

Spring 1967

ASPECTS OF MEMBRANE STRUCTURE AND FUNCTION IN STREPTOCOCCUS FAECIUM

JAMES JOSEPH BURKE JR.

Follow this and additional works at: <https://scholars.unh.edu/dissertation>

Recommended Citation

BURKE, JAMES JOSEPH JR., "ASPECTS OF MEMBRANE STRUCTURE AND FUNCTION IN STREPTOCOCCUS FAECIUM" (1967). *Doctoral Dissertations*. 870.
<https://scholars.unh.edu/dissertation/870>

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.

**This dissertation has been
microfilmed exactly as received 68-14,971**

**BURKE, Jr., James Joseph, 1940-
ASPECTS OF MEMBRANE STRUCTURE AND FUNCTION
IN STREPTOCOCCUS FAECIUM.**

**University of New Hampshire, Ph.D., 1967
Microbiology**

University Microfilms, Inc., Ann Arbor, Michigan

ASPECTS OF MEMBRANE STRUCTURE AND FUNCTION
IN STREPTOCOCCUS FAECIUM

BY

JAMES JOSEPH BURKE, Jr.

B.S., Merrimack College, 1961

A THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirements for the Degree of
Doctor of Philosophy

Graduate School
Department of Microbiology

June, 1967

This thesis has been examined and approved.

William R. Chesbro

L. V. Slonetz

J. F. Metcalf

Edward J. Harris

Paul R. Jones

13 July 1967

Date

ABSTRACT

ASPECTS OF MEMBRANE STRUCTURE AND FUNCTION IN STREPTOCOCCUS FAECIUM

by

JAMES JOSEPH BURKE, Jr.

The aim of this investigation was to develop techniques for the study of the bacterial membrane. The areas studied were: the effects of homologous membrane antiserum on the growth and metabolism of protoplasts; the antigenic composition of the membrane; the mode of growth of the protoplast membrane; the properties of the membrane-bound ATPase and ATP-pool driven transport of amino acids.

The organism used in these studies was Streptococcus faecium, strain HF8AG. Membranes were produced by osmotic lysis of muramidase induced protoplasts.

The growth of protoplasts of S. faecium was inhibited by treatment with homologous membrane antiserum. This activity was confined to the γ -globulin fraction of the immune serum and was also displayed by univalent antibody, prepared by papain treatment of purified immune globulin. The inhibition appeared due to interference with metabolite transport. Glucose transport was completely inhibited, while the transport of the amino acids arginine, alanine and glutamic acid was inhibited 60%, 28% and 18%, respectively. Normal sera were

ineffective in preventing growth, but a heat resistant, dialyzable factor, causing lysis of protoplasts, was present.

Application of gel-immunodiffusion techniques to membranes solubilized by ultrasound or detergents demonstrated the antigenic heterogeneity of the structure. Three antigens were detectable, two of which were heat labile and susceptible to proteolysis. The third antigen was identified as a teichoic acid characteristic of the Group D streptococci.

Study of the membrane bound ATPase of S. faecium indicated that the enzyme was Mg^{++} dependent, although Mn^{++} or Co^{++} could relieve the requirement to some extent. Stimulation of the enzyme by Na^+ and K^+ could not be detected. Of various alternative substrates used, only ITP was hydrolyzed to a significant extent. The sole products of the reaction with ATP were ADP and orthophosphate. Due to competitive inhibition by ADP, the reaction would cease when 20% of the ATP present was hydrolyzed. Thiol binding agents had no effect on the activity of the enzyme. Of a variety of enzyme inhibitors used, only borate ion produced significant inhibition, indicating the involvement of the 2',3' hydroxyl groups of the ribose moiety of ATP in the catalytic process. Mild trypsin treatment of intact protoplasts resulted in complete loss of ATPase activity, although trypsin was shown not to penetrate the membrane under the experimental conditions. Since intact protoplasts show no ATPase activity, it was concluded that a part of the enzyme, other than the ATP-binding site present on the outer surface of the membrane.

Direct vital staining of the protoplasts with

fluorescein isothiocyanate was performed in such a way that the membrane was specifically labelled. Such labelled protoplasts were demonstrated to grow in a fashion indistinguishable from unlabelled protoplasts. Fluorescence microscopy of fluorescein-labelled protoplasts, carried out at various stages of the growth process, indicated that insertion of newly synthesized membrane material occurred by the process of intercallation. Loss of fluorescein label by enzymic processes was not detectable.

Non-growing suspensions of S. faecium cells, when incubated with glucose, accumulated intracellular ATP. If further glycolysis were inhibited after formation of the ATP, either by F^{4-} or AsO_4^{3-} the cells could be shown capable of active transport of amino acids. The rate and extent of this transport was directly related to the concentration of ATP in the cells. The stoichiometry of the reaction indicated that one mole of amino acid was transported for each mole of ATP hydrolyzed. Thiol binding agents completely inhibited the active transport process, while leaving passive transport unaffected, which indicated the involvement of sulfhydryl groups in the process of active transport.

ACKNOWLEDGMENT

I wish to express my appreciation to Dr. William R. Chesbro, without whose advice and criticism this work could not have been done.

TABLE OF CONTENTS

LIST OF FIGURES.....	iv
LIST OF TABLES.....	vi
INTRODUCTION.....	1
REVIEW OF LITERATURE.....	4
METHODS AND MATERIALS.....	15
I. Organism and Cultural Conditions.....	15
II. Formation and Fractionation of Protoplasts.....	16
III. Production and Fractionation of Anti-membrane Antiserum.....	17
IV. Effect of Antiserum Fractions on Protoplast Growth.....	20
V. Analysis of Membrane Antigens.....	24
VI. Production and Growth of Fluorescein-labelled Protoplasts.....	26
VII. ATP-pool-driven Transport in <u>Streptococcus</u> <u>faecium</u>	29
VIII. Properties of Membrane ATPase of <u>Streptococcus</u> <u>faecium</u>	32
RESULTS	
I. Formation and Fractionation of Anti-membrane Antiserum.....	35
II. Effects of Serum Fractions on Protoplasts.....	36
III. Analysis of Membrane Antigens.....	49
IV. Growth of the Protoplast Membrane.....	59
V. ATP-pool-driven Transport of Amino Acids.....	74
VI. Properties of Membrane ATPase of <u>Streptococcus</u> <u>faecium</u>	80
DISCUSSION	
I. Effects of Antiserum Fractions on Protoplast Growth.....	97
II. Analysis of Membrane Antigens.....	99

TABLE OF CONTENTS

DISCUSSION

III. Growth of the Protoplast Membrane..... 101

IV. ATP-pool-driven Transport of Amino Acids..... 104

V. Properties of the Membrane ATPase of Streptococcus
faecium.....107

LITERATURE CITED..... 113

LIST OF FIGURES

Number	Page
1. Effects of normal serum and membrane antiserum on suspensions of protoplasts in 0.5 M sucrose....	37
2. Reaction between membrane antiserum and a suspension of protoplasts in 0.5 M sucrose.....	39
3. Turbidity changes associated with protoplast growth.....	41
4. Comparison of changes in turbidity and membrane protein occurring during protoplast growth.....	42
5. Effects of fractions of membrane antiserum on protoplast growth.....	44
6. Changes in exogenous pH associated with glucose metabolism of protoplasts.....	46
7. Utilization of glucose by sucrose suspensions of protoplasts.....	47
8. Effects of antiserum treatment on amino acid transport in protoplasts.....	50
9. Gel-diffusion analysis of membrane antigens.....	51
10. Turbidity changes accompanying growth of FITC-labelled protoplasts.....	60
11. Penetration of protoplasts by FITC during the labelling process.....	62
12. Changes in turbidity and membrane dry weight during growth of FITC-labelled protoplasts.....	64
13. Growth of FITC-labelled protoplasts followed by fluorescence microscopy.....	65
14. Changes in FITC:membrane ratio in growing and non-growing FITC-labelled protoplasts.....	73
15. Formation of ATP pool in cells incubated with 20 μ M/ml glucose.....	75
16. Effect of inhibitors on glucose utilization by non-growing cell suspensions.....	77
17. Rate of hydrolysis of ATP by membrane fragments...	82
18. Rate of hydrolysis of ATP by cytoplasm.....	85

LIST OF FIGURES

Number		Page
19.	The effect of Mg:ATP ratio on the activity of membrane ATPase.....	86
20.	Response of membrane ATPase to temperature....	87
21.	Response of membrane ATPase to pH.....	89
22.	Effects of trypsin treatment on the ATPase activity of cell fractions.....	94
23.	Loss of membrane protein during trypsin treatment.....	96

LIST OF TABLES

Number		Page
I.	Effects of antiserum treatment on amino acid transport in protoplasts.....	48
II.	Antigens used in gel-diffusion.....	58
III.	Transport of alanine by cells pre-incubated with glucose.....	78
IV.	Alanine incorporation during ATP-pool-driven transport.....	79
V.	Effect of exogenous alanine on the endogenous ATP pool level.....	81
VI.	Hydrolysis of ATP by cell fractions of <u>Streptococcus faecium</u>	84
VII.	Hydrolysis of substrates other than ATP by membranes.....	90
VIII.	Effectiveness of divalent cations as cofactors of ATPase.....	91
IX.	Effects of various agents on membrane ATPase activity.....	92

INTRODUCTION

The discovery of an osmotic barrier in the bacterial cell, distinguishable from the cell wall, was made at the beginning of this century. Although this structure could not be directly observed until the advent of electron microscopy, its semipermeable, lipophilic nature was inferred from its behavior in the presence of various solutes; high concentrations of lipophobic solutes caused retraction of the cytoplasm from the wall, while lipophilic solutes produced no apparent effect. By mid-century, observations of this type had been made on a wide variety of bacterial cells, confirming and extending the initial observations.

However, attempts to isolate the cell component to which these osmotic properties could be attributed were complicated by the fact that mechanical disintegration of cells produced a wide variety of physically separable particles, none of which could be unambiguously associated with the structure present in intact bacteria.

This methodological difficulty was overcome a decade ago by the development of mild and specific enzymatic procedures for the lysis of the cell wall. When such enzymatic digestions are carried out in the presence of high concentrations of an impermeant solute, the disintegration of the wall results in the production of a spherical body, the protoplast. Bacterial protoplasts possess most of the metabolic capabilities of the intact cell and all of the osmotic properties.

If protoplasts are suspended in a hypotonic medium, all osmotic properties are lost and the cytoplasmic components are released, resulting in the production of spherical 'ghosts'. It is now generally conceded that the 'ghost' fraction of osmotically disrupted protoplasts is mainly composed of fragments of the osmotic barrier.

Careful chemical and cytological investigations of protoplast 'ghosts' have demonstrated the close structural relationship with the so-called 'unit membrane' of plant and animal cells, and most investigators refer to these structures as membranes.

Isolated bacterial membranes have since been the object of considerable study. The physical structure, chemical composition and enzymic activities of membranes from several gram-positive species have been catalogued. Similar work with gram-negative bacteria has been hampered due to the more complex cell wall composition of this group; the presence of lipoproteins in the wall making it difficult both to separate the wall completely from the membrane and to categorize any isolated component as 'pure' membrane.

It is not in categorizing the components of membranes, however, that the greatest challenge lies, but in correlating the structure of membranes with their in vivo functions. A substantial literature concerned with the kinetics and specificity of transport phenomena in bacteria exists. These data indicate the existence, in the membrane, of a number of systems capable of selectively translocating certain solutes

into the cell. When coupled to the energy metabolism of the cell, these systems can transport materials against a concentration gradient. The isolation of a functioning transport system and the direct characterization of its components represent a major obstacle to the ultimate clarification of the nature of membrane transport.

Much of the material presented in this dissertation is either directly concerned with the mechanisms of transport phenomena, or contains implications concerning such mechanisms. The study of ATP pool-driven transport was undertaken, primarily, to develop a new approach to the problem of active transport in bacteria.

The investigations into the antigenic structure of the membrane were undertaken because almost nothing was known about this subject beyond the observation that this structure contained antigenic components, but also to see whether the application of immunological techniques could be used in the study of membrane function. As a sequel to this work, the effects of membrane antiserum on metabolizing protoplasts were studied.

Finally, a study of the mechanism of membrane growth was undertaken, first because no information existed on this topic, but also in the expectation that such knowledge would contain implications of membrane structure and provide insight into its dynamics.

REVIEW OF THE LITERATURE

The first indication of the existence of an osmotic barrier in the bacterial cell, distinguishable from the cell wall was provided by Fischer in 1903 (25). In his studies of plasmolysis, he found that high concentrations of hydrophilic solutes caused a retraction of the cytoplasm from the wall, while similar concentrations of lipophilic solutes did not. This was interpreted as evidence for the presence of a semipermeable, lipid membrane in bacteria, similar to those previously found in plant and animal cells. Subsequent investigations of a similar nature confirmed these results and extended their scope to include a wide variety of bacterial species.

It was not until the mid-1950's, however, that a definitive demonstration of a plasma membrane in bacteria was made. Weibull found that treatment of B. megaterium with lysozyme would result in the lysis of the cell wall and the emergence of the cytoplasm as a spherical body, the protoplast (74). He found that the presence of 0.3M. sucrose, or isoosmotic concentrations of impermeant salts, would prevent the lysis of the protoplast. Similar osmotically sensitive forms have since been produced from a variety of gram-positive and gram-negative cells, not only by lysozyme treatment, but also by penicillin treatment, nutritional deprivation, and the use of phage-associated lytic enzymes, or a combination of these methods (51,74).

Although all the procedures mentioned above are capable of producing osmotically fragile bodies from the parent cell, they are not all equally effective in removing completely the cell wall. This situation has induced a recommendation by a group of workers that the term protoplast be applied only to an osmotically fragile form having no residual cell wall, as judged by chemical, immunological and cytological study, failure to interact with specific phage, and inability to regenerate the parent cell (9). The use of the same term in quotation marks was suggested for use when a smaller number of tests indicated the absence of wall components, and spheroplast was offered to designate osmotically sensitive forms which still retain wall material. These recommendations will be adhered to in this dissertation, when referring to specific organisms. In general discussion, the designation protoplast will be used.

The properties of protoplasts, a subject extensively investigated in the past decade, will be discussed in a later section of this review. For present purposes, it is only necessary to indicate that protoplasts retain the osmotic properties attributed to the plasma membrane of the intact cell (51,74).

When bacterial protoplasts are exposed to a hypotonic medium, they lyse, releasing the soluble cytoplasm and leaving behind an optically pale, sac-like structure, the 'ghost' (74). Weibull observed, in studies of controlled lysis of B. megaterium protoplasts, a one-to-one correspondence between the

initial number of protoplasts and the number of 'ghosts' remaining after lysis (74). Since this is the sole structure remaining after protoplast lysis, and always appears concomitantly with lysis, Weibull concluded that it was the protoplast membrane. Most investigators are now of the same opinion (3,33,39,51,54,63,68,74).

It has been suggested that the 'ghost' is a cytological artifact, resulting from complexing between lysozyme and cytoplasmic components (55). Weibull, Zacharias, and Beckman, however have determined that I¹³¹-labelled lysozyme comprises less than 2% of the dry weight of the 'ghost' (75); and Shockman (68) has been able to reduce the lysozyme content of Streptococcus faecalis membranes to undetectable levels, while retaining the structural integrity of the membranes. There is little doubt that protoplast ghosts are true cell organelles, the bacterial cell membranes.

From its inception, the chemical and physiological investigation of the bacterial membrane has been plagued by interpretational ambiguities. To characterize the material of the plasma membrane it is necessary, not only to isolate it from the other components of the cell, but also to demonstrate that this material has neither gained nor lost components during the process.

The problem of isolation was the major obstacle prior to the introduction of enzymatic techniques. The inherent fragility of the plasma membrane and its tendency to form vesicles upon mechanical disintegration of the cell led early

workers to conclude that these structures represented miniature counterparts of the mitochondria and microsomes of higher cells (47,52). It was subsequently demonstrated that protoplast 'ghosts' could be converted to such vesicles by brief mechanical disintegration (74), indicating that they are part of a larger membranous structure.

Although modern techniques have alleviated to some extent the difficulties of membrane isolation, the problem of demonstrating purity still remains. The problem is not entirely solvable, as it requires an a priori decision concerning what the membrane should or should not contain. Gentle preparation may maintain the membrane in a native state, but fail to remove mechanically included cytoplasmic components, while rigorous preparative procedures include the risk of loss of membrane components. Increasing numbers of reports indicate that a variety of material may be bound to the membrane via divalent cations (7,8,34,53,54,66). Since most of the published procedures for the preparation of membranes (33,63,67,74) include multiple washings in the absence of divalent ions, some caution should be used in interpreting the results obtained.

The enzymic capabilities of membranes have been studied intensively in the past ten years. The results have been summarized by several reviewers (49,51,52). In general, all aerobes and facultative aerobes have their electron transport system localized in the membrane, in many cases still capable of oxidative phosphorylation in the isolated state

(49). In addition, a number of dehydrogenases have been found in the membrane fractions of aerobic organisms. Dehydrogenases for Tricarboxylic Acid Cycle intermediates are most often found, e.g. succinic and malic dehydrogenase (39, 52) and NADH oxidase is consistently found.

The membrane-bound enzymes of facultative and obligate anaerobes have received much less attention than those of aerobes. Those that have been studied show a more modest complement of activities than displayed by aerobes. Moore and O'Kane (53) have discovered a membrane-bound particle in Streptococcus faecalis containing hexokinase, galactokinase, 2-ketogluconate kinase and glucose-6-phosphate dehydrogenase. The particle, released from the membrane by EDTA or citrate, migrated as a single electrophoretic band. The release process is prevented by the presence of Mg^{++} or Ca^{++} in amounts equimolar to EDTA or citrate, indicating some form of ionic binding. A weak hexokinase activity has also been found in hulls (wall+membrane) of Lactobacillus spp. (39).

One enzyme that has been found in membranes of a variety of bacteria is ATPase. E. coli, Ps. fluorescens, L. arabinosis, Vitreoscilla spp. and V. parahaemolyticus all show this activity in their membranes (76,50,4,49,16,36). These enzymes are all most active against ATP, although those that have been tested show some activity against GTP and ITP. In the case of the aerobic organisms, the activity may be a result of the derangement of the oxidative phosphorylating system, leading to hydrolysis of ATP. In facultative

anaerobes, such as Streptococcus faecium, the function of this enzyme is completely unknown. It has been suggested, in analogy to the situation found in mammalian erythrocytes (79), that the enzyme is involved in transport (4,52). However, the ATPase involved in ion transport in erythrocytes is a Na^+ , K^+ -activated enzyme, while, with one possible exception (36), none of the bacterial ATPases are stimulated by these ions.

A recent development has been the discovery of the presence of complete metabolic pathways in the bacterial membrane. Cronenwett and Wagner (18) have reported the complete synthesis of leucine and isoleucine by membranes of S. typhimurium. The capability of amino acid synthesis has not been reported, and may not have been looked for, in any other membrane system.

It has been known for some time that isolated bacterial membranes have the capacity to incorporate C^{14} -labelled amino acids into a form precipitable by hot trichloroacetic acid (49). Until recently, the significance of this incorporation has been blurred by ignorance of its mechanism. Moore and Umbreit have shown that the ability is associated with membrane-bound polyribosomes, and further that the synthetic activity of the bound ribosome fraction is considerably higher than that of 'soluble' ribosomes (54). The authors suggest that in the intact cell the polyribosomes are normally bound to the membrane, and that the bulk of protein synthesis occurs in these structures. It was also found that the

polyribosomes were bonded to the membrane by magnesium ions and could be easily dislodged by washing in the absence of this ion.

In view of the foregoing, it is ironic that many investigators (4,33,68) have used a low RNA content as a criterion of membrane purity. In spite of this attempt to reduce RNA content to a minimum, most reports indicate the presence in the membrane preparations of a firmly bound RNA fraction, which could not be further reduced by non-enzymatic means. Shockman (68), the investigator most sedulous in attempts to reduce membrane RNA, still had 5% of the membrane of Streptococcus faecalis as RNA. Using the same organism, Abrams found that 15% of the membrane was RNA (6). The latter worker also established that C¹⁴-labelled nucleotides are incorporated into this RNA fraction in the isolated membrane (6). The 'nascent' RNA has the same base composition as the RNA found in untreated membranes, but different from ribosomal RNA. This ability has not been reported for any other bacterial membrane.

One recent report (32) indicates that the membrane may be capable of DNA synthesis. Carefully lysed B. subtilis protoplasts were found to contain the entire genome in the membrane fraction. Incubation with isotopically-labelled thymine demonstrated incorporation of this base into the 'nascent' DNA of the membrane.

