STUDIES ON LIPOIC ACID UPTAKE

By

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INTRODUCTION

The mechanism by which vitamins, or indeed any compounds, are taken into the living cell is unknown. The bacterial cell, with its rigid cell wall and well-defined membrane, presents an ideal system for a study of this mechanism, particularly since the growth and handling of these organisms present fewer problems than the much more elegant techniques required for comparable work with mammalian cells. Therefore, most of the current work in this field, as well as the best defined theories of mechanism, deal specifically with the bacterial cell.

The two principal theories on the mechanism of uptake are best represented by the work of Georges Cohen and Jacques Monod of the Pasteur Institute and that of Peter Mitchell of the University of Edinburgh.

Cohen and Monod support the theory that the transport of various compounds across the membrane and into the bacterial cell is due to the function of a group of enzymes called "permeases" which are located in the membrane complex (1). They describe certain cells which lack the ability to metabolize a specific substrate, even though they possess the relevant enzyme system in the cell-free extract. This inability or "crypticity" is attributed to the lack of a permease which would allow the substrate to be taken into the cells and thereby become vulnerable to metabolic enzymes.

Cohen and Monod further state that where the cryptic state concerns an entire class of chemical compounds such as the phosphorylated

nucleotides and hexose phosphates, the solubility or electrical properties of the class may forbid their passage through the membrane. The transport mechanism (permease) is inducible. They found that a labeled thiogalactoside was not taken up by cells grown in the absence of a galactoside, but was taken up by cells which had been induced. The accumulation was reversible in the presence of unlabeled thiogalactoside.

Monod's permease theory (1) would require specific permeases to catalyze the accumulation of the compounds rather than to serve as final acceptors. In the older stoichiometric theory, the accumulation within the cell would be due to a stoichiometric combination of the compound with specific final acceptor sites. The permease theory provides for an intracellular concentration which would be a steady-state intermediate between an entry reaction (catalyzed by stereospecific sites) and an independent exit reaction. If this exit rate remains constant, then the intracellular level of the substrate at equilibrium would be proportional to the activity of the permease.

Cohen and Monod argue that the stoichiometric theory, in highly induced cells, would require one binding site per cellular protein molecular weight of 2000 in the case of galactoside accumulation. Also, in this scheme, the number of specific sites would determine the saturation value for a compound and should be the same for all galactosides. Actually, Monod found that the saturation value varied by as much as a 5:1 ratio (1). Further, the rate of entry of galactosides should be proportional to the galactoside concentration and the number of free sites in the cell, while experimental evidence indicated that the initial rate of entry of a galactoside was not much faster

than its rate of exchange during the steady state. This remained true even at saturating concentrations when few binding sites remained free. Also, there appeared to be no significant differences in uptake when the concentration of the thiogalactoside was increased above a saturating concentration and therefore, the initial rate of entry appeared to be independent of concentration above saturation. The accumulation of galactosides was inhibited by typical uncoupling agents such as dinitrophenol and azide. Since these inhibitors have little effect on the in vivo hydrolysis of galactosides by intracellular galactosidases, the rate of hydrolysis is presumably controlled by the permeases and the function of the uncoupling agents is against the energy coupling which allows the permease reaction to function.

Monod also noted that when the concentration gradient is in favor of entry, as when the intracellular hydrolase splits the substrate on entry, the uncoupling agents do not inhibit (1). He cited further evidence for the permease theory by showing how normal Escherichia coli cells grown and induced in the presence of p-fluorophenylalanine form normal amounts of β-galactosidase but only traces of a permease, indicating that the analog had been incorporated into and had inactivated the permease.

Cohen and Rickenberg have shown that the uptake of the amino acid, valine, in E. coli K12 was quite rapid, even under conditions where protein synthesis was blocked (2, 3). Here also, the accumulation was inhibited by azide and 2,4-dinitrophenol and was optimal in the presence of an external energy source. Exchange of the accumulated valine was accomplished by several structurally related amino acids as well as unlabeled valine itself. The stereospecificity of the system

was illustrated by the effective competition of only the L-isomers, while the system showed little or no affinity for peptides containing valine or structurally similar amino acids.

The stereospecificity of the transport mechanism is further illustrated by the work of Leach and Snell who established the existence of three separate uptake systems in Lactobacillus casei (4). One of these systems was specific for glycine; another had equal affinity for either D- or L-alanine; while a third catalyzed the uptake of the peptides, glycyl-L-alanine and L-alanylglycine. The rate of uptake and accumulation of glycine-C^{14} from the peptides was substantially higher than from free glycine, amounting to about 7- and 10-fold respectively.

Mitchell disputes the permease theory and conceives that the activities ascribed to the permease may be due to ordinary metabolic enzymes located on the surface of the cell membrane (5). He envisions the cell wall functioning as a molecular sieve which allows the substrate molecules to pass freely through it into a space between the cell wall and the membrane which he calls the "periplasm." He also feels that some of these enzymes may be free in the periplasm, retained by the cell wall from diffusing into the medium outside the cell.

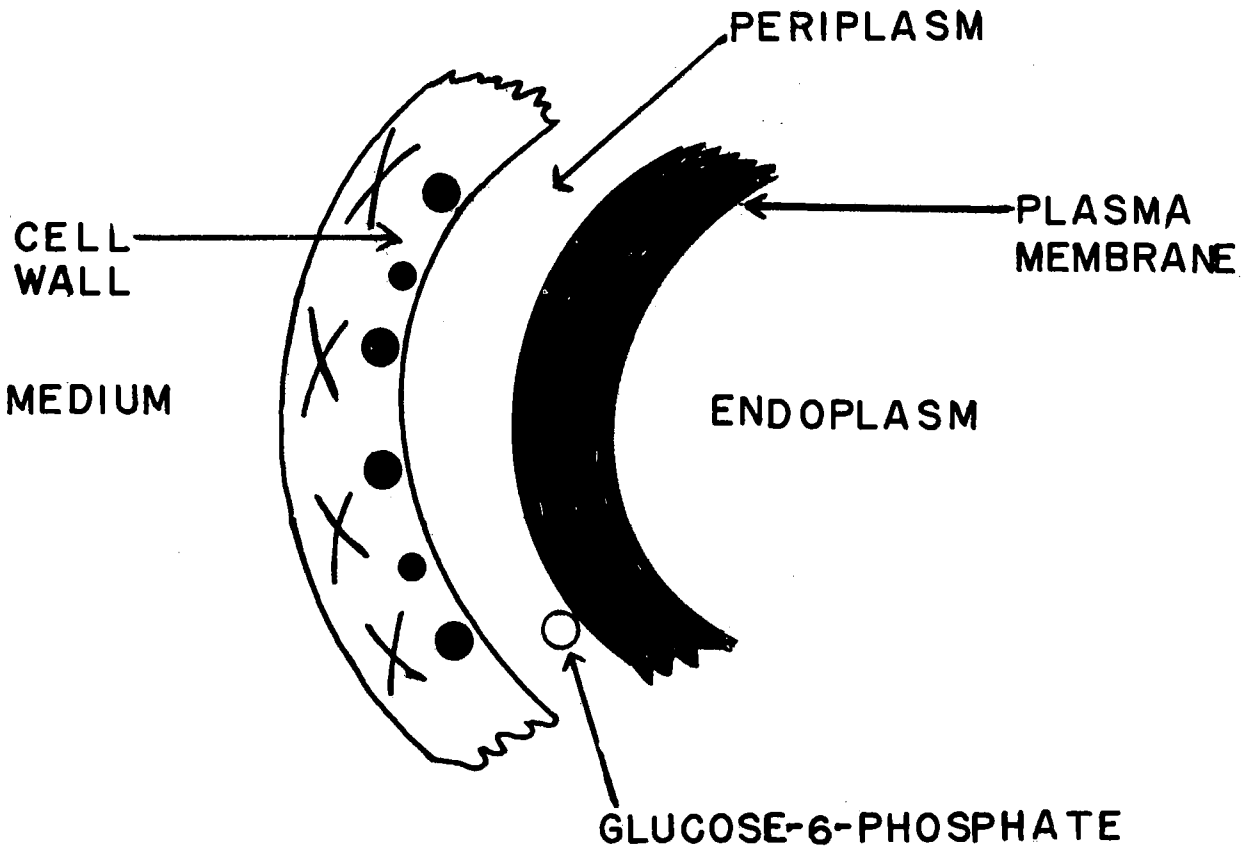
Mitchell noted that membrane fragments obtained from the staphlococci contained a number of enzyme activities, in many cases up to 90 per cent or more of the total enzymatic activity in the cell (5). He then posed the questions, "Why are these metabolic systems organized on the surface of the membrane" and What is the function of the enzymes and catalytic carriers located in the membrane complex?"

Mitchell proposes that in normal metabolism, the enzymes are specifically oriented in an organized membrane structure, and that as catalysis occurs, an actual microscopic vectorial movement into, and eventually across, the membrane is initiated. If we accept this picture of transport on the level of molecular dimensions, then the need to recognize the locational specificities of the enzymes as well as their substrate specificities becomes apparent.

Mitchell selected the enzyme glucose-6-phosphatase to illustrate the reason for the apparent crypticity of certain cells to specific substrates of which Monod has spoken. Only about 6 per cent of the total activity of this enzyme in E. coli seems to be associated with the cell membrane and it is therefore classed as a "soluble" enzyme (5). Mitchell found that although glucose-6-phosphate did not penetrate the plasma membrane of intact cells, the cells fermented it at a rate equivalent to the total glucose-6-phosphatase in the cell. The phosphatase of the intact-cell suspensions liberated the inorganic phosphate from the externally added glucose-6-phosphate into the medium, and not within the area confined by the membrane. This result led to the hypothesis that glucose-6-phosphatase in this organism is confined to the periplasm as illustrated in Scheme 1.

The existence of the periplasm has been confirmed microscopically. Anoptral contrast micrographs show that serum albumin (M.W. 70,000) does not penetrate the cell wall into the periplasm, which was enlarged by plasmolysis with 0.4 M sodium chloride.

