STUDIES ON THE FORMATION AND CHARACTERIZATION OF PROTOPLASTS OF *STREPTOCOCCUS FAECALIS* 10C1

By

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STUDIES ON THE FORMATION AND CHARACTERIZATION OF

PROTOPLASTS OF STREPTOCOCCUS FAECALIS 10C1

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INTRODUCTION

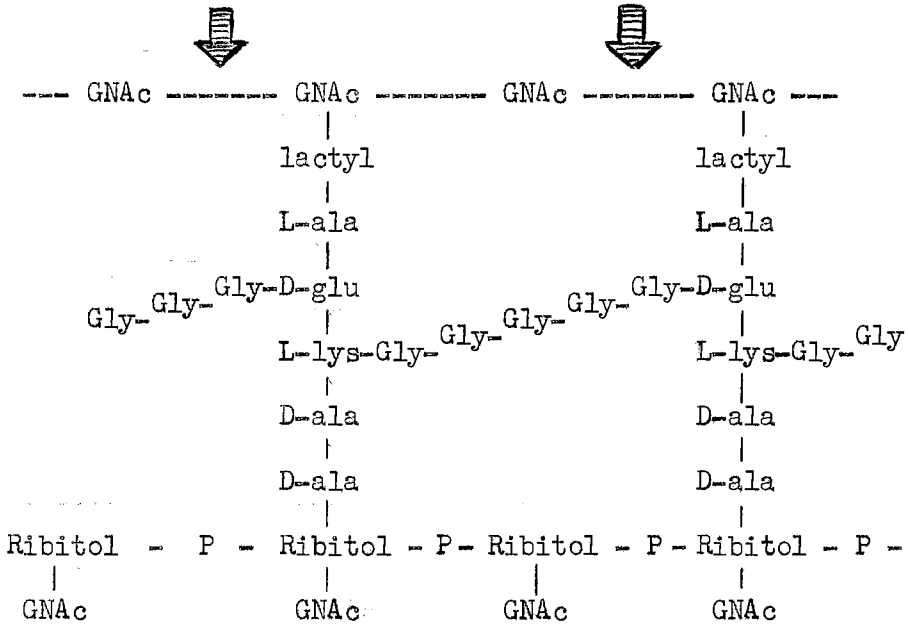
Tomcsik and Guex-Holzer (1) and Weibull (2) formed protoplasts by the action of lysozyme on suspensions of Bacillus megaterium in buffered sucrose solution. The protoplasts appear as osmotically sensitive spheres from which the cell wall has been removed (2). Formation of protoplasts of gram-negative organisms is more difficult. However, addition of versene and tris-(hydroxymethyl)-aminomethane buffer to the lysozyme system allows formation of osmotically sensitive structures (3).

The rigidity of the cell wall is due to a polymer of muramic acid and glucosamine in which a β-1,4 linkage is the site of lysozyme action (4). This polymer is the backbone of a glycopeptide present in the cell wall of Staphylococcus aureus, the structure of which is shown in Scheme 1 (5).

The formation of protoplasts has made available a valuable tool to study the role of the cell wall in bacterial growth and metabolism and as a starting point for the isolation of various bacterial structures.

Certain problems in transport have been studied through the comparison of the activities of whole cells and protoplasts. The general state of both amino acid and carbohydrate transport will be reviewed. Gale and his colleagues (6) first studied the absorption of

A POSSIBLE STRUCTURE OF THE GLYCOPEPTIDE POLYMER IN THE CELL WALL OF <u>STAPHYLOCOCCUS AUREUS</u> (TWO REPEATING UNITS ARE SHOWN)



A pentaglycl peptide links the lysine of one peptide bridge with the glutamic acid of another.

------ indicates the polymer chain responsible for the rigidity of the cell wall

⇩ indicates the site of lysozyme activity

GNAc indicates acetylglucosamine

GNAc indicates acetylmuramic acid
|
lactyl

Scheme 1

amino acids by bacterial cells using the amino acid decarboxylases for amino acid determinations. These findings resulted in the development of the concept of an intracellular pool which contains compounds absorbed from the external medium as well as those synthesized intracellularly. Later studies on the absorption of amino acids (7, 8, 9) and on the uptake of vitamins (10, 11) used radioactive substrates to follow accumulation. Other vitamin uptake studies used microbiological assays to determine the amount of substrate taken up by the cell (12, 13). Leach and Snell (14) point out that the occurrence of an exchange diffusion process in addition of the net uptake of amino acids complicates the interpretation of kinetic measurements of uptake using radioactive tracers. Cohen and Rickenberg (9), Britten, Roberts, and French (15), and Leach and Snell (7) have presented evidence that the amino acid transport systems which concentrate the amino acid in the cell pool are energy-requiring and temperature-dependent. Metabolic inhibitors such as 2,4-dinitrophenol and sodium azide inhibit the transport process. There are several systems, some of which may be specific for a single amino acid or a family of structurally related amino acids. Other systems may vary widely in structural specificity.

Mora and Snell (16) have compared the transport of glycine and alanine in both whole cells and protoplasts of Streptococcus faecalis. In this organism, one system for the accumulation of glycine, L-alanine, and D-alanine was demonstrated by competition studies. This system required an energy source, possessed a high temperature coefficient, and was readily saturated at a high amino acid concentra-

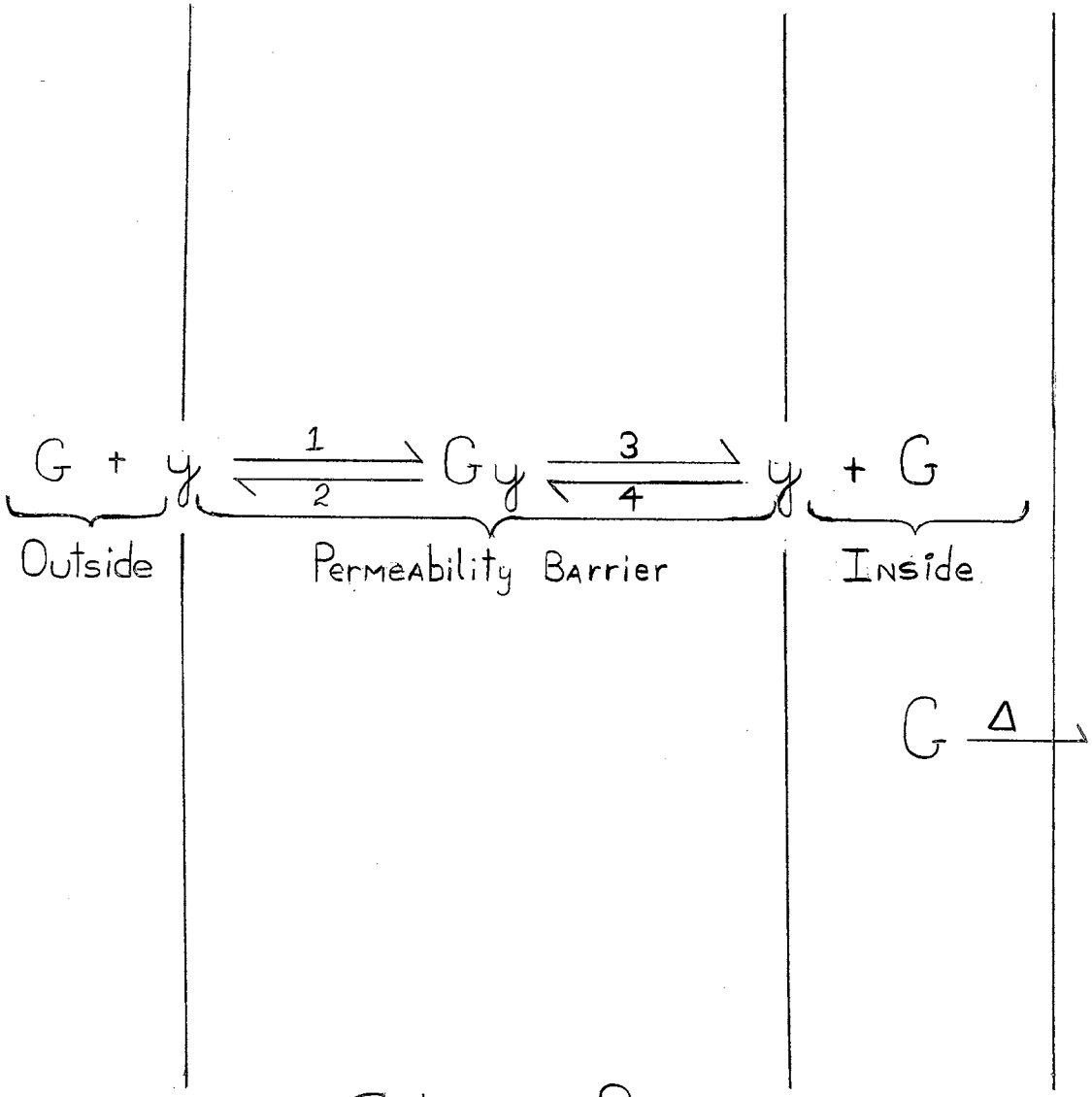
tion. The Michaelis constants for protoplasts and whole cells were similar. Uptake of glycine and L-alanine by protoplasts is stimulated by K^+ and by pyridoxal but these substances have no effect on uptake by whole cells.

The transport of carbohydrates has been demonstrated to be due to systems with properties similar to those described for the amino acids. Protoplasts have been used for two studies on carbohydrate accumulation. Rickenberg (17) observed that non-metabolizable galactosides were transported into and accumulated by protoplasts. He concluded that the site of galactoside-permease activity resides in the cytoplasmic membrane and not in the cell wall. Sistrom (18) confirmed and extended these findings. The β-thiomethyl-galactosides are transported into cells but are not metabolized. The accumulation of these compounds by Escherichia coli protoplasts results in a swelling of the protoplasts while metabolizable sugars provoke only a slight response. The swelling which occurred was the amount expected from the accumulation of the requisite amount of carbohydrate in the free form as calculated from the total amount known to be accumulated by whole cells.

Abrams (19) has shown that the metabolism of glucose by protoplasts of S. faecalis (ATCC # 9790) caused a swelling that was maximal when the concentration of lactic acid within the protoplast was maximal. The presence of K^+ was necessary for the swelling, while Na^+ was inhibitory. The swelling was found to be reversed upon completion of glucose metabolism and return of lactic acid to the external medium with contraction of the protoplasts.

Currently, two theories for the mechanism of transport of compounds across the permeability barrier must be considered. These theories were proposed by Cohen and Monod (20) and by Mitchell (21). Cohen and Monod suggested that the crypticity, or lack of ability to metabolize a substrate for which the metabolic enzymes are present in cell-free extracts, observed in certain bacteria might be explained as the lack of a specific transport system. A strain of E. coli possessed the enzyme, β-galactosidase, which metabolized lactose in cell-free extracts, but the cells lacked the ability to transport β - galactosides across the permeability barrier and hence could not be grown on a lactose medium. With other cultures, growth in the presence of a β-galactoside resulted in gaining the ability to accumulate β-thiogalactosides. This inducible system was highly stereospecific; glucosides and other carbohydrates do not compete with the galactosides. The amount of galactoside accumulated was found to be incompatible with the stoichiometric theory that the accumulated compound was loosely bound to some nondiffusible cell constituent; it was compatible with the catalytic theory in which the accumulated compound exists in the cell in the free state.