It is evident from the foregoing that the study of bacterial membrane function is still in its infancy. A final

judgment concerning the extent of membrane involvement in bacterial metabolism cannot be made until a much greater breadth and depth of knowledge has been accumulated.

Information concerning the fine details of membrane composition is scanty. Gross chemical analyses of a few bacterial membranes have been performed, including those of S. aureus, M. lysodeikticus, B. megaterium, and Strep. faecalis (52,33,74,68). The results of these analyses are remarkably consistent and can be easily summarized. The major components of the membrane fractions are protein (presumably lipoprotein) and lipid, the former comprising between 40% and 65% of the membrane dry weight, the latter representing 13% to 40% of membrane substance. In addition there are small and variable amounts of carbohydrate (1-20%) and nucleic acid (0-10%), depending on the method of membrane preparation.

Of the membrane lipid, the major component is phospholipid, comprising 50-70% of the total membrane lipid and representing the entire phospholipid content of the gram-positive cell. The composition of this fraction has been the object of several investigations. These studies have revealed the relative simplicity of this fraction, generally composed of phosphatidyl glycerols and their amino acid esters (40,52,65,73). Thus far analysis has failed to demonstrate the presence of sterols or sphingolipids in bacteria (79), although lecithins have been found in certain highly membranous bacterial species (37).

In contrast to the interest in the lipid fraction, almost no work has been done on the protein. An amino acid analysis of the total protein of the membrane of Strep. faecalis has been performed by Shockman (68). The results are not particularly startling, the full range of amino acids usually found in protein being present. The amounts of aspartic acid and glutamic acid are fairly high, but not excessively so. This analysis, of course, is of a fairly heterogeneous group of proteins, including not only the protein(s) of the membrane, but also any enzymes and ribosomal proteins remaining attached. Analysis of individual protein fractions may reveal special characteristics.

The physical characteristics of bacterial membranes have been investigated much less intensively than their chemical properties. The electrophoretic mobility of various membrane fractions of M. lysodeikticus has been studied (23). The results indicated that the lipid component contributes little to the surface charges of the intact membrane and is probably covered by the protein layer in the whole membrane. No similar studies have been reported for any other bacterial membrane.

The lethal effects of ultrasound and detergents on bacteria have been known and applied for many years. Recently, these effects have been employed as tools for the investigation of membrane structure. In a study of isolated membranes of S. lutea and M. lysodeikticus, Salton and Freer found that treatment with ultrasound or detergents yielded

ultracentrifugally homogeneous subunits (65). The sedimentation coefficients obtained by sonic disintegration were 4.2 S for M. lysodeikticus and 1.18 S and 3.56 S for S. lutea. In a similar study of S. lutea membranes, Brown (8) obtained subunits having sedimentation coefficients of 5 S and 70 S, the lighter converting to the heavier in the presence of Mg^{++} . The failure of Salton to obtain the heavier fragment is not surprising, since divalent cations were absent from his preparations. These results suggest that the membrane is composed of subunits of equal size, probably held together by non-covalent bonds.

Of the properties of the plasma membrane, the most characteristic is selective permeability, or transport. Two general categories of transport are recognized on the basis of kinetics and specificity; diffusion and facilitated transport. The former category, as the name implies, includes the permeation of substances to which the membrane does not form a physical barrier, e.g. urea and glycerol, which are soluble in the lipid component of the membrane as well as in an aqueous environment; and water, which freely passes in or out of the cell via aqueous channels through the membrane.

The distinguishing characteristic of facilitated transport is its specificity; the ability to make distinctions between closely related compounds. Two types of facilitated transport exist. The division is based on the fact that one, passive transport, does not require an energy source for its operation, while the other, active transport, does. The

operation of an active transport system may lead to the production of an apparent concentration gradient between the interior of the cell and the external environment.

Although a great deal is known about the kinetics and specificity of transport, virtually nothing is known about the structure of the transport systems. What little is known about the organization of these systems, which are sometimes referred to as 'permeases', in analogy to enzymes, was deduced from indirect evidence, rather than from direct study of the system. This indirect approach, epitomized by the continuing studies of the β -galactoside permease of E. coli (44,45), has, until now, been the only way of studying the structure of transport systems. The major obstacle to direct study has been, and continues to be, the inability to isolate a functional permease.

Recently, some progress has been made in the direct study of some components of transport systems. Fox and Kennedy (30), using an ingenious combination of radio-labelling and genetic induction, have succeeded in isolating one of the components of the β -galactoside permease of E. coli. The function of this component is not yet known. Almost simultaneously, Pardee and Prestidge (60), succeeded in isolating the binding site of a transport system of Salmonella typhimurium. It appears, that the methodological impasse which had prevented isolation and characterization of cell components involved in transport, has been, at least partially, superseded.

METHODS AND MATERIALS

Organism and Cultural Conditions

The organism used throughout these investigations was Streptococcus faecium, strain HF8AG.

The medium used had the following composition per L: tryptone (Difco, Detroit, Michigan), 8 g; yeast extract (Difco), 5 g; dextrose (Fisher Chemical Company, Fair Lawn, New Jersey), 10 g; Tween 80 (polyoxyethylene sorbitan mono-oleate), 3 drops; K_2HPO_4 , 2 g; Na_2CO_3 , 3 g. The latter two components were dissolved in a volume of distilled water equal to 1/5th of the final culture volume; the remaining components were dissolved in distilled water equivalent in volume to 4/5ths of the desired culture volume. The two solutions were autoclaved separately and recombined aseptically immediately before inoculation.

Starter cultures were prepared by inoculation from stab cultures of the organism. The medium used for both starter and stab cultures is identical to that given above, except for the deletion of the buffer salts, and the addition of 1.5% agar to the medium used for stab cultures.

Batch cultures were inoculated by the addition of 5% (v/v) of a turbid starter culture to the reconstituted medium at 37 C. Incubation was carried out statically at 37 C for 16-18 hours. The organisms were harvested by centrifugation at 10,000 x g for 5 minutes.

Formation and Fractionation of Protoplasts

Cells, harvested by centrifugation, were washed once with 30 ml of 0.075 M phosphate buffer, pH 6.8, centrifuged and resuspended in sucrose-phosphate buffer (0.5 M sucrose, 0.075 M potassium phosphate, pH 6.8) at 37 C to a volume equivalent to 1/10th of the original culture volume. To this suspension, sufficient egg white muramidase (lysozyme, B grade; Calbiochem, Los Angeles, California) was added, as a solution in 1 ml of sucrose-phosphate buffer, to produce a final concentration of 200 µg/ml. Incubation at 37 C was carried out for a period sufficient to produce maximum conversion to protoplasts, usually 60-90 minutes.

The extent of conversion to protoplasts was assessed in two ways; the development of osmotic fragility, and the loss of positive gram reaction. In the first method, successive 0.5 ml portions of the cell suspension were withdrawn at intervals subsequent to the addition of muramidase, mixed with 5 ml of distilled water and the turbidity of the suspension read on a Klett-Summerson Photoelectric Colorimeter (Klett Manufacturing Co., New York, New York) using a filter with a bandpass of 580-640 mµ. The process of conversion to protoplasts is indicated by a progressive lowering of turbidity over a period of time. When a sample failed to demonstrate a turbidity lower than that of the previous sample, the conversion was considered complete. In the second method, gram stains were made of samples of the suspension and examined microscopically. The absence of gram-positive diplococci in several randomly selected fields was considered evidence for

conversion to protoplasts.

Subsequent to formation, the protoplasts were washed in sucrose-phosphate buffer and resuspended in the same medium if intact protoplasts were called for.

Protoplasts were disrupted by osmotic lysis for the production of membranes. Washed protoplasts were suspended in 0.1 M Tris buffer pH 7.5 at a temperature of 5 C to a volume equivalent to one half of the original culture volume. The suspension was vigorously agitated with the aid of a magnetic stirrer for a period of 30 minutes, at 5 C. Lysis was followed by centrifugation at 12,000 x g for 20 minutes at 5 C. The supernatant of this centrifugation consisted mainly of non-sedimentable internal components of the cell and was designated as the 'cytoplasm'; the pellet contained mainly membranes. The membrane fraction was then washed three times with 0.1 M Tris pH 7.5 at 5 C, containing 0.001 M/L $MgCl_2$ and once with 0.1 M Tris pH 7.5 at 5 C. Subsequent treatment of the membranes was dependent on the use to which they were to be put and will be discussed in the appropriate sections.

Production and Fractionation of Anti-membrane Antiserum

The procedure used for the estimation of protein concentration throughout this work is that of Lowry, as modified by DeMoss and Bard (20). Commercially available Folin-Ciocalteu reagent (Fisher Chemical Co., Fair Lawn, New Jersey) was used. The standard curve was prepared using crystalline bovine serum albumin (Calbiochem, Los Angeles,

California). Optical density measurements were made with the Klett Colorimeter, using a filter with a range of 580-640 mu.

A suspension of membranes containing 40 mg of protein was centrifuged at 15,000 x g for 20 minutes at 5 C, washed twice in 0.68% NaCl and resuspended in 10 ml of Freund's Complete Adjuvant (Difco). The suspension was homogenized in Servall High Speed Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). Homogenization was considered complete when a drop of the material maintained its coherence on the surface of ice cold water.

Immunization was begun immediately after the preparation of the antigen. Six simultaneous 0.5 ml injections were made subcutaneously, followed by a three week rest period. Blood was obtained by incision of the marginal ear vein. Anti-membrane titers were determined by means of tube agglutination. The test antigen was a physiological saline suspension of membranes adjusted to an optical density equivalent to that of McFarland Nephelometry Standard #3.

Serum was fractionated by the method of Kekwick (42). This procedure involves three precipitations with $(\text{NH}_4)_2\text{SO}_4$ and yields three fractions: albumin, α - and β -globulins and γ -globulin. The fractions thus obtained were dialyzed for 24 hours at 5 C against 0.1 M potassium phosphate buffer, pH 7.0.

Univalent antibodies were prepared by papain digestion of the immune globulin fraction, according to the methodology

of Porter (58). The γ -globulin fraction of the antiserum was adjusted to a concentration of 15 mg/ml with 0.1 M potassium phosphate buffer pH 7.0. To this were added 0.15 mg/ml mercuripapain (Worthington Biochemical Corp., Freehold, New Jersey), 0.01 mM/ml cysteine (Mann Research Laboratories, New York, New York), 0.2 mM/ml ethylenediaminetetraacetic acid (EDTA) (Fisher) and 0.1 ml/ml toluene. The mixture was incubated for 16 hours at 25 C, then dialyzed against 0.04 N acetic acid for 48 hours, with several changes of acetic acid. The solution was frozen and stored at -10 C.

Electrophoretic distribution patterns of the various serum fractions were determined using a Spinco model R-series D Durrum Cell paper electrophoresis unit. The power of 2.5 milliamperes was supplied by a Spinco Duostat. Protein migration took place in veronal buffer (Spinco B-2) at pH 8.6 for 16 hours. The paper strips were stained with bromphenol blue (Spinco B-4) and scanned with the aid of a Spinco Analytrol. The Analytrol was equipped with the B-5 cam and a 500 m μ neutral density filter. The tracings were made using a slit width of 1.5 mm.

The presence of residual multivalent antibody in the papain-digested γ -globulin fraction was tested for, using the double-gel-diffusion technique of Ouchterlony (57). The antigen used was produced by digestion of a suspension of membranes containing 1 mg/ml protein in 0.1 M potassium phosphate, pH 7.5, by 0.1 mg/ml Grade A pancreatic trypsin (6 Kunitz units/mg) (Calbiochem, Los Angeles) for 4 hours at 37 C, followed

by centrifugation at 15,000 g for 15 minutes; the supernatant being employed as the antigen. Residual trypsin was inactivated by the addition of 0.1 mg/ml ovomucoid inhibitor (Mann Research Laboratories, New York, New York). Undiluted antigen was placed in the central well and dilutions of the papain digested γ -globulin were placed in the peripheral wells. Controls were run using undigested γ -globulin and unfractionated antiserum.

The preparation of the gel diffusion plates is discussed in a later section.

The titer of univalent antibody was assessed by its ability to neutralize antigen preparations to the action of complete, precipitating antibody. The antigen used was that described above. The papain digested γ -globulin was serially diluted and 1.0 ml portions of each dilution were mixed with an equal volume of trypsin-solubilized antigen. These were incubated for 2 hours at 37 C and subsequently for 12 hours at 5 C. Samples of the various mixtures were placed in the peripheral wells of gel diffusion plates, antiserum was introduced into the central wells, and the plates incubated for 48 hours, at 20 C. The titer of the univalent antibody was considered to be that dilution which permitted the formation of the first visible line of precipitation.

Effect of Antiserum Fractions on Protoplast Growth

The medium used for the cultivation of protoplasts had the following final composition per L: tryptone, 8 g; yeast extract, 5 g; dextrose, 10 g; Tween 80, 3 drops;

sucrose, 0.1 g. The medium was adjusted to a volume 4/5ths of the calculated volume and adjusted to pH 7.0 using a Beckmann Zeromatic pH Meter (Beckmann Instruments, Palo Alto, California). Fresh medium was prepared at weekly intervals. The medium was not sterilized. Reconstitution of the medium to the desired concentration was accomplished immediately before inoculation by the addition of serum or serum fractions, the effects of which were to be tested. Control media were reconstituted with distilled water.

Before inoculation into the growth medium, protoplasts were treated with the serum fraction under investigation. Serum fractions were made 0.5 M with respect to sucrose and diluted 2:5 with 0.5 M sucrose. The protoplast suspension used had a concentration ten times that of the culture from which it was prepared. Sufficient protoplast suspension was pipetted into the diluted serum to produce a turbidity equal to 100 Klett units (O.D. = 0.2), using a Klett colorimeter and a red filter with a range of 580-640 m μ . These suspensions were incubated at 37 C for 90 minutes, the turbidity being monitored at 15 minute intervals during the incubation. The incubation period was followed by centrifugation at room temperature for 5 minutes at 10,000 x g. The sediment was resuspended in the reconstituted growth medium, containing the appropriate serum fraction, and the culture incubated as previously described.

Membrane protein was determined by pipetting samples of the protoplast culture into ten times the sample volume

of ice cold 0.075 M potassium phosphate buffer, pH 7.0, followed by incubation at 5 C for a period of 30 minutes. The lysed protoplasts were centrifuged at 15,000 x g for 20 minutes at 5 C, washed twice with phosphate buffer and resuspended in a convenient volume for application of the Lowry method.

Total protoplast protein was determined by centrifugation of samples of protoplast cultures, washing with 0.5 M sucrose-0.075 M potassium phosphate, pH 7.0, at room temperature and resuspension in a convenient volume of 0.075 M phosphate buffer, pH 7.0 for use in the Lowry determination.

Glucose was determined by the colorimetric, enzymatic method of Teller (71). The developing reagent had the following composition: horse radish peroxidase, Grade B (Calbiochem), 10 mg; fungal glucose oxidase, Grade B (Calbiochem), 250 mg; o-dianisidine, purified (Sigma Chemical Co., St. Louis, Mo.), 1.0 ml of a 1% ethanolic solution; 0.005 M potassium phosphate buffer, pH 7.0, to 200 ml. The o-dianisidine solution was added after the buffer in order to avoid possible protein denaturation. The optical density of the reaction mixture was determined using the Klett equipped with a filter having a bandpass of 380-460 m μ . Samples were obtained by centrifugation of aliquots of protoplast cultures at 10,000 x g for 10 minutes at 5 C. The clear supernatant was decanted and used in the determination.

In order to determine the extent of acid production in glucose metabolizing protoplasts, a suspension having a

turbidity of 200 Klett Units was prepared using unbuffered 0.5 M sucrose. The pH of the suspension was adjusted to 7.0 with KOH and sufficient 1 M glucose solution was added to produce a final concentration of 100 mM/ml. The pH was measured at intervals with a Beckmann Zeromatic pH Meter and the turbidity was monitored as previously described. Lysed protoplast suspensions were prepared by substituting distilled water for sucrose solution.

Amino acid uptake was determined with the aid of L-alanine- 1-C^{14} (Calbiochem), L-arginine- 1-C^{14} (Calbiochem) and L-leucine- 1-C^{14} (Calbiochem). The incubation mixtures had the following composition: protoplasts, 0.5 mg/ml protein; amino acid, 300 μM /ml, containing 0.02 mc/50 μM of labelled amino acid; glucose, 50 μg /ml. All stock solutions were prepared in 0.5 M sucrose-0.75 M potassium phosphate, pH 7.0. When passive uptake was being studied, 0.5 M sucrose was substituted for the glucose. Incubation was carried out at ambient temperature for 5 minutes. One milliliter samples were withdrawn at the beginning and end of the incubation period, filtered through Millipore membrane filters, pore size 0.4 μ (Millipore Filter Corp., New Haven, Conn.), washed with 1 ml of 0.5 M sucrose and the filters transferred to planchets equipped with holders for membrane filters (Atomic Accessories Inc., Spring Valley, New York). Radioactivity measurements were made with a Baird Atomic Gas Flow Proportional Counter (Baird Atomic, Cambridge, Mass.). The samples were positioned 3-5 mm from the window, the pulse height

discriminator was set at 2.5 and the operating voltage was 2,000 v.

Analysis of Membrane Antigens

The methods used for the preparation of anti-membrane antiserum were described in an earlier section.

Membranes were converted to a 'soluble' form by treatment with ultrasound. The membranes were washed once and resuspended in 0.85% NaCl-0.075 M potassium phosphate, pH 7.0. The final suspension contained 1 mg/ml membrane protein. The suspension was cooled to 5 C and treated with ultrasound in a Branson Model S-75 sonifier (Branson Instruments, New York, New York) at maximum output for a period of twenty minutes. The sonicated material was centrifuged at 60,000 x g for one hour in a Beckman-Spinco Model L ultracentrifuge. The pellet was discarded and the supernatant stored aseptically at 5 C.

Treatment of the membranes with detergents was carried out at 37 C using a suspension of membranes in 0.85% NaCl-0.075 M potassium phosphate, pH 7.0, containing 1 mg/ml protein. The detergents employed were: sodium lauryl sulfate, pure (Fisher); cetyltrimethylammonium bromide, CTBA (Fisher); Surfactol-100, Triton X (Huron Chemicals, Ann Arbor, Michigan). Sufficient 10% (w/v) detergent solution in 0.85% NaCl-0.075 M potassium phosphate, pH 7.0 was added to the membrane suspension to produce a final concentration of detergent of 1%. This mixture was incubated for two hours at 37 C, then centrifuged at 30,000 x g for one hour in a Beckman-Spinco Model

L ultracentrifuge. The supernatant was freed from detergent by passage through a column of Sephadex-G-25 (Pharmacia, Upsala, Sweden). The column dimensions were 1 cm in diameter and 10 cm in height and the eluant was 0.01 M potassium phosphate, pH 7.0. Following elution, the solution was concentrated by preevaporation, using Aquacide II (DEAE-cellulose), (Calbiochem), to a final protein concentration of 1 mg/ml.

Immunological analysis was carried out using a modification of the double-diffusion-in-gel technique of Ouchterlony (49). The vessels used were 55 mm x 14 mm Petri dishes. Wells were formed by positioning 0.5 cm diameter stainless steel cylinders in the plate, with the aid of a template, so that a central well was surrounded by six peripheral wells at a distance of one centimeter, measured from the outer edges of the cylinders. Five milliliters of molten agar, cooled to 55 C, were pipetted into the prepared Petri dishes and allowed to solidify at room temperature. After solidification, the cylinders were removed as well as any agar that had entered the cylinders.

The agar used in immunodiffusion had the following composition: Ionagar No. 2 (Consolidated Laboratories, Chicago Heights, Illinois), 0.5%; NaCl 0.85%, NaN_2 0.01 M; potassium phosphate, 0.01 M, pH 7.0.

Following preparation of the plates, the central wells were filled with antiserum, the peripheral wells with antigens or 0.85% NaCl, and the covered plates incubated in a moist chamber at 20 C for 48-72 hours.

Lines of precipitation were stained using Buffalo Black. In the procedure used, the plates are first rinsed with 0.85% NaCl, to remove unreacted antiserum and antigen from the wells, and then allowed to stand overnight at room temperature, filled with 0.85% NaCl. After decanting the NaCl solution, the plates were rinsed with distilled water for a period of one hour, and the water decanted. The plates were then completely covered with moistened filter paper, in intimate contact with the gel, and incubated at 70 C until completely dehydrated. The filter paper was moistened and removed and the gel was submerged in 0.01% Buffalo Black (Allied Chemical Corp., New York, New York) for a period of three minutes. The plates were then washed at three minute intervals with 2% acetic acid, until all excess dye was removed.

