VITAMIN TRANSPORT IN *ESCHERICHIA COLI*

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VITAMIN TRANSPORT IN ESCHERICHIA COLI

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

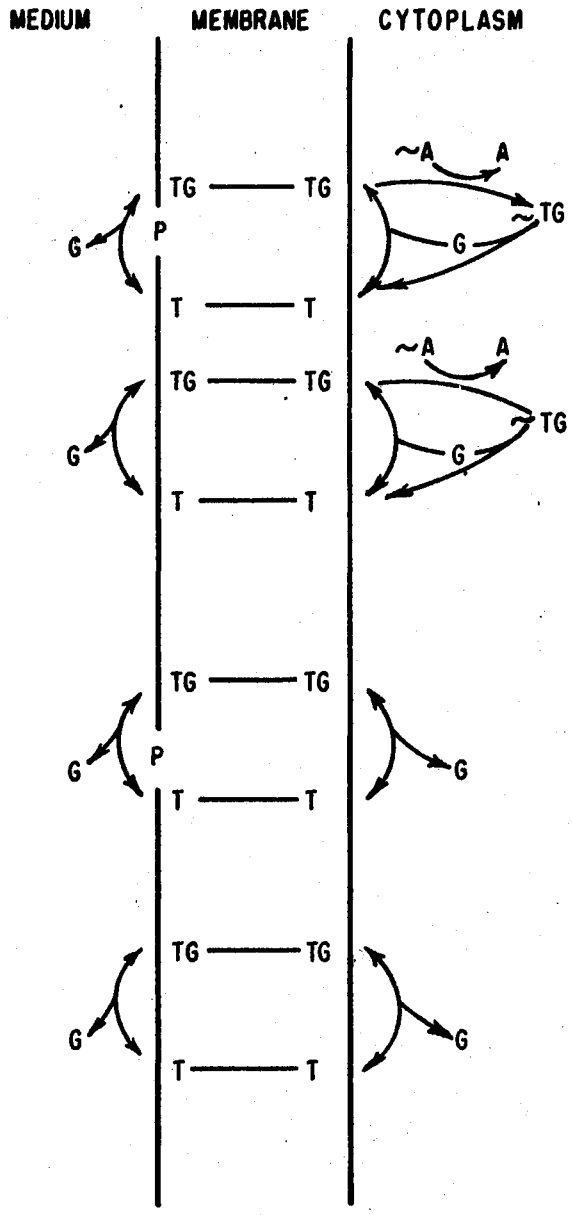
CHAPTER I

INTRODUCTION

The nature and mechanism of membrane transport in bacteria are important topics in understanding basic life processes. References to reviews are (1,2). Prior to the mid-1960's nearly all transport studies were performed with whole cells. This type of study yielded information on the influx and efflux of substrates with the resultant kinetic measurements providing the foundation for several transport models. A model described by Koch (3) for sugar transport in Escherichia coli is a typical example of this type of research (Fig. 1). Although these studies were valuable in that they limited speculation on the mechanisms of transport, the enzymatic basis could not be defined at the whole-cell level. Two types of transport mechanisms resulted: 1) vectorial synthesis where the substance is transported in the course of a covalent transformation, epitomized by the sugar phosphotransferase and 2) transport coupled to metabolic energy sources, notably the lactose and amino acid permeases studied by Cohen and Monod (4) and more recently by Kaback (5,6). In addition a number of binding proteins have been isolated from osmotic shock fluid and characterized. Although the proteins are strongly implicated in the

Figure 1. Model for Sugar Transport in E. coli

Koch (3) describes his model as follows: G = sugar, P = permease - inducible, stereospecific, fixed in position (several P's of same or different specificity may react with same T). T = transporter or carrier element which crosses the barrier in an unspecified manner with or without sugar. A = immediate energy source, reserves of ~A are not large. ~TG = "activated" transporter sugar (must be in or on membrane).



(a) Complete process

(b) Energy coupled cryptic process

(c) Permease mediated facilitated diffusion process

(d) Facilitated diffusion process

transport process, their mechanism of function is not understood.

Kundig and Roseman (7) have described a system for the vectorial phosphorylation of sugars in bacteria involving three components (E_I,E_{II} and HPr) and utilizing phosphoenolpyruvate (PEP) as an energy source. This is shown in the following scheme.

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

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

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

E_I is a soluble enzyme catalyzing the phosphorylation of HPr by PEP. HPr is a histidine-containing protein, also soluble, with a molecular weight of about 10,000. E_{II} is the name given for several different membrane bound enzymes, each specific for a different carbohydrate. In some cases four factors are involved (8). Characteristics of this PEP phosphotransferase system have been reviewed recently (2) and it was concluded "almost unequivocally to be responsible for the translocation and accumulation of at least glucose and related sugars". Several investigators (Mitchell (9), Christiansen (10), and Kaback (2)) have pointed out that this kind of group translocation mechanism could be applicable to the transport of any organic molecule. It could be invoked even where unchanged substrate is apparently being concentrated if the phosphorylation mechanism (for example)

were tightly coupled to a dephosphorylation mechanism, all part of the same group translocation machinery. The transport can also be visualized as being coupled with oxidation-reduction, acetylation-deacetylation, etc.

Although vectorial covalent reactions are appealing mechanisms for transport, little support has been found for their general existence outside the phosphotransferase system. For example, Mitchell (9) suggested that the α-ketoglutarate-lipoic acid oxidoreductase in the cell membrane is responsible for the transport of α-ketoglutarate. Although one would expect an analogous requirement for pyruvate, Leach et al. (11) have shown that pyruvate is taken up by an Streptococcus faecalis mutant with an inactivated pyruvate-lipoic acid oxidoreductase. However, Overath and coworkers (12) have shown good evidence for vectorial acylation of fatty acids in E. coli. Membrane bound acyl-coenzyme A synthetase is partially responsible for the transport process which is tightly coupled to further fatty acid metabolism. This is in agreement with the findings that only a small part of the fatty acid taken up can be identified as the acyl-coenzyme A derivative and that there is no efflux reaction even in the presence of excess exogenous fatty acid. Group translocation mechanisms cannot account for all transport processes, however. Both glycine transport in E. coli membrane preparations (13,14) and sugar transport in erythrocyte membranes (15), among others, occur by facilitated diffusion. These systems do not concentrate

substrates against a gradient and competition for transport is exhibited by similar compounds. Glycerol and glucose enter erythrocytes at a rate 10^2-10^4 times greater than could be accounted for on the basis of simple diffusion through a lipid layer. Mutants defective in facilitated glycine transport have been characterized. The transport of lactose and β-galactosides is not a straightforward example of vectorial phosphorylation. Kaback has shown that the transport of β-galactosides via the lactose transport system is similar to the transport of several amino acids (6). The transport of both β-galactosides and the amino acids is increased several fold upon the conversion of D-lactate to pyruvate in isolated membrane preparations. Succinate, α-hydroxybutyrate and L-lactate partially replace D-lactate but are less effective. ATP, PEP, glucose and several other compounds are ineffective in increasing transport. It is proposed that transport of β-galactoside and the amino acids is coupled to a membrane-bound D-lactate dehydrogenase via a respiratory chain. This is based on the requirement for oxygen and on several respiratory chain inhibitor studies. Kaback finds that the PEP phosphotransferase system is not involved in β-galactoside transport since PEP failed to stimulate lactose transport in membrane preparations which readily utilized PEP for the transport of methylglucoside. In addition a mutant defective in enzyme I of the PEP phosphotransferase system accumulated thiomethylgalactoside through D-lactate coupled transport. Stable

high-energy phosphate compounds are not involved since β-galactoside transport is not sensitive to arsenate or oligomycin and added ATP is not stimulatory. Klein et al. (16) found that similar membrane preparations are unable to conduct oxidative phosphorylation by Pavlosova and Harold (17) indicated that uncouplers of oxidative phosphorylation block thiomethylgalactoside transport but do not alter ATP levels.

The exact function of binding proteins in the transport process is not known despite the isolation and characterization of several binding proteins. Indeed, whether they function in transport at all is debated by some (2). In addition binding proteins have been implicated in bacterial chemotaxis and regulatory functions which may be the true functions of binding proteins (2). Bacterial chemotaxis is a surface phenomenon and involves the same degree of specificity and type of genetic control as binding proteins (2,18). The chemoreceptors for chemotaxis are not the enzymes for metabolism or the transport machinery. Adler (18) suggests that the chemoreceptors are possibly components of the transport machinery which are unchanged in the transport mutants studied or a new entity which functions only in chemotaxis. The R_2 protein from E. coli is involved in regulating alkaline phosphatase synthesis. It is similar to binding proteins in that it is lost upon osmotic shock, has a molecular weight of 30,000 and binds phosphate firmly (19). Mutants lacking the protein transport phosphate

normally.

Binding proteins are membrane associated and mutants lacking a specific binding protein also lack the corresponding transport ability. The ability of a cell to transport several compounds has been associated with the loss of the corresponding binding proteins upon osmotic shock (2). Behavior of the binding proteins regarding repression, inhibition and affinity for substrate are often analogous to properties of the transport system. All of this evidence, even though voluminous, is indirect and circumstantial. Restorations of transport functions by a specific binding protein were often unsuccessful, involved impure preparations or were treated with reservations. For example, Pardee (20) as well as Piperno and Oxender (21) failed to reactivate uptake of sulfate and leucine in shocked cells by the addition of purified binding protein. Anraku (22) and Wilson and Holden (23) partially restored transport of galactose and arginine to shocked systems but used impure fractions of the shock fluid. Since several components that can effect transport other than binding proteins are lost upon osmotic shock such as the HPr (2) and fatty acids (24), these results while demonstrating restoration, do not indicate which component(s) is active. Recently however, Medveczky and Rosenberg (25) showed conclusively that a phosphate binding protein was involved in phosphate transport in E. coli by restoring transport in both shocked cells and a mutant defective in the binding

protein by the addition of pure binding protein. In addition, no stimulation of transport was imparted to normal cells or to a mutant defective in phosphate transport through a defect other than the binding protein.

In general, the majority of evidence indicates that binding proteins are involved in transport. However, this does not rule out the involvement of similar proteins in chemotaxis or regulatory functions. In addition, one should not get the impression that binding proteins are necessarily involved in all transport systems. For example, Kaback has found no evidence of binding proteins for proline or glycine (2). However, a proline binding protein released by osmotic shock from E. coli has been reported by Anraku (26) but is only found when the cells are grown in a tryptone medium. Because of this evidence and the fact that several mutants defective only in transport have yielded normal binding proteins (25,27-29), one must concede that where binding proteins are involved in transport, they do not act alone. Kaback and Milner (5) have shown that D-lactate stimulates the transport of leucine, isoleucine and valine (amino acids with well-characterized binding proteins) and glycine (transported by facilitated diffusion in a different strain). Continued study of these, the proline transport-binding protein and similar systems should yield information on the relationship of binding proteins and facilitated diffusion to active transport.

Binding proteins have been demonstrated for 15 differ-

ent compounds. The different proteins bind lysine (26), threonine (26), proline (26), cystine (26,30), arginine (23, 30), glutamine (31), histidine (29,32), phenylalanine (33), branched chain amino acids (isoleucine, leucine and valine) (21,22), leucine (34), galactose (22,35), arabinose (36), sulfate (37) and phosphate (25). All are derived from E. coli except the sulfate and histidine binding proteins (Salmonella typhimurium) and the phenylalanine binding protein (Comamonas sp). All are released by osmotic shock and have similar molecular weights (25,000 - 42,000). Their dissociation constants range from 0.02 to 12 μM.

Comparatively few subcellular studies in vitamin transport have been performed. Bradbeer et al. (38,39) have shown that B_{12} uptake in E. coli occurs in two sequential steps, an initial rapid phase of binding to the membrane and a second energy-requiring slower phase of transport into the cell. Although osmotic shock reduced transport overall, no B_{12} binding material was released and the initial binding phase was unaffected. It was suggested that the reduced uptake was due to a loss of energy producing potential by the cell. The energy-donor system is unknown. Both glucose and D-lactate stimulate uptake equally in whole cells while neither glucose, D-lactate or PEP stimulated transport in membrane vesicles.

The only other significant subcellular study of vitamin transport was performed by Oh and Leach with lipoic acid (46,47). The intracellular distribution of the lipoic acid

activating enzymes was investigated since these enzymes were likely candidates for a vectorial adenylation mechanism for lipoic acid transport. The enzymes were not found associated with the membrane, however. This suggests they are not involved in transport. They also reported that lipoic acid uptake is reduced 50% upon osmotic shock and that membrane preparations actively take up the vitamin.

Kawasaki et al. (40-42) have reported that thiamine is accumulated very early as thiamine pyrophosphate (TPP) with only a small percentage as free thiamine. The percentage of free internal thiamine decreases further with increasing time. They found that a mutant defective in thiamine transport has normal levels of thiamine kinase and that exogenous thiamine is exchangeable with internal thiamine. On this basis it was suggested that thiamine transport in E. coli occurs in two steps, the facilitated diffusion across the membrane and accumulation of the thiamine as TPP by the membrane-bound thiamine kinase. Older work by Neujahr (43-45) in Lactobacillus showed that the percentage of free thiamine compared to TPP is greatly increased when cells were grown in limiting amounts of phosphate while the total thiamine concentration remains approximately constant. In addition the percentage of free thiamine to bound thiamine increases during the uptake process and free endogenous thiamine exists several hundred fold concentrated over the exogenous thiamine. On this basis Neujahr suggests that thiamine is actively transported and that thiamine kinase is

independent of transport and accumulation in Lactobacillus. Kawasaki's group have not performed any experiments comparable to those of Neujahr using limiting amounts of phosphate.

Kinetic and inhibitor studies of other vitamin transport systems have been performed in whole cells for biotin (48-51), folic acid (52,53) and nicotinamide (54). Transport of pantotheine has been observed but not investigated (55). No reports concerning transport of other vitamins such as riboflavin and pyridoxine could be found.

One goal of this research was to survey for the existence of binding proteins for several vitamins including lipoic acid and characterize those found. In addition because of this research group's strong interest in lipoic acid, further studies were performed on its transport characteristics and protein-bound exchange potential in intact cells.

Lipoic acid is bound covalently in both pyruvate and α-ketoglutarate dehydrogenase complexes through an amide linkage to the ω-amino group of lysine (56). Enzymes have been described in E. coli and S. faecalis which catalyze the covalent addition of lipoic acid to the apopyruvate dehydrogenase complex converting it to a functional system (57). An enzyme which hydrolyzes this amide bond yielding free lipoic acid and the apopyruvate dehydrogenase complex has also been described (58). Powell et al. (59) found that the rate of 4-phosphopantotheine turnover of the acyl carrier protein in E. coli was four times greater than the

growth rate. This reaction would involve the formation and breakage of a serine hydroxyl phosphate ester linkage. They suggested that a similar turnover might occur for both lipoic acid and biotin involving the amide linkage. Therefore, experiments were performed investigating this possibility in growing E. coli cultures with respect to lipoic acid. Reed et al. (58) have previously shown that there is no turnover of lipoic acid during in vitro functioning of the pyruvate dehydrogenase complex.

CHAPTER II

EXPERIMENTAL PROCEDURE

Materials

D-Biotin (carbonyl-^{14}C, 58 mc/mmole), D-riboflavin-2-^{14}C (61 mc/mmole), pyridoxine·HCl (^{3}H-generally labelled, 950 mc/mmole) and ^{35}S-thiamine (55 mc/mmole) were from Amersham/Searle. Nicotinamide(carbonyl-^{14}C, 67 mc/mmole), L-leucine (248 mc/mmole) and L-proline (220 mc/mmole) were products of New England Nuclear. The nicotinamide and leucine were gifts respectively from R. K. Gholson and G. V. Odell. ^{35}S-Lipoic acid (35 mc/mmole) was prepared as previously described (60).

Other materials and their sources are: bovine serum albumin, α-chymotrypsinogen, mercaptoethanol, oxidized glutathione, iodoacetic acid and sodium azide from Sigma; catalase, pepsin, trypsin and lysozyme from Worthingtom Biochemical Corp.; ovalbumin from Mann Research Laboratories; coenzyme A and dithiothreitol from P-L Biochemicals, Inc.; reduced glutathione, cysteine, cystine and folic acid from Nutritional Biochemicals Corp.; N-ethylmaleimide from Eastman Kodak; thiamine pyrophosphate, flavin adenine dinucleotide, and flavin mononucleotide from Calbiochem; thiamine from Merck and Co.; chloramphenicol from Parke,

Davis and Company; DEAE cellulose (DE 32) from Whatman; hydroxylapatite (HTP) and molecular seive gel (P-150) from Bio-gel; and dialysis and Paulus cell membranes (48 A pore diameter from Aurther Thomas Company. β-lactalbumin was prepared and donated by R. Mawal. Lipoic acid and 8-methyl lipoic acid were gifts of D. S. Acker. The C-7 and C-9 analogs of lipoic acid were gifts of L. J. Reed.

Methods

Preparation of Cells for Uptake Studies

E. coli (Crookes) was used in all phases of this study. Ten ml of cells grown overnight in a 0.2% glucose-minimal medium (M-9) (61) was diluted 20-fold with the same medium at 37° and incubated with shaking for 3 hours. The culture was diluted with an equal volume of fresh medium and grown for another 45 minutes. Chloramphenicol was added to a final concentration of 200 μg/ml and incubated with shaking for 10 minutes. The mid-log phase cells were harvested by centrifugation at 7500 x g for 15 minutes and washed once in M-9 at 4°. The cells were divided into two equal portions. One portion was suspended in cold M-9 plus 100 μg/ml chloramphenicol and stored at 4° (normal cells). The other portion was suspended in 20% sucrose, 30 mM Tris-HCl, 1 mM EDTA buffer at pH 8.0 and incubated at room temperature (20-25°) for 10 minutes. The cells were centrifuged at 13,000 x g for 14 minutes and the well-drained cell pellet was rapidly suspended in 100 ml of ice-cold 0.5 mM $MgCl_2$. The

osmotically shocked cells were centrifuged as before and the pellet was suspended and stored as described above. Both preparations were used within 2 hours.

Uptake Procedure

Cell preparations containing 1.1 to 1.3 x 10^9 cells/ml were incubated for 10 min in 0.2% glucose M-9 plus chloramphenicol (0.1 mg/ml) at 37°. The radioactive substrate was added to a final concentration of 1 μM and was incubated with shaking for 10 min at 37°. The uptake process was stopped by pipetting 0.5 ml of the sample into one ml of crushed, frozen M-9. After centrifugation at 4° and one 4° wash in M-9, the cell pellet was suspended in one ml of 5% trichloroacetic acid, transferred to scintillation vials and counted in 10 ml of Bray's scintillation cocktail (62). Samples in all experiments were counted to a 1% standard counting error.

Cell viability of the normal and shocked cells was determined by plating on 1% tryptone.

Shock Fluid Preparation

Cells were grown aerobically in 0.2% glucose M-9 at 37° and were harvested in a continuous flow Sharples refrigerated centrifuge. Thirty-six liters of early stationary culture yielded about 550 g of cell paste. The cells were shocked by a procedure modified from that of Nossal and Heppel (63).

The cell paste was suspended in about 800 ml of 20% sucrose, 30 mM Tris·HCl, 1 mM EDTA buffer at pH 8.0. This was stirred for 10 minutes at room temperature and then centrifuged at 13,000 x g for 15 minutes. The well-drained pellet was smeared on the inside of a 2 liter Ehrlenmeyer flask, one liter of ice cold 0.5 mM $MgCl_2$ was added and the cells were rapidly suspended by vigorous manual shaking. After standing for 10 minutes, the suspension was centrifuged for 30 minutes at 16,000 x g. The resultant shock fluid was decanted and concentrated by ultrafiltration using an Amicon UM-10 membrane to a protein concentration of approximately 4 mg/ml. The typical yield for this procedure was about 500 mg of protein.

Binding Assay Methods

Most of the binding studies except those for lipoic acid were performed with an ultrafiltration cell developed by Paulus (64). Small circles of dialysis tubing (Arthur Thomas Co., 48 Å pore diameter) were used as membrane filters. Data for saturation curves were collected by equilibrium dialysis. Samples were equilibrated at least 12 hours before sampling, except where otherwise noted. The standard assay was performed at room temperature and 1 μM vitamin concentration except where otherwise noted.

Protein Determination

All protein determinations were performed by the method

of Lowry et al. (65) using bovine serum albumin as the standard protein.

Determination of Molecular Weight

The molecular weight of the binding proteins was determined by polyacrylamide disc gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (66). Standard proteins used as molecular markers were bovine serum albumin (66,500), catalase (60,000), ovalbumin (43,000), pepsin (35,000), α-chymotrypsinogen (25,700) and β-lactoglobulin (18,400). Other methods of molecular weight determination included the ratio of migration in 6 and 9% gels by the method of Davis (67) and gel filtration in Bio-gel P-150. The gel column was 1.5 x 60 cm.

Amino Acid Analysis

Purified binding protein was hydrolyzed 22 hours in vacuo at 60° in the presence of 6 N HCl. After removal of the aqueous and volatile components by rotary evaporation, the residue was analyzed on a Beckman Model 120C amino acid analyzer by J. Dahlem.