Mitchell further proposed that the enzymes may be poised in an equilibrium that favors their segregation into the periplasm, the plasma membrane, or the cytoplasm, and that the designation of a

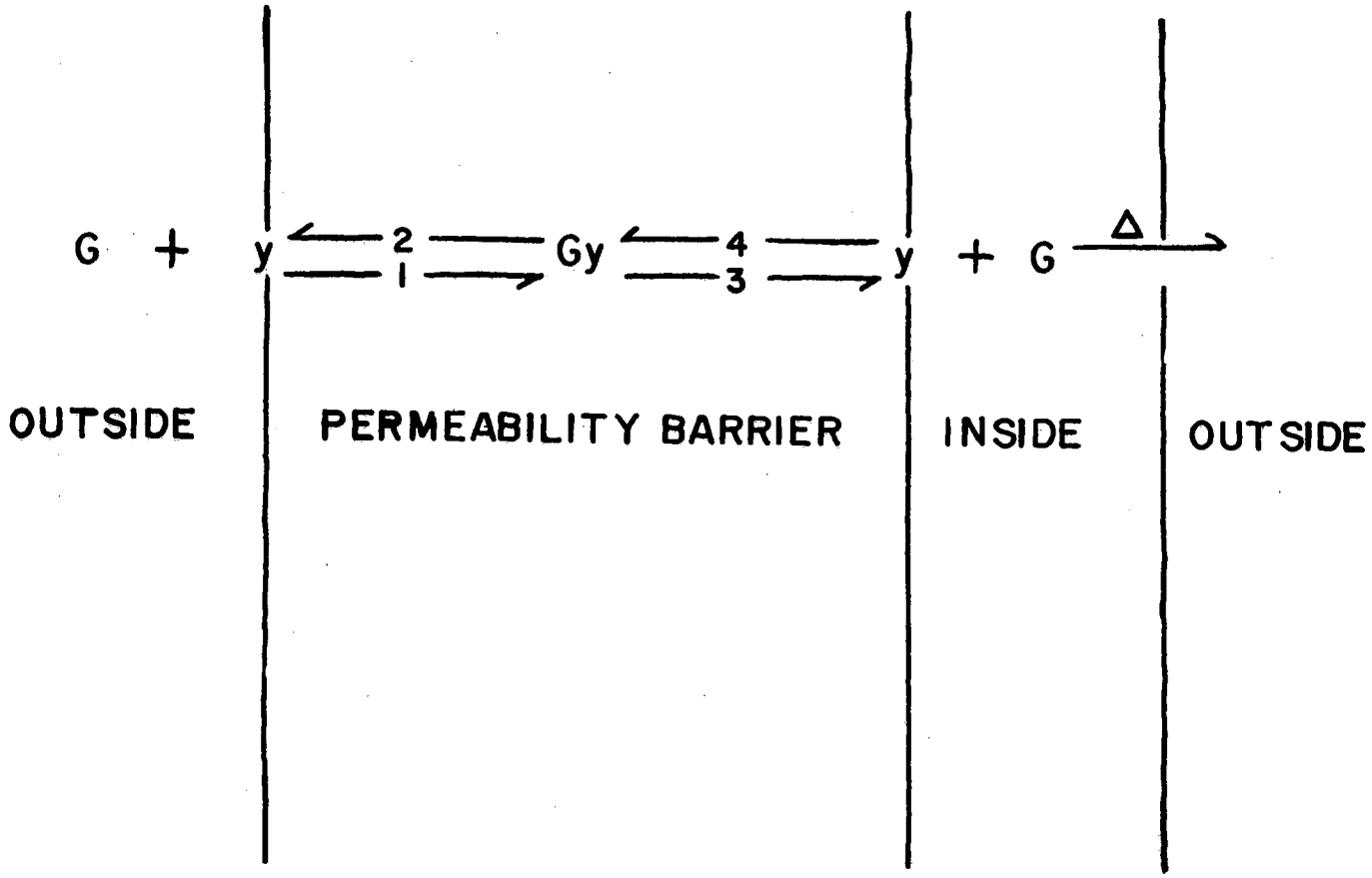

PERIPLASM
CELL WALL
PLASMA MEMBRANE
MEDIUM
ENDOPLASM
GLUCOSE-6-PHOSPHATE

SCHEME I

given enzyme as soluble or insoluble may be an expression of locational affinities for bonding the protein to complimentary "substratum" sites in the cell (5). He found that the α-ketoglutarate dehydrogenase activity of Micrococcus lysodeikticus is about equally distributed between a soluble fraction and a fraction tightly bound to the plasma membrane. When the membrane bound enzyme was inactivated with an irreversible inhibitor (iodoacetate at pH 8), and then treated with a normal protoplasm fraction, the membranes were found to have somewhat more than doubled their activity, while a like amount of activity was lost by the protoplasm fraction with which it was treated. This "reactivation" would imply that the distribution of the α-ketoglutarate dehydrogenase activity is dependent on the locational affinities of the soluble enzyme and specific substratum sites in the plasma membrane complex.

With these basic ideas, we may then attempt to summarize the somewhat conflicting hypotheses of Monod and Mitchell by briefly itemizing the basic tenets of each.

Monod assumes the existence of an osmotic barrier enclosing the entire cell, which is impermeable to most compounds. He assumes the existence of independent, stereospecific permeases which are functionally specialized for the transport of specific compounds through the osmotic barrier, and which are distinct from the ordinary metabolic enzymes dealing with these compounds. Lastly, he assumes that the permease may be coupled to some energy-yielding reaction when acting as a pump against a concentration gradient, or uncoupled, when functioning as an equilibrator of outside and inside concentrations. This concept is expressed in Scheme 2 where G represents the substrate, y is the permease, and the complex Gy is the hypothetical intermediate of

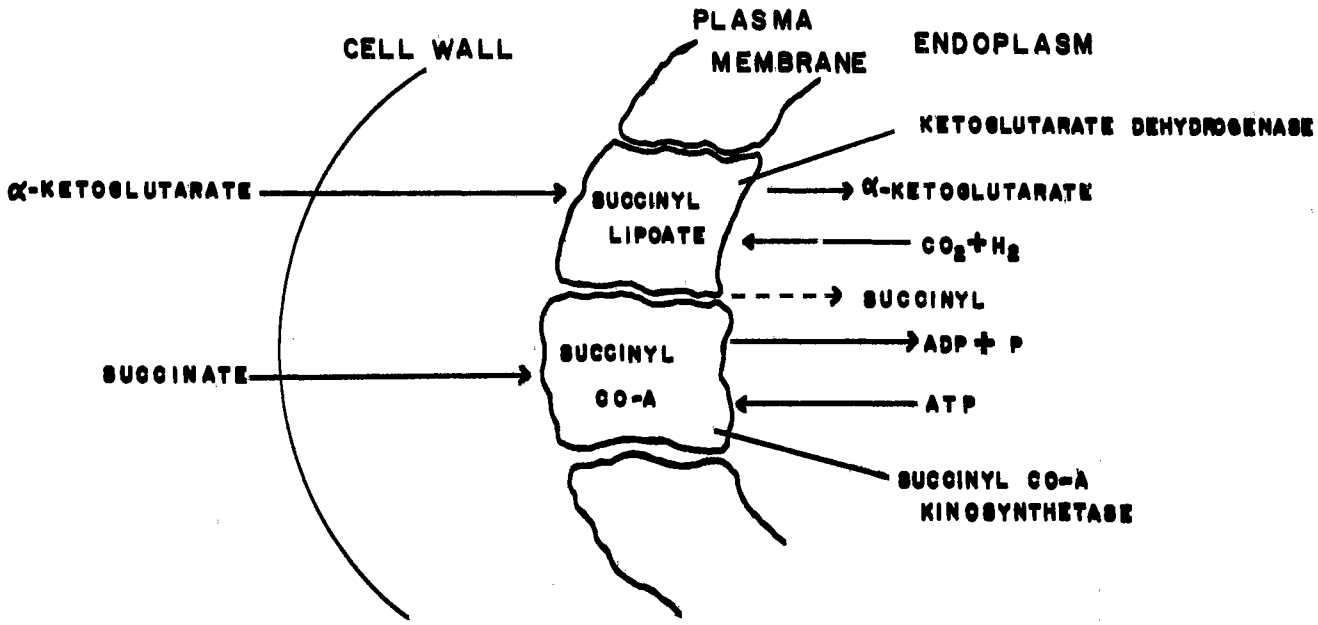

G + y
2
1
Gy
4
3
y + G
Δ
OUTSIDE
PERMEABILITY BARRIER
INSIDE
OUTSIDE

SCHEME 2

transfer within the cell membrane. He also suggests that the net effect of coupling with an energy donor is to inhibit, in some cells, the internal association reaction (reaction 4) allowing the substrate to accumulate until the concentration is high enough for non-specific leakage to occur. Horecker also suggests that accumulation may depend on a retention mechanism functionally different from the entry process (6).

Mitchell, on the other hand, assumes a molecular sieve action on the part of the cell wall, a periplasm between the plasma membrane and the cell wall in which ordinary metabolic enzymes can function either in solution or attached to the plasma membrane, and finally the osmotic barrier itself. He further assumes that the determining factor between soluble and insoluble enzymes is the affinity for bonding between the protein and the substratum sites in the organized plasma membrane as well as the number of sites available. This idea can best be expressed by his hypothetical diagram for succinate and α-ketoglutarate translocation through the membrane of M. lysodeikticus as shown in Scheme 3. The dehydrogenases are depicted as being part of the plasma membrane and anchored to each other by specific residual bonds.

So far we have discussed only mechanisms for generalized metabolites or their analogs. What mechanism might be predicted for the vitamins which are required in catalytic amounts and, in general, are not broken down by the cellular enzyme complement? Certainly the stepwise passage from one metabolic enzyme to another through the cell membrane would not be applicable to the vitamins. Very little information on the uptake of vitamins in microorganisms has been obtained. However, three vitamins have been studied to a limited extent.


CELL WALL
PLASMA MEMBRANE
ENDOPLASM
KETOGLUTARATE DEHYDROGENASE
α-KETOGLUTARATE
SUCCINYL LIPOATE
α-KETOGLUTARATE
$CO_2 + H_2$
SUCCINYL
SUCCINATE
SUCCINYL CO-A
ADP + P
ATP
SUCCINYL CO-A KINOSYNTHETASE

SCHEME 3

Oginsky studied the uptake of Co^{60} labeled vitamin B_{12}, noting that an extremely rapid uptake occurred in resting cells (7). She found that 87 per cent of the total uptake occurred even without incubation. A time lag of about 30 minutes was noted before manometric determination was possible but it was apparently not due to slow absorption. Respiring cells showed a somewhat slower uptake compared to the resting cells and some loss of B_{12} was observed when glucose was added to cells after absorption which was not due to exchange.

Lichstein and Ferguson investigated the permeability of L. arabinosus to biotin but did not measure the velocity of the uptake (8). They found that the uptake increased appreciably in the presence of glucose while iodoacetate and homobiotin inhibited the reaction.

In 1959, Wood and Hitchings studied the uptake of folic acid by a variety of microorganisms (9). In all cases where uptake occurred, they found that glucose was required. Considerable variation in the ability of specific organisms to absorb folic acid was apparent. Streptococcus faecalis showed a rapid uptake from which a quantitative release of the absorbed compound could be obtained by heating the washed cells. L. casei degraded folic acid to a diazotizable amine while Pediococcus cerevisiae produced the amine only when high concentrations of folic acid were used. E. coli, in contrast, showed no uptake. In all cases where uptake occurred, a pH maximum of about 6.5 and a temperature optimum of 37° C was observed; a maximum level of accumulation was reached within 5 to 10 minutes.

The uptake of the vitamin, lipoic acid, has not been studied. This compound presents an excellent subject for study from several standpoints. First, it is a relatively simple molecule, the synthesis

of which has been established. It does only a few things within the bacterial system and these functions are well defined. Its method of binding to the enzyme is known. In short, it presents a simplified approach to the study of vitamin uptake.

