction was suspended in 0.02 M phosphate buffer at pH 7.0. The membrane supensions were subjected to sonic oscillation for 5 minutes in a Raytheon 10 KC sonic oscillator at maximum power to solubilize the membrane bound enzymes (SM).

Preparation of Whole Cell Extract (CE)

One gram of S. faecalis 10C1 and E. coli were individually suspended in 0.02 M potassium phosphate buffer of pH 7.0 to the final volume of 5 ml each. The cell suspension of E. coli was subjected to sonic vibration in the presence of 0.4 g/ml of fine glass beads for 10 minutes in the same sonic oscillator. Both cellfree extracts were then centrifuged at 44,000 x g for 30 minutes in Sorvall RC-2 centrifuge at 2° and the supernatant solution were used for the enzymatic assays.

Preparation of PS-2A, PS-2B and PP-1

About 25 grams of S. faecalis cell paste suspended in 50 ml of 0.02 M potassium phosphate buffer, pH 7.0 were subjected to sonic vibration for 45 minutes at maximum power with a Raytheon 10KC oscillator and then centrifuged for 40 minutes at 45,000 x g in Sorvall RC-2 centrifuge at 2°. The cell-free extract diluted to a protein concentration of 20 mg per ml with 0.02 M potassium phosphate buffer, pH 6.0 was fractionated with approximately 0.12 volume of 2 per cent protamine sulfate, pH 5.0 and centrifuged for 45 minutes at 44,000 x g in Sorvall RC-2 centrifuge. To the supernatant solution 0.02 volume of the protamine sulfate solution was added and the precipitate was collected by centrifugation. The precipitate was suspended in a volume of 1 M potassium chloride equal to 0.1 the volume of the diluted extract and then centrifuged at 65,000 x g for 30 minutes in a Spinco preparative ultracentrifuge. The clear supernatant solution (designated PP) and the supernatant solution from the protamine sulfate fractionation (designated PS) was further fractionated with ammonium sulfate as below.

Fraction PP was brought to 60% saturation with a saturated ammonium sulfate solution at 3°. The precipitate collected by centrifugation at 44,000 x g for 30 minutes in Sorvall RC-2 centrifuge was dissolved in a volume of 0.02 M potassium phosphate buffer, pH 7.0 equal to that of fraction PP. The solution (28 ml) was dialyzed for 4 hours with stirring against 2 l of 0.02 M potassium phosphate buffer, pH 7.0 at 3°. The dialyzed solution was PP-1. Fraction PS was brought to 60% saturation with solid ammonium sulfate (390 g/l). The supernatant solution after centrifugation was saturated with solid ammonium sulfate (300 g/l). The precipitate collected by centrifugation was dissolved in a volume of 0.02 M potassium phosphate buffer, pH 7.0 equal to 0.1 the volume of fraction PS. The solution (36 ml) was dialyzed with stirring for 4 hours against 2 l of the same buffer. This was the preparation of fraction PS-2.

To 40 ml of fraction PS-2, 93 ml of a 4 molal solution of ammonium sulfate adjusted to pH 8.0 with concentrated ammonium hydroxide was slowly added and then 14 g of ammonium sulfate were added to the mixture. After the precipitate (PS-2A) was removed by centrifugation, the supernatant solution was saturated with solid ammonium sulfate. The precipitate (PS-2B) was collected by centrifugation. The precipitates, PS-2A and PS-2B were dissolved in 0.02 M potassium phosphate buffer, pH 7.0 and the dialyzed for 4 hours with stirring against the same buffer (59). These fractionation scheme is shown in Fig. 4.

Enzymatic Assays

The assay procedure consisted of two steps. The first involved activation of the apopyruvate dehydrogenase system (56). The complete

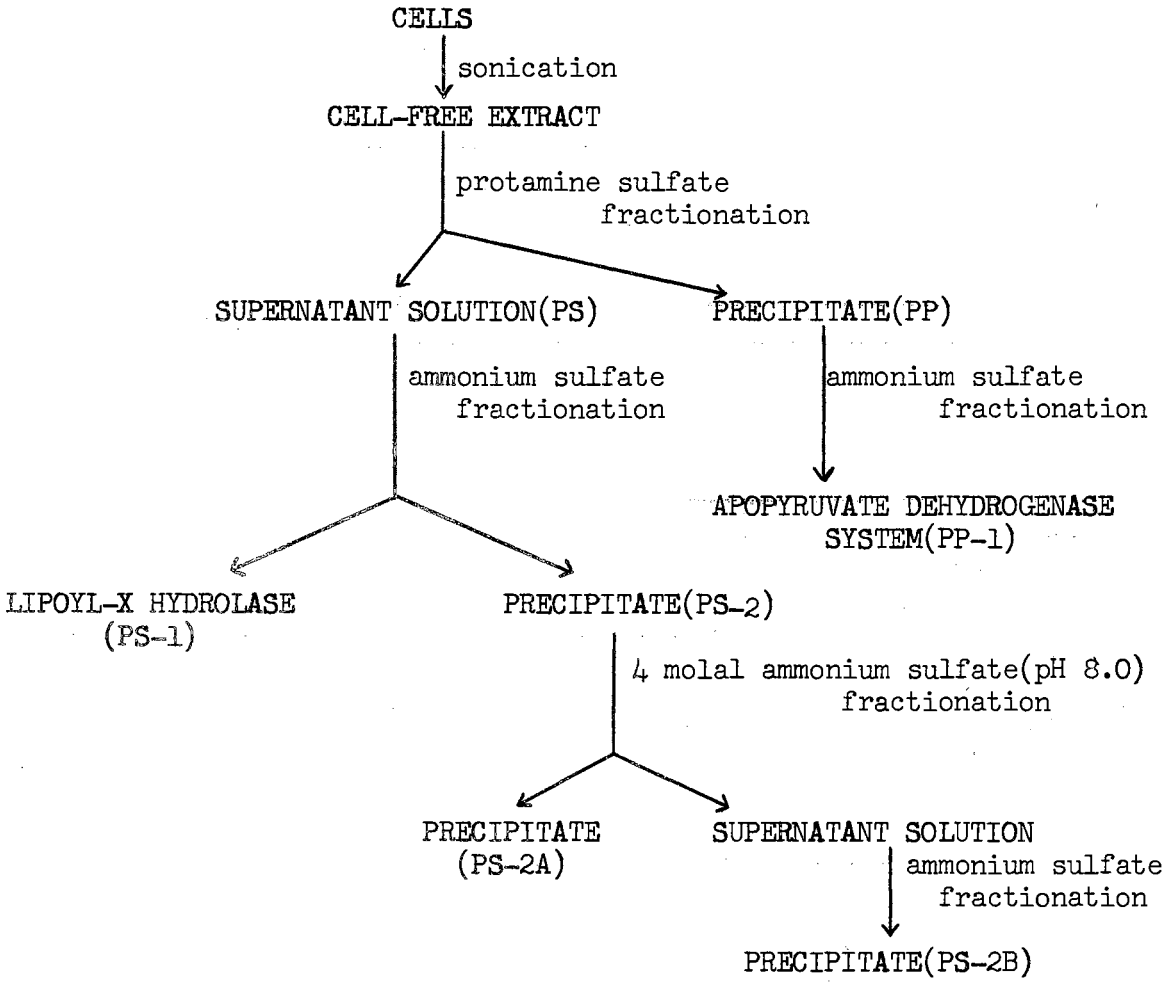


Figure 4. Fractionation Scheme of Lipoic Acid Activating System from S. faecalis 10C1

reaction mixture contained 0.005 μmole of dl-lipoic acid, 0.05 μmole of TPP, 0.02 μmole of ATP, 0.8 μmole of $MgSO_4$, 6 μmoles of potassium phosphate buffer at pH 7.0, and an excess of the apopyruvate dehydrogenase system or the lipoic acid-activating enzymes, depending on the activity to be determined. The total volume of 0.25 ml of the mixture was incubated for one hour at 37°. The second step involved an assay for pyruvate dehydrogenase activity wherein, to the incubated mixture, 100 μmoles of potassium phosphate buffer of pH 7.0, 50 moles of potassium pyruvate, 0.8 μmole of CoA, 0.27 μmole of NAD^+, 6.5 μmoles of L-cysteine, 12 units of phosphotransacetylase, prepared from a dry culture of Clostridium kluyveri (60) and 2,000 units of lactate dehydrogenase (Worthington Biochemical Corp. E.C. No. 1.1.1.27) were added to a final volume of 1 ml. This mixture was incubated for 30 minutes at 37° and then assayed for the formation of acetyl phosphate (61).

In the assays of malic dehydrogenase, NADH oxidase, and glucose-6-phosphate dehydrogenase, one unit of enzyme equals the amount of enzyme for 0.01 change in absorbance at 340 mμ per minute. The reaction mixture for malic dehydrogenase determination contained 0.1 ml of 0.0015 M NADH solution, 0.3 ml of 0.25 M phosphate buffer (pH 7.4), 0.1 ml of 0.0076 M oxalacetate (pH 7.4) and diluted enzyme fractions in a total of 3.0 ml (62).

For the assay of NADH oxidase, the reaction mixture contained 0.1 ml of 0.002 M NADH solution, 1.0 ml of 0.1 M phosphate buffer (pH 7.4), and diluted enzyme fractions in a total of 3.0 ml; this reaction mixture is as described for lactate dehydrogenase (63) with the omission of pyruvate. For the assay of glucose-6-phosphate dehydrogenase, the reaction mixture 1.0 ml of 0.1 M Tris-HCl buffer (pH 7.8),

0.2 ml of 0.1 M $MgCl_2$ solution, 0.05 ml of 0.2 M glucose-6-phosphate solution, 0.1 ml of 0.0015 M NADP solution, diluted enzyme reactions and was made up to 3.0 ml (64).

For the protein determinations a sample was made up to 3.0 ml and 4.0 ml of 12% of trichloroacetic acid solution were added. The readings were taken at 600 mμ in a Spectronic 20 (65).

Uptake Experiments

Cells of E. coli (Crookes strain) were grown on minimal medium, M-9 (66). Ten milliliters of a stationary culture were added to 200 ml of fresh sterile medium and incubated for 5 hours at 27°. The harvested cells were washed twice with M-9 and used within 2 hours.

The concentration of cells was determined by reading the absorbance at 630 mμ in a Bausch and Lomb Spectronic-20 spectrophotometer and comparing the readings to a standard curve of mg of dry weight per ml versus absorbance. The cell suspension was used to yield 2 mg per ml dry weight final concentration. The cells were equilibrated at 20° for 15 minutes. Glucose was added to a final concentration of 1 mg per ml to insure an adequate energy source and the incubation was continued for 15 minutes longer. At this time, labeled lipoic acid was added and aliquots were removed at appropriate intervals using a Cornwall syringe with the cannula set for 0.5 ml.

The uptake reaction was stopped by ejecting an aliquot (0.5 ml) into mushy ice (1 ml of frozen minimal medium). Termination of the uptake by filtration on a Millipore filter was attempted but the high background due to adsorption of the labeled lipoic acid on the filter made further measurements impractical. Table I shows some of the

TABLE I

VARIOUS METHODS FOR STOPPING UPTAKE OF LIPOIC ACID BY *E. COLI*

Treatments	The amount of lipoic acid taken up (CPM)
100-fold dilution	7
Quick freezing in Liq. N_2	1280
Slow freezing	1004
Mushy ice	1092
Millipore Filter	40
Ice-cold 25% glycerol	51

The uptake experiment procedure is described in the Materials and Methods section. The amounts of radioactivity were measured after the incubation of the cells in the presence of 20 µg of radioactive lipoic acid per ml for 5 minutes. For the measurement of background the uptake experiments were performed without the cells and appropriate corrections under the same conditions were made.

results under various conditions in stoppint the uptake. After stopping the uptake on mushy ice, the aliquots were thawed slowly in the cold (2^{o}). The cells were removed by centrifugation and washed twice with the cold minimal medium. The two 1 ml washings were found to be sufficient to remove essentially all of the radioactivity from the surface of the cells (67). After the final washing, the cell pellet was suspended in 0.5 ml of distilled water and transferred to vials by using a Pasteur pipette. Bray's scintillation solution (68) was used to count the radioactivities in a Packard Tri-Carb Liquid Scintillation Counter.

During the course of stopping the uptake and washing the cells, the viabilities of the cells at each step were measured by plating each aliquots on Bacto-tryptone agar plates. Table II shows the results.

Osmotic Shocking Procedure

Ten milliliters of cells grown on M-9 to the early stationary phase were added to 200 ml of fresh M-9 and incubated for 4-5 hours to obtain midlog phase cells. The cells were harvested and washed twice with 0.03 M Tris-HCl (pH 7.1). The washed cells (250 mg, wet weight) were suspended in 20 ml of 20% sucrose in 0.03 M Tris-HCl-10^{-4}M EDTA and slowly rotated at room temperature for 10 minutes. The cell suspension was then centrifuged at 10,000 x g for 10 minutes in the cold. The supernatant fluid was removed. The cell pellet was immediately subjected to shocking by adding 20 ml of ice-cold 5 x 10^{-4} M $MgCl_2$ solution. After 3 minutes, an equal volume of 0.05 M Tric-HCl (pH 7.1) containing 1 x 10^{-3} M $MgCl_2$ was added to the cell suspension and

TABLE II

CELL VIABILITIES DURING OSMOTIC SHOCK EXPERIMENT

Treatments	Length of time of osmotic shock(min.) 2	4	6	10
Control cells	100	100	100	100
Shocked cells	100	97	97	97
Shocked cells in mushy ice	94	91	91	94
Shocked cells after washing	91	80	63	63

Osmotic shock treatment is described in the Materials and Methods section. The viability of the control cell was taken as the 100% value. The shocked cells in mushy ice was determined after the frozen cells were thawed slowly at 0^{o}. The cells were then washed once with ice-cold M-9 media by centrifugation at 7,500 x g and the viabilities were determined on Bacto-trypton agar plates.

centrifuged at 10,000 x g for 10 minutes. To count the viable cells, aliquots from the untreated and shocked cells were plated on agar plates containing 1% tryptone broth plus 0.5% NaCl (18). Measurement of cell viability following osmotic shock under various conditions indicated that 2-3 minutes of osmotic shocking was sufficient.

For the preparation of shock fluid from E. coli the same method was applied. The supernatant solution from the shocked cells was lyophilized and dissolved in 0.1 M potassium phosphate buffer, pH 6.6, and then dialyzed against the same buffer. Approximately 3.7 mg of protein was usually obtained from 1 g of starting cells.