Sodium azide and 2,4-dinitrophenol inhibited the accumulation of galactosides but did not inhibit the in vivo hydrolysis of the galactosides by the intracellular galactosidase. These inhibitors evidently uncouple an external source of energy needed by the transport system to function against a concentration gradient, since the entry of galactosides per se is not inhibited. The model proposed by Cohen and Monod, as shown in Scheme 2, involves a permease, (y), functioning to catalyze

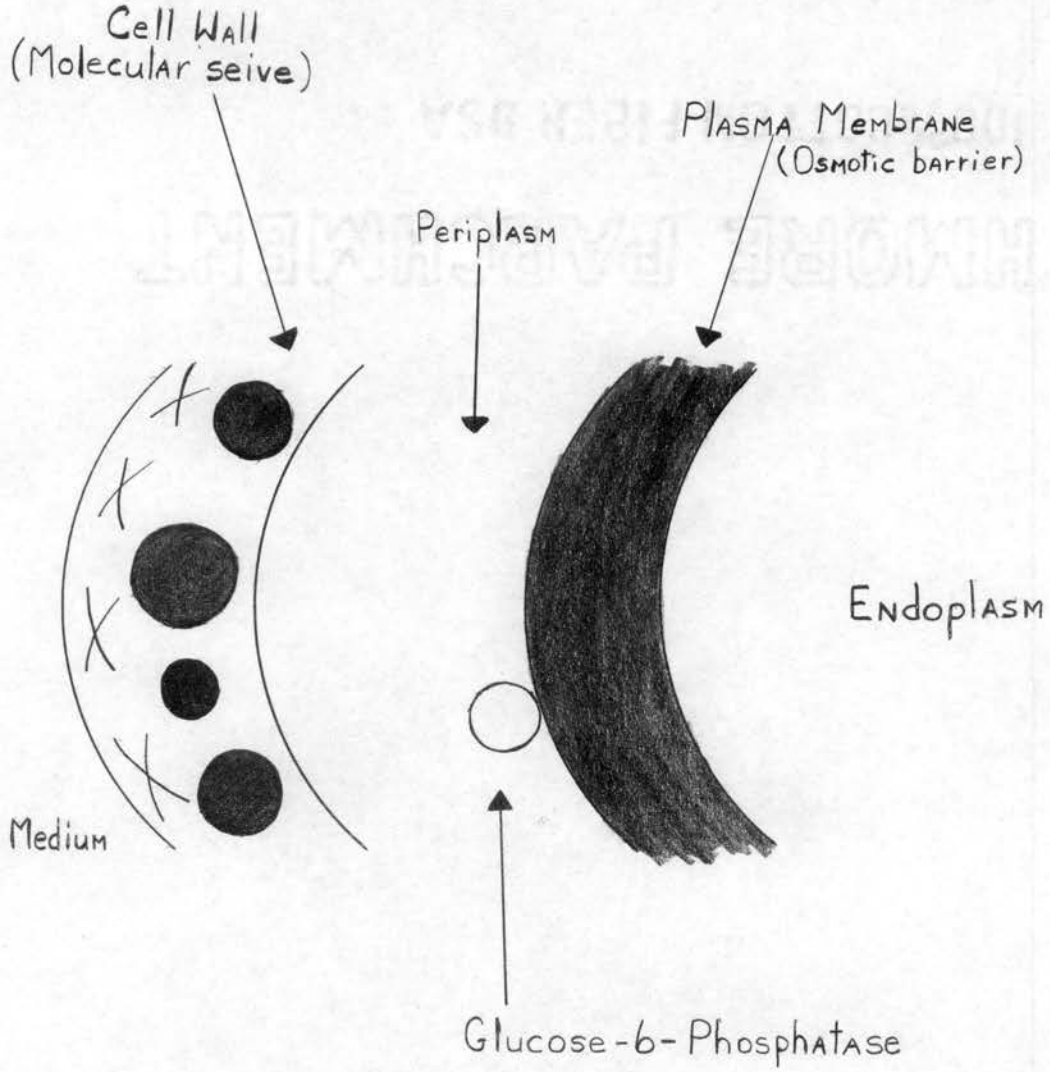


Scheme 2

the reversible entry of compound (G) into the cell. This enzyme is presumably located in the cell membrane. An independent exit system, (Δ), also exists. When tyrosine or phenylalanine is replaced by the amino acid analogue, p-fluorophenylalanine, permease formation is inhibited but a normal amount of intracellular galactosidase is produced. The permease is thus seen as a specific transport enzyme which requires an energy source for the accumulation of compounds, is stereospecific, and is inducible.

Mitchell (21) believes the transport system contains normal metabolic enzymes located at certain sites or substrata on or near the cell membrane and that vectorial transport is catalyzed by their functioning. The cell wall is envisioned as resembling a molecular seive which encloses a space called the periplasm located between the cell wall and the cell membrane as shown in Scheme 3. Some of the transport enzymes, such as glucose-6-phosphatase which is theorized to be responsible for the transport of glucose across the membrane, may be found in this periplasm. The proof offered for the existence of the periplasm results from a study in which bovine serum albumin is added to a hypertonic saline solution for the observation of whole cells by anoptral contrast micrography; the periplasm appears as a darkened area between the cell wall and the rest of the cell.

The cell membrane contains metabolic enzymes for about one-half of the α-ketoglutarate dehydrogenase activity of the cell; the enzyme was found to be tightly bound to the membrane fraction of Staph. aureus. The enzyme from the membrane fraction was shown to be identical with the enzyme from the "soluble" fraction by inactivating the membrane


Cell Wall
(Molecular seive)
Plasma Membrane
(Osmotic barrier)
Periplasm
Endoplasm
Medium
Glucose-6-Phosphatase

Scheme 3

fraction with iodoacetate, adding active enzyme from the "soluble" fraction, and noting the reactivation of the membrane bound dehydrogenase activity.

Relatively few studies on the accumulation of vitamins have been made. One would expect that the systems responsible for the uptake of a vitamin would be very efficient and specific because of the low concentration of the vitamin required in the external medium for growth. This might be an advantage in the study of transport problems. Amino acids and carbohydrates are rapidly metabolized by cells and their atoms are converted to many other substances or otherwise spread over many cellular substances. The metabolism of vitamins is simpler and it is possible to select a compound which has very limited metabolism. For these reasons our laboratory is studying the uptake of lipoic acid. The function of this vitamin in S. faecalis 10C1 is limited to pyruvate and α-ketoglutarate dehydrogenases as shown in Scheme 4. Reed and co-workers (22) have established the reaction sequency by which lipoic acid is converted to the enzymatically functional, protein-bound form. This reaction sequence in S. faecalis requires ATP, a divalent metal, two enzymes, and time for the reaction to occur at the physiological temperature. A process similar to amino acid activation occurs since lipoyl adenylate is formed as an enzyme bound intermediate and pyrophosphate is produced. The dihyrolipoic transacetylase (2.3.1.12 acetyl-CoA:dihydrolipoate S-acetyltransferase) is the enzyme of the dehydrogenation complex to which lipoic acid is bound by a peptide bond through the ϵ-amino group of lysine. A specific enzyme, lipoyl-X hydrolase, exists which is capable of removing

SCHEME 4

EXPLANATION OF SYMBOLS

E_1, E_2 } Enzymes of the Lipoic Acid Activating System

E_3 = Pyruvic Carboxylase

E_4 = Dihydrolipoic Transacetylase

E_5 = Dihydrolipoic Dehydrogenase

ATP = Adenosine Triphosphate

PP = Pyrophosphate

AMP = Adenosine Monophosphate

Lys = Lysine

TPP = Thiamin Pyrophosphate

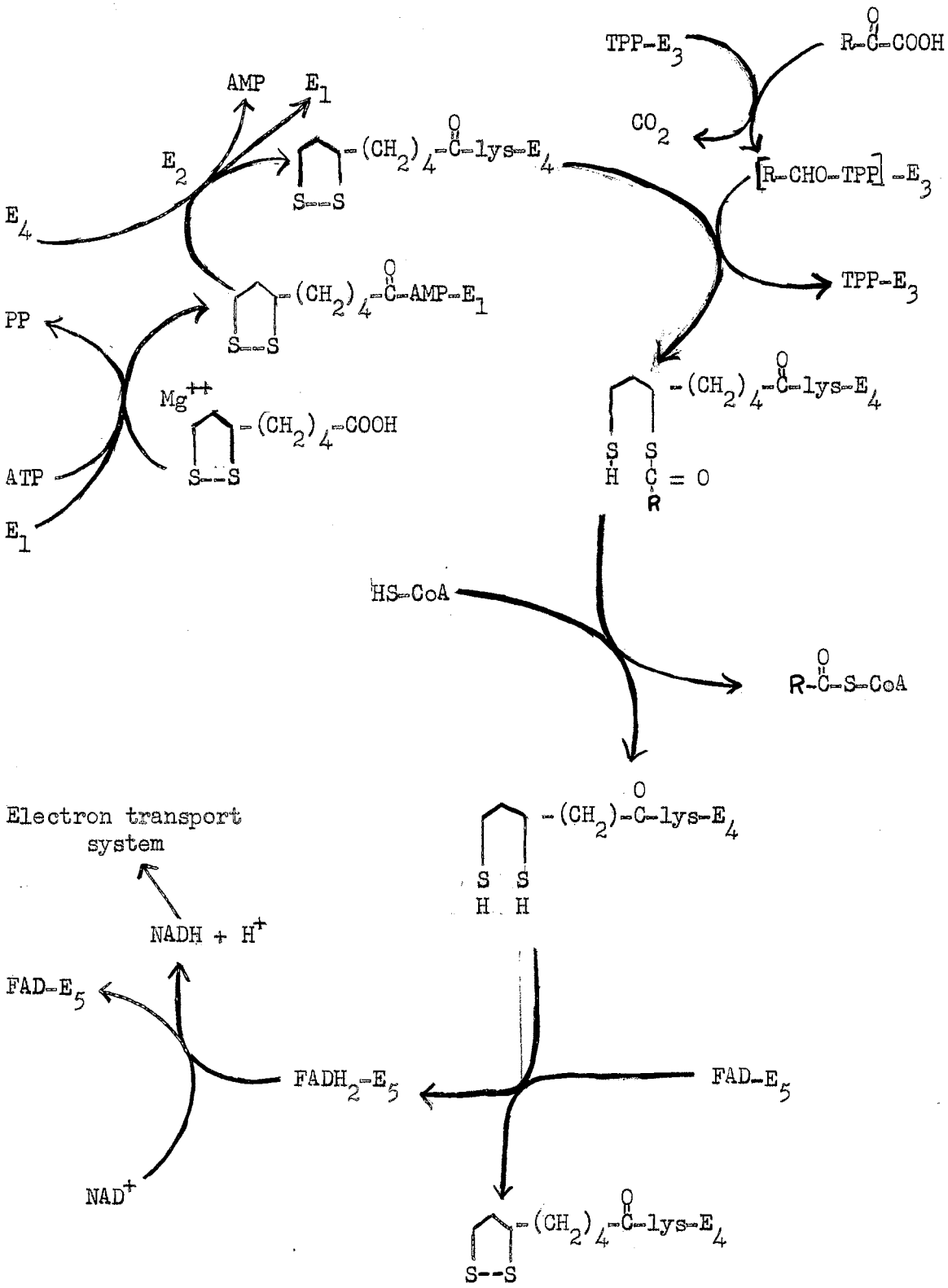
$R-\overset{O}{\overset{\|}{C}}-COOH$ = Pyruvic Acid (or α-Ketoglutaric Acid)

HS-CoA = Coenzyme A

FAD = Flavin Adenine Dinucleotide

NAD = Nicotinamide Adenine Dinucleotide

$-(CH_2)_4-\overset{O}{\overset{\|}{C}}-COOH$ = Lipoic Acid


TPP-E3
R-C(=O)-COOH
CO2
[R-CHO-TPP]-E3
AMP
E1
E2
E4
-(CH2)4-C(=O)-lys-E4
S--S
-(CH2)4-C(=O)-AMP-E1
TPP-E3
PP
Mg++
-(CH2)4-COOH
ATP
E1
-(CH2)4-C(=O)-lys-E4
S H
S
C = O
R
HS-CoA
R-C(=O)-S-CoA
Electron transport system
NADH + H+
FAD-E5
FADH2-E5
NAD+
-(CH2)-C(=O)-lys-E4
S H
S H
FAD-E5
-(CH2)4-C(=O)-lys-E4
S--S

Scheme 4

the bound lipoic acid, inactivating the enzyme. The above knowledge demonstrates that more is known about the metabolism and functioning of lipoic acid than any other vitamin. Thus this system appears to be the one of choice for a study of vitamin uptake.

