Studies on the Biosynthesis of Lipoic Acid

by

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(ABSTRACT)

A rapid, chemical method for the detection and quantitation of lipoic acid has been developed. Lipoic acid produces a yellow color when reacted with $PdCl_4^{-2}$ in 1 N HCl. The colored complex formed is extractable into methylene chloride, which can be readily concentrated, increasing the color intensity. The limit of sensitivity of this assay for lipoic acid detection in supernatants from Escherichia coli K-12 cell cultures is 2.5×10^{-5} M, giving an absorbance of 0.10 at 408 nm. Using this assay, it would be possible to screen for a mutant of Escherichia coli K-12 which excretes 100-fold more lipoic acid than the parent strain.

Cellular lipoic acid in an anaerobic Escherichia coli K-12 culture remained constant (5 to 6 μg/g dry weight) during growth in a minimal salts medium containing 1% glucose. However, cellular lipoic acid in an aerobic Escherichia coli K-12 culture increased from 15 μg/g dry weight to 20 to 25 μg/g dry weight during cell growth in the same medium. Aeration (2 L air/L medium/ minute) of a mid-log phase anaerobic Escherichia coli K-12 culture resulted in a doubling of cellular lipoic acid levels within the first thirty minutes and a four fold increase over the next five hours of aerated cell growth.

Chinese hamster ovary cells were found not to incorporate the two known bacterial precursors, [$^2H_3$]-acetate and [U-$^2H_{15}$]-octanoate, into lipoic acid. Further studies with both Chinese hamster ovary and mouse

fibroblast cells which were designed to demonstrate that these two transformed cell lines require lipoic acid for maximal growth were inconclusive.

DEDICATION

The work and thought that went into this research is dedicated to my wife, Jennifer, whose advice, support and compassion kept me working even after I had given up. Words can never express my feelings for the way you helped me these past two years, and only my love for you can ever repay you.

This work is also dedicated to my parents Patricia Sweeney Wolfe and Henry R. Wolfe, Sr, who instilled in my four brothers and me the desire to be the best we can at whatever we do. The example which they set has been an inspiration for me in both my personal and professional life.

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The use of Dr. Brian Storrie's equipment, time and cell culture facilities are appreciated greatly. I am grateful to both Dr. Storrie and Mrs. Meena Sachdeva for introducing me to the intricacies of mammalian cell culture. Without their help, the mammalian cell studies would not have been possible.

I would like to thank the members of my committee, Dr. Richard E. Ebel, Dr. E. M. Gregory, Dr. Joseph O. Falkinham, and especially my advisor Dr. Robert H. White for the numerous helpful and stimulating discussions.

I would like to thank Mr. Kim Harich for performing GC-MS analyses and for instruction in the operation of the mass spectrometer.

I am very grateful for the independence which Dr. Robert H. White has given me in the laboratory over the past two years. His open-minded approach has taught me to examine natural phenomena from many different angles.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

INTRODUCTION

In the characterization of biological systems, it is essential to determine an organism's growth requirements. By selectively removing nutrients and cofactors from bacterial cultures, it has been possible to identify the growth requirements for many organisms. In 1941, Virginia Dewey determined that Tetrahymena pyriformis (gelii) requires lipoic acid for growth (Dewey, 1941).

Lipoic acid was first isolated in crystalline form in 1951 by Lester Reed, et al (1951). It was his research group which first named the molecule lipoic acid, owing to its solubility in organic solvents and its acidity (pK_a=4.7). Two years later, its structure was determined to be 6,8-dithiooctanoic acid (Reed, et al 1953).

Lipoic acid is a covalently bound cofactor in four known multienzyme complexes: (1) pyruvate dehydrogenase (Reed, et al 1966, Reed, 1974, Collins, et al 1977); (2) α-ketoglutarate dehydrogenase (Reed, 1974); (3) branched chain α-keto acid dehydrogenase (Sokatch, et al 1981a, Sokatch, et al 1981b); and (4) glycine synthase (Sagers, et al 1973, Kochi, et al 1976, Motokawa, et al 1979, Kikuchi, et al 1980). In each case, lipoic acid has been proposed to transfer the specific intermediates for each of these reactions within its multienzyme complex. During the catalytic cycle of these enzymes, lipoic acid alternates between its reduced (dithiol) and oxidized (disulfide) states.

Since octanoic acid forms the backbone of the lipoic acid it is not surprising that both acetate and octanoate are lipoic acid precursors in E. coli K-12 (Parry, 1977, White, 1980a). However, the mechanism(s) of sulfur introduction from cysteine, the most likely sulfur source (White, 1982), at C-6 and C-8 of octanoic acid during the formation of lipoic acid are still unknown. Sulfur introduction at C-6 of octanoic acid occurs with a net inversion of stereochemistry (Reed, 1964, Parry, et al 1978, White, 1980a), based on the absolute configuration of lipoic acid (Mislow, et al 1956, Golding, in press). This observed inversion of configuration would be consistent with an S_N2 reaction at a hydroxylated carbon of a possible 6-hydroxyoctanoic acid intermediate. The introduction of sulfur at C-8 could proceed in an analogous manner. Such intermediates could be formed by a hydroxylation of a saturated carbon with retention of configuration, as described for hydroxyproline biosynthesis (Fujita, 1964). However, these hydroxyoctanoic acids are unlikely intermediates since they are not biosynthetically incorporated into lipoic acid (White, 1980b). However, both [6-R,S]-mercaptooctanoic and 8-mercaptooctanoic acids are precursors for lipoic acid (White, 1980b).

The biosynthesis of lipoic acid (Parry, 1977, White, 1980b), biotin (Parry, et al 1980) and penicillin (Baxter, et al 1982) are similar in that no unsaturation occurs at the site of sulfur introduction and that hydroxylated intermediates are not involved. The introduction of sulfur at C-4 of dethiobiotin, forming biotin (Figure 1) and at C-3 of L-(L-

-aminoadipyl)-L-cysteinyl-D-valine to form isopenicillin N (Figure 2) occurs with retention of configuration.

Low cellular concentrations of lipoic acid and the lack of a rapid assay have been the major obstacles in studies on lipoic acid biosynthesis. This thesis describes a rapid, chemical assay for lipoic acid, a method for increasing cellular lipoic acid concentrations in E. coli K-12 and results of studies which indicate that Chinese hamster ovary cells do not incorporate two known bacterial precursors into lipoic acid.

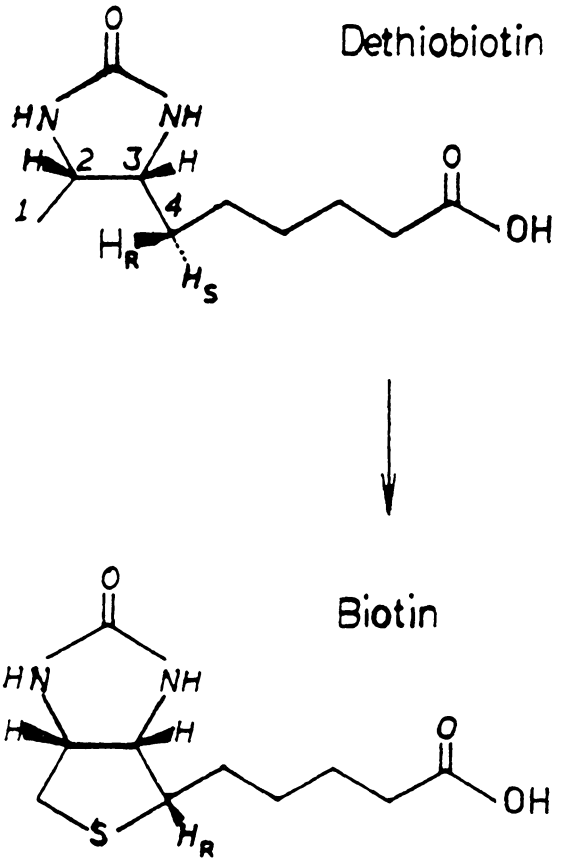

Figure 1: Incorporation of Sulfur into Biotin

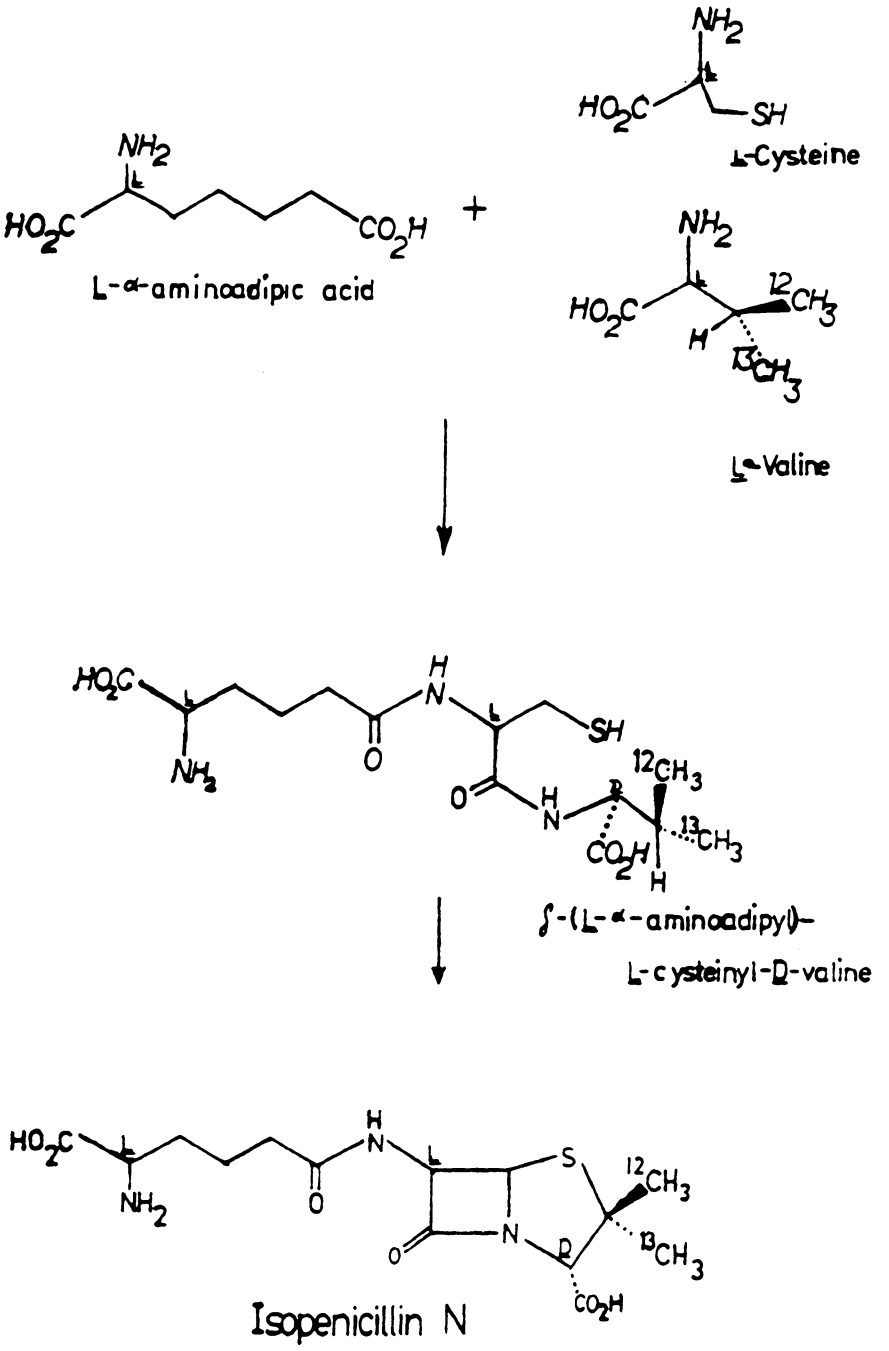

Figure 2: Incorporation of Cysteine Sulfur into Penicillin

Chapter II

LITERATURE REVIEW

ROLE OF LIPOIC ACID IN CELL METABOLISM

Lipoic acid is a covalently bound enzyme cofactor of four known multienzyme complexes, pyruvate dehydrogenase (Reed, et al 1966, Reed, 1974, Collins, et al 1977), α-ketoglutarate dehydrogenase (Reed, 1974), branched chain α-keto acid dehydrogenase (Sokatch, et al 1981a, Sokatch, et al 1981b) and glycine synthase (Sagers, et al 1973, Kochi, et al 1976, Motokawa, et al 1979, Kikuchi, et al 1980). In all four complexes, the lipoic acid transfers catalytic intermediates between active sites of the multienzyme complex.