Although lipoic acid as a specific compound has been known for only a little over ten years, substances exhibiting a similar biological activity were recognized independently by several workers as early as 1937, when Snell and co-workers observed that extracts of several natural materials stimulated the growth of lactic acid bacteria (10, 11). In 1947, Gunsalus demonstrated that a factor in yeast extract was required by the bacterial mutant, S. faecalis 10C1, for the oxidation of pyruvate which he called the pyruvate oxidation factor (12).

In 1949, Stokstad reported that the growth factor, protogen, was required by the protozoan Tetrahymena geleii and developed a specific assay for the factor (13). Two years later, Snell and Broquist noted a high protogen and pyruvate oxidation factor activity in material being assayed for the acetate replacing factor and suggested that the three factors might be identical (14). Their hypothesis was confirmed in 1951 when Reed, Gunsalus and co-workers in collaboration with the Eli Lilly Research Laboratories first isolated crystalline lipoic acid from beef liver hydrolysate (15). The "B. R." factor necessary for the growth of Butyribacterium rettgeri on a lactate medium, which was first described by Kline and Barker, was also found to be replacable by lipoic acid (16, 17).

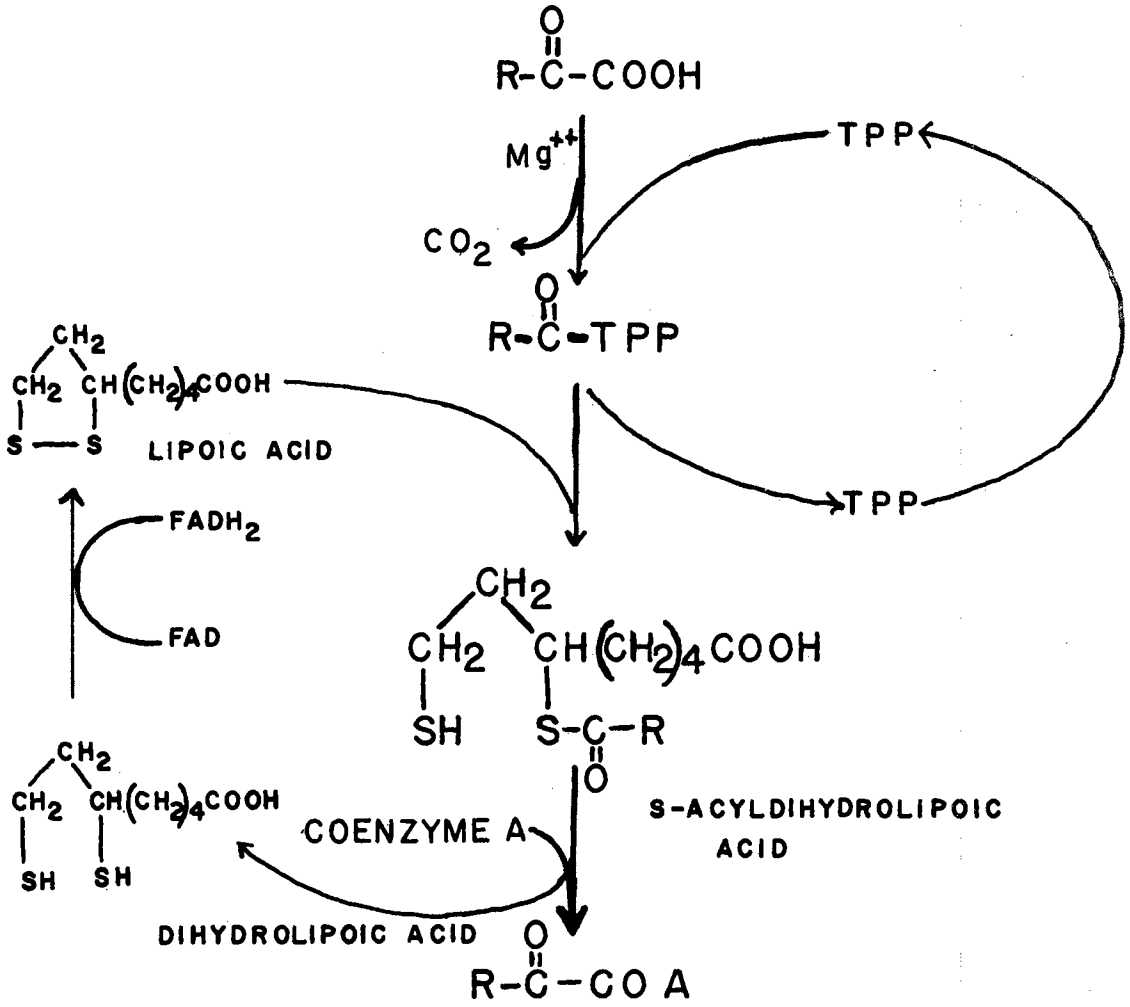
The active form of lipoic acid was found to be (+)-α-lipoic acid (18). Although lipoic acid has been suggested as a direct electron

acceptor from the illuminated chlorophyll system in photosynthesis (19) and as a cofactor for the liver enzyme sulfite oxidase, which oxidizes inorganic sulfite to sulfate (20), its best defined functions are in the acyl generation, acyl transfer, and oxidative reactions of the α-keto acids (21). Nawa, Brady, Koike and Reed found lipoic acid was bound to the enzyme, dihydrolipoic transacetylase (2.3.1.12 acetyl-CoA: dihydrolipoate S-acetyltransferase), by a peptide bond between the carboxyl of lipoic acid and the ε-amino group of lysine on the enzyme (22). The reaction sequence for oxidative decarboxylation of α-keto acids is shown in Scheme 4.

After the α-keto acid has been decarboxylated by the thiamine pyrophosphate-carboxylase complex, the acyl residue is transferred to the sulfur atom attached to the 6-carbon of the bound lipoic acid forming a thioester bond. The acyl residue is then attached to co-enzyme A to form the acyl-CoA derivative while the bound lipoic acid is oxidized in a flavoprotein mediated step.

Since the uptake of lipoic acid remains one of the few unsolved problems in the biochemistry of this compound, these studies were undertaken to establish some of the properties of the uptake system with the additional hope of isolating a carrier-lipoic complex.

FUNCTION OF LIPOIC ACID IN THE DECARBOXYLATION OF α-KETO ACIDS



SCHEME 4

EXPERIMENTAL PROCEDURE

A. Materials.

Unlabeled DL-α-lipoic acid, 1,2-dithiolane-3-butyric acid, and 1,2-dithiolane-3-caproic acid were generously supplied by Dr. Lester J. Reed of the University of Texas. Other unlabeled compounds were of reagent grade and were obtained from regular commercial sources.

Adipic acid-monoethylester was prepared by the method of Fichter and Laurie in a 57.4 per cent yield (23). This compound reacted with thionyl chloride as described by Berg to produce ethyl δ-chloroformyl-valerate in an over-all yield of 54.1 per cent (24). Ethyl DL-6,8-dichlorooctanoate was prepared by reacting ethyl δ-chloroformyl-valerate with ethylene in a Friedel-Crafts type reaction followed by reduction with sodium borohydride and halogenation via thionyl chloride as described by Reed and Niu (25). Over-all yield at this stage was 11.6 per cent. The ester was hydrolyzed by refluxing for 18 hours in 12 N HCl as described by Acker and Wayne producing DL-6,8-dichloro-octanoic acid in a 5.6 per cent over-all yield (26).

Elemental S^{35} (25 mc) was reacted with Na_2S in alcohol to form the polysulfide, Na_2S_x, which was combined *in situ* with the DL-6,8-dichlorooctanoic acid to form DL-α-lipoic acid-S^{35} (26). The crude lipoic acid (yellow oil) was placed in a sublimation tube and distilled onto a dry-ice filled finger under a high vacuum as described by Thomas and Reed (27). Over-all yield for the entire synthesis was 2.1 per cent based on adipic acid.

The final product had a melting point (uncorrected) of 57-58°

and showed an ultra-violet absorption spectrum identical to a known sample of DL-α-lipoic acid. Ascending paper chromatography in two solvent systems followed by color development using sodium cyanide (1 M) followed by sodium nitroprusside (1 M) yielded a single spot. This spot contained about 99 per cent of the total radioactivity, about 1 per cent remaining at the origin. Specific activity of the final product was 46 μc/mg.

B. Methods.

1. Growth of Cells

Cells of S. faecalis were grown for 8-10 hours at 37° in the lipoic acid-free synthetic medium described by Gunsalus and Razzell (28), harvested by centrifugation, washed twice with cold salts solution, and then suspended in the neutral salts solution described by Leach and Snell (29). The cells were used within 2 hours after preparation and the suspension was kept in an ice bath until experiments were begun.

2. Uptake Studies

The concentration of cells was determined by reading the optical density in a Bausch and Lomb Spectronic-20 spectrophotometer and comparing the reading to a standard curve of mg of dry weight/ml vs. optical density. A quantity of cell suspension sufficient to give a final concentration of 0.3 mg per ml dry weight was added to a tube containing the neutral salts solution (29) previously described. The cells were allowed to equilibrate at the temperature of the experiment for 15 minutes. Glucose was added to a final concentration of 1 mg/ml to insure an adequate energy source and the incubation was continued for 15 minutes longer. At this time, labeled lipoic acid was added and

aliquots were removed at appropriate intervals using either a blow-out pipette or a Cornwall syringe with cannula set for 0.5 ml.

With the exception of preliminary studies to determine the effect of temperature on uptake, all regular uptake studies were done at 20°. This temperature was selected in preference to the growth temperature (37°) since the lowered uptake made the reaction easier to control and allowed study of the kinetics. When a higher accumulation of intracellular lipoic acid was desired, as in the fractionation studies, a temperature of 37° was used. It was found desirable to perform the energy requirement and 2,4-dinitrophenol inhibition studies at 37°, since the higher temperature made the depletion of endogeneous energy sources more rapid.

Stopping the uptake was accomplished by ejecting the aliquot either into mushy ice (1 ml of frozen salts solution) or into a centrifuge tube previously cooled to the temperature of liquid nitrogen and suspended in a Dewar flask of the coolant.

An experiment was designed to determine the relative damage caused to the whole cells by each process. Using the optical density at 260 mμ as a measure of the cytoplasmic contents released, cells were frozen rapidly, slowly (-24°), and ejected into mushy ice, then thawed, centrifuged, and the supernatant assayed on a Beckman DU spectrophotometer. To obtain a measure of total cell contents, another aliquot was sonicated for 45 minutes using a Raytheon 10KC sonic oscillator. The results of this experiment are shown in Table I.

Collection of the cells using the millipore filter technique proved inapplicable. In the concentration range from 10 μg/ml to 500 μg/ml, the lipoic acid in the medium was absorbed by the filter

TABLE I

RELEASE OF CELL CONTENTS

Five 5 ml cell suspensions containing 1 mg/ml of cells and 1 mg/ml of glucose were treated as shown in table, thawed in cold (if frozen), centrifuged, and the optical density of the supernatant read at 260 mμ. A measure of total release was obtained by sonication of one aliquot for 45 minutes using a Raytheon 10KC sonic oscillator.