This study was undertaken to characterize the formation and properties of S. faecalis 10C1 protoplasts. The effect of swelling due to transport and metabolism of compounds related to lipoic acid metabolism and certain other compounds required for cell growth was determined. A lipoic acid dependent swelling due to pyruvate metabolism was observed.

EXPERIMENTAL PROCEDURE

A. Materials

The DL-α-lipoic acid used in these studies was generously supplied by Dr. D. S. Acker of the Central Research Department of E. I. du Pont de Nemours & Company. The lysozyme (3.2.1.17) was obtained from the Worthington Biochemical Corporation.

B. Methods

1. Growth of Cells

Stock cultures of S. faecalis strain 10C1 (ATCC # 11700) and S. faecalis strain R (ATCC # 8043) were carried in stab cultures containing 1.0 per cent Bacto-yeast extract, 1.0 per cent Bacto-tryptone, 0.5 per cent K_2HPO_4, 0.1 per cent glucose, and 2.0 per cent Bacto-agar.

S. faecalis 10C1 and S. faecalis R were grown for 12 hr in the synthetic medium of Gunsalus, Dolin, and Struglia (23). The cells were harvested by centrifugation at 1800 x g for 5 min and washed once with distilled water.

2. Formation of Protoplasts

The washed cells were suspended in a 0.6 M sucrose-0.075 M KH_2PO_4-K_2HPO_4 solution (pH 6.2) at a cell concentration of approximately 2 mg per ml. At this cell concentration an aliquot of 2 ml of the cell suspension diluted with 3 ml of the sucrose solution gives an optical density of approximately 0.35 at 630 m μ. After the addition of crys-

talline lysozyme to a concentration of 350 μg per ml, the suspension was incubated at 37°C for 1 hr. Lysozyme action was followed by measuring the decrease in optical density at 630 mμ; a decrease to about 80 per cent of the original optical density was usually observed in 1 hr. Measurements were made by diluting 2 ml of the suspension with 3 ml of sucrose solution and reading the resulting value on a Bausch and Lomb Spectronic 20.

3. Characterization of Protoplasts

The formation of protoplasts was demonstrated by their lysis in distilled water. A 2 ml aliquot of the above suspension was centrifuged and suspended in 5 ml of distilled water. A resulting optical density of 0.10 or less was considered to be indicative of extensive protoplast formation.

4. Stabilization of Protoplasts

To demonstrate the stability of protoplasts formed in 0.6 M sucrose, 2 ml aliquots of the suspension were centrifuged and suspended in 5 ml of 0.6 M sucrose-0.075 M phosphate buffer, pH 6.2. The optical density at 630 mμ was followed for 1 hr.

The effect of the refractive index on optical density readings of sucrose and other stabilizing agents at various concentrations was determined with whole cells of S. faecalis 10C1. Cells were suspended in 5 ml samples of each concentration of the stabilizing agents. Cell densities for all samples were the same and equivalent to an optical density of 0.60 at 630 mμ in distilled water. Refractive indices of each stabilizer system was read on a Zeiss refractometer at room temperature and optical densities of the suspended cells were determined.

5. Studies on the Metabolic Swelling of Protoplasts

Two ml aliquots of the protoplast suspension were centrifuged and suspended in 5 ml of the buffered sucrose solution. Swelling of the protoplasts was followed by measuring the optical density at 630 mμ for at least 60 min after addition of the compounds. Among the compounds added were the components of the manometric assay system for lipoic acid which were at the same final concentration used in the manometric assay. The concentration of these compounds was adjusted so that addition of 0.1 ml to the tube gave the desired final concentration.

6. Manometric Assay for Lipoic Acid with Whole Cells and Protoplasts of S. faecalis 10C1

The method used was essentially that of Gunsalus, Dolin and Struglia (23). A supplement of riboflavin, thiamin, $MgSO_4$, adenosine, and glutamic acid was made at a concentration such that 0.3 ml placed in each Warburg flask gave the desired final concentration. The lipoic acid was added to the main compartment in varying concentrations. In the assay with protoplasts, 1 ml of 1.2 M sucrose-0.15 M phosphate buffer (pH 6.2) was placed in each flask as an osmotic stabilizer. Each center well contained 0.15 ml of 20 per cent KOH and a filter paper wick for the absorption of carbon dioxide. After equilibration at 37°C, pyruvate was added to the system from the side arm to a concentration of 0.03 M. Whole cells were assayed both in the presence and absence of sucrose.

RESULTS

A. Formation of Protoplasts

1. Effect of Lysozyme Concentration

Lysozyme action on a 12 hr culture of S. faecalis 10C1 in 0.6 M sucrose-0.075 M phosphate buffer (pH 6.2) was measured at 630 mμ for concentrations from 50 μg to 350 μg per ml of lysozyme as shown in Table I. Cultures earlier than 12 hr were less susceptible to lysozyme action. A concentration of 350 μg per ml, similar to the amount used by Mora and Snell (16) for S. faecalis (ATCC # 9790), was found to be most effective at a cell concentration of 2.0 mg per ml (dry weight). S. faecalis R was lysed at a concentration of 50 μg per ml lysozyme at a cell concentration of 0.5 mg per ml as described by Brown, Sandine, and Elliker (24); S. faecalis 10C1 at a concentration of 0.5 mg per ml (dry weight) was also lysable by lysozyme at a concentration of 50 μg per ml and these levels were used in some experiments.

2. Effect of Versene on Lysozyme Action

Versene has been found to enhance lysozyme action in some organisms (3). The optimal ratio of versene to lysozyme for maximum lysis varies with the organism. It was found with S. faecalis 10C1 and S. faecalis R that a ratio of 3:1 increased the rate of lysis. Figure 1 shows the effect of the addition of versene to the lysozyme system; at

TABLE I

EFFECT OF LYSOZYME CONCENTRATION

Whole cells of S. faecalis 10C1 were suspended in 0.6 M sucrose solution, pH 6.2, at a cell concentration of about 2.0 mg per ml. Lysozyme was added to the concentrations shown below and incubated at 37°C for 1 hr. The optical density at 630 mμ was measured by diluting 2 ml of the suspension with 3 ml of 0.6 M sucrose solution and reading the resulting value on the Bausch and Lomb Spectronic 20. Two ml aliquots of each solution were centrifuged and suspended in 5 ml distilled water; the resulting optical density was read at 630 mμ.

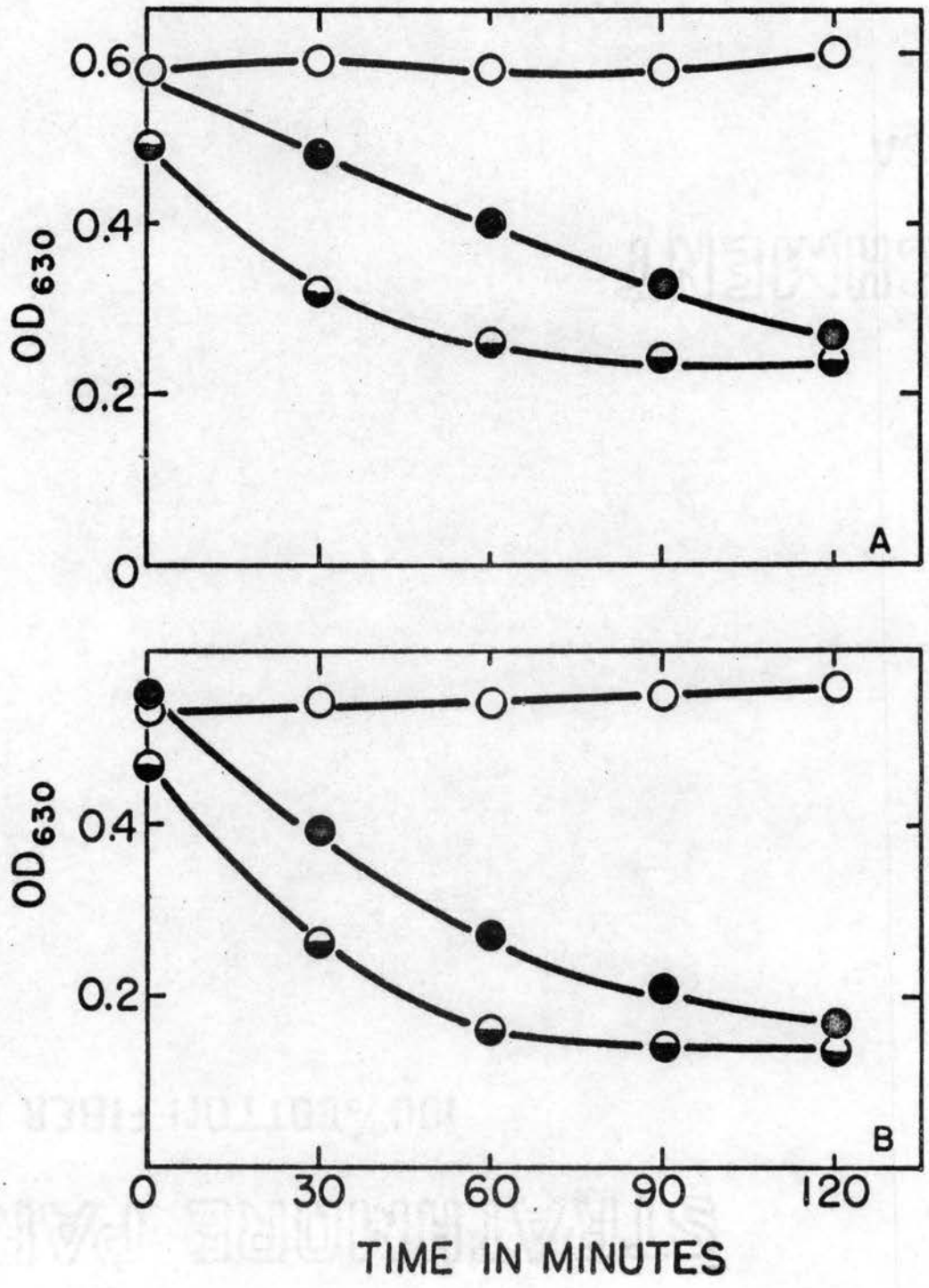
Lysozyme Conc. μg/ml	% Orig. OD_{630} after 1 hr	% Orig. OD_{630} after dilution at 1 hr
0	97	124
50	97	103
100	100	79
150	97	50
200	100	43
250	85	23
300	82	20
350	85	15

Figure 1

EFFECT OF LYSOZYME AND VERSENE ON LYSIS OF

S. FAECALIS 10C1 AND S. FAECALIS R

Whole cells of S. faecalis 10C1 and S. faecalis R were suspended in distilled water at an optical density of approximately 0.6 and incubated at 37°C. The control tube is designated by O, while ● shows the effect of lysozyme at 50 μg per ml, ◐ shows the effect of the addition of lysozyme plus 150 μg per ml versene. Part A shows the lysis of S. faecalis 10C1 while Part B shows S. faecalis R.