Production and Growth of Fluorescein-labelled Protoplasts

Protoplasts were prepared as previously described and suspended at ten times the culture concentration in sucrose-phosphate buffer at room temperature.

Protoplast labelling was carried out using solutions of fluorescein isothiocyanate in 0.5 M sucrose-0.075 M potassium phosphate, pH 6.8, prepared immediately before use at room temperature. The fluorescein isothiocyanate used was prepared by Calbiochem (Los Angeles, California).

The volume of protoplast suspension added to the labelling solution was that which would produce an optical density equal to 100 Klett units in an equivalent volume of 0.5 M

sucrose-0.075 M potassium phosphate, pH 6.8. The protoplasts were distributed evenly throughout the labelling solution by gentle swirling and the resulting mixture allowed to stand for 5 minutes. The mixture was then centrifuged at 10,000 x g for 3 minutes at room temperature and washed with successive 30 ml. portions of 0.5 M sucrose-0.075 M potassium phosphate, pH 6.8, until the supernatant was colorless (at a concentration of 0.5 mg/ml fluorescein isothiocyanate, four washings were sufficient). The pellet was then resuspended in sucrose-phosphate buffer, pH 6.8, to 1/5th of the volume of protoplast growth medium was added and incubation carried out. The composition of the growth medium and cultural conditions have been described in a previous section.

The penetration of fluorescein isothiocyanate into the cytoplasmic portion of the protoplast was tested for by measuring the optical density of protoplast lysates at 450 m μ using a Klett. The lysates were adjusted to the same volume as the original protoplast suspension and membrane fragments were removed by centrifugation prior to optical density measurements.

In order to test for the formation of inducible enzymes capable of hydrolyzing bonds between fluorescein and protein, labelled protoplasts were incubated in growth medium until growth terminated, the protoplasts lysed in 0.075 M potassium phosphate, pH 6.8, and the lysate made 0.5 M with respect to sucrose. The lysate was adjusted to the volume of the growth medium used and freshly labelled protoplasts were added to a

final turbidity of 100 Klett units. The suspension was incubated at 37 C for two hours and samples were removed, fixed, mounted and the protoplasts photographed as described below.

Protoplasts were fixed by pipetting a sample into an equal volume of 20% formalin containing 0.5 M sucrose and 0.075 M potassium phosphate, pH 6.8. After standing at room temperature for five minutes, the mixture was centrifuged at 10,000 x g for five minutes, washed once with distilled water and resuspended in distilled water.

Fixed protoplasts were smeared on a No. 00 glass coverslip in a thin film and allowed to air dry. The dried films were mounted on glass slides having a maximum thickness of 0.9-1.0 mm, using 90% glycerol-0.85% NaCl-0.01 M potassium phosphate, pH 7.1 as mounting medium. Coverslips were sealed to the slides using molten wax.

Microscopy and photomicrography were accomplished with the aid of a 'Binolux' microscope (Reichert, Austria), equipped with a darkfield condenser. Adjustment and focusing of the instrument as well as the choice of filters were according to the recommendations of the manufacturer. Photographs were taken using a 35 mm camera, mounted on the barrel of the microscope. The film used was Kodak Tri-X Pan, black and white. Exposure times used were 3 minutes for visible light illumination and 60 minutes for ultraviolet illumination.

Development of the film was accomplished using Microdol-X (Easkman Kodak, Rochester, N. Y.) at one third of the usual concentration for 15 minutes at 75 F.

ATP-Pool-Driven transport in Streptococcus faecium

Methods used in the cultivation and harvesting of cells have already been described. The cells used in these experiments were washed twice after harvesting with 0.075 M potassium phosphate, pH 7.0 and resuspended in that buffer to one half of the culture volume harvested.

Cells were preincubated with 20 mM/ml glucose for a period of one hour at room temperature, the glucose being added as a 200 mM/ml solution to a selected volume of a concentrated cell suspension, described in the previous paragraph.

Following the glucose incubation, further glycolysis was inhibited by the addition of NaF, Na_2HAsO_4 , or Iodoacetic acid as 0.1 M solutions, producing a final concentration of 0.01 M/L of the inhibitor. Incubation was allowed to proceed for five minutes at room temperature.

Subsequent to treatment with inhibitor, the amino acid alanine was added to the mixture, producing a final concentration of 600 mM/ml. The amino acid solution contained enough C^{14} -Alanine to produce a final activity of 0.022 mc/50 mM of amino acid. Uptake was allowed to proceed for ten minutes. Samples for radioactivity measurements were taken as described in a previous section and their activity measured.

The final concentration of cells in the determination was equal to that of the original culture. The dry weight of the cells was determined by filtering aliquots of the suspension through weighted 'Selas' metal membrane filters

(Flowtronics, Inc., Norwalk, Conn.), having a pore diameter of 0.45 μ , followed by heating at 105 C for four hours. The filters were then cooled in a desiccator and weighted.

The extent of amino acid binding in the cells was determined by extraction of the membrane filters containing radioactive cells with five successive one milliliter portions of tert-butanol-isopropanol-water (10:25:65, v:v:v) at room temperature, followed by a second determination of radioactivity.

Studies of passive uptake were conducted in the same manner as those of active transport, except for the substitution of buffer for glucose in the preincubation.

The procedure used for the extraction of ATP from cells was that of Forrest and Walker (29). A volume of cells containing 2-3 mg dry weight was centrifuged and the cell paste resuspended in 0.5 ml of 0.3 M H_2SO_4 . This suspension was allowed to stand for 30 minutes at room temperature; the extract was neutralized with 0.6 M NaOH, and the volume was made up to 10 ml. The extract was then centrifuged and an ATP assay performed on the supernatant.

The method used for the ATP assay was a modification of the methods of Strehler and Totter (70) and Forrest and Walker (29), based on the quantitative detection of light emitted by extracts of firefly tails in the presence of ATP. The light detector used was a Beckman DU spectrophotometer (Beckman Instruments, Palo Alto, Cal.), with settings modified as described below.

The enzyme extract was prepared by crushing 20 mg of dried firefly tails (Sigma, St. Louis, Mo.) in the presence of 10 ml of 0.025 M Na_2HAsO_4 , pH 7.5 for 10-15 minutes at 5 C. Sufficient solid $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was added, following the extraction, to produce a final concentration of 1.5 mg/ml and the extract was centrifuged at 30,000 x g for 20 minutes at 5 C. The supernatant was decanted and maintained at 5 C until immediately prior to the performance of the assay.

The reaction mixture had the following composition: 0.025 M Na_2HAsO_4 , pH 7.4, 1.0 ml; Firefly tail extract, 1.0 ml; ATP, 0.1-1.0 mg in a volume of 1.0 ml. The reaction is carried out at room temperature in a quartz cuvette and light emission is recorded 30 seconds after the addition of the ATP.

Standard solutions of ATP were prepared with ATP disodium salt (Sigma, St. Louis, Mo.). The amount of ATP present in this commercial preparation was determined by measuring the extinction coefficient of solutions of the preparation in 0.1 M KCl, pH 2.0 at 245 m μ using the Beckman DU, and comparing the figures thus obtained with known values.

The control settings of the spectrophotometer and power supply were as follows: Lamp Controls, off; Sensitivity (power supply), 9; Sensitivity (spectrophotometer), full clockwise; Zero Suppression, off; Screen Bias, 4; Slit Width, 0.01 mm; Load Resistor, 2; Selector Switch, 0.1; Phototube Positioning Knob, out (photomultiplier); Wavelength, 600 m μ ; Transmittance Scale, 0%. Null balance was achieved using the Dark Current control. Under these conditions, the instrument responds only to light emitted from the cuvette.

Properties of the Membrane ATPase of Streptococcus faecium

Membranes were prepared as previously described and suspended in 0.1 M Tris buffer, pH 7.5 to 1/5th of the original culture volume. Such suspensions generally contained between 0.1 and 0.3 mg/ml protein, as determined by the Lowry method.

Assays of ATPase were usually carried out by incubating the sample at 37 C in a solution containing 0.1 M Tris, pH 7.5, 0.005 M disodium ATP, and 0.005 M MgCl₂. The reaction was stopped by adding sufficient 17.5% trichloroacetic acid solution to produce a final concentration of 7%. Denatured protein was removed by low speed centrifugation. The same procedure was used in testing for activity against ADP (Sigma), AMP (Calbiochem), ITP (Sigma), GTP (Sigma) and sodium pyrophosphate.

Detection of inorganic phosphate was carried out by the method of Potter (59). Between 0.1 and 0.3 ml of sample were mixed with a volume of 0.5 M acetate buffer, pH 4.2, to produce a final volume of 2.5 ml. To this were added in sequence, 0.25 ml of fresh 1% ascorbic acid in 0.05 M sodium acetate and 0.25 ml of 1% ammonium molybdate in 0.05 M sodium acetate. The mixture was shaken and incubated for 10 minutes. The resulting color was read at 580-640 m μ in a Klett colorimeter. All operations, except colorimetry, were carried out at a temperature of 5-10 C.

Protein determinations were performed using samples of membrane that had been sedimented by centrifugation, washed

with 0.01 M phosphate buffer, pH 7.0 and resuspended in a known volume of this buffer. This procedure is necessary in order to remove Tris from the medium, since it was found to interfere in the Lowry determination.

In assessing the effects of various agents on the activity of the membrane ATPase, an incubation period of 10 minutes was used. These agents were added to the membrane suspension prior to the addition of ATP or magnesium chloride. In determining the effectiveness of divalent cations in activation of the enzyme, magnesium was omitted from the assay medium.

Trypsin treatment of protoplasts, membrane fragments and cytoplasm was carried out at 37 C in 0.075 M potassium phosphate buffer, pH 7.0 and 0.5 M sucrose, in the case of protoplasts. In the cases of membrane and cytoplasm treatment, the substrate concentration was adjusted to 1 mg/ml protein. The concentration of protoplasts used was such that, when lysed, approximately 1 mg/ml of membrane protein would be released. This generally corresponded to a turbidity of 150-200 Klett Units.

Trypsin (Worthington, 3x recrystallized) was added to a concentration of 0.1 mg/ml.

The reaction was stopped by addition of ovomucoid inhibitor (Worthington) equivalent to the amount of trypsin present. Membranes and protoplasts were washed twice with 0.01 M phosphate buffer, pH 7.0, supplemented with 0.5 M sucrose in the case of protoplasts. Cytoplasm was stored at 5 C until assayed for ATPase.

Protoplasts were lysed by resuspension in 0.01 M phosphate buffer, pH 7.0 to the same volume as used in the trypsin treatment, and allowed to stand, with occasional agitation, at 5 C for 30 minutes. The membrane fraction of this lysate was washed three times in 0.1 M Tham, pH 7.5 and resuspended in this buffer to a volume containing 1.0 mg/ml protein.

The cytoplasmic fraction of the protoplast lysate was concentrated by preevaporation with Aquacide and adjusted to contain 1 mg/ml protein.

RESULTS

Formation and Fractionation of Anti-membrane Antiserum

The agglutinin titer of the antiserum used in these studies was 1:640. Normal serum produced no titer under the conditions of the test. Neither antiserum nor normal serum demonstrated any activity against whole cells.

Electrophoretic analysis of the serum fractions produced by $(\text{NH}_4)_2\text{SO}_4$ precipitation indicated almost complete separation of the major components. Comparison of Analytrol tracings of the serum fractions with that of unfractionated normal or immune sera indicated less than 5% contamination of the major component by other serum factors.

Papain digestion of the V-globulin fraction of the antiserum resulted in complete loss of precipitin activity in both gel diffusion and tube precipitation tests. The retention of active binding sites on the digested immune globulin was indicated by a titer of 1:100 in the neutralization test.

Effects of Serum Fractions on Protoplasts

Figure 1 indicates the effects of normal and immune sera on suspensions of protoplasts in 0.5 M sucrose. The rapid and profound loss of turbidity in the case of the undialyzed, normal serum indicated that considerable lysis was occurring. Some loss of turbidity was also noticed during treatment with undialyzed antiserum, but this effect was considerably less extreme. It is possible that the serum component(s) responsible for this effect were present in smaller amounts in the antiserum than in the normal serum, or that

the lytic process was counteracted by the reaction of the protoplasts with immunoglobulin. On the basis of the available data, it was not possible to make a definite choice between these alternatives.

In an attempt to characterize the lytic factor, the effects of the various serum fractions on suspensions of protoplasts were studied. No change in the turbidity of the suspensions was observed with any fraction of either normal or immune serum. The possibility that the lytic factor had been lost during dialysis was confirmed by the absence of lytic activity in dialyzed, normal serum and the loss of turbidity change in the case of dialyzed antiserum.

Since both normal and immune sera had been treated, previous to use, at 60 C for one hour, the participation of complement in the lytic process was ruled out.

Further characterization of the lytic factor was not attempted, as it was subsidiary to the main purpose of the investigation.

The course of the reaction between antiserum and protoplasts is indicated in Figure 2. The process was essentially complete in 90 minutes and resulted in a virtual doubling of the apparent protein content of the protoplasts. Although simple adsorption of serum components plays some part in this increase, inspection of Figure 4 will indicate that most of the acquired protein was not adsorbed. The amounts of membrane protein present at the initiation of the growth process indicated that, although normal serum treatment resulted in a

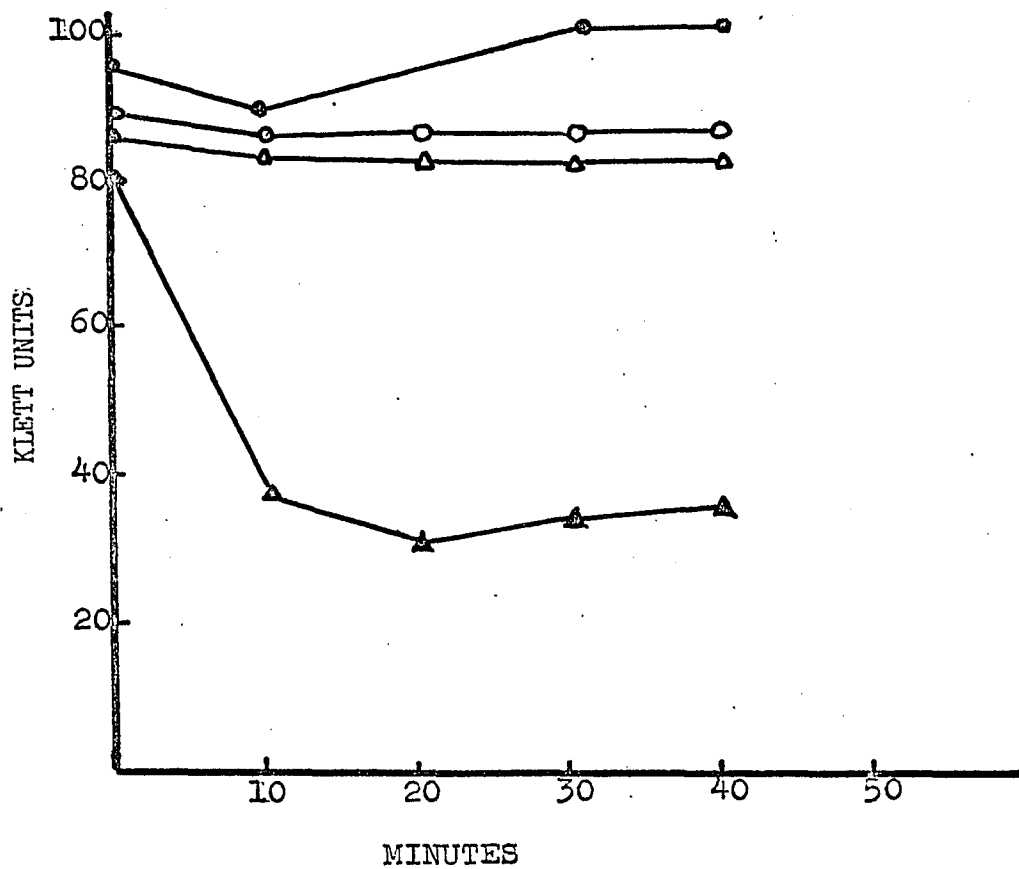


FIGURE 1. Effects of normal serum and membrane antiserum on suspensions of protoplasts in 0.5 M_r sucrose. Symbols: ●, undialyzed membrane antiserum; ○, dialyzed membrane antiserum; ▲, undialyzed normal serum; △, dialyzed normal serum.

twofold increase of membrane protein, compared to untreated protoplasts, antiserum treatment resulted in a fourfold increase, on the same basis. Figure 3 is a growth curve, typical of those obtained in the course of this investigation. The normal growth curve consists of three portions: an initial drop in turbidity; a fairly consistent linear increase, generally terminating at a turbidity somewhat higher than the initial density; and a period of slow loss of turbidity. That such curves do indicate the occurrence of growth, is demonstrated by the optical density profile produced by the introduction of 100 $\mu\text{g/ml}$ chloramphenicol into the culture medium. This antibiotic, a specific inhibitor of protein synthesis, when added, causes a rapid lysis of the protoplasts. Equal concentrations of chloramphenicol have no lytic effect on sucrose suspensions of protoplasts. Generally, for normally metabolizing protoplasts, the maintenance of an optical density greater than that of the chloramphenicol-treated suspension was considered ad hoc evidence of growth.

Comparison with the control shows the protoplasts treated with normal serum grew normally, although the growth response was somewhat enhanced. The apparently superior growth may be ascribed to a greater osmotic stability, contributed by adsorbed serum proteins. Alternatively, it may result from toxic components of the medium being sequestered by serum components.

The response of the antiserum-treated protoplasts is obviously anomalous. There are two likely explanations of

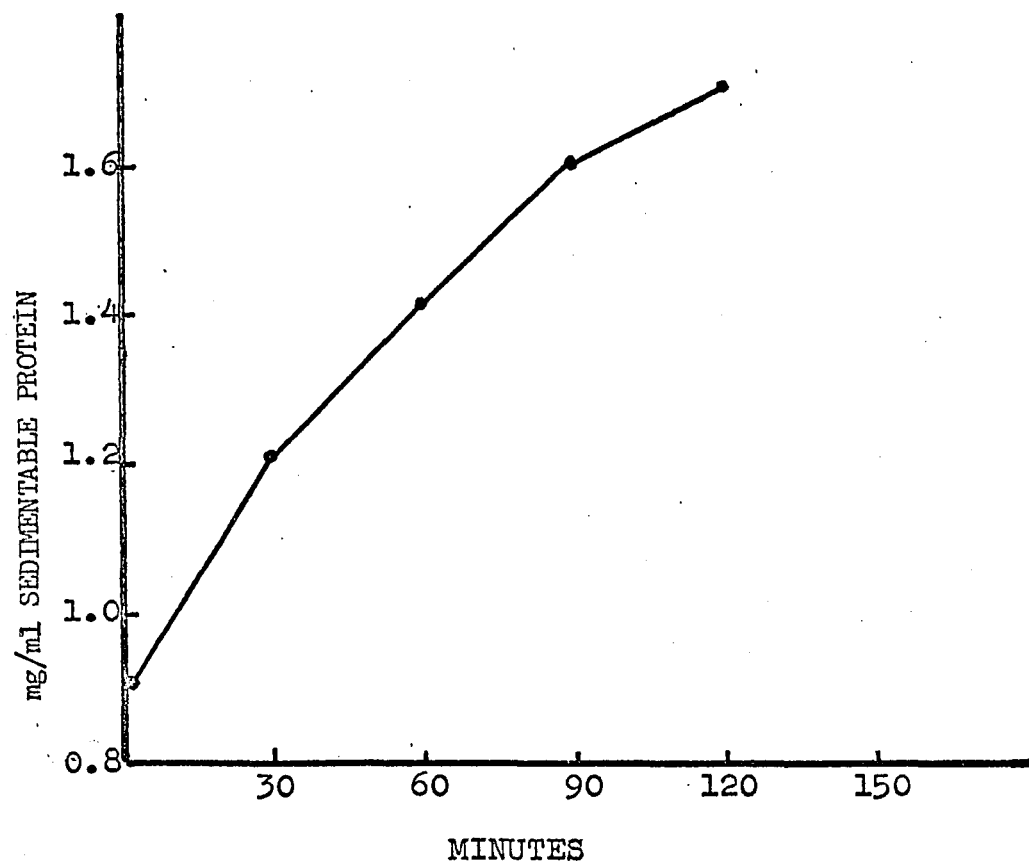


FIGURE 2. Reaction between membrane antiserum and a suspension of protoplasts in 0.5 M. sucrose.

this phenomenon: first, the immune globulins had formed 'bridges' between surface components of the membrane, preventing normal osmotic response; second, antibody molecules were preventing transport of some vital nutrient. The first possibility is obviated for two reasons: first, as Figure 5 shows, univalent immune globulin, incapable of forming 'bridges' between membrane components, still prevented normal growth; second, Figure 4 indicates that no net increase in membrane protein occurred in antiserum-treated protoplasts. The second possibility, i.e. a derangement of transport, seemed the more likely, and was studied. However, before this investigation was undertaken, it was necessary to establish criteria for growth, or lack of growth, other than turbidimetry, and also to establish definitely that immune globulin was the serum factor responsible for the effect.