In the α-keto acid dehydrogenases, lipoic acid functions to carry an acyl group from its thiamin pyrophosphate conjugate to the coenzyme A binding site where acyl-coenzyme A is released (Figure 3) (Reed, 1974). Regeneration of the disulfide form of lipoic acid is necessary for activity of the multienzyme complex and is accomplished by oxidation of the dithiol by flavin adenine dinucleotide (Walsh, 1978).

In the glycine synthase found in bacteria, plants and various animals (Sagers, et al 1973, Kochi, et al 1976, Motokawa, et al 1979, Kikuchi, et al 1980), lipoic acid transfers the aminomethyl moiety of glycine from its pyridoxal phosphate conjugate, with the concomitant release of CO_2, to an active site of another enzyme of the complex. This enzyme cleaves the group from lipoic acid, forming NH_3 and N^5, N^{10}-methylenetetrahydrofolate (Figure 4).

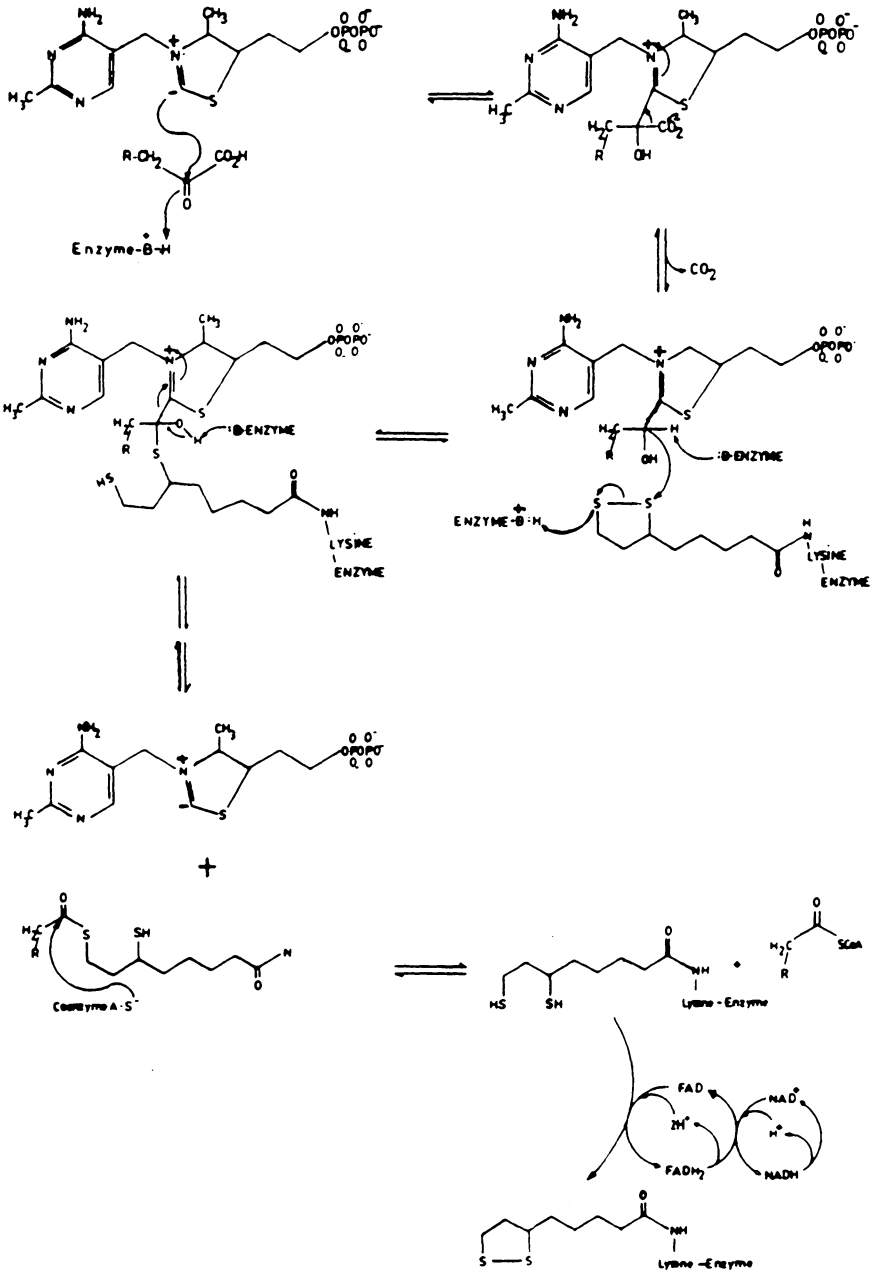

Figure 3: General Reaction Sequence for α-Keto Acid Dehydrogenases

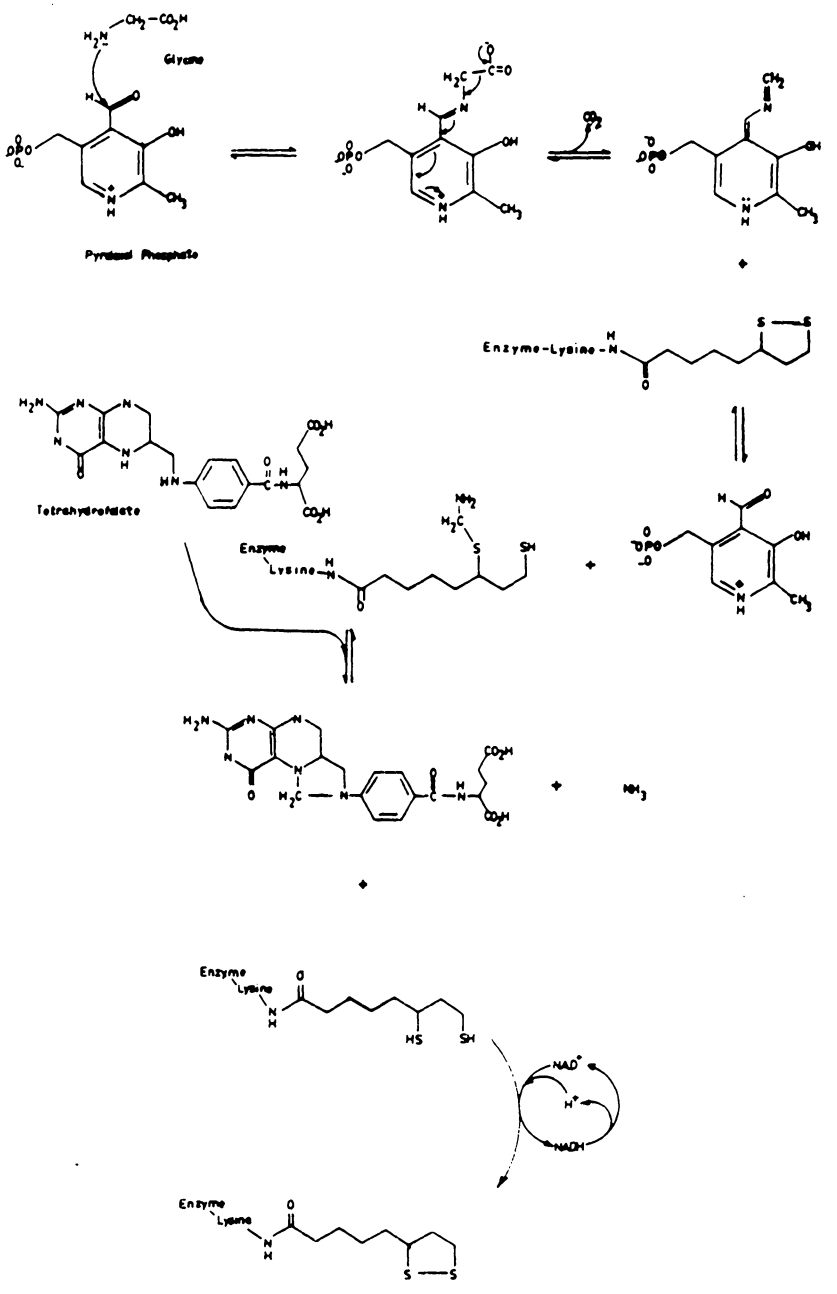

Figure 4: Glycine Synthase Reaction Sequence

EFFECTS OF OXYGEN ON BACTERIAL CELL METABOLISM

The activities of the enzymes of the central metabolic pathways of bacteria have been found to be tightly regulated. Activation or inhibition of branch point enzymes has been observed to change cell physiology from an anabolic to a catabolic system (Gest, 1980, Gest, 1981).

Anaerobically grown E. coli metabolizes glucose mainly by glycolysis (Amarasingham, et al 1965, Gray, et al 1966, Wimpenny, et al 1966, Reicheldt, et al 1971, Wimpenny, et al 1971, Thomas, et al 1972, Gest, 1980). Under anaerobic conditions , activity of the α-ketoglutarate dehydrogenase complex is repressed (Amarasingham, et al 1965, Thomas, et al 1972, Cole, et al 1979a, Neidhardt, et al 1983), and the Krebs cycle becomes a branched pathway (Amarasingham, et al 1965, Gest, 1981).The tricarboxylic acid branch of this pathway serves the catabolic and anabolic needs of the cell. The dicarboxylic acid branch maintains the redox balance of the cell by dehydration of malate to fumarate and subsequent reduction of fumarate to succinate (Figure 5) (Gest, 1981). The addition of glutamate to anaerobically grown E. coli increases the activity of α-ketoglutarate dehydrogenase (Cole, et al 1979b).

Aerobically grown E. coli metabolizes glucose by the hexosemonophosphate pathway and the aerobic form of the Krebs cycle. Activities of enzymes associated with the hexosemonophosphate pathway, the Krebs cycle and also the respiratory chain (NADH oxidase, succinate oxidase) are seen to increase in the presence of O_2 or NO_3^-, when ac-

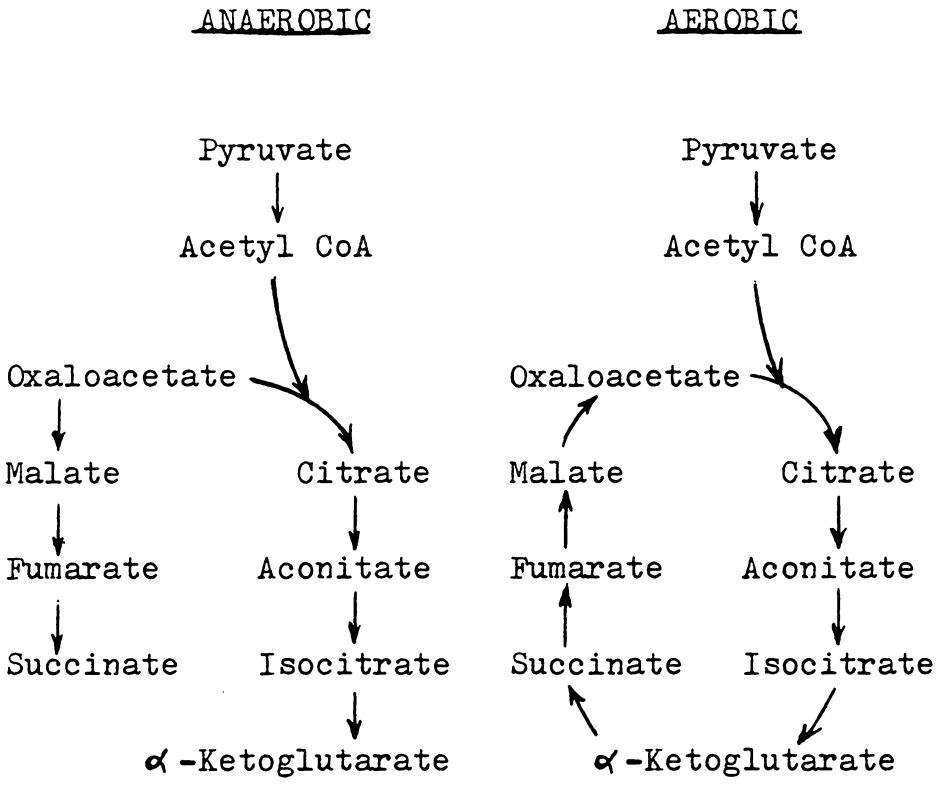

Figure 5: Anaerobic and Aerobic Forms of the Krebs Cycle

tivites of several enzymes of the glycolytic pathway are seen to decrease (Cavari, et al 1968, Wimpenny, et al 1971, Thomas, et al 1972, Niedhardt, et al 1983b). The activity of α-ketoglutarate dehydrogenase increases when anaerobically grown Citrobacter freudii cells are exposed to O_2 or NO_3^- (Cole, et al 1979a, Cole, et al 1979b). These changes are responsible for converting bacterial metabolism from the branched, anaerobic type to the cyclic, aerobic type.