0.6
0.4
0.2
0
OD_{630}
A
0.4
0.2
OD_{630}
B
0
30
60
90
120
TIME IN MINUTES

the end of the 2 hr incubation, the same end-point was reached.

3. Comparison of the Lysis of S. faecalis R and S. faecalis 10C1

The lysis of S. faecalis R at a lysozyme concentration of 50 μg per ml has been described (24). Figure 1 shows a comparison of the lysis of S. faecalis R and S. faecalis 10C1 with lysozyme and with lysozyme and versene at a lysozyme concentration of 50 μg per ml. The rate of lysis of S. faecalis R was found to be greater than that of S. faecalis 10C1 under the same conditions as well as the final per cent of lysis at 2 hr.

4. Effect of Osmotic Stabilizers on Protoplast Formation

The osmotic stabilizers, polyethylene glycol (PEG), sucrose, KCl, and combinations of these were used as suspending media for protoplast formation as shown in Table II. After incubation with lysozyme at 37°C for 1 hr, the optical density at 630 mμ was recorded and the suspension was centrifuged. The aliquot was suspended in distilled water at a dilution of 1:4. Those solutions containing sucrose were found to give more extensive protoplast formation. A sucrose concentration of 0.6 M was noted to give both good protoplast formation and stabilization. This concentration of sucrose was used in subsequent experiments for the formation and stabilization of protoplasts.

5. Characterization of Protoplasts

Disruption occurs when the lysozyme-treated cells are suspended in a hypotonic solution. Protoplast formation was characterized by lysis of the suspension in distilled water as shown in Figure 2. It can be seen that complete formation of protoplasts from S. faecalis 10C1 was not achieved since there were probably some whole cells left

TABLE II

EFFECT OF OSMOTIC STABILIZERS ON PROTOPLAST FORMATION

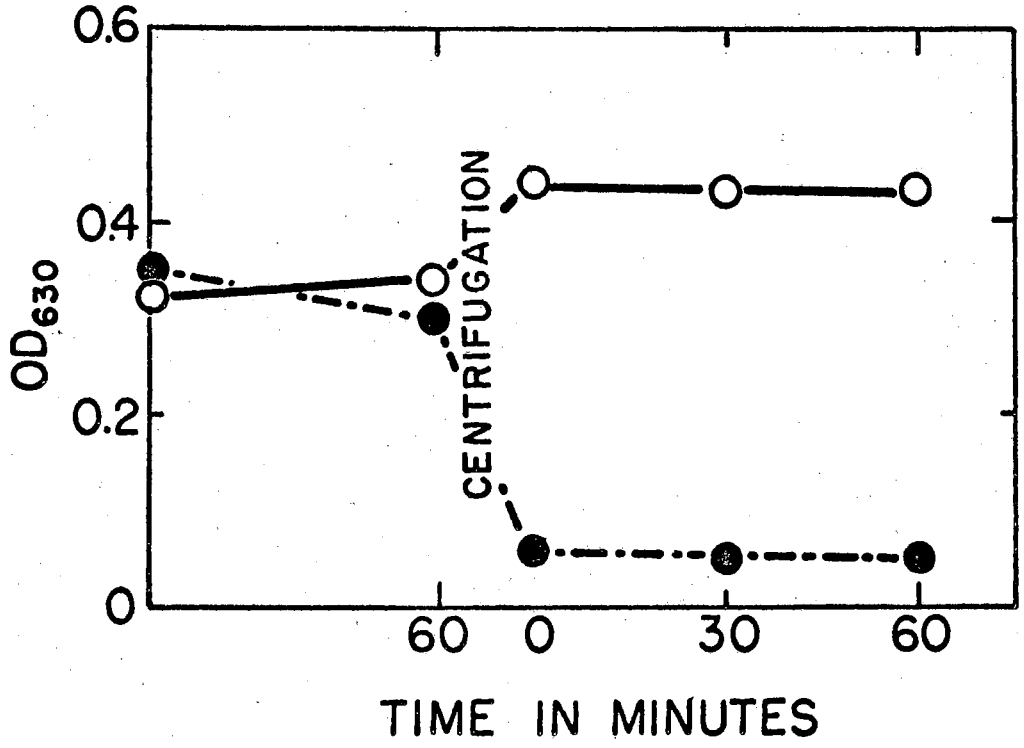
Whole cells of S. faecalis 10C1 were suspended in the osmotic stabilizers listed below at a cell concentration of 0.5 mg per ml. Lysozyme was added to a concentration of 50 μg per ml and the optical density was followed at 630 mμ for 1 hr. At the end of 1 hr, the suspensions were centrifuged and suspended in distilled water at a dilution of 1:4.

Stabilizer	Concentration	% Orig. OD_{630} after 1 hr	% Orig. OD_{630} after dilution at 1 hr
H_2O		61	16
Polyethylene glycol (PEG)	5%	90	19
	10%	104	26
KCl	0.85%	95	18
Sucrose	0.5M	67	9
	0.6M	80	10
	0.7M	89	8
	1.0M	97	11
PEG + sucrose	5%+ 0.5M	72	13
	5%+ 1.0M	89	15
	10%+ 0.5M	82	11
	10%+ 1.0M	100	12

Figure 2

CHARACTERIZATION OF PROTOPLASTS

Cells of S. faecalis 10C1 were suspended in 0.6 M sucrose at a concentration of 2 mg per ml. Crystalline lysozyme was added to one flask at a concentration of 350 μg per ml. The cells were incubated at 37°C for 1 hr. At the end of 1 hr, 2 ml aliquots were centrifuged and suspended in 5 ml distilled water. The control tube is shown as O, while the lysozyme treated cells are shown as ●.


0.6
0.4
0.2
0
OD_{630}
CENTRIFUGATION
60
0
30
60
TIME IN MINUTES

in the suspension, giving an optical density greater than 0; however, cell membranes might be responsible for some of the light scattering. Extensive protoplast formation was indicated by an optical density of about 20 per cent of the original density upon dilution in distilled water.

6. Stabilization of Protoplasts

Aliquots of a protoplast suspension were centrifuged and suspended in sucrose solutions of varying concentrations; whole cells were also suspended in these sucrose solutions. The resulting optical densities at 630 mμ are shown in Figure 3. At lower concentrations, the volume of the protoplast increases greatly and many of them are lysed. As the concentration becomes greater, the protoplast volume correspondingly decreases, the stability increases, and the optical density rises. A plateau of optical density is reached at about 0.6 M; this point was taken to be the point at which maximum stability is reached.

The refractive index of the suspending medium also has an effect on the optical density of the suspension. The correlation between refractive index and optical density is seen in Figure 4. The suspensions of whole cells in Figure 3 were noted to give a decreased optical density with increased concentration of sucrose. This phenomenon was observed with PEG and polyvinylpyrollidinone (PVP) as well. Increasing concentrations of these compounds show an increased refractive index. The ability of a particle to scatter light depends on the difference in the refractive index of the medium and the particle (24). As the concentration of the stabilizer increases the refractive indices of the

Figure 3

EFFECT OF SUCROSE MOLARITY ON OPTICAL DENSITY OF CELLS AND PROTOPLASTS

Suspensions of whole cells and protoplasts were suspended in 5 ml aliquots of varying concentrations of sucrose. The optical density at 630 mμ of the resulting suspension was read and plotted as per cent of the original optical density of the suspension.