Figure 4 presents results obtained from an experiment in which both turbidity changes and alterations in protein content were studied. It was decided to use measurements of membrane protein changes as the basis for growth estimation, because this structure remains sedimentable after protoplast lysis, unlike cytoplasmic components, and thus provides a clear index of net protein synthesis, uncomplicated by lytic phenomena. The most striking feature of these results is the parallelism between changes in turbidity drop corresponded, in duration, with an initial loss of membrane protein, and increases in protein were reflected in like increases in optical density. It is probable that the two processes are

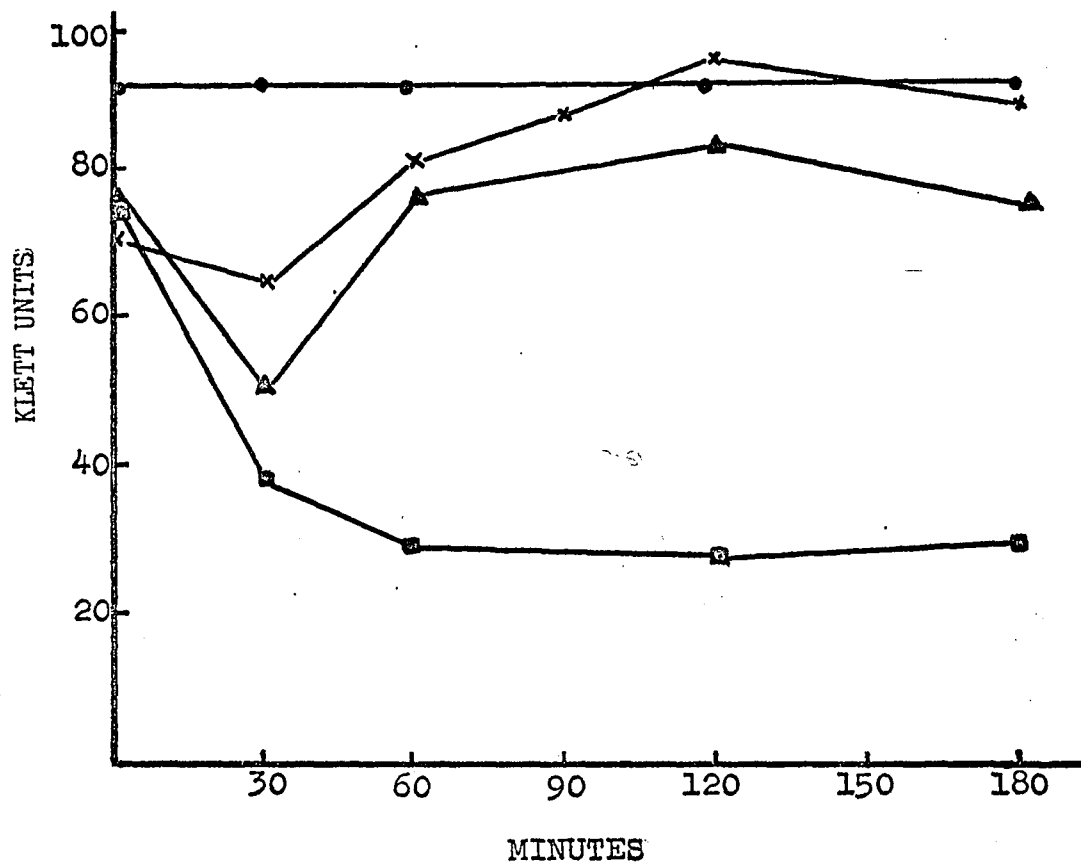


FIGURE 3. Turbidity changes associated with protoplast growth. Symbols: ●, antiserum-treated protoplasts; ×, normal serum-treated protoplasts; ▲, untreated control; ■, protoplasts incubated in the presence of 50 $\mu\text{g.}/\text{ml}$. chloramphenicol.

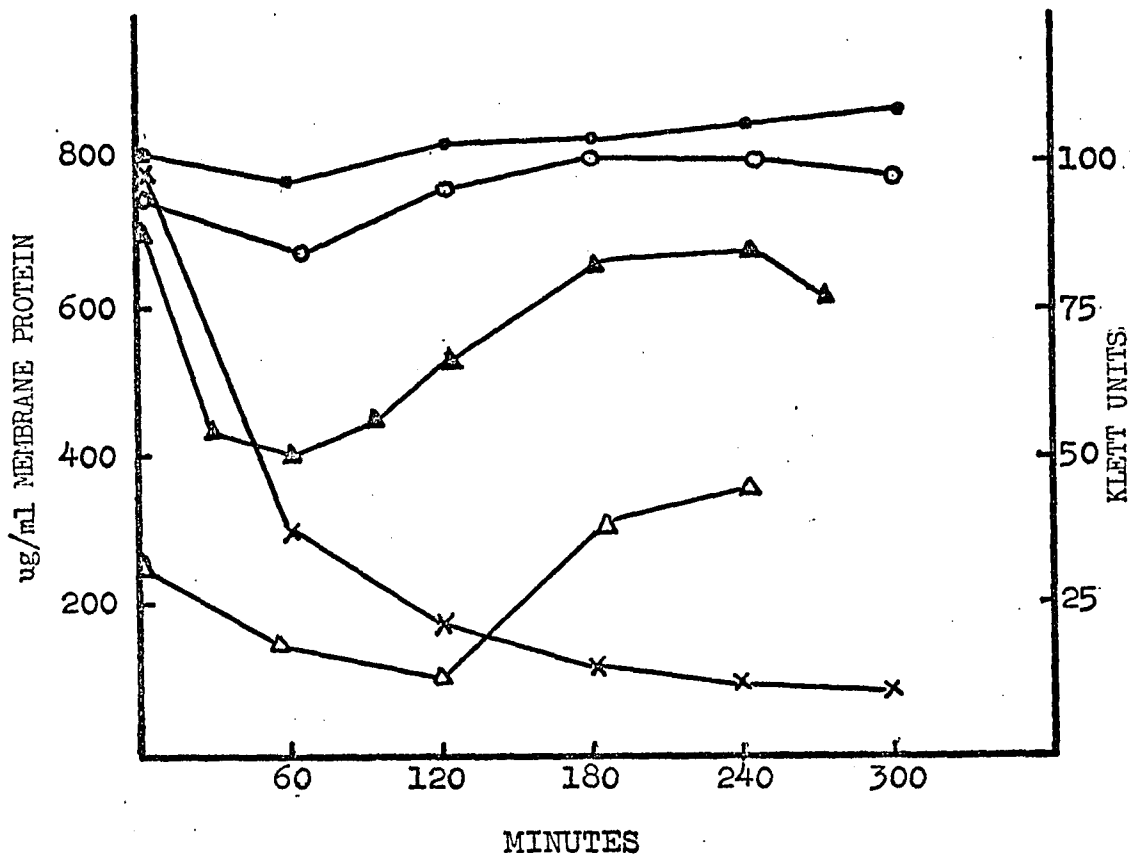


FIGURE 4. Comparison of changes in turbidity and membrane protein occurring during protoplast growth. Symbols: ●, turbidity of antiserum-treated protoplasts; ○, membrane protein of antiserum-treated protoplasts; ▲, turbidity of untreated control; △, membrane protein of control; ×, culture treated with 50 ug./ml. chloramphenicol; turbidity.

linked, either directly, or through a third process that governs both.

In the case of antiserum-treated protoplasts, Figure 4 clearly shows no net protein synthesis is taking place, at least so far as membrane protein changes can be used as a criterion. Again, the two curves are parallel. Thus, two criteria indicate that antiserum-treated protoplasts will not grow.

In Figure 5, data are presented which indicate that the γ -globulin fraction of the immune serum was responsible for the inhibition of growth. The albumin fraction and the α -globulin, β -globulin fraction of both normal and immune sera produced growth responses identical to the control curve. The significance of the response of protoplasts to treatment with univalent immune γ -globulin has already been mentioned.

The likelihood that the growth inhibition was due to an inhibition of transport led to an investigation of this possibility. Since the inhibition of growth was total, the transport of glucose was investigated, since this nutrient is required for all energy dependent processes in Strep. faecium. It was impossible to study glucose uptake directly, since this compound does not accumulate in the cell. It was, however, possible to infer the existence or inhibition of glucose transport indirectly. This was done in two ways: first, the pH changes occurring in unbuffered sucrose suspensions of antiserum-treated and untreated protoplasts were measured and compared, and; second, the amounts of unmetabolized glucose

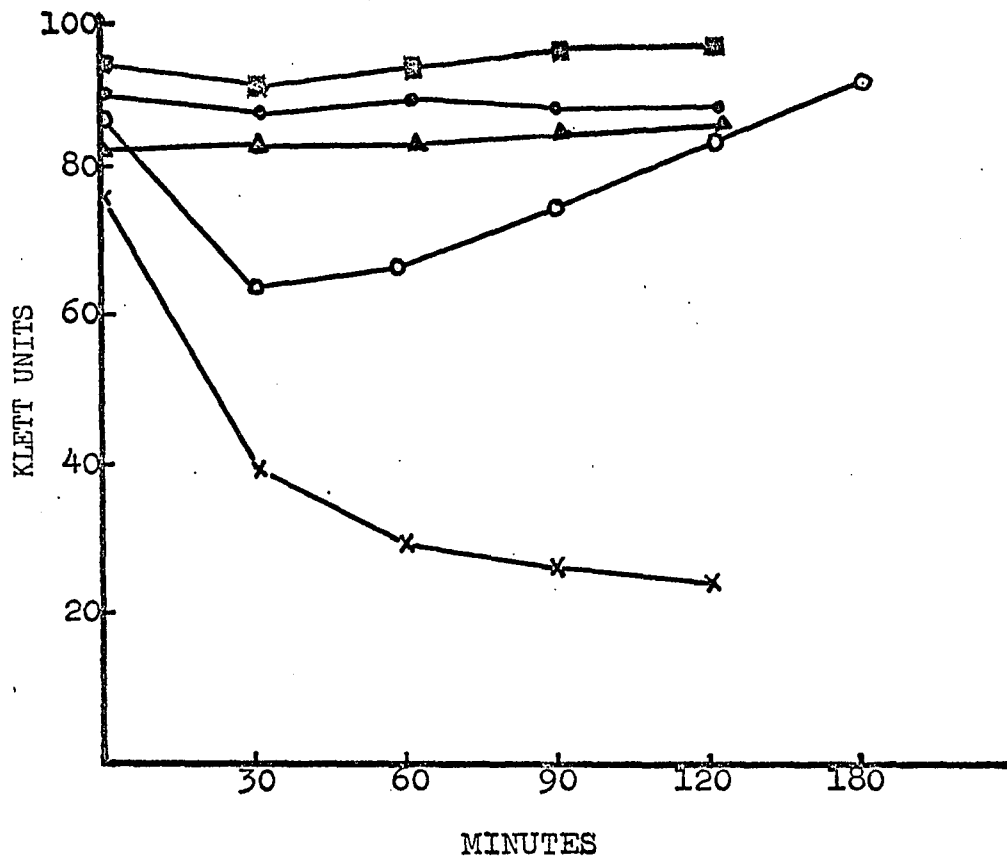


FIGURE 5. Effects of fractions of membrane antiserum on protoplast growth. Symbols: ○, protoplasts treated with unfractionated membrane antiserum; △, protoplasts treated with antiserum γ -globulin; ■, protoplasts treated with univalent antibody; ●, untreated control; ×, culture containing 50 μ g./ml. chloramphenicol.

remaining in sucrose suspensions of treated and untreated protoplasts were compared.

Figure 6 shows that a substantial difference existed between acid production of antiserum-treated protoplasts and that of untreated, or normal-serum-treated protoplasts. The data of Figure 7 confirm the hypothesis that glucose dissimilation is inhibited in the presence of antiserum.

There remained the possibility that the inhibition of glucose metabolism was the result of inhibition by antiserum of some cytoplasmic enzymes(s). If such were the case, protoplasts treated with antiserum, then lysed in the absence of unreacted antiserum, would not metabolize glucose. Such lysed suspensions were prepared and it was found, as Figure 7 shows, that their ability to metabolize glucose is the same as lysed suspensions of untreated protoplasts. It may therefore be concluded that the primary point of action of anti-membrane antiserum is on the glucose transport system.

It was of interest to determine whether glucose transport was the only permeation affected by antiserum. For this reason, the transport of three amino acids was studied. The three were glutamic acid, alanine and arginine, representing the acidic, neutral and basic classes, respectively. These were selected both because they represent three separate chemical and metabolic groups, and also because all can be transported by Strep. faecium without energy supplied by glucose metabolism. The results are presented in Table I.

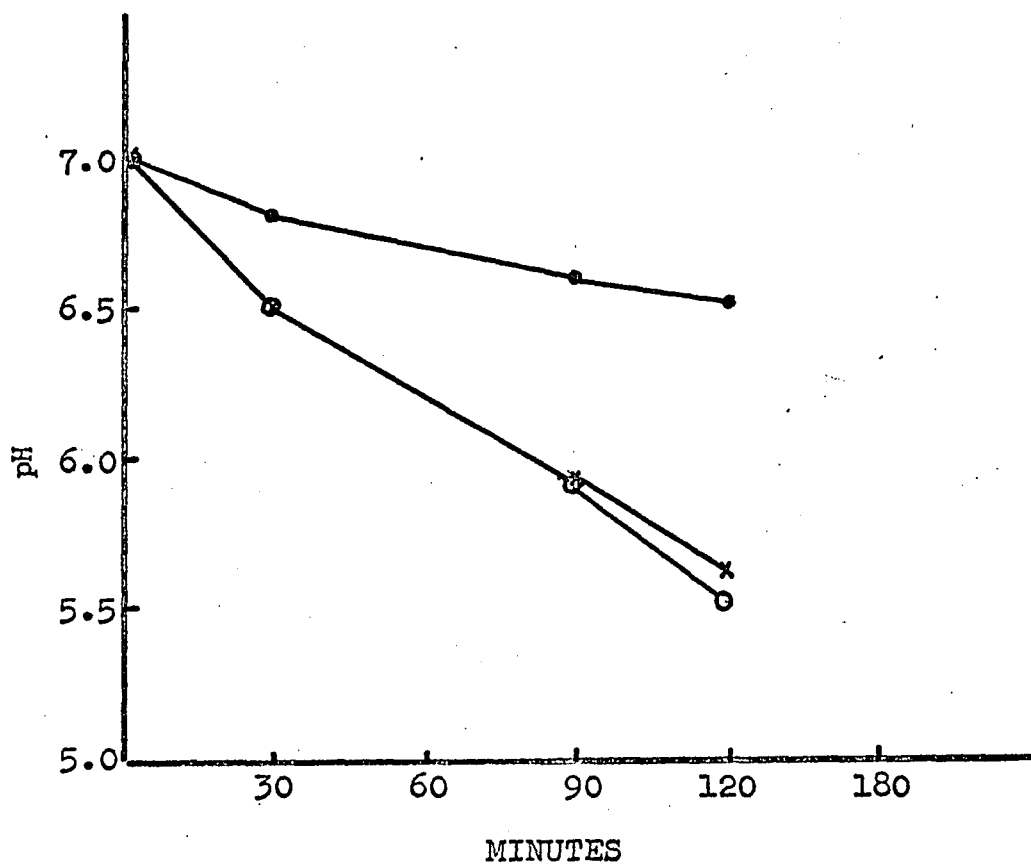


FIGURE 6. Changes in exogenous pH associated with glucose metabolism of protoplasts. Symbols: ●, protoplasts treated with anti-serum; ○, untreated control; ×, protoplasts treated with normal-serum.

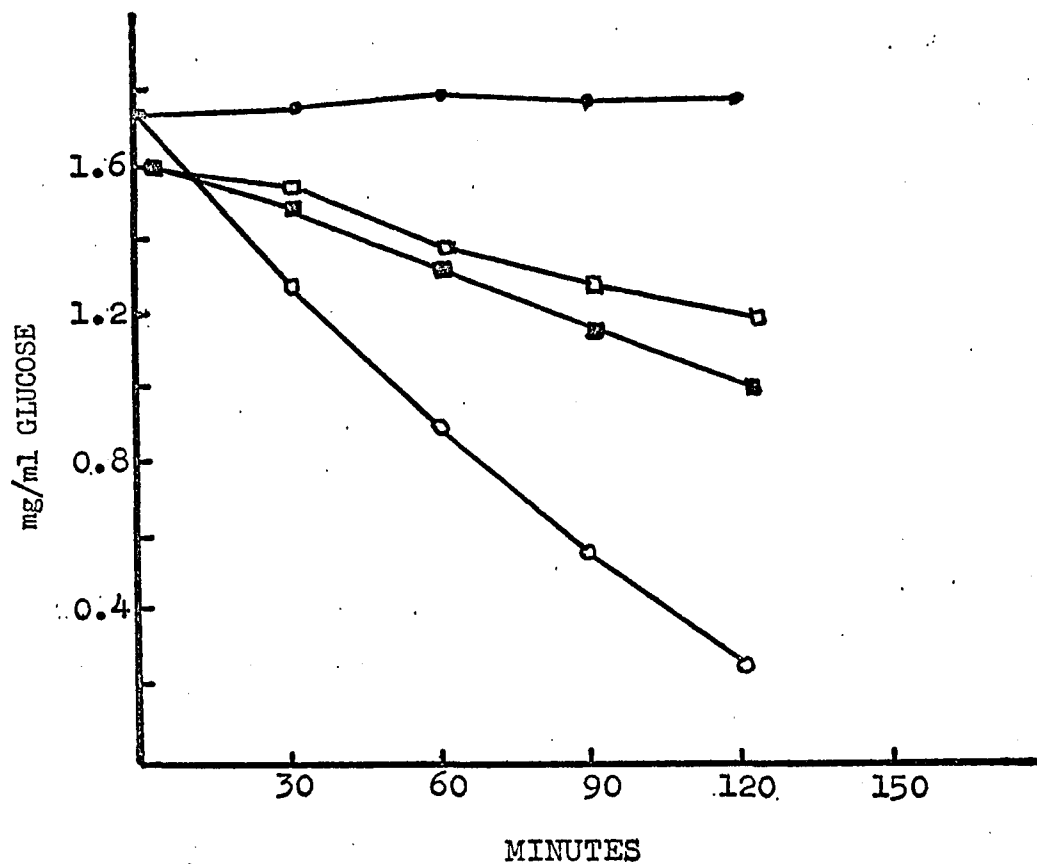


FIGURE 7. Utilization of glucose by sucrose suspensions of protoplasts. Symbols: ●, antiserum-treated protoplasts; ○, untreated control; ◻, lysate of antiserum-treated protoplasts; ◼, lysate of untreated protoplasts.

TABLE I
EFFECTS OF ANTISERUM TREATMENT ON AMINO ACID TRANSPORT
IN PROTOPLASTS

Amino Acid	counts/min./mg, dry weight	
	Control	Treated
Arginine	260	150
Alanine	510	370
Glutamic Acid	620	582

It is apparent that the transport of all three amino acids is inhibited to some extent by antiserum. The extent of inhibition seems to be related to the chemical class to which the amino acid belongs. Thus, the inhibition is greatest (60%) in the case of arginine (basic), less (28%) with alanine (neutral) and least (12%) in the case of glutamic acid (acidic). However, these results, although providing a basis for speculation and further experiment, should be interpreted conservatively. It is certain that treatment with membrane antiserum does cause some inhibition of amino acid transport and further work may clarify the relationship between the chemical class of the amino acid and the extent of the inhibition.