Purification of Thiamine and Riboflavin Binding Proteins

The thiamine and riboflavin binding proteins precipitated between 430 and 600 g/liter of ammonium sulfate. The ammonium sulfate fractionation and all the following steps in the purification were performed at 4°. The ammonium

sulfate fraction was dialyzed against TMM buffer (10 mM Tris·HCl, 1 mM $MgCl_2$, 0.5 mM mercaptoethanol, pH 8.0), concentrated via Diaflo filtration and then loaded onto a DEAE column (1.3 x 15 cm, Whatman DE 32) equilibrated in the same buffer. The column was then eluted with a linear 0-0.15M NaCl gradient in TMM buffer (from equal volumes of 0 and 0.15 M solutions). The thiamine binding protein eluted between 0.04 and 0.06 M and the riboflavin binding protein between 0.07 and 0.1 M NaCl (Fig. 1). The fractions containing high thiamine binding activity were pooled, dialyzed against 10 mM KPO_4 buffer, pH 7.5, and concentrated as before. This preparation was placed onto a hydroxylapatite column (1.5 x 20 cm, Bio-Gel Cellex-HPT) and eluted with a linear 0.05-0.20 M gradient of the same buffer, also from equal volumes of each. The thiamine binding activity was again pooled, concentrated as before and passed through a molecular sieve column (1.5 x 60 cm, Bio-Gel P-150).

Lipoic Acid Exchange Procedure

Cultures of E. coli were initiated by diluting stationary cells 20-fold with fresh M-9 containing 0.2% glucose at 37°. One culture was grown to mid-log phase (A_{600} = 0.6-0.8) in the presence of ^{35}S-lipoic acid, harvested by centrifugation at 10,000 x g for 15 minutes, the well-drained pellet suspended in fresh medium at 37° and unlabeled lipoic acid added. Control experiments were performed in two other parallel cultures. One culture was grown in the presence of

unlabeled lipoic acid during the first part of the experiment while the other control was grown in M-9 with no additions. Both cell preparations were incubated with ^{35}S-lipoic acid after the centrifugation step. The generation time for each culture was 60 to 70 minutes. Incorporation was stopped by pipetting 1 ml samples into 2 ml of 95% ethanol. Samples were then centrifuged (ten minutes at 10,000 x g and 4°) and the supernatant solution removed. The pellet was washed by suspension in 1 ml of water and 2 ml of ethanol added before centrifugation as described above. The precipitate was suspended in one ml of water, transferred to scintillation vials and counted to a 1% counting error in 10 ml of Bray's (62) scintillation fluid. In experiments where both the free pool and protein-bound lipoic acid were measured, the uptake was stopped by pipetting 0.5 ml aliquots into 1 ml of crushed, frozen M-9 and centrifugation as above. The cells were then washed by suspension in M-9 at 4° and centrifugation. The cells were then suspended in 0.5 ml of distilled water and 1 ml of 95% ethanol added with vigorous mixing. Centrifugation as above yielded the soluble lipoic acid in the supernatant solution and the precipitated protein-bound lipoic acid. Both were counted as above. The ethanol fractionation proved to be the most convenient and accurate method for this type of measurement. Comparative results of other techniques are shown in Table I.

TABLE I

METHODS FOR MEASURING FREE POOL AND PROTEIN-BOUND LIPOIC ACID

	Treatments			
	Boil Cells (5 min). in Aqueous Suspension	Suspend in Cold 5% TCA	Suspend in 70% Ethanol	None (Total pmoles)
pmoles in Supernatant Solution	16.6	7.9	15.2	--
pmoles Pellet	54.4	27.7	55.8	--
pmoles Total	71.0	35.6	71.0	72.8

Mid-log phase cells were exposed to 2 μM ^{35}S-lipoic acid for 10 minutes before stopping the uptake in slushy M-9. After washing the cells in M-9 at 4°, the above treatments were performed. The treated cells were separated from their supernatant fluid and both portions measured as described for the 70% ethanol treatment in the methods section.

CHAPTER III

RIBOFLAVIN AND THIAMINE UPTAKE IN WHOLE CELLS

Time Course of Uptake

The uptake kinetics of riboflavin, thiamine and valine was determined at a concentration of one μM. The mid-log phase cells were treated with chloramphenicol to inhibit incorporation into protein (especially for valine). The pmoles of substrate contained in the free cell pool plotted vs time is shown in Figure 2. Although not shown in Figure 2, thiamine uptake was linear with time for more than 50 minutes. The thiamine uptake is slower to reach equilibrium than reported in the literature (20 minutes) by Kawasaki (40). Riboflavin uptake apparently exhibits a large overshoot and then proceeds to a low level. However, the data on riboflavin are difficult to reproduce. In general one observes a low level of uptake as in Figure 2 with an occasional (0-3/experiment) spuriously high value.

Time Course of Uptake in Normal and Shocked Cells

The effect of osmotic shock on the time course of thiamine uptake is shown in Figure 3. Although the loss in viability for this preparation was 14%, the rate of uptake is reduced by more than one-half. Results of a similar

Figure 2. Time Course of Uptake for Thiamine, Riboflavin and Valine in E. coli

Cells were prepared for uptake in the usual manner in the presence of chloramphenicol. The uptake was performed at 37° with shaking and a substrate concentration of 1 μM. Aliquots (0.5 ml) were pipetted into 1 ml of slushy M-9 to stop the uptake. After washing in M-9 at 4°, the cells were disrupted in cold 5% trichloroacetic acid. The pmoles of TCA soluble thiamine (O-O), riboflavin (●-●) or valine (Δ-Δ) in 0.5 ml of cells is plotted against the sampling time. The cell viability was 1 x 10^9 cells/ml.

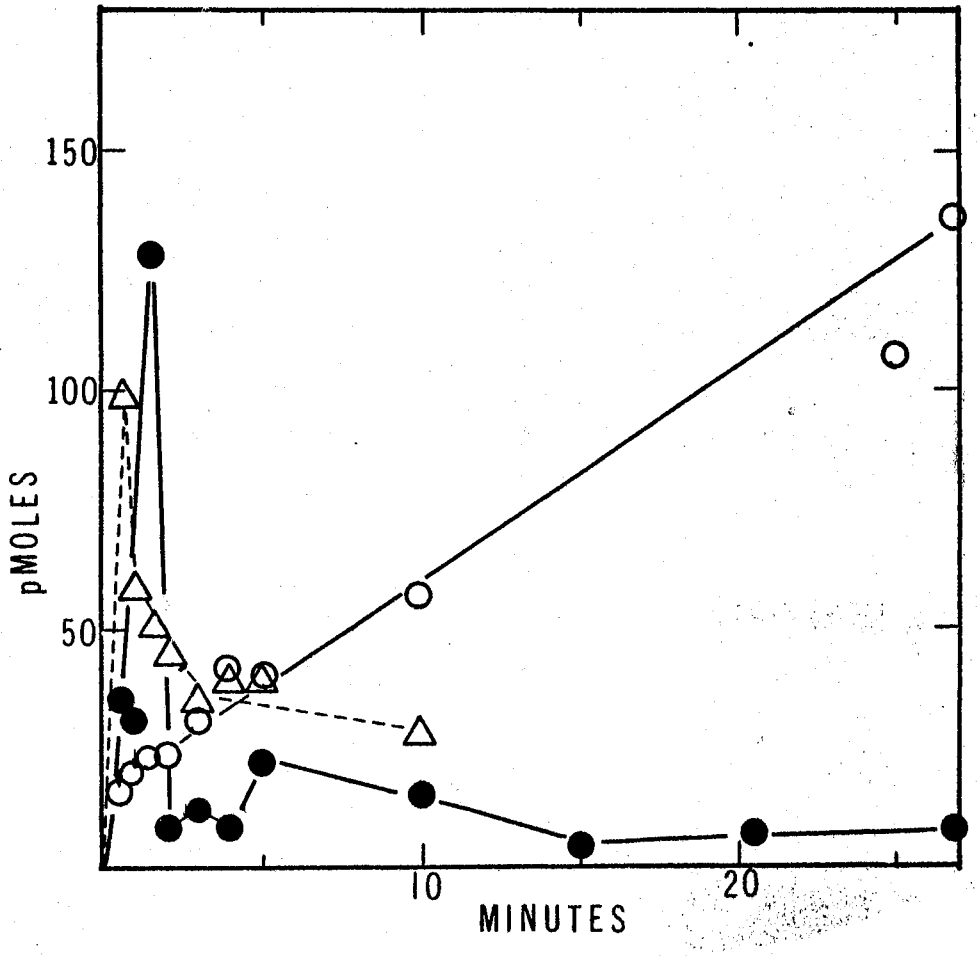
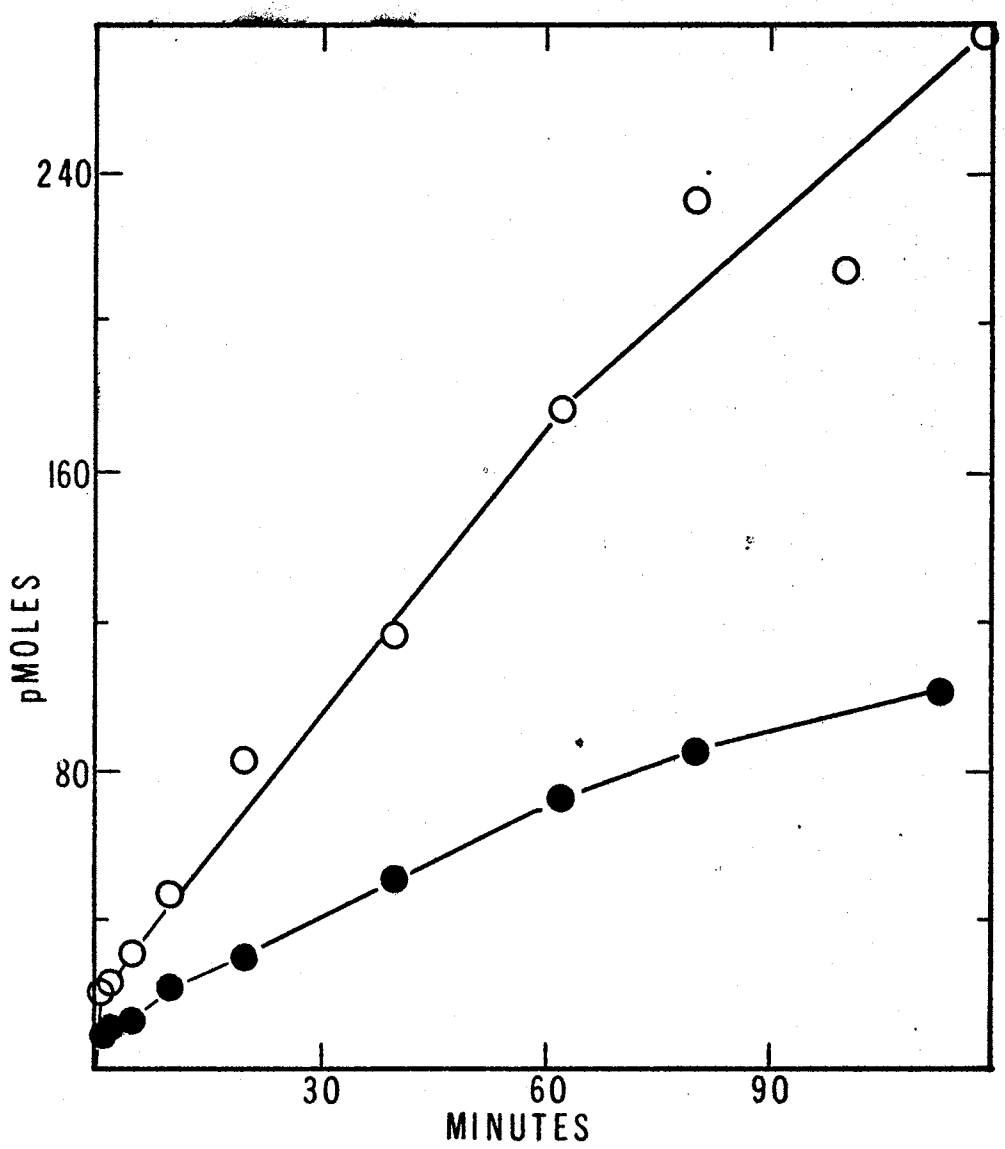

150
100
50
pMOLES
10
20
MINUTES

Figure 3. Time Course of Uptake for Thiamine in Normal and Shocked Cells

Cells were prepared for uptake, treated with chloramphenicol and subjected to osmotic shock as described in the methods section. Both normal and shocked cells were suspended in fresh M-9 at 37^{o} for 10 minutes before ^{35}S-thiamine was added to 1 μM. At the times shown, 0.5 ml was withdrawn from the culture and pipetted into 1 ml of slushy M-9. After washing one time in 4^{o} M-9, the entire cell pellet was transferred in 1 ml of water to a scintillation vial and counted. The pmoles of thiamine contained in 0.5 ml of cells are plotted against the time of sampling for both the shocked (●-●) and normal (0-0) cells. The viabilities for each preparation were: normal, 4.2×10^{8} cells/ml; shocked 3.6×10^{8} cells/ml.


240
160
80
pMOLES
30
60
90
MINUTES

experiment with riboflavin are shown in Figure 4. The unshocked preparation shows the typical spurious uptake plot. However, the shocked cells in this and other experiments are not found to exhibit any spuriously high values.

Saturation Curve for Riboflavin

An attempt to demonstrate a saturation curve for riboflavin yielded the results shown in Figure 5. Stopping the uptake on slushy ice or by Millipore filtration gave similar results. In both cases, the uptake turned sharply upward at about 10 μM riboflavin. Although saturation was not reached, the K_m for uptake is much higher than that reported for thiamine, 0.8 μM (40).

Identification of Thiamine Uptake Products in Shocked and Normal Cells

Since thiamine is converted to thiamine pyrophosphate inside the cell, an experiment was performed to check for any difference in the levels of thiamine compounds in normal and shocked cells. Samples from the experiment shown in Figure 3 were boiled to extract the soluble thiamine compounds and the supernatant solution analyzed by paper chromatography. Three radioactive spots emerged with R_f values similar to authentic thiamine pyrophosphate, thiamine monophosphate and thiamine. The results are shown in Figure 6. Although much less radioactive label is found in the shocked preparation, the ratios of the three compounds are not

Figure 4. Time Course of Riboflavin Uptake in Normal and Shocked Cells

Chloramphenicol treated cells were prepared for uptake and shocked in the usual manner. Uptake was stopped and the cells washed as described in the methods section. The entire cell pellet was suspended in 1 ml of water and transferred to a scintillation vial. The pmoles of riboflavin in 0.5 ml of normal (O-O) and shocked (●-●) cells are plotted against the time of sampling. The cell viability was the same for both preparations, 4×10^8 cells/ml.

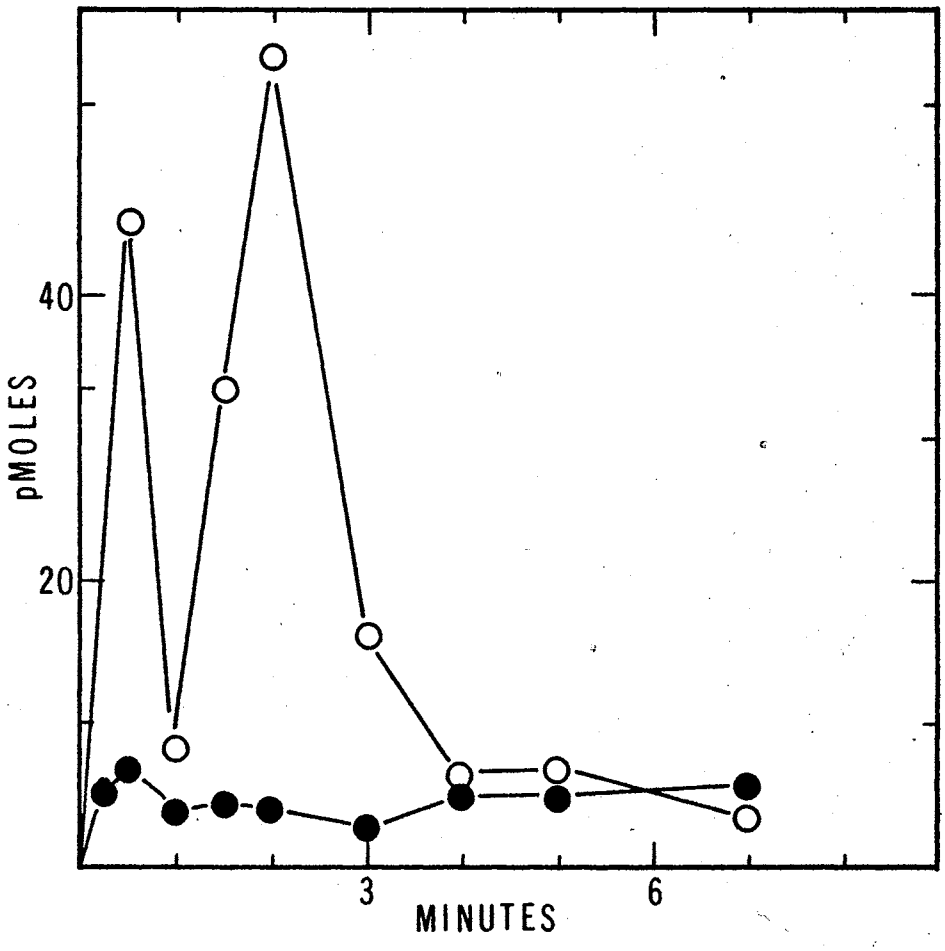

40
20
pMOLES
3
6
MINUTES

Figure 5. Riboflavin Saturation of E. coli

A mid-log phase culture of E. coli in M-9 was incubated with different concentrations of riboflavin at 37° for 10 minutes. Two methods for measuring the uptake were used. Aliquots were pipetted into mushy M-9 and washed in the usual manner in cold M-9 or were filtered rapidly on millipore filter pads (4.5 μ) and washed immediately with 5 ml of M-9 at 4°. In either case the washed whole cells were transferred to scintillation vials and counted. The millipore (●) and mushy M-9 (O) preparations gave similar results and are shown as one line.