Treatment	O.D.$_{260\ m\mu}$
Not Frozen	0.086
Quick Frozen (N_2)	0.121
Slow Freezing (-24°)	0.131
Mushy Ice	0.062
Total Release	1.70

material to such an extent as to make the method useless. In each case, the zero time aliquot (no cells) had a higher activity than those aliquots containing cells.

After collecting the cells as described, the aliquots were allowed to thaw slowly in the cold (2°). The cells were removed by centrifugation and washed twice with cold neutral salts solution which was used as the original suspension medium. The two 1 ml washings were found to be sufficient to remove essentially all the radioactivity from the surface of the cells as indicated by the assay of the original medium and the two successive washings shown in Table II.

After the final washing, the cell pellet was suspended in 0.5 ml of distilled water and transferred to 1.25 in. planchets using a Pasteur pipette. The water was removed using radiant heat and the radioactivity was determined using a Baird Atomic thin-window gas flow counter. All aliquots were counted to 3,000 total counts. On an average sample counting 300 counts per minute, this method gave approximately 2 per cent counting error. Efficiency of counting was 20-25 per cent based on a C^{14} standard.

3. Fractionation of Cells

To determine the distribution of the lipoic acid in the cell, a modification of the fractionation method described by Park and Hancock was used (30). Early fractionations by the original method indicated a high percentage of the total radioactivity in the lipid (alcohol soluble) fraction and a very low percentage in the pool (trichloroacetic acid soluble) fraction. This artifact was found to be due to the insolubility of the lipoic acid in the acidic solution. In all later fractionations, the pool lipoic acid was extracted by

TABLE II

REMOVAL OF ABSORBED RADIOACTIVITY BY WASHING

Cells from a typical uptake experiment were thawed in the cold (2°) and separated from the supernatant (uptake medium) by centrifugation. The cell pellet was washed twice by centrifugation using 1 ml quantities of cold neutral salts solution. Radioactivity in the supernatant and washings was measured to determine the efficiency of the washing process.

Fraction	Volume	Total Counts Per Minute	Per Cent of Total
	ml		
Supernatant	0.5	65,500	70.5
1st Washing	1.0	27,258	29.4
2nd Washing	1.0	99	0.1

boiling the cells in distilled water for 15 minutes; the remainder of the extraction procedure was carried out as described in the literature. A comparison of fractionation by each method is shown in Table III.

Cells used in the fractionation were exposed to lipoic acid as described in the uptake procedure except that the cell concentration of 1 mg/ml was used. An aliquot was removed for determination of total radioactivity after the cells had been washed and suspended in distilled water prior to boiling. At least 5 mg of washed cells were used for each fractionation.

TABLE III

A COMPARISON OF FRACTIONATION BY THE METHOD OF PARK AND HANCOCK AND A MODIFIED METHOD

Two 5 ml suspensions of cells (1 mg/ml) and glucose (1 mg/ml) in neutral salts solution were exposed to radioactive lipoic acid (10 μg/ml) for 30 minutes at 20° as described under normal uptake conditions. After washing, one cell pellet was fractionated by the method of Park and Hancock (30). The other pellet was first boiled in 5 ml of distilled water for 15 minutes to remove the pool lipoic acid; the remainder of the fractionation was the same as described for the first pellet.

Fraction	Per Cent of Total Radioactivity	
	Method of Park and Hancock	Modified Method
Water (boiling)	----	57.2
Cold trichloroacetic acid (5% at 0°)	0.36	2.2
Ethanol: water 3:1 v/v	54.9	11.4
Hot trichloroacetic acid (5% at 90°)	0.22	3.9
Trypsin soluble	18.2	9.4
Residue	21.1	14.6

RESULTS

A. Effect of Cell Concentration on Uptake.

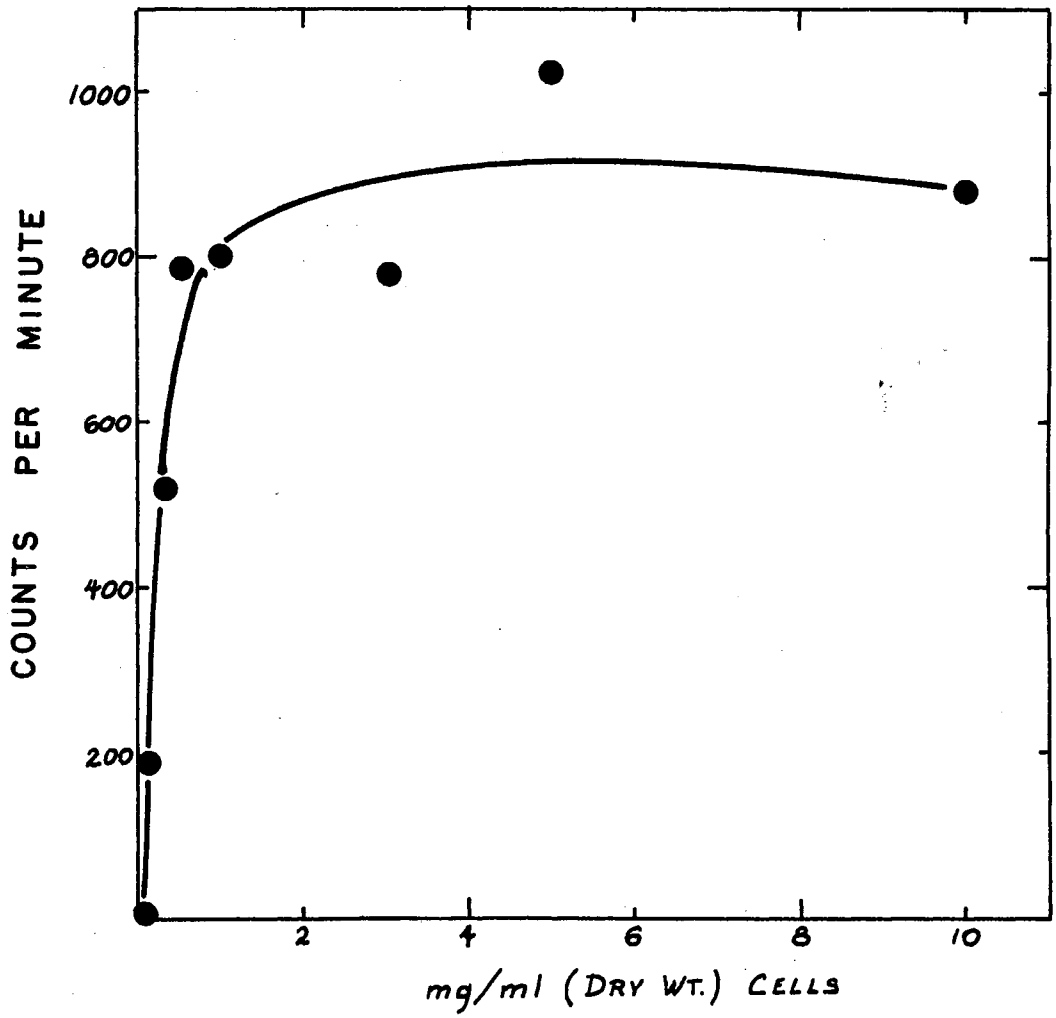
To find the region of cell concentration over which lipoic acid uptake was proportional to the number of cells, washed cells in varying concentrations from 0 to 10 mg/ml were exposed to a glucose concentration of 1 mg/ml for 15 minutes at 37°. Then radioactive lipoic acid was added to a final concentration of 10 μg/ml as previously described. Uptake was proportional to cell concentration up to 500 μg/ml. Then a plateau was reached, above which an increase in cell concentration failed to produce a corresponding increase in uptake. This result, shown in Fig. 1, may be due to an equilibrium-volume relationship at the higher concentration or to lack of some component in the uptake system. A cell concentration of 300 μg/ml was used in most of the following experiments.

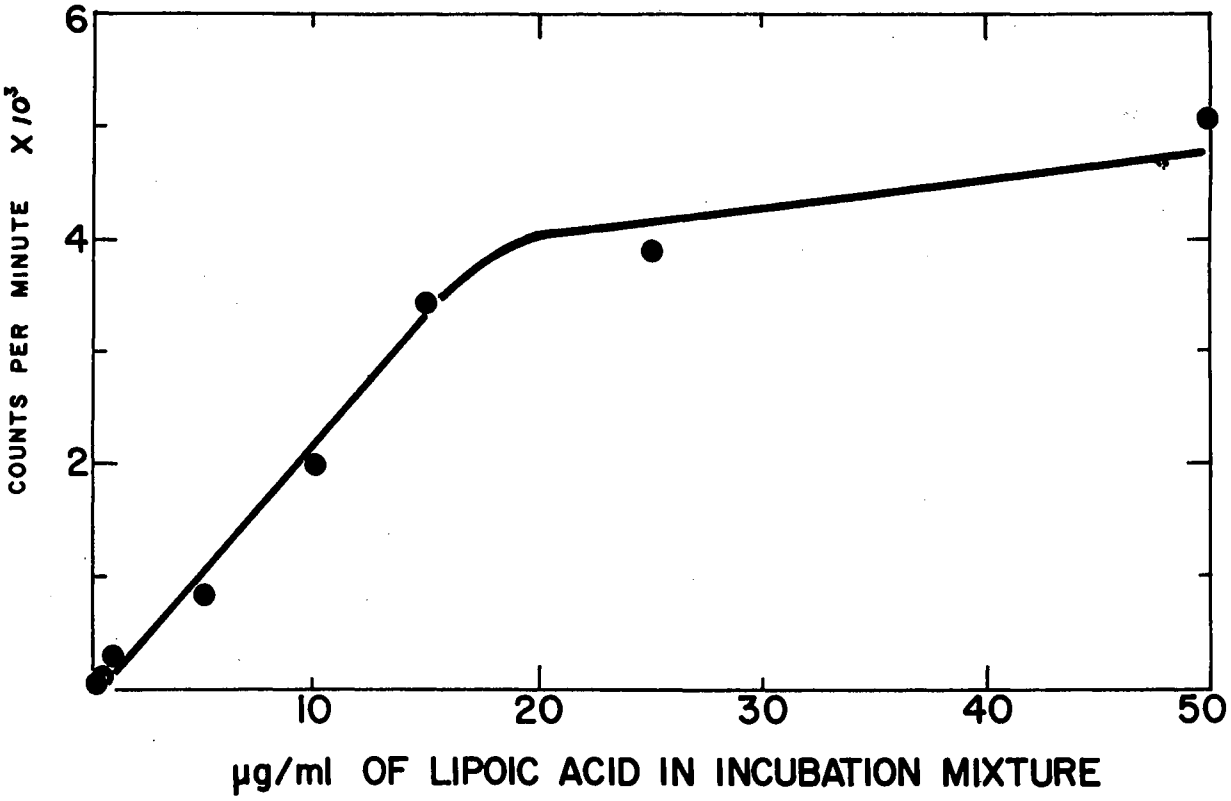
B. Effect of Lipoic Acid Concentration on Uptake.