Analysis of Membrane Antigens

Using the procedures described for the preparation of soluble antigens, the following percentages of membrane protein were found in the ultracentrifugal supernatant: sonicated membrane (S-M), 99%; sodium lauryl sulfate-treated membrane (SLS-M), 95%; Triton-X-treated membrane (T-M), 92%; cetyltrimethylammonium bromide-treated membrane (CTAB-M), 40%. The poor performance of CTAB was the result of its tendency to cause precipitation of the membrane, an effect probably related to its cationic nature.

The results of the gel diffusion studies are presented in Figure 9. The contents of the various wells are listed in Table II. In order to facilitate designation of the plate and well being described, the following system will be used:

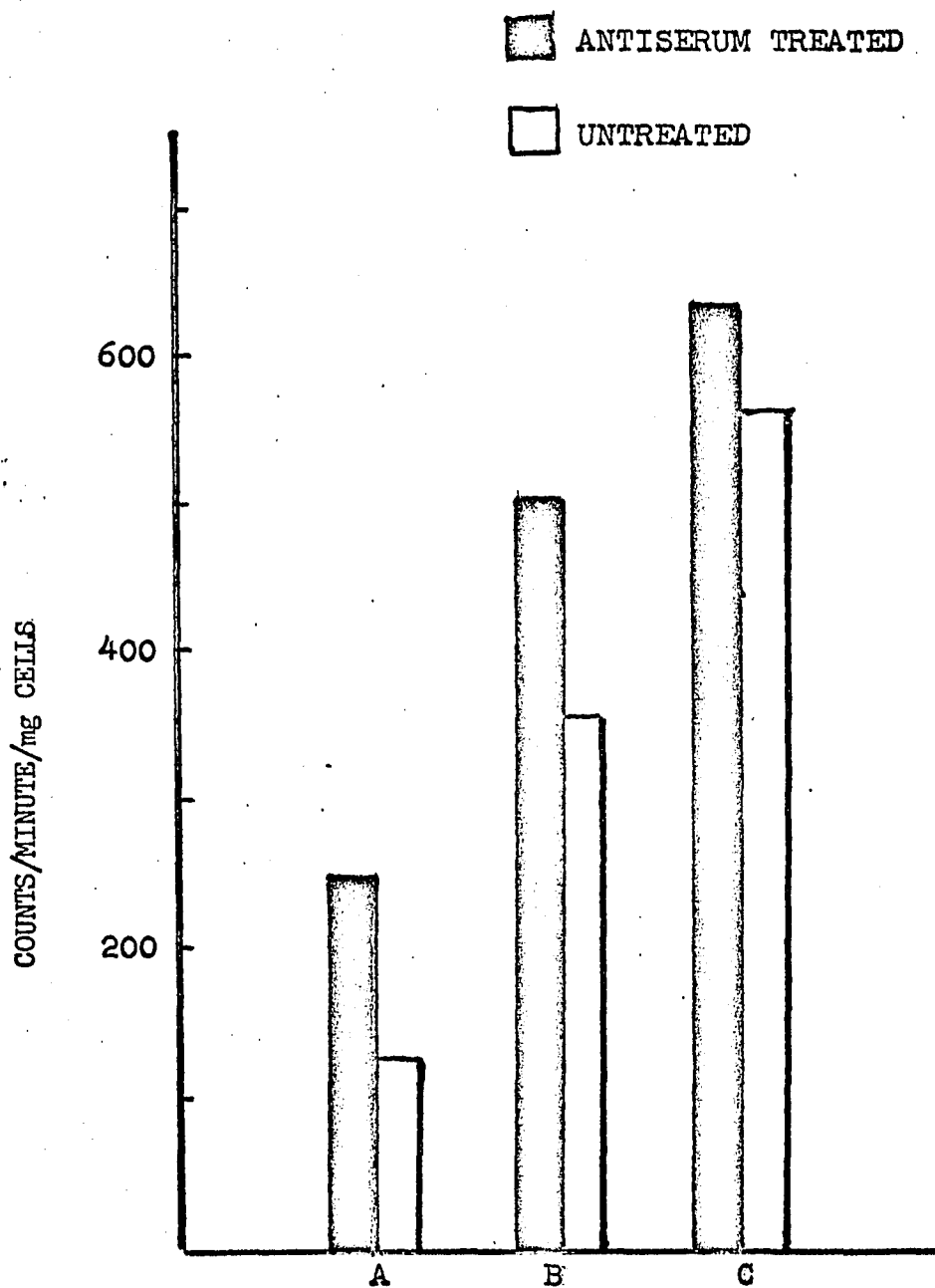
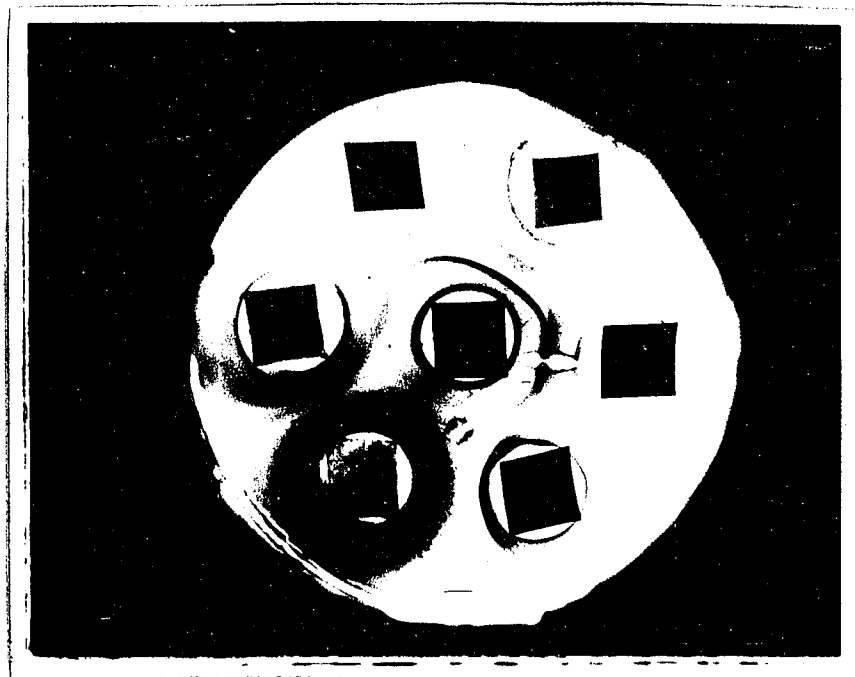
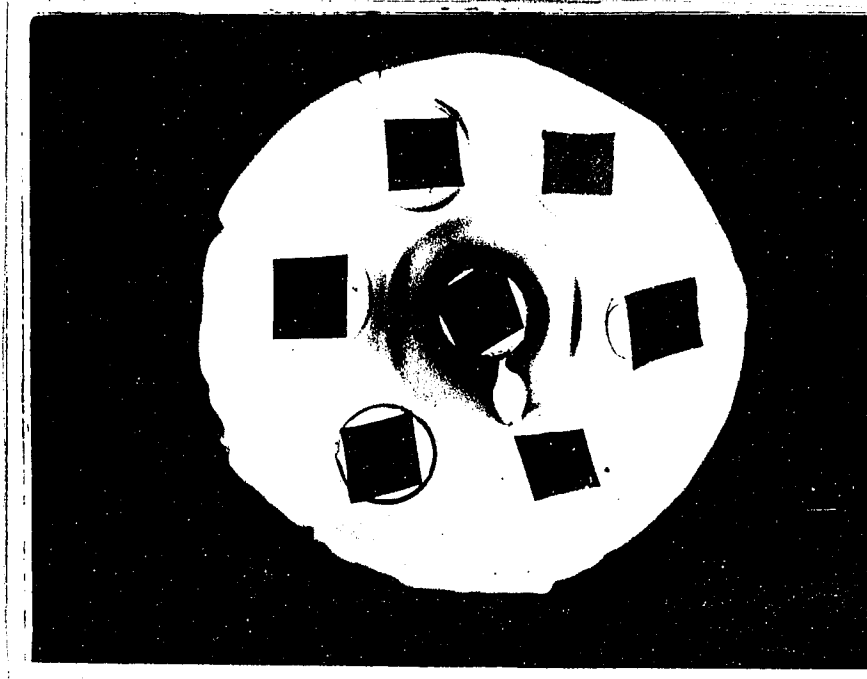


FIGURE 8. Effect of antiserum treatment on the ability of pro-
toplasts to transport amino acids. Symbols: A, arginine trans-
port; B, alanine transport; C, glutamic acid transport.

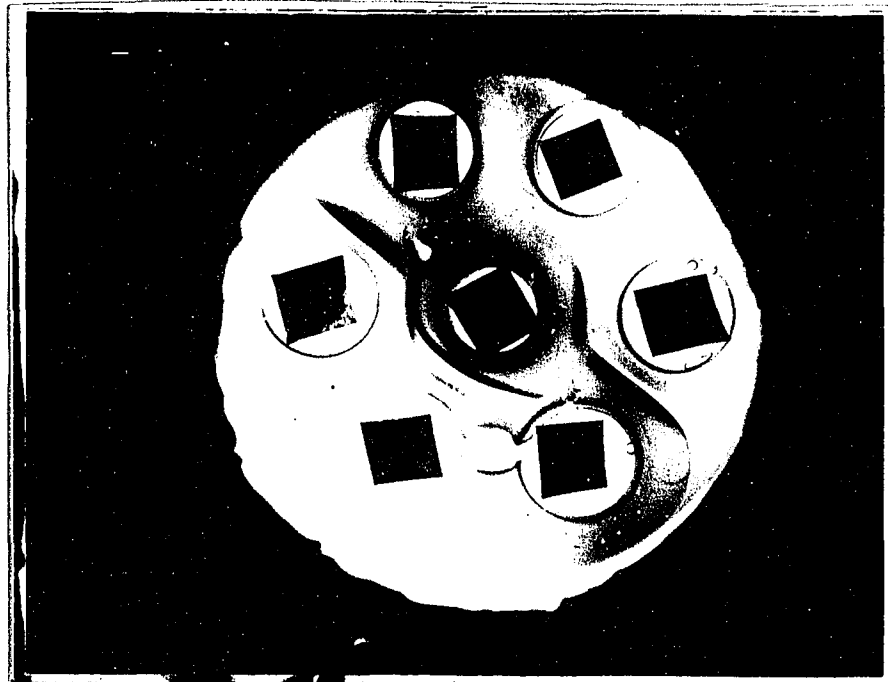


a

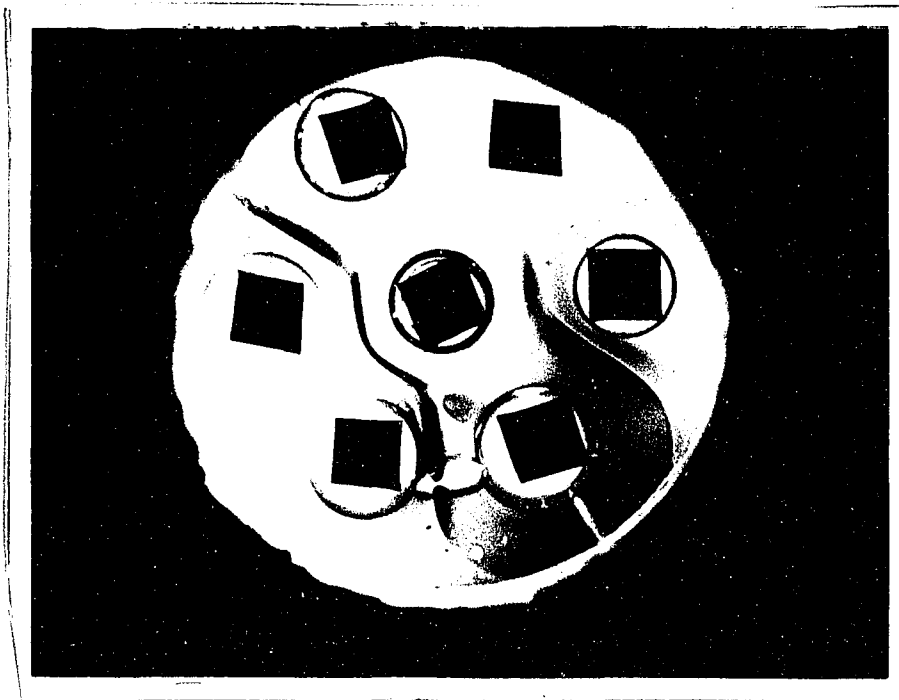


b

FIGURE 9. Gel-diffusion analysis of membrane antigens. Symbols: a, plate a; b, plate b.

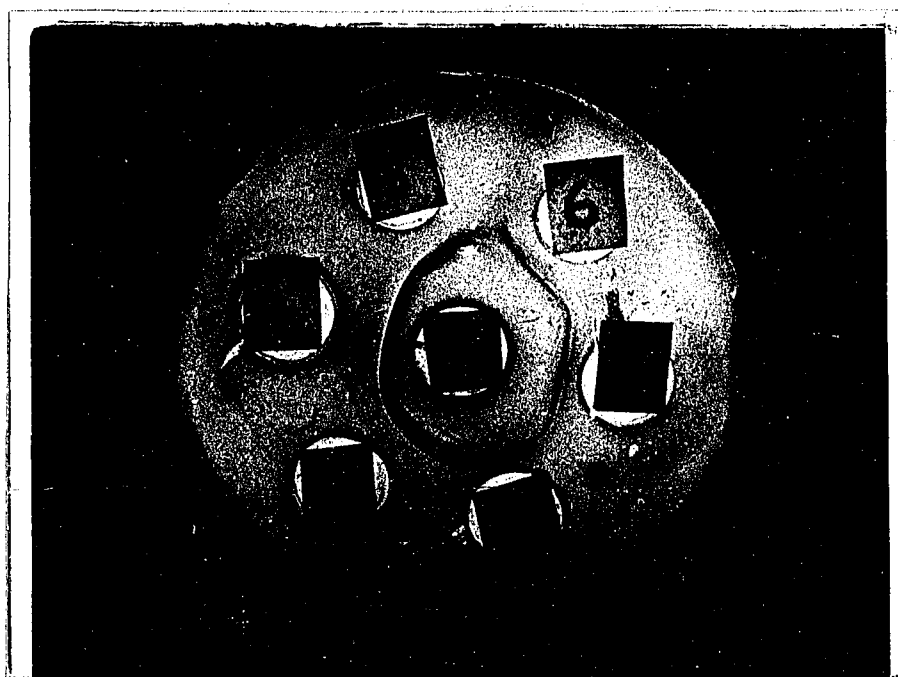


c

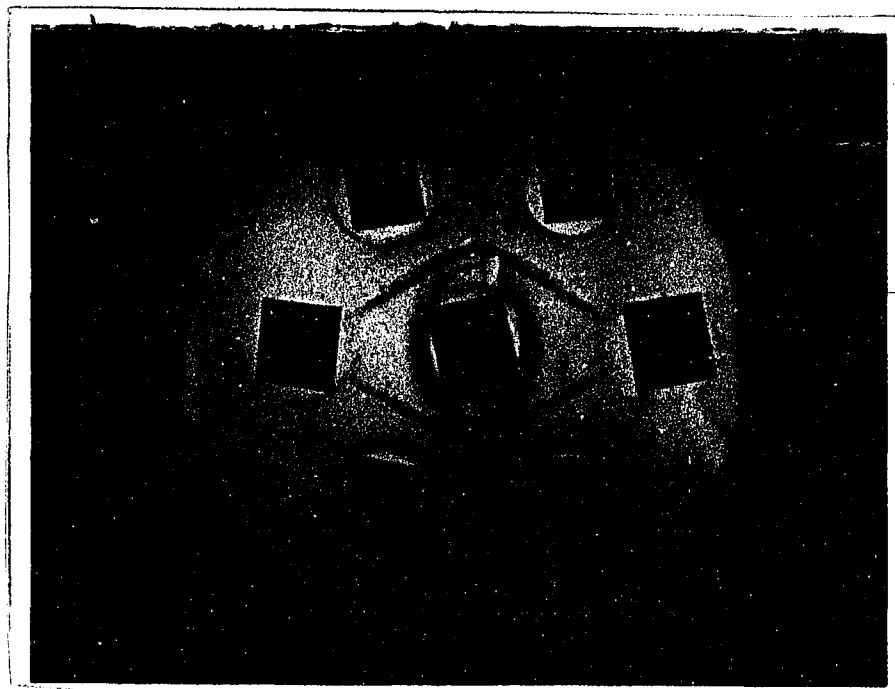


d

FIGURE 9 (cont'd). Gel-diffusion analysis of membrane antigens. Symbols: c, plate c; d, plate d.

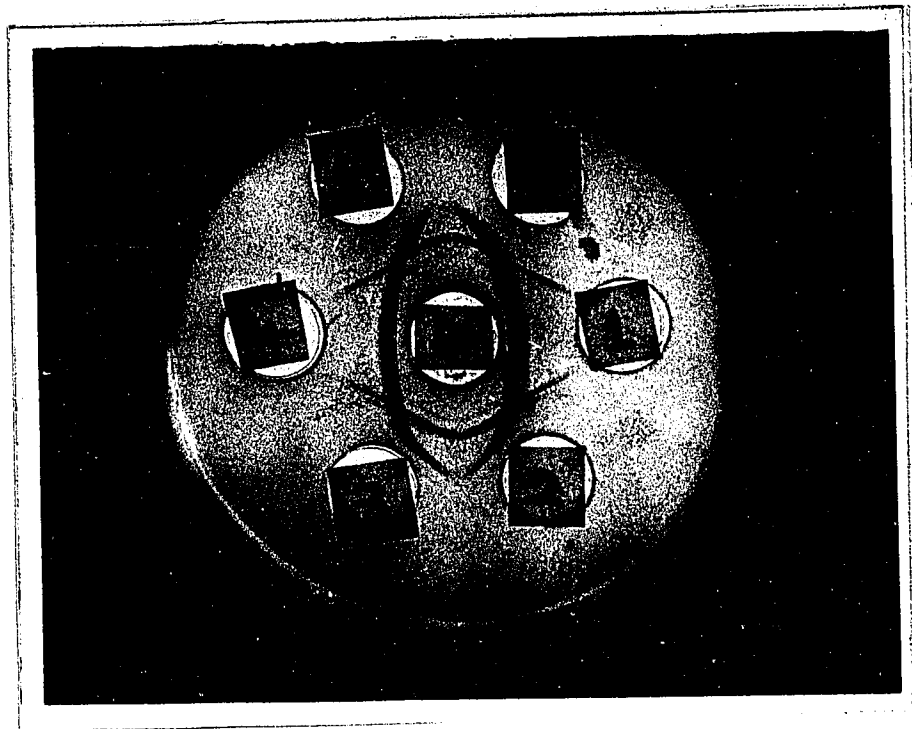


e



f

FIGURE 9 (cont'd). Gel-diffusion analysis of membrane antigens. Symbols: e, plate e; f, plate f.



g

FIGURE 9 (cont'd). Gel-diffusion analysis of membrane anti-
gens. Symbols. g, plate g.

TABLE II
ANTIGENS USED IN GEL DIFFUSION

Well Number	Plate						
	a	b	c	d	e	f	g
1	S-M	S-M	S-M	S-M	S-M	Mu	Mu
2	SLS-M	L-M ³	Mu ⁸	Mu	Gy-H ⁹	T-M	T-M
3	GTAB-M	Tr-M ⁴	W	SLS-M	W-H	Mu	Mu
4	S-M	S-M	S-M	S-M	S-M	T-M	T-M
5	Cy ²	R-M ⁵	Mu	Mu	S-M-H	Mu	Mu
6	W ¹¹	D-M ⁶	Cy	Tr-M	Tr-M	T-M	T-M
7 ¹	As-A	As-A ⁷	As-A	As-A	As-A	As-A	As-U ¹⁰

- 1 central well.
2 cytoplasmic fraction.
3 lipase-treated membrane.
4 trypsin-treated membrane.
5 ribonuclease-treated membrane.
6 deoxyribonuclease-treated membrane.
7 membrane antiserum, absorbed with muramidase.
8 muramidase.
9 the suffix, H, indicates heat treatment of the antigen.
10 membrane antiserum, unabsorbed with muramidase.
11 wall fraction.

to the abbreviation of the type of antigen contained in the well, will be appended both the letter designating the photograph and the number of the well, e.g. sonicated membrane in well 1 of plate photograph a, will be abbreviated S-m, a1.