Induction of α-ketoglutarate dehydrogenase activity upon aeration of an anaerobically grown E. coli culture would require the incorporation of lipoic acid into the newly formed apoenzyme. This lipoic acid may be obtained from preexisting cellular sources (Leach, 1979) or may require de novo biosynthesis. To test the hypothesis that cellular lipoic acid levels would increase as a result of such a transition, an anaerobically grown culture of E. coli K-12 was aerated and analyzed for changes in the cellular lipoic acid concentration.

DETECTION OF LIPOIC ACID

The major difficulty encountered in studies of lipoic acid biosynthesis has been low cellular concentration. Methods are available which allow us to detect these low levels (O'Kane, et al 1948, Stokstad, et al 1953, Gunsalus, et al 1957, Herbert, et al 1970, Herbert, et al 1975, White, 1981). To more conveniently study the mechanisms involved in lipoic acid biosynthesis and to identify intermediates involved in lipoic acid biosynthesis, it may be necessary to isolate an organism which

produces higher levels of lipoic acid than wild type strain of E. coli K-12. Isolation of such a strain would be facilitated by a rapid assay for lipoic acid.

A gas chromatographic assay for lipoic acid provides both the accuracy and versatility required for a screening assay (White 1981), but is lengthy (1-2 hours per sample) and thus impractical for screening thousands of cell colonies which might be necessary to identify a lipoic acid-overproducer. However, the gas chromatographic assay has been used for measurement of lipoic acid levels throughout the research discussed in this thesis because of its accuracy.

LIPOIC ACID BIOSYNTHESIS IN MAMMALIAN CELL CULTURE

The degradation of lipoic acid in both humans (Jukes, et al 1956, Kato, et al 1965) and rats (McCormick, et al 1974, McCormick, et al 1977) has been studied. However, the mechanism(s) by which mammals synthesize lipoic acid, if at all, are unknown.

The study of lipoic acid biosynthesis in mammals is complicated by the presence of lipoic acid-producing intestinal flora. Two methods which could be used to avoid this problem are the use of mammalian cell (or tissue) culture and the germ-free animal.

Past work done on lipoic acid requirements of mammalian cells in culture has resulted in the inclusion of 1 μM lipoic acid in some commercial media. This addition of lipoic acid to culture media is based on the following statement appearing in a 1963 paper by Ham:

> "small amounts of copper, zinc, and lipoic acid either singly or together frequently improve growth of strain CHD-3."

Strain CHD-3 which was used in that study is a Chinese hamster ovary cell line. Later studies suggested inclusion of 10 nM lipoic acid as a component of growth media, and indicated that 1 μM, the level suggested in 1963 actually inhibits growth of Chinese hamster ovary cells (Ham, et al 1977). Although it has been noted that inclusion of lipoic acid in a growth medium will frequently improve cell growth, there has never been a study which demonstrates that lipoic acid is required to get maximal growth of cells.

Chapter III

EXPERIMENTAL PROCEDURE

MATERIALS

Reagent grade chemicals obtained from Fisher Scientific Company are as follows: $NaBH_4$, $(NH_4)_2SO_4$, $Ca(NO_3)_2 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot 7H_2O$, KH_2PO_4, D-glucose, pyrogallol (1,3,5-trihydroxybenzene), $MgSO_4 \cdot 7H_2O$, Na_2SO_4, $NaHCO_3$ and Na_2CO_3. HPLC-grade methylene chloride and methanol were obtained from Burdick-Jackson Laboratories. Benzyl chloride and n-heptane were purchased form the Eastman Company. Tryptamine, 1,3-propanedithiol and [$^2H_3$]-acetic acid were purchased from Sigma Chemical Company. Bacto-Agar was obtained from Difco Biological Supply. The [U-$^2H_{15}$]-octanoic acid was purchased from Merck, Sharp & Dohme of Canada and 5 x 20 cm. silica gel thin layer (0.25 mm.) plates were from E. Merck. Complete alpha-modified minimal essential medium was obtained from Flow Laboratories, and lipoic acid-deficient alpha-modified minimal essential medium was made from its component chemicals. Fetal calf serum was purchased from Biofluids, Inc. and heated at 60°C for 15 minutes to destroy complement activity. Grand Island Biological Company, Inc. supplied sterile, frozen trypsin in a 2.5 % solution. Mammalian cells were grown on 100 x 20 mm polystyrene tissue culture dishes supplied by Corning Glass Works.

Diazomethane was prepared as follows: 10 ml of 50% (w/v) potassium hydroxide in a 125 ml Erlenmeyer flask was cooled in an ice-water

bath and first HPLC-grade methanol (30 ml) was added, then diethyl ether (15 ml) was added. The flask was fitted with bent glass tubing through a rubber stopper. At one end of the glass tubing was the flask described and at the other, in the ice-water bath was a 30 ml test tube containing diethyl ether (2-5 ml). DIAZALD® (N-methyl-N-nitroso-p-toluenesulfonamide) (1-2 g) was added to the flask which was swirled in a 90° C water bath, allowing the diazomethane produced to be distilled into the chilled test tube. The product is a bright yellow solution of diazomethane in diethyl ether.

The Li_2PdCl_4 used was a gift from Kenneth Natalie, Department of Chemistry, Virginia Polytechnic Institute and State University. The complex was synthesized as follows: 0.17 moles of $PdCl_2$ was added to 0.34 moles of LiCl in 100 ml of water and the solution was heated until homogeneous. The water was removed by rotary evaporation and the product dried to a constant weight in an Abderhalden apparatus, giving a dark brown solid in 98% yield.

Fifty milliliters of heat-treated (60°C for 15 min) fetal calf serum was dialyzed against running tap water for 24 hours and then against 2 liters of quartz distilled water for four hours. The serum was then made 0.9% in NaCl and filter-sterilized before storage in glass bottles at 4°C. The lipoic acid concentration of complete fetal calf serum was determined by gas chromatography to be 2.1×10^{-7} M whereas dialyzed fetal calf serum was 8.6×10^{-9} M.

The culture of Escherischia coli K-12 used in these studies was a gift from Dr. Joseph O. Falkinham, III of the Department of Biology, Virginia Polytechnic Institute and State University.

METHODS

Li_2PdCl_4 Assay for Lipoic Acid

Lipoic acid was quantitated as the Pd-lipoic acid complex as follows: microliter quantities of 10 mM lipoic acid in ethanol were added to 1 N HCl. This solution was mixed and then made 0.13 mM in Li_2PdCl_4, yielding a yellow color. The final volume was 3.275 ml. The absorbance of the aqueous solution at 358 nm was used to measure the lipoic acid concentration. The yellow colored complex which formed in 1 N HCl was extractable into methylene chloride; in this solvent, the lipoic acid concentration could be determined from the absorbance at 408 nm.

The stoichiometry of the Pd-lipoic acid complex was determined by titration of the Li_2PdCl_4 with increasing amounts of lipoic acid. A model study of the product formed between palladium and lipoic acid was performed by obtaining a spectrum of the complex formed when Li_2PdCl_4 reacts with 1,2-dithiolane in 1 N HCl. The 1,2-dithiolane was formed from 1,3-dithiopropane as a result of air and photo-oxidation and could be quantitated at 330 nm, using the molar absorptivity (ϵ =147 M cm) determined by M. Calvin (1951).

The accuracy of the palladium assay relative to the gas chromatographic assay for lipoic acid was determined. Twelve ml of a stationary phase (1.8 A_{550}) culture of E. coli K-12 was acidified with three ml of 6 N HCl and 195 µl of 10^{-2} M lipoic acid was then added. Twelve ml of cell-free supernatant from the same culture was treated identically. Each test solution was divided into three 5 ml aliquots and each was analyzed for lipoic acid in three different ways (Methods A,B,C).

Method A involved the addition of 40 µl of 10 mM Li_2PdCl_4 to 5 ml of the test solution. The absorbance of the solution was then measured at 358 nm and the samples were extracted with 2 ml of methylene chloride. The absorbance at 408 nm of these extracts was measured to determine extractable Pd-lipoic acid complex.

Method B involved extraction of the lipoic acid from either of the test solutions into methylene chloride (three 1 ml portions) before lipoic acid quantitation. The methylene chloride was removed and the residue diluted in 5 ml of 1 N HCl. Forty microliters of 10 mM Li_2PdCl_4 was then added and the absorbance at 358 nm was obtained. The Pd-lipoic acid complex was then extracted into 2 ml of methylene chloride and the absorbance at 408 nm was measured. A Cary model 219 spectrophotometer was used for all absorbance measurements; with a spectral band width of 1.0 nm and a period of 0.5 seconds.

Method C was analogous to method B in that the lipoic acid was removed from the sample by extraction into methylene chloride before measurement. However, the amount of lipoic acid present was determined by the gas chromatographic lipoic acid assay (White, 1980b).

Changes in Lipoic Acid Levels in E. coli K-12

All E. coli K-12 cultures were grown in a minimal salts medium containing the following components: 57 mM $Na_2HPO_4 \cdot 7H_2O$, 19.8 mM KH_2PO_4, 406 μM $MgSO_4 \cdot 7H_2O$, 7.6 mM $(NH_4)_2SO_4 \cdot 7H_2O$, 4.5 μM $FeSO_4 \cdot 7H_2O$ and 125 μM $Ca(NO_3)_2 4H_2O$ in tap distilled water at pH 7.2 (Adelberg, et al 1965). The carbon source was 1% D-glucose. All E. coli K-12 cultures, except inocula, were grown in a 4.8 L bottle with two fritted glass spargers for gas input.

Low oxygen tension for "anaerobic" growth was achieved by sparging the growth bottle with commercial nitrogen gas for 15 minutes prior to inoculation. E. coli K-12 cultures were maintained at room temperature on 2% agar slants of the minimal salts medium containing 1% glucose, and were transferred monthly to new slants.

Aerobic inocula were prepared by washing the cells from a 24 hour slant of E. coli K-12 with 5 ml of sterile minimal salts medium into 400 ml of sterile minimal salts medium containing 1% glucose at pH 7.2 in a Fernbach flask which was shaken at 37°C in a rotary shaker at 150 rpm. The aerobic inoculum was added to 4.3 liters of sterile, aerated minimal salts medium containing 1% glucose when the absorbance was between 1.5 and 2.0 (550 nm). Medium intended for aerobic cultures was aerated at 2.0 L air/ L medium/minute for 15 minutes prior to inoculation. This aeration rate was continued throughout growth of the culture.

"Anaerobic" inocula were prepared by washing the cells from a 24-hour slant of E. coli K-12 with 5 ml of sterile minimal salts medium into 500 ml of sterile, non-degassed minimal salts medium containing 1% glucose in a 500 ml bottle, and sealing the bottle. "Anaerobic" inocula were grown in the sealed bottle at 37°C on a rotary shaker at 150 rpm to an absorbance of 0.5 to 0.7 (550 nm) at which time it was transferred anaerobically to the growth bottle containing 4.3 liters of degassed, sterile minimal salts medium + 1 % glucose.

The growth flask was incubated at 37°C and was continuously stirred. "Anaerobic" samples (100-500 ml) were removed under nitrogen pressure and collected in in 2.8 liter Fernbach flasks in a salt/ice bath. All samples were stored at -10°C in the salt/ice bath (for less than 2 hours) until centrifugation at 4°C. Cells were centrifuged at 4°C at 23,300 x g for 10 minutes. The anaerobic culture was aerated by sparging with 6.6 liters of air per minute when the culture reached an absorbance of 0.4 to 0.5 (550 nm).

Lipoic acid was analyzed according to the method of White (White, 1981). Cell pellets were suspended in approximately three pellet volumes of 0.4% tryptamine in 6 N HCl for a 12 hour hydrolysis at 115°C. Dry cell weights were determined by drying 10% of the resuspended pellet to a constant weight.

Lipoic Acid Biosynthesis in Mammalian Cell Culture

Chinese hamster ovary (sc2) cells and mouse fibroblast (L929) cells were kindly donated by Dr. Brian Storrie, Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University.

Inocula were grown in commercial alpha minimal essential medium containing 10% (v/v) fetal calf serum. The cell pellets were washed twice with lipoic acid-deficient alpha minimal essential medium prior to use for inoculation.

Cells for experiments on the incorporation of deuterated lipoic acid precursors were grown in 300 ml bottles containing 250 ml of lipoic acid -deficient alpha minimal essential medium containing 10% (v/v) dialyzed fetal calf serum. These cultures were incubated for 3-4 days at 37 °C in an atmosphere of 95% air, 5% CO_2 with continuous stirring. Cells were harvested by centrifugation at 4°C at 3,320 x g for 10 minutes. The cell pellet was hydrolyzed and analyzed for lipoic acid as described above. The derivatized lipoic acid homologs were analyzed by mass spectrometry for incorporation of deuterium into lipoic acid. The fatty acids isolated from cells grown in the presence of 0.45 mM acetate were analyzed by gas chromatography-mass spectrometry for deuterium incorporation.