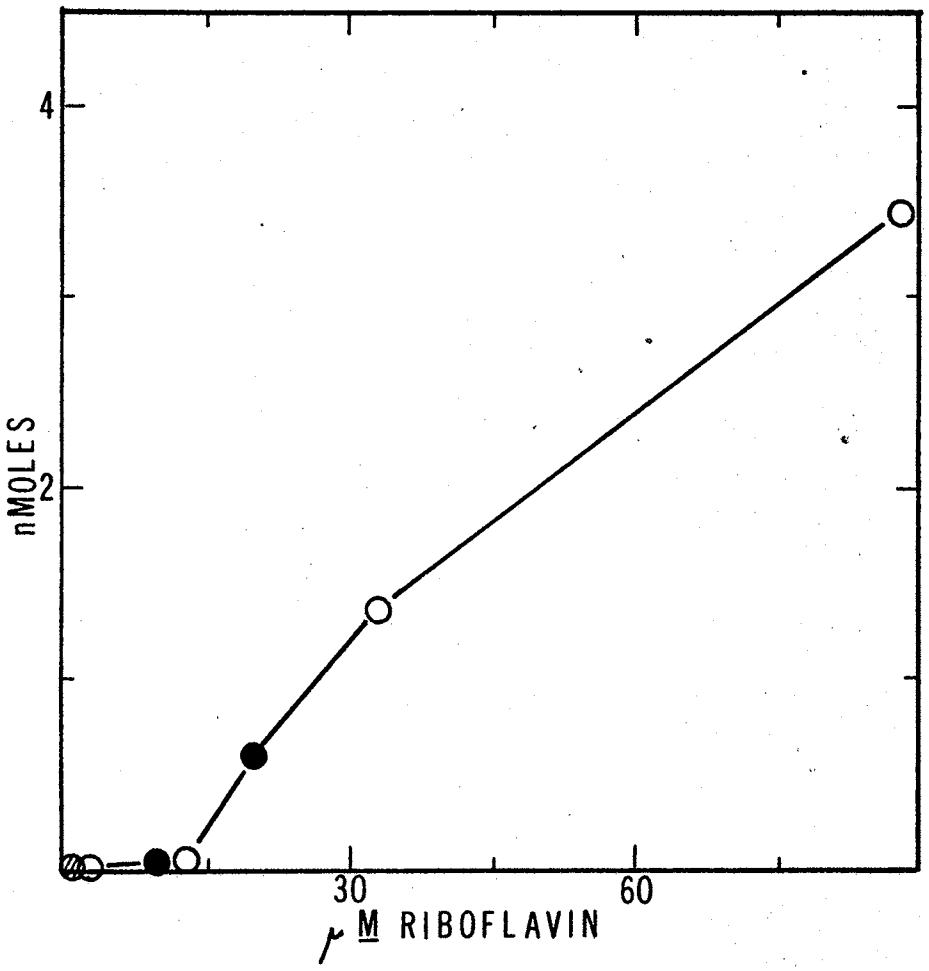
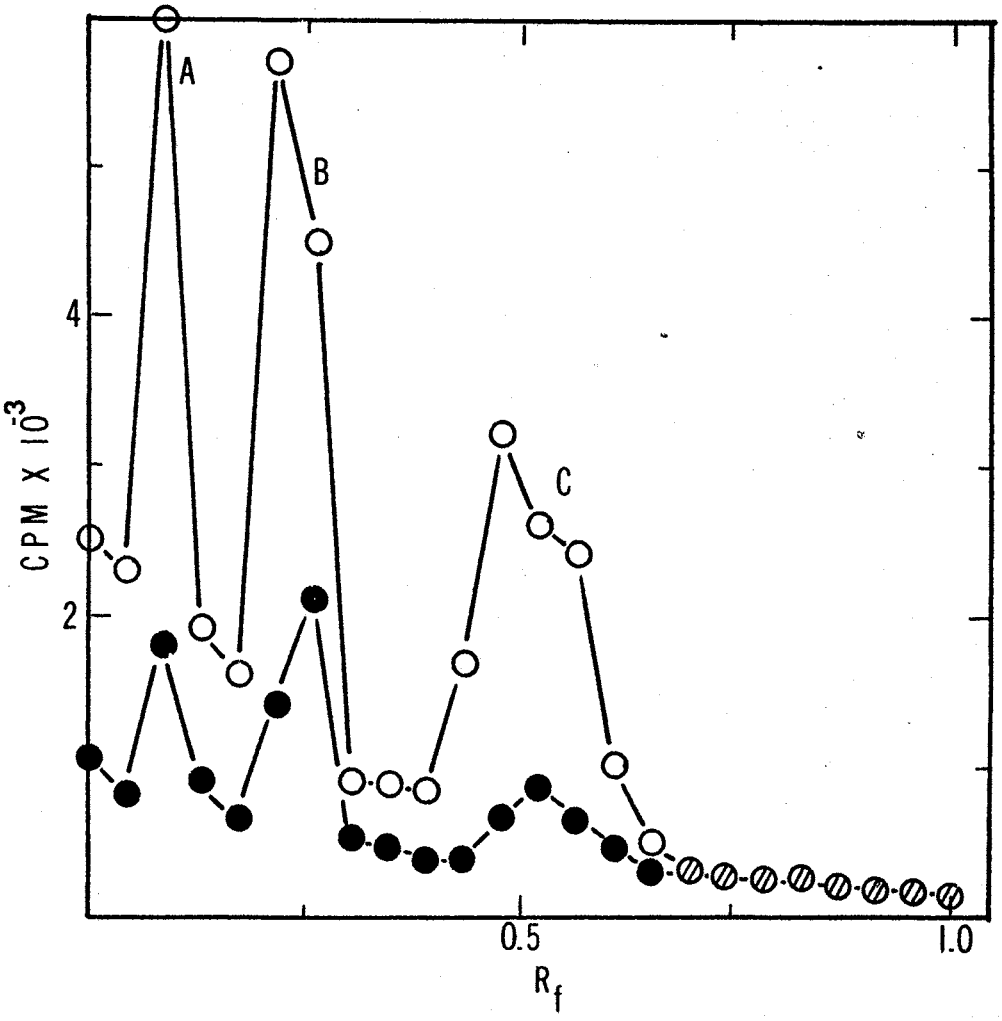

4
2
nMOLES
30
60
μM RIBOFLAVIN

Figure 6. Identification of the Soluble Endogenous Forms of Thiamine

Four ml of cells from Figure 16, after being exposed to 1 μM ^{35}S-thiamine for 20 minutes were pooled and prepared for uptake analysis as described in the methods section. However, instead of lysis in 5% trichloroacetic acid, the cells were placed in a boiling water bath in acetate buffer pH 4.5 for 10 minutes to extract the soluble thiamine compounds (40). The boiled suspension was centrifuged at 10,000 g for 10 minutes and the supernatant solution removed. Equal amounts of this preparation from both normal and shocked cells were spotted on Whatman no. 1 paper and chromatographed by the method of Kawasaki (40). After development and drying, the strips were cut into 0.5 cm pieces, placed in scintillation vials and counted in 10 ml of Bray's scintillation fluid. The cpm in each piece from both normal (O-O) and shocked (●-●) cells is shown vs its R_f value. The peaks A, B and C have R_f values similar respectively to thiamine pyrophosphate, thiamine monophosphate and thiamine.


A
B
C
4
2
CPM X 10^{-3}
0.5
1.0
R_f

significantly different from the normal preparation.

Effect of Osmotic Shock on Uptake

The effect of osmotic shock on the uptake of several vitamins is shown in Table II. Of the compounds investigated, thiamine uptake was by far the most reduced (85%) upon osmotic shock. Biotin uptake was unaffected while the uptake of the remaining vitamins was reduced 20-30%. Riboflavin uptake was increased about two fold.

TABLE II

EFFECT OF OSMOTIC SHOCK ON VITAMIN UPTAKE

Vitamin	pmoles taken up/6.5 x 10^8 cells		% Shocked Normal
	Normal Cells	Shocked Cells	
Thiamine	40	6.0	15
Nicotinamide	194	132	68
Nicotinic Acid	67	52	77
Biotin	1.3	1.3	100
Pyridoxine	1.1	0.8	73
Riboflavin	1.7	3.8	220
Lipoic Acid	70	54	77

Mid-log phase cells were prepared for uptake and shocked as described in the methods section. The amount of vitamin taken up during a 5 minutes exposure to 1 μM vitamin (except lipoic acid - 10 μM) was determined for an equal number of normal and shocked cells. The uptake was stopped by pipetting 0.5 ml of cell suspension into 1 ml of crushed, frozen M-9 and trichloroacetic acid soluble material measured as described in the methods section.

CHAPTER IV

BINDING TO SHOCK FLUID

Binding to Crude Osmotic Shock Fluid

Since osmotic shock reduced the uptake of most vitamins, shock fluid was concentrated and tested for its ability to bind the vitamins and leucine. Binding data of the compounds at 1 μM by the Paulus (64) method of membrane filtration are shown in Table III. Thiamine was bound to a greater extent than the other compounds (72 pmoles/mg). The other vitamins were bound between 0.2 and 7.0 pmoles/mg. Proline was not bound. The absence of a proline binding protein has been reported in shock fluid (2) unless the cells are grown in a special medium (26). Although 9 pmoles of leucine are bound per mg of protein, this is much less than reported by Anraku (22) (580 pmoles/mg). If the concentrated shock fluid is dialyzed overnight, the amount of leucine bound/mg of protein is increased about 10 fold. Therefore, the apparent low value is in part due to competition of labeled leucine with unlabeled leucine in the crude shock fluid. Free amino acids are known to be lost upon osmotic shock (68).

TABLE III

BINDING OF VITAMINS AND AMINO ACIDS TO CONCENTRATED SHOCK FLUID

Vitamin or Amino Acid	pmoles Bound/mg Protein
Thiamine	72
Nicotinamide	0.2
Riboflavin	6.2
Lipoic acid	7.0
Biotin	2.5
Pyridoxine	5.2
Leucine	9.0
Proline	0.01

Binding to unpurified shock fluid was measured with a Paulus cell using 1 μM substrate and about 2 mg/ml protein.

Separation of Binding Proteins

To determine if a specific binding protein or proteins exist for the vitamins, the shock fluid was fractionated with ammonium sulfate, dialyzed and binding experiments performed. These data are shown in Table IV. Of the vitamins and amino acids tested, only leucine and thiamine binding activities were separated to a significant extent. Some separation of binding activity was observed with riboflavin and the 40-60% ammonium sulfate saturation fractions were further fractionated by DEAE cellulose column chromatography. However, the binding in these two fractions could not be associated with any DEAE fraction as the riboflavin binding was spread throughout the protein pattern. A check of riboflavin binding in the 70-90% ammonium sulfate fraction upon DEAE cellulose chromatography did yield a separate fraction with the majority of the riboflavin binding activity (see riboflavin binding protein purification, page 78). Although the lipoic acid binding was not significantly separated, shock fluid was subjected to numerous other fractionation procedures to insure no binding protein escaped detection. Concentrated shock fluid was fractionated by DEAE column chromatography and each fraction checked for lipoic acid binding via equilibrium dialysis at 1 μM lipoic acid. These data are shown in Figure 7. Although the results were spurious, any group of connected fractions which contained a relatively high lipoic acid binding activity was pooled and passed through a gel filtration

TABLE IV

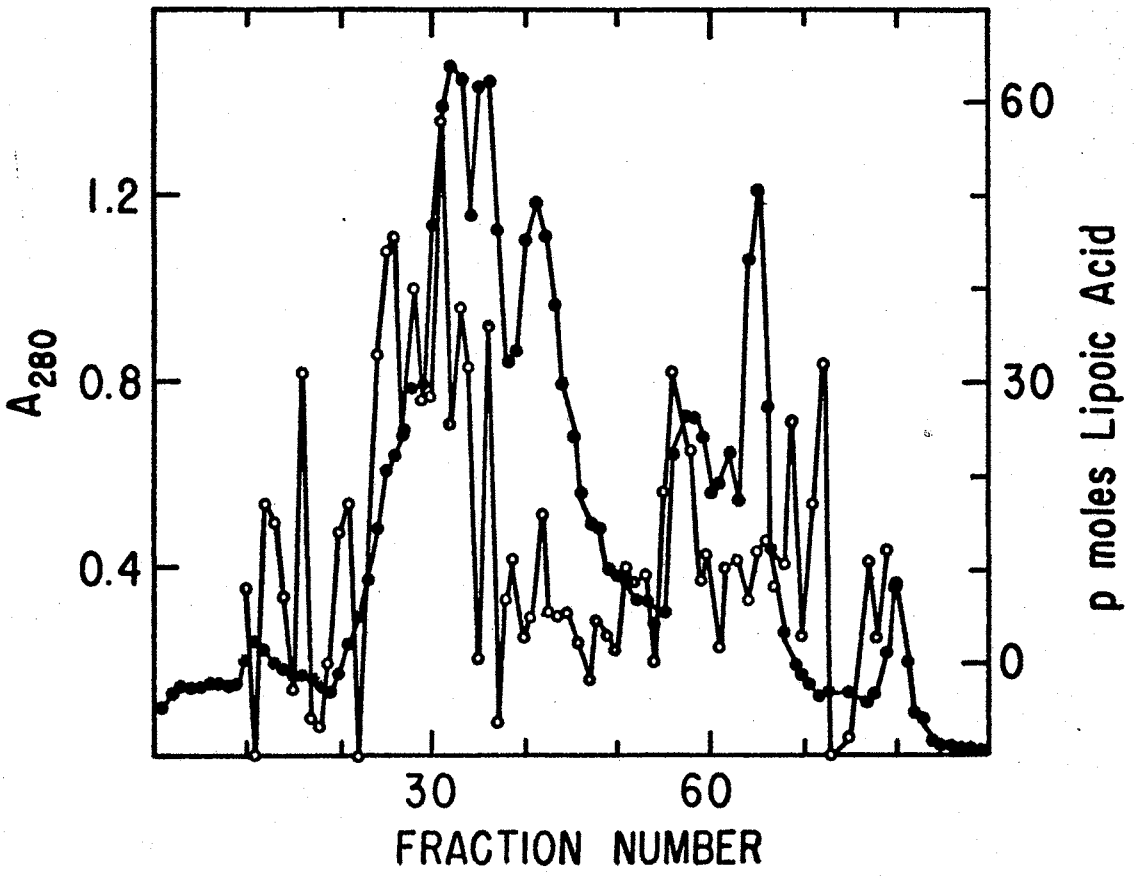
BINDING OF VITAMINS AND AMINO ACIDS TO FRACTIONATED SHOCK FLUID

Ammonium Sulfate Fraction	pmoles Bound/mg Protein								
	leucine (0.8)	thiamine (1.0)	ribo-flavin (1.4)	lipoic acid (1.0)	biotin (1.0)	nicotinic acid (1.0)	nicotin-amide (1.0)	proline (0.2)	pyrid-oxine (1.0)
0-30	0.8	8.2	0.7	13.1	0.7	4.1	0.1	0.09	10.7
30-40	1.4	6.4	1.3	7.0	0.6	4.1	0.2	0.01	5.3
40-50	0.7	7.2	2.0	7.5	0.6	3.1	0.2	0.02	3.1
50-60	0.7	4.0	2.0	7.5	0.6	3.8	trace	0.03	3.1
60-70	1.6	4.2	1.2	10.1	0.6	2.0	0.1	0.04	5.5
70-80	11.5	6.8	1.1	6.9	0.6	3.2	0.1	0.05	5.0
80-100	24.2	25.8	0.8	2.7	0.5	3.1	0.1	trace	3.3
unfrac-tionated	1.8	14.7	1.3	3.0	0.5	1.3	trace	0.01	4.5

Each ammonium sulfate fraction was assayed at about 2 mg/ml protein by the Paulus method of membrane filtration. The assay concentration for each compound is shown in parenthesis below the compound name. The pmoles of substrate bound per mg of protein are shown for each compound and fraction of shock fluid.

Figure 7. Lipoic Acid Binding Assay of DEAE Fractionated Shock Fluid

Unpurified concentrated shock fluid was subjected to DEAE (Mannex) column chromatography (1.5 x 20 cm). After washing the column in TMM buffer (5m$\underline{M}$ tris, 1m$\underline{M}$ $MgCl_2$, 0.5 m$\underline{M}$ mercaptoethanol, pH 8.0), a 0-0.15 $\underline{M}$ linear NaCl gradient (80 ml each) in TMM buffer was started at fraction 10. This was followed by 35 ml of 0.3 $\underline{M}$ NaCl buffer (fraction 48), 35 ml of 0.6 $\underline{M}$ NaCl buffer (fraction 57) and 130 ml of 1.0 $\underline{M}$ NaCl buffer (fraction 65). Lipoic acid binding activity was assayed at 1 $\mu\underline{M}$ lipoic acid via equilibrium dialysis. The net pmoles of lipoic acid bound to 20 μl of each fraction (O-O) and the absorbance at 280 nm (●-●) are shown for each fraction.


1.2
0.8
0.4
A_{280}
60
30
0
p moles Lipoic Acid
30
60
FRACTION NUMBER

column and the fractions again assayed for activity. Again spurious data were obtained indicating that there was insufficient binding to override the fluctuations in experimental background. In addition, shock fluid was fractionated initially by gel filtration at 4° and also ammonium sulfate fractions were subjected to gel filtration and DEAE cellulose chromatography. These modified procedures gave similar results to those shown.

Lipoic Acid Binding to Shock Fluid

Although lipoic acid binding activity could not successfully be fractionated, the nature of lipoic acid binding to the shock fluid was investigated. The attempted saturation of shock fluid by lipoic acid is shown in Figure 8. Although no points are shown for concentrations below 100 μM, other experiments using concentrations as low as 0.5 μM up to 100 μM indicate that this region is indeed linear as shown in Figure 8. The above data were collected at 4° by equilibrium dialysis. Although low concentrations of lipoic acid (below 20 μM) came to equilibrium with the protein in 24 hours, higher concentrations had not reached equilibrium in three days. This is shown in Figure 9. At the highest concentration shown in Figure 9, about one mole of lipoic acid is bound per 2.5 kg of protein. Since mercaptoethanol does not interfere with the binding (Table V), disulfide interchange is unlikely to be involved in the binding. Therefore, the lipoic acid is likely involved in hydrophobic

Figure 8. Lipoic Acid Saturation of Shock Fluid

Unpurified shock fluid (270 μg/ml) was subjected to equilibrium dialysis with varying lipoic acid concentrations. After 70 hours at 4^{o} 0.1 ml aliquots were removed and counted. The nmoles of lipoic acid bound to 270 μg of protein are plotted against the free lipoic acid concentration.

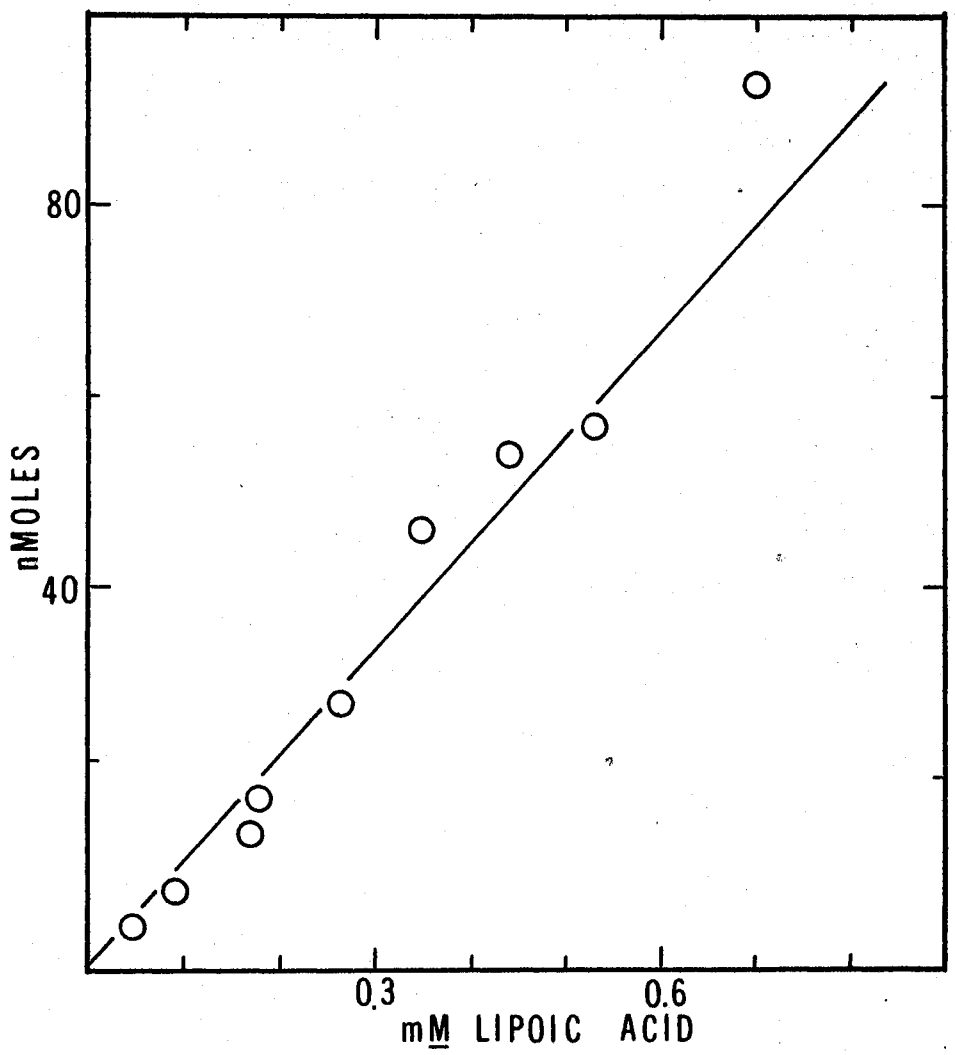

80
40
nMOLES
0.3
0.6
mM LIPOIC ACID

Figure 9. Time for Equilibration of Lipoic Acid and Shock Fluid

Unpurified shock fluid (270 μg/ml) was subjected to equilibrium dialysis with varying lipoic acid concentrations at 4°. At the times shown 0.1 ml aliquots were removed from each side of each cell. The excess cpm on the protein side are plotted against the time of removal for each concentration. The lipoic acid concentrations in μM for the five curves are: 1) 170, 2) 130, 3) 90, 4) 50, and 5) 20.

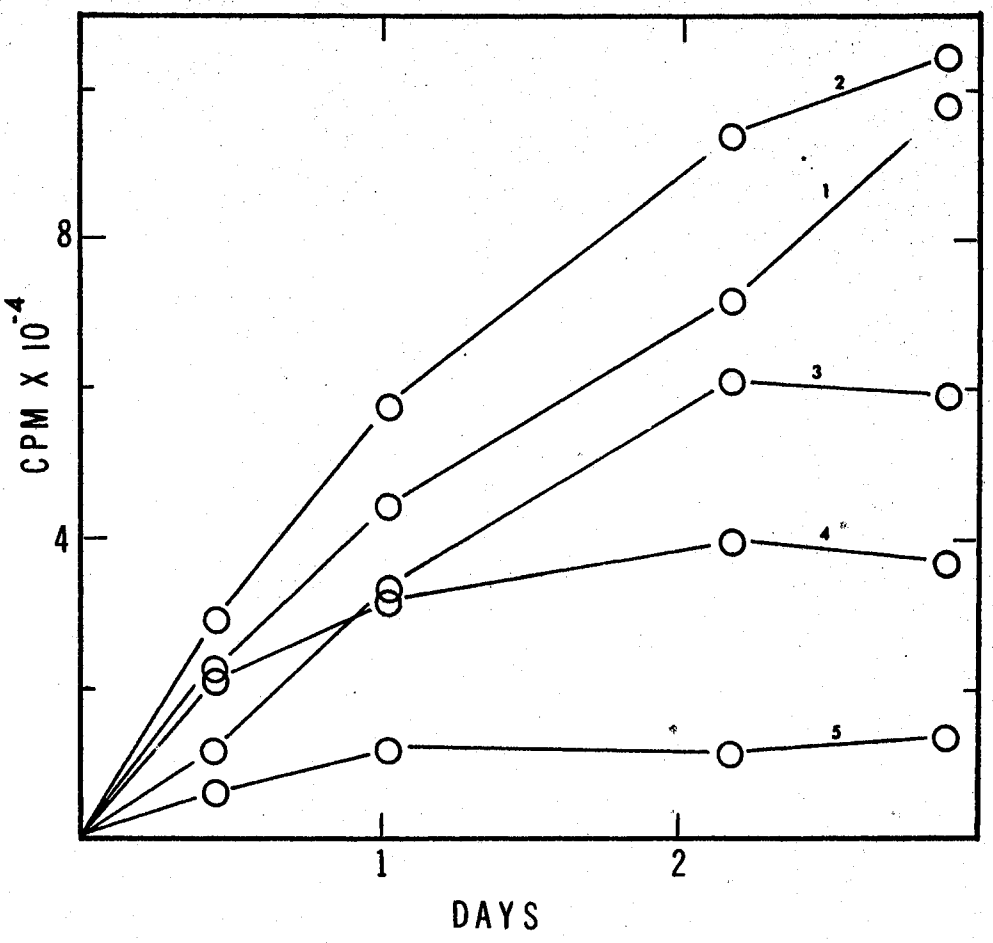

CPM X 10^{-4}
8
4
1
2
DAYS
1
2
3
4
5

TABLE V

EFFECT OF MERCAPTOETHANOL ON LIPOIC ACID BINDING TO SHOCK FLUID

Mercaptoethanol Conc. (mM)	cpm Lipoic Acid Bound	
	14 Hours	38 Hours
0	4100	3800
200	4600	3140

Unpurified shock fluid (135 μg/ml) was subjected to equilibrium dialysis with 200 μM ^{35}S-lipoic acid in the presence or absence of 0.2 M mercaptoethanol. After 14 and 38 hours at 4°, 0.1 ml aliquots were removed and counted. The cpm bound to 135 μg of protein are shown for each condition.

interactions with shock fluid proteins and perhaps forms micelles with the lipids that are also lost upon osmotic shock (24). This may in part explain why equilibrium was reached so slowly, since hydrophobic interactions are decreased greatly by lower temperatures.