The number of reactive antigenic sites released by the various treatments can be determined by examination of Figure 9 a, d and f. Sonication solubilized three reactive sites, as can be seen by examination of S-M, a1 and c4. The innermost line was the most sharply defined and intense, the second rather diffuse, and the outermost line, almost invisible in A-M, a1, but apparent in S-M, c4, is extremely faint. All three lines were quite close to each other, the outer two not being completely separated, except in special cases. Thus only two lines were distinguishable in S-M, a4, b1, b4, c1, d1, d4, e1, and e4. It is also possible that the additional line represents an artifact, although the cause is not apparent.

SLS-M, a2 and d3 show two clear lines of precipitation, with traces of a third, intermediate line in d3. As with the sonicated preparation, the innermost line is the stronger.

The pattern produced by CTAB treatment is more difficult to interpret, due to the non-specific precipitation apparent in CTAB-M, a3. Two lines of approximately equal intensity can be seen. Because of the tendency to produce artifacts, study of this antigen was not pursued.

The effects of Triton-X treatment can be seen in T-M, f5 and f6. Two slightly separated, sharply defined lines of almost equal intensity are apparent.

In addition to studying the effects of different solubilization procedures on the antigenic picture, it was of interest to look for antigens in the muramidase solubilized material of the cell (the so-called 'wall' fraction) and in the centrifugally non-sedimentable portion of the protoplast lysate (the 'cytoplasmic' fraction). The reactive components of the 'wall' fraction are shown in a5. The number and intensity of the lines are similar to those of S-M, a1. The 'cytoplasmic' fraction, in a5, displays a similar pattern, although the intermediate line is of lesser intensity than in a1 or 26. The protein concentration of the 'wall' fraction used in this test was 1.2 mg/ml, rather than the usual 1.0 mg/ml. The higher concentration was used in order to compensate for the 0.2 mg/ml of muramidase known to be present. The protein concentration of the 'cytoplasmic' fraction was adjusted to 1.0 mg/ml. It is not possible to conclude, with the data at hand, whether these antigens are native to the wall and cytoplasm of the cell, or represent membrane components, solubilized during protoplast formation. The profound effects of membrane antiserum on the native protoplast membrane argue for the latter alternative.

It has long been recognized that a certain amount of the muramidase used to lyse the cell wall remains bound to the underlying membrane. As a result, the membrane antiserum prepared for these studies had a capacity to react with this enzyme. This reaction can be seen at g1 and g4. In order to simplify the situation, the serum was absorbed with

muramidase prior to use in other gel diffusion tests. The results of this absorption can be seen at f1 and f4. No visible reaction is apparent. Thus, precipitin lines appearing in the other plates can be ascribed to native antigens.

Of greater importance than the number of lines are the interactions of the lines of adjacent wells. By studying these reactions, an index of the similarities and differences between antigens may be obtained. Four general types of interactions are recognized, based on the extent to which lines of precipitation from adjacent wells interfere with one another (49). Type I is characterized by mutual deviation of the lines, leading to complete coalescence. The result is generally a single smooth arc of precipitation between the two wells. A type I reaction indicates that the two antigens are identical. A type II reaction, characteristic of totally dissimilar antigens, results in the absence of any interference; the two lines cross one another. When two similar, but not identical, antigens interact a type III reaction occurs. The pattern produced by this interaction is characterized by partial coalescence of the lines, with formation of a small, projecting 'spur' at the apex of the arc. A type IV reaction is characterized by the inhibition of the development of one line by another. When this reaction occurs, there is no coalescence of the lines and one or both of the lines are prevented from crossing the other. Several factors are involved in the production of a type IV reaction. The two antigenic particles must contain multiple determinants, at least one of which is common to both. The antiserum must be

polyvalent and have a higher titer for an unshared antigenic determinant than for the shared determinant.

Inspection of Figure 9 will reveal that reaction type I is the most prominent. Only in Figure 9 g is type II apparent, indicating antigenic dissimilarity between muramidase, g1 and g4 and T-M, g2, g3, g5 and g6. No instance of type III or type IV reactions is evident.

Treatment of S-M with 0.1 mg/ml trypsin for a period of four hours, followed by the addition of an equal amount of ovomucoid inhibitor, causes substantial degradation of the ability of the preparation to form lines of precipitation. The effect can be clearly seen in d6 and to a lesser extent in b3. On the other hand, autoclaving samples of S-M for five minutes at 15 pounds pressure, followed by centrifugation and gel diffusion of the supernatant, produces the pattern seen at e5. Similar treatment of the 'wall' fraction and the 'cytoplasmic' fraction result in the patterns e3 and e2, respectively. It is apparent that at least one of the antigens is fairly heat stable. The most likely possible identity is a teichoic acid. It is difficult to reconcile the results obtained by heating the antigen with those obtained by trypsin treatment, except by postulating that one of the membrane antigens is a heat-stable protein or polypeptide.

Growth of the Protoplast Membrane

Figure 10 presents a series of growth curves of protoplasts labelled with fluorescein isothiocyanate (FITC). As can be seen, low levels of FITC (0.02-0.5 mg/ml in the

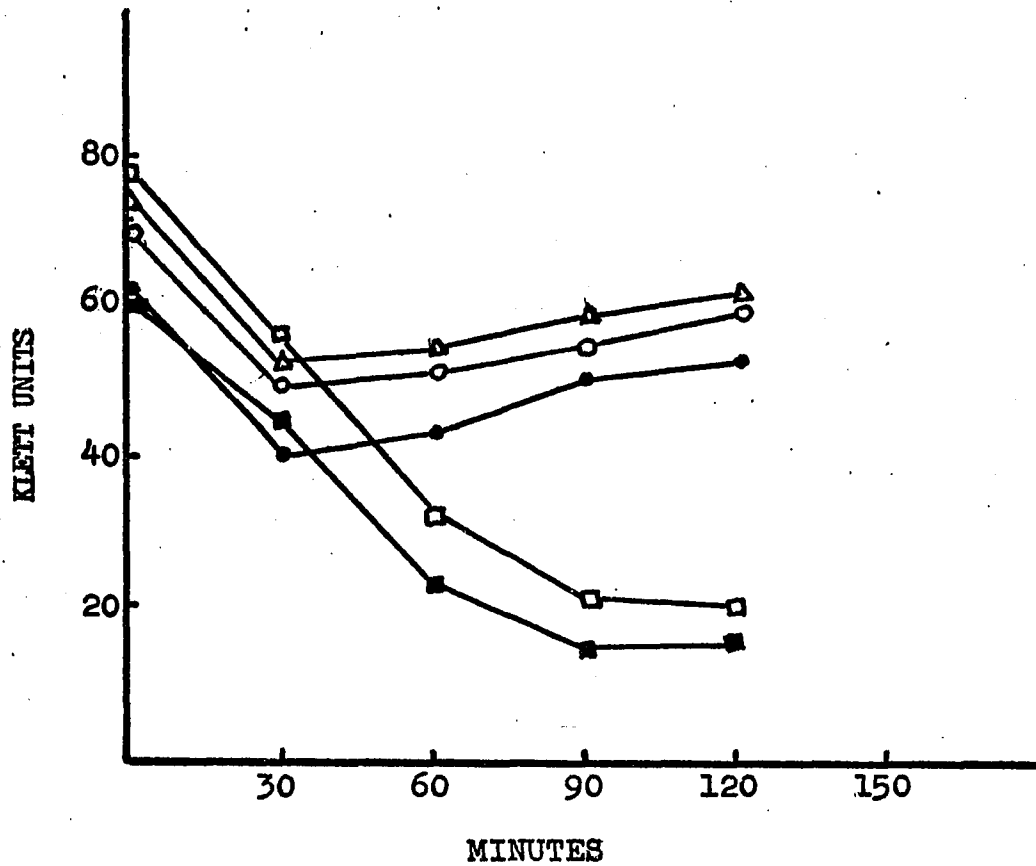


FIGURE 10. Turbidity changes accompanying growth of FITC-labeled protoplasts. Symbols: ●, untreated control; ○, protoplasts labeled with 0.2 mg./ml. FITC; ▲, protoplasts labeled with 0.4 mg./ml. FITC; ■, protoplasts labeled with 0.8 mg./ml. FITC; ◻, protoplasts labeled with 1.6 mg./ml. FITC.

labelling solution) have very little effect on the growth process, compared to unlabelled protoplasts. Higher levels (0.8-1.6 mg/ml) of FITC cause rapid lysis of the protoplasts in the growth medium, similar to that induced by chloramphenicol. Thus, there seems to be a definite level of FITC labelling beyond which protoplast growth ceases, but below which the growth proceeds in a seemingly normal fashion.

In comparing the growth curves of Figure 10 with those of Figure 3, it is apparent that the increase in turbidity, following the initial period of optical density drop, was less in the former than in the latter. This effect is probably a result of the multiple washings to which the FITC-labelled protoplasts and the unlabelled control were subjected (at least four), doubtless causing some lysis. However, growth did occur, as comparison with the chloramphenicol-treated sample will show.

Figure II indicates the amount of FITC penetration into the cytoplasmic portion of the protoplasts resulting from labelling with various concentrations of FITC. Labelled protoplast lysates were adjusted to a concentration of 1 mg/ml dry weight and the optical density at 450 m μ determined, using an untreated lysate as a blank. The standard curve was prepared using protoplast lysates to which known amounts of FITC were added. It can be seen that below an FITC concentration of 0.8 mg/ml in the labelling solution, penetration was nil. Above this point, penetration increased rapidly. The inflection point of this curve corresponds well with the point

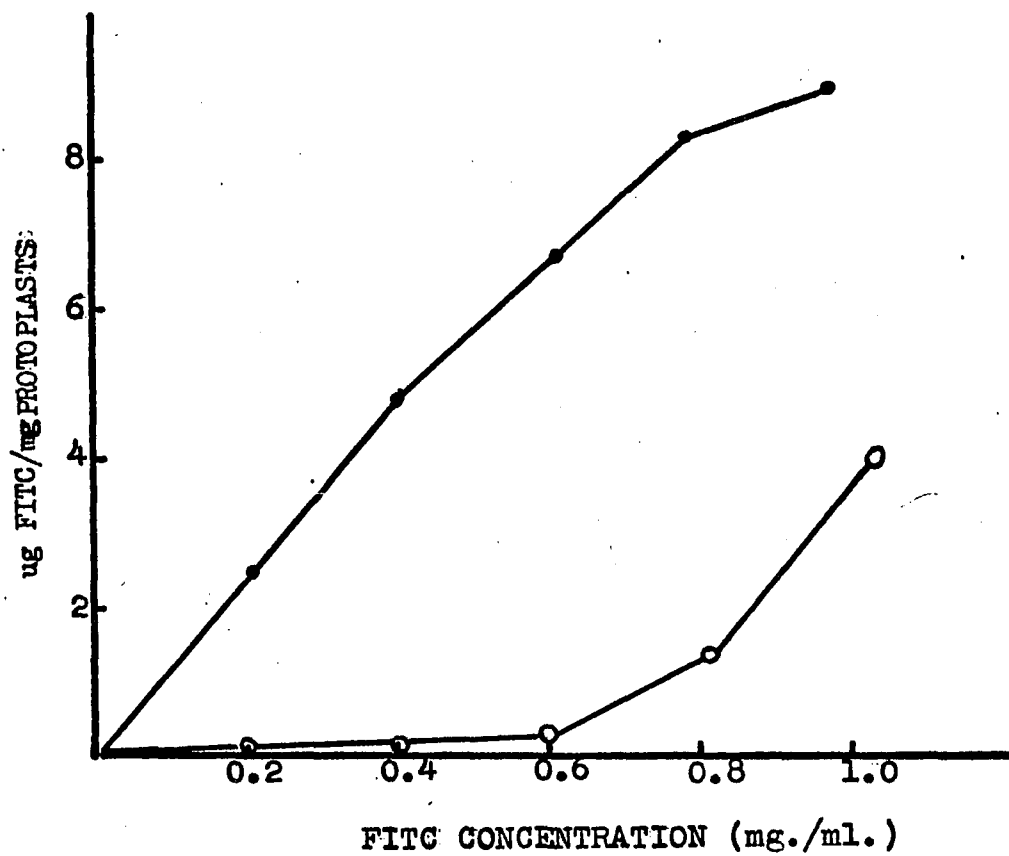


FIGURE 11. Penetration of protoplasts by FITC during the labeling process. Symbols: ●, membrane-bound FITC; ○, cytoplasmic FITC.

in Figure 10 beyond which the labelled protoplasts no longer grew. This indicates that cessation of growth was caused by penetration of FITC into the cytoplasm and its reaction with some vital cell component(s).

Similar determinations, carried out with sonicated FITC-labelled membranes, are presented in Figure II. The point at which the slope of this curve changes, indicating that most readily reactive sites have been conjugated, also corresponds to the point at which significant FITC appears in the cytoplasm. It is, therefore, probable that FITC does not penetrate the membrane in detectable amounts until available sites on the membrane have been exhausted.

The concentration of FITC chosen for labelling protoplasts for the study of membrane growth was 0.5 mg/ml. This concentration produces the maximum labelling of the membrane consistent with minimum penetration. Figure 12 shows the growth curves produced by such labelled protoplasts. Both the turbidity and the membrane dry weight compared favorably with the unlabelled control. During the incubation period, the membrane dry weight doubled.

Figure 13 contains photomicrographs of samples of such labelled protoplasts, showing the changes occurring during the growth process. Each microscopic field was photographed twice, once with visible light (indicated by the subscript 'v' in Figure 13) and subsequently with ultraviolet light ('u' in Figure 13). Thus, within a sample taken at a given time, individual protoplasts may be studied and the

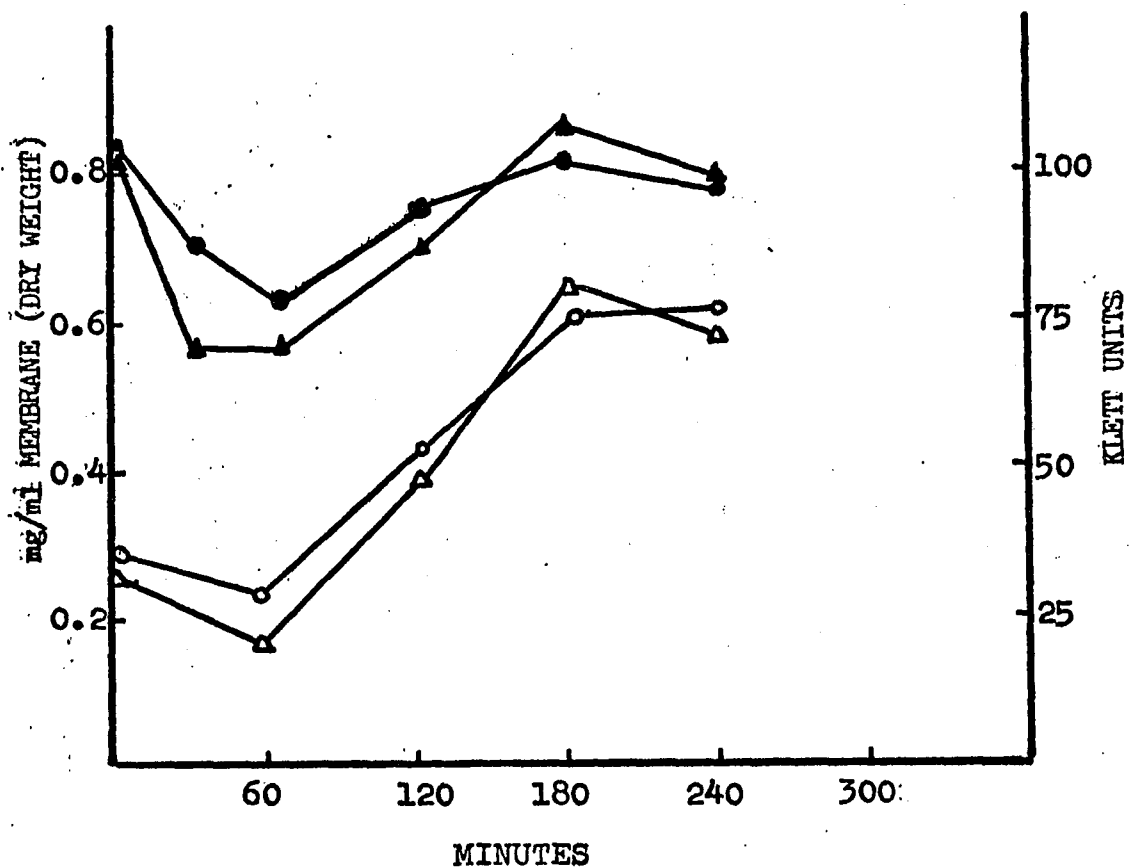
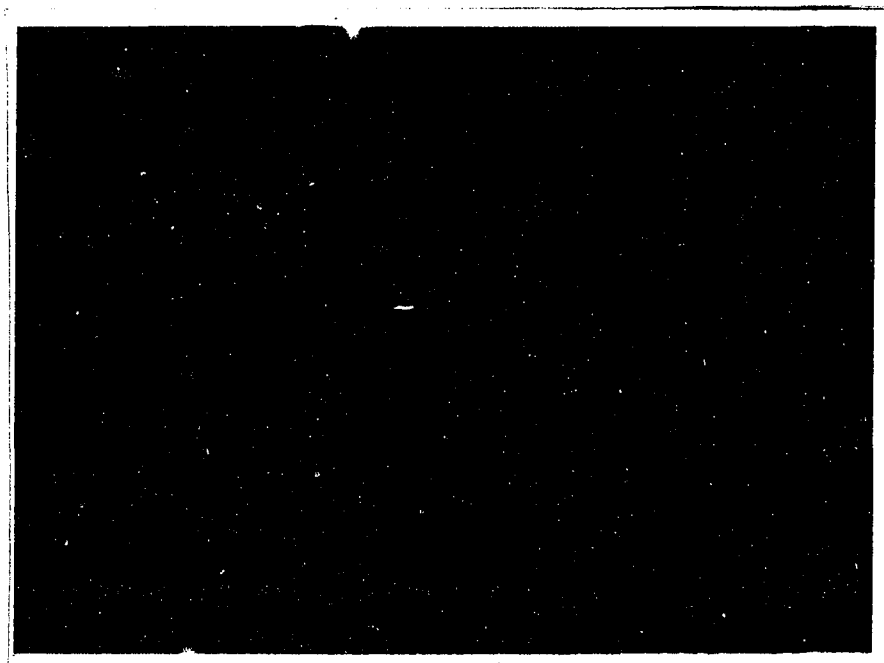


FIGURE 12. Changes in turbidity and membrane dry weight during growth of FITC-labeled protoplasts. Symbols: ●, turbidity of FITC-labeled culture; ○, membrane dry weight of FITC-labeled protoplasts; ▲, turbidity of untreated control; △, membrane dry weight of control.



O_v



O_u

FIGURE 13. Growth of FITC-labelled protoplasts. Symbols: O_v, 0 minutes, visible light; O_u, 0 minutes, ultraviolet light.