Membrane Preparations for Binding Studies

Cells of E. coli were grown in minimal medium to the midlogarithmic phase and washed two times with ice-cold 0.01 M Tris-HCl, pH 8.0 after harvesting by centrifugation. The cells were suspended in 0.03 M Tris-HCl, pH 8.0, containing 20% sucrose plus EDTA (10 mM final concentration) at room temperature and the mixture was incubated with 500 μg/ml of lysozyme at room temperature for 30 minutes while stirring. After centrifuged at 16,000 x g for 30 minutes in Sorvall RC-2 centrifuge, the spheroplast pellet was homogenized by sing a teflon-glass homogenizer and suspended in a small volume of 0.1 M potassium phosphate, pH 6.6 containing 20% sucrose plus 20 mM $MgSO_4$, then 10 μg/ml of deoxyribonuclease were added. The lysate was diluted 200-fold in 0.05 M potassium phosphate, pH 6.6 and centrifuged at 16,000 x g for 30 minutes. The pellet was washed five times with 0.1 M potassium phosphate, pH 6.6 containing 10 mM EDTA and finally suspended in the same buffer (69). An aliquot of the membrane preparation was taken for the measurement of

whole cell contamination and plated according to Neu and Heppel (18).

Measurement of Binding

The binding measurements were made using a multicavity equilibrium dialysis cell (Chemical Rubber Co.). One cavity contained the substrate (i.e., ^{35}S-lipoic acid in 0.1 M potassium phosphate buffer, pH 6.6, containing 1 mM $MgSO_4$ and 10 mM glucose) and the other cavity contained the membrane preparation of E. coli in the same buffer. The compartments were separated by a cellulose dialyzing membrane(Arthur H. Thomas Co. product, 4.8 mμ pore size) that was equilibrated in a solution of 0.1 M potassium phosphate, pH 6.6 containing 1 mM $MgSO_4$ at room temperature. After equal volumes of the substrate and the membranes were introduced into each cavity by using a hydrodermic syringe, the dialysis cell was incubated with shaking at 37° until equilibrium was reached (approximately 12 hours). The length of time for equilibrium was determined by using controls, i.e. without the cell membrane preparation.

CHAPTER III

INTRACELLULAR DISTRIBUTION OF ENZYMES

The existence and overall properties of a lipoic acid transport system have been established by Sanders and Leach (50) and Wilson and Leach (51) as a prelude to the isolation of the lipoic acid-carrier complex. The system is present in a number of microorganisms including both S. faecalis and E. coli. A study of the distribution into intracellular and membrane bound enzymes of those enzymes known to be concerned with lipoic acid metabolism and function might incriminate one of these enzymes in the transport process. Two enzymes are involved in the activating system (55). One of the enzymes (PS-2A) catalyzes the ATP-dependent activation of lipoic acid to form enzyme bound lipoyladenylate while the second enzyme (PS-2B) functions in transferring of the enzymes bound lipoic acid to apopyruvate dehydrogenase complex to form the holopyruvate dehydrogenase complex. The reactions catalyzed the these two enzymes resemble what would be expected for an energy-dependent transport reaction. If these enzymes were located in or on the cell membrane, the possibility of their participation in active transport would be enhanced. To determine the localization of the activating enzymes and apopyruvate dehydrogenase system in the cell, the whole cell extracts, the solubilized membrane fractions and membrane supernatant solutions from both E. coli and S. faecalis were prepared and assayed for pyruvate dismutation activities. Marker enzymes, malic

dehydrogenase localized in both the membrane fraction and its supernatant solution (70), NADH oxidase known to be associated with the membrane fraction (71) and glucose-6-phosphate dehydrogenase present only in the membrane supernatant solution (71) were assayed for their distribution to establish that typical membrane preparations were obtained.

Preparation of Lipoic Acid-Activating Enzymes and Apopyruvate Dehydrogenase Complex from S. faecalis 10C1

The two activating enzymes (PS-2A and PS-2B) and apopyruvate dehydrogenase complex were required to determine the distribution of the various enzymes in the whole cell extracts, the solubilized membrane fractions and membrane supernatant solutions. By protamine sulfate fractionation a lipoic acid-activating fraction (PS) was separated from the apopyruvate dehydrogenase complex (PP) and further fractionated into two enzymes (PS-2A and PS-2B) by ammonium sulfate precipitations. The apopyruvate dehydrogenase complex of lipoic acid-deficient cells is inactive due to the lack of lipoic acid and must be incubated with lipoic acid, ATP, Mg^{++} and the two enzymes to gain catalytic activity (55). A summary of the fractionation is presented in Table III. Fraction PS-2A was heat-labile whereas Fraction PS-2B could be heated in boiling water for 10 minutes without loss of activity. These fractions could be stored in a deep freeze (-20°) for several months. After 3 months, 45% of the PS-2A and 80% of the PS-2B activity remained.

The Distribution of Pyruvate Dismutation Enzymes in S. faecalis 10C1

The whole cell extract (CE), the solubilized membrane fraction (SM)

TABLE III

FRACTIONATION OF LIPOIC ACID-DEFICIENT
S. FAECALIS 10C1 EXTRACTS

Fraction	Total Volume (ml)	Total Protein (mg)	Specific Activity*	Total Units	%Yield
Diluted Extract	315	6145	0.78	4690	100
PP	28	280	14.6	4088	86
PS	340	1860	2.2	4105	87
PP-1	29	135	16.0	2160	46
PS-2	46	180	14.0	2520	53
PS-2A	27	95	23.1	2185	46
PS-2B	26	104	20.0	2080	44

One unit of lipoic acid-activating system corresponds to the production of one μmole of acetyl phosphate in 30 minutes by the "activated" apopyruvate dehydrogenase system. Each fraction was assayed in the presence of an excess of the other essential fractions by the two step procedure described under the Materials and Methods section. All operations were done at 0° - 5°.

* Units per mg of protein

TABLE IV

DISTRIBUTION OF THE ENZYMES FOR PYRUVATE DISMUTATION IN S. FAECALIS 10C1

Preparation	Total μmoles of acetyl-phosphate formed	μmoles of acetyl-phosphate formed per mg of protein	% Activity
CE	828	2.76	100
SM	0	0	0
MS	805	4.70	97

Each fraction was incubated in the two step manner described in the Materials and Methods section with the activity of the holo-pyruvate dehydrogenation complex being the ultimate activity determined. The protein contents were; whole extract, 60 mg/ml; membrane fraction, 20 mg/ml, and membrane supernatant solution, 12 mg/ml.

and membrane supernatant solution (MS) were prepared from the same batch using equal weights of cells to provide a valid comparison of enzymes distribution. As shown in Table IV, the activity of the pyruvate dehydrogenase system was found exclusively in the membrane supernatant solution, that is, the soluble portion which is not membrane bound. This experiment did not involve supplementation of the individual fractions with the lipoic acid activating enzymes or apopyruvate dehydrogenase complex and thus detects only where both enzyme systems are in the same fraction.

Pyruvate Dismutation by the Fractions from *S. faecalis* 10C1 upon Supplementation

The whole extract (CE), the solubilized membrane fraction (SM) and its supernatant solution (MS) were assayed for pyruvate dismutation activity with the additions of APDH, PS-2A and PS-2B separately, or PS-2A + PS-2B. The results are shown in Table V. The complete system plus APDH would detect the activating enzymes that might be present in the solubilized membrane preparation whole the additions of PS-2A and PS-2B either separately or together to the complete system would detect either one of the activating enzymes plus APDH dehydrogenase system or APDH only in the membrane preparation. However, neither the activating enzymes nor the apopyruvate dehydrogenase system was found in the solubilized membrane fraction.

The Distribution of Pyruvate Dismutation Enzymes in *E. coli* (Crookes strain)

The whole cell extract (CE), the solubilized membrane fraction

TABLE V

PYRUVATE DISMUTATION BY THE FRACTIONS FROM *S. FAECALIS* 10C1 UPON SUPPLEMENTATION

Components	μmole of acetyl-phosphate formed per mg of protein		
	CE	SM	MS
Complete	2.8	0	4.7
No lipoic acid	0	0	0
Complete + APDH	2.6	0	4.5
Complete + PS-2A	2.7	0	4.7
Complete + PS-2B	2.8	0	4.7
Complete + PS-2A + PS-2B	2.6	0	4.4

The complete system contained the reaction mixture as described in the Materials and Methods section. The fractions to be tested: whole extract, 55 mg/ml protein; membrane fraction, 61 mg/ml; and membrane supernatant solution, 18 mg/ml were prepared as described in the Materials and Methods section. Partially purified APDH, PS-2A and PS-2B were prepared from *S. faecalis* 10C1 (59), and in these assay APDH (400 μg), PS-2A (450 μg) and PS-2B (120 μg) were added to the assay mixture in the first incubation as indicated in the left column. Neither PS-2A + PS-2B nor APDH itself gave any pyruvate dismutation activity, whereas this activity was observed when all three enzymes were incubated together in the two step assay. The specific activities observed were PS-2A, 28.0; PS-2B, 21.9; and APDH, 18.3 when each was the respective limiting component.

TABLE VI

DISTRIBUTION OF ENZYMES FOR PYRUVATE DISMUTATION IN E. COLI (CROOKES STRAIN)

Fraction	Total μmoles of acetyl-phosphate	μmoles of acetyl-phosphate formed per mg protein	% Activity
CE	989	2.2	100
SM	0	0	0
MS	812	2.6	82

Since the holopyruvate dehydrogenation complex is already formed in E. coli, these reactions were started at the second stage. The E. Coli cells were grown as described in the Materials and Methods section and divided into two portions one of which was for the preparation of whole extract and the other was for the preparation of membrane and its supernatant fraction. The protein contents were: whole extract, 105 mg/ml; membrane fraction, 15 mg/ml; and supernatant fraction, 18 mg/ml.

(SM) and its supernatant solution (MS) were prepared from the same batch using equal weights of cells to provide a valid comparison of enzyme distribution for pyruvate dismutation. No pyruvate dehydrogenase complex activity was observed with the solubilized membrane fraction, whereas all of the enzyme activity was found in the membrane supernatant solution as shown in Table VI. This is consistent with the S. faecalis 10C1 findings in which all of the pyruvate dismutation activity was found in the membrane supernatant solution.

Pyruvate Dismutation by the Fractions from E. coli (Crookes strain) upon Supplementation

That the lipoic acid-activating system i.e., fractions PS-2A and PS-2B, is not unique for S. faecalis 10C1 was indicated by the observation that the enzymes can be replaced by an enzyme fraction prepared from extracts of E. coli (Crookes strain) (72). As shown in Table VII, neither the activating enzymes nor the apopyruvate dehydrogenase system was found in the solubilized membrane fraction. The supplementation of the membrane supernatant solution with APDH exhibited an increased pyruvate dismutation activity.

Distribution of Marker Enzymes in S. faecalis 10C1

To establish that typical membranes were being obtained, the distribution of marker enzymes (malic dehydrogenase, NADH oxidase and glucose-6-phosphate dehydrogenase) was measured. As shown in Table VIII, the L-malate oxidizing system is associated with both fractions (SM and MS). The NADH oxidase known to be associated with membranes was exclusively found in the solubilized membrane whereas

TABLE VII

PYRUVATE DISMUTATION BY THE FRACTIONS FROM E. COLI (CROOKES STRAIN) UPON SUPPLEMENTATION

Components	μmoles of acetyl-phosphate formed per mg of protein		
	CE	SM	MS
Complete, no first step	2.2	0	2.6
Complete + APDH	2.8	0	3.8
Complete + PS-2A	2.2	0	2.4
Complete + PS-2B	2.1	0	2.3
Complete + PS-2A + PS-2B	2.2	0	2.4

For the first line no lipoic was added to the reaction mixture and no first step incubation for activation was included. In all other mixtures the first step incubation with lipoic acid was done as described in the Materials and Methods section. The protein contents of the preparations were: whole extract, 105 mg/ml; membrane fraction, 15 mg/ml; and supernatant solution, 18 mg/ml. Partially purified APDH, PS-2A and PS-2B were prepared from S. faecalis 10C1 (59) and amounts of the protein used were: APDH, 650 μg of protein; PS-2A 400 μg of protein; and PS-2B, 350 μg of protein. The specific activities observed were APDH, 16.1; PS-2A, 24.7; and PS-2B, 18.5 when each was the respective limiting component. The enzyme activity was the average values of two experiments.

TABLE VIII

DISTRIBUTION OF MARKER ENZYMES IN
S. FAECALIS 10C1

Enzymes	Total units[1]			Specific Activity[2]			%Activity[3]	
	CE	SM	MS	CE	SM	MS	SM	MS
Malic dehydrogenase	2700	550	1600	5.0	1.8	5.2	23.7	59.3
NADH oxidase	315	225	0.2	0.58	0.73	0	71	0.06
G-6-P dehydrogenase	332	0.1	262	0.6	0	0.86	0.03	80

The enzyme activities were determined from initial rates (linear and within the first three minutes), and were proportional to the concentration of enzyme being determined in the presence of the respective substrates. Each reaction mixtures contained either 1.8 mg protein (CE) or 0.6 mg protein (SM), or 0.36 mg protein (MS). The rates of oxidation of NADH for malic dehydrogenase and NADH oxidase activities, and reduction of NADP for G-6-P dehydrogenase activity were measured at 340 mμ by using a Gilford model 2000 system as described in the Material and Methods section.

[1] One unit of enzyme euqals the amount of enzymes for 0.01 change in absorbance per minute.

[2] Units activity per mg protein of each fraction.

[3] The activity of the CE was taken as the 100% value.

glucose-6-phosphate dehydrogenase was localized in the membrane supernatant solution.

Distribution of Marker Enzymes in *E. coli* (Crookes Strain)

The distributions of the marker enzymes were determined in *E. coli*. Table IX shows that malic dehydrogenase were present in both the membrane fraction and its supernatant solution. Based on total activity, NADH oxidase appeared to be associated with the membrane fraction while glucose-6-phosphate dehydrogenase was located in the membrane supernatant fraction.

Distribution of Enzymes for Pyruvate Dismutation in Membranes of *S. faecalis* 10C1 Prepared in Two Methods

The possibility that using the detergent Brij 58 to disrupt the *S. faecalis* protoplasts changes the membrane-bound enzyme activities, perhaps due to destruction of the membrane structure or release of the enzymes, was studied. To find whether or not the detergent has any effect on the membrane preparation, the protoplasts were disrupted by osmotic lysis or by detergent lysis and then the solubilized membrane preparations were assayed for the activities of the indicated enzymes as shown in Table X. If the membrane preparation contained either the activating enzymes or apopyruvate dehydrogenase complex, the supplementations of the enzymes to the assay mixtures would increase the pyruvate dismutation activity. However the results indicate no increase in either membrane preparation.