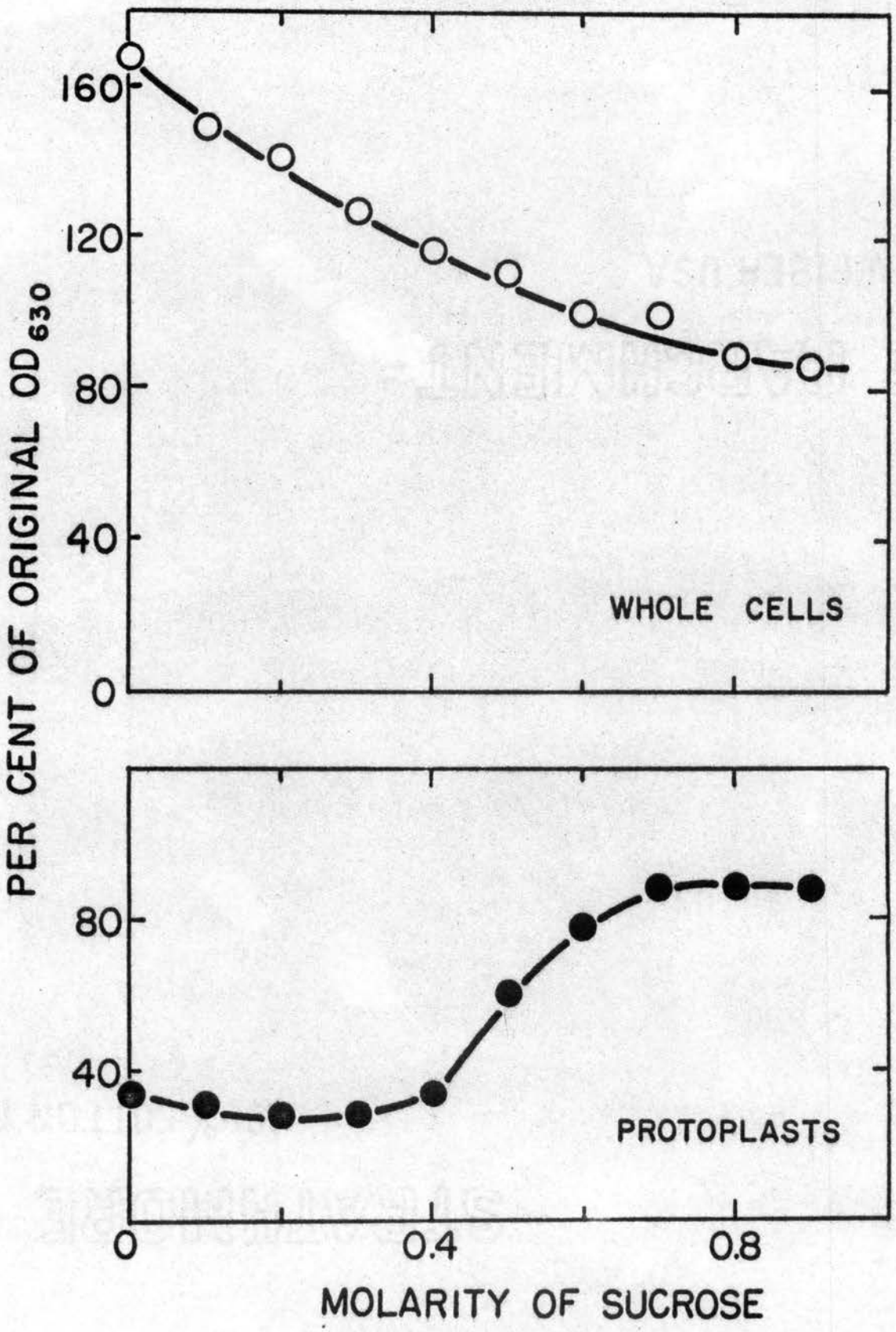

160
120
80
40
0
WHOLE CELLS
80
40
PROTOPLASTS
0
0.4
0.8
PER CENT OF ORIGINAL OD630
MOLARITY OF SUCROSE

Figure 4

EFFECT OF REFRACTIVE INDEX OF THE SUSPENDING MEDIUM ON OPTICAL DENSITY OF WHOLE CELLS OF S. FAECALIS 10C1

Whole cells of S. faecalis 10C1 were suspended in various concentrations of some osmotic stabilizers at a concentration equaling an optical density of approximately 0.6 in distilled water. The optical density at 630 mμ at each concentration was read immediately. The refractive index of each concentration was read on the refractometer and plotted against the optical density of the suspension at that concentration.

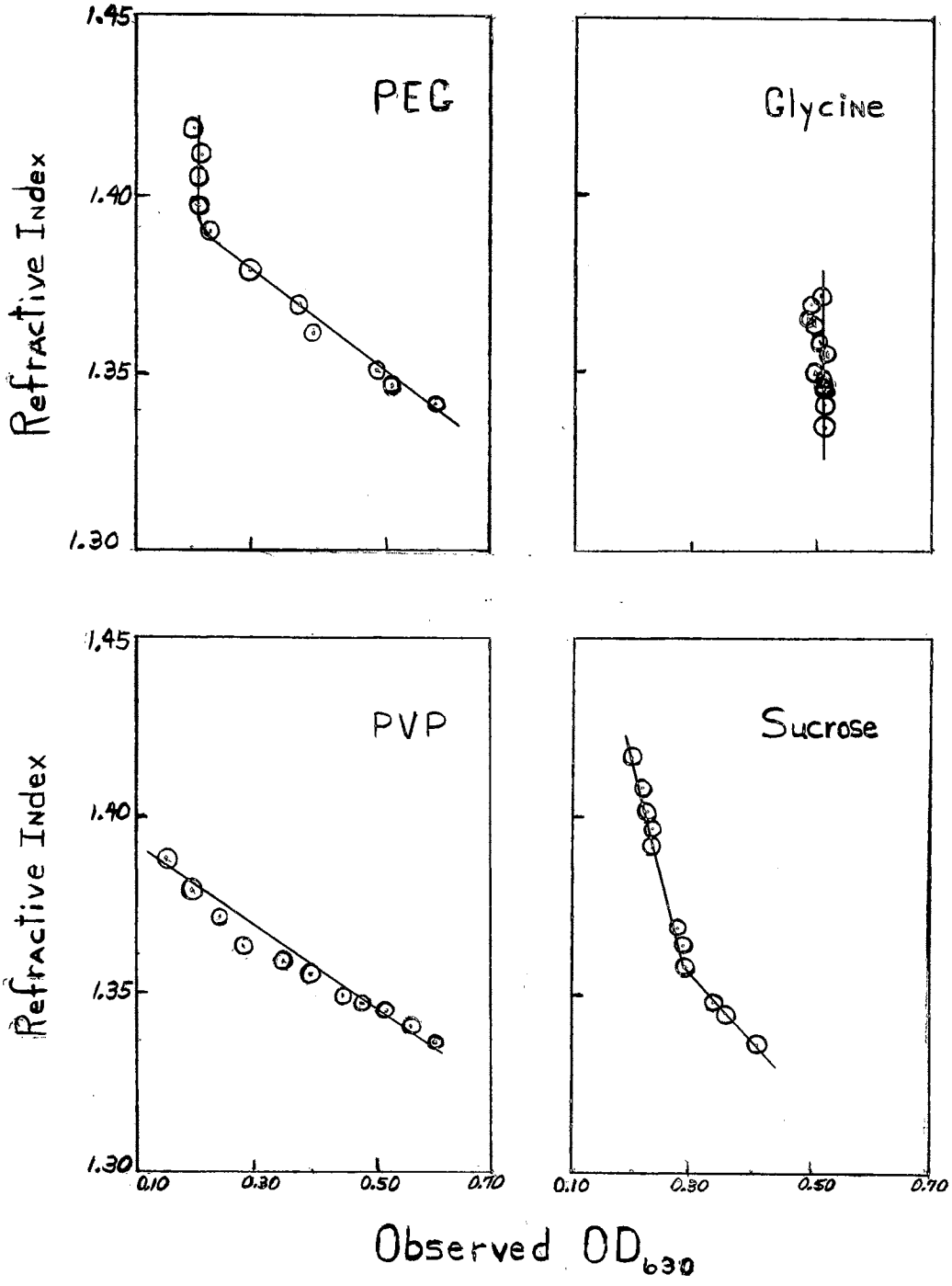

PEG
Glycine
PVP
Sucrose
Refractive Index
Refractive Index
1.45
1.40
1.35
1.30
0.10
0.30
0.50
0.70
Observed OD630

Figure 4

medium and the cell converge with a decrease in light scattering ability. A study comparing refractive index of a glycine solution (pH 6.2) and alanine (not shown) with the resulting optical density of cells suspended in it, however, shows that the refractive index of the glycine solution increased as much as that of PVP while the optical density of the cell suspension did not change. Therefore, some factor other than refractive index must play a role in the change of optical density.

An aliquot of protoplast suspension was centrifuged, suspended in 0.6 M sucrose solution, and the optical density was followed for 1 hr as shown in Figure 5. The protoplast suspension is shown to be stable in 0.6 M sucrose.

B. Studies on Metabolic Swelling

1. Effect of Glucose and Pyruvate on Metabolic Swelling of S. faecalis R

Abrams (18) has shown metabolic swelling with glucose in S. faecalis (ATCC # 9790), but this strain did not swell with added pyruvate (0.01 M). Figure 6 shows the effect of addition of glucose and pyruvate on protoplasts of S. faecalis R. With this strain of S. faecalis, swelling is produced with both glucose and pyruvate. The reversal of swelling has been described by Abrams as a return to the external medium of products of metabolism of the substrate and occurs after the glucose has disappeared completely. It would be expected that disappearance of pyruvate would also result in shrinkage.

2. Effect of Lipoic Acid on Swelling of S. faecalis 10C1

Studies with S. faecalis 10C1 have shown that this lipoic acid deficient mutant can concentrate S^{35}-labelled lipoic acid in the interior

Figure 5

STABILIZATION OF PROTOPLASTS

Cells of S. faecalis 10C1 were suspended in 0.6 M sucrose at a concentration of 2 mg per ml. Crystalline lysozyme was added at a concentration of 350 μg per ml to one flask. The flasks were incubated at 37°C for 1 hr. At the end of 1 hr, 2 ml aliquots were centrifuged and suspended in 5 ml sucrose solution. O shows the control tube, while ● shows the lysozyme treated cells. The dotted arrows show the characterization of protoplasts after each 1 hr period.

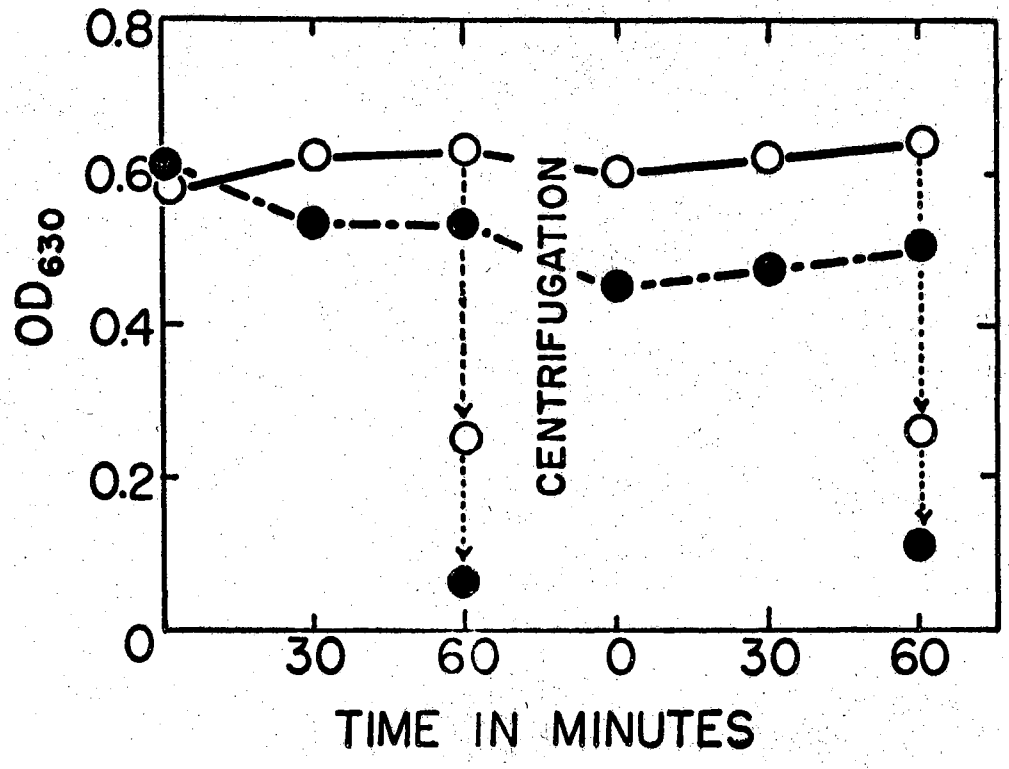
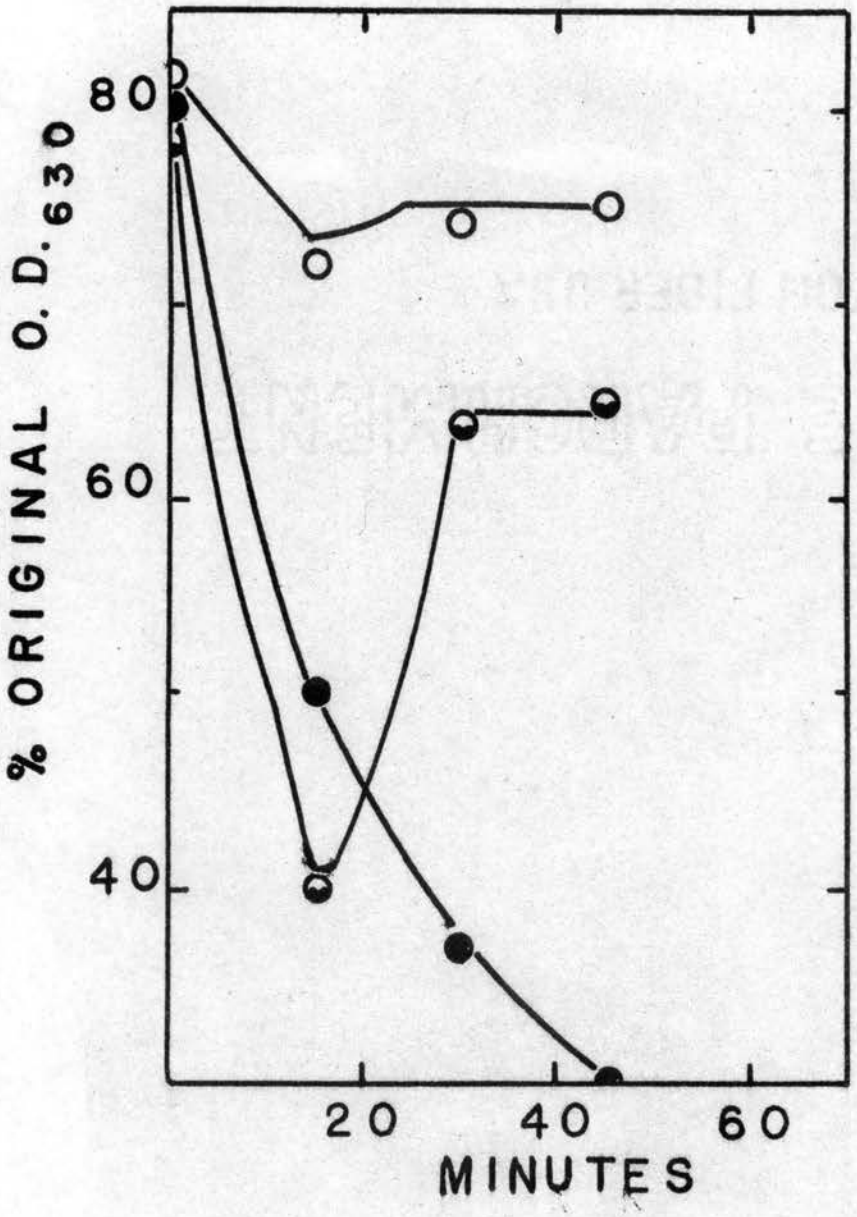