Plate cultures of mammalian cells were grown in 15 ml of alpha modified minimal esssential medium in 100 x 20 mm polystyrene tissue culture dishes. The inocula were grown in commercial alpha-modified minimal essential medium and were removed from their culture dishes by a 10 minute, 37°C incubation with 0.05 % trypsin (Bashor, 1979). The cells were washed with lipoic acid deficient alpha-modified minimal essential medium before adding 5 x 10 cells to each tissue culture dish. The cultures were grown for 92 - 93 hours at 37°C in an atmosphere of 95% air 5% CO_2 and harvested by trypsin dispersion as before.

Chapter IV

RESULTS

ASSAY FOR LIPOIC ACID USING PALLADIUM SALTS

Li_2PdCl_4 reacted with lipoic acid in 1 N HCl to produce a yellow-colored complex with two absorption maxima (Figure 6). Since the longer wavelength band was at 430 nm, the solution appeared yellow. Åckerfeldt and Lövegren (1964) obtained similar results from the reaction of palladium salts with various thiols and disulfides . The yellow Pd-lipoic acid complex was extractable into methylene chloride from 1 N HCl. The complex has a different absorption spectrum in methylene chloride relative to that seen in 1 N HCl, suggestive of either the existence of different chromophores in the two solvents or of a large solvent effect (Figure 6).

Titration of Li_2PdCl_4 with lipoic acid in 1 N HCl or acidified (1 N in HCl) *E. coli* supernatant was consistent with a 1:1 complex of palladium and lipoic acid (Figures 7 and 8). In addition, the curves indicated that the limit of detection for this assay is 2.5×10^{-5} M lipoic acid in 1 N HCl and in acidified (1 N in HCl) *E. coli* K-12 supernatant, giving an absorbance of 0.1 at 358 nm in either solvent. Titration of Li_2PdCl_4 with lipoic acid followed by extraction of the Pd-lipoic acid complex into methylene chloride was consistent with the extraction of a 1:2 complex of palladium and lipoic acid into methylene chloride (Figure 9 and 10). These titration curves indicate a limit of detection of 5×10^{-5} M for li-

Legend

Figure 6: Curve A is the spectrum of a 1 N HCl solution containing 1.3 x 10^{-4}M Li_2PdCl_4. Curve B is the spectrum of a 1 N HCl solution containing 1.3 x 10^{-4}M Li_2PdCl_4 and 1.3 x 10^{-4}M lipoic acid. Curve C is the spectrum of a methylene chloride extract of the sample used in curve B. The extraction was done using 1.0 ml of methylene chloride per 3.0 ml of curve B solution.

Molar absorptivities were calculated from these spectra. For the chromophore(s) in aqueous solution (curve B), the molar absorptivities are 3.5 x $10^3 M^{-1}cm^{-1}$ at 358 nm and 2.4 x 10^3 $M^{-1}cm^{-1}$ at 430 nm. The molar absorptivity for the chromophore in the methylene chloride extract is 1.4 x 10^4 $M^{-1}cm^{-1}$ at 408 nm.

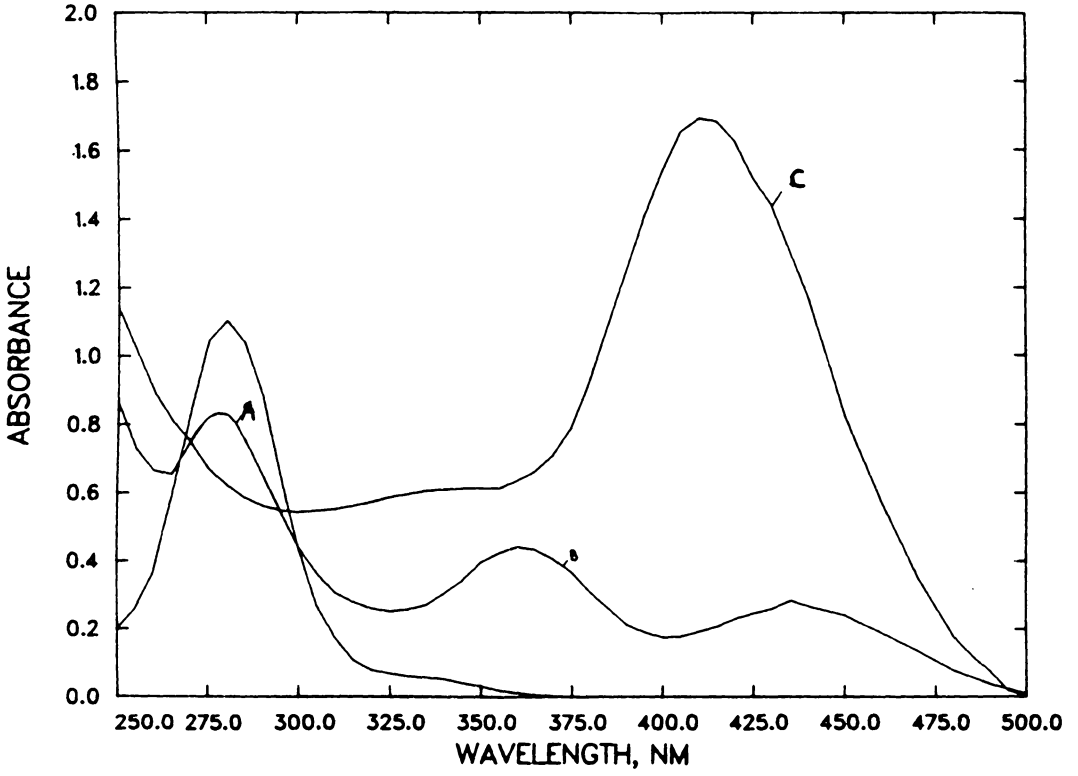

Figure 6: Spectra of the Pd-Lipoic Acid Complexes

poic acid after extraction into methylene chloride, giving an absorbance of 0.2 at 408 nm.

Several sulfur-containing biological molecules interfere with the palladium-dependent lipoic acid assay in 1 N HCl (Figure 11) or in acidified (1 N in HCl) E. coli K-12 cell free medium (Figure 12). In each case, the intereference could be greatly reduced by extraction into CH_2Cl_2 (Figures 13 and 14).

The reaction product between $PdCl_4^{-2}$ and 1,2-dithiolane In 1 N HCl gave a complex with the same spectrum as that produced from the reaction of $PdCl_4^{-2}$ with lipoic acid (Figure 15). This indicates that only the sulfur atoms of the lipoic acid are involved in the formation of the complex.

The accuracy of the palladium assay relative to the gas chromatographic lipoic acid assay demonstrated good agreement between the two methods at 0.1 mM lipoic acid the single time this comparison was made (Table 1). The palladium assay gave results within 20% of values obtained by the GC assay when the lipoic acid was quantitated by measuring the absorbance at 358 nm in the aqueous solution (Method B). Quantitation of lipoic acid in the CH_2Cl_2 layer at 408 nm provided results within 9% of the GC value when an extract of the whole culture was used and when an extract of the culture supernatant was measured, 100% agreement with the gas chromatography assay was found. Although this comparison was only done once, this method seems to be useful for the quantitation of free lipoic acid in supernatants from cultures of lipoic-acid overproducing cells. The free lipoic acid level in

LEGEND

Figure 7: The absorbance of the Pd-lipoic acid complex at 358 nm was obtained after titration of the lipoic acid solution with Li_2PdCl_4 as follows: A) 30 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCl was added to 3.0 ml of a solution of 1.3 to 20 x 10^{-5}M lipoic acid in 1 N HCl. B) 15 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCl was added to 3.0 ml of a solution of 1.3 to 10 x 10^{-5}M lipoic acid in 1 N HCl. C) 9 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCl was added to 3.0 ml of a solution of 1.3 to 8.0 x 10^{-5}M lipoic acid in 1 N HCl. D) 3 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCl was added to 3.0 ml of a solution of 0.65 to 3.3 x 10^{-5}M lipoic acid in 1 N HCl. The final volume of each solution was adjusted to 3.10 ml with 1 N HCl before reading the absorbance.

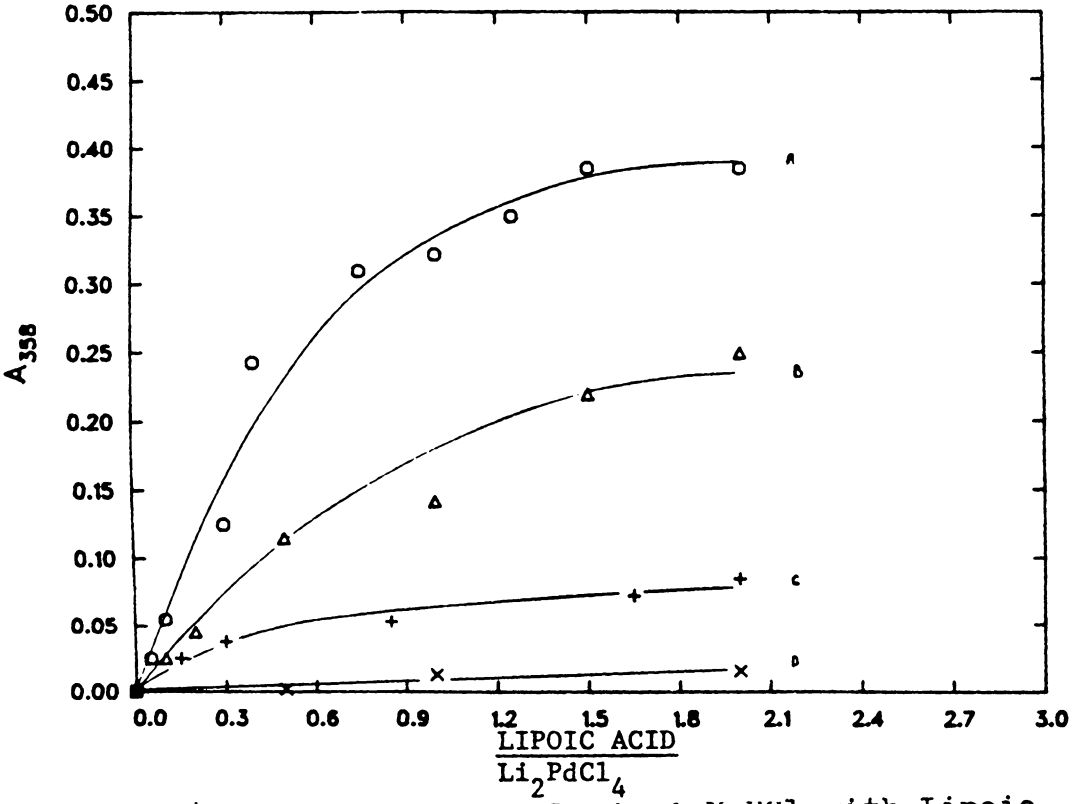

Figure 7: Titration of Li_2PdCl_4 in 1 N HCl with Lipoic Acid

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Figure 8: The absorbance of the Pd-lipoic acid complex at 358 nm was obtained after titration of the lipoic acid solution with Li_2PdCl_4 as follows: A) 30 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCL was added to 3.0 ml of a solution of 1.3 to 20 x 10^{-5}M lipoic acid in acidified (1 N in HCl) E. coli K12 supernatant. B) 15 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCl was added to 3.00 ml of a solution of 1.3 to 10 x 10^{-5}M lipoic acid in acidified (1 N in HCl) E. coli K12 supernatant. C) 9 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCl was added to 3.0 ml of a solution of 1.3 to 8.0 x 10^{-5}M lipoic acid in acidified (1 N in HCl) E. coli K12 supernatant. D) 3 µl of 10^{-2}M Li_2PdCl_4 in 1 N HCl was added to 3.0 ml of a solution of 0.65 to 3.3 x 10^{-5}M lipoic acid in acidified (1 N in HCl) E. coli K12 supernatant. The final volume of each solution was adjusted to 3.10 ml with 1 N HCl before reading the absorbance.