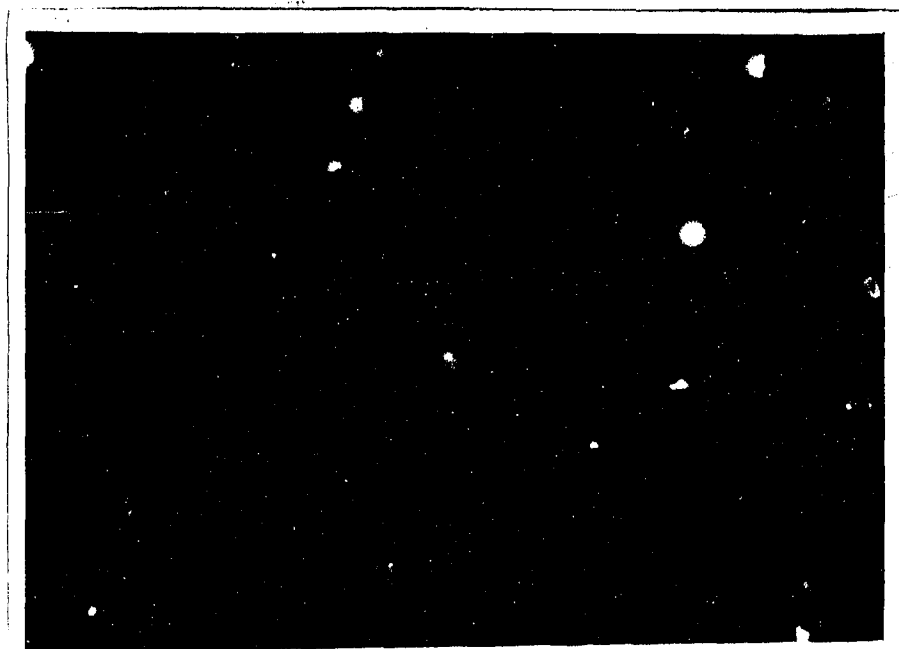
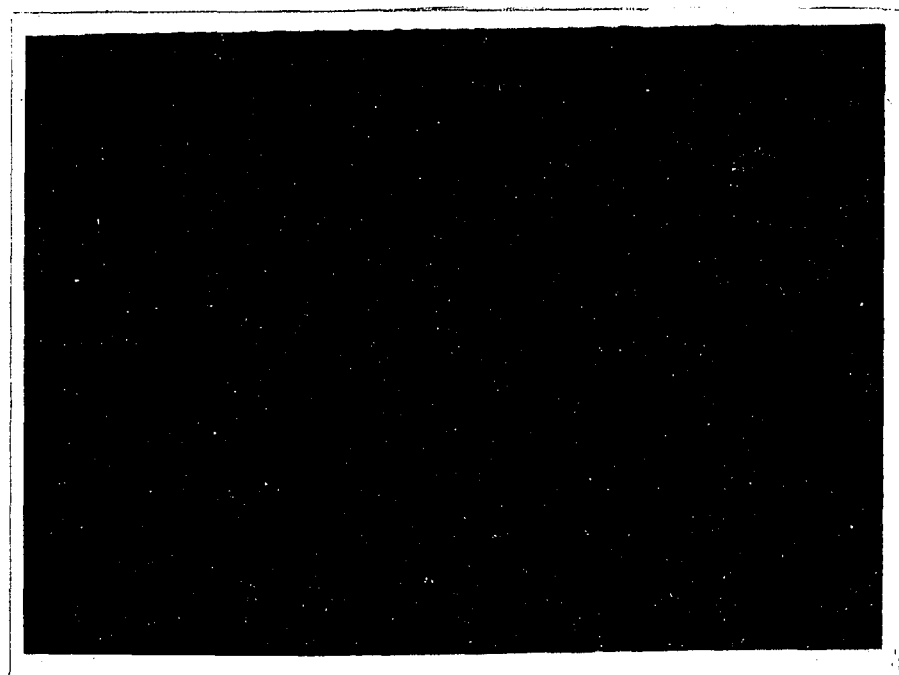
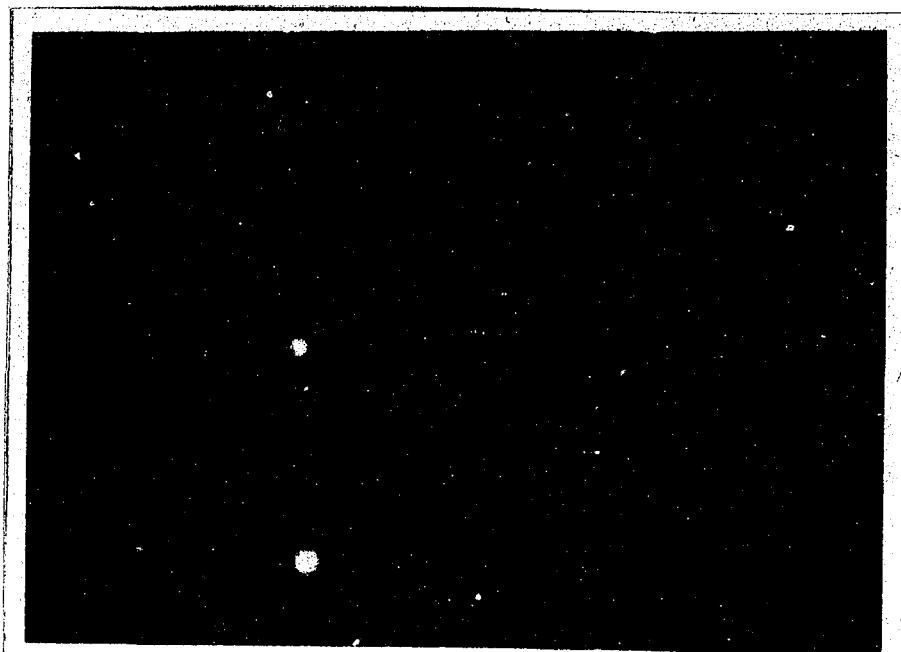
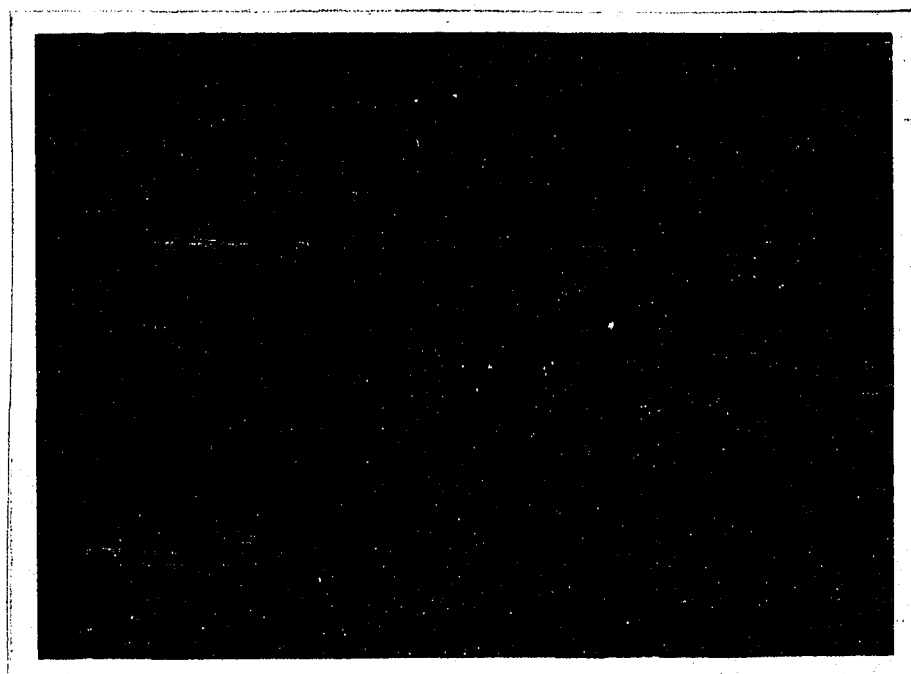
30_v30_u

FIGURE 13 (cont'd). Growth of FITC-labelled protoplasts. Symbols: 30_v, 30 minutes, visible light; 30_u, 30 minutes, ultraviolet light.



60v



60u

FIGURE 13 (cont'd). Growth of FITC-labelled protoplasts. Symbols: 60v, 60 minutes, visible light; 60u, 60 minutes, ultra-violet light.

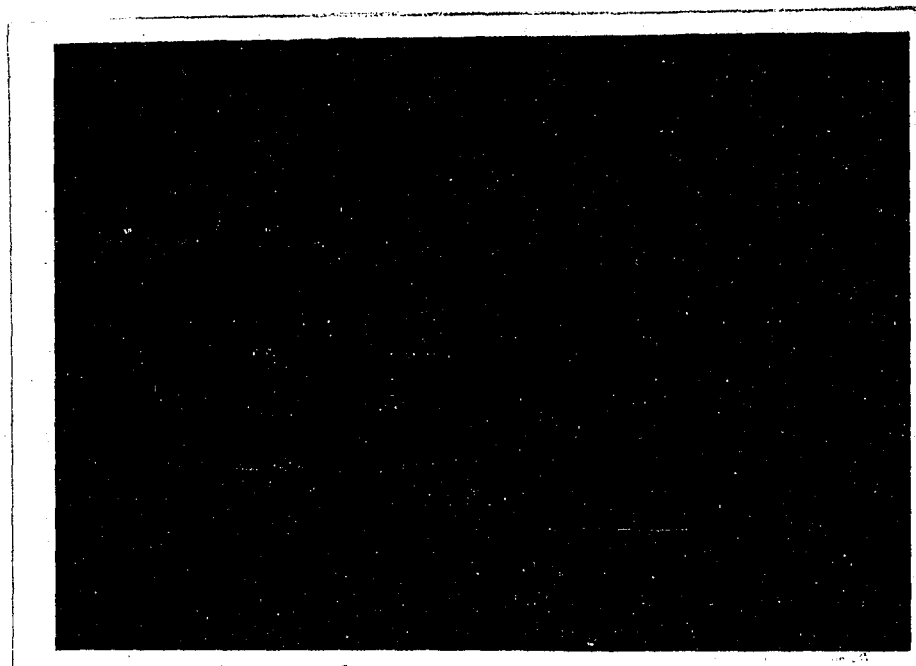
120_v120_u

FIGURE 13 (cont'd). Growth of FITC-labelled protoplasts. Symbols: 120_v, 120-minutes, visible light; 120_u, 120 minutes ultraviolet light.

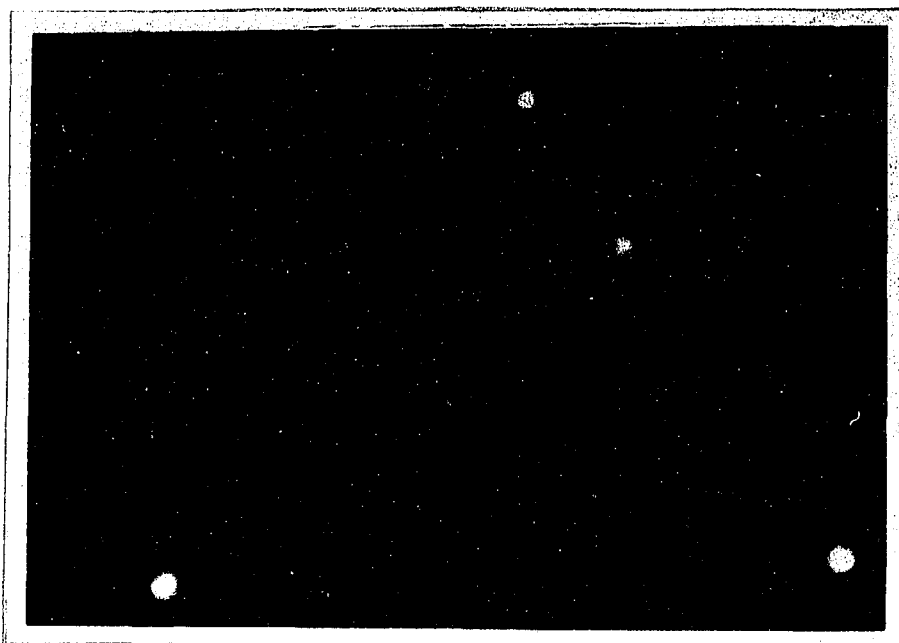
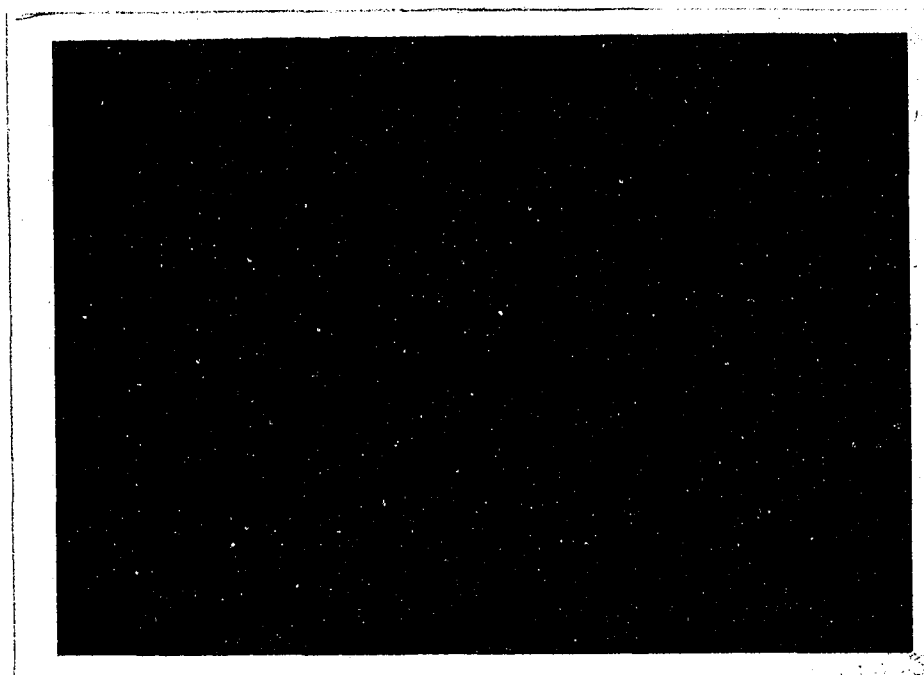
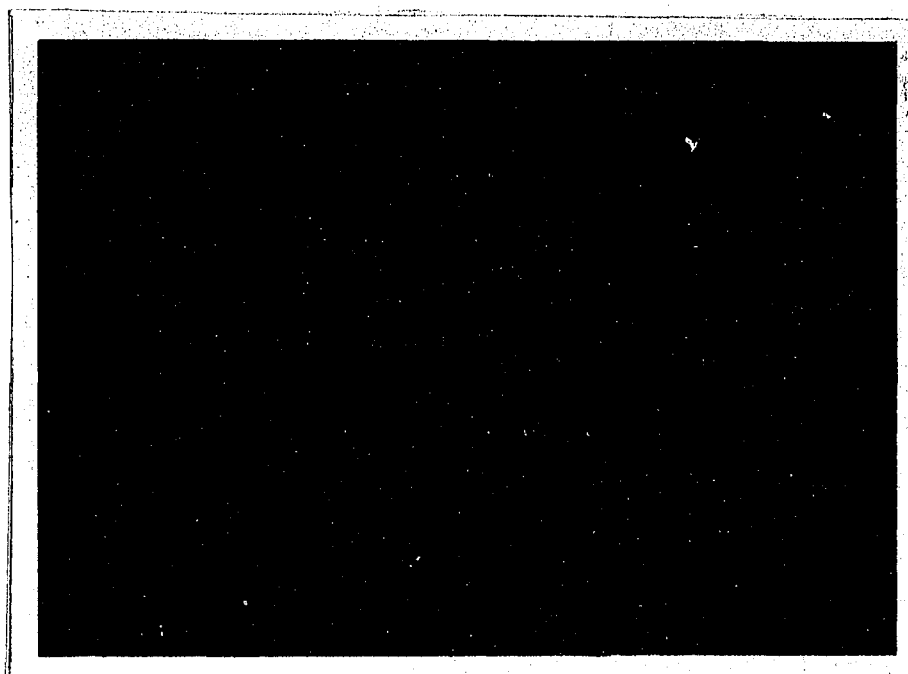
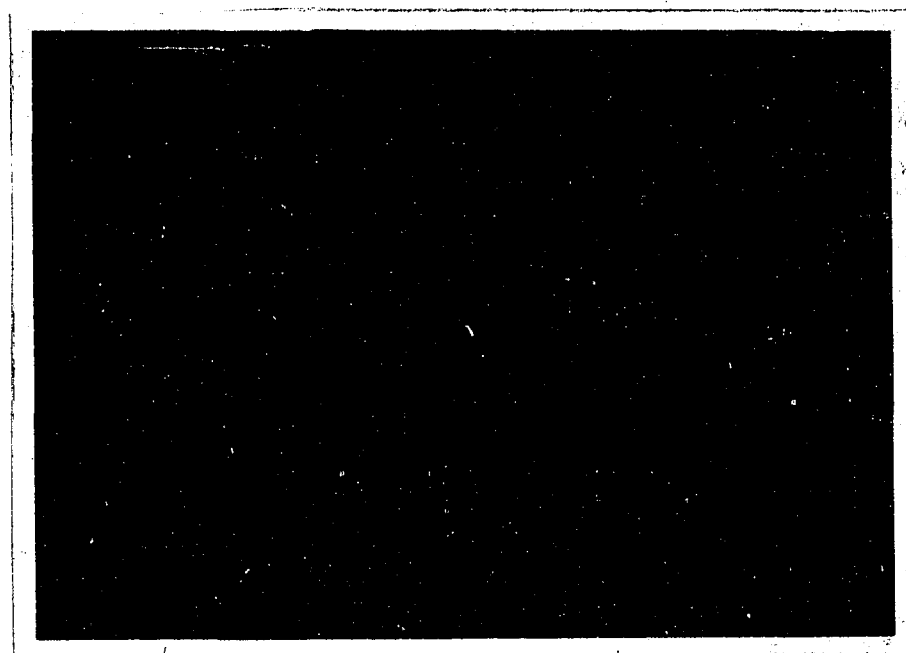
180_v180_u

FIGURE 13 (cont'd). Growth of FITC-labelled protoplasts. Symbols: 180_v, 180-minutes, visible light; 180_u, 180 minutes, ultraviolet light.



0-su



180-su

FIGURE 13 (cont'd). Growth of FITC-labelled protoplasts. Symbols: 0-su, 0 minutes, sucrose-suspension of protoplasts, ultraviolet light; 180-su, 180 minutes, sucrose suspension of protoplasts, ultraviolet light.

structure revealed by visible light compared to its ultraviolet light-induced fluorescence.

The most important feature of the sequence of photomicrographs is that, as growth proceeded, there was an increase in the apparent diameter of the protoplasts and a corresponding decrease in the intensity of fluorescence. After 180 minutes, the diameter of the protoplasts had virtually doubled and the fluorescence had almost completely disappeared. These results are predictable consequences of protoplast growth.

The process of fluorescence loss is also of great interest, since it provides information on the localization of membrane biosynthesis. If, for example, membrane biosynthesis occurred only at the equator of the protoplast, fluorescence would be lost from this region first, the fluorescence of the poles remaining undiminished. If membrane growth occurred at a single point, the result would be loss of fluorescence from an ever-widening circle, with a peripheral area of undiminished fluorescence, if seen from directly above that point. Of course, the pattern produced depends on the orientation of the observer to the growing area, but these two mechanisms can be deduced unambiguously from inspection of a suitable number of photomicrographs of growing protoplasts.

A third mechanism involves biosynthesis at many points on the membrane. The result of such biosynthesis would be a gradual diminution of fluorescence over the entire visible surface of the protoplast. This process is known as

'intercalation', and appears to be the mechanism we are dealing with in Streptococcus faecium, as inspection of Figure 13 will show.

However, it is possible that the observed loss of fluorescence is the result of enzymatic hydrolysis of the bond between fluorescein and protein, or the excision of the conjugated protein from the membrane, and not a result of growth. To test this possibility, a sucrose suspension of FITC-labelled protoplasts was incubated in the presence of a lysate of FITC-labelled protoplasts that had been allowed to grow for 180 minutes. The final concentration of the lysate protein in the sucrose was 1 mg/ml. Incubation was carried out at 37 C for 180 minutes and samples were removed at the beginning and end of the period for photomicrography and determination of the ratio of fluorescein to protein in the membrane. Figure 13, 0-Sm and 180-Sm shows that the intensity of fluorescence did not appreciably diminish during the incubation. Figure 14 shows that sonicated membranes, prepared from the incubated protoplasts, retain their initial optical density, indicating little or no loss of label during the process. These results indicate that there was neither a constitutive nor an induced enzyme in the protoplasts, capable of removing the label. Thus, the loss of fluorescence is due to the growth of the membrane.