To determine the substrate concentration necessary to saturate the uptake system, cell concentrations of 1 mg/ml were incubated with varying concentrations of radioactive lipoic acid after incubation with glucose. The curve shown in Fig. 2 exhibits the typical hyperbolic form of a Michaelis saturation curve and indicates that a concentration of 20 μg/ml is sufficient to saturate the system under the conditions of study.

C. Effect of Temperature on Uptake.

A series of uptake studies at varying temperatures was undertaken to determine whether the uptake mechanism might be temperature dependent.


COUNTS PER MINUTE
1000
800
600
400
200
2
4
6
8
10
mg/ml (DRY WT.) CELLS


6
4
2
COUNTS PER MINUTE X 10^3
10
20
30
40
50
µg/ml OF LIPOIC ACID IN INCUBATION MIXTURE

The extent of uptake was proportional to temperature as shown in Fig. 3. A slight but significant uptake occurred at 0° which was about 1/10 of that accumulated at 37°. Uptake at all temperatures was characterized by a very rapid accumulation up to about 30 seconds followed by a slight decline and then a plateau. The effect of temperatures above 37° was not studied.

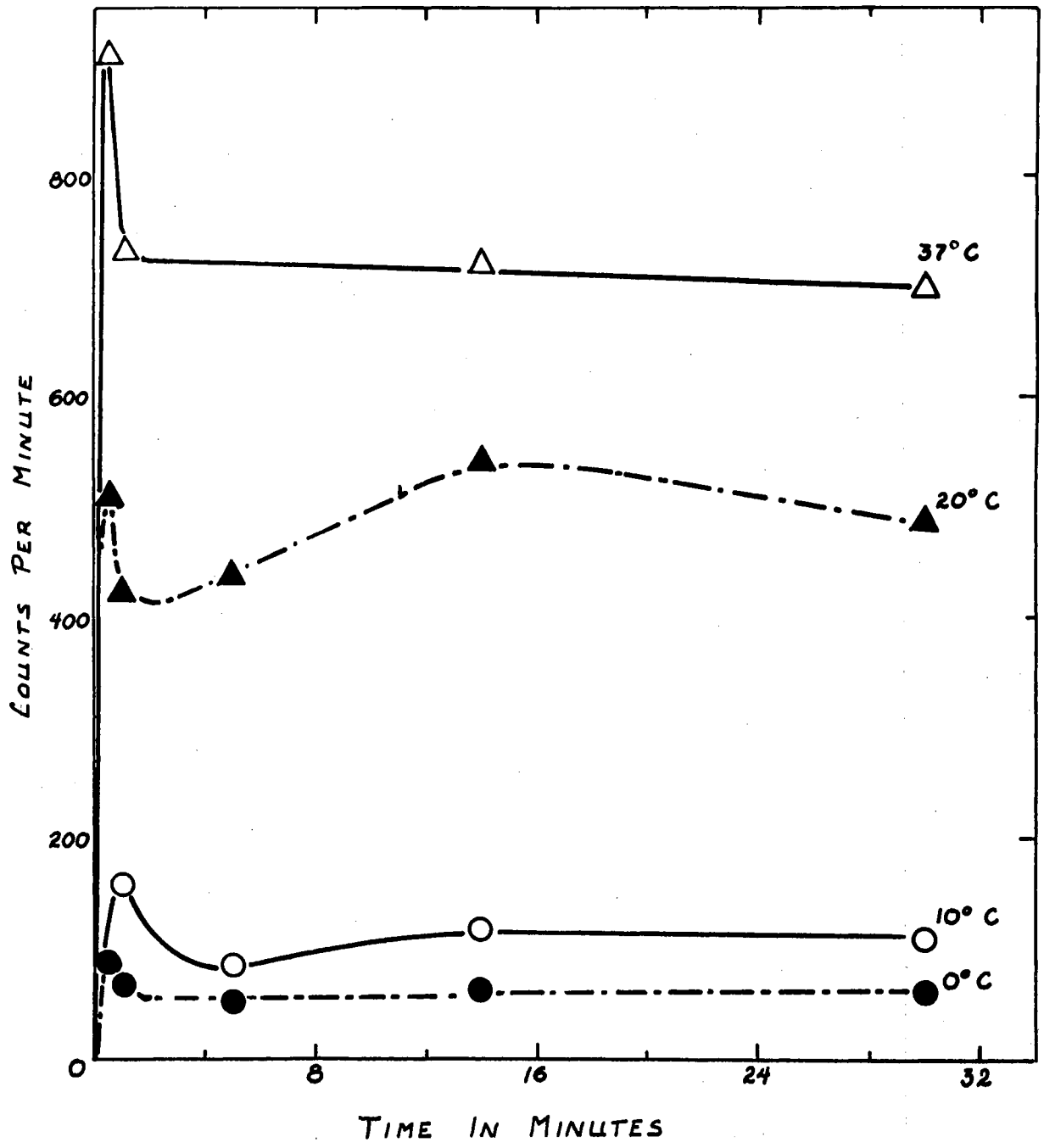
D. Uptake by Cells Grown in the Presence of Lipoic Acid.

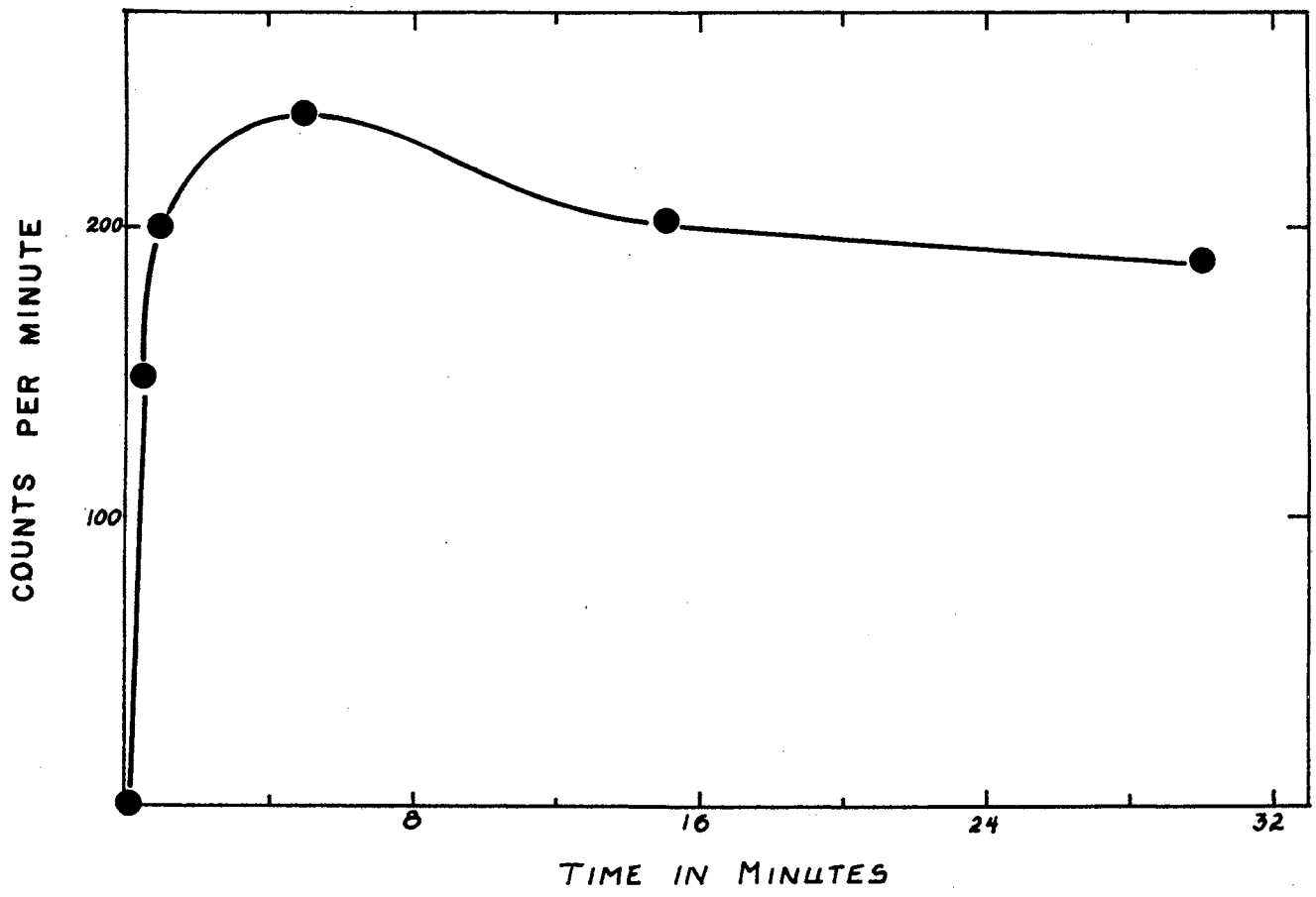
When cells were grown in the defined medium supplemented with 50 μg/ml of unlabeled lipoic acid and exposed to standard uptake conditions at 20°, the curve shown in Fig. 4 resulted. In this curve, the maximum accumulation time was somewhat later than when the cells were grown in a lipoic acid-free medium, occurring at about 5 minutes instead of 30 seconds. The total accumulation was approximately half of that noted in lipoic acid-free cells under the same uptake conditions. In this case, the maximum accumulation was only slightly higher than the plateau value indicating that an exit system was already present.