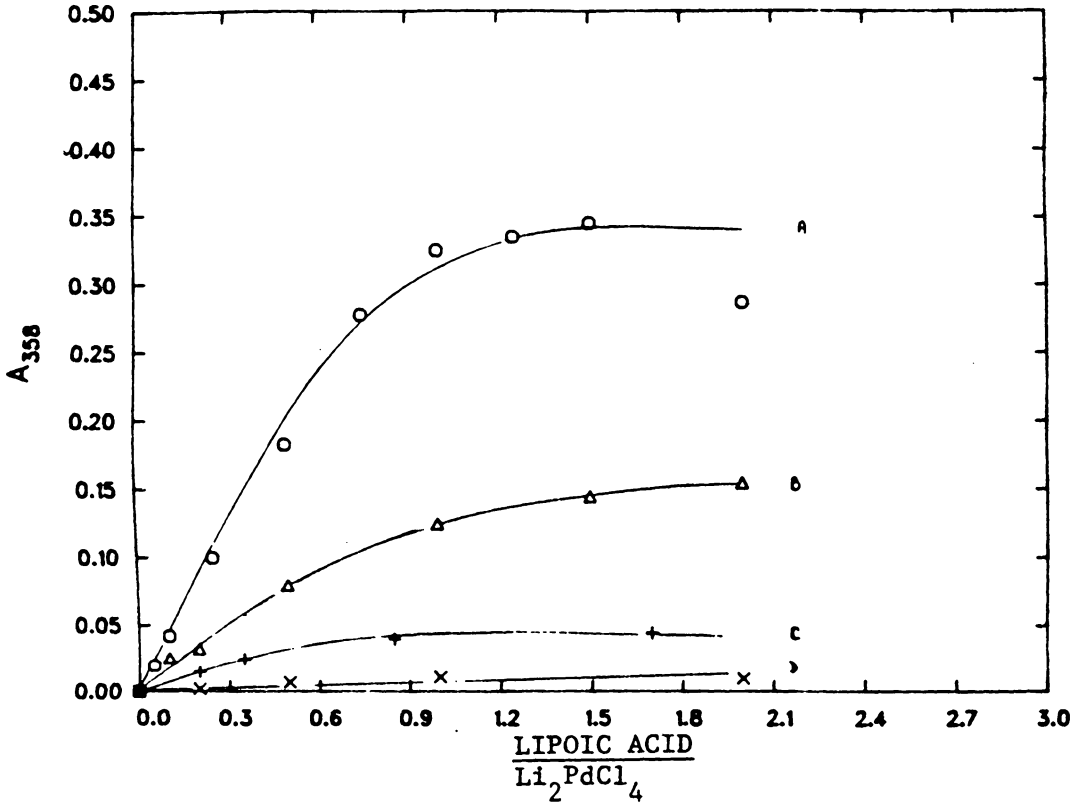

Figure 8: Titration of Li_2PdCl_4 in Acidified *E. coli* K-12 Cell-Free Supernatant with Lipoic Acid

Legend

Figure 9: These data were obtained by extraction of the samples from Figure 7 with 1 ml of methylene chloride and then measuring the absorbance of the methylene chloride layer at 408 nm. After the final volumes of the solutions in Figure 7 had been adjusted to 3.10 ml, they were then extracted with methylene chloride. As shown in Figure 6, 408 nm is the absorbance maximum for the Pd-lipoic acid complex in methylene chloride.

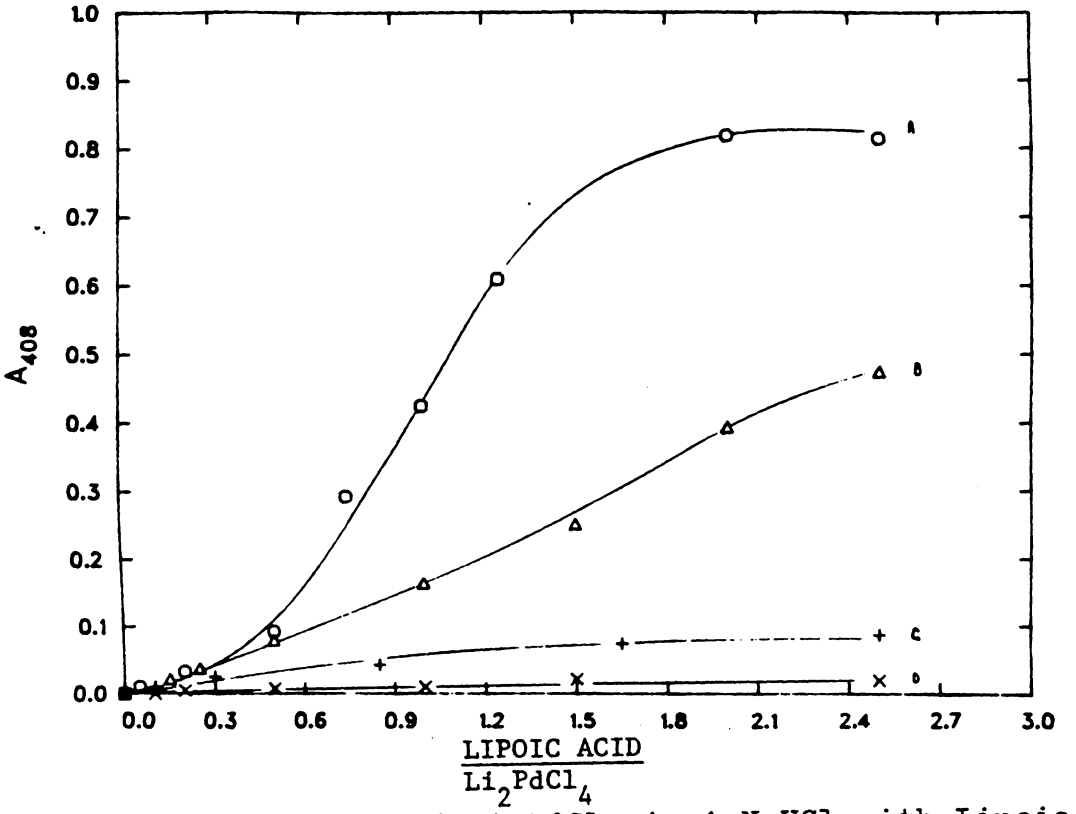

Figure 9: Titration of Li_2PdCl_4 in 1 N HCl with Lipoic Acid, CH_2Cl_2 Extract

Legend

Figure 10: These data were obtained by extraction of the samples from Figure 7 with 1 ml of methylene chloride and then measuring the absorbance of the methylene chloride layer at 408 nm. After the final volumes of the solutions in Figure 7 had been adjusted to 3.10 ml, they were then extracted with the methylene chloride.

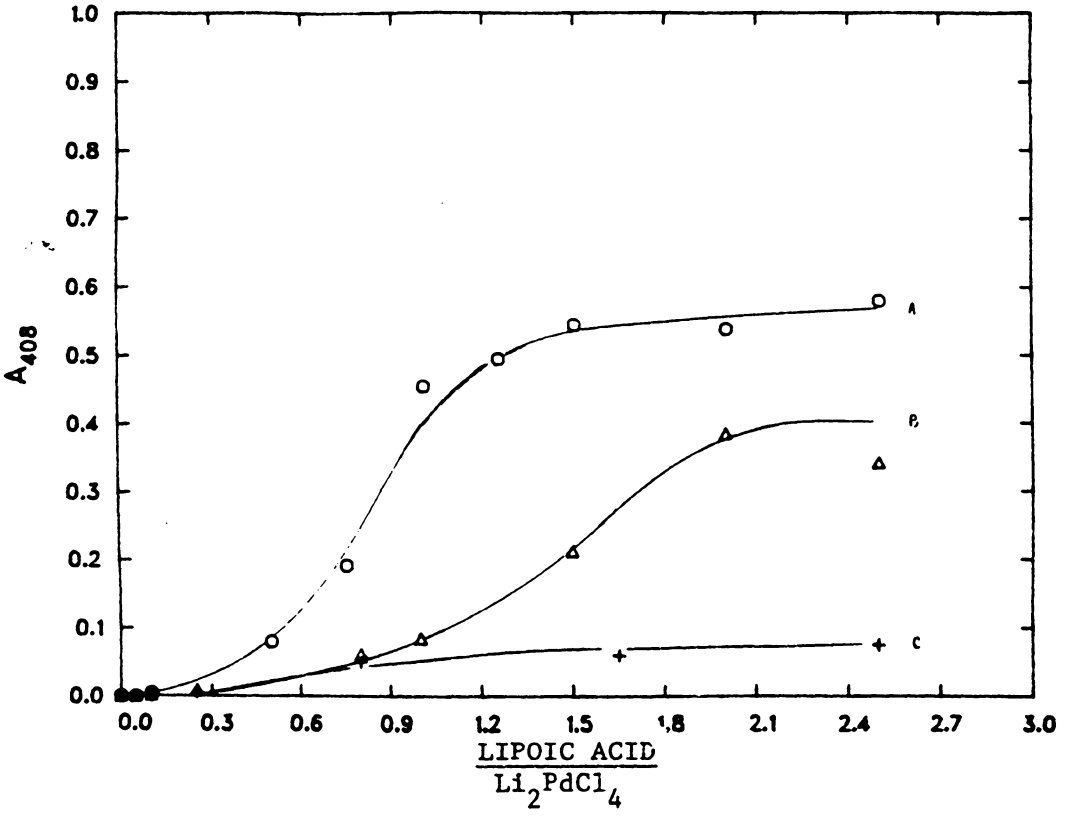

Figure 10: Titration of Li_2PdCl_4 in Acidified E. coli K-12 Cell-Free Supernatant with Lipoic Acid, CH_2Cl_2 Extract

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Figure 11: To 3.0 ml of 1 N HCl was added 30 µl of 10^{-2}M sample solution and 30 µl of 10^{-2}M Li_2PdCl_4. The sample solutions listed below gave the spectra as lettered: a) dithiothreitol, b) lipoic acid, c) cysteine, d) cyteamine, e) reduced glutathione, f) β-mercaptoethanol, g) methionine, h) cysteic acid, i) cystine, j) 1 N HCl. The spectra were obtained with a Cary model 219 spectrophotometer, as described in the Methods section.

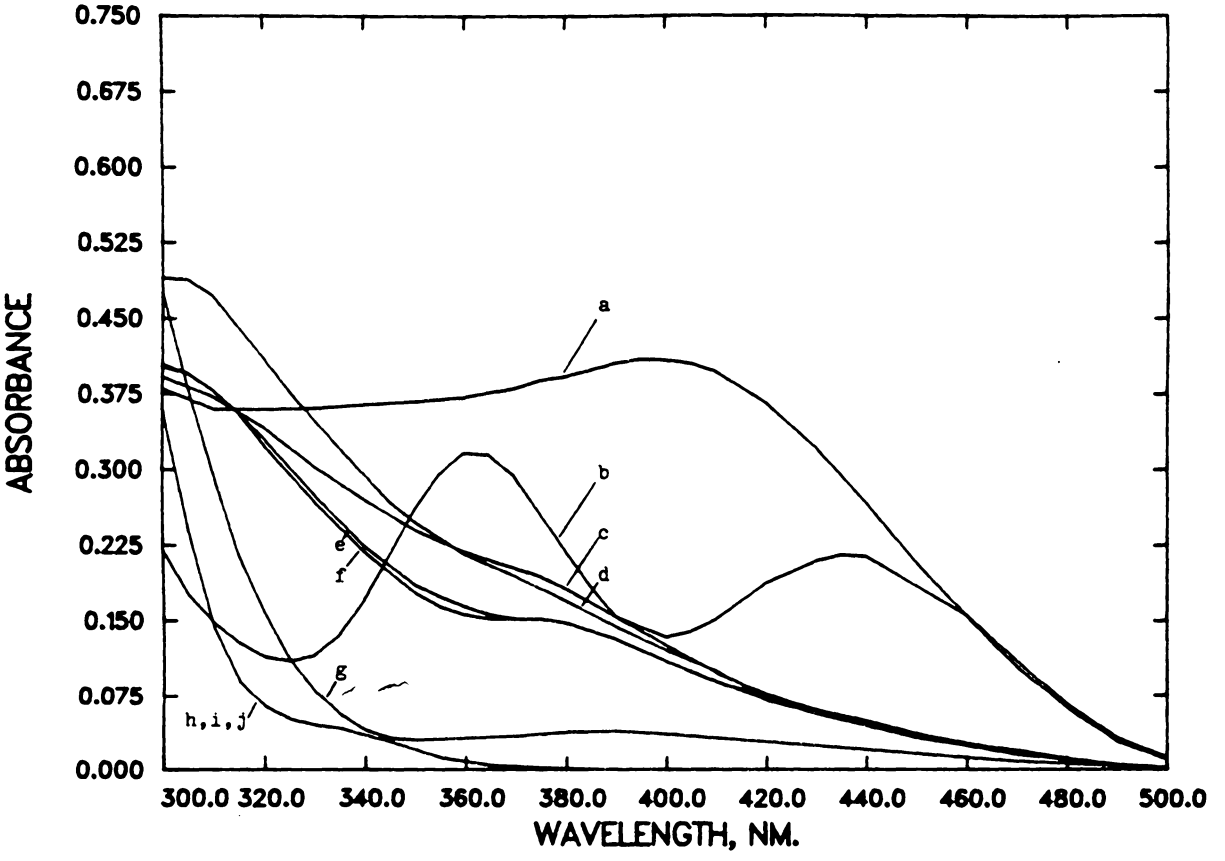

Figure 11: Absorption Spectra of Pd Complexes with Various Biological Molecules in 1 N HCl

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Figure 12: To 3.0 ml of acidified (1 N in HCl) *E. coli* K-12 supernatant was added 30 µl of 10^{-2}M sample solution and 30 µl of 10^{-2}M Li_2PdCl_4. The sample solutions listed below gave the spectra as lettered: a) dithiothreitol, b) lipoic acid, c) cysteine, d) cysteamine, e) reduced glutathione, f) β-mercaptoethanol, g) methionine, h) cysteic acid, i) cystine, j) acidified (1 N in HCl) *E. coli* K-12 supernatant. The acidified *E. coli* K-12 supernatant was prepared by centrifuging a 16-hour culture of *E. coli* K-12 (grown on the minimal salts medium described + 1% glucose) for ten minutes at 23,300xg at 4°C, and then acidifying the solution with HCl to a 1 normal concentration.