Figure 14 also shows the change in the fluorescein to protein ratio that occurs during the growth process. Following an initial 30 minute period, during which the ratio remained constant, a linear fall in the ratio was observed. At

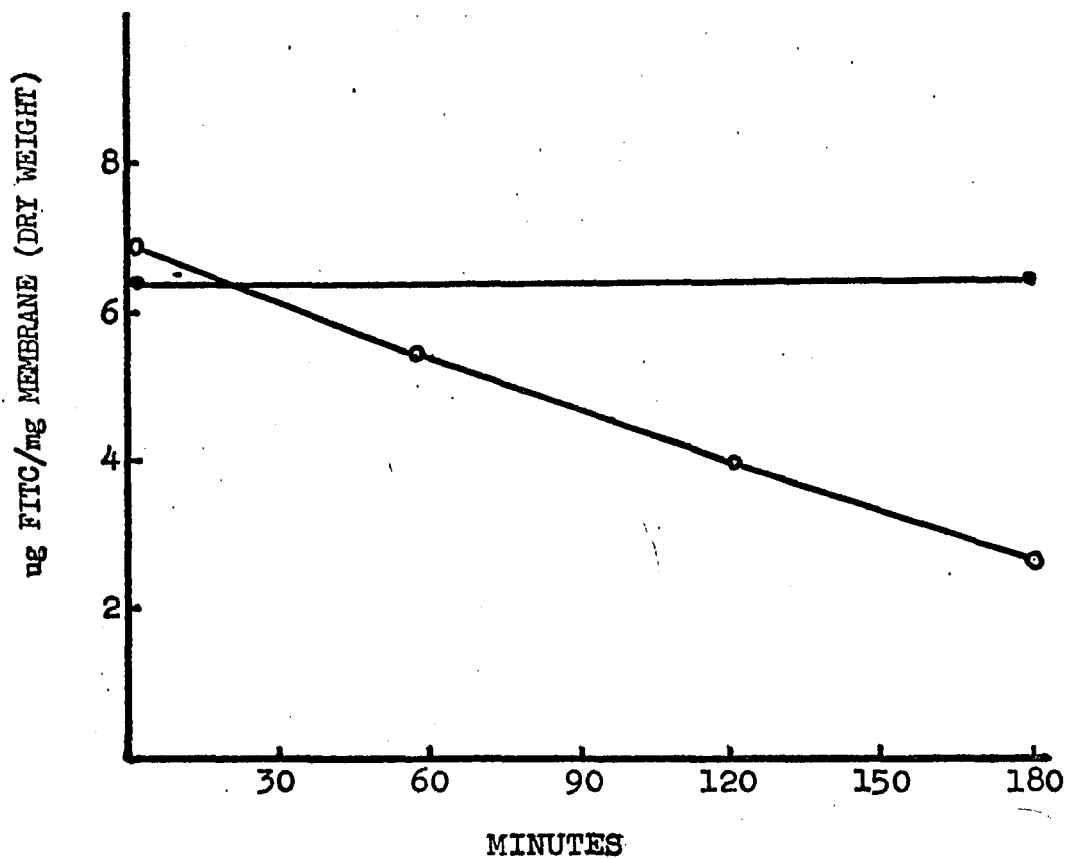


FIGURE 14. Changes in FITC:membrane ratio in growing and non-growing FITC-labeled protoplasts. Symbols: ●, sucrose suspension of labeled protoplasts incubated with a lysate of fully grown, labeled protoplasts; ○, growing culture of FITC-labeled protoplasts.

180 minutes, the figure was 40% of the initial value. This change is consistent with the growth of the membrane, since the amount of membrane protein would increase, while the amount of fluorescein would remain constant.

ATP-pool-driven Transport of Amino Acids

Figure 15 shows the formation of an ATP pool in cells of Streptococcus faecium incubated in the presence of 20 mM/ml glucose, 0.01 mM/ml potassium phosphate, pH 7.0. The maximum pool level, achieved after 60 minutes incubation, is almost 12 $\mu\text{g}/\text{mg}$ dry weight, a level which evidently represents the maximum pool capacity, since nearly 8 mM/ml glucose were still present at that time.

The effects of the three inhibitors, NaF, Na_2HAsO_4 and triiodoacetic acid, on the metabolism of glucose are shown in Figure 16. All three inhibitors caused complete inhibition immediately after addition. The high concentration of the inhibitors (0.01 mM/ml) insured rapid cessation of glycolytic activity.

As a consequence of these results, it is possible to induce the formation of large amounts of endogenous ATP and also to inhibit the formation of further ATP by the addition of inhibitors at any chosen time. Thus, it became possible to make a direct test of the hypothesis that ATP is the direct energy source for active transport, study the stoichiometry between ATP and the transported substance, in the absence of ATP and the transported substance, in the absence of ATP biosynthesis, and test the effects of inhibitors on the transport system directly.

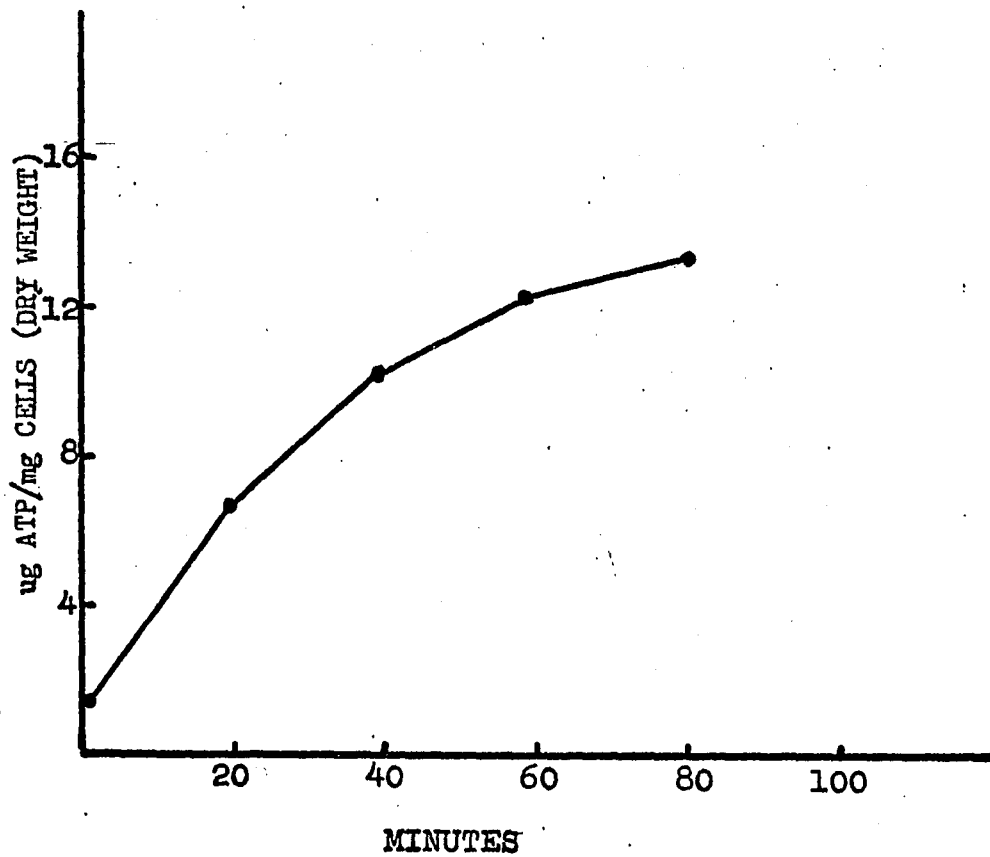


FIGURE 15. Formation of ATP pool in cells incubated with 20 mM./ml. glucose.

If the belief that ATP drives active transport is correct, then it should be possible to use an endogenous pool of ATP for the energy source, in the absence of glycolysis. The results of such an experiment are presented in Table III. It can be seen that cells preincubated with glucose and treated with fluoride or arsenate, displayed an enhanced transport of alanine, compared to controls which had not been preincubated. On the other hand, samples treated with triiodoacetic acid did not show enhanced transport. The most probable explanation of this observation is that this agent inhibits active transport of alanine. Unlike fluoride or arsenate, which act as competitive inhibitors of phosphate, triiodoacetic acid exerts its inhibitory powers by reaction with sulfhydryl groups, a much less specific mode of action. The inhibition of at least one permease system by sulfhydryl inhibition has been established in E. coli (30).

The extent of alanine transport in untreated cells, incubated with glucose for the same period as the treated cells, was somewhat higher, as Table III shows. This is not an unexpected result, since the supply of ATP in the preincubated cells is limited, while normal cells can continue to generate it until the glucose is exhausted. This result does indicate, however, that the factor limiting the extent of alanine transport in the preincubated cells was not saturation of the endogenous alanine pool, but depletion of the energy source.

Table IV shows the amounts of TBIP-insoluble alanine present in the variously treated samples. No significant

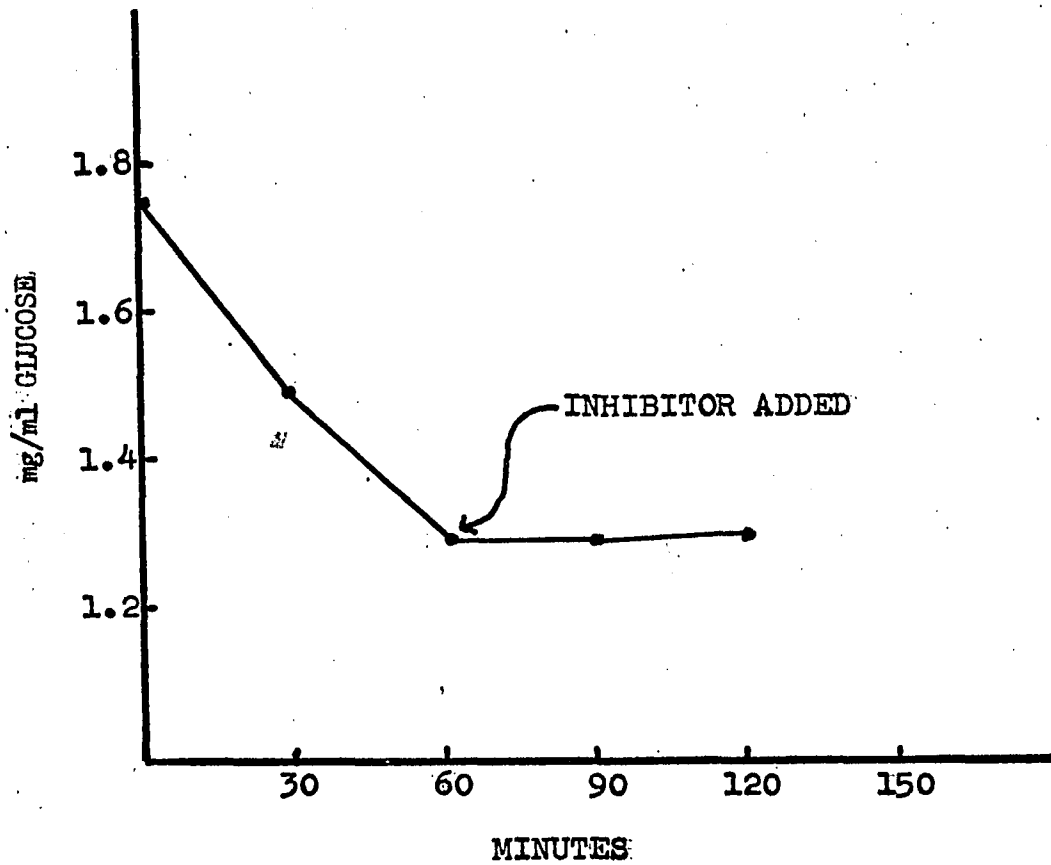


FIGURE 16. Effect of inhibitors on glucose utilization by non-growing cell suspensions.

TABLE III

TRANSPORT OF ALANINE BY CELLS PRE-INCUBATED WITH GLUCOSE

Inhibitor Added	mM Alanine Transported/mg cells	
	Control	Glucose Pre-incubated
None	16.3	36.5
Fluoride	17.0	25.7
Arsenate	12.5	29.2
Iodoacetate	10.4	10.2

TABLE IV
ALANINE INCORPORATION DURING ATP-POOL-DRIVEN TRANSPORT

Inhibitor Added	mM TBIP-Insoluble Alanine	
	Control	Glucose Pre-incubated
None	2.7	3.8
Fluoride	3.1	3.9
Arsenate	2.5	2.9
Iodoacetate	2.2	2.0

differences in the amounts of bound alanine were found. Thus the enhanced transport in glucose preincubated cells cannot be attributed to ATP-powered binding of the amino acid.

The fate of ATP during the transport process is indicated in Table V. It can be seen that during the incubation period in the presence of exogenous alanine, the pool level of ATP fell from 12 $\mu\text{g}/\text{mg}$ cells to an undetectable level. During the same interval, the pool level in control cells fell only 2 $\mu\text{g}/\text{mg}$ cells. It is apparent that the ATP was consumed in driving transport. These data provide some basis for establishing a molar relationship between the amount of alanine transported and the amount of ATP consumed, but since the situation is complicated by a number of conceptual and experimental ambiguities, consideration will be reserved for the Discussion.

Properties of the Membrane ATPase of Streptococcus faecium

The relationship between ATP concentration and the rate and extent of orthophosphate release in the presence of membrane fragments is illustrated in Figure 17. The shapes of these curves are typical of those produced in enzyme catalyzed reactions. The maximum observed rate of ATP hydrolysis, expressed as inorganic phosphate release, was 0.5 μM $\text{P}_i/\text{min}/\text{mg}$ protein. This rate obtains during the first 15 minutes of the reaction in the presence of 0.005 M ATP and during the first 25 minutes in the presence of 0.010 M ATP. When the substrate concentration was 0.001 M, a rate of 0.3 μM $\text{P}_i/\text{min}/\text{mg}$ protein was found during the first 5 minutes of the reaction. In all three cases, the slope of the curve

TABLE V
EFFECT OF EXOGENOUS ALANINE ON THE ENDOGENOUS ATP-POOL LEVEL

Time (min.)	mg ATP/mg cells, dry weight	
	Control	600 mM/ml Alanine
0	11.7	11.7
10	9.1	<0.1

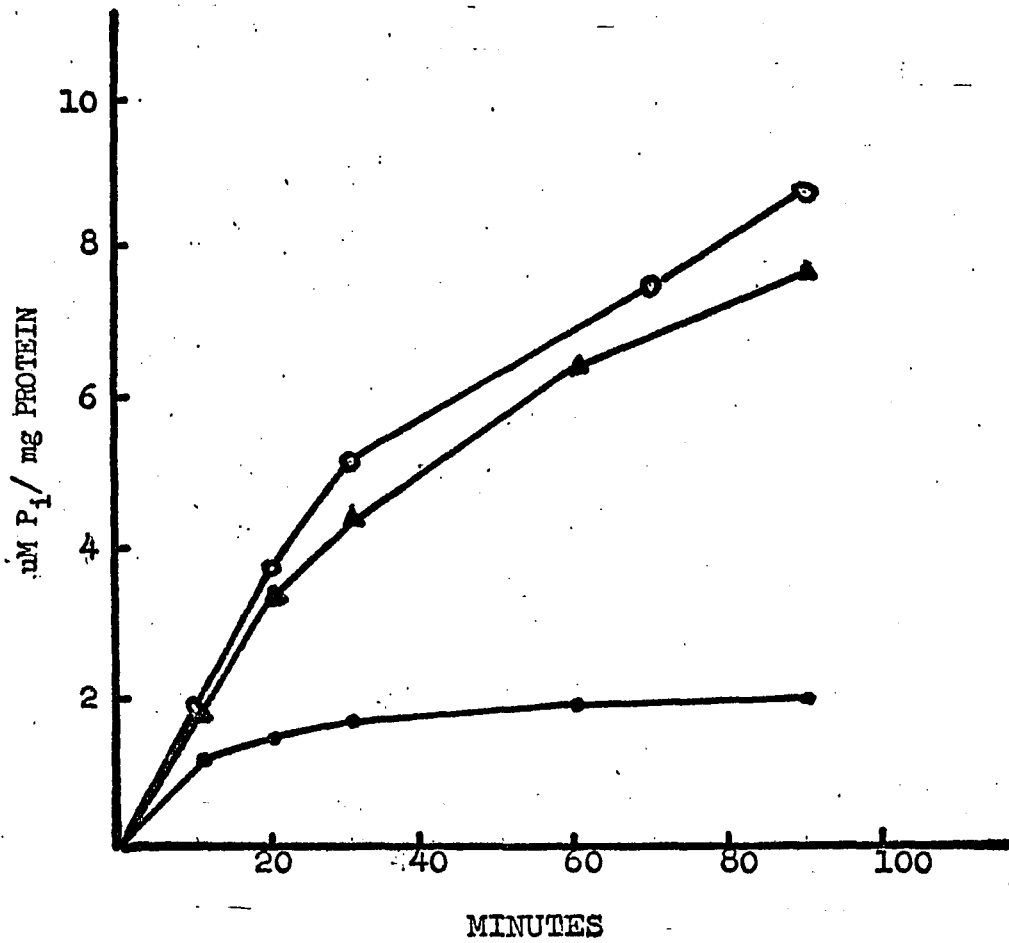


FIGURE 17. Rate of hydrolysis of ATP by membrane fragments. Symbols: ●, 0.001 M ATP; ▲, 0.005 M ATP; ○, 0.01 M ATP.

changed when approximately 15% of the substrate had been hydrolyzed, assuming that only the terminal phosphate group of ATP was released in this reaction.

Electrophoretic analysis of the products of the reaction indicated the presence of only ATP and ADP. This fact, when combined with the observations, presented in Table VII, that neither ADP nor AMP were hydrolyzed by membranes, indicates that the enzyme releases only the terminal phosphate group from ATP.

The ability of various cell fractions to catalyze this reaction can be seen in Table VI. Neither whole cells nor intact protoplasts were found to hydrolyze ATP to any appreciable extent. The small amount of activity found in protoplasts was most probably due to the presence of some membrane 'ghosts' in the suspension. About 90% of the ATPase actually present in the cell was found in the membrane fraction, the remainder being found in the cytoplasm.

The time course of ATP hydrolysis by the cytoplasmic fraction is shown in Figure 18. The rate of the reaction was apparently linear for the first 15 minutes. During this interval, the specific activity was 0.05 $\mu\text{M}/\text{min}/\text{mg}$ protein.

Figure 19 shows the relationship between the molar ratio $\text{Mg}^{++}:\text{ATP}$ and the rate of the membrane ATPase. The maximum rate is achieved when equimolar quantities of the two species are present, although an excess of Mg^{++} does not apparently retard the reaction.

Temperature effects on the rate of the reaction are shown in Figure 20. The maximum observed rate was at 37 C.

TABLE VI
HYDROLYSIS OF ATP BY CELL FRACTIONS OF STREPTOCOCCUS FAECIUM

Cell Fraction	$\mu\text{M P}_i/\text{min.}/\text{mg protein}$
Whole Cells	0
Protoplasts	0
Membrane Fragments	0.5
Cytoplasm	0.05

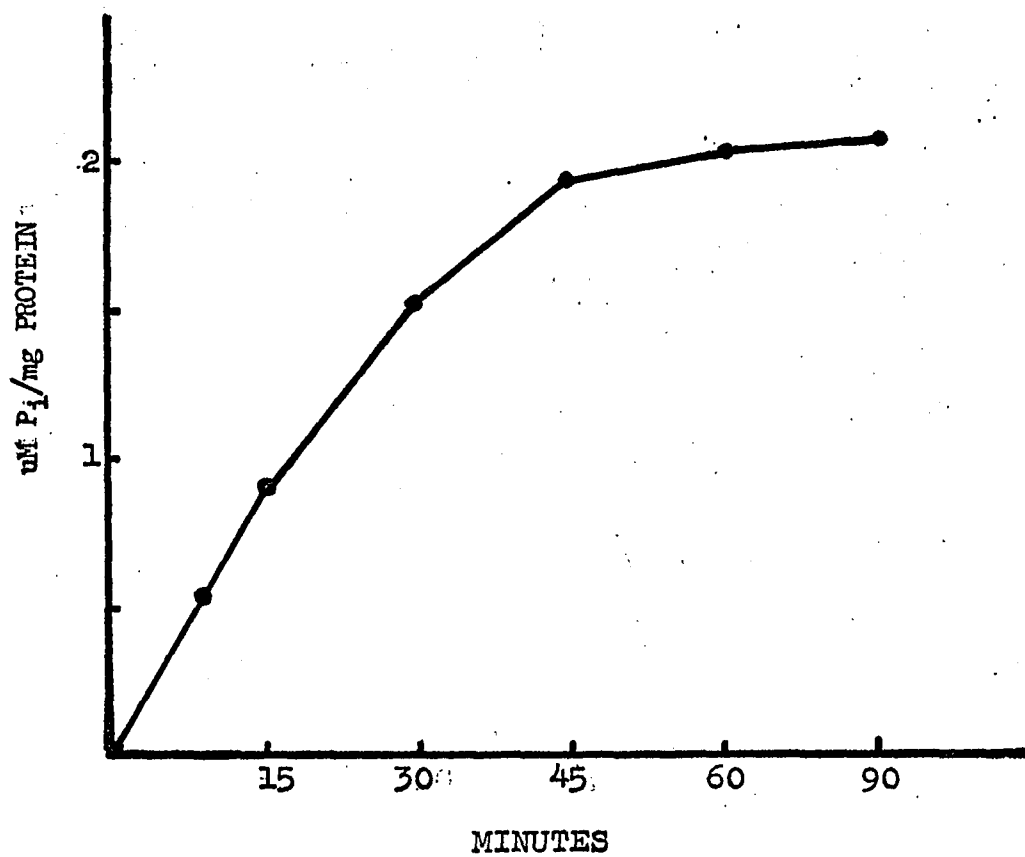


FIGURE 18. Rate of hydrolysis of ATP by cytoplasm.