TABLE IX

DISTRIBUTION OF MARKER ENZYMES IN
E. COLI (CROOKES STRAIN)

Enzymes	Total Units [1]			Specific Activity [2]			%Activity [3]	
	CE	SM	MS	CE	SM	MS	SM	MS
Malic dehydrogenase	780	92.5	634	3.0	1.9	2.0	12	81
NADH oxidase	39.6	32.4	0.2	0.17	0.68	0	81	0.5
G-6-P dehydrogenase	148	0.37	94.6	0.64	0.01	0.30	0.2	64

The enzyme activities were determined from initial rates (linear and within the first three minutes), and were proportional to the concentration of enzyme being determined in the presence of the respective substrates. Each reaction mixture contained either 2.8 mg protein (CE) or 0.39 mg protein (SM), or 0.45 mg protein (MS). The rates of oxidation of NADH for malic dehydrogenase and NADH oxidase, and the rate of reduction of NADP for glucose-6-phosphate dehydrogenase were measured at 340 mμ by using Gilford model 2000 system as described in the Materials and Methods section.

[1] One unit of enzyme equals the amount of enzyme for 0.01 change in absorbance per minute.

[2] Units of activity per mg protein of each fraction.

[3] The activity of the CE was taken as the 100% value.

TABLE X

DISTRIBUTION OF ENZYMES FOR PYRUVATE DISMUTATION, IN MEMBRANES, OF S. FAECALIS 10C1 PREPARED BY TWO METHODS

Additional Components	Assayed for	Enzyme Specific Activity	
		Brij 58 Treatment	Osmotic Lysis
A. Membrane Assay			
APDH	PS-2A+PS-2B	0.01	0.01
PS-2A	APDH + PS-2B	0	0.01
PS-2B	APDH + PS-2B	0	0.01
APDH + PS-2A	PS-2B	0	0.01
APDH + PS-2B	PS-2A	0.01	0
PS-2A+PS-2B	APDH	0.01	0
B. Control Assay		No Membrane	
APDH + PS-2A		0.02	
APDH + PS-2B		0.01	
PS-2A + PS-2B		0	
APDH + PS-2A + PS-2B		1.28	

The Brij 58 treatment is described in the Materials and Methods section. The osmotic lysis was conducted in a volume of 6.0×10^{-3} M $MgSO_4$ solution at room temperature for 30 minutes. The solubilized membrane preparations were supplemented with the indicated enzyme preparation(3) and incubated in the two step assay for determination of the specific activities of the other components. Thus when supplemented with APDH the assay is for PS-2A+PS-2B activity, etc. In control, the incubation mixtures contained APDH, 650 µg protein; PS-2A, 400 µg protein, and PS-2B, 350 µg protein. The enzyme specific activity was the average values of two experiments.

Distribution of Marker Enzymes in Membranes of *S. faecalis* 10C1 Prepared in Two Methods

Because the use of Brij 58 to disrupt the protoplasts might result in the removal of enzymes normally associated with the membranes, the membranes were also prepared by osmotic lysis as in the previous experiment. Since the same total amount of the marker enzymes were found in the membrane fractions prepared by either method, the detergent probably had no marked effect on the membrane preparations.

TABLE XI

EFFECT OF DETERGENT BRIJ 58 ON MEMBRANE PREPARATION OF *S. FAECALIS* 10C1

Enzymes	Total Units	
	Brij 58 treatment	Osmotic lysis
Malic Dehydrogenase	550	580
NADH Oxidase	225	231
G-6-P Dehydrogenase	0.1	0.1

The Brij 58 treatment is described in the Material and Methods section. The osmotic lysis was carried out in a volume of 6.0×10^{-3} M $MgSO_4$ solution at room temperature for 30 minutes. Assay for the enzymes was the same as described in Table VIII.

CHAPTER IV

UPTAKE OF LIPOIC ACID BY WHOLE *E. COLI* CELLS

To characterize the transport system of whole *E. coli* cells, certain kinetic properties of the uptake of lipoic acid were studied. This work established that the *E. coli* system was similar to that which Sanders and Leach (50) studied in *S. faecalis*. Some parallel studies of the valine uptake system were performed to compare the two systems.

Effect of Cell Concentration on the Uptake of Lipoic Acid

The uptake of lipoic acid by varying concentrations of *E. coli* cells was measured. The washed cell suspensions in the M-9 medium at concentrations from 0 to 6 mg/ml were equilibrated in a water bath at 20° for 15 minutes and then incubated in the presence of glucose (1 mg/ml) for another 15 minutes. Radioactive lipoic acid was added to the final concentration of 10 µg/ml and samples were taken after 5 minutes incubation at 20°. As shown in Fig. 5, the amount of radioactive lipoic acid taken up was proportional to cell concentrations up to 3 mg/ml and the uptake curve was of the Michaelis-Menten type.

Effect of Lipoic Acid Concentration on Uptake

The substrate concentration necessary to saturate the uptake system was determined by varying the concentration of lipoic acid from

Figure 5. Effects of Cell Concentration on the Uptake of Lipoic Acid by *E. coli*

Washed cells in the M-9 medium at the indicated concentrations were incubated for 15 minutes at 20° and then glucose was added to the final concentrations of 1 mg/ml. After 15 minutes incubation at 20°, the cells were incubated with 10 ug/ml of radioactive lipoic acid for 5 minutes. Then aliquots (0.5 ml) of the cell suspension were taken out and ejected into mushy ice to stop the uptake reaction. The frozen cells in the mushy ice were slowly thawed at 2° and then washed twice with the same ice cold medium by centrifugation at 6000 x g. The radioactivity of the washed cell was measured in a Packard scintillation spectrometer.

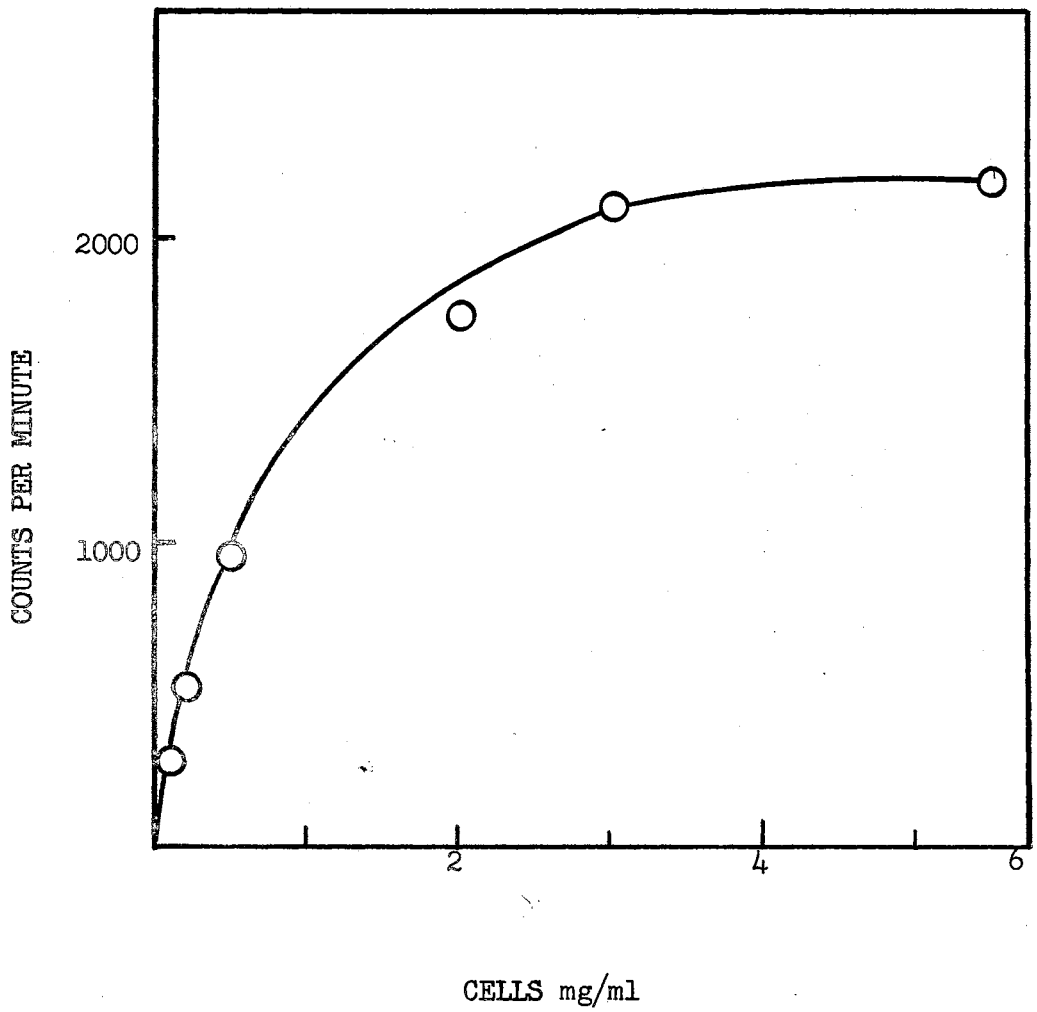
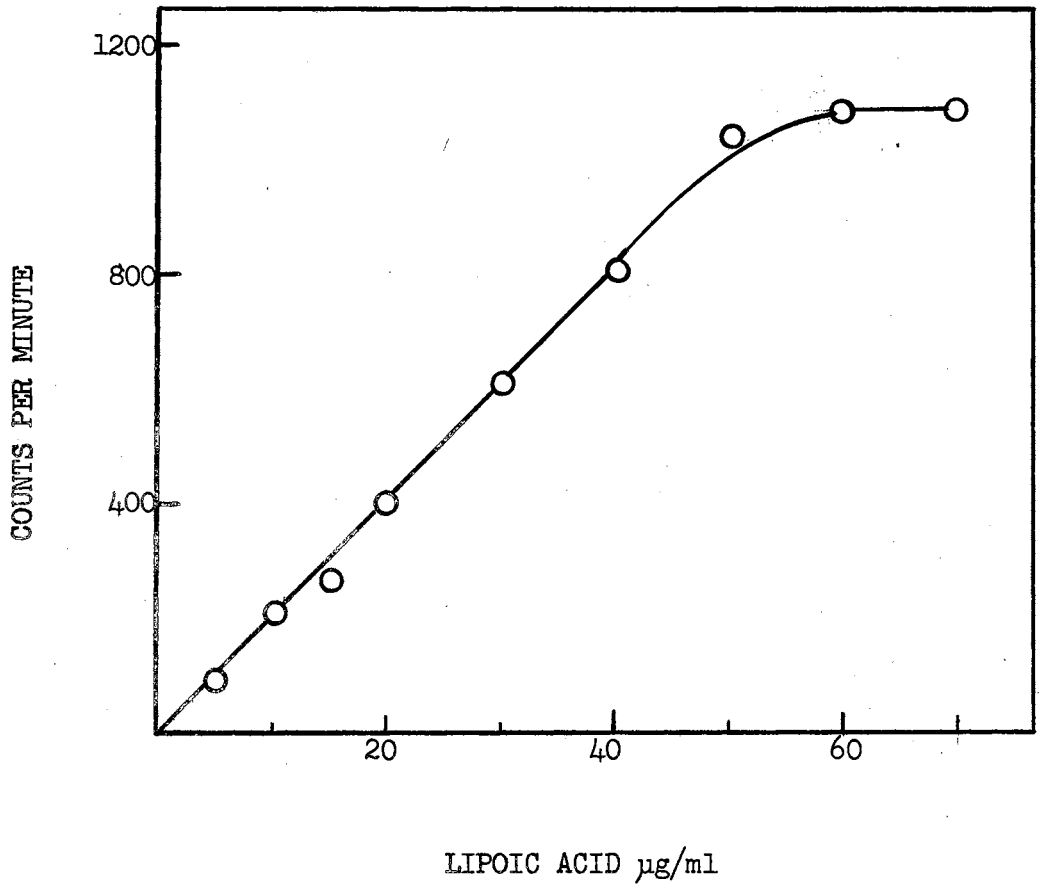

COUNTS PER MINUTE
2000
1000
2
4
6
CELLS mg/ml

Figure 6. Effects of Lipoic Acid Concentration on the Uptake by *E. coli*

The washed cell suspensions (2 mg/ml) in the M-9 medium were incubated at 20° for 15 minutes and in the presence of glucose (1 mg/ml) for another 15 minutes. Radioactive lipoic acid at the indicated concentrations was then added to the cell suspension. After the incubation at 20° for 5 minutes, the uptake was stopped in mushy ice. The radioactive cells were washed twice with the ice cold M-9 medium before the measurement of the radioactivity.


1200
800
400
COUNTS PER MINUTE
20
40
60
LIPOIC ACID µg/ml

0 to 70 µg/ml. The washed cell suspensions in the M-9 medium (2 mg/ml) were incubated at 20° for 15 minute. After incubation at 20° in the presence of glucose (1 mg/ml) for 15 minutes, the cells were incubated with the various concentrations of lipoic acid at 20° for 5 minutes. The uptake reaction was stopped by ejecting the cells into mushy ice. As shown in Fig. 6, the lipoic acid uptake curve indicates a concentration of 50 µg/ml being sufficient to saturate the uptake system. The apparent K_m value is 4.3×10^{-5} M.

Kinetics of Lipoic Acid Uptake

The time course of uptake was determined at 20°. The washed cell suspension in the M-9 medium was incubated at 20° for 15 minutes and then in the presence of glucose (1 mg/ml) for another 15 minutes. After the addition of radioactive lipoic acid (10 µg/ml) to the cells, samples were taken at various times, and the uptake was stopped by the procedure used in the previous experiment. The results shown in Fig.7, indicate that the maximum uptake occurred rapidly within one minute, and after 3 minutes the uptake decreased gradually to a plateau where equilibrium was reached. A similar time course of lipoic acid uptake was observed with S. faecalis 10C1 with the maximum uptake being obtained at about 30 seconds and equilibrium reached after one minute (50).

Kinetics of L-Valine Uptake

The time course of valine uptake was determined under conditions similar to those used with lipoic acid to provide valid comparisons of the two transport systems. The washed cell suspension in the M-9

Figure 7. Time Course of Lipoic Acid Uptake by *E. coli*

The washed cell suspension (1 mg/ml) was equilibrated at 20° for 15 minutes and incubated in the presence of glucose (1 mg/ml) for another 15 minutes. After radioactive lipoic acid (10 μg/ml) was added, samples were taken at the indicated times and ejected into mushy ice to stop the uptake reaction. The cells were then washed twice with the ice cold M-9 medium and the radioactivity of the cells was determined as in the previous experiments.