0.8
0.6
0.4
0.2
0
OD_{630}
CENTRIFUGATION
30
60
0
30
60
TIME IN MINUTES

Figure 6

METABOLIC SWELLING OF S. FAECALIS R WITH GLUCOSE AND PYRUVATE

Two ml aliquots of a protoplast suspension of S. faecalis R were centrifuged and suspended in sucrose solution. Glucose and pyruvate to concentrations of 200 μg per ml and 0.03 M respectively were added in 0.1 ml amounts. The optical density at 630 mμ was followed for 1 hr. The control tube is shown by O, while the effect of glucose is shown by ●, and the effect of pyruvate is shown by ◒.


% ORIGINAL O.D.630
80
60
40
20
40
60
MINUTES

of the cell at a concentration of about 100-fold above that of the exterior medium (11). Lipoic acid was added to a protoplast suspension to study its effect on swelling. Figure 7 shows that 0.01 M lipoic acid does not produce swelling.

3. Effect of Amino Acids on Swelling of S. faecalis 10C1

Uptake of amino acids by cells has been shown with several amino acids. Mora and Snell (16) studied the uptake of glycine and alanine in S. faecalis (ATCC # 9790) protoplasts. The uptake of lysine in S. faecalis whole cells has also been studied (26). The amino acids shown in Table III were added in solution at pH 6.2. Swelling was not observed under the conditions used for the swelling with glucose in S. faecalis 10C1. It is probable that the amino acids, when taken into the cell, enter the amino acid pool and become bound. Since compounds which are not free in the interior of the cell do not contribute to the internal osmotic pressure, the bound amino acids would not cause swelling.

4. Effect of Glucose and Pyruvate on Metabolic Swelling of S. faecalis 10C1

Metabolic swelling was produced in protoplasts of S. faecalis R by both glucose and pyruvate; swelling was observed in S. faecalis 10C1 with glucose, but pyruvate alone did not produce swelling. This strain requires the addition of both lipoic acid and pyruvate for swelling. Figure 8 shows the metabolic swelling produced by the addition of pyruvate, lipoic acid, and thiamin to protoplasts of S. faecalis 10C1. Pyruvate alone or pyruvate and thiamine produce no metabolic swelling, while the addition of 0.001 μg per ml of lipoic acid to pyruvate gives swelling. The addition of thiamin to pyruvate and lipoic acid produced further

Figure 7

EFFECT OF LIPOIC ACID ON PROTOPLASTS OF S. FAECALIS 10C1

Two ml aliquots of a protoplast suspension were centrifuged and suspended in 5 ml of 0.6 M sucrose solution (pH 6.2). Lipoic acid was added to a concentration of 0.01 M. The optical density was followed at 630 mμ for 1 hr. The control is shown by O, while the effect of lipoic acid is shown by ●.

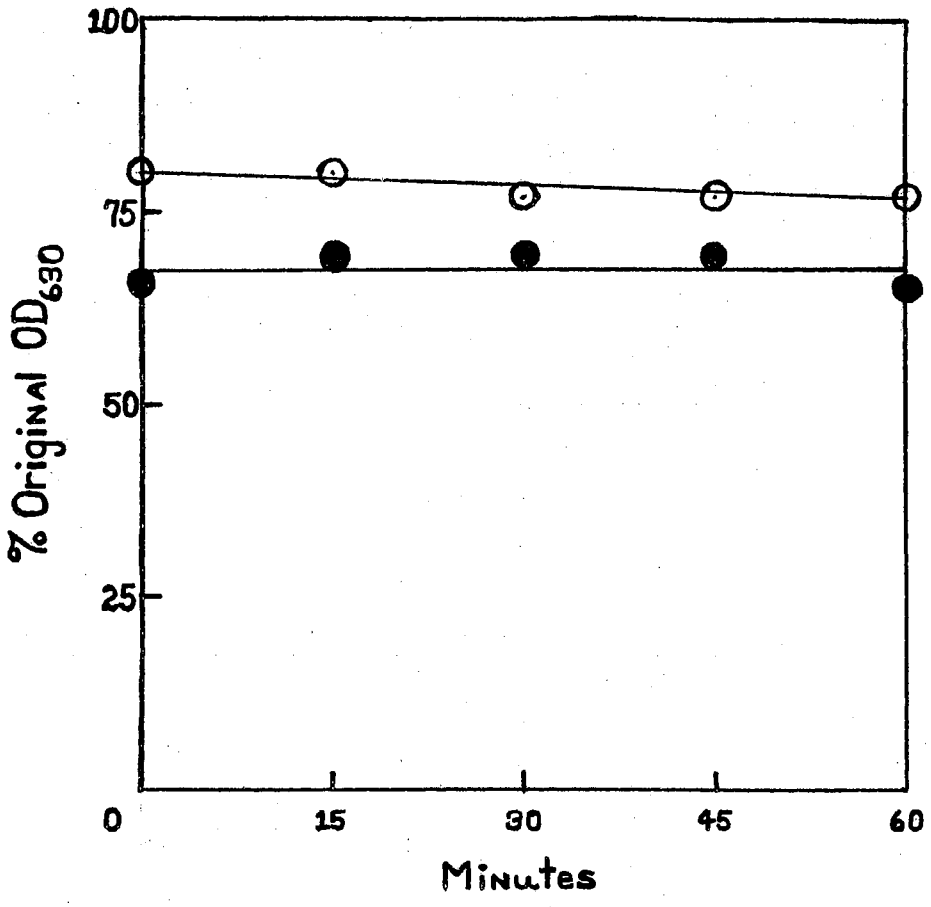

100
75
50
25
0
15
30
45
60
% Original OD_{630}
Minutes

TABLE III

EFFECT OF AMINO ACIDS ON SWELLING OF S. FAECALIS 10C1

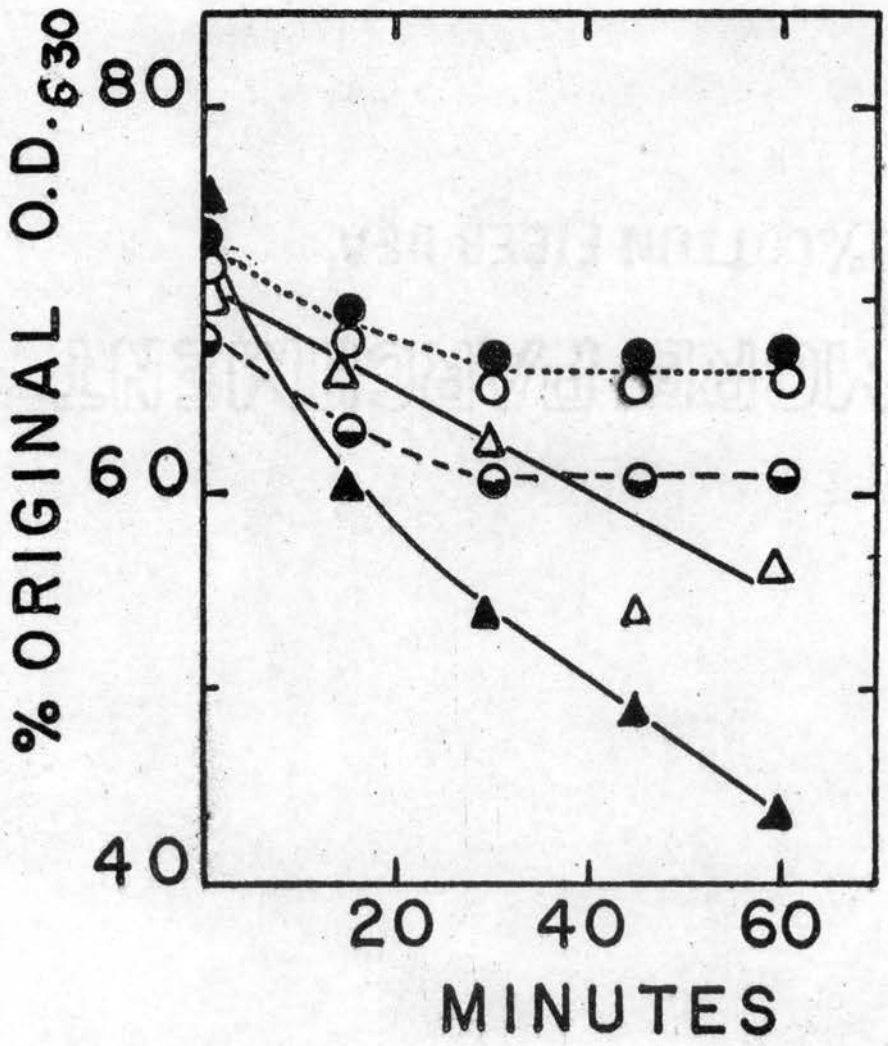
Two ml aliquots of a protoplast suspension were centrifuged and suspended in sucrose solution. The following amino acids were added to a concentration of 400 μg per ml in 0.1 ml. The optical density of the protoplast suspension was followed at 630 mμ for 1 hr.