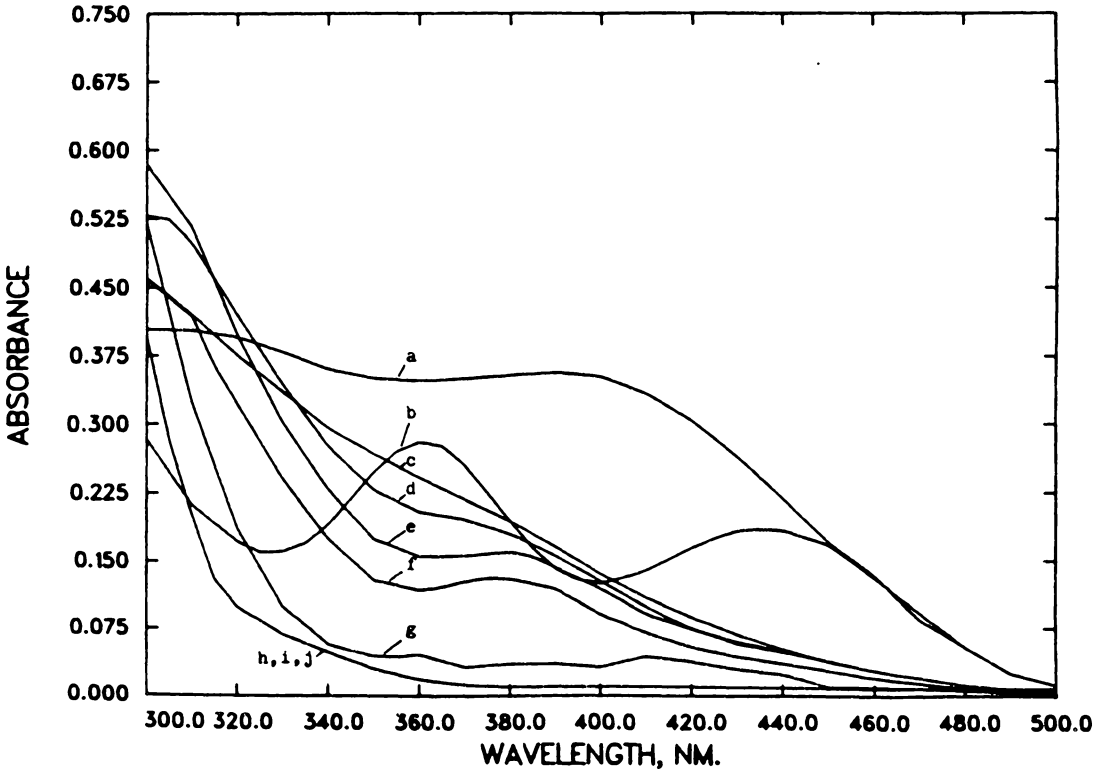

Figure 12: Absorption Spectra of Pd Complexes with Various Biological Molecules in Acidified E. coli K-12 Supernatant

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Figure 13: These data were obtained by extraction of the samples from Figure 11 with 1.0 ml of methylene chloride and measuring the absorbance of that layer at 408 nm. Extraction of the samples gave solutions having spectra as lettered below: a) lipoic acid, b) β-mercaptoethanol, c) 1 N HCl, d) cystine, e) dithiothreitol, f) cysteic acid, g) methionine, h) cysteamine, i) cysteine, j) reduced glutathione. As can be seen in the figure, samples d through j gave spectra virtually indistinguishable from background.

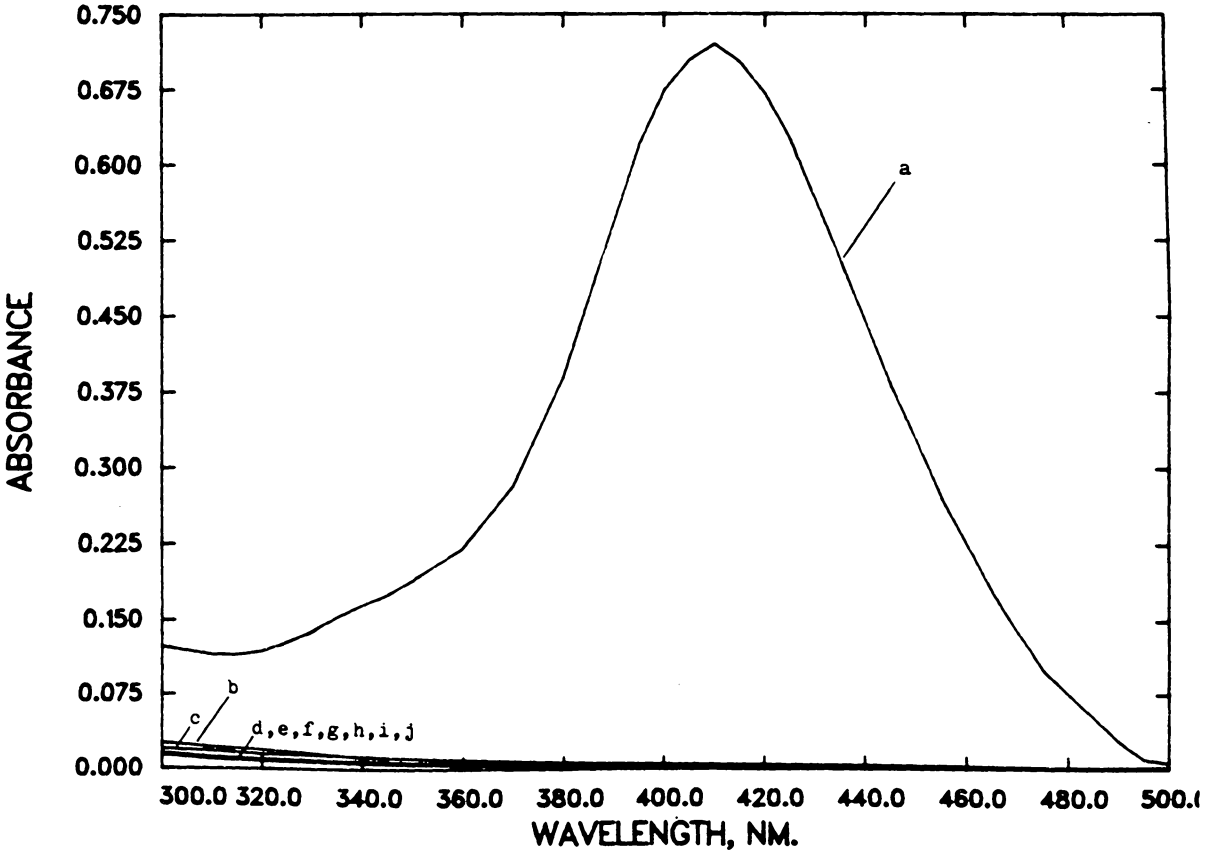

Figure 13: Absorption Spectra of Pd Complexes with Various Biological Molecules in HCl, CH_2Cl_2 Extract

Legend

Figure 14: These data were obtained by extraction of the samples from Figure 12 with 1.0 ml of methylene chloride and measuring the absorbance of that layer at 408 nm. Extraction of the samples gave solutions having spectra as lettered below: a) lipoic acid, b) β-mercaptoethanol, c) cystine, d) cysteic acid, e) methionine, f) cysteine, g) reduced glutathione, h) cysteamine, and i) acidified (1 N in HCl) *E. coli* K-12 supernatant. As can be seen from the figure, samples c through i have indistinguishable spectra.

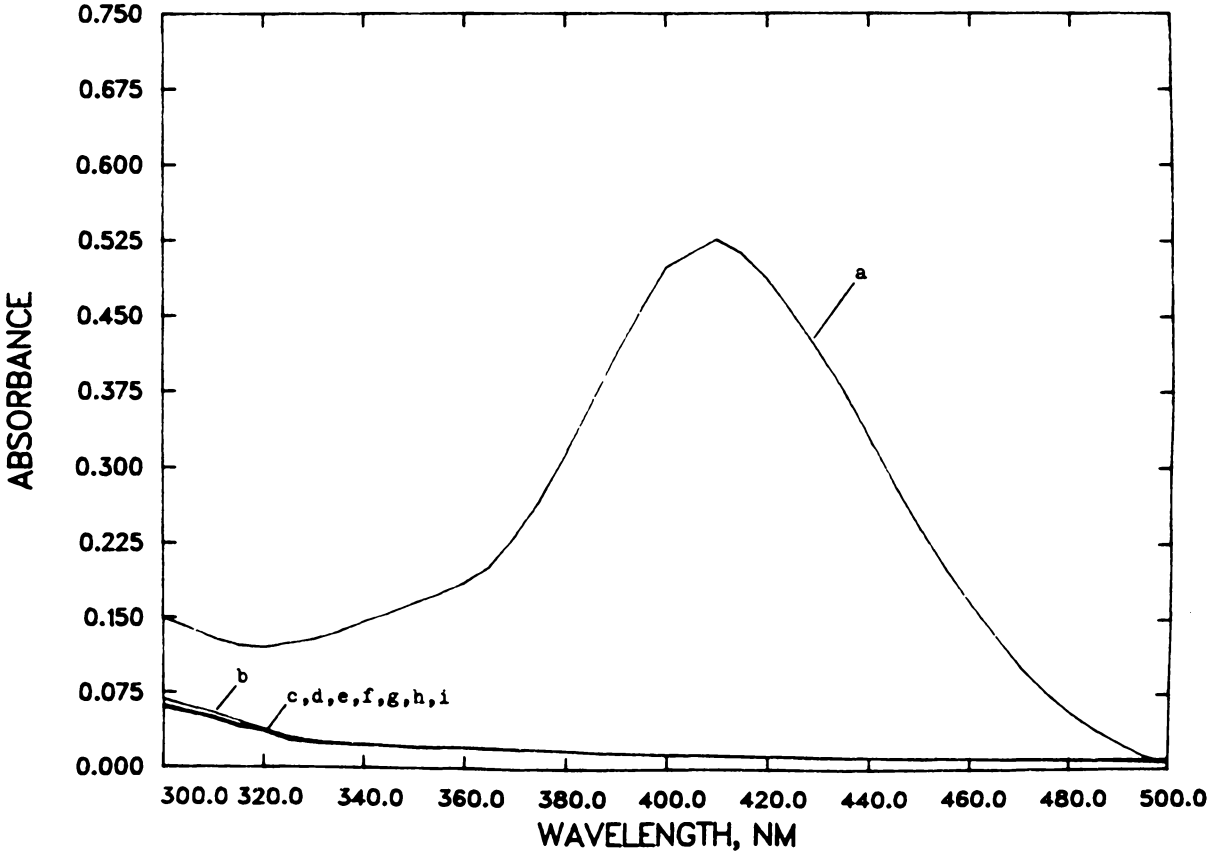

Figure 14: Absorption Spectra of Pd Complexes with Biological Molecules in Acidified E. coli K-12 Supernatant, CH_2Cl_2 Extract

LEGEND

Figure 15: The spectra shown are a comparison of the Pd-lipoic acid complex formed in 1 N HCl with the Pd-dithiolane complex formed in 1 N HCl. Spectrum A was obtained by adding 40 µl of 10^{-2}M Li_2PdCl_4 to a solution of 1.3 x 10^{-4}M lipoic acid in 1 N HCl. Curve B was obtained by adding 40 µl of 10^{-2}M Li_2PdCl_4 to 2.7 x 10^{-5}M 1,2-dithiolane in 1 N HCl.