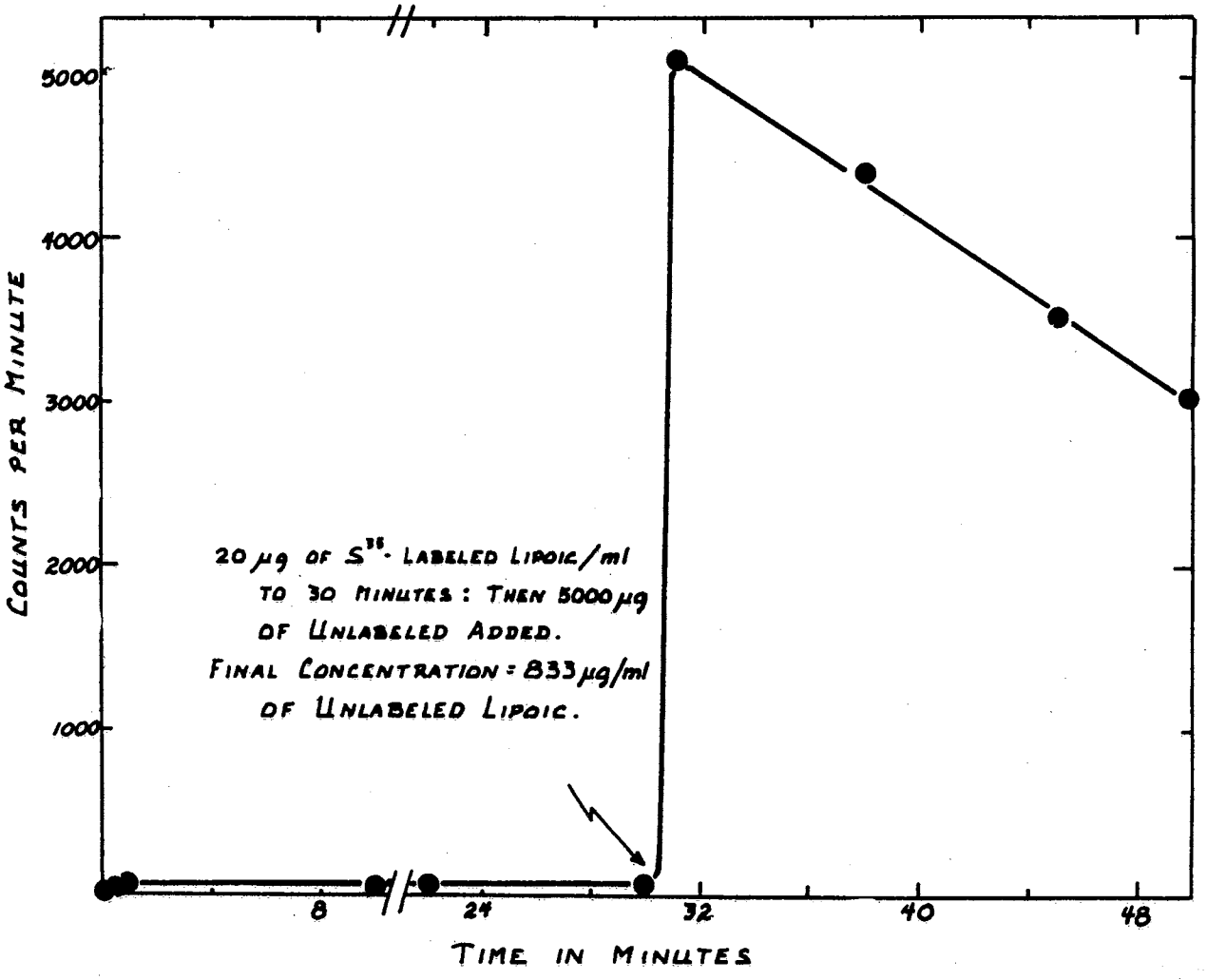
E. Effect of Unlabeled Lipoic Acid Addition During Uptake.

To determine whether an exchange mechanism operated in the system, a standard uptake study was performed for 30 minutes; then unlabeled lipoic acid was added to a final concentration of 830 μg/ml. Instead of the expected decrease in the accumulated radioactivity, a 50-fold increase was noted, followed by a linear decline. These results are shown in Fig. 5.

When this experiment was performed using two incubation mixtures from the same batch of cells, but adding an equal volume of neutral salts solution to one sample and to the other the unlabeled lipoic


800
600
400
200
0
8
16
24
32
37° C
20° C
10° C
0° C
Counts Per Minute
Time In Minutes


COUNTS PER MINUTE
200
100
8
16
24
32
TIME IN MINUTES


5000
4000
3000
2000
1000
COUNTS PER MINUTE
20 μg OF S35- LABELED LIPOIC/ml
TO 30 MINUTES: THEN 5000 μg
OF UNLABELED ADDED.
FINAL CONCENTRATION = 833 μg/ml
OF UNLABELED LIPOIC.
8
24
32
40
48
TIME IN MINUTES

acid (in salts solution at pH 7), no stimulation was noted. The increase in the accumulated radioactivity in the cells treated with the unlabeled lipoic acid was similar to that in Fig. 5.

F. Studies of Kinetics of Uptake.

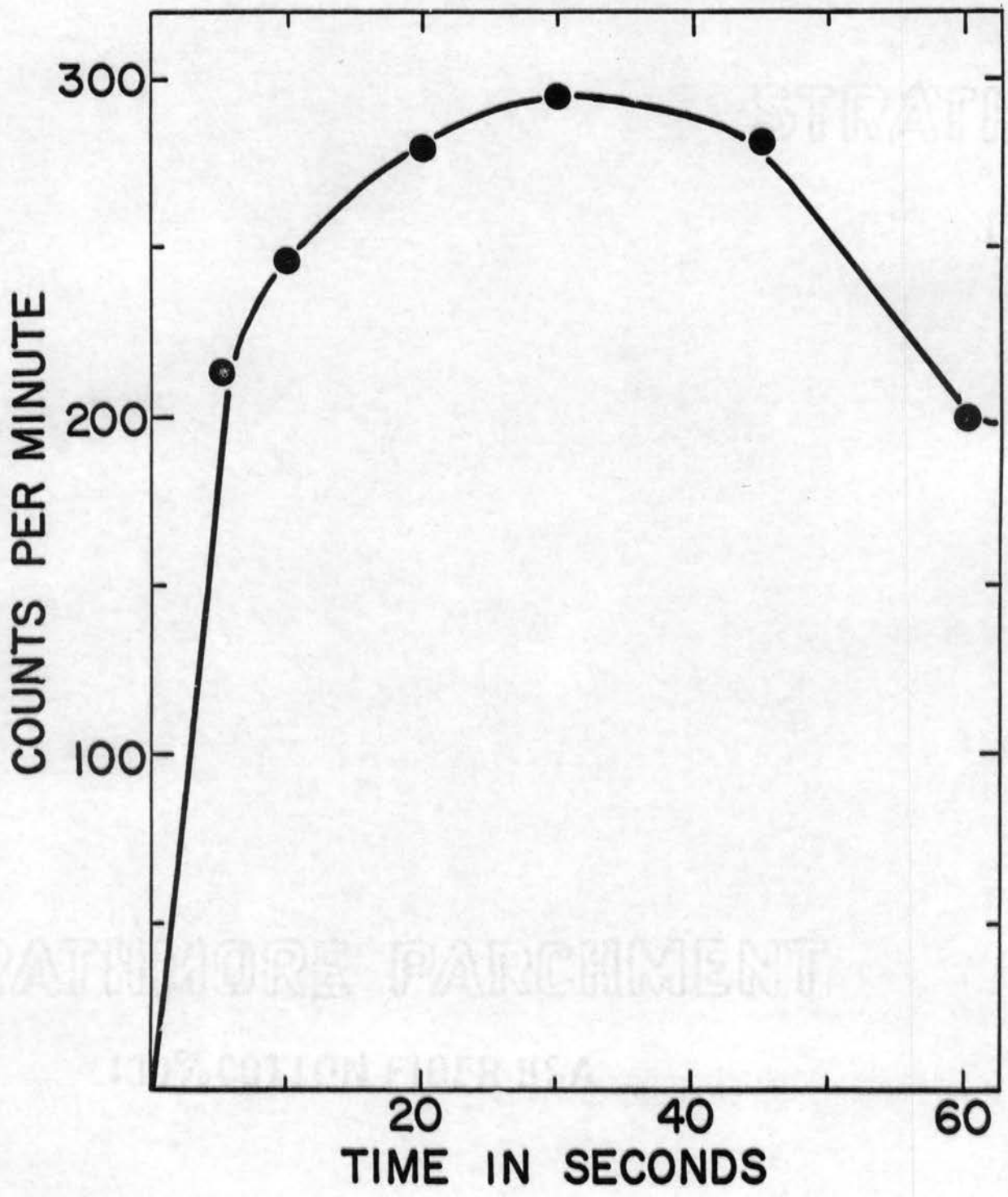
Since the uptake of lipoic acid in the early stages was extremely rapid, an experiment was performed to determine the time course of accumulation during the first minute. Aliquots were ejected into centrifuge tubes suspended in liquid nitrogen to stop the uptake. Freezing under these conditions required less than 2 seconds. The time study, the results of which are shown in Fig. 6, indicated that the equilibrium value was reached in about 5 seconds, after which uptake slowed gradually until a maximum was reached at 30 seconds. After 30 seconds, a decline was noted until 1 minute after which an equilibrium value was reached. (See Fig. 3.)

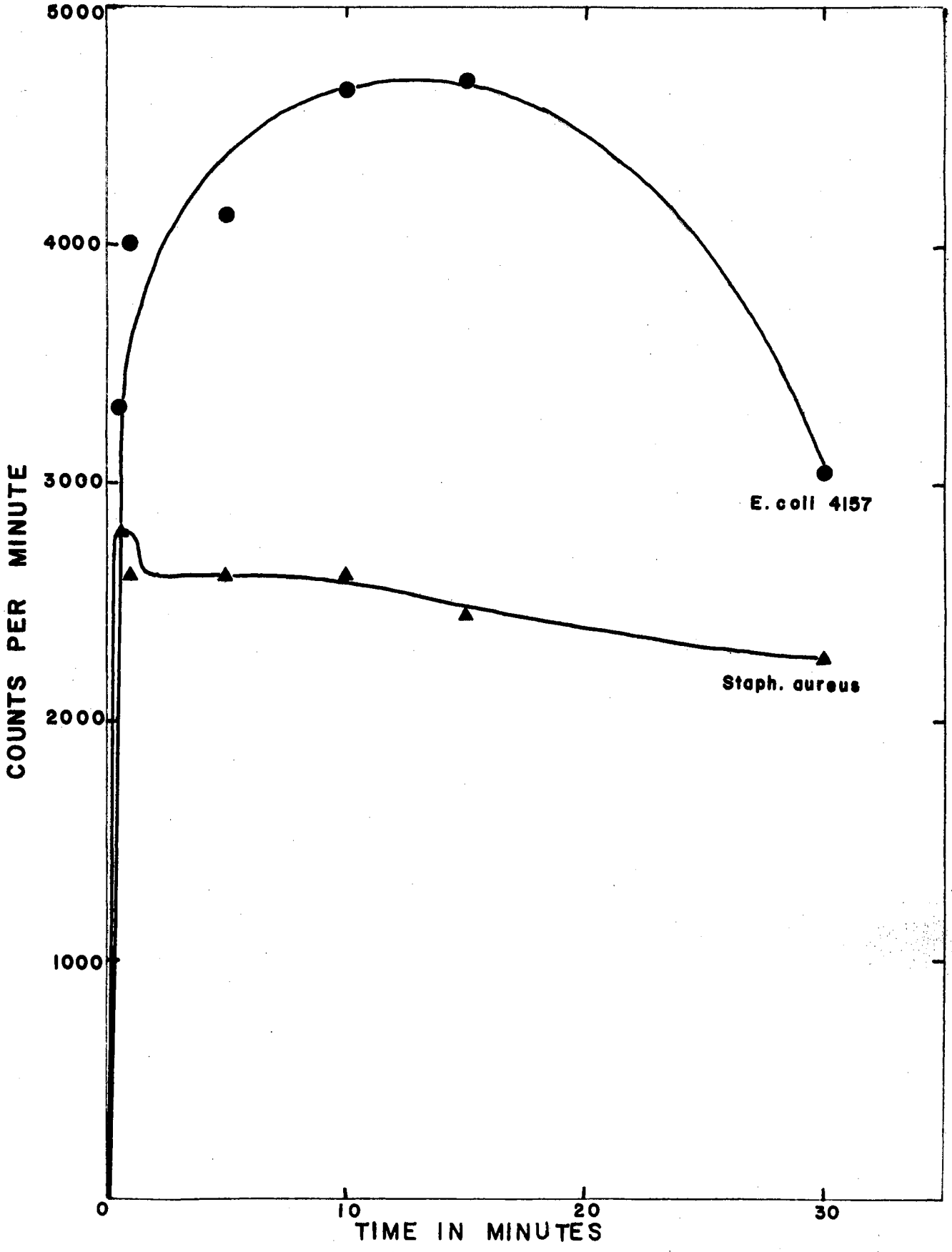
G. Uptake of Lipoic Acid in E. coli and Staph. aureus.