CHAPTER V

THIAMINE BINDING PROTEIN

Purification

A thiamine binding protein was purified by conventional means as shown in Table VI. The purification procedure is described in detail in the methods section. The protein pattern from the DEAE cellulose step is shown in Figure 10 along with the binding activities associated with both thiamine and riboflavin. The thiamine binding protein has a specific activity of 18.2 nmoles bound/mg protein at the final step shown in the purification scheme. Later duplications of this procedure yielded a final product in which 85% of the protein migrated as a single band on polyacrylamide disc gel electrophoresis. The specific activity was 13.9. In an attempt to purify further the protein it was passed through the last two steps of the procedure. This yielded a protein about 92% pure (Figure 11) and a specific activity of 11.7.

Identification of the Thiamine Binding Protein

Polyacrylamide disc gel electrophoresis of the binding protein after hydroxylapatite chromatography is shown in Figures 11 and 12. An identical gel was immersed in a 1 μM

TABLE VI

PURIFICATION OF THE THIAMINE BINDING PROTEIN

Fraction	Volume ml	Protein mg/ml	Total Activity (pmoles x 10^{-3})	Specific Activity (pmoles/mg)	Purification
Crude Shock Fluid	250	4.0	80	80	1
$(NH_4)_2SO_4$ (0.7-0.9)	29	13	48	130	1.6
DEAE	16	1.4	27	1200	3.0
Hydroxyl-apatite	20	0.3	25	4800	60
Gel Filtration	7.7	0.15	21	18200	230

The thiamine binding activity was followed during its purification by Paulus cell assay at 1 μM thiamine. All steps in the purification were carried out at 4° as described in the methods section.

Figure 10. Purification of Riboflavin and Thiamine Binding Proteins by DEAE Chromatography

The 0.7 - 0.9 saturated ammonium sulfate fraction was loaded onto the column (1.3 x 15 cm) and washed with 20 ml of TMM buffer. A 200 ml linear NaCl gradient 0 - 0.15 M, (marked by the two arrows) was passed through the column followed by 1.0 M NaCl, each in TMM buffer. The protein elution (●-●) was followed by its absorption at 280 nm. The binding affinities were determined at 1 μM vitamin concentration using the membrane filtration technique of Paulus. Some fractions were not assayed for binding activity since previous experiments showed only one band of activity. Thiamine binding (O-O) and riboflavin binding (□-□) are shown as pmoles bound/0.1 ml of fraction.

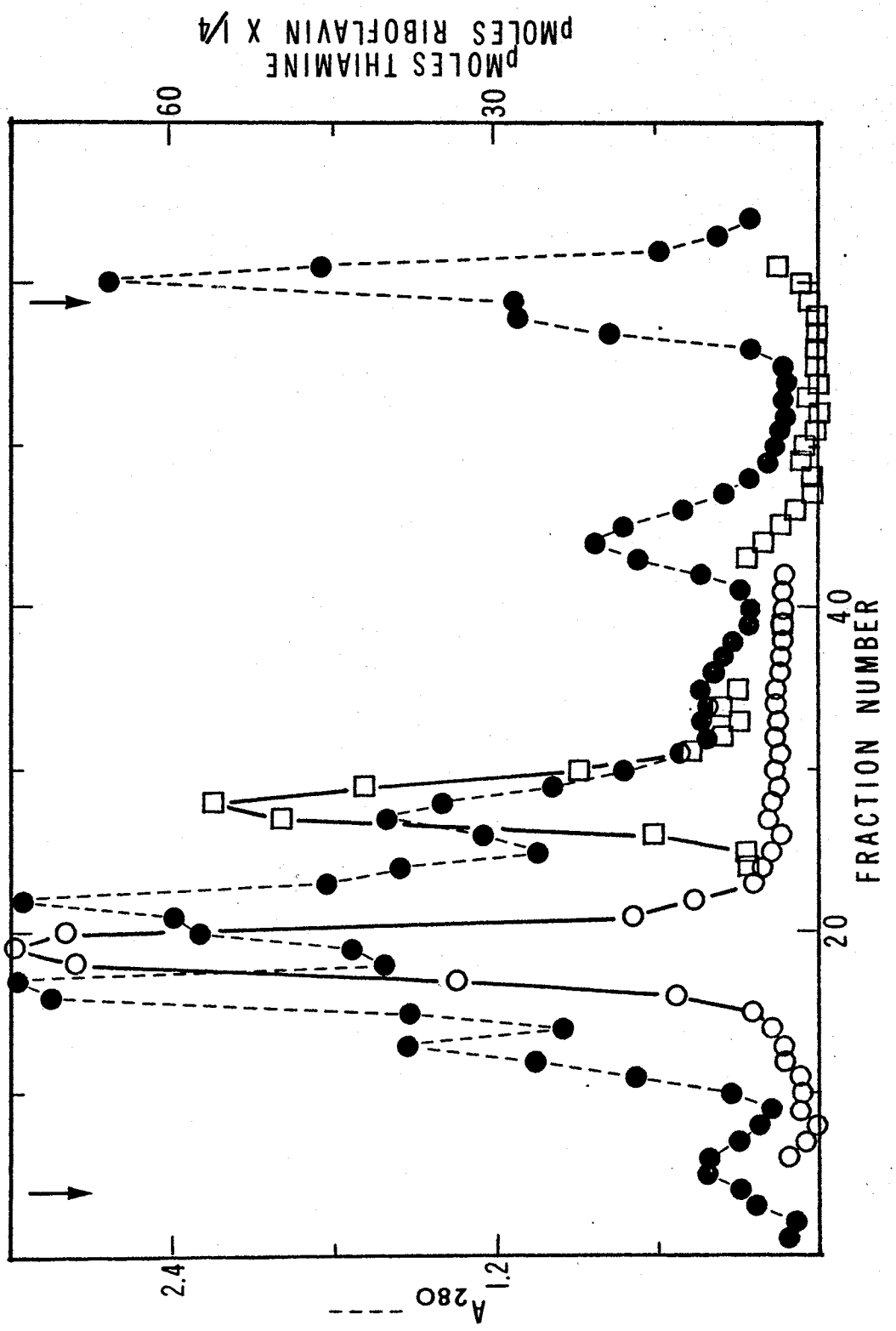

pMOLES THIAMINE
pMOLES RIBOFLAVIN X 1/4
60
30
2.4
1.2
A_{280}
20
40
FRACTION NUMBER

Figure 11. Disc Gel Electrophoresis Patterns of Binding Proteins

The binding proteins were subjected to polyacrylamide disc gel electrophoresis under various conditions. All normal gels were 7% polyacrylamide. The sodium dodecylsulfate gels were 6% polyacrylamide. Gel scans were performed and recorded using a linear transport Gilford attachment and a Beckman DU spectrophotometer. The numbers represent the relative area under each absorbing band as judged by scanning the gel at 550 nm. D- represents dye front.

A) Thiamine binding protein (30 μg) after the hydroxylapatite purification step.

B) Thiamine binding protein (25 μg) after the gel filtration purification step.

C) Thiamine binding protein (9 μg) with about 0.5 m$\underline{M}$ thiamine in the protein sample.

D) Thiamine binding protein (30 μg) in the presence of 0.1% sodium dodecylsulfate. A trace impurity is shown.

E) Riboflavin binding protein (50 μg) after the DEAE purification step. Several trace impurities are shown.

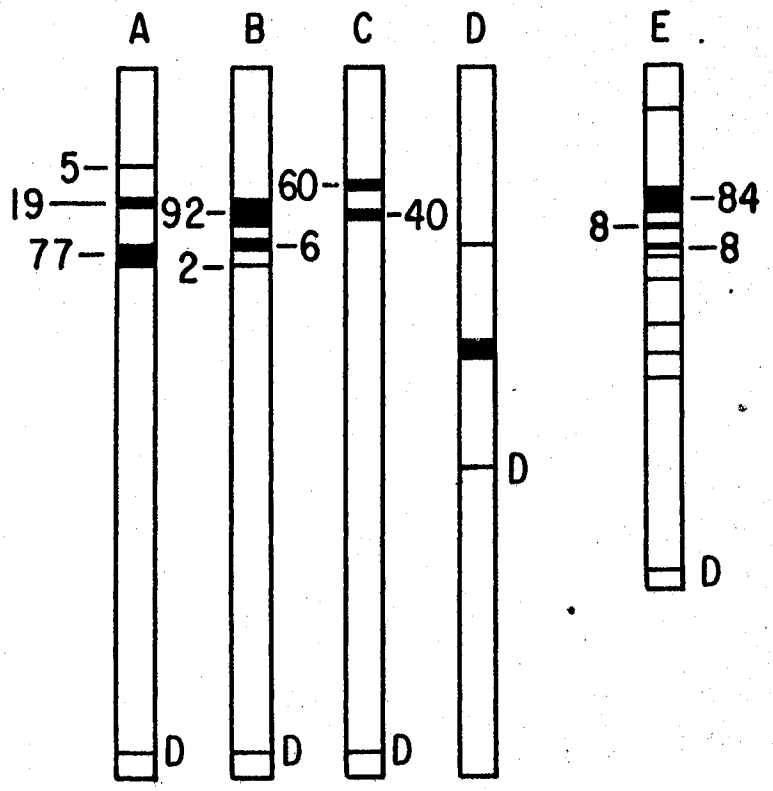
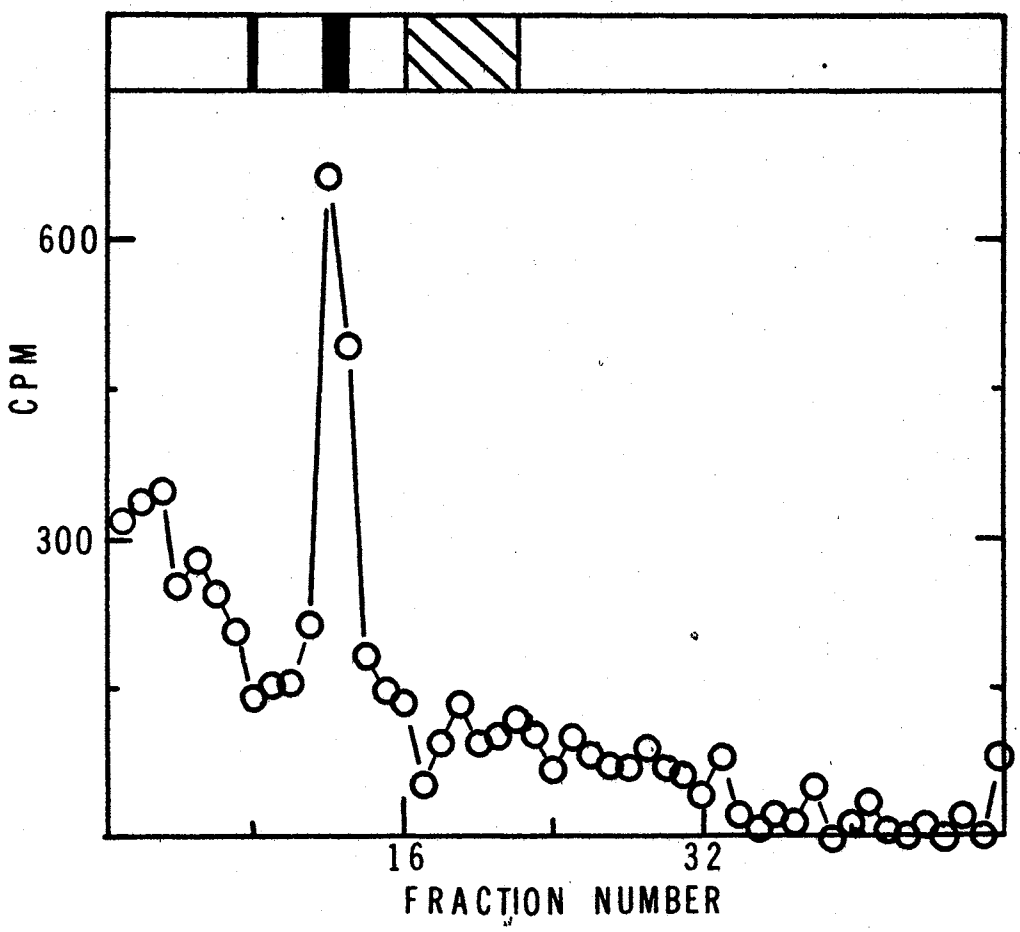

A
B
C
D
E
5
19
77
92
60
2
6
40
84
8
8
D
D
D
D
D

Figure 12. Binding of Thiamine to Electrophoresed Thiamine Binding Protein in Polyacrylamide Gel

The thiamine binding protein fraction (30 μg) after hydroxylapatite chromatography was electrophoresed in a 7% polyacrylamide gel and stained in aniline blue black. An identical gel was immersed immediately in a 1 μM solution of ^{35}S-thiamine. After one hour the gel was removed, rinsed with water and immediately sliced into 48 equal parts. The cpm in each slice (minus background) is plotted against the fraction number. A reproduction of the protein pattern corresponding to the fraction numbers is also shown.


600
300
CPM
16
32
FRACTION NUMBER

solution of thiamine for one hour and then immediately sliced into 2 mm sections. The cpm bound to each section after subtraction of background cpm are indicated in Figure 12. The gel pattern of an identical gel stained in amido blue-black is related to the binding curve. A majority of the thiamine bound is associated with the protein band which is purified by gel chromatography (Figure 11).

pH Optimum for Binding

The optimum binding pH was determined by using the Paulus membrane filtration apparatus and a 0.15 M citrate-phosphate-tris buffer (0.05 M in each) over the entire pH range. The pH vs binding activity curve is shown in Figure 13. The thiamine binding protein exhibited a definite and rather sharp pH optimum at 9.2.

Exchange of Bound Thiamine

The ability of labeled, bound thiamine to exchange with an excess of unlabeled compound was determined by equilibrium dialysis (Figure 14). After equilibration for 11 hours at 4° in the presence of 0.50 μM labeled thiamine, unlabeled thiamine was added to 500 μM to both sides of the dialysis cell. Within 10 hours equilibrium was accomplished and the binding appears completely reversible. Although other thiamine binding protein assays were performed at 25°, the difference in the amount of binding with respect to temperature was insignificant.

Figure 13. pH Optimum for the Binding Proteins

Binding experiments were performed by the membrane filtration technique of Paulus at 1 μM vitamin. A citrate, phosphate, tris buffer, 0.05 M in each component, was used over the entire pH range. The relative amount bound for each pH is shown for thiamine (O-O) and riboflavin (●-●).

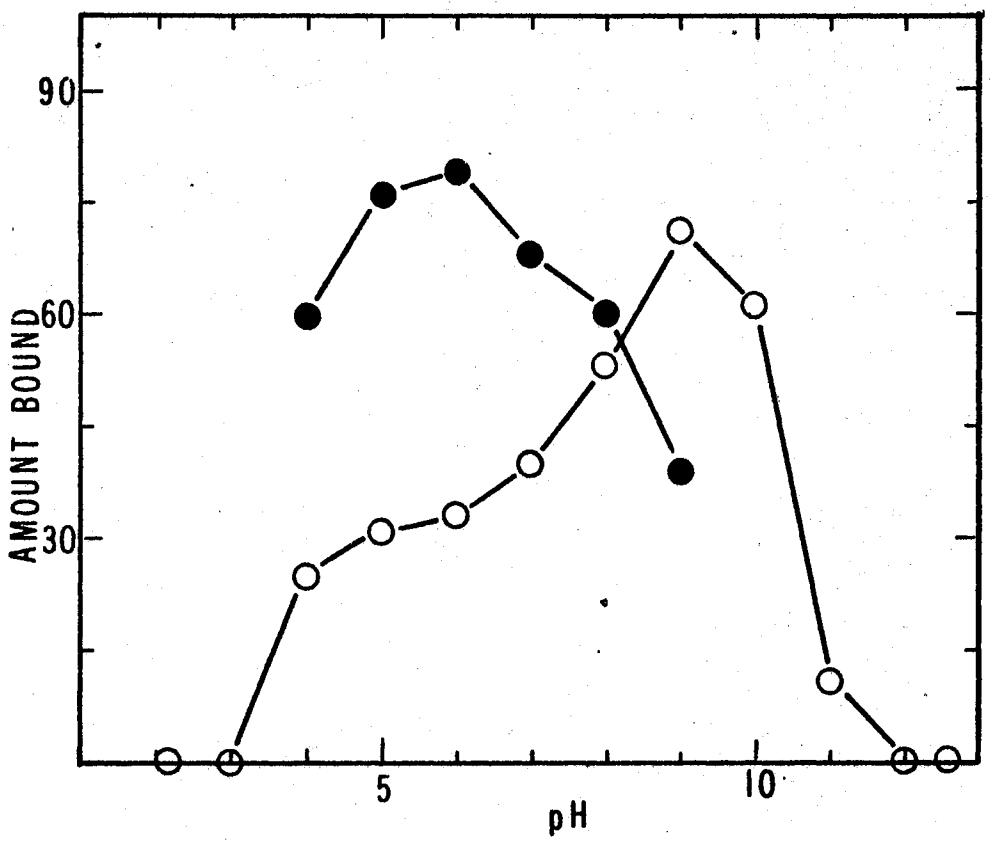
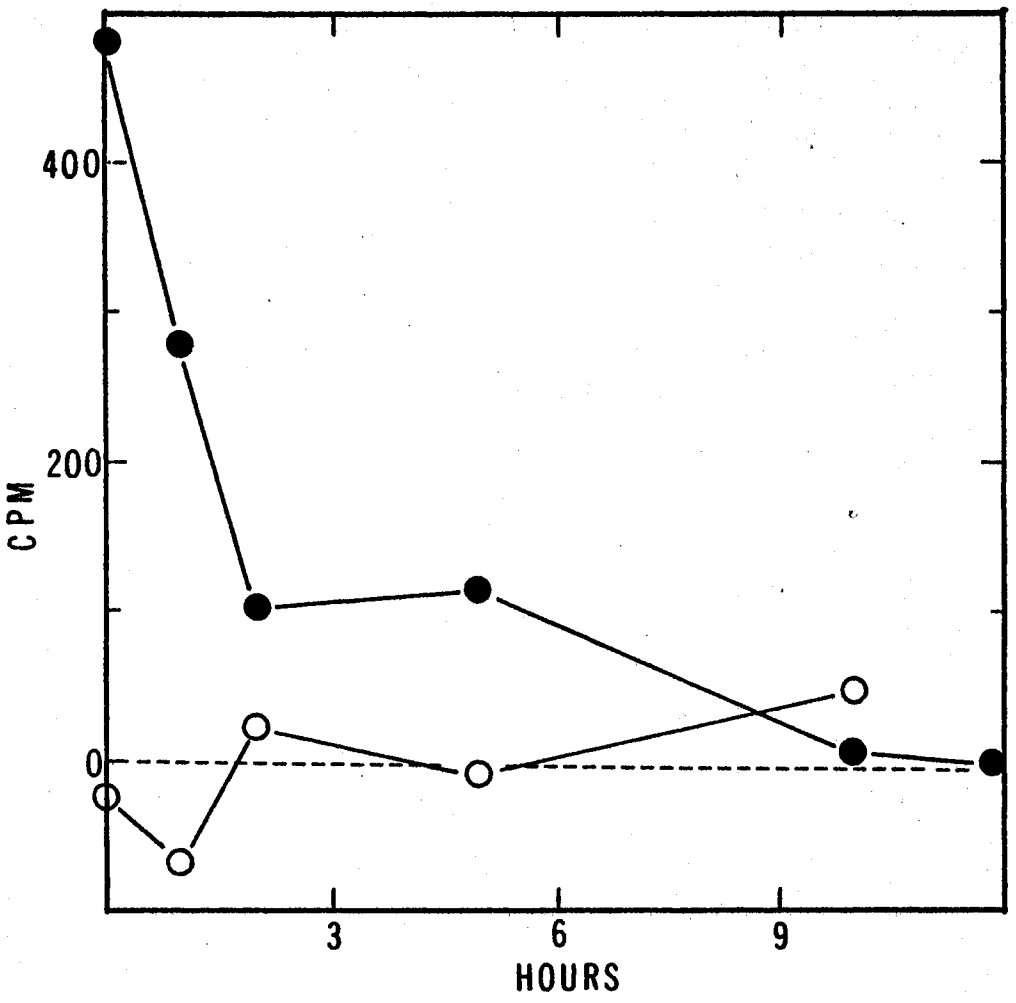

90
60
30
AMOUNT BOUND
5
10
pH

Figure 14. Exchange of Thiamine to the Thiamine Binding Protein

Purified thiamine binding protein was placed in equilibrium dialysis cells and ^{35}S-thiamine added to 0.5 μM. To an identically treated cell additional thiamine unlabeled was added to 500 μM. This served as a control. After shaking 11 hours at 4°, both cells were sampled and unlabeled thiamine added to the first cell to 500 μM. Additional samples were taken at the times shown. The net cpm on the protein side are plotted against the time of sampling for the control (O-O) and the exchange (●-●) cells.