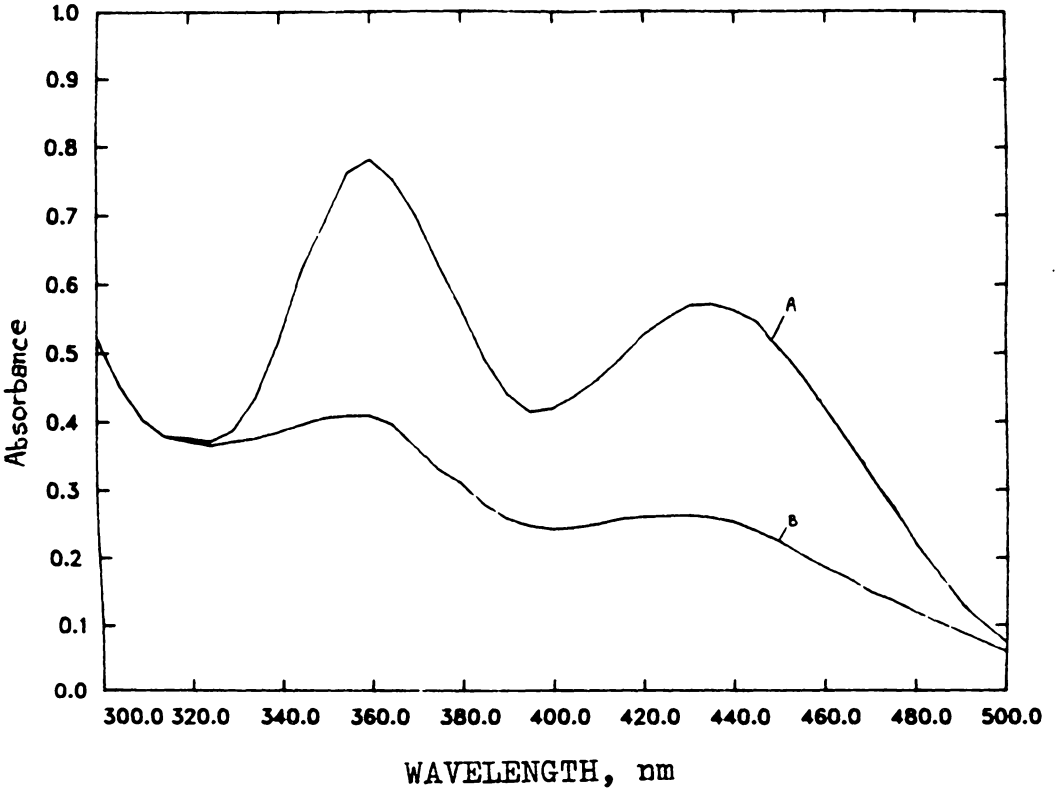

Figure 15: Comparison of the Pd-Lipoic Acid Complex with the Pd-1,2-dithiolane Complex, in 1 N HCl

the supernatant of an aerobic stationary phase culture of E. coli K-12 culture grown in the minimal salts medium is 9.97×10^{-9} M by gas chromatographic assay.

CHANGES IN LIPOIC ACID LEVELS IN E. COLI K-12 DURING CHANGING GROWTH CONDITIONS

Aeration of an anaerobic culture of E. coli increases the specific activities (Amarasingham, et al 1965, Gray, 1966, Thomas, 1972) and levels (Neidhardt, et al 1983a) of lipoic acid-containing enzymes. The following experiments were conducted to determine if lipoic acid biosynthesis was increased upon aeration of an anaerobic culture.

An anaerobic culture of E. coli K-12 was analyzed for total cellular lipoic acid over a 13 hour period. Cellular lipoic acid levels remained low (5 to 6 µg/g dry weight) throughout growth of the culture (Figure 16). During the growth of an aerobic culture of E. coli K-12, cellular lipoic acid levels increased steadily from 15 µg/g dry weight in lag phase to 20 to 25 µg/g dry weight during stationary phase (Figure 17).

An anaerobic culture of E. coli K-12 was aerated (6.6 l air/minute) at mid-log phase. Aeration resulted in a 2.5 to 4-fold increase in cellular lipoic acid levels. (Figure 18).

Legend

Table I: Method A - 40 µl of 10^{-2}M Li_2PdCl_4 was added to 5.0 ml of test solution. The absorbance at 358 nm was measured and the solutions then extracted with 1.0 ml of methylene chloride, each. The absorbance of the extracts was measured at 408 nm (one trial only).

Method B - Each of the test solutions (5.0 ml) was extracted with 1.0 ml of methylene chloride. The methylene chloride was removed under a stream of N_2 gas and the residue diluted to 5.0 ml with 8 x 10^{-5}M Li_2PdCl_4 in 1 N HCl. The absorbance at 358 nm was measured and the solutions then extracted with 1.0 ml of methylene chloride each. The absorbance of the extracts was measured at 408 nm (one trial only).

Method C - Each of the test solutions (5.0 ml) was extracted with 1.0 ml of methylene chloride. The free lipoic acid in the extracts was then quantitated by the gas chromatographic method (White, 1981)

Concentrations of the Pd-lipoic acid complex in aqueous solution and methylene chloride were determined using the respective molar absorptivity values, 3.5 x $10^3 M^{-1}cm^{-1}$ and 1.3 x $10^4 M^{-1}cm^{-1}$, determined previously.

TABLE I

Comparison of the Palladium Assay with the Gas Chromatography Assay for Lipoic Acid

SAMPLE*	LIPOIC ACID CONCENTRATION		
	By A_{358}	By A_{408}	By G.C.
E. coli K-12 Culture			
Method A	9.1×10^{-5}M	2.1×10^{-6}M	**
Method B	1.3×10^{-4}M	1.2×10^{-4}M	1.1×10^{-4}M
E. coli K-12 Supernatant			
Method A	6.6×10^{-5}M	2.9×10^{-6}M	**
Method B	1.3×10^{-4}M	1.0×10^{-4}M	1.0×10^{-4}M

* All samples contained 10^{-4}M authentic lipoic acid.

** The gas chromatographic method is not comparable to method A.

Legend

Figure 16: A 10% (v/v) inoculum of stationary phase (A_{550}=1.0) E. coli K-12 was added under anaerobic conditions to a degassed growth flask containing 4.3 liters of minimal medium plus 1% (w/v) glucose. The flask was kept anaerobic throughout growth and samples were removed under N_2 gas pressure.

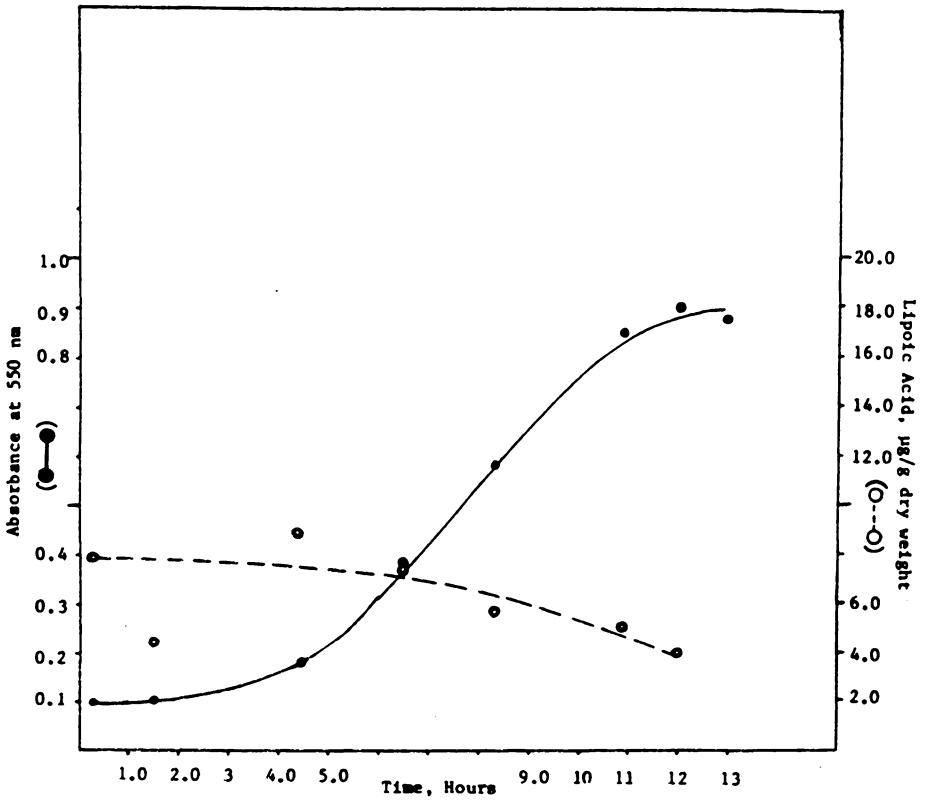

Figure 16: Anaerobic Growth of E. coli K-12 and Lipoic Acid Production

Legend

Figure 17: A 10% (v/v) stationary phase (A_{550}=2.0) aerobic inoculum of E. coli K-12 was added to 4.3 liters of minimal salts medium plus 1% (w/v) glucose. The medium had been aerated at 2.0 L air/L medium/minute for fifteen minutes prior to inoculation. The culture was aerated at 6.6 L air/minute throughout growth and samples were removed under air pressure.

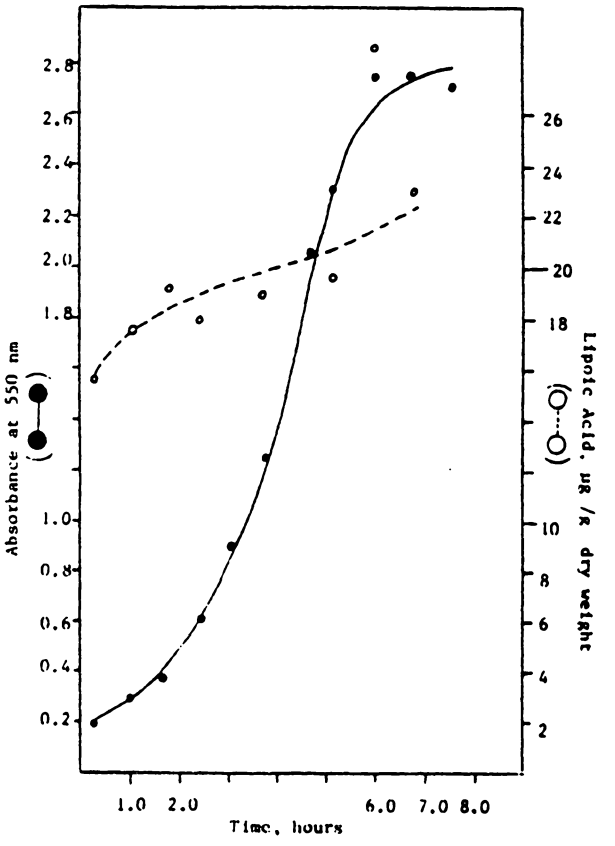

Figure 17: Aerobic Growth of E. coli K-12 and Lipoic Acid Production

Legend

Figure 18: A 10% (v/v) stationary phase (A_{550}=1.0) anaerobic inoculum of E. coli K-12 was added under anaerobic conditions to a degassed growth of minimal salts medium plus 1% (w/v) glucose. Samples were removed under N_2 pressure until the point indicated by an arrow in the figure. At this point, the sterile air supply was abruptly turned on at 6.6 L/minute and from then on samples were collected under air pressure.