When E. coli No. 4157 and Staph. aureus (wild type) were incubated with glucose and lipoic acid in the manner described for S. faecalis, two distinctly different patterns of uptake were noted. Cells of Staph. aureus exhibited a very rapid uptake to 30 seconds followed by a decline like that of S. faecalis. E. coli No. 4157 also showed a rapid uptake during the first minute which gradually slowed to give a maximum value at about 15 minutes after which it declined. The peak accumulation value for E. coli was considerably higher than for Staph. aureus. The results of these studies are shown in Fig. 7.

H. Energy Requirement and 2,4-Dinitrophenol Inhibition.

When S. faecalis 10C1 cells were depleted of endogeneous


300
200
100
COUNTS PER MINUTE
20
40
60
TIME IN SECONDS


5000
4000
3000
2000
1000
0
10
20
30
COUNTS PER MINUTE
TIME IN MINUTES
E. coli 4157
Staph. aureus

substrates by a 3.5 hour incubation in neutral salts solution at 37° and uptake measured with and without an energy source (glucose, 1 mg/ml) and in the presence and absence of 2,4-dinitrophenol (5×10^{-3} M), the results shown in Fig. 8 were noted. Cells supplied with glucose in the presence of 2,4-dinitrophenol showed some uptake after a 15 minute lag period while cells supplied with glucose but no inhibitor took up lipoic acid rapidly.

No change in uptake was noted when pyruvate or glutamate was added in a standard uptake experiment.

I. Inhibition Studies With Octanoate and Lipoic Acid Analogs.

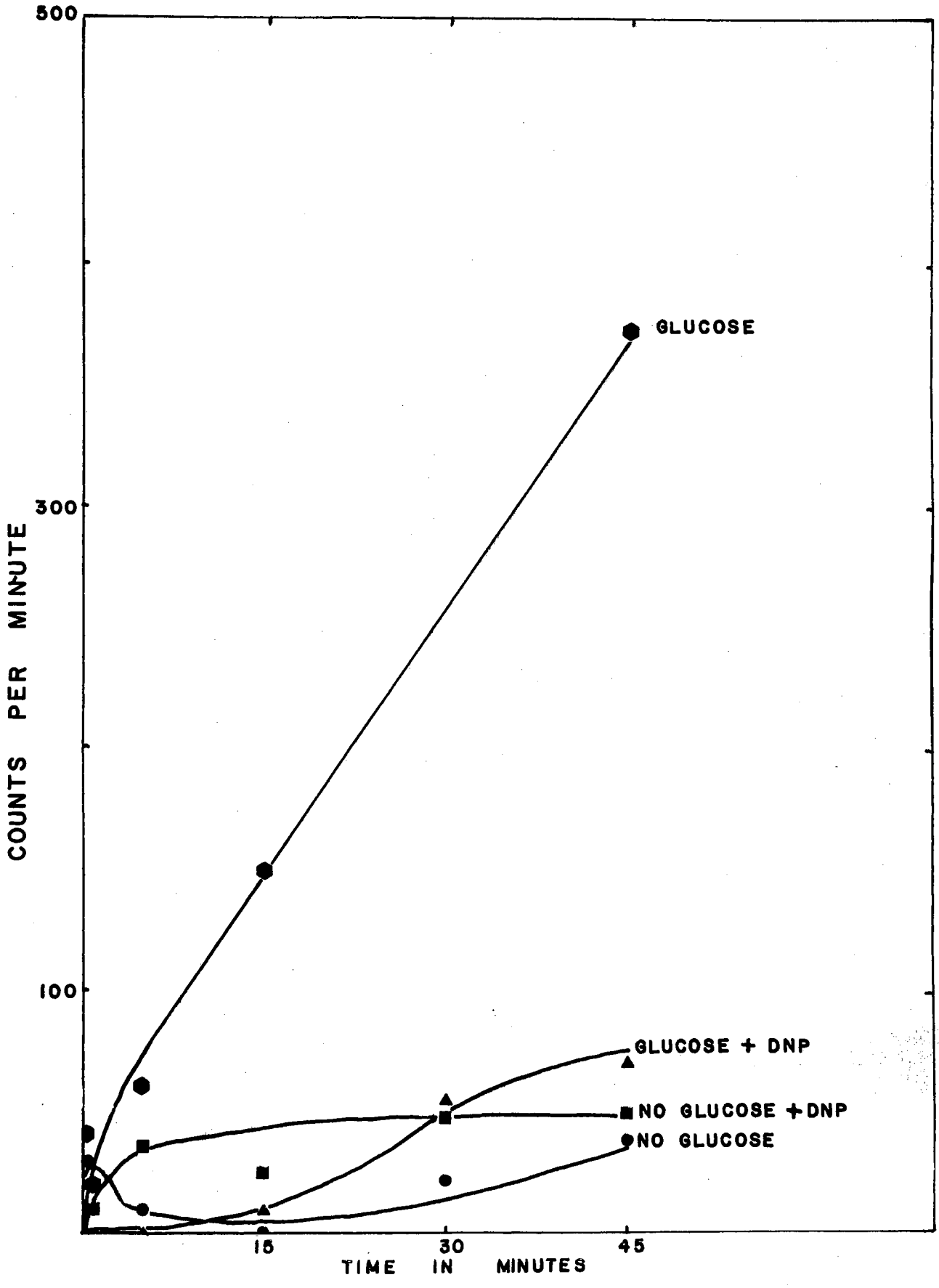
To determine whether octanoate might influence the uptake of lipoic acid, the uptake reaction was measured in the presence and absence of a neutral solution of potassium octanoate (final concentration 10 μg/ml) which was introduced just before adding the lipoic acid. The results of this study are shown in Fig. 9.

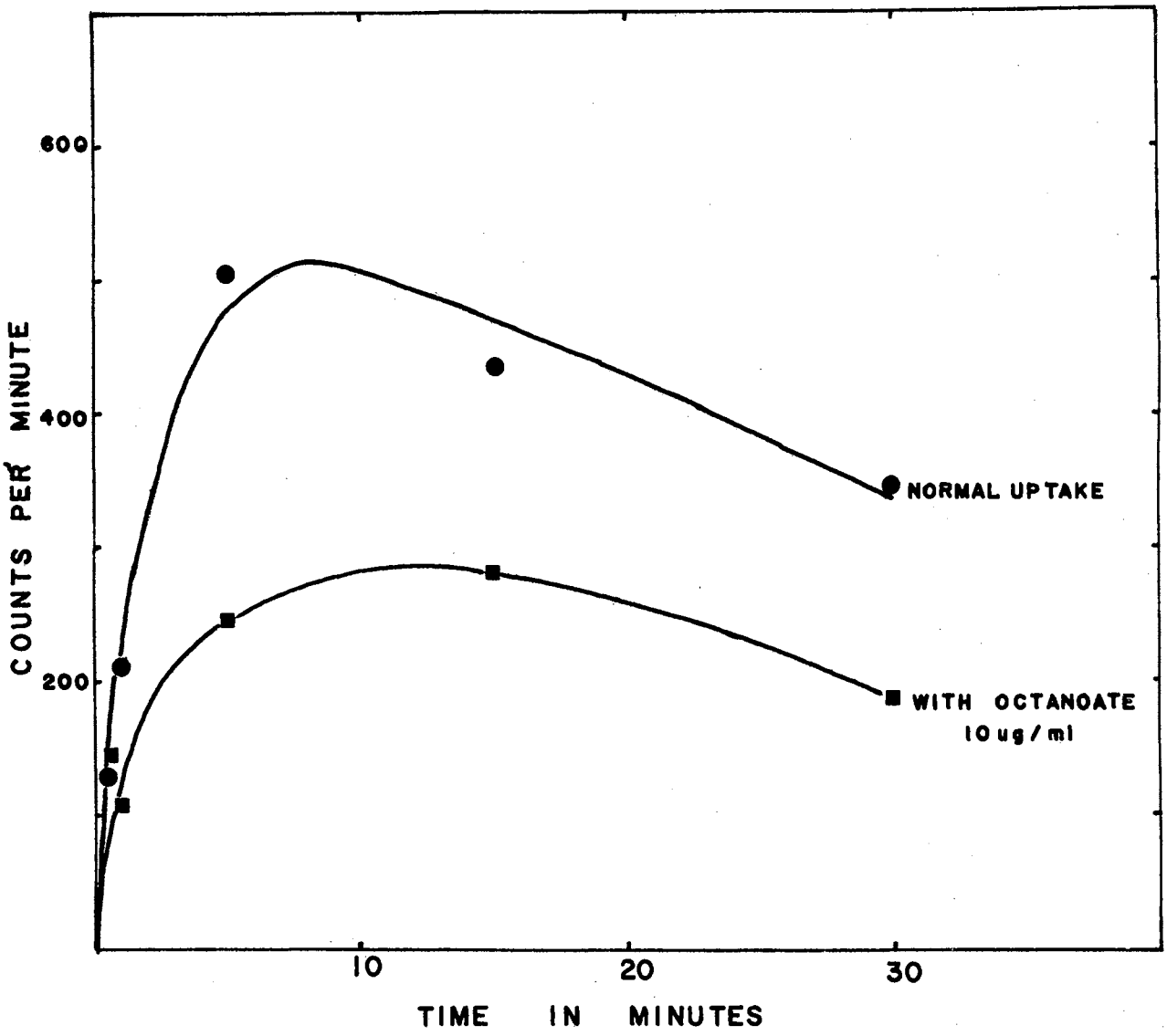
While the pattern of uptake was essentially unaltered, the accumulation of lipoate in the presence of the octanoate was depressed to approximately half.

When the 7- and 9-carbon analogs of lipoic acid, 1,2-dithiolane-3-butyric acid and 1,2-dithiolane-3-caproic acid, were studied in the same manner, no discernible inhibition was noted.

J. Fractionation Studies.

To investigate the distribution of the lipoic acid in the cell, both after a normal uptake and after the stimulation of uptake caused by the addition of unlabeled lipoic acid, cells were fractionated according to the modofied method of Park and Hancock (30) previously described. In each case, over half of the total radioactivity


500
300
100
COUNTS PER MINUTE
GLUCOSE
GLUCOSE + DNP
NO GLUCOSE +DNP
NO GLUCOSE
15
30
45
TIME IN MINUTES


600
400
200
COUNTS PER MINUTE
NORMAL UPTAKE
WITH OCTANOATE
10 ug/ml
10
20
30
TIME IN MINUTES

accumulated appeared in the pool. The results of these studies are shown in Table IV.