400
200
0
CPM
3
6
9
HOURS

Determination of the Binding Constant

The dissociation constant was determined via equilibrium dialysis. The data are shown in Figure 15. The K_D is about 0.05 μM.

Inhibition Constant of Thiamine Pyrophosphate

Thiamine pyrophosphate competitively inhibits thiamine binding to the binding protein as shown in Table VII. The K_I was calculated from the binding data assuming all reactions to be in equilibrium. The K_I was determined to be 0.5 ± 0.1 μM.

Effect of Other Compounds on Thiamine Binding

The effect of various compounds on thiamine binding was performed by Paulus membrane filtration. The results are shown in Table VIII. The inorganic compounds potassium phosphate, sodium pyrophosphate and magnesium chloride had little effect on thiamine binding. Similarly mercaptoethanol and the sulfhydryl reagents N-ethylmaleimide and iodoacetic acid had no effect. Some inhibition by the pyrimidines thymine and cytosine occurred at 100 μM. From these data the approximate K_I for the pyrimidines is 100 μM.

Determination of Molecular Weight

The molecular weight of the thiamine binding protein was determined from its mobility in 6% polyacrylamide gels upon electrophoresis in the presence of sodium dodecyl-

Figure 15. Thiamine Saturation of Thiamine Binding Protein

The binding experiments were performed by equilibrium dialysis for 12 hours at room temperature. Theoretical lines for both the hyperbola and its reciprocal plot were calculated using K_D of 0.05 μM and a saturation value of 13.5 pmoles. The experimental points were then added to the graph.

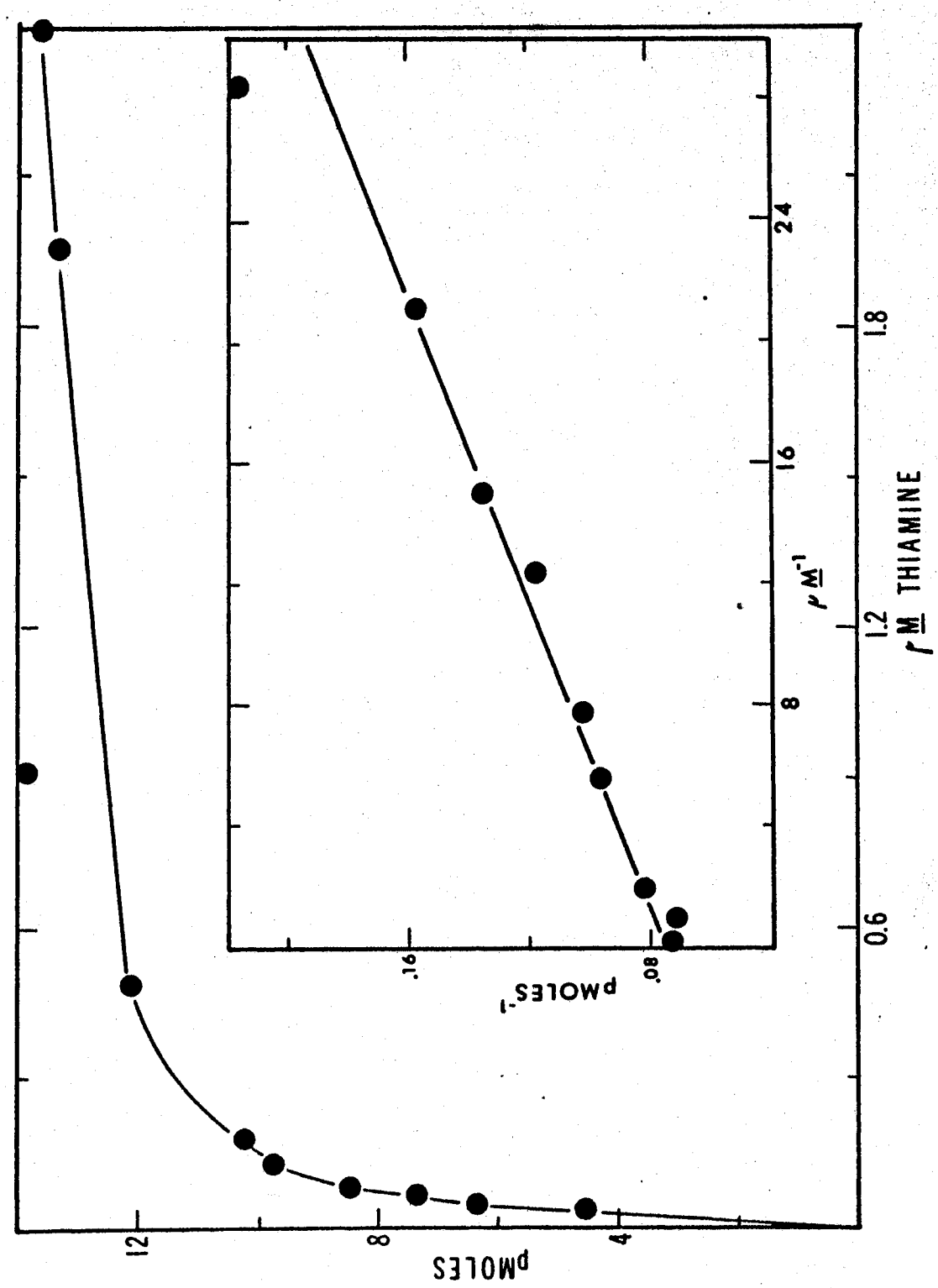

pMOLES
12
8
4
μM THIAMINE
0.6
1.2
1.8
pMOLES⁻¹
.08
.16
μM⁻¹
8
16
24

TABLE VII

THIAMINE BINDING INHIBITION BY THIAMINE PYROPHOSPHATE

Thiamine Pyrophosphate Concentration total (μM)	pmoles Thiamine Bound	Calc. K_I (μM)
0	50.0	--
0.5	47.2	0.626
1.0	44.2	0.539
1.5	41.6	0.507
2.0	40.5	0.579
5.0	27.6	0.355
10.0	21.2	0.400
	Aver.	0.5 ± 0.1

Experiments were performed in duplicate using Paulus cell membrane filtration and using the citrate-phosphate-tris buffer, pH 9.0. Labeled thiamine was added to all samples to a final concentration of 1.0 μM and TPP was added to the concentrations shown. The reaction was visualized as follows:

$$EI \overset{I}{=} E + S = ES.$$

The K_I was calculated assuming complete equilibrium of all steps.

TABLE VIII

EFFECT OF VARIOUS COMPOUNDS ON THIAMINE BINDING

Additions	% of Control
None (control)	100
1 mM $MgCl_2$	96
0.5 M KPO_4, pH 7.5	94
1 mM Na pyrophosphate	101
0.1 M mercaptoethanol	94
10 mM N-ethylmaleimide	96
10 mM iodoacetic acid	85
10 μM thymine	104
100 μM thymine	81
10 μM cytosine	104
100 μM cytosine	87

Thiamine binding protein was exposed to the compounds listed before assaying via the Paulus binding assay at 2 μM thiamine. All values shown are the average of duplicate experiments and are listed as per cent of the control.

sulfate. The procedure was essentially that of Weber and Osborn (66). Several proteins of known molecular weight served as molecular markers. The data are shown in Figure 16. Molecular weight determinations by relative migration in 6 and 9% gels by the method of Davis (67) or by the elution volume from Biogel P-150 (Figures 17 and 18), although not as definite, gave comparable values. The molecular weight of the thiamine binding protein is approximately 34,000.

Behavior Upon Electrophoresis

The thiamine binding protein shows a single band (exclusive of impurities) in both 6 and 9% gels and in sodium dodecylsulfate polyacrylamide gel electrophoresis (Figure 11). Since molecular weight determinations in both kinds of gels gave similar results, no subunit association is suggested under these conditions. The addition of thiamine to the binding protein before addition to the gel divides the protein into two bands (Figure 11), one traveling slower than the normal protein. To determine what effect thiamine had on the apparent molecular weight of the thiamine binding protein under such conditions, the experiment was performed in 6 and 9% gels with 0.5 mM thiamine present or absent in the protein sample. The migration distance and ratios are shown in Table IX. The data indicate the slower migrating band is from 2000-4000 smaller in apparent molecular weight as estimated from Figure 17.

Figure 16. Molecular Weight Determination of Binding Proteins by SDS Polyacryamide Gel Electrophoresis

The molecular weights were determined by a method essentially that of Weber and Osborn (66). The mobilities in 0.1% sodium dodecylsulfate are proportional to the log of the molecular weight. The following proteins were used as molecular weight standards: 1, catalase (60,000); 2, ovalbumin (43,000); 5, pepsin (35,000); 6, α-chymotrypsinogen (25,700); 7, trypsin (23,000) and 8, β-lactoglobulin (18,400). The binding proteins are identified as that of 3, riboflavin and 4, thiamine.

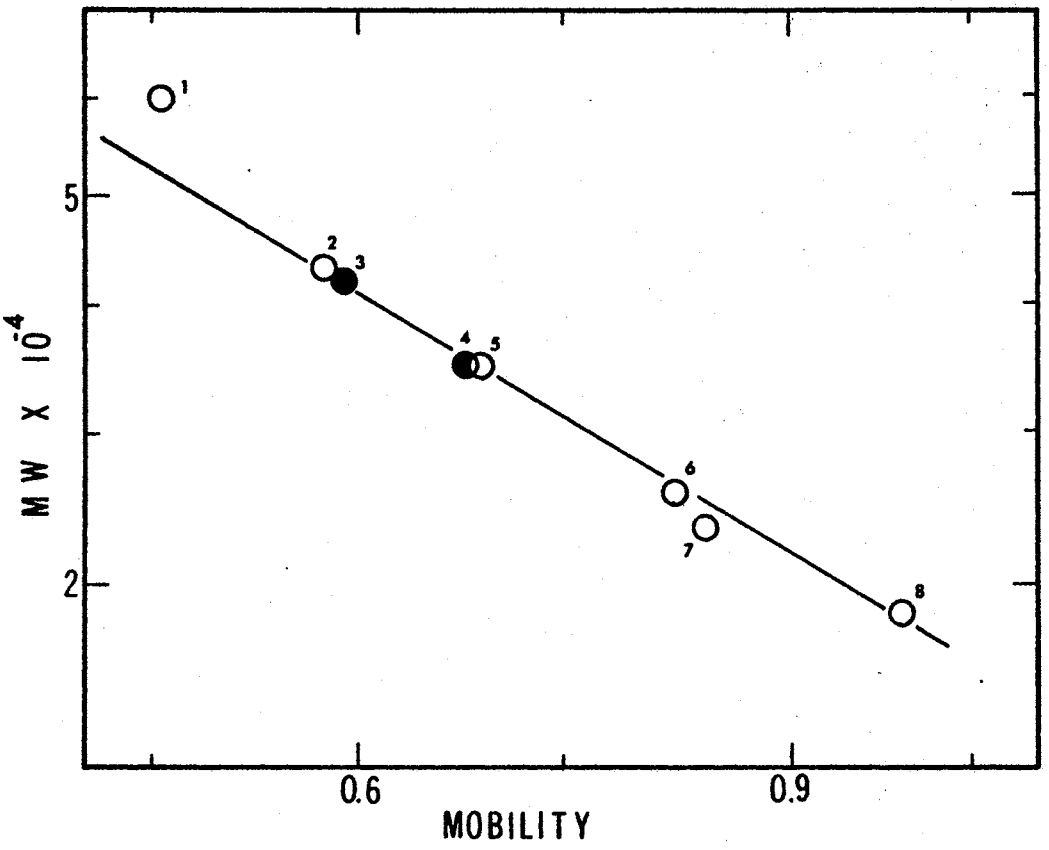

MW X 10^{-4}
5
2
0.6
0.9
MOBILITY
1
2
3
4
5
6
7
8

Figure 17. Molecular Weight Determination by Migration Rate in 6 and 9% Gels

The molecular weight of the riboflavin and thiamine binding proteins was determined by the method of Davis (67). The ratio of the distance known proteins migrated in 6 and 9% gels were graphed vs their molecular weights. The 6/9 ratio of the binding proteins was placed on the experimental line and their molecular weights estimated. The identity of the proteins and respective molecular weights are: 1, bovine serum albumin (66,500); 2, ovalbumin (45,000); 3, riboflavin binding protein (47,000); 4, thiamine binding protein (43,000) and 5, β-lactoglobulin (35,000).

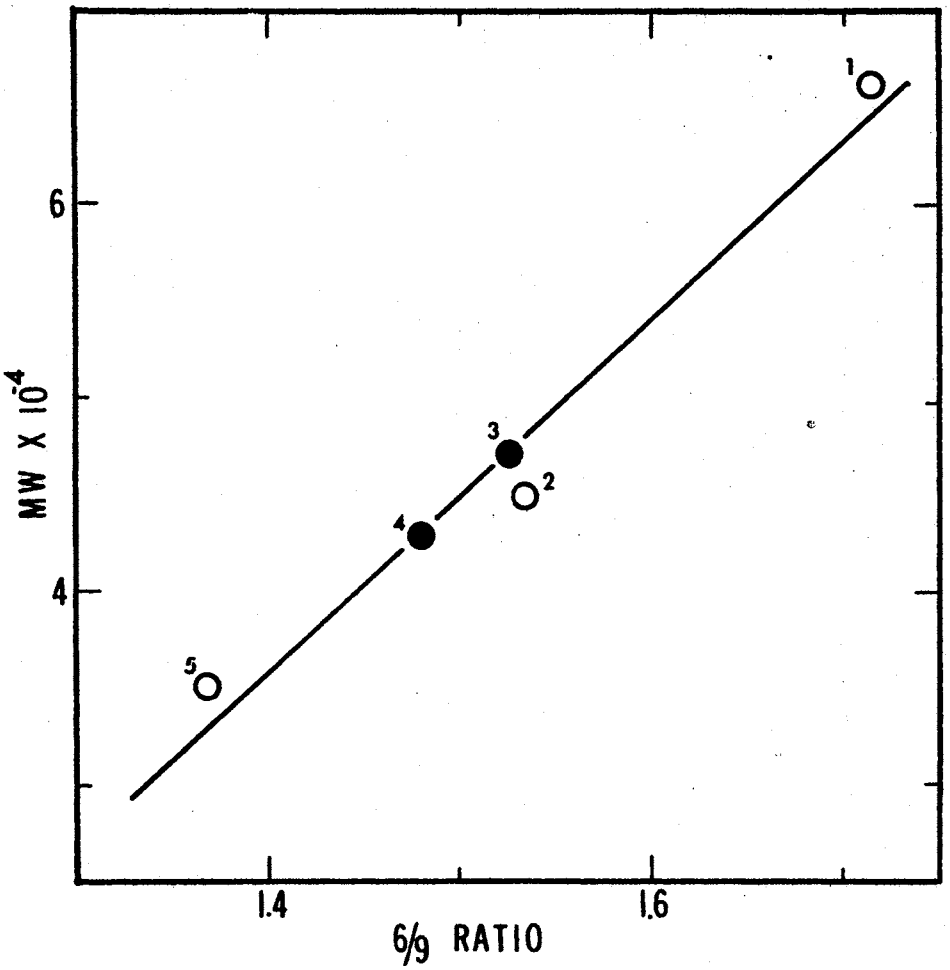

MW X 10^{-4}
6
4
1.4
1.6
6/9 RATIO
1
2
3
4
5

Figure 18. Gel Chromatography Molecular Weight Determination

The exclusion volume from a Bio-gel P-150 column (1.5 x 60 cm) for several known proteins was plotted against their molecular weight. The exclusion volumes for the riboflavin and thiamine binding proteins were placed on the line and their molecular weights determined. The known proteins were detected by the absorbance at 280 nm and the binding proteins by the Paulus method of membrane filtration in the presence of 1 μM substrate. The identity of the proteins and their molecular weights are: 1, bovine serum albumin (66,500); 2, ovalbumin (45,000); 3, riboflavin binding protein (44,000); 4, thiamine binding protein (41,000); 5, β-lactoglobulin (35,000); 6, α-chymotrypsinogen (23,500) and 7, lysozyme (13,900).

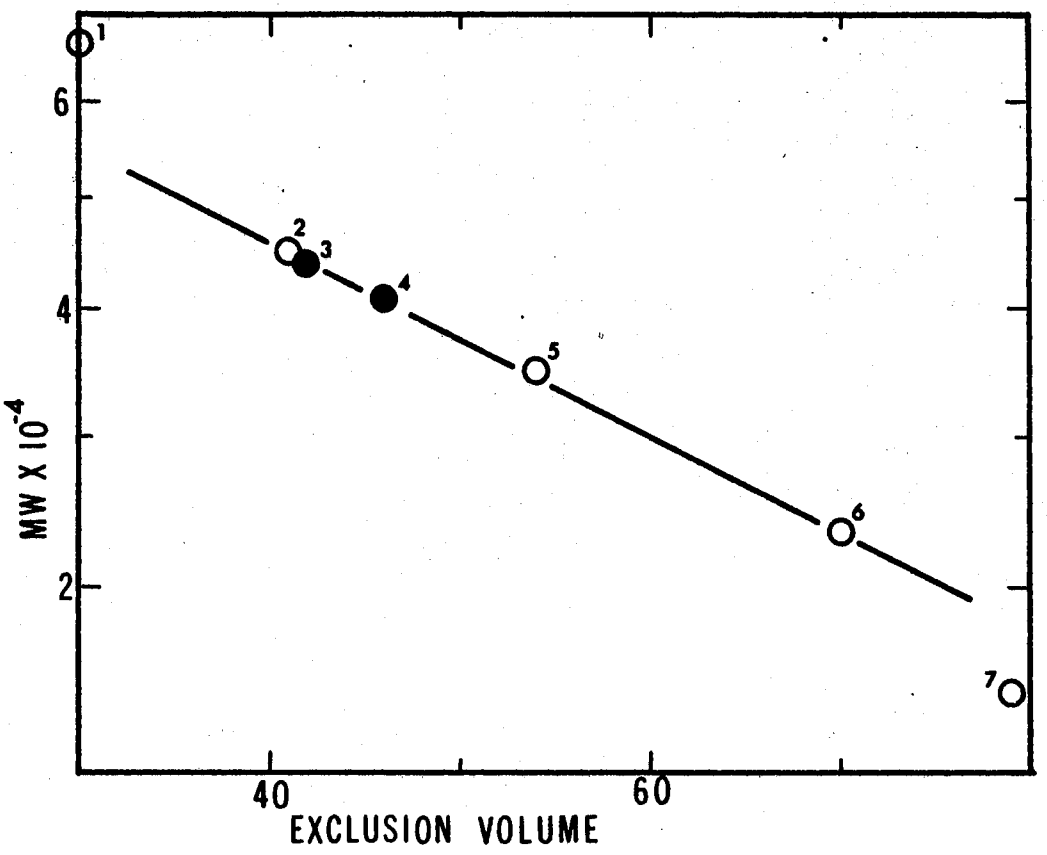

MW X 10^{-4}
6
4
2
40
60
EXCLUSION VOLUME
1
2
3
4
5
6
7

TABLE IX

EFFECT OF THIAMINE BINDING ON THE APPARENT MOLECULAR WEIGHT

0.5 mM Thiamine	Migration Distance (mm) 6%	9%	Ratio of 6/9 Distances
Absent	265	166	1.60
Present	237	155	1.53

Equal amounts of thiamine binding protein in the presence or absence of 0.5 mM thiamine were placed on 6 and 9% 10 cm polyacrylamide gels. After electrophoresis the distance each band traveled from the top of the gel was measured. The ratios of 6 and 9% migration distances are listed for both conditions.