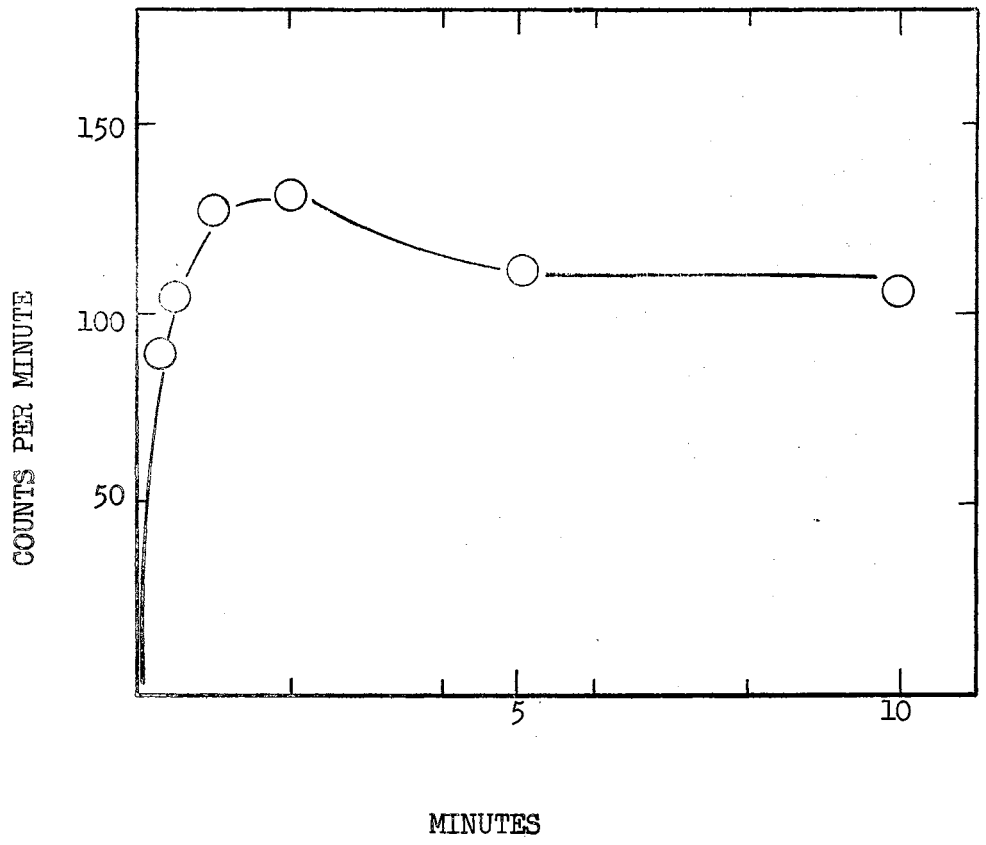
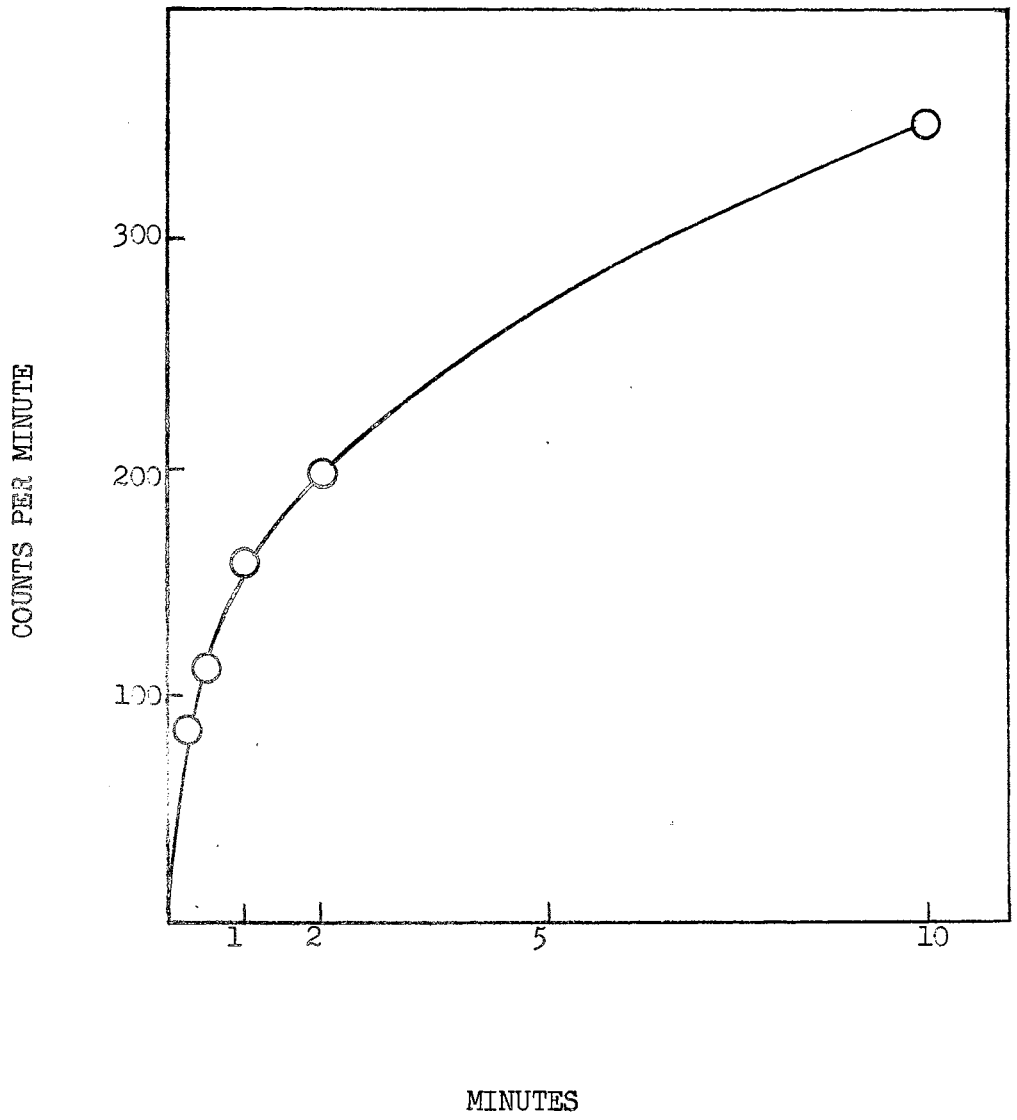

150
100
50
COUNTS PER MINUTE
5
10
MINUTES

Figure 8. Time Course of L-Valine Uptake by E. coli

The cell suspension in the M-9 medium concentration of cell (1 mg/ml) was incubated for 15 minutes at 20° and then glucose (1 mg/ml) was added. After incubation for 15 minutes, radioactive L-valine (0.025 μmole/ml) was added to the cell suspension. Aliquots were taken at the indicated times and radioactivity of the samples was measured as in the previous experiments.


COUNTS PER MINUTE
300
200
100
1
2
5
10
MINUTES

medium was equilibrated at 20° for 15 minutes and the incubated in the presence of glucose for 15 minutes. Radioactive L-valine was added to the cell suspension and then samples were taken at various times as in the previous experiment. The results shown in Fig. 8 indicate that the curve was typical for amino acid uptake (38).

Effect of Temperature on Lipoic Acid Uptake

Washed cells in the M-9 medium were equilibrated at 10° and 20°, and then incubated in the presence of glucose (1 mg/ml) for 15 minutes. After radioactive lipoic acid was added, the time course of uptake was studied as in the previous experiment. In both cases there was a rapid uptake which reached a maximum at one minute, followed by a slight decline in the amount of lipoic acid uptake as shown in Fig. 9.

Effect of Temperature on L-Valine Uptake

The effect of temperature was demonstrated in the uptake of L-valine. Washed cells in the M-9 medium were equilibrated for 15 minutes at 10° and 20° respectively, and incubated in the presence of glucose for another 15 minutes. After radioactive L-valine was added, the time course studies of uptake were performed as in the previous experiment. The uptake at 20° was significantly greater than that at 10°, as shown in Fig. 10. Both uptake curves at 10° and 20° exhibited the same type of hyperbolic curves which were typical for amino acid uptake.

Figure 9. Effect of Temperature on the Uptake of Lipoic Acid by E. coli

The washed cell suspensions (1 mg/ml) were incubated for 15 minutes in water baths at 10^{o} and 20^{o} respectively, and then incubated in the presence of glucose (1 mg/ml) for another 15 minutes. Radioactive lipoic acid (10 µg/ml) was added to each suspension and then samples was taken out at the indicated times and the uptake reaction was stopped in mushy ice. The cells were washed twice with ice cold M-9 medium and then radioactivity of the cells was measured as in the previous experiment. Background (non-specific binding at 0^{o}) was subtracted from the uptakes at 10^{o} and 20^{o}. Uptake at 10^{o} (●); uptake at 20^{o} (○).

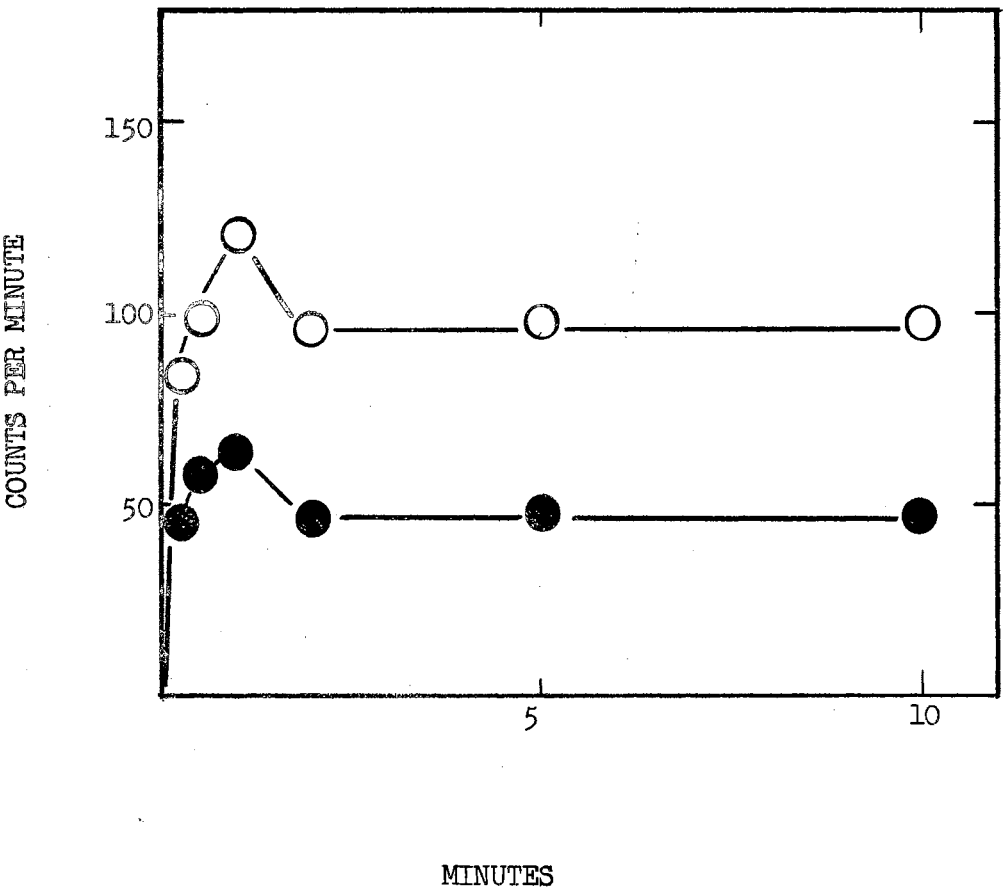
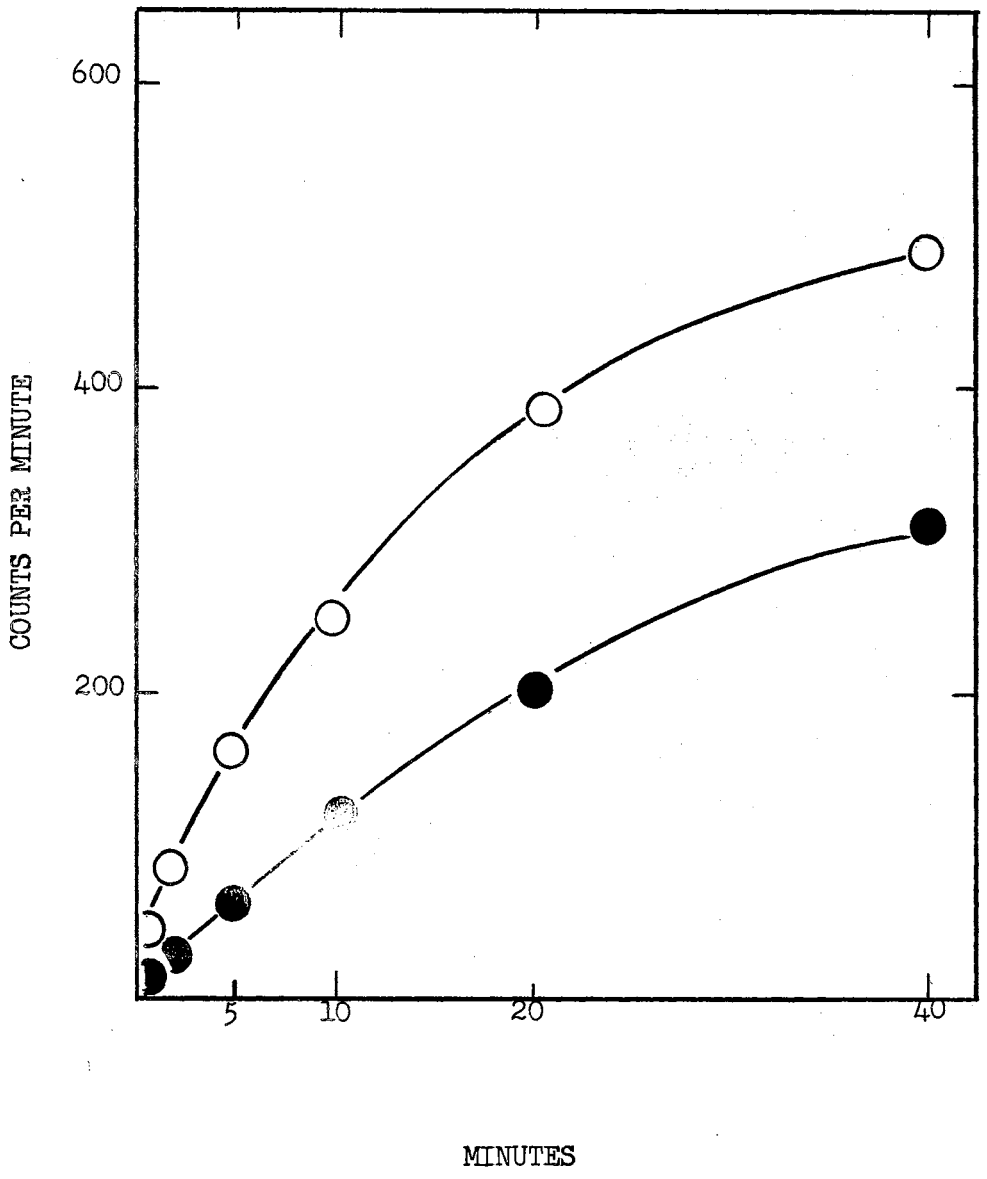

150
100
50
5
10
COUNTS PER MINUTE
MINUTES

Figure 10. Effect of Temperature on the Uptake of L-Valine by E. coli

The washed cell suspensions (1 mg/ml) were incubated for 15 minutes in water baths at 0°, 10° and 20° respectively, and then incubated in the presence of glucose (1 mg/ml) for another 15 minutes. Radioactive L-valine (0.025 μmole/ml) was added to each suspension and then samples were taken out at the indicated times and the uptake reaction was stopped in mushy ice. The cells were washed twice with ice cold M-9 medium and then radioactivity of the cells was determined as in the previous experiments. Background (non-specific binding at 0°) was subtracted from the uptakes at 10° and 20°. Uptake at 10° (●); uptake at 20° (○).