Amino Acid	% Decrease in OD_{630} in 1 hr Control	Amino Acid
	5	
Phenylalanine		7
Leucine		4
Cysteine		7
Glycine		7
Aspartic Acid		7
Lysine		7
Glutamic Acid		0

Figure 8

METABOLIC SWELLING OF PROTOPLASTS OF S. FAECALIS 10C1

Two ml aliquots of a protoplast suspension were centrifuged and suspended in 0.6 M sucrose solution (pH 6.2). The effects of the addition of pyruvate, lipoic acid and thiamin were noted by following the optical density at 630 mμ for 1 hr. O shows the control, ● shows the effect of the addition of 0.03 M pyruvate, ◐ shows the effect of the addition of 0.03 M pyruvate and 10 μg per ml thiamin, Δ shows the effect of the addition of 0.03 M pyruvate plus 0.001 μg per ml lipoic acid, and ▲ shows the effect of the addition of pyruvate, lipoic acid, and thiamin.


% ORIGINAL O.D.630
80
60
40
20
40
60
MINUTES

stimulation of swelling.

The addition of various concentrations of lipoic acid shows that there is a concentration effect upon the swelling of protoplasts with pyruvate as shown in Table IV.

5. Effect of Lipoic Acid Assay Components on Metabolic Swelling of S. faecalis 10C1

Components of the lipoic acid assay system of Gunsalus, Dolin, and Struglia (23) were added to the protoplast suspension in all possible combinations to test for metabolic swelling. It was found that for swelling to occur, lipoic acid and pyruvate must be present. Under these conditions, thiamin stimulated swelling. Table V shows the effect of the components on S. faecalis 10C1 protoplasts.

6. Effect of Inhibitors on Metabolic Swelling of S. faecalis 10C1

Studies with the inhibitors of oxidative phosphorylation, 2,4-dinitrophenol (DNP) and sodium azide, show that protoplast swelling with either glucose or pyruvate was decreased. As shown in Table VI, the inhibition is not complete. Inhibition by potassium thiocyanate and potassium fluoride was also studied in the presence of glucose and pyruvate. Thiocyanate inhibition is attributed to its reaction with postively charged groups on proteins. A decrease in swelling was noted with this inhibitor. Potassium fluoride is an inhibitor of glycolysis; however, no decrease was noted in the presence of this compound.

C. Manometric Measurements of Uptake

1. Establishment of the Assay Method

For whole cells of S. faecalis 10C1, the assay method of Gunsalus, Dolin, and Struglia (23) was used to determine the amount of

TABLE IV

EFFECT OF LIPOIC ACID CONCENTRATION ON METABOLIC SWELLING

Two ml aliquots of a protoplast suspension were centrifuged and suspended in 5 ml sucrose solution. Pyruvate was added to 0.03 M. Lipoic acid was added to the concentrations shown and the decrease in optical density at 630 mμ was followed for 1 hr.

Concentration of lipoic acid μg per ml	% change in optical density produced in 1 hr
0 (no pyruvate)	8
0 (pyruvate added)	5
0.0004	15
0.001	23
0.004	28
0.04	32
0.2	35

TABLE V

METABOLIC SWELLING OF PROTOPLASTS BY COMPONENTS OF THE MANOMETRIC ASSAY SYSTEM

Two ml of a protoplast suspension were centrifuged and suspended in sucrose solution. The following compounds were added to the desired concentration in 0.1 ml of solution. Swelling of the protoplasts was followed at 630 mμ for 45 min. The swelling is expressed as per cent of decrease in optical density during this time. The following abbreviations are used. G = glutamic acid at 5 mg per ml, R = riboflavin at 20 μg per ml, T = thiamin at 10 μg per ml, L = lipoic acid at 0.002 μg per ml, A = adenosine at 200 μg per ml, Mg = $MgSO_4$ at 1.25 mg per ml, P = pyruvate at 0.03 M.

Swelling in One Component Systems		
Component	Control	With Added Component
T	5	10
L	6	9
P	5	7
A	5	4
R	5	2
Mg	5	2
G	5	0

TABLE V (Continued)

Swelling in Two Component Systems		
Components	Control	With Added Compounds
P + L	7	27
A + P	0	8
R + L	3	10
P + R	5	11
G + T	5	10
G + A	0	5
A + L	3	7
A + T	0	3
L + T	3	6
G + P	5	8
G + R	3	5
R + T	3	5
G + Mg	10	11
P + Mg	10	11
G + L	3	3
T + Mg	5	5
A + Mg	5	5
A + R	5	5
L + Mg	10	10
P + T	5	3
R + Mg	5	0

TABLE V (Continued)

Swelling in Three Component Systems		
Components	Control	With Added Compounds
R + P + L	0	18
T + P + L	0	17
Mg + P + L	5	21
A + P + L	5	20
G + P + L	5	20
A + T + L	0	10
G + A + P	2	12
P + R + T	3	11
R + T + L	0	8
R + T + Mg	3	8
A + P + R	3	8
G + T + L	0	5
G + A + L	5	8
G + L + Mg	3	6
G + P + T	5	8
A + P + Mg	3	6
G + A + Mg	2	4
G + R + Mg	3	5
G + P + R	5	7
A + R + Mg	3	5
A + P + T	3	5

TABLE V (Continued)

Swelling in Three Component Systems		
Components	Control	With Added Compounds
A + R + L	0	2
P + T + Mg	3	5
R + L + Mg	0	2
P + R + Mg	5	6
G + A + R	2	2
G + A + T	2	2
G + P + Mg	5	5
G + R + T	3	3
G + R + L	0	0
A + T + Mg	5	5
A + L + Mg	3	3
A + R + T	3	3
T + L + Mg	3	3

Swelling in Four Component Systems		
Components	Control	With Added Compounds
R + T + P + L	5	35
R + A + P + L	5	35
A + T + P + L	5	33
Mg + T + P + L	6	31

TABLE V (Continued)

Swelling in Four Component Systems		
Components	Control	With Added Compounds
A + Mg + P + L	5	30
G + Mg + P + L	9	34
R + Mg + P + L	6	28
G + T + P + L	9	28
G + A + P + L	9	26
G + R + P + L	9	18
R + A + P + Mg	6	15
R + P + G + Mg	6	15
T + R + G + L	5	13
T + A + P + G	9	17
R + A + G + L	5	13
T + R + P + G	9	14
T + P + G + Mg	6	12
R + A + P + G	9	14
T + R + A + P	7	10
T + R + A + L	9	12
T + A + P + Mg	6	9
T + G + L + Mg	6	9
T + A + G + L	5	8
R + A + L + Mg	6	9
A + P + G + Mg	9	12

TABLE V (Continued)

Swelling in Four Component Systems		
Components	Control	With Added Compounds
A + G + L + Mg	5	8
T + R + A + G	7	8
T + R + P + Mg	3	3
T + R + G + Mg	3	3
T + R + L + Mg	3	3
T + A + G + Mg	6	6
T + A + L + Mg	6	6
R + A + G + Mg	6	6
T + R + A + Mg	3	0

Swelling in Five Component Systems		
Components	Control	With Added Compounds
R + G + T + P + L	3	30
A + Mg + G + P + L	9	37
G + Mg + T + P + L	9	34
A + G + T + P + L	9	31
R + A + T + P + L	5	26
R + G + Mg + P + L	3	24
A + Mg + T + P + L	5	24
R + A + Mg + P + L	5	24

TABLE V (Continued)

Swelling in Five Component Systems		
Components	Control	With Added Compounds
R + Mg + T + P + L	2	19
R + A + G + P + L	9	21
R + A + T + P + L	9	20
T + A + R + L + Mg	2	9
R + A + P + G + Mg	9	14
T + R + A + P + Mg	7	11
T + R + P + G + Mg	7	11
T + R + G + L + Mg	2	5
T + A + P + G + Mg	9	12
T + A + G + L + Mg	9	12
T + R + A + P + G	7	10
R + A + G + L + Mg	9	10
T + R + A + G + Mg	7	7

Swelling with Six Component Systems		
Components	Control	With Added Compounds
R + A + G + Mg + P + L	0	29
R + A + G + T + P + L	0	26
R + G + Mg + T + P + L	0	23

TABLE V (Continued)

Swelling with Six Component Systems		
Components	Control	With Added Compounds
A + G + Mg + T + P + L	0	23
R + A + Mg + T + P + L	3	25
T + R + A + P + G + Mg	3	9
T + R + A + G + L + Mg	3	6

Swelling with Seven Component Systems		
Components	Control	With Added Compounds
R + Mg + A + G + T + P + L	0	29

TABLE VI

EFFECT OF INHIBITORS ON METABOLIC SWELLING

The metabolic inhibitors listed below were added to suspensions of protoplasts of S. faecalis 10C1 before the addition of substrates or after swelling had proceeded for 15 min. Inhibitors were added at 15 min by centrifuging the suspension and suspending in sucrose solution containing the inhibitor. The optical density at 630 mμ was followed for 1 hr to determine whether these inhibitors would have any effect on the metabolic swelling with glucose and pyruvate.

Inhibitor	Concentration (Molar)	Substrate	% Change OD_{630}	
			Inhibitor Added 0	15 min
None		Pyruvate + Lipoic Acid	-16	
		Glucose	-35	
DNP	10^{-4}	Pyruvate + Lipoic Acid	-11	- 8
	10^{-3}		- 3	-12
	10^{-4}	Glucose	-27	-27
	10^{-3}		-20	-27

TABLE VI (Continued)

Inhibitor	Concentration (Molar)	Substrate	% Change OD_{630}	
			Inhibitor Added	
			0	15 min
NaN_3	10^{-4}	Pyruvate + Lipoic Acid	-5	- 5
	10^{-3}		0	+ 2
	10^{-4}	Glucose	-18	-15
	10^{-3}		-16	-14
KCNS	10^{-4}	Pyruvate + Lipoic Acid	-13	-13
	10^{-3}		-10	-11
	10^{-4}	Glucose	-21	-24
	10^{-3}		-11	-27
KF	10^{-4}	Pyruvate + Lipoic Acid	-20	-37
	10^{-3}		-20	-37
	10^{-4}	Glucose	-31	-40
	10^{-3}		-35	-34

oxygen uptake resulting from the addition of lipoic acid in the presence of pyruvate in this strain. A linear response of oxygen uptake was found with added lipoic acid as shown in Figure 9. Whole cells were assayed in the presence of sucrose to determine the effect of this osmotic stabilizer on the system. A slight rise in oxygen uptake may be noted in the control flask with the addition of sucrose.

The duration of incubation before the addition of pyruvate on oxygen uptake was studied. Times of 15 to 45 min after the addition of lipoic acid all gave the maximum rate of oxygen uptake. It was assumed that the lipoic acid entered the cell and was bound during this time. In subsequent assays, a 15 min incubation time was used.