Amino Acid Analysis

An amino acid analysis of the thiamine binding protein yielded the results shown in Table X. The data for the isoleucine (69) and sulfate (37) binding proteins are shown for comparative purposes. Based on 0.458 mg of protein (0.0135 μmoles), the protein has 189 amino acids and a calculated molecular weight of 29,200 (exclusive of tryptophan). The thiamine binding protein is typical of other binding proteins in that it is low in sulfur containing amino acids and histidine. The only major difference is that it is unusually low in isoleucine.

Reconstitution of the Transport System

Purified thiamine binding protein was incubated ten minutes with normal and shocked cells before addition of thiamine. After an additional ten minutes samples were taken and the uptake determined. The results are shown in Table XI. No reconstitution of transport was apparent under these conditions.

TABLE X

AMINO ACID COMPOSITION OF BINDING PROTEINS

Amino Acid	Number of Amino Acids/Mole Binding Protein		
	Thiamine	Isoleucine	Sulfate
Asp	24	38	31
Thr	12	15	11
Ser	11	15	10
Glu	25	33	21
Pro	11	9	9
Gly	13	30	17
Ala	23	38	24
Val	16	21	19
Met	2	4	0
Ileu	3	19	16
Leu	20	22	16
Tyr	8	9	8
Phe	9	9	Not Given
Lys	15	26	20
His	1	4	3
Arg	5	6	10
Cys	1	1	(0.2)
Trp	Not Determined	12	5
NH_3	31	Not Given	Not Given

Thiamine binding protein (0.458 mg, 0.0135 μmoles at MW 34,000) was hydrolyzed *in vacuo* in the presence of 6 N HCl for 22 hours at 60°. After removal of the liquid on a rotary evaporator, the residue was subjected to an amino acid analysis. The number of amino acids per molecule of thiamine binding protein are shown assuming 0.0135 μmoles was placed on the column. The composition of the isoleucine (69) and sulfate (37) binding proteins are shown for comparative purposes.

TABLE XI

RECONSTITUTION OF BINDING PROTEINS TO OSMOTICALLY SHOCKED CELLS

Cell type	Thiamine Uptake	
	pmoles Bound	% of Control
Normal	56	100
Normal + Binding Protein	58	105
Shocked	17	30
Shocked + Binding Protein	17	30

The cell preparation and uptake procedure are as listed in the methods section, except that the cells were not subjected to chloramphenicol. The cells were allowed to incubate with the thiamine binding protein for 10 minutes at 37° before the labeled vitamin was added to 1 μM. After an additional 10 minutes the uptake was stopped and the entire cell contents measured for uptake of label as outlined in the methods section. The pmoles taken up per 1.2×10^9 cells are shown for each condition.

CHAPTER VI

RIBOFLAVIN BINDING PROTEIN

Purification

A riboflavin binding protein was purified from the same ammonium sulfate fraction as yielded the thiamine binding protein. Although other ammonium sulfate fractions had specific activities more than two times higher (Table IV), these fractions did not yield a separable binding protein on DEAE cellulose chromatography. DEAE chromatography of the 70-90% saturated ammonium sulfate fraction yielded only one peak of significant riboflavin binding material (Figure 10). Gel filtration of the resulting riboflavin binding material indicates that the protein is nearly pure (Figure 19). In addition a quantitative determination by disc gel electrophoresis indicates that 84% of the protein migrated in one band after separation on the DEAE column (Figure 11). The purification scheme is shown in Table XII.

pH Optimum for Binding

The optimum pH for binding was determined exactly as for the thiamine binding protein utilizing a citrate-phosphate-tris buffer and Paulus cell filtration for the binding assay. A plot of binding activity vs pH is shown in

Figure 19. Gel Filtration of the Riboflavin Binding Protein

One ml of riboflavin binding protein after DEAE cellulose chromatography was passed through a Bio-gel P-150 column (1.3 x 60 cm) and the one ml fractions assayed for riboflavin binding by the Paulus method of membrane filtration at 1 μM riboflavin. The pmoles bound/0.1 ml (●-●) and A_{280} (O-O) vs ml are shown in the figure.

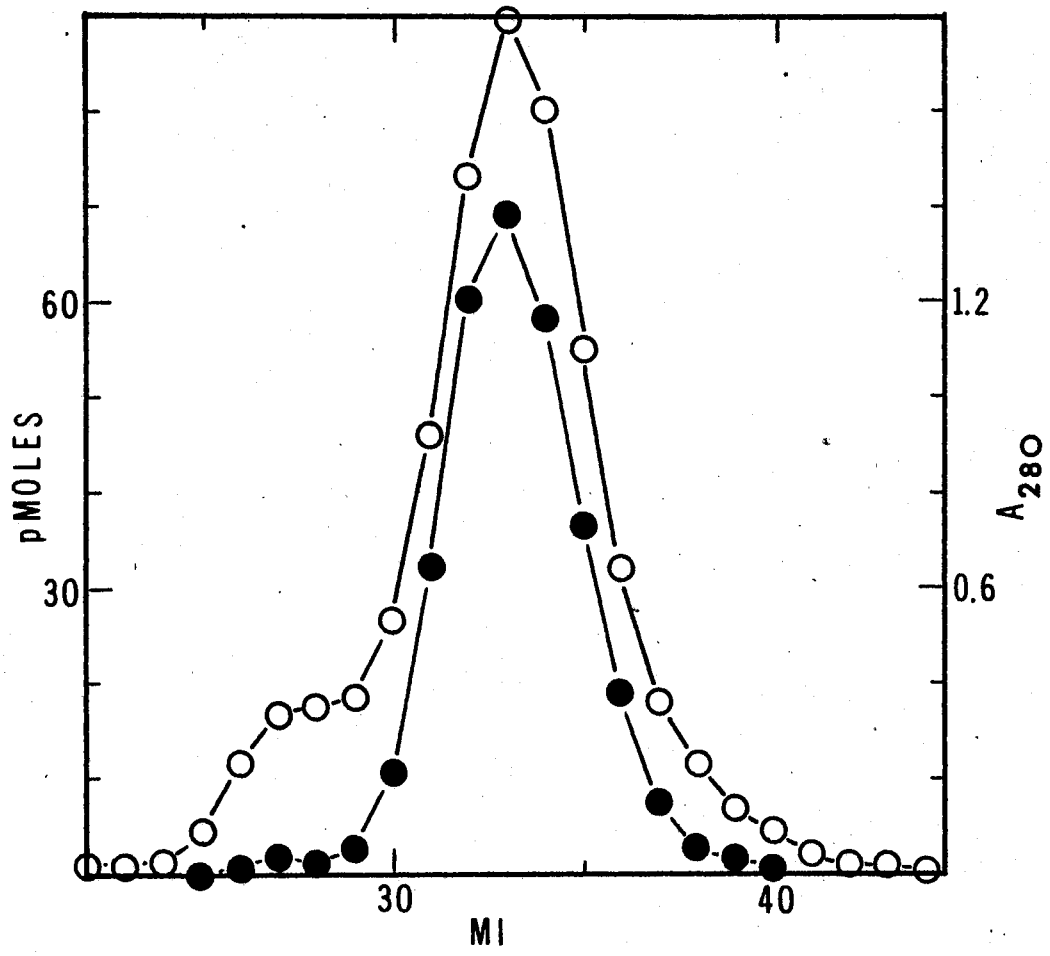

60
30
pMOLES
1.2
0.6
A_{280}
30
40
MI

TABLE XII

PURIFICATION OF THE RIBOFLAVIN BINDING PROTEIN

Fraction	Volume (ml)	Protein (mg/ml)	Total Activity (pmoles x 10^{-3})	Specific Activity (pmoles/mg)	Purification
Crude Shock Fluid	250	4.0	6.5	6.5	1
$(NH_4)_2SO_4$ (0.7-0.9)	29	13	1.8	4.8	0.7
DEAE	41	0.8	9.0	273	42

The riboflavin binding activity was followed during its purification by Paulus cell assay at 1 μM riboflavin. All steps in the purification were carried out at 4° as described in the methods section.

Figure 13. The optimum is near pH 6 but is not as sharp as for the thiamine binding protein.

Determination of the Binding Constant

The determination of the binding constant was performed by equilibrium dialysis. The plot of riboflavin concentration vs bound riboflavin at a constant protein concentration is shown in Figure 20. The binding is much weaker than found for the thiamine binding protein and isestimated to have a K_D in the vicinity of 100 μM. The assay was not carried out to saturation due to the spurious nature of the data and the insolubility of riboflavin.

Inhibition by Analogs

The possibility of inhibition of riboflavin binding by flavin adenine dinucleotide, flavin mononucleotide or folic acid was tested. Riboflavin binding measured by Paulus cell filtration was unaffected by any of these compounds at concentrations up to 100 μM (Table XIII).

Determination of Molecular Weight

The molecular weight was determined as for the thiamine binding protein and the experimental results are shown in Figure 16. The molecualr weight is approximately 42,000 and is comparable to less definite determinations by other methods (Figures 17 and 18).

Figure 20. Riboflavin Saturation of Riboflavin Binding Protein

Binding of riboflavin was measured by equilibrium dialysis at concentrations up to 80 μM. The data from two similar experiments (●) (○) are shown. Two theoretical lines were calculated using a maximum binding value of 370 pmoles and dissociation constants of 80 (line A) and 125 (line B) μM.

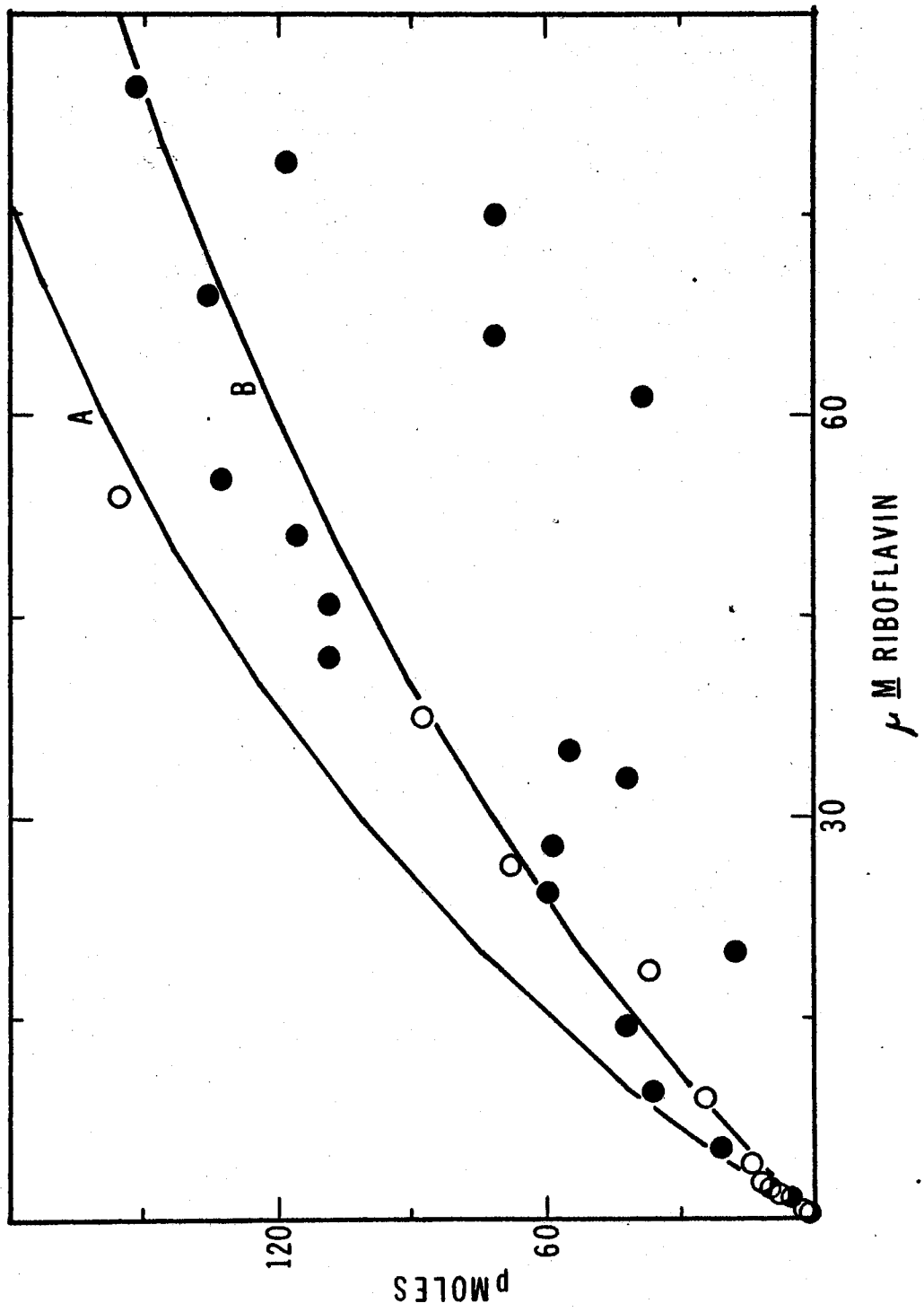

A
B
120
60
pMOLES
30
60
μM RIBOFLAVIN

TABLE XIII

EFFECT OF POSSIBLE INHIBITORS ON RIBOFLAVIN BINDING

Inhibitor	Concentration (μM)	^{14}C-Riboflavin Bound to Riboflavin Binding Protein (cpm)
Flavin Adenine Dinucleotide	0	19800
	100	19000
Flavin Mononucleotide	0	23100
	100	25800
Folic Acid	0	860
	100	850

The riboflavin binding protein was assayed for binding activity via Paulus cell filtration in the presence and absence of three possible inhibitors. Riboflavin concentration was 5 μM for the analog experiment and 1 μM for the folic acid experiment.

Reconstitution of the Transport System

Purified riboflavin binding protein was incubated ten minutes with normal and shocked cells before addition of riboflavin. After an additional ten minutes samples were taken and the uptake determined. The results are shown in Table XIV. No reconstitution of transport was apparent under these conditions.

TABLE XIV

RECONSTITUTION OF RIBOFLAVIN BINDING PROTEIN TO OSMOTICALLY SHOCKED CELLS

Cell type	Riboflavin Uptake	
	pmoles Bound	% of Control
Normal	4.6	100
Normal + Binding Protein	4.2	91
Shocked	5.9	128
Shocked + Binding Protein	4.2	91

The cell preparation and uptake procedure are as listed in the methods section, except that the cells were not subjected to chloramphenicol. The cells were allowed to incubate with the riboflavin binding protein for 10 minutes at 37° before the labeled vitamin was added to 1 μM. After an additional 10 minutes the uptake was stopped and the entire cell contents measured for uptake of label as outlined in the methods section. The pmoles taken up per 1.2×10^9 cells are shown for each condition.

CHAPTER VII

LIPOIC ACID UPTAKE IN WHOLE CELLS

Uptake Saturation in Whole Cells

Soluble and protein-bound lipoic acid were measured in intact E. coli at varying external concentrations. The saturation curves for both lipoic acid binding to protein and accumulation in the free cell pool are shown in Figure 21. The protein incorporating system is one-half saturated at a lipoic acid concentration of about 300 μM. The apparent lipoic acid transport exhibits saturation at 100 μM and one-half maximal uptake at 50 μM. This is followed (above a concentration of 300 μM) by a region which changes linearly with concentration at least to 1 mM. This line, possibly representing passive diffusion of lipoic acid, extrapolates through zero.

Time Course of Uptake

Oh (47) has shown the time course of lipoic acid uptake for the entire cell in E. coli. This experiment was repeated with a modified procedure. The free pool and protein-bound fraction were separated before measurement and the measurements taken of actively growing cells for nearly three generations. The results are shown in Figure 22. As

Figure 21. Lipoic Acid Saturation of Intact E. coli

A mid-log phase culture (A_{600} = 0.9) of E. coli was exposed to different concentrations of ^{35}S-lipoic acid for 10 minutes at 37°. The uptake was stopped and the free pool and protein-bound lipoic acid measured as described in the methods section. Duplicate samples were taken and averaged for each lipoic acid concentration. The nmoles of free lipoic acid (O-O) and protein-bound lipoic acid (●-●) from 0.5 ml of cells are shown for each external lipoic acid concentration.

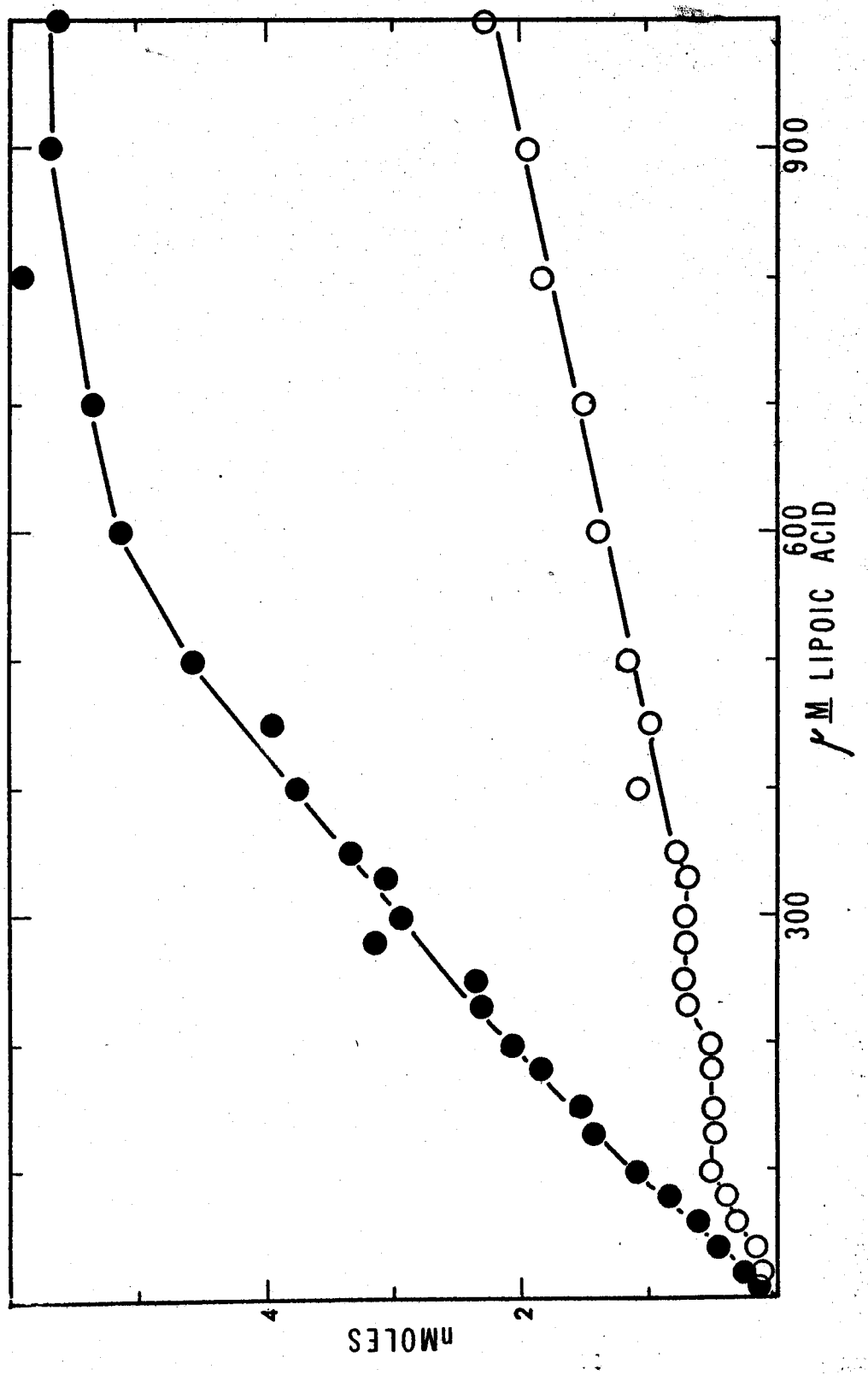
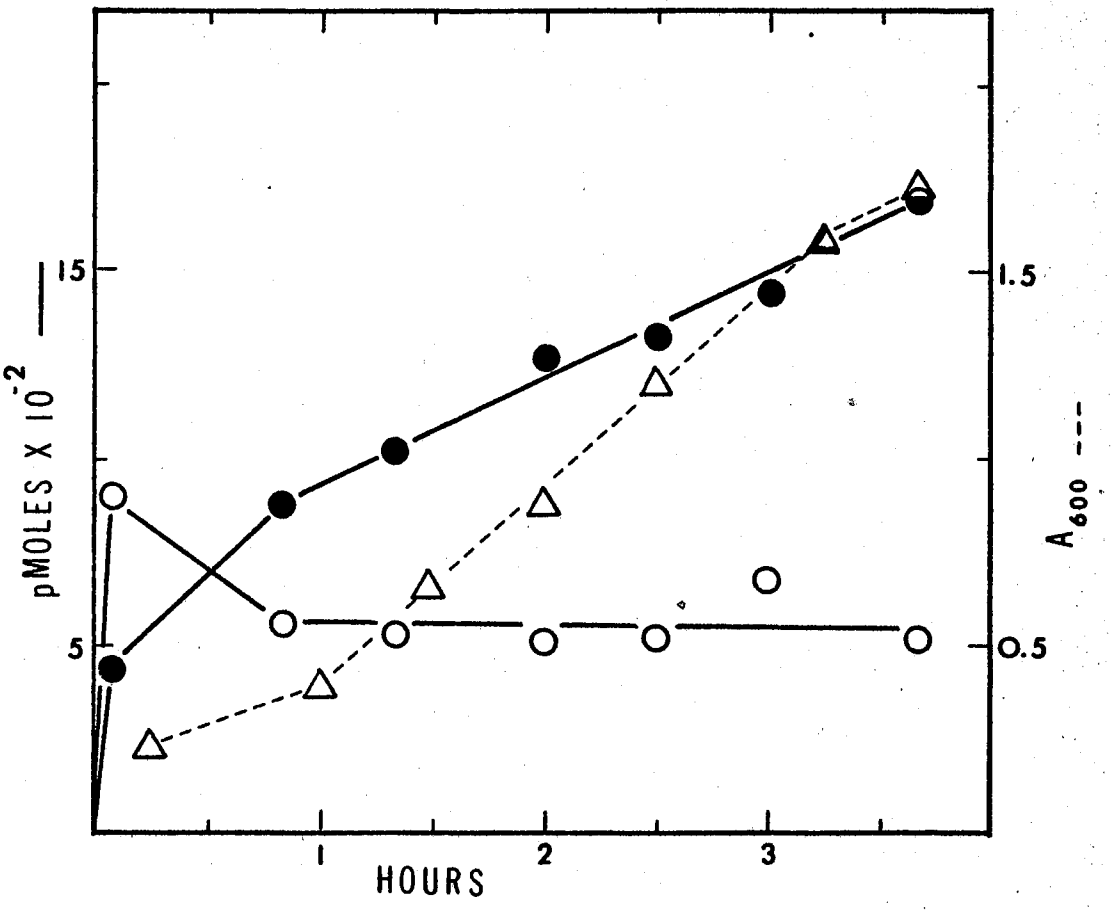

nMOLES
2
4
300
600
900
μM LIPOIC ACID

Figure 22. Time Course of Lipoic Acid Uptake

Normal cells were prepared as for lipoic acid exchange experiments. The cells were suspended at an A_{600} of 0.2 in fresh M-9 at 37° with the addition of ^{35}S-lipoic acid to 10 μM. At the time indicated 4 ml aliquots were pipetted in duplicate into chilled 4° centrifuge tubes and centrifuged at 10,000 g for 10 minutes at 4°. The normal procedure for measurement of free pool and protein-bound lipoic acid was then followed. The pmoles of lipoic acid in 4 ml of cells (free pool, O-O; bound, ●-●) and the absorbance at 600 nm (Δ-Δ) are shown as a function of time.


pMOLES X 10^{-2}
15
5
1
2
3
HOURS
1.5
0.5
A_{600} ---

expected, the free pool exhibits the typical overshoot phenomenon and the incorporation into protein is slower than uptake. Although the protein-bound fraction increases with cell density, the free pool remains fairly constant.