600
400
200
COUNTS PER MINUTE
5
10
20
40
MINUTES

CHAPTER V

EFFECT OF OSMOTIC SHOCK ON LIPOIC ACID UPTAKE

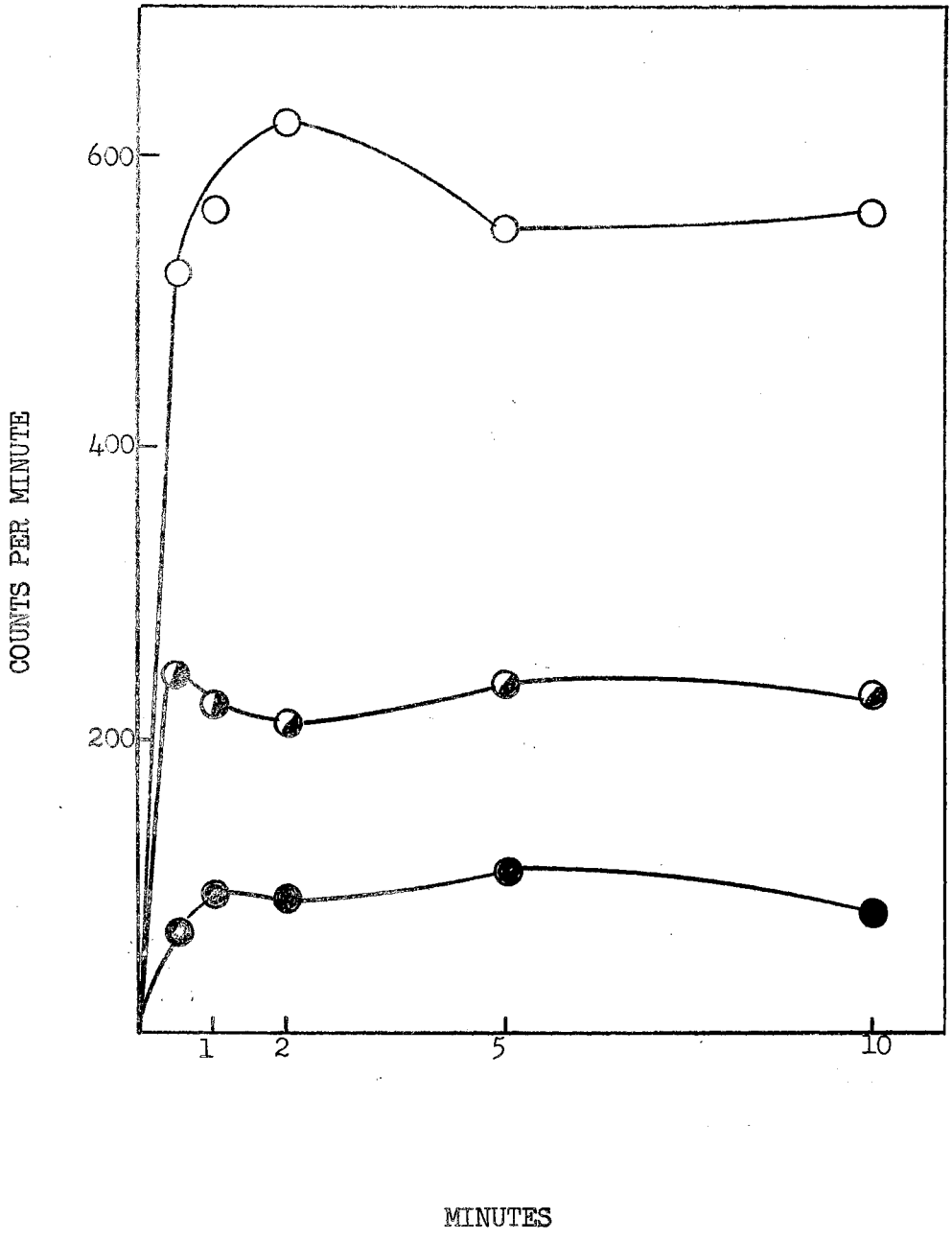
Several binding proteins of transported substrates have been identified in the protein fractions released by osmotic shock (70). Cells are shocked by suspending them at room temperature in hypertonic sucrose solution. Following sedimentation by centrifugation, they are exposed to an ice-cold magnesium solution which releases proteins from the membrane-wall space of the cell. A number of hydrolytic enzymes and proteins (5-10% of the cellular protein) found in the cell periplasmic space are released without significant loss of viability of the shocked cells (71). The effect of osmotic shocking on the transport of lipoic acid and valine was determined.

Effect of Osmotic Shock on Lipoic Acid Uptake

The washed cells were subjected to the following osmotic shock treatment: the cells suspended in a hypertonic sucrose solution were immediately centrifuged and then transferred to a ice-cold hypotonic solution. The shocked cells and untreated cells were equilibrated at 20° for 15 minutes and then glucose was added. One batch of the untreated cells was incubated with 2,4-dinitrophenol. After 15 minutes incubation, radioactive lipoic acid was added to each cell suspension and then samples were taken at various times for the measurement of radioactivity. As shown in Fig. 11, the amount of lipoic acid taken

Figure 11. Effect of Osmotic Shock on Lipoic Acid Uptake by E. coli

The cells were washed with cold 0.03 M Tris-HCl buffer solution pH 7.1. One gram (wet weight) of cells was suspended in 80 ml of 20% sucrose-0.033 M Tris-HCl - 1 x 10^{-4} M EDTA, pH 7.1 at 24°, and the suspension was stirred slowly for 10 minutes. Then the cells were centrifuged for 10 minutes at 13,000 x g. The well drained pellet was rapidly dispersed in 80 ml of ice cold, 5 x 10^{-4} M $MgCl_2$ solution. After 3 minutes, the suspension was centrifuged and a sample was taken and plated on tryptone agar containing 0.5% NaCl for viability measurement. The amount of lipoic acid taken up by the shocked cells was corrected on the basis of an equal viability for the untreated cells and the shocked cells after the uptake experiments. The shocked cells, untreated cells and untreated cell plus 2,4-dinitrophenol (4 x 10^{-3} M) were incubated at 20° for 30 minutes. Radioactive lipoic acid (20 μg/ml) was added and then samples were taken at the indicated times. Rate of uptake by the control cells (○); rate of uptake by the shocked cells (◐); rate of uptake by 2,4-dinitrophenol treated cells (●).


600
400
200
COUNTS PER MINUTE
1
2
5
10
MINUTES

up by the shocked cells was approximately 50 percent of that taken up by the untreated cells. Treatment of cells with 2,4-dinitrophenol gave the level of interaction not due to the energy-dependent transport system.

Effect of Osmotic Shock on L-Valine Uptake

The osmotic shock treatment reduces the transport activity for branched amino acids in E. coli (40). To correlate the extent of release of the component for valine transport with that for the lipoic acid transport, valine uptake was determined with the untreated cells and the osmotic shock treated cells. Fig. 12 shows a marked reduction of valie uptake by osmotic shocking. Treatment of cells with 2,4-dinitrophenol gave the level of interaction not due to the energy-dependent transport system.

Reactivation Attempts for Lipoic Acid Uptake Using Concentrated Shock Fluid in E. coli

Restorations of galactose uptake (27) and sugar uptake (26) in shocked E. coli cells were demonstrated when the cells were treated with shock fluid. The washed cells were subjected to osmotic shock as in the previous experiment. The fluid obtained from the cold water shock containing Mg^{++} was dialyzed against cold distilled water overnight. The fluid was then centrifuged to remove whole cells, lyophilized and then the residue was dissolved in M-9 medium. One batch of the shocked cells was incubated without the concentrated shock fluid and another batch of the shock cells was incubated with the concentrated shock fluid. The uptake curves are shown in Fig. 13. The

Figure 12. Effect of Osmotic Shock on L-Valine Uptake by E. coli

Washed cells in cold 0.03 M Tris-HCl buffer solution, pH 7.1 were suspended in 20% sucrose-0.033 M Tris-HC-1 x 10^{-4} M EDTA solution, pH 7.1. The suspension (1 g wet weight of cells/80 ml) was centrifuged after 10 minutes and then cell pellet was immediately dispersed in 80 ml of ice cold, 5 x 10^{-4} M $MgCl_2$ solution. The suspension was centrifuged after 3 minutes and viability was measured. Corrections for viabilities were made as in the previous experiment. The shocked cells, untreated cells and untreated cells plus 2,4-dinitrophenol were incubated for 30 minutes. Radioactive L-valine (0.025 μmole/ml) was added and then samples were taken at the indicated times. Rate of uptake by the control cells (○); rate of uptake by the shocked cells (⊖); rate of uptake by 2,4-dinitrophenol treated cells (●).

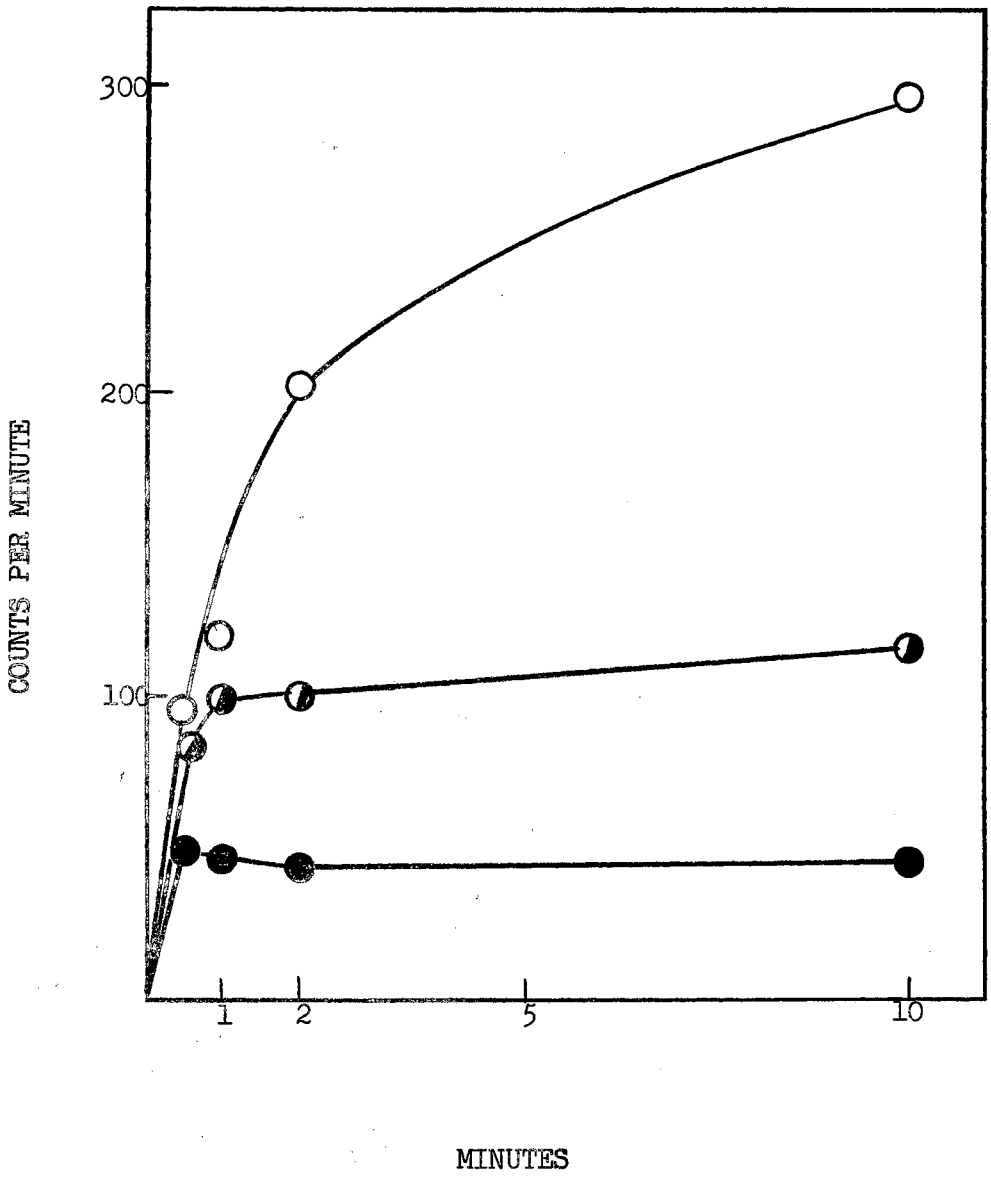
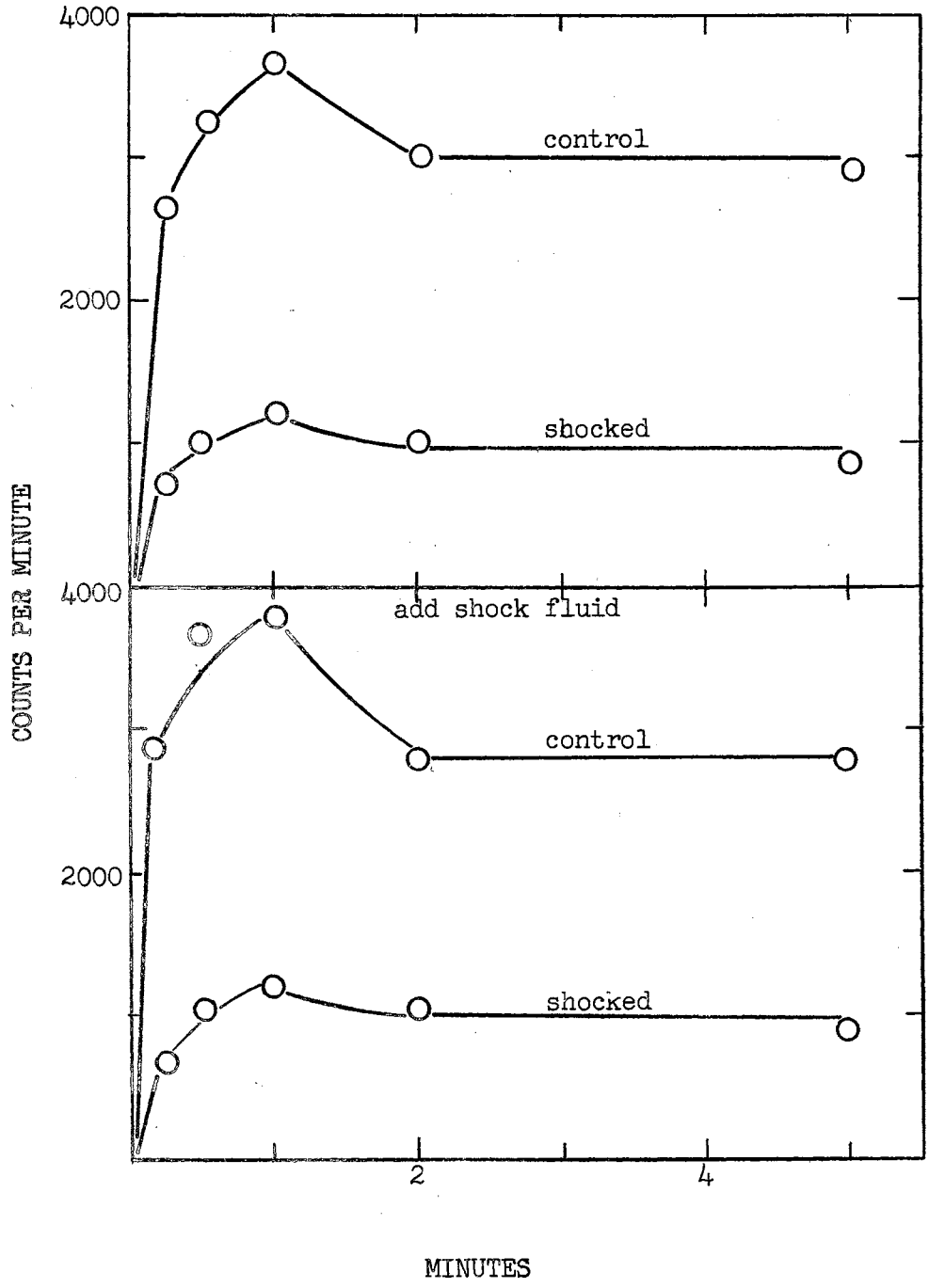