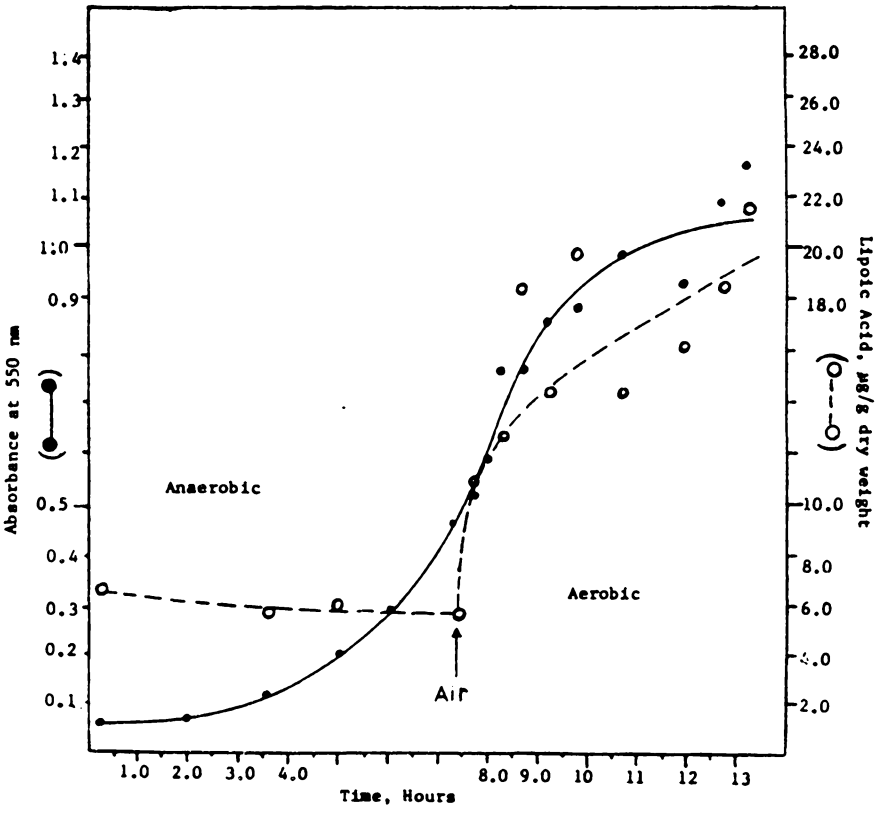

Figure 18: Anaerobic to Aerobic Transition of E. coli K-12: Effect on Lipoic Acid Production

STUDIES ON LIPOIC ACID BIOSYNTHESIS IN MAMMALIAN CELLS

The biosynthesis of lipoic acid in mammalian cell culture was investigated in two transformed cell lines, Chinese hamster ovary cells and mouse fibroblasts.

Acetate and octanoate are lipoic acid precursors in bacteria (Parry, 1977, White, 1980a). The incorporation of label from either [$^{2}H_{3}$]-acetate or [U-$^{2}H_{15}$]-octanoate into the lipoic acid of mammalian cells would therefore demonstrate that such cells can make lipoic acid by a similar pathway.

Chinese hamster ovary cells were relatively unaffected by millimolar concentrations of acetate and octanoate (Figure 19). After a three days of growing Chinese hamster ovary cells in media containing 6 mM [$^{2}H_{3}$]-acetate or 0.45 mM [U-$^{2}H_{15}$]-octanoate, the cells were harvested and the cellular lipoic acid was analyzed by mass spectrometry for deuterium incorporation (White, 1981). Less than 1% of the total cellular lipoic acid was labelled. Analysis of the fatty acids of the cells grown in 0.45 mM [$^{2}H_{3}$]-acetate by gas chromatography-mass spectrometry showed no deuterium incorporation above background levels.

To determine if glucose (5.5 mM) present in the cell culture media prevented the uptake and/or incorporation of the labelled substrates into either the lipoic or fatty acid pools, an attempt was made to grow Chinese hamster ovary cells in glucose-free media supplemented with acetate (10^{-7} M to 10^{-2} M). No increase in cell number was observed under these conditions.

Legend

Figure 19: 200 Chinese hamster ovary cells were grown in 5.0 ml of α-modified minimal essential medium, containing 10% (v/v) dialyzed fetal calf serum. The cultures were grown in various concentrations of acetate and octanoate by adding 100 ul of the appropriate sterile stock solution to each tissue culture dish. The cell colonies were counted macroscopically after fixing with 75% ethanol in glacial acetic acid and then staining with crystal violet. (o——o) Acetate concentrations. (▲- - -▲) Octanoate concentrations.

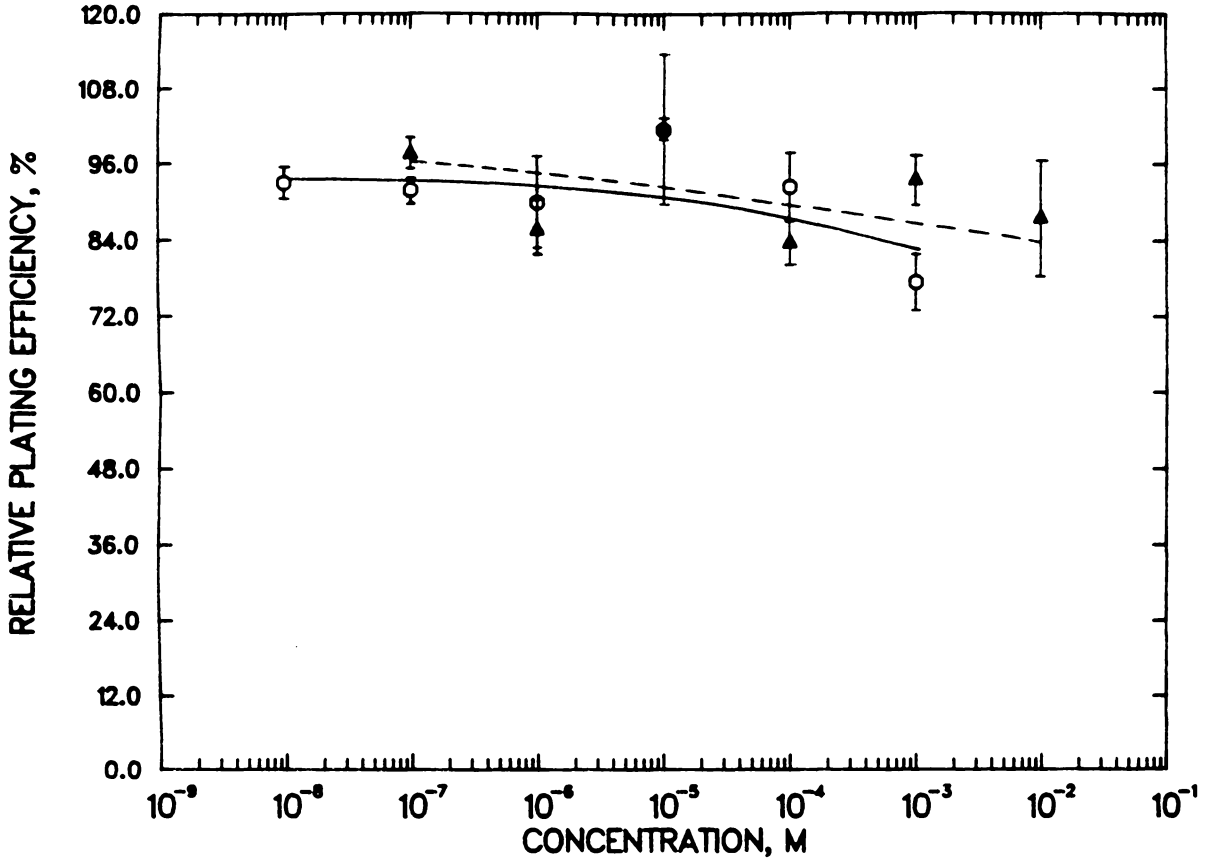

Figure 19: Viability of Chinese Hamster Ovary Cells in the Presence of Acetate and Octanoate

To test the hypothesis that Chinese hamster ovary cells, and another transformed cell line, the mouse fibroblast require lipoic acid for growth, the growth medium was varied with respect to its lipoic acid content. Under these conditions, both cell lines showed a requirement for lipoic acid. Unfortunately, the experiment could not be repeated after numerous attempts. This experiment therefore, did not determine, conclusively, if mammalian cells require lipoic acid for maximal growth.

Chapter V

DISCUSSION

A rapid assay for detection of lipoic acid in bacterial cultures has been developed. Extraction of the yellow Pd-lipoic acid complex into methylene chloride improves the specificity of the assay for lipoic acid, since it eliminates interferences from other sulfur-containing biological molecules. Standard curves have shown that the limit of sensitivity of the assay for free lipoic acid at an absorbance of 0.1 in acidified E. coli K-12 supernatant is 2.5×10^{-5} M, and 5×10^{-5} M in a methylene chloride extract of acidified E. coli K-12 supernatant.

Titration data are consistent with a one-to-one palladium to lipoic acid complex as the chromophore in 1 N HCl. Involvement of the sulfur atoms in this complex is supported by inspection of the absorption spectrum of a palladium complex formed with 1,2-dithiolane, which is nearly identical to the spectrum seen for the Pd-lipoic acid complex.

Extraction of lipoic acid-titrated samples of Li_2PdCl_4 into methylene chloride provided evidence that the chromophore in this solvent has a palladium to lipoic acid ratio of one-to-two.

Comparison of palladium assay with the gas chromatographic assay for lipoic acid has shown that at 0.1 mM concentrations of lipoic acid, the more rapid palladium assay can be used for quantitative as well as qualitative purposes. The quantitation of lipoic acid at 358 nm following a reaction of Li_2PdCl_4 with lipoic acid in either an acidified bacterial

culture or an acidified cell-free supernatant, will serve as a rapid screening assay for the isolation of a lipoic acid overproducer. Extraction of the free lipoic acid from either the acidified culture or the acidified cell-free supernatant before its reaction with Li_2PdCl_4 in 1 N HCl is useful as a quantitative screening assay for high (0.1 mM) concentrations of lipoic acid.

The mutagenesis and screening of E. coli K-12 led to the isolation of a mutant which excretes 1000-fold more biotin into the medium than the parent strain (Pai, 1983). Using the palladium-dependent lipoic acid assay, it would be possible to screen for a mutant which would similarly excrete 100-fold more lipoic acid into the culture medium. The free lipoic acid can be extracted from the culture and concentrated before its reaction with palladium. Therefore, it is possible to detect a mutant which excretes 100-fold more lipoic acid than the parent strain (10^{-8}M) by extracting the free lipoic acid from a 200 ml culture of the mutant and reacting the residue with palladium in 2 ml of 1 N HCl.

The four-fold increase in lipoic acid seen upon aeration of an anaerobic E. coli K-12 culture could prove useful in developing a cell-free system for studying lipoic acid biosynthesis. A cell-free system could be used to establish the true precursors in lipoic acid biosynthesis, which in turn may shed some light on the mechanism of lipoic acid biosynthesis.

Data obtained from studies with mammalian cells demonstrate that Chinese hamster ovary cells do not incorporate label into lipoic acid

from the two known bacterial precursors, [$^{2}H_{3}$]-acetic acid and [U-$^{2}H_{15}$]- octanoic acid. Therefore, if these cells do make lipoic acid, it occurs by a different route than that observed in bacteria.

Chapter VI

CONCLUSIONS

A rapid, colorimetric assay for lipoic acid has been developed. Since it is such a rapid assay, it will be useful for screening large numbers cells in an attempt to identify and isolate one which excretes large amounts (50 to 100 μM) of lipoic acid.

It was observed that cellular lipoic acid levels remain constant throughout the growth of an anaerobic culture of E. coli K-12. However, cellular lipoic acid levels in an aerobic culture of E. coli K-12 increased throughout cell growth. A further study demonstrated that aeration of an anaerobic culture of E. coli K-12 resulted in a rapid increase in cellular lipoic acid which continued throughout cell growth. This identification of a factor which regulates cellular lipoic acid levels in E. coli K-12 will be useful in further studies of lipoic acid biosynthesis.

Studies of lipoic acid biosynthesis in the transformed Chinese hamster ovary cell have demonstrated that this cell does not incorporate either acetate or octanoate, two known bacterial precursors of lipoic acid, into its lipoic acid. Therefore, if this cell makes its own lipoic acid, it does so by a different mechanism than that which occurs in bacteria.

Chapter VII

SUMMARY

A rapid assay for lipoic acid in bacterial cultures has been developed by reacting free lipoic acid with Li_2PdCl_4 in 1 N HCl. This assay has a limit of sensitivity of 2.5 to 5 x 10^{-5} M lipoic acid. It is possible to reduce the interference from other biological molecules by extracting free lipoic acid from the sample before reacting it with palladium. The chromophore in aqueous solution is a 1:1 palladium to lipoic acid complex whereas in methylene chloride it is a 1:2 palladium to lipoic acid complex.

Lipoic acid biosynthesis has been studied during the anaerobic and aerobic states of E. coli K-12. Production of lipoic acid during anaerobiosis is slower than the cell growth rate but during aerobiosis it is more rapid than the cell growth rate. Approximately 3-4 times more cellular lipoic acid per gram dry weight of cells is made during aerobic growth than during anaerobic growth. Upon aeration, cellular lipoic acid levels in an anaerobic culture of E. coli K-12 rapidly increased four-fold.

Studies with Chinese hamster ovary cells have demonstrated that neither acetate nor octanoate, two known bacterial precursors for lipoic acid, were incorporated into cellular lipoic acid. In addition, acetate was found not to be incorporated into the cellular fatty acids.

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