Effect of Sulfhydryl Compounds

Chloramphenicol treated mid-log phase cells were incubated with various concentrations of the sulfhydryl compounds before addition of 10 μM lipoic acid. Although the procedure used did not separate bound and free lipoic acid, in the presence of chloramphenicol very little incorporation occurs (see following section). The data are shown in Table XV. Of the compounds tested, cysteine and cystine are the only potent inhibitors of the uptake process. Mercaptoethanol stimulates uptake approximately 20%. Although this is not a large change, it was observed consistently in other similar experiments. Data with glutathione were less reproducible, causing up to 50% inhibition at 1mM in other similar experiments.

Effect of Inhibitors and Lipoic Acid Analogs

Mid-log phase cells were incubated with the various compounds for 10 minutes before addition of lipoic acid to 10 μM. After an additional 10 minutes an aliquot of cells was treated as for the saturation experiment in this chapter. This yielded the effect of the inhibitor on both incorporation into protein and accumulation of free lipoic acid

TABLE XV

EFFECT OF SULFHYDRYL COMPOUNDS ON LIPOIC ACID UPTAKE

Addition Compound	Conc. (mM)	pmoles Lipoic Acid	% of Control
None (control)	--	128	100
Mercaptoethanol	1.0	154	120
Mercaptoethanol	10.0	152	119
Cysteine	1.0	15	12
Cystine	0.01	137	107
Cystine	0.1	25	19
Cystine	1.0	11	9
Coenzyme A (red.)	0.01	123	96
Coenzyme A (red.)	1.0	101	79
Red. Glutathione	0.01	124	97
Red. Glutathione	1.0	127	99
Oxid. Glutathione	0.01	114	89
Oxid. Glutathione	1.0	108	84
Dithiothreitol	0.01	104	81
Dithiothreitol	0.1	121	95
Dithiothreitol	1.0	137	107

The sulfhydryl compounds were added to mid-log phase cells (prepared for uptake studies in the presence of chloramphenicol) 10 minutes before the addition of ^{35}S-lipoic acid to 10 μM. After an additional 10 minutes the uptake was stopped in slushy M-9 and the cells washed as described in the methods section. The entire cell pellet was then suspended in 1 ml of water transferred to scintillation vials and counted. The pmoles taken up by 0.5 ml of cells are shown for each condition and are the average of triplicate samples.

(Table XVI). A similar experiment was performed with lipoic acid analogs and fatty acids (Table XVII). Unlabeled lipoic acid (4mM) caused a 60% reduction of the soluble lipoic acid pool and a 30% reduction of the label in protein-bound form. Chloramphenicol decreased the incorporation into protein by 93%. The accumulation of free lipoic acid was unaffected. Sodium azide (1mM) had no effect on lipoic acid uptake. Potassium cyanide (1mM) affected both lipoic acid measurements about equally (35%). At 0.1 mM dinitrophenol, free accumulation was inhibited 3.5 times more than incorporation.

Since cysteine and cystine were good inhibitors in previous experiments under conditions where lipoic acid incorporation into protein was inhibited, its effect on the separated systems was investigated here. Cystine was found to inhibit both free cellular accumulation (68%) and incorporation (92%). Ammonium sulfide inhibited incorporation to the same extent as cystine but did not affect accumulation of free lipoic acid.

In the second experiment unlabeled lipoic acid (1mM) inhibits both phenomenon (about 20% each). The C-7 and C-9 analogs of lipoic acid inhibit accumulation in the free pool. The C-9 compound also inhibits incorporation of lipoic acid into protein. Oleic acid is the best inhibitor of incorporation in this series (42% inhibition) but does not effect free pool accumulation. No inhibition was observed with octanoate, hexanoate or acetic acids. Similar

TABLE XVI

EFFECT OF INHIBITORS ON LIPOIC ACID UPTAKE

Additions	Concentration (mM)	% of Control	
		Free Pool	Protein-Bound
Potassium Octanoate	0.7	86	97
Cystine	1.0	32	8
Dinitrophenol	0.1	65	90
KCN	1.0	60	67
NaN_3	1.0	99	103
Chloramphenicol	400 (μg/ml)	99	7
Unlabeled Lipoic Acid	4.0	38	67
$(NH_4)_2S$	1.0	97	7

The uptake of 10 μM ^{35}S-lipoic acid by mid-log phase cells was performed exactly as described in the previous table except in the general absence of chloramphenicol. The inhibitors were incubated with the cells 10 minutes before addition of labeled lipoic acid to 10 μM. The uptake was stopped after 10 minutes on mushy ice (M-9) and washed as before. However, the washed cells were measured for both free pool and protein-bound lipoic acid as described in the methods section. All values are the average of two experiments each with triplicate samples except the ammonium sulfide data which was collected only once in triplicate. The per cent of uptake relative to the control (no additions) is shown for each condition.

TABLE XVII

EFFECT OF LIPOIC ACID ANALOGS ON UPTAKE

Additions	% of Control	
	Free Pool	Protein-Bound
None	100	100
Unlabeled Lipoic Acid	80	83
Oleic Acid	102	58
Octanoic Acid	106	101
Hexanoic Acid	105	96
Acetic Acid	116	100
C-7 Lipoic Acid	71	90
C-9 Lipoic Acid	78	75
8-Methyl Lipoic Acid	105	91

The experiment was performed exactly as stated in the previous table except that the compounds were added only three minutes before the addition of 10 μM ^{35}S-lipoic acid. All compounds were dissolved in 95% ethanol and were added to 1 mM. The control contained no additions except an equal volume of 95% ethanol. Although the data shown here were collected 10 minutes after the addition of the labeled lipoic acid, samplings 3 minutes after gave similar results. Each value shown is the average of triplicate samples.

data were obtained whether sampling occurred three minutes or ten minutes after addition of the labeled lipoic acid.

CHAPTER VIII

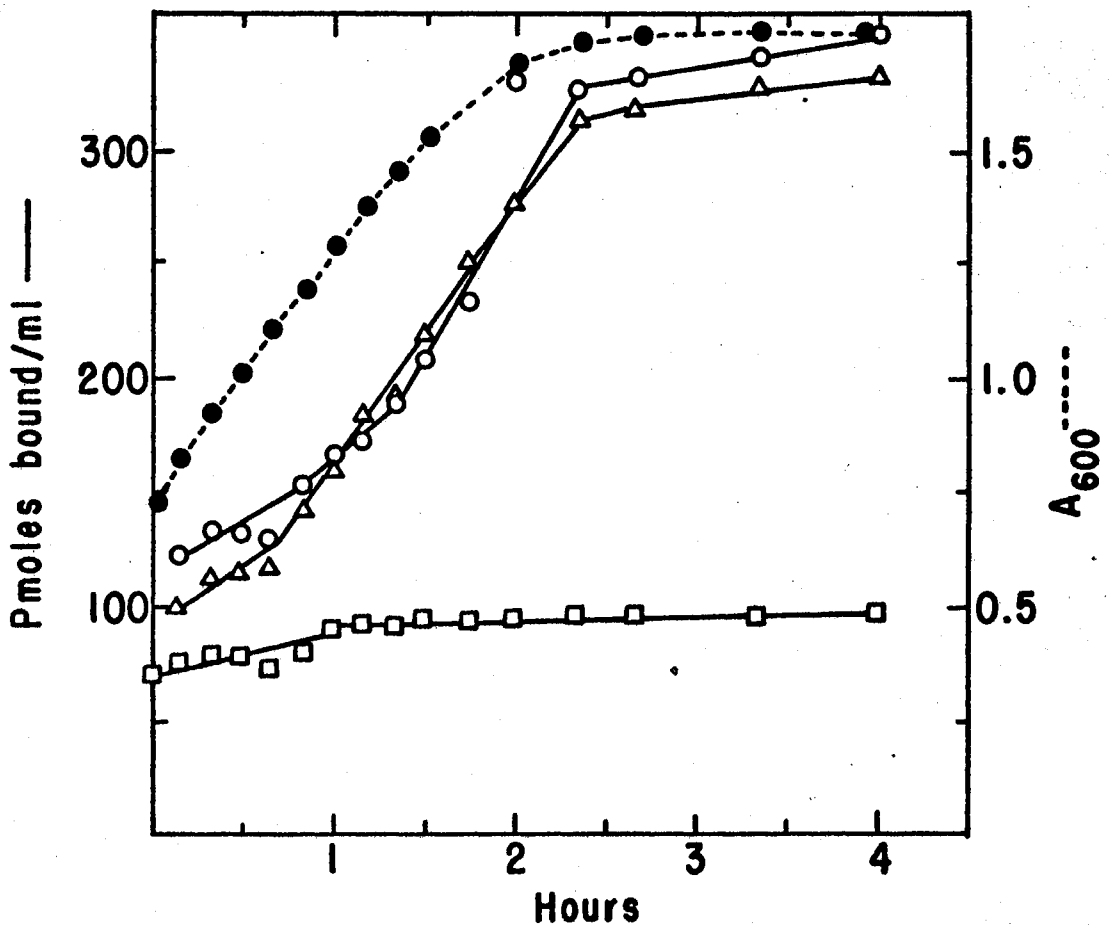
EXCHANGE OF COVALENTLY BOUND LIPOIC ACID

Vagelos' work on pantothenate turnover and his suggestion that lipoic acid may turn over in the α-keto acid dehydrogenase complexes (59) initiated the investigation of protein-bound lipoic acid exchange in E. coli. Mid-log phase cells were grown three hours in the presence of 2 μM labeled lipoic acid. After preparation as described in the methods section, the cells were suspended at 37° in fresh medium and an aliquot taken before the addition of unlabeled lipoic acid to a concentration of 50 μM. Growth was followed by measuring A_{600} and further aliquots were taken at the times indicated in Figure 23. As controls, two parallel cultures (one grown in normal medium, one in 50 μM unlabeled lipoic acid) were similarly treated and suspended but with the addition to 2 μM of labeled lipoic acid. The uptake was stopped by pipetting 1 ml of cells into 2 ml of 95% ethanol. One wash of this precipitate in 70% ethanol yielded a protein-bound lipoic acid fraction.

Both control cultures incorporated lipoic acid roughly parallel to their growth. If exchange occurs, one might expect a more rapid labeling of the cells grown in lipoic acid. However, no significant difference could be distin-

Figure 23. Protein-Bound Lipoic Acid Exchange at Low Lipoic Acid Concentrations

Mid-log phase cells were prepared for exchange as explained in the methods section. Cells were grown in 2 μM ^{35}S-lipoic acid (□-□), 50 μM unlabeled lipoic acid (△-△) or normal medium (○-○). After centrifugation, the cells were suspended in fresh M-9 at 37° at zero time. Unlabeled lipoic acid (50 μM) was added to the labeled cells to measure the exchange rate (□-□) and 2 μM ^{35}S-lipoic acid was added to the control cultures (○-○, △-△). The pmoles of labeled lipoic acid bound to protein were measured as described for each 1 ml sample. All samples were taken in duplicate and averaged. The growth was followed by measuring the absorbance of the cell suspension at 600 nm (●-●).


Pmoles bound/ml
300
200
100
1.5
1.0
0.5
A_{600}
1
2
3
4
Hours

guished between the cells grown in the presence of 50 μM lipoic acid and those grown in normal medium. The cells grown in labeled lipoic acid did not lose any label upon prolonged growth in the presence of unlabeled lipoic acid. In fact a small rise in the amount of bound lipoic acid often occurred. This may be a small amount of labeled pool material being incorporated. Since the data shown in Figure 23 were obtained with lipoic acid concentrations below saturation, a similar experiment was performed using 0.5 mM lipoic acid for both labeled and unlabeled conditions (Figure 24). Similar results were obtained except that far more substrate was incorporated.

Since lipoic acid is not easily exchanged between the external medium and the free endogenous pool, any exchange between protein-bound lipoic acid and the labeled pool could be partially masked. To alleviate this possibility similar experiments were performed on osmotically shocked cells. Osmotic shock should release some of the free cell pool. These data are shown in Figure 25. About 20% of the free endogenous pool is lost upon osmotic shock. Although the shocked cells showed a lag in their growth, upon recovery they were able to take up and incorporate lipoic acid as well as the normal cells (Figure 26). The results show no dilution of the protein-bound label through three generations. Similar results were obtained in either the presence or absence of excess unlabeled compound. Osmotic

Figure 24. Protein-Bound Lipoic Acid Exchange at Saturating Levels of Lipoic Acid

The procedure used in this experiment was exactly the same as for Figure 22 except that 0.5 m$\underline{M}$ lipoic acid was used for all lipoic acid steps. The control cells grown in normal medium or with the addition of 0.5 m$\underline{M}$ unlabeled lipoic acid gave similar results upon addition of ^{35}S-lipoic acid and were averaged. The pmoles of protein-bound lipoic acid per ml of labeled cells upon addition of 0.5 m$\underline{M}$ unlabeled lipoic acid (O-O) and control cells (●-●) are plotted against the time of sampling. The growth of the cells was followed by absorbance at 600 nm (Δ-Δ).

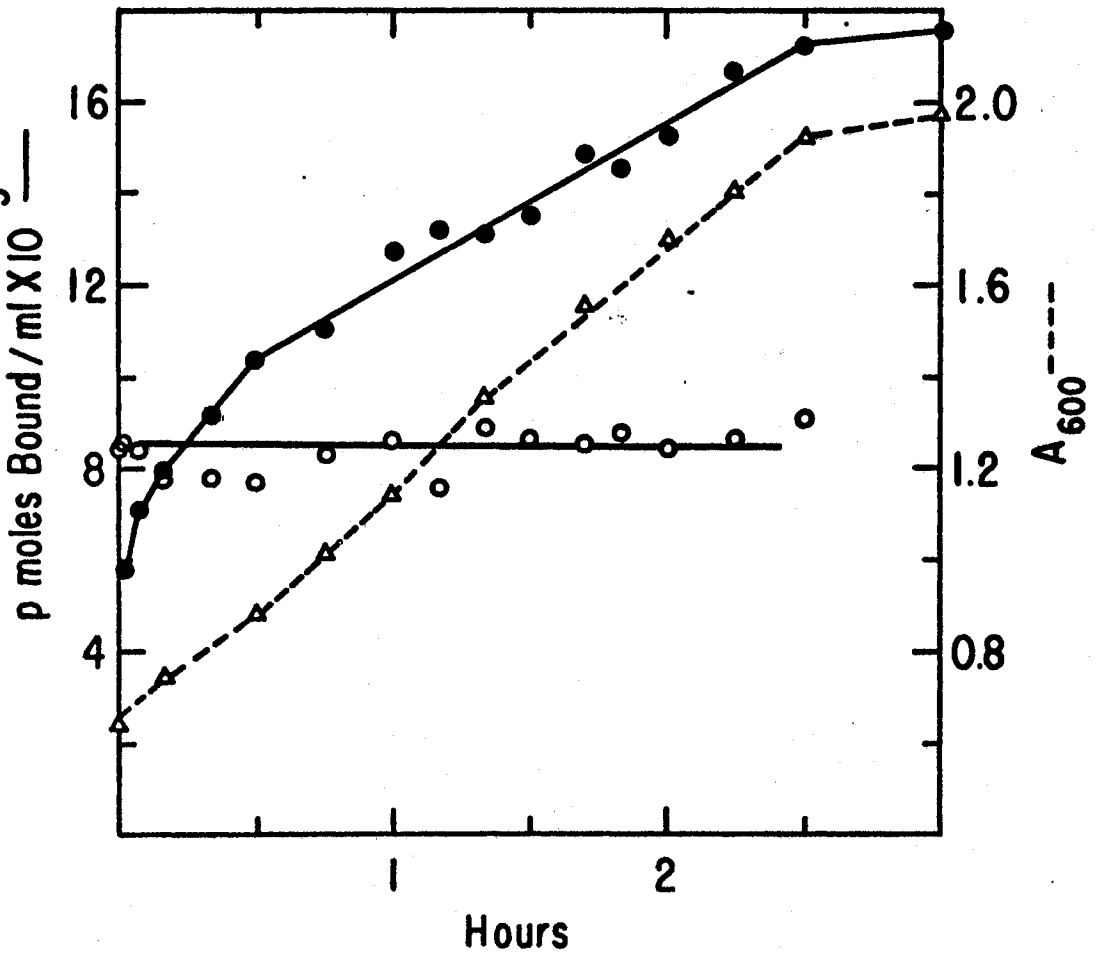

p moles Bound/ml X10^{-3}
16
12
8
4
1
2
Hours
2.0
1.6
1.2
0.8
A_{600}

Figure 25. Protein-Bound Lipoic Acid Exchange in Normal and Shocked Cells

Mid-log phase cells grown in the presence of 2 μM ^{35}S-lipoic acid were prepared for the exchange experiment and half of these were subjected to osmotic shock as described in the methods section. A 4 ml aliquot of cells was taken at zero time while the cells were suspended in fresh M-9 at 0°. The cells were then placed in 37° bath and either no addition made or unlabeled lipoic acid added to 10 μM. Four ml aliquots were removed at each time and placed in ice cold centrifuge tubes. The normal procedure for measurement of both free pool and protein-bound lipoic acid was then followed. The pmoles of protein-bound lipoic acid in 4 ml of cells are plotted against the sampling time for the normal (O-O) and shocked (●-●) cells. The presence or absence of unlabeled lipoic acid gave similar results and the date were averaged to give one line. Each point is the average of 4 experimental samples. The growth of the cells was followed by measuring the absorbance of the normal (□-□) and shocked (■-■) cell suspensions at 600 nm.