300
200
100
COUNTS PER MINUTE
1
2
5
10
MINUTES

Figure 13. Reactivation Attempts for Lipoic Acid Uptake Using Concentrated Shock Fluid in *E. coli*

For osmotic shock treatment, the cells in a hypertonic sucrose solution were centrifuged at 13,000 x g for 10 minutes and then the well drained pellet was immediately dispersed in cold, Mg^{++} containing solution. Three to four minutes after dispersion, the cells were centrifuged at 10,000 x g and the supernatant solution, shock fluid was dialyzed overnight against cold distilled water. The dialyzed solution was centrifuged and lyophilized. The residue was dissolved in M-9 medium. The shocked cells in M-9 medium (2 mg/ml) were incubated with the shock fluid protein (500 µg/ml) for 30 minutes at 20°. The incubated shocked cells and shocked cells were then incubated at 20° for the uptake studies.


4000
2000
control
shocked
4000
add shock fluid
control
2000
shocked
2
4
COUNTS PER MINUTE
MINUTES

top figure indicates that there was a reduction of more than 50% in the lipoic acid uptake due to the osmotic shock. The bottom figure shows that there was no restoration of the reduced uptake of the shocked cells that were incubated with the concentrated, dialyzed shock fluid.

Effect of Sulfhydryl Reagents on Lipoic Acid Uptake

7-diazonium-1,3-naphthalenedisulfonate (2.5×10^{-3} M), *p*-chloromercuric benzoate (3×10^{-4} M) and N-ethylmaleimide (1×10^{-3} M) were incubated with the cell suspension (2 mg/ml) at 20° to determine inhibition of the lipoic acid uptake. After 30 minutes, the cells were washed twice with the M-9 medium. The washed cells were incubated at 20° for 15 minutes and then glucose was added. After 15 minutes, radioactive lipoic acid (3×10^{-5} M) was added to the cell suspension and samples were taken at one minute for measurement of radioactivity. Table XII shows that there were no inhibitions in the lipoic acid uptake. These reagents that were applied in determining the location of sulfate binding protein (44) are probably not suitable reagents for the lipoic acid uptake system.

TABLE XII

EFFECTS OF SULFHYDRYL REAGENTS ON UPTAKE OF LIPOIC ACID BY *E. COLI*

Reagents	Uptake by the cells Counts per Minute
Control	3255
7-amino-1,3-naphthalenedisulfonic acid, 2.5×10^{-3} M	3223
p-chloromercuribenzoate, 3.0×10^{-4} M	3100
N-ethylmaleimide, 1.0×10^{-3} M	3090

The procedure for the uptake is described in the Materials and Methods section. A 55 mg amount of 7-amino-1,3-naphthalenedisulfonic acid(NDS) was dissolved in 3.5 ml of water plus 0.05 ml of concentrated HCl and was cooled on ice-salt; then 0.25 ml of 0.5 M $NaNO_2$ solution was added at -3°. After 30 minutes, 55 mg of urea was added to destroy excess nitrite. The diazo-NDS, *p*-chloromercuribenzoate and N-ethylmaleimide were incubated with cells for 30 minutes and then the cells were washed twice with the M-9 medium. The washed cell suspension (2 mg/ml) was incubated at 20° for 15 minutes. Aliquots were taken to stop the uptake in mushy ice after the addition of radioactive lipoic acid. For background, the same procedure was followed without cells and appropriate corrections were made.

CHAPTER VI

BINDING OF LIPOIC ACID TO MEMBRANE

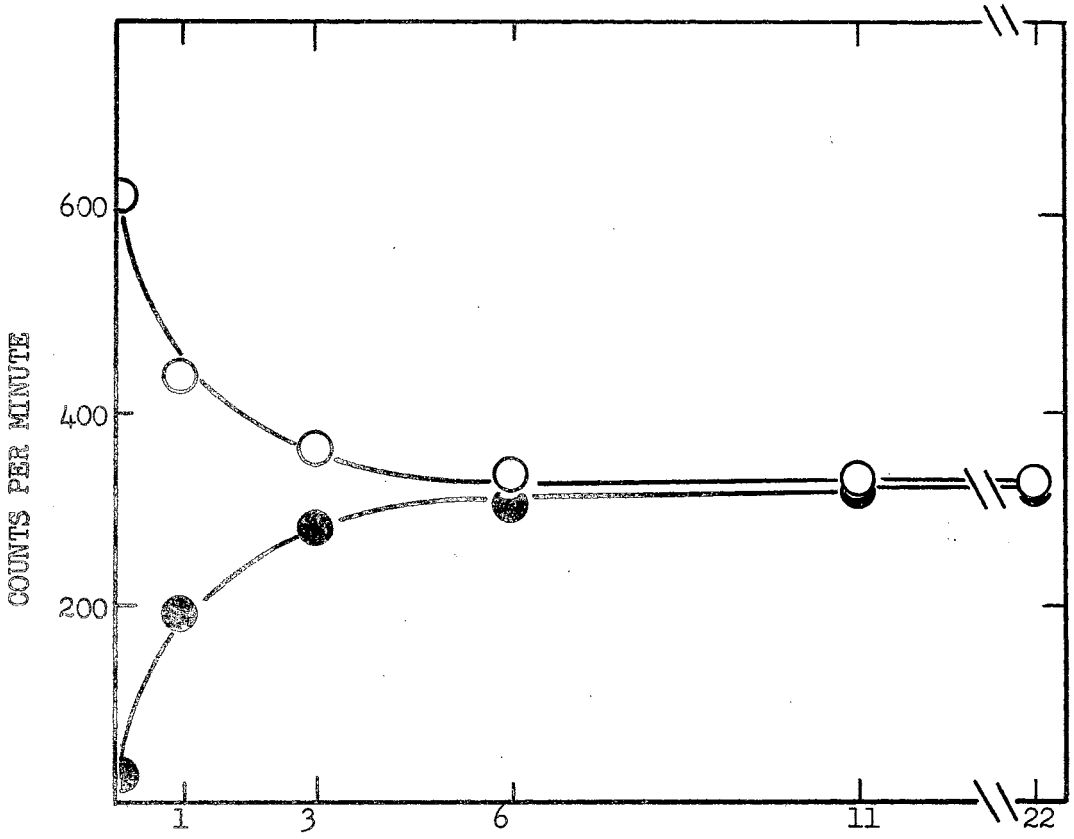
The membrane-bound M protein for β-galactoside transport (28), the membrane-bound enzymes E_{II} in sugar transport (30) and the concentrative uptake of proline by the membranes suggest a functional role of the membranes in transport processes. Membrane fractions were prepared by treatment of cells with lysozyme and EDTA, followed by osmotic lysis of the spheroplasts. The membrane pellet was washed four times with EDTA solution. Binding studies were done using a CRC Equilibrium Dialysis Cells (1 ml cavity) to detect the lipoic acid binding activity. An energy-dependent binding was demonstrated which was inhibitable by metabolic uncoupling agents and structural analogs, and the bound lipoic acid was exchangeable.

Time-Course of Dialysis to Equilibrium by Means of CRC Dialysis Cell

To find the time required to reach equilibrium using the dialysis cell, a time course study of dialysis without membrane preparation was conducted at 37°. The dialysis mixture of 1×10^{-3} M $MgSO_4$-0.1 M potassium phosphate, pH 6.6 plus glucose (10 mM) was dialyzed against radioactive lipoic acid (1.5×10^{-6} M) in the same buffer solution. The compartments were separated by cellulose dialyzing membrane (Arthur H. Thomas Co. product, average pore size, 48 Å) that was

Figure 14. Dialysis to Equilibrium by Means of CRC Dialysis Cell

A dialysis solution of 1×10^{-3} M $MgSO_4$ - 0.1 M potassium phosphate, pH 6.6 plus glucose (10 mM) in one cavity was dialyzed against radioactive lipoic acid (1.5×10^{-6} M) in the other cavity. The compartments were separated by cellulose dialyzing membrane that was equilibriated in the same dialysis solution. The dialysis was carried out with slow shaking at 37° and aliquots (0.05 ml) from each cavity were taken by using a hydrodermic microsyringe at the indicated times for the measurement of radioactivity. Aliquots (0.1 ml) were plated on Bacto-tryptone agar plates before and after the dialysis to check for bacterial contamination. Radioactive lipoic acid in the cavity (O); no lipoic acid in the cavity (●).


COUNTS PER MINUTE
600
400
200
1
3
6
11
22
HOURS

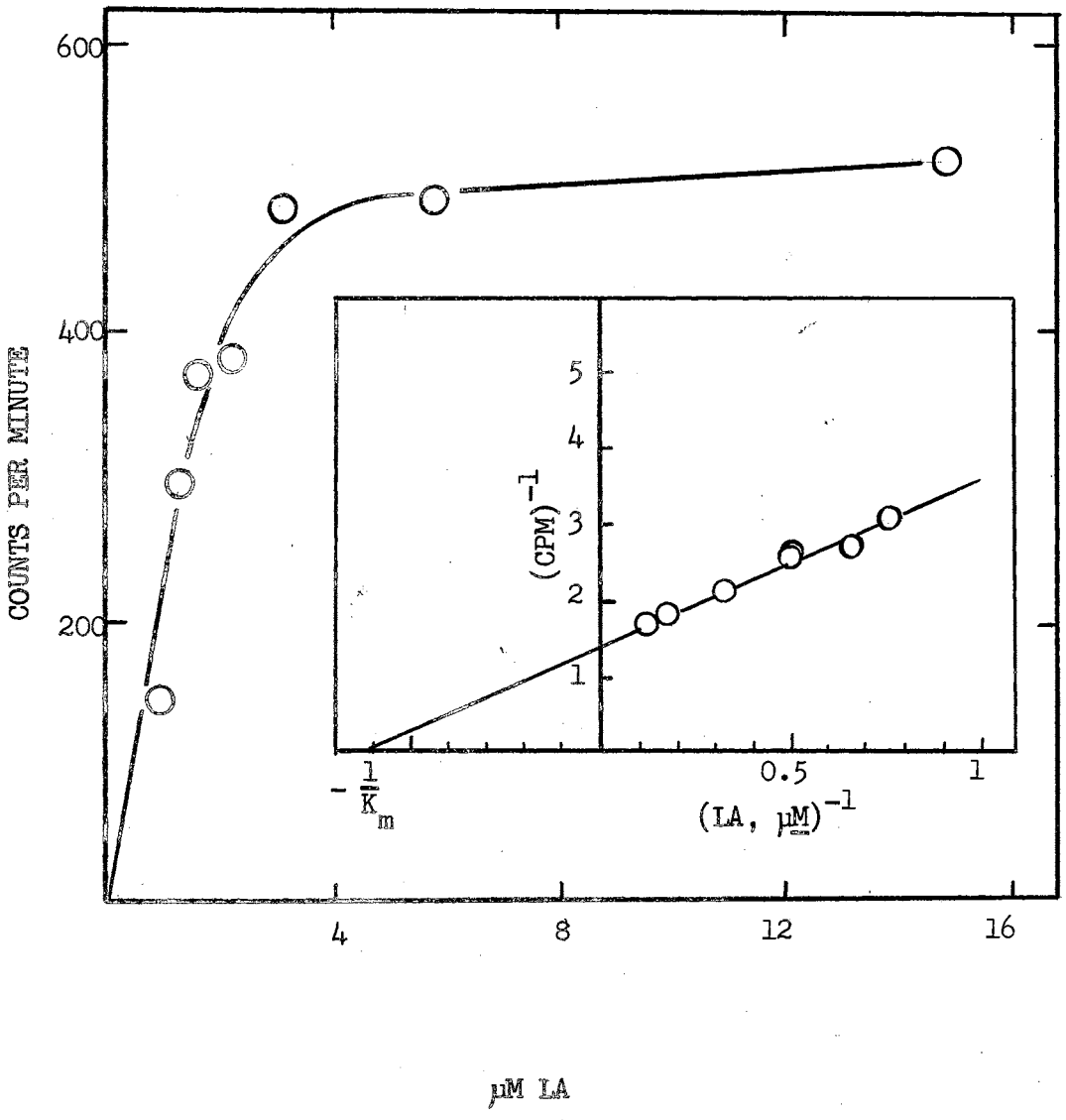
equilibrated in the same dialysis buffer solution. The dialysis was carried out with shaking at 37°. During the course of dialysis aliquots were taken out from each cavity at different times to measure the radioactivity. As shown in Fig. 14, a rapid movement of radioactive lipoic acid across the semipermeable membrane occurred within 3 hours and equilibrium was reached after 10-11 hours. Aliquots were plated on tryptone agar plates before and after the dialysis to detect bacterial contamination. No contamination was observed.

Effect of Lipoic Acid Concentration on Binding to the Membrane Preparation of *E. coli*

The effect of varying lipoic acid concentration on binding to membrane preparation was determined. Spheroplasts prepared by treatment of cells with lysozyme and EDTA were osmotically lysed and the membrane pellet after centrifugation was washed four times with 0.1 M phosphate buffer solution, pH 6.6 containing EDTA (1×10^{-4} M). The membrane suspension in 1×10^{-3} M $MgSO_4$ - 0.1 M potassium phosphate buffer, pH 6.6 containing 10 mM glucose was dialyzed to equilibrium with varying concentrations of radioactive lipoic acid (0 to 15 M). The dialysis cell was slowly shaken during the dialysis at 37° for 12 hours. As shown in Fig. 15, the binding activity was proportional to the lipoic acid concentration up to 3 μM. The binding curve was of the typical Michaelis-Menten type. A Lineweaver-Burke plot indicated a K_m value of 1.4×10^{-6} M.

Figure 15. The Effect of Lipoic Acid Concentration on Binding to Membrane Preparation of *E. coli*

The membrane preparation in 1 m*M* $MgSO_4$ - 0.1 *M* potassium phosphate buffer solution, pH 6.6 (110 µg/ml) was dialyzed in the presence of glucose (10 m*M*) against radioactive lipoic acid at the indicated concentrations of from 0 to 15 *M*. After 12 hours dialysis with shaking at 37°, aliquots (0.1 ml) were taken out for measurement of radioactivity. A Lineweaver-Burk plot for the lipoic acid binding shows K_m value of 1.4×10^{-6} *M*.


600
400
200
COUNTS PER MINUTE
4
8
12
16
μM LA
5
4
3
2
1
(CPM)$^{-1}$
$-\frac{1}{K_m}$
0.5
1
(LA, μM)$^{-1}$

Effect of Mg^{++} on Lipoic Acid Binding to the Membrane Preparation

To determine the optimum concentration of Mg^{++} for the lipoic acid binding to the membrane preparation, the equilibrium dialysis was performed with varying concentrations (0 to 9 m$\underline{M}$) of $MgSO_4$ under the same condition. The results shown in Fig. 16, indicates that the maximum binding of lipoic acid to the membrane preparation occurred at the concentration 1 m$\underline{M}$ $MgSO_4$ in 0.1 $\underline{M}$ potassium phosphate buffer (ph 6.6).

Effects of pH on Lipoic Acid Binding to the Membrane Preparation

Membrane preparations were suspended in 0.1 $\underline{M}$ potassium phosphate buffer solution and 0.1 $\underline{M}$ Tris-HCl buffer solution of different pH's to determine the optimum pH for lipoic acid binding. The pH 5.5,6 and 6.6 were made of 0.1 $\underline{M}$ potassium phosphate buffer solutions and pH 7,7.5 and 8.1 were 0.1 $\underline{M}$ Tris-HCl buffer solutions. As shown in Fig. 17, the optimum pH was at pH 6.5. The binding activity was lower at pH 8.0 than at pH 5.5.

Exchange Reaction in Lipoic Acid Binding to the Membrane Preparation

The ability of unlabeled lipoic acid to exchange for radioactive lipoic acid bound to the membrane preparation is shown in Fig. 18. The dialysis reaction mixture contained the membrane preparation (125 µg/ml) in 1 m$\underline{M}$ $MgSO_4$ - 0.1 $\underline{M}$ potassium phosphate-10 m$\underline{M}$ glucose solution pH 6.6

Figure 16. Effect of Mg^{++} Concentration on Lipoic Binding Acid to Membrane Preparation of E. coli

The membrane preparation (125 µg/ml) was suspended in 0.1 M potassium phosphate containing different Mg^{++} concentrations (0 to 9 mM) and then the mixtures plus glucose (10 mM) were dialyzed against 3×10^{-5} M radioactive lipoic acid at 37° for 12 hours. Aliquots (0.1 ml) were taken from each cavity for measurement of the radioactivity.

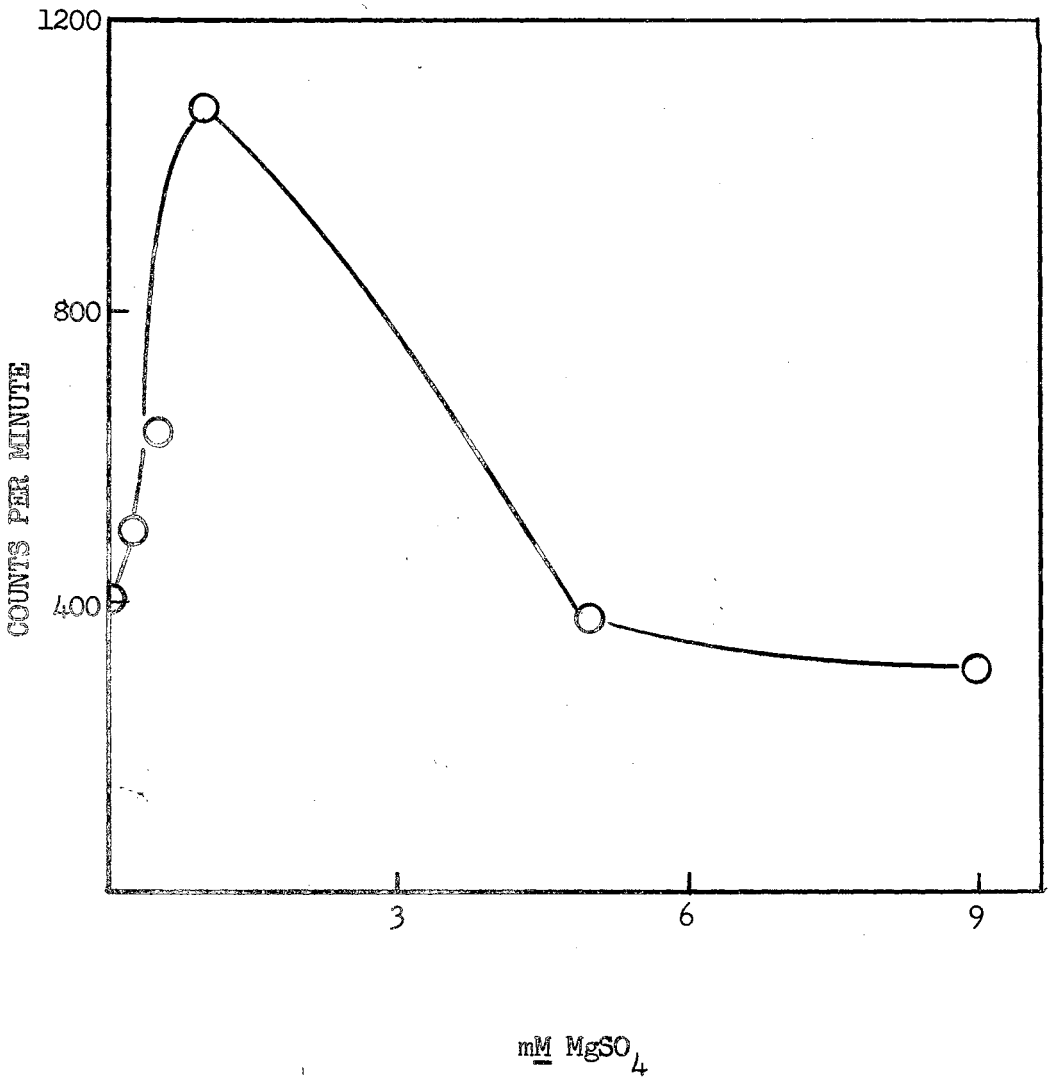

1200
800
400
COUNTS PER MINUTE
3
6
9
mM MgSO4

Figure 17. Effects of pH on Lipoic Acid Binding to the Membrane Preparation of E. coli

The procedures for binding measurement are described in the Materials and Methods section. The buffer solution below pH 7.0 was 0.1 M phosphate buffer and 0.1 M Tris-HCl buffer solutionwas used for above pH 7.0. The concentration of membrane protein and ^{35}S-lipoic acid were 125 μg/ml and 3×10^{-5} M. The dialysis cellwas thaken at 37° during dialysis for 12 hours. Aliquots (0.1 ml) from each cavity were taken to measure radioactivity.

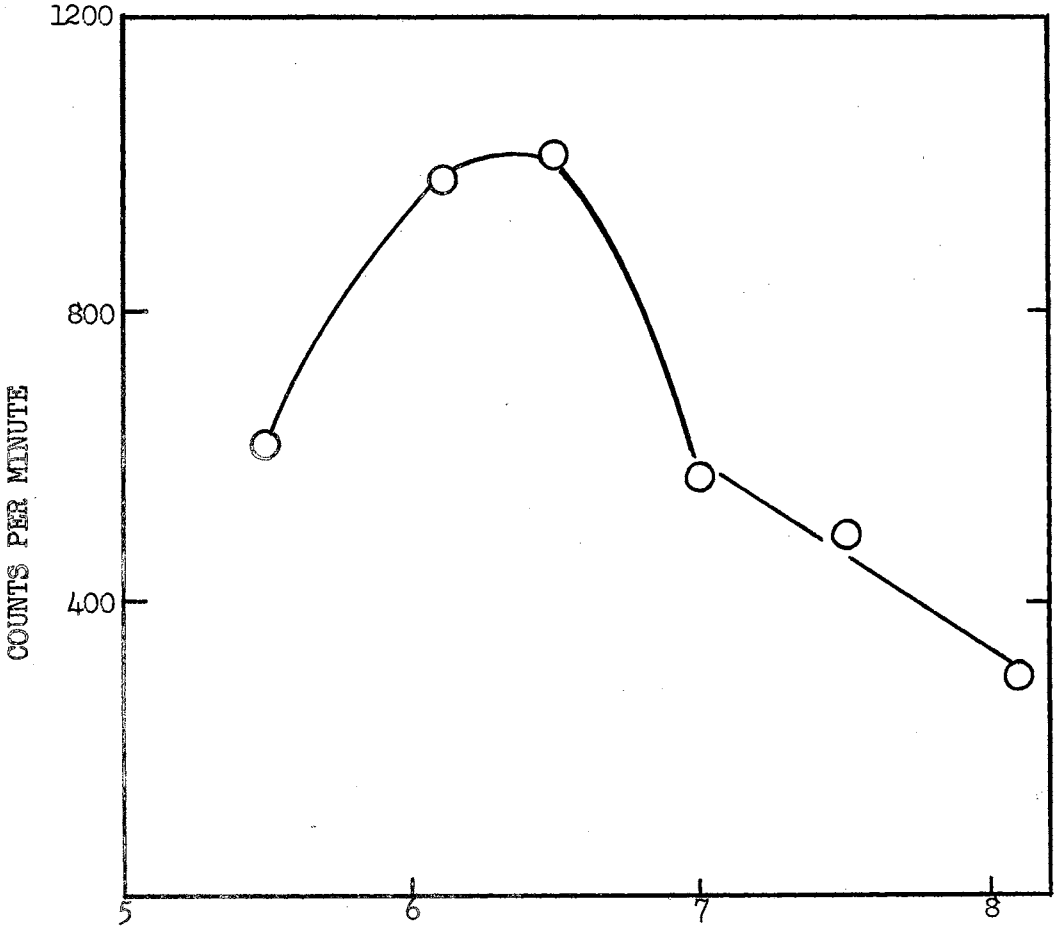
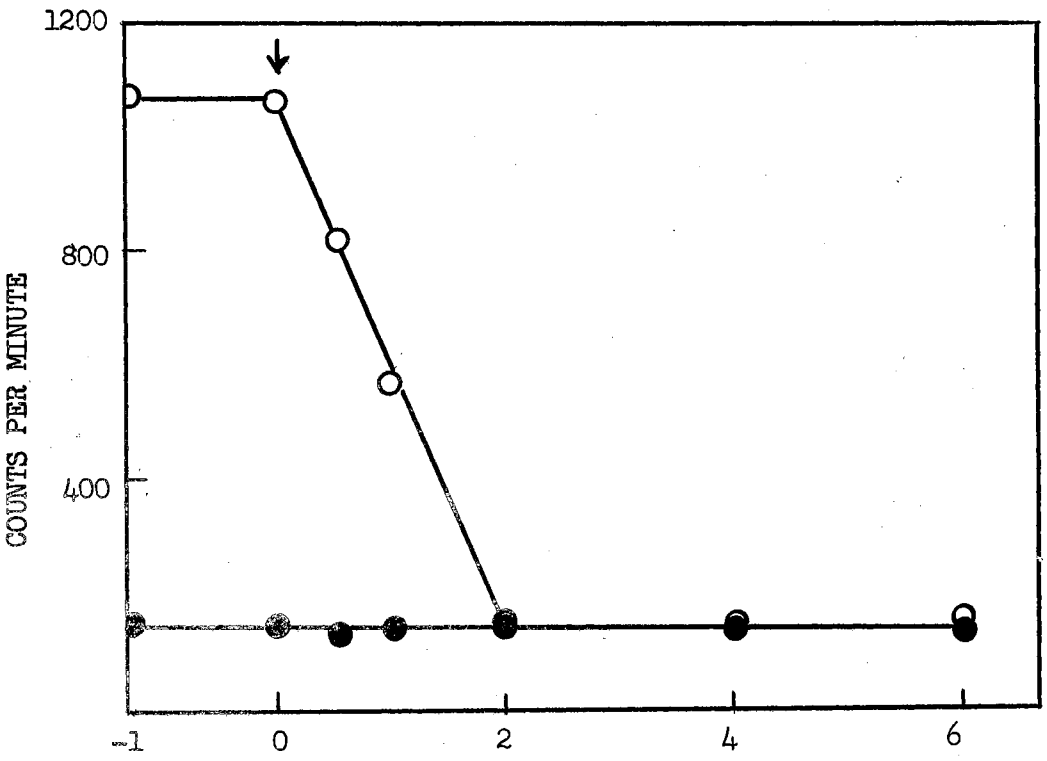

1200
800
400
COUNTS PER MINUTE
5
6
7
8
pH

Figure 18. Exchange Reaction in Lipoic Acid Binding to Membrane Preparation

Two dialyses of membrane preparation (125 μg/ml) in 1 m$\underline{M}$ $MgSO_4$ - 0.1 $\underline{M}$ potassium phosphate buffer solution plus glucose (10 m$\underline{M}$), pH 6.6 were performed against 3×10^{-5} $\underline{M}$ radioactive lipoic acid with and without 200-fold unlabeled lipoic acid. At equilibrium after 12 hours, 200-fold unlabeled lipoic acid was introduced to the cell dialyzed without unlabeled lipoic acid at zero time. Aliquots (0.1 ml) from each cavity were taken out at the indicated times to measure the radioactivity. Dialysis without unlabeled lipoic acid (O); dialysis with unlabeled lipoic acid (●).


1200
800
400
COUNTS PER MINUTE
-1
0
2
4
6
HOURS

and was dialyzed against 3 x 10^{-5} M radioactive lipoic acid. At equilibrium after 12 hours 200-fold excess unlabeled lipoic acid was introduced to the dialysis mixture at zero time. Within two hours the radioactivity of the membrane bound lipoic acid was decreased to the baseline of that which was obtained from the reaction mixture dialyzed to equilibrium with 200-fold excess unlabeled lipoic acid. The results indicate that the membrane bound radioactive lipoic acid was exchangeable with the unlabeled free lipoic acid.

Binding of L-Proline, L-Valine and Lipoic Acid to Membrane Preparation and Shock Fluid of *E. coli*

The concentrative binding of L-proline to the membrane fraction which were prepared by treatment of cells with lysozyme and EDTA, fllowed by osmotic lysis of the spheroplasts and washing of the membrane pellet with EDTA containing buffer solution was demonstrated (43). When *E. coli* cells were subjected to osmotic shock, L-valine binding components were readily released into shock fluid (40). As control experiments, the proline binding to membrane preparation and valine binding to concentrated shock fluid were conducted to establish a validity of the binding technique used in the lipoic acid binding studies. The concentrated shock fluid was prepared through dialysis and lyophilization and the membrane preparation was washed with EDTA contining phosphate buffer solution. The results shown in Table XIII, indicate that lipoic acid binding components were present in both membrane preparation and shock fluid.

TABLE XIII

BINDING OF L-PROLINE, L-VALINE AND LIPOIC ACID TO MEMBRANE AND SHOCK FLUID OF E. COLI

Preparation	Counts per Minute Bound		
	L-proline	L-valine	Lipoic acid
Membrane (180 µg/ml)	27,800	-	980
Membrane (360 µg/ml)	57,000	-	1,700
Shock Fluid (230 µg/ml)	-	128,000	520
Shock Fluid (349 µg/ml)	-	134,000	860

The procedure for equilibrium dialysis is described in the Materials and Methods section. The membrane and shock fluid were prepared according to the procedures in the Materials and Methods section. The concentrations of the substrates, i.e. ^{14}C-L-proline, ^{14}C-L-valine and ^{35}S-lipoic acid were 1×10^{-4} M, 5×10^{-6} M and 3×10^{-5} M respectively.

Effects of Inhibitors on Lipoic Acid Binding to the Membrane Preparation

To measure the effects of metabolic inhibitors and structural analogs on the lipoic acid binding activity to the membrane preparation of E. coli, KCN, NaN_3 and 2,4-dinitrophenol were added to the dialyzing mixtures. As shown in Table XIV, KCN produced a 54% inhibition at 10^{-3} M; NaN_3, 66% inhibition at 10^{-3} M; and 2,4-dinitrophenol, 51% inhibition at 10^{-4} M. The control contained no inhibitors. When no glucose was added 43% inhibition was observed. Octanoic acid inhibited the lipoic acid binding by 70% at 10^{-4} M, where as propionic acid and acetic acid produced 69% and 44% inhibitions at 10^{-4} M, respectively. The results suggest that the binding reaction is dependent on metabolic energy and the structural analogs in order of octanoic, propionic and acetic acids inhibit the lipoic acid binding.

Effect of Osmotic Shock on the Membrane Preparation of E. coli

The membrane preparation made as described in the Materials and Methods section was subjected to the osmotic shock to determine if the lipoic acid binding system was released from the membrane. The membrane preparation (11.5 mg) was suspended in hypertonic sucrose solution and after centrifugation, the membrane pellet was immediately suspended in ice cold distilled water. The shock fluid contained 500 μg of protein. The untreated membrane, shock fluid protein and shocked membrane were suspended in 1 x 10^{-3} M $MgSO_4$ - 0.1 M potassium phosphate, pH 6.6 plus glucose (10 mM) for equilibrium dialysis. Each fraction was dialyzed against radioactive lipoic acid (3 x 10^{-5} M)

TABLE XIV

EFFECT OF INHIBITORS ON LIPOIC ACID BINDING TO MEMBRANE PREPARATION OF E. COLI

Conditions during dialysis	Bound Lipoic Acid(CPM)	% Inhibition
Control	1071	0
No glucose	610	43
10^{-3} M KCN	486	55
10^{-3} M NaN_3	360	67
10^{-4} M 2,4-dinitophenol	520	52
10^{-4} M octanoic acid	315	71
10^{-4} M propionic acid	333	69
10^{-4} M acetic acid	603	44

The membrane preparation in 0.1 M potassium phosphate buffer, pH 6.6 (125 µg/ml) were dialyzed against radioactive lipoic acid (3×10^{-6} M) at 37° for 14 hours. Aliquots (0.1 ml) were taken out from each cavity to measure radioactivity. "Control" did not contain inhibitor and the inhibition was taken as 0% value. Glucose was not added to the dialyzing mixture in the second line experiment.

TABLE XV

EFFECT OF OSMOTIC SHOCK ON LIPOIC ACID BINDING TO MEMBRANE PREPARATION OF *E. COLI*

Preparation	Bound Lipoic Acid (CPM)
Membrane Preparation (150 μg)	780
Shock Fluid protein (125 μg)	200
Shocked membrane preparation (150 μg)	740

The membrane preparation (11.5 mg) was suspended in 10 ml of 20% sucrose-0.033 M Tris-HCl-1 x 10^{-4} M EDTA, pH 7.1 at 24° and the suspension was stirred slowly for 10 minutes. After centrifugation at 36,000 x g for 20 minutes, the membrane pellet was immediately suspended in 10 ml of ice cold distilled water for 10 minutes. Each fraction was suspended in 0.1 M potassium phosphate buffer solution, pH 6.6. The procedure for equilibrium dialysis is described in the Materials and Methods section.

for 12 hours. Samples were then removed for measurement of radioactivity. The results shown in Table XIV indicate no effect of the osmotic shock treatment of the membrane preparation on the lipoic acid binding.

CHAPTER VII

DISCUSSION

Cohen and Monod (1) visualized that the transport systems are specific for that function and under genetic control. Recently certain substrate-binding proteins presumably concerned in transport have been characterized. The most fully characterized of these is a protein (MW 32,000) which binds sulfate and was crystallized from S. typhimurium by Pardee (36). Sulfate transport is under the control of the cys A region of the chromosome which consists of at least three cistrons, a mutation in any of the three citrons results in impaired sulfate transporting ability. The Lac operon in E. coli contains a gene which codes for the synthesis of β-galactoside permease. Fox and Kennedy (28) have isolated a protein component (MW 31,000), called M protein of the β-galactoside transport system. This protein is absent in uninduced cells and in transport-negative mutants. Kundig, Ghosh and Roseman (30) characterized a phosphotransferase system in E. coli for the uptake of a variety of sugars. One component, the heat stable protein HPr, is phosphorylated on a histidine residue by enzymes E_I using phosphoenolpyruvate, as the phosphate donor. The sugars are released inside the cell as sugar phosphates in a reaction catalyzed by a family of enzymes E_{II}, each specific for a particular sugar. The molecular weight of the protein HPr is 9,400. A protein component for branched amino acid transport in E. coli was isolated and its molecular

weight is 36,000 (25).

In contrast Mitchell's view (10) is that normal metabolic enzymes located in the cytoplasmic membrane vectorially transport the substrate to the inside of the cell through their catalytic action. Honas and Gourley (75) implied an enzyme phosphokinase catalyzing the phosphate exchange system in rabbit erythrocytes and Prankerd (76) suggested a direct participation of 3-phosphoglyceraldehyde dehydrogenase in phosphate uptake by the erythrocyte. Mitchell (8) proposed that the oxidoreduction and group-transferring enzymes catalyze the movement of a substrate on or within the enzyme molecule present in the cell membrane. Skou (24) explained the movement of K^+ and Na^+ by the ATPase systems of cell membranes.

Many enzymes located in membranous structure within the cell require lipid components for activity. The enzymes, β-hydroxybutyrate dehydrogenase, cytochrome oxidase or succinic dehydrogenase in mitochondria (77,78) and the enzymes for biosynthesis of the cell envelope lipopolysaccharides of S. typhimurium (79) are enzymes of this type. Also the presence of lipids is essential for the enzymatic activity of ATPase (24).

Sanders and Leach (50,51) and Wilson and Leach (52) have chaacterized the overall properties of lipoic acid transport system in S. faecalis strain 10C1. The reactions through which the free lipoic acid is converted to the protein-bound, enzymatically functional form are known (55). Also the nature of its binding to the protein through the ε-amino group of lysine (54) and its sole role in S. faecalis and E. coli as an essential component of α-keto acid oxidases have been established (54).

There are two enzymes concerned in the activation reaction in lipoic acid metabolism. The first enzyme (PS-2A) catalyzes the activation reaction of lipoic acid in the presence of ATP to form the enzyme-bound lipoyladenylate. This activated lipoic acid is transferred to the second enzyme (PS-2B) to form the enzyme-bound lipoic acid and then the lipoic acid is transferred to apopyruvate dehydrogenase to form holopyruvate dehydrogenase complex. Thus the reaction process from the free lipoic acid to the protein-bound lipoic acid requires metabolic energy and the enzyme (PS-2B) serves a transfer function. A working hypothesis was that the reactions catalyzed by the two activating enzymes resemble what would be expected for an energy-dependent transport reaction. If these enzymes were located in or on the cell membrane, the possibility of their participation in active transport would be enhanced by the study of the intracellular distribution of the activating enzymes and apopyruvate dehydrogenase complex. However, the results showed that the activating enzymes and apopyruvate dehydrogenase complex were not found in the membrane fraction but were found exclusively in the membrane supernatant solution. The distribution of the marker enzymes (malic dehydrogenase, NADH oxidase and glucose-6-phosphate dehydrogenase) indicated that the membranes isolated were typical preparations. Thus it is unlikely that the lipoic acid activating enzymes function in the transport of lipoic acid. Likewise, Leach, Winter and Wilson (80) observed that the pyruvate dehydrogenase complex is not a functional requirement for the transport of pyruvate in S. faecalis. These findings are not consistent with the assumption that enzymes function in substrate transport systems as Mitchell (10) had proposed.

Kinetic studies have demonstrated the existence of the lipoic

acid transport system in E. coli. The results were almost parallel to the findings with S. faecalis (50). The rapid initial uptake within 30 seconds was followed by a slight decline to a plateau. The valine uptake curve was typical for amino acid transport.

Neu and Heppel (19) found that the osmotic shock treatment in which bacteria are rapidly transferred from sucrose solution of high osmotic strength to a dilute salt solution releases a number of hydrolytic enzymes and proteins located on or near the cell membrane. This osmotic shock treatment released the sulfate binding protein (36), galactose binding protein (27), leucine binding protein (25) and the heat stable protein HPr for sugar transport (30). When E. coli cells are osmotically shocked, the uptake of lipoic acid is reduced to 50% of the normal value.

A definite demonstration that the binding proteins are involved in the transports would be to reconstitute the system; that is add the proteins to cells incapable of transport and obtain transport. The uptake of galactose by the shocked cells was restored to the normal value (27) and the heat stable protein HPr that was released by the osmotic shocking was successfully used in reconstituting normal transport (26). The ability for lipoic acid uptake has not been restored by the addition of the concentrated shock fluid to the shocked cells. Likewise, the restoration of the uptake of amino acids in osmotically shocked cells has not been demonstrated (25).

Not all the binding proteins for transport are released by osmotic shock. The M protein (28) and Na^+ - and K^+-activated ATPase (24) are firmly bound to the cell membrane. The enzyme E_{II} of sugar transport (30) and concentrative proline uptake system (43) are firmly associated

with the cell membrane. The equilibrium dialysis binding experiments demonstrate the existence of lipoic acid binding component in the membrane preparation.

The binding activity of the membrane preparation is an energy-dependent process; metabolic uncoupling agents such as potassium cyanide, sodium azide, or 2,4-dinitrophenol inhibit the lipoic acid binding activity. When glucose is not added to the membrane preparation, only 56 percent of the binding is observed. The uptake of proline by isolated membrane preparation of E. coli was stimulated by glucose and was inhibited by a variety of compounds known to uncouple oxidative phosphorylation (43). Similarly, glycine uptake by the membrane preparation was dependent on pH and on the presence of Mg^{++} and glucose (81).

Octanoic acid is an effective inhibitor for the binding of lipoic acid to the membrane preparation. This result is consistent with the inhibition studies of whole cell uptake of lipoic acid by octanoic acid (50). Both results indicate that the inhibition by octanoic acid is probably due to the binding to the same site as lipoic acid in the transport system. Apparently the 1,2-dithiolane ring of lipoic acid molecule is not functional for the binding or transport, but rather the side chain might play an important role in this reaction. Since acetic and propionic acids also are inhibitory, possibly the carboxyl group of the acids is functional in the reaction.

The observations of the optimum concentration of $MgSO_4$ (1.5 mM) and pH 6.6 for the binding activity, and the exchange reaction were similar to the glycine and proline uptake system.

Isolations of lipoic acid transport-negative mutant of E. coli and

lipoic binding protein would enhance a further convincing evidence that the binding component present in the membrane preparation is responsible for the transport system.

SUMMARY

Both the lipoic acid activating system and the pyruvate dehydrogenase complex were found in the soluble fraction when cells were fractionated to separate soluble enzymes and the cell membrane. Since the distribution of the marker enzymes (malic dehydrogenase, NADH oxidase and glucose-6-phosphate dehydrogenase) was consistent with typical membrane preparations, the failure to find either of the enzymes of the lipoic acid activating system in the S. faecalis and E. coli membranes makes unlikely their function in the transport of lipoic acid.

The lipoic acid uptake by E. coli was characterized by a rapid accumulation for 30 seconds followed by a slight decline before a plateau was reached. The uncoupling reagent, 2,4-dinitrophenol, inhibited both lipoic acid and valine uptakes.

Osmotic shock treatment reduced the uptake of either lipoic acid or valine to 50 percent of the normal values. It has not been possible to restore lipoic acid transport ability by incubation of the treated cells with concentrated shock fluid.

Isolated membrane preparations from E. coli exhibited a glucose-dependent binding of radioactive lipoic acid. Membranes were prepared by treatment of cells with lysozyme and EDTA and then osmotic lysis of the spheroplasts. Lipoic acid binding to the membrane preparations had a pH optimum (6.6) and an optimum Mg^{++} (1.5 mM $MgSO_4$). The binding reaction was inhibited 40 to 70% by uncoupling reagents such as KCN,

NaN_3, 2,4-dinitrophenol, and by structural analogs, octanoic, propionic and acetic acids. The exchange reaction of unlabeled lipoic acid for membrane bound lipoic acid was demonstrated.

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