Cells were also fractionated after freezing at zero time and after a 30 minute uptake period. A substantially greater percentage of the accumulated radioactivity was present in the pool of the cells collected at zero time than after 30 minutes. The slightly greater percentage in the lipid (alcohol soluble) fraction at zero time may also be significant. As might be expected, considerably more of the radioactivity was incorporated into the protein (trypsin soluble) fraction after 30 minutes than at zero time. A comparison of these fractionations is shown in Table V.

K. Identification of Intracellular Lipoic Acid.

Five mg of cells in a concentration of 1 mg/ml were exposed to standard uptake conditions for 30 minutes at 20°, washed and collected. The cell pellet was hydrolyzed in 1 ml of 6 N HCl for 2 hours at 100°. The residue was taken to dryness in vacuo, diluted with water and evaporated twice. Chromatography in a lutidine:water (65:35 v/v) solvent system with both labeled and unlabeled lipoic acid indicated that only a single radioactive compound was present in the hydrolysate and that the Rf value of the compound was the same as that of lipoic acid.

TABLE IV

FRACTIONATION OF CELLS BEFORE AND AFTER ADDITION OF UNLABELED LIPOIC ACID

Two 7 ml suspensions of cells (1 mg/ml) and glucose (1 mg/ml) in neutral salts solution were incubated with radioactive lipoic acid for 15 minutes at 37° as described for normal uptake conditions. Unlabeled potassium lipoate was added to one suspension to a final concentration of 666 μg/ml and an equal volume of neutral salts solution (2 ml) was added to the other suspension. Incubation was continued for 4 minutes after which the cells were collected by centrifugation, washed, and fractionated by the modified method of Park and Hancock (30) described in experimental procedure. One ml of the suspension of washed cells was taken for determination of total radioactivity before boiling.

Fraction	Per Cent of Total Radioactivity[a]	
	Salts Solution	Lipoic Acid 666 μg/ml
Water (boiling	65.1	68.1
Cold trichloroacetic acid (5% at 0°)	0.6	0.5
Ethanol:water 3:1 v/v	6.0	5.2
Hot trichloroacetic acid (5% at 90°)	1.2	1.1
Trypsin soluble	17.6	13.9
Residue	9.8	11.1

[a]Per cent of total radioactivity based on 96.0 per cent recovery for the suspension to which salts solution was added and 99.9 per cent recovery for the suspension containing 666 μg/ml of unlabeled lipoic acid.

TABLE V

CHANGES IN DISTRIBUTION OF ACCUMULATED LIPOIC ACID WITH TIME

Two 6 ml suspensions of cells (1 mg/ml) and glucose (1 mg/ml) in neutral salts solution were exposed to radioactive lipoic acid (10 μg/ml) for the time intervals shown at 37° as described for normal uptake conditions. The zero time suspension was frozen in liquid nitrogen before adding the lipoic acid; the 30 minute suspension was frozen after 30 minutes incubation with lipoic acid. After thawing and washing in the cold (2°), the cell pellets were diluted to 6 ml with distilled water and 1 ml was taken to assay for total radioactivity. The remaining 5 mg of cells were fractionated by the modified method of Park and Hancock (30) described in experimental procedure.

Fraction	Per Cent of Total Radioactivity[a]	
	Zero Time	30 min
Water (boiling)	57.2	33.0
Cold trichloroacetic acid (5% at 0°)	2.2	1.4
Ethanol:water 3:1 v/v	11.4	7.1
Hot trichloroacetic acid (5% at 90°)	3.9	13.6
Trypsin soluble	9.4	15.3
Residue	14.6	36.6

[a]Per cent of total radioactivity based on 74.0 per cent recovery for the zero tube and 86.5 per cent recovery for the 30 minute tube.

DISCUSSION

The time-temperature studies indicate a temperature sensitivity which might be linked either to an optimum temperature for the permease, or to an energy requirement. The slight uptake at 0° C and the maximum at 30 seconds are consistent with a two-step process for uptake. The first step would be a non-enzymatic adsorption of the charged molecule to the oppositely charged sites on the surface of the cell membrane; the second step would be the transport of the lipoic acid molecule across the membrane by the permease. The slight decline noted after 30 seconds is probably due to an independent exit system which does not function at peak efficiency until somewhat later.

The reduced uptake by cells grown in the presence of unlabeled lipoic acid indicates that the permease is probably constitutive. The occurrence of uptake in cells grown without lipoic acid in a resting condition is in agreement with this theory.

The lack of uptake observed in cells depleted of their endogeneous substrates and without an external energy source, as well as the inhibition of uptake by the uncoupling agent, 2,4-dinitrophenol, is an indication of an energy requirement for the permease reaction.

The inhibition of uptake by octanoic acid is probably due to simple competition for the active sites in the transport mechanism. Apparently the bond angles involved or other stereospecific requirements prevent the 7- and 9-carbon analogs of lipoic acid from being effective antagonists.

The unusual stimulation of uptake noted upon the addition of

unlabeled lipoic acid illustrates that we are not observing a simple case of exchange. Two possible explanations may be considered: 1. Linear disulfide polymers of lipoic acid are known to be formed at high concentrations under rather mild, non-enzymatic conditions and the reaction might have an enzymatic counterpart functioning in the periplasm of the cell (31). 2. The addition of a large amount of unlabeled lipoic acid to the medium may cause a "streaming effect" through the cell wall forcing the labeled compound from the periplasm into the membrane.

The distribution of the lipoic acid in the cell merits some discussion. Chromatography of cell hydrolysates indicates that lipoic acid is not broken down to any discernible extent in its passage through the membrane. The regularly occurring activity in the lipid fraction would be consistent with the hypothesis of a permease, the labeling occurring from the transitory binding of the lipoic acid to the enzyme during its passage through the membrane.

It would be particularly informative to study the distribution of proteins labeled by lipoic acid and to determine the intracellular distribution of the known enzymes involved. Some difficulty has been encountered in the preparation of protoplasts from the strain of S. faecalis used in these experiments. However, if protoplasts can be successfully formed, then osmotic lysis followed by centrifugation would effectively separate the cell wall and periplasm, the soluble cytoplasm, and the proteins attached to the membranes. Ideally, only the pyruvate dehydrogenation system, the α-ketoglutarate dehydrogenation system, and the transport or permease system would be labeled. The possible isolation of a lipoate-permease complex would effectively

establish a permease type of transport mechanism for this compound.

SUMMARY

Lipoic acid (1,2-dithiolane-3-valeric acid) labeled with S^{35} was synthesized. A system was established for the study of the uptake of this compound in the lipoic acid-deficient bacterial mutant, S. faecalis 10C1. The effect of varying concentrations of lipoic acid and cells on uptake was established.

The uptake was characterized by a rapid accumulation for 30 seconds followed by a slight decline and a plateau. An effect of temperature was observed. The uptake at 0° was about 10 per cent of the uptake at 37°. The transport mechanism proved to be dependent on an external energy source and 2,4-dinitrophenol inhibited uptake in cells previously depleted of their endogeneous substrates.

Octanoic acid partially inhibited uptake, but the 7- and 9-carbon analogs of lipoic acid showed little or no inhibition.

Cellular distribution of the absorbed lipoic acid was studied by established fractionation methods. Approximately 50-60 per cent of the absorbed material was found in the pool fraction while the lipid fraction represented about 6 per cent. About 15-20 per cent each was found in the trypsin soluble and mucopeptide fractions.

A SELECTED BIBLIOGRAPHY

1. Cohen, G. N. and Monod, J., Bacteriol. Rev., 21, 169 (1957).

2. Cohen, G. N. and Rickenberg, H. V., Compt. rend., 240, 2086 (1955) via Bacteriol. Rev., 21, 169 (1957).

3. Cohen, G. N. and Rickenberg, H. V., Ann. inst. Pasteur, 91, 693 (1956) via Bacteriol. Rev., 21, 169 (1957).

4. Leach, F. R. and Snell, E. E., Biochim. Biophys. Acta, 34, 292 (1959).

5. Mitchell, P. 1961. Approaches to the analysis of specific membrane transport, p. 581-603. In T. W. Goodwin and O. Lindberg (ed.), Biological structure and function, II, Academic Press, New York.

6. Osborn, M. J., McLellan, W. L. and Horecker, B. L., J. Biol. Chem., 236, 2585 (1961).

7. Oginsky, E. L., Arch. Biochem., 36, 71 (1952).

8. Lichstein, H. C. and Ferguson, R. B., J. Biol. Chem., 233, 243 (1958).

9. Wood, R. C. and Hitchings, G. H., J. Biol. Chem., 234, 2381 (1959).

10. Snell, E. E., Strong, F. M. and Peterson, W. H., Biochem. J., 31, 1789 (1937).

11. Snell, E. E., Tatum, E. L. and Peterson, W. H., J. Bacteriol., 33, 207 (1937).

12. O'Kane, D. J. and Gunsalus, I. C., J. Bacteriol., 54, 20 (1947).

13. Stokstad, E. L. R., Hoffman, E. E., Regan, M. A., Fordham, D. and Jukes, T. H., Arch. Biochem., 20, 75 (1949).

14. Snell, E. E. and Broquist, H. P., Arch. Biochem., 23, 326 (1949).

15. Reed, L. J., Gunsalus, I. C., Schnakenberg, G. H. F., Soper, Q. F., Boaz, H. E., Kern, S. F. and Parke, T. V., J. Am. Chem. Soc., 75, 1267 (1953).

16. Kline, L. and Barker, H. A., J. Bacteriol., 60, 349 (1950).

17. Kline, L., Pine, L., Gunsalus, I. C. and Barker, H. A., J. Bacteriol., 64, 467 (1952).

18. Reed, L. J. 1957. The chemistry and function of lipoic acid, p. 319-347. In F. F. Nord (ed.) Advances in Enzymology, XVIII, Interscience Publishers Inc., New York.

19. Bassham, J. A., J. Am. Chem. Soc., 78, 4120 (1956).

20. Fridovich, I. and Handler, P., J. Biol. Chem., 223, 321 (1956).

21. Gunsalus, I. C., Federation Proc., 13, 715 (1954).

22. Nawa, H., Brady, W. T., Koike, M. and Reed, L. J., J. Am. Chem. Soc., 81, 2908 (1959).

23. Fichter, F. and Laurie, S., Helv. Chim. Acta, 16, 887 (1933).

24. Berg, H., Ber. 67B, 1622 (1934).

25. Reed, L. J. and Niu, C., J. Am. Chem. Soc., 77, 416 (1955).

26. Acker, D. S. and Wayne, W. J., J. Am. Chem. Soc., 79, 6483 (1957).

27. Thomas, R. C. and Reed, L. J., J. Am. Chem. Soc., 77, 5446 (1955).

28. Gunsalus, I. C. and Razzell, W. D. 1957. Preparation and assay of lipoic acid and derivatives, p. 941-946. In S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, III, Academic Press, New York.

29. Leach, F. R. and Snell, E. E., J. Biol. Chem., 235, 3523 (1960).

30. Park, J. T. and Hancock, R., J. Gen. Microbiol., 22, 249 (1960).

VITA

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