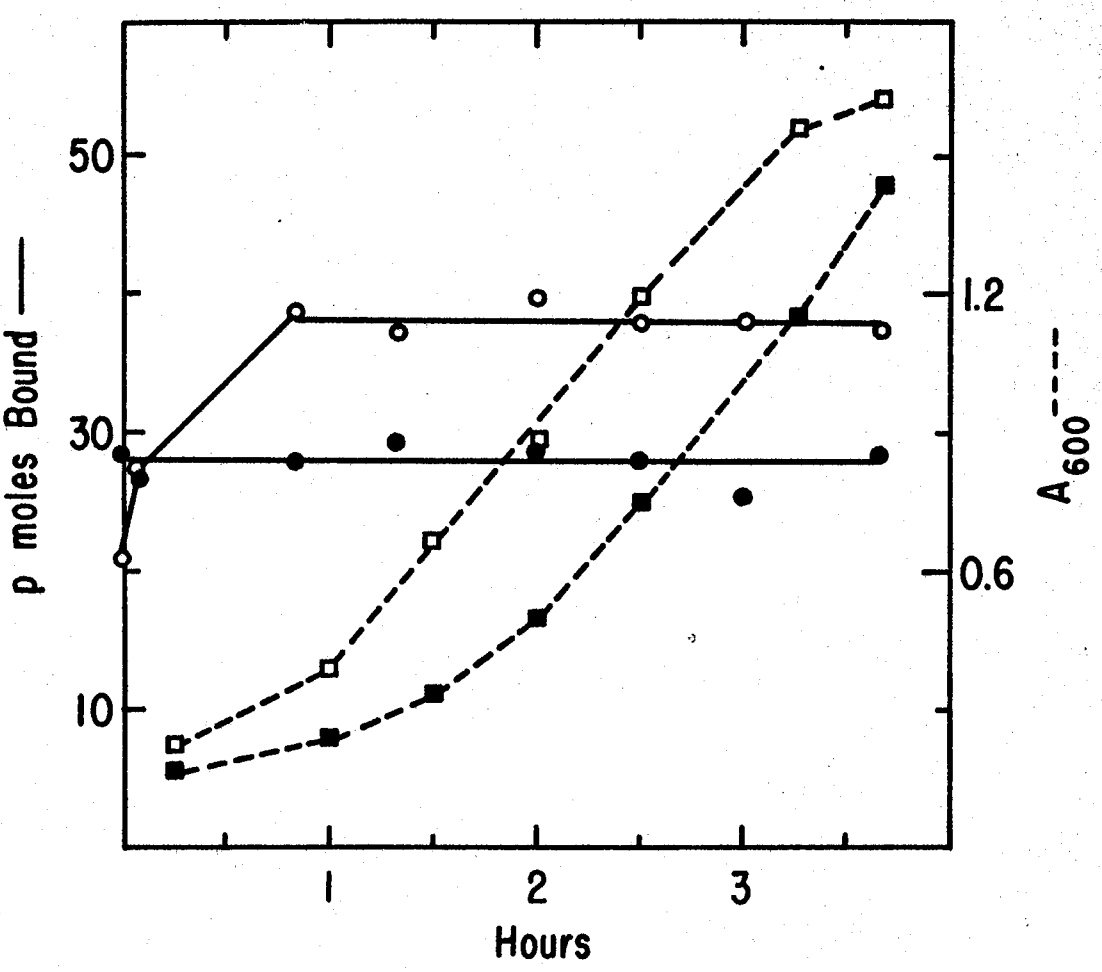
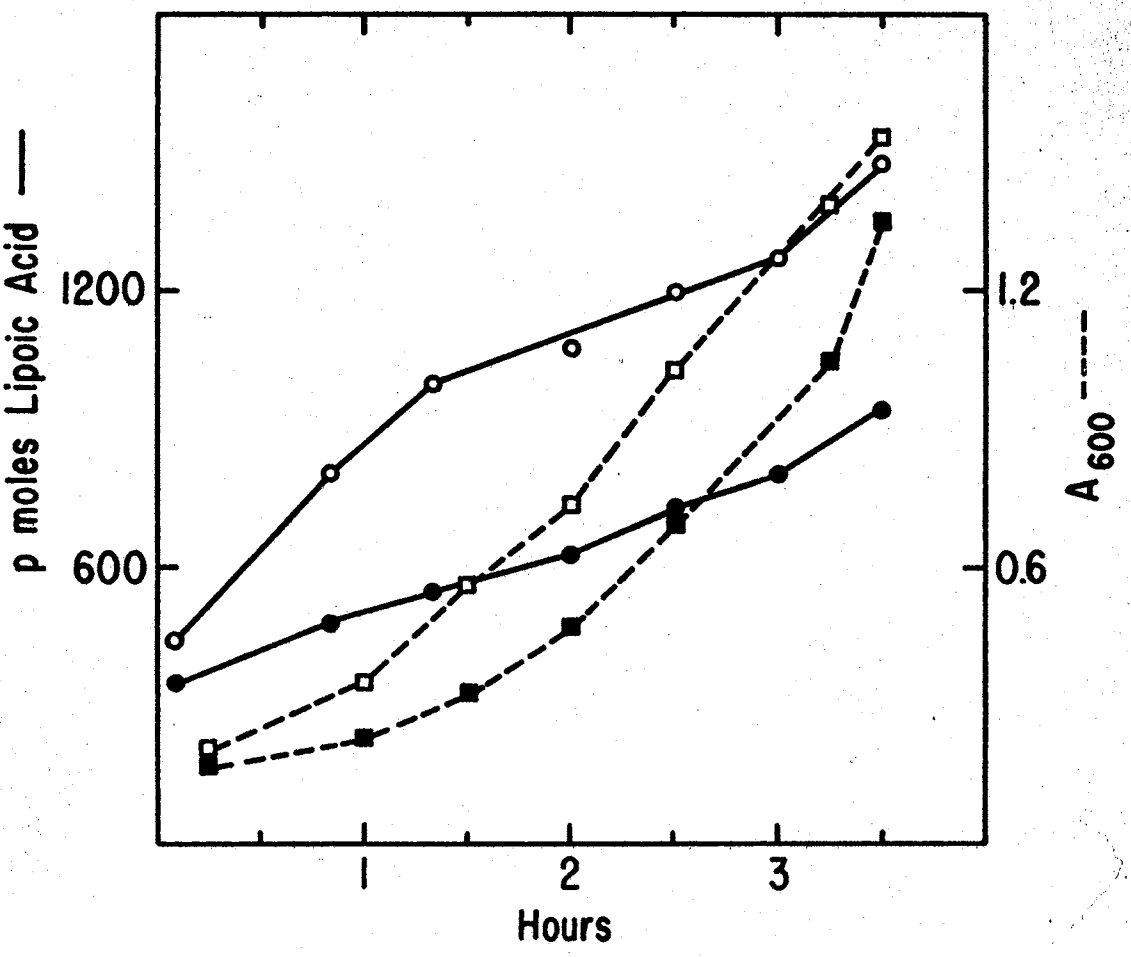

50
30
10
p moles Bound
1.2
0.6
A_{600}
1
2
3
Hours

Figure 26. Incorporation of Lipoic Acid into Normal and Shocked Cells

Mid-log phase cells were prepared for the exchange experiment and half of these were subjected to osmotic shock as described in the methods section. The cells were placed in a 37° bath and labeled lipoic acid added to 10 μM. Four ml aliquots were removed at the times indicated and placed in ice cold centrifuge tubes. The normal procedure for measurement of both free pool and protein-bound lipoic acid was then followed. The pmoles of protein-bound lipoic acid in 4 ml of cells are plotted against the sampling time for the normal (O-O) and shocked (●-●) cells. Each point is the average of duplicate samples. The growth of the cells was followed by measuring the absorbance of the normal (□-□) and shocked (■-■) cell suspensions at 600 nm.


p moles Lipoic Acid —
1200
600
1.2
0.6
A_{600} ----
1
2
3
Hours

shock does eliminate the small rise in protein-bound material usually observed.

CHAPTER IX

DISCUSSION

The experiments reported herein constitute the first demonstration of vitamin binding proteins in bacteria. Although the role of binding proteins in transport is unclear (2), evidence continues to support their involvement (25). However, binding proteins are not the sole component in transport. The mechanism by which they function is still unknown. The continued investigation of binding proteins and the search for new binding proteins should help solve this problem. Bradbeer (39) has shown that osmotic shock reduces B_{12} transport but claims that no binding protein is released and that the initial binding site for the B_{12} transport system is firmly bound to the cell membrane. We have sought binding proteins for six vitamins in the osmotic shock fluid from E. coli. Two binding proteins were found, one for thiamine and one for riboflavin. Although the remaining vitamins were bound by crude shock fluid, we were unable to purify their corresponding binding proteins.

Both the effect of osmotic shock on transport and binding to crude shock fluid strongly implicated existence of the thiamine binding protein. However, evidence for a riboflavin binding protein remained obscure until further

purification.

The thiamine binding protein exhibits a rather sharp pH optimum at 9.2. Other binding proteins for which there are pH optimum data in the literature are affected little over a broad pH range: leucine, pH 4-9 (2) and glutamine, pH 3-9 (31). As expected, the bound thiamine is freely exchangeable. The dissociation constant of thiamine (0.05 μM) is lower than most binding proteins. The K_D values reported for other binding proteins range from 0.15 to 12 μM (21-23, 26-36) except for the sulfate binding protein (20) for which the K_D is 0.02 μM.

Neujahr has reported that thiamine pyrophosphate competes with labeled thiamine for uptake in whole cells but is not as good a competitor as unlabeled thiamine (43). Thiamine pyrophosphate also competes with thiamine for binding to the thiamine binding protein. However, the K_I is 0.5 μM which means that the binding protein has only one-tenth the affinity for the pyrophosphate compared to thiamine.

The binding is not affected by magnesium, high phosphate concentrations or pyrophosphate. Similarly mercaptoethanol or the sulfhydryl reagents N-ethylmaleimide and iodoacetic acid have no effect. This suggests that sulfhydryl groups are not involved in binding thiamine. Some inhibition by thymine and cytosine occurred at 100 μM. From the data the approximate K_I for these compounds is 100 μM. Therefore, the binding site for thiamine likely has some affinity for the pyrimidine moeity.

The molecular weight determined by disc gel electrophoresis in SDS, 34,000, is well within the range of those reported previously for other binding proteins, 25,000-42,000 (25,30,36).

Assuming a molecular weight of 34,000 and that one mole of substrate is bound for one mole of protein, the specific activity in nmoles bound/mg is 29.4. Although nearly pure the thiamine binding protein has a specific activity, depending on the preparation, between 11.7 and 18.2. This is roughly one-half what it theoretically should be. Recycling of an 85% pure preparation (specific activity 13.9) through two purification steps gives a 92% pure protein with a specific activity of 11.7. This suggests that inactivation occurs upon purification manipulations.

Disc gel electrophoresis of the thiamine binding protein in the presence of thiamine produces a slower moving protein than is exhibited by the normal protein. This is indicative of either the neutralization of negative charges or an apparent increase in molecular weight. Since dimerization upon binding could explain both the slower moving band in the presence of thiamine and the low specific activity of the purified preparation, this possibility was investigated. The thiamine binding protein was run in the presence and absence of thiamine in both 6 and 9% gels. The ratio of migration distances in 6 and 9% gels should vary greatly if a large change occurs in molecular weight and should be identical if only a charge difference occurs.

From the ratios found and by estimation from a previous similar molecular weight determination, the slower moving band had an apparent molecular weight 2,000 to 4,000 less than the normal binding protein. This is within the error (10%) usually associated with this type of study (67) and a large difference due to dimerization can be ruled out.

The amino acid composition of the thiamine binding protein is similar to that of other binding proteins except that it is unusually low in isoleucine. Since thiamine is a requirement for isoleucine synthesis (three steps from the final product), it is appealing to speculate on an evolutionary explaination for its absence. It would be to the cell's advantage not to have isoleucine in a protein required for the uptake of a compound necessary for isoleucine synthesis. However, this hypothesis is not supported by the fact that valine occurs in relatively normal amounts but requires thiamine in its synthesis in a manner exactly analogous to isoleucine.

The riboflavin binding protein has not been characterized to the same extent as the thiamine binding protein. DEAE chromatography of the 0.7 to 0.9 ammonium sulfate saturated shock fluid yields a riboflavin binding protein well separated from the bulk of the protein. The protein is about 84% pure at that point. The binding of riboflavin was not carried out to saturation due to the insolubility of the compound at concentrations approaching 1 m$\underline{M}$ and the more spurious nature of the results at these higher concentra-

tions. However, the K_D is approximately 100 μM. The high dissociation constant and non-specific binding by riboflavin may be responsible in part for the latent appearance of the riboflavin binding protein in its purification since these assays were performed at 1 μM riboflavin - far below saturation. The K_D is much higher than reported for other binding proteins (0.02-12 μM) (20-23,26-36). Since the dissociation constant is so high, it seemed possible that the true substrate for the protein may be a derivative of riboflavin such as flavin mononucleotide or flavin adenine dinucleotide. Folic acid also shows some similarity to the ring structure of riboflavin and could fit into this possibility. However, these possibilities were ruled out on the basis of a competition experiment, all being poorer substrates than riboflavin.

The pH optimum is more typical of binding proteins, exhibiting a broad bell-shaped curve with a maximum near pH 6. The molecular weight (42,000) is on the high end of the range reported for other binding proteins, 25,000-42,000 (25,30,36).

Kawasaki (40) has reported that the K_m for thiamine uptake in *E. coli* is 0.8 μM and that the thiamine is accumulated as thiamine pyrophosphate. The rate of thiamine uptake was maximal at pH 6.5. Kawasaki (40) proposed that thiamine is transported across the membrane by facilitated diffusion and is subsequently concentrated as thiamine pyrophosphate through the action of membrane-bound thiamine

kinase. In addition, Kawasaki and Esaki (42) have shown that the formation of the thiamine transport system is controlled by repression and derepression.

A limited number of experiments utilizing whole cells were performed to support the binding protein findings. The time course for thiamine uptake is roughly similar to that reported by Kawasaki (40). Results with riboflavin uptake were spurious and poorly reproducible. Similar uptake experiments comparing osmotically shocked and normal cells showed that the rate of thiamine uptake was reduced by about one-half by osmotic shock. On the other hand, riboflavin uptake in shocked cells did not exhibit spurious values. In addition, in static riboflavin uptake assays the shocked cells took up about twice as much label as normal cells. This apparent stimulation may be an artifactual interaction involving modified structures on the cell membrane.

In order to correlate the affinity of riboflavin for the binding protein and the transport K_m of riboflavin, preliminary experiments were performed to characterize a saturation curve for riboflavin uptake. Experiments were conducted utilizing two different methods of stopping the uptake (slushy M-9 and Millipore filtration) in case one of the methods was subject to artifactual errors. However, both methods gave similar results. The saturation was not complete at 90 μM and higher concentrations were not used due to the insolubility of the compound. Although the

apparent high K_m value for uptake correlates with the high K_D for binding, neither seems realistic. Intuitively one would expect transport of the vitamins to be highly specific.

Since thiamine accumulates endogenously as thiamine pyrophosphate (40), the endogenous level of the thiamine derivatives was compared in normal and shocked cells after uptake had proceeded for about 20 minutes. If the thiamine binding protein has any effect on the nature of the endogenous material, the ratio of the thiamine compounds may be different in normal and shocked cells. Although the total amount transported into the shocked cells was much less, the relative amounts of thiamine pyrophosphate, monophosphate and thiamine were unchanged. This suggests that the thiamine binding protein has little, if any, effect on how the thiamine is accumulated inside the cell.

A preliminary attempt to reconstitute the transport system after osmotic shock by the addition of purified binding protein showed no increase in transport ability. This is not particularly disturbing however, since similar attempts by other investigators have often not been successful (20,21).

Sanders and Leach (60) characterized the transport of lipoic acid in S. faecalis and established the existence of lipoic acid transport in E. coli. Oh (47) further characterized the system in E. coli. However, the lipoic acid measurements involved in his work included the amount taken up by the whole cell and did not distinguish the free pool

and protein-bound material. The uptake into the free pool and incorporation into protein are saturated differently. While the free pool of lipoic acid saturates at about 100 μM, the incorporation into protein saturates near 1 mM. Oh's demonstration of saturation at 300 μM for the whole cell is probably reasonable, however, since the uptake was performed at 20°. The uptake studies in the present investigation were performed at 37°. Oh has shown that total uptake varies greatly with temperature.

Cystine and cysteine were potent lipoic acid inhibitors for both accumulation into the free pool and incorporation into protein. Since other sulfhydryl and disulfide compounds do not show this effect, the entire structure of cysteine and cystine must be involved. These two amino acids are both probably transported as cystine since a binding protein exists specific for cystine (26). It is possible that cystine and lipoic acid compete for the same transport system. The addition of ammonium sulfide to the cells prior to uptake potently inhibits lipoic acid incorporation into protein but does not effect free pool accumulation. Cysteine is known to be degraded into alanine and hydrogen sulfide (70) and the smell of the latter compound was very evident upon addition of either of these amino acids to the metabolizing cells. Therefore, the inhibition by cystine of lipoic acid incorporation into protein could be a function of either cystine itself or the de novo hydrogen sulfide.

Several fatty acids and lipoic acid analogs were tested

as potential inhibitors of lipoic acid uptake. Of oleic, octanoate, hexanoate and acetic, only oleic showed any inhibitory action. Incorporation into protein was inhibited about 40% while free pool accumulation was uneffected. Both C-7 and C-9 lipoic acid analogs inhibited free pool accumulation and the C-9 compound also inhibited incorporation into protein.

As expected potassium cyanide and dinitrophenol are inhibitory to both uptake processes. However, 1 m$\underline{M}$ sodium azide has no effect on either process. Chloramphenicol inhibits incorporation into protein by 93% while not affecting the free pool accumulation. This suggests that the covalent addition of lipoic acid occurs rapidly and only with de novo protein.

Vagelos (59) has suggested that bound lipoic acid and biotin could turn over as pantothenate does. However, we have found that lipoic acid is extremely resistant to turn over. Cells labeled with both low (2 $\mu\underline{M}$) and high (0.5 m$\underline{M}$) levels of lipoic acid showed equal ability to retain the protein-bound label when suspended in fresh medium in the presence of unlabeled lipoic acid. In fact a small rise in the amount bound usually occurred. Since exchange between exogenous lipoic acid and the free pool occurred with difficulty, this increase in activity probably arises from the endogenous labeled pool carried through the procedure of suspension in fresh unlabeled medium. If true, this could apparently decrease any exchange that occurs with

protein-bound lipoic acid, since this label would be available to combine with the de novo apo α-keto acid dehydrogenase complexes. To avoid this possible problem, cells were osmotically shocked to remove the labeled free pool and then subjected to the exchange procedure. Again lipoic acid remained constant through three generations of growth. The slight increase in activity was not seen however, suggesting that this phenomenon was due to the carry over of the labeled free pool.

Turnover in vivo of enzyme cofactors provides a basis for controlling the activity of the enzyme. Although turnover of the 4-phosphopantotheine of the acyl carrier protein could possibly affect fatty acid synthesis, on the basis of the experiments shown here no such control is likely with the α-keto acid dehydrogenase complexes.

CHAPTER X

SUMMARY

Several vitamins were utilized in the search for vitamin binding proteins in E. coli. Binding proteins for thiamine and riboflavin were released by cold osmotic shock. Binding of the other vitamins to shock fluid was not separable. Involvement of the thiamine binding protein is implicated in the transport process since osmotic shock greatly reduced the cell's ability to transport thiamine compared to the other vitamins, including riboflavin. The form in which thiamine was accumulated in the free cell pool is not influenced by osmotic shock.

Thiamine binding is reversible, has a pH optimum near 9 and has a dissociation constant of 0.05 μM. The binding has no requirement for Mg^{++} and is not influenced by N-ethylmaleimide or iodoacetic acid. Pyrimidines (cytosine and thymine) and thiamine pyrophosphate compete with thiamine for binding and have K_D's respectively of 100 and 0.5 μM. The thiamine binding protein has a molecular weight of 35,000 and shows no evidence of subunit association. The amino acid composition is similar to other binding proteins except that it is low in isoleucine content. The thiamine binding protein has been purified by conven-

tional means to a specific activity of 18 pmoles bound/mg of protein and a purity of 92%. Polyacrylamide gel electrophoresis in the presence of thiamine reduced the protein's mobility but has little or no effect on its molecular weight.

The riboflavin binding protein has a pH optimum near pH 6 and a K_D near 100 μM. The protein was purified by conventional means to a point where 84% of the protein produces a single band on polyacrylamide gel electrophoresis. The molecular weight is near 42,000. Riboflavin binding is not influenced by flavin adenine dinucleotide, flavin mononucleotide or folic acid. The involvement of the riboflavin binding protein in transport is less tenable than the involvement of the thiamine binding protein since the riboflavin data on reduction of transport upon osmotic shock is inconclusive and the dissociation constant for binding seems unreasonably high.

Attempted reconstitution of either binding protein with osmotically shocked cells had no influence on uptake characteristics.

About 80% of the lipoic acid taken up by E. coli was rapidly incorporated into a protein-bound form in the cell. This incorporation is one-half saturated at a lipoic acid concentration of 300 μM while the accumulation in the free lipoic acid pool was one-half saturated at 50 μM.

The time course of lipoic acid uptake was similar to that reported by Oh. Measurement of uptake during active growth shows the incorporation of lipoic acid into protein

increases more than accumulation in the free lipoic acid pool with respect to the number of cells.

Sulfhydryl containing compounds in general have no effect on lipoic acid uptake. However, cystine and cysteine inhibit both incorporation into protein and accumulation in the free pool. Ammonium sulfide inhibits lipoic acid incorporation into protein.

Of several fatty acids and lipoic acid analogs tested as lipoic acid antagonists only oleic acid and C-9 lipoic acid inhibited lipoic acid incorporation into protein. The C-7 and C-9 analogs inhibited free pool accumulation.

Lipoic acid, once bound to protein, does not turn over in E. coli. The cells were incubated with high and low lipoic acid concentrations with similar results. Labeled cells whose pool was subjected to osmotic shock were also resistant to exchange with unlabeled lipoic acid.

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