2. Comparison of Uptake in Whole Cells and Protoplasts

Whole cells, whole cells in sucrose solution, and protoplasts of S. faecalis 10C1 when assayed manometrically were noted to have almost identical responses to the addition of lipoic acid. Table VII shows the amount of oxygen uptake for whole cells and protoplasts of S. faecalis 10C1 with various levels of lipoic acid. This similarity between oxygen uptake in whole cells and protoplasts indicates that the cell wall contributed no rate-limiting features to the transport of pyruvate and lipoic acid into the cell.

Figure 9

LIPOIC ACID UPTAKE IN WHOLE CELLS OF S. FAECALIS 10C1 BY MANOMETRIC METHODS

The response of whole cells of S. faecalis 10C1 to lipoic acid concentrations from 0.0005 μg to 0.005 μg per ml was determined manometrically by the method of Gunsalus, Dolin, and Struglia (23) as modified in the Experimental Procedure, section 6. Oxygen uptake was followed for 1 hr after the addition of pyruvate to the system.

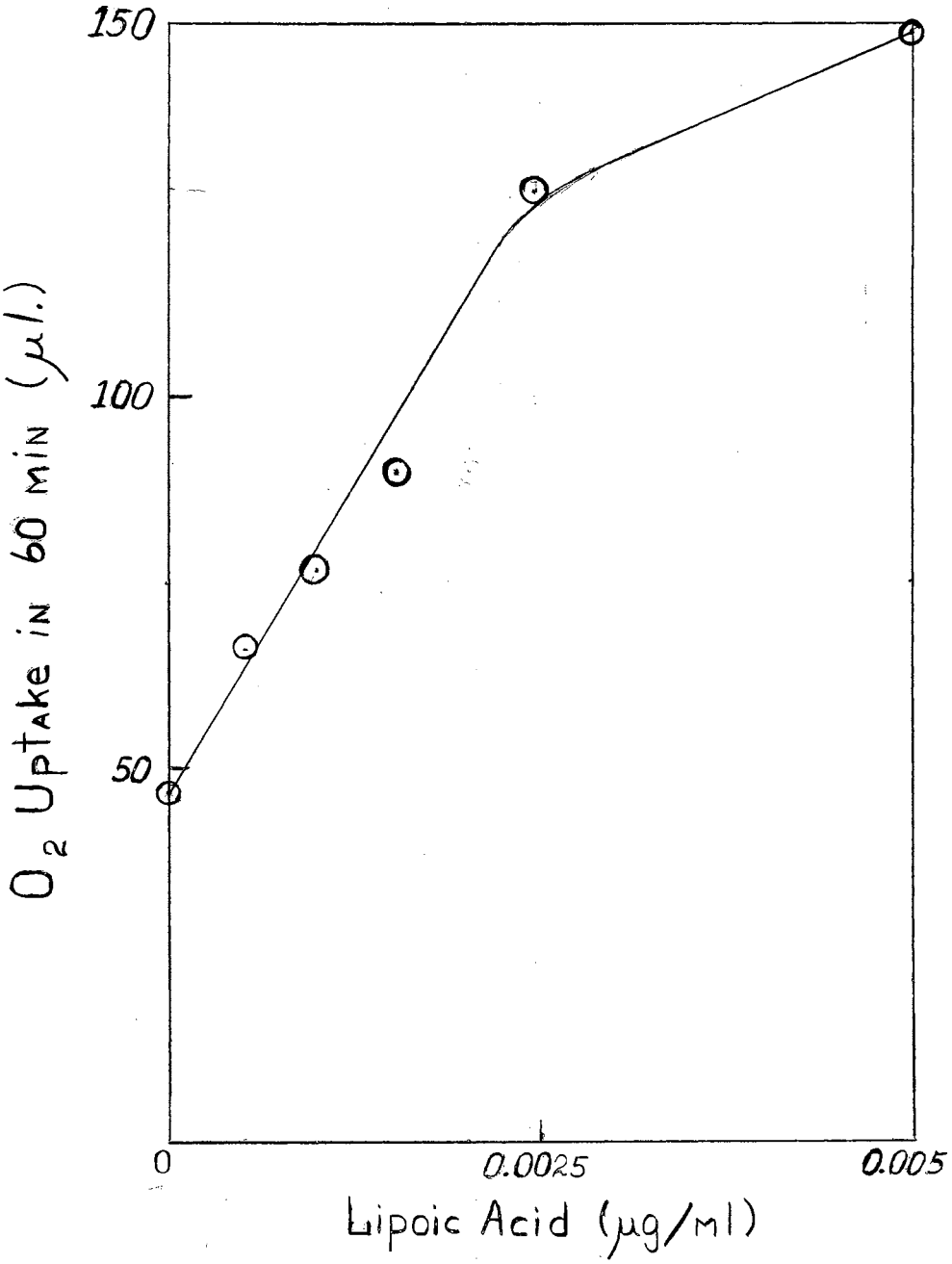


Figure 9

TABLE VII

MANOMETRIC MEASUREMENT OF LIPOIC ACID WITH WHOLE CELLS AND PROTOPLASTS OF S. FAECALIS 10C1

Whole cells and protoplasts were assayed by the method of Gunsalus, Dolin, and Struglia (23) as modified in the Experimental Procedure, section 6. Ten mg of cells were used per flask. Protoplasts formed from 10 mg of cells were centrifuged and suspended in 1 ml of 1.2 M sucrose 1.5 M phosphate buffer, pH 6.2, for each flask. Oxygen uptake was followed for 1 hr after addition of pyruvate.

System Assayed	Lipoic Acid Concentration (μg per ml)	Amount of Oxygen uptake in 1 hr (μl)
Whole cells	0	6.7
	0.001	65.5
	0.0025	96.8
Whole cells in sucrose	0	17.7
	0.001	66.8
	0.0025	96.6
Protoplasts	0	11.7
	0.001	67.7
	0.0025	101.4

DISCUSSION

Mucopeptides composed of acetylglucosamine and acetylmuramic acid are responsible for the rigidity of the cell wall and are the site of action of lysozyme. Salton has attempted correlation of cell wall composition and lysozyme sensitivity (27,28,29). It appears that with increasing complexity of the cell wall structure as reflected by an increase in the number of components, sensitivity to lysozyme decreases. Micrococcus lysodeikticus possesses a cell wall composed mainly of mucopeptide (29) and is most sensitive to lysozyme action. S. faecalis (30) cell walls contain mucopeptide, mucopolysaccharide, and both ribitol and glycerol phosphate teichoic acids and are more resistant to lysozyme action. The variation in sensitivity of the strains of S. faecalis used in this study might reflect a difference in cell wall composition. Quantitative analysis of the cell walls of these two strains are not available; however, it has been demonstrated that substitution of hydroxylysine for lysine in cell walls of S. faecalis makes them more resistant to lysozyme (31). Gram-negative organisms contain a lipoprotein layer in their cell walls in addition to most of the constituents listed above. Lysis requires participation of factors other than lysozyme.

Removal of the rigid cell wall in the presence of a stabilizer results in the unmasking of the osmotically responsive portion of the cell. Protoplasts swell and contract with variations in the osmotic pressure.

This osmotic response to substances allows determination of permeability. Swelling due to the metabolism of compounds such as glucose has been observed and this would be expected from the production of several molecules from one during glycolysis. Abrams noted that maximum swelling occurred when glucose was completely removed from the external medium and that at this time the lactic acid concentration in the cell was maximum (19). As lactic acid slowly returned to the external medium, the protoplasts contracted to approach their initial volume. In E. coli, non-metabolizable carbohydrates can produce osmotic swelling which presumably represents their concentration in the free state inside the cell. However, in these studies no osmotic response to vitamins and amino acids was noted under conditions which should result in an accumulation. This may reflect the binding of the "pool" material by some macromolecular species so that the osmotic pressure is not changed. A model developed by Britten and McClure (32) with the assumption that specific receptor sites exist for pool amino acids in E. coli fits the available data better than a simple permease or a site model.

This thesis describes the swelling of S. faecalis protoplasts due to pyruvate metabolism. Under conditions where lipoic acid is required for pyruvate oxidation it is required for metabolic swelling of protoplasts. This swelling is also stimulated by thiamin. Pyruvate can give rise to lactate by the dismutation reaction shown below.

$$2\ CH_3COCOOH + \tfrac{1}{2}O_2 \longrightarrow CH_3COOH + CO_2 + CH_3CHOHCOOH$$

S. faecalis strain 10C1 contains all the enzymes necessary for the above reaction. If the carbon dioxide is released from the cell, there is no net change in the number of molecules following pyruvate dismutation and

a mere increase in the number of intracellular molecules can not be the mechanism of metabolic swelling. The above reaction requires the regeneration of certain cofactors through coupling to electron transport and oxidative phosphorylation. To determine if uncoupling of the above two processes has any effect on metabolic swelling, dinitrophenol, an uncoupling agent, was used to inhibit metabolic swelling. The question remains unsettled as to a direct involvement of oxidative phosphorylation in metabolic swelling since energy is required for the transport of substrates to the interior of the cell. If the hypothesis of Mitchell is correct, thiamin and lipoic acid should function in the transport of pyruvate to the interior of the cell.

Measurement of osmotic response by optical density determination is defined by the equation shown below (33).

$$A = \frac{32\pi^3}{2.3\ (3)} \left(\frac{\frac{dn}{dc}}{n_o} \right)^2 \frac{q^2 v}{\lambda^4}$$

in which

A = absorbancy

n = index of refraction of the particle

n_o = index of refraction of the medium

$\frac{dn}{dc}$ = specific refraction index increment

dc = change of concentration of substance in the particle

q = anhydrous mass of material in a single particle

v = number of particles per unit volume

λ = wavelength of light

As the refractive index of the medium approaches that of the cells, less

light scattering occurs and the optical density observed decreases. Generally the optical density observed with a non-permeable solute should be inversely proportional to the concentration and hence to the refractive index. The observation that a change in the refractive index of glycine or alanine solutions fails to produce a change in the optical density of the cell suspension could be interpreted as being due to the penetration of the solute into the cell in such a manner to keep the difference of the refractive indices of the medium and cells constant. However, S. faecalis cells have been shown to have a definite, limited capacity for these amino acids. Also one would expect to measure an osmotic response of the protoplasts to concentration of the free amino acids. These two observations suggest that the theory of light scattering does not hold for all types of bacterial suspensions in all solutions.

This study has suggested various questions concerning transport problems and has made certain points clear. The intracellular form of transported lipoic acid and amino acids appears to be a complex which is broken rapidly by mild treatments. The mechanism for selective transport is situated in the cell membrane and the cell wall does not influence transport in any rate-limiting manner. A choice between the theories of Cohen and Monod and of Mitchell is not possible from the data presented, but a system for testing the hypotheses using labeled pyruvate has been suggested. Methods have been established for the formation of protoplasts and preliminary experiments have been done on the preparation of the cell membranes. A study of the distribution of enzymes concerned with lipoic acid metabolism and function should provide another test of Mitchell's hypothesis.

SUMMARY

Methods for the preparation, stabilization and characterization of protoplasts formed from S. faecalis 10C1 were established.

These protoplasts show swelling in response to glucose metabolism and it was demonstrated that swelling due to pyruvate metabolism depended upon the presence of lipoic acid. Protoplasts and whole cells respond in a similar manner to varying lipoic acid concentrations in the manometric assay system.

The evidence is consistent with the supposition that the cell wall does not influence transport in a rate limiting fashion and that the pool lipoic acid is not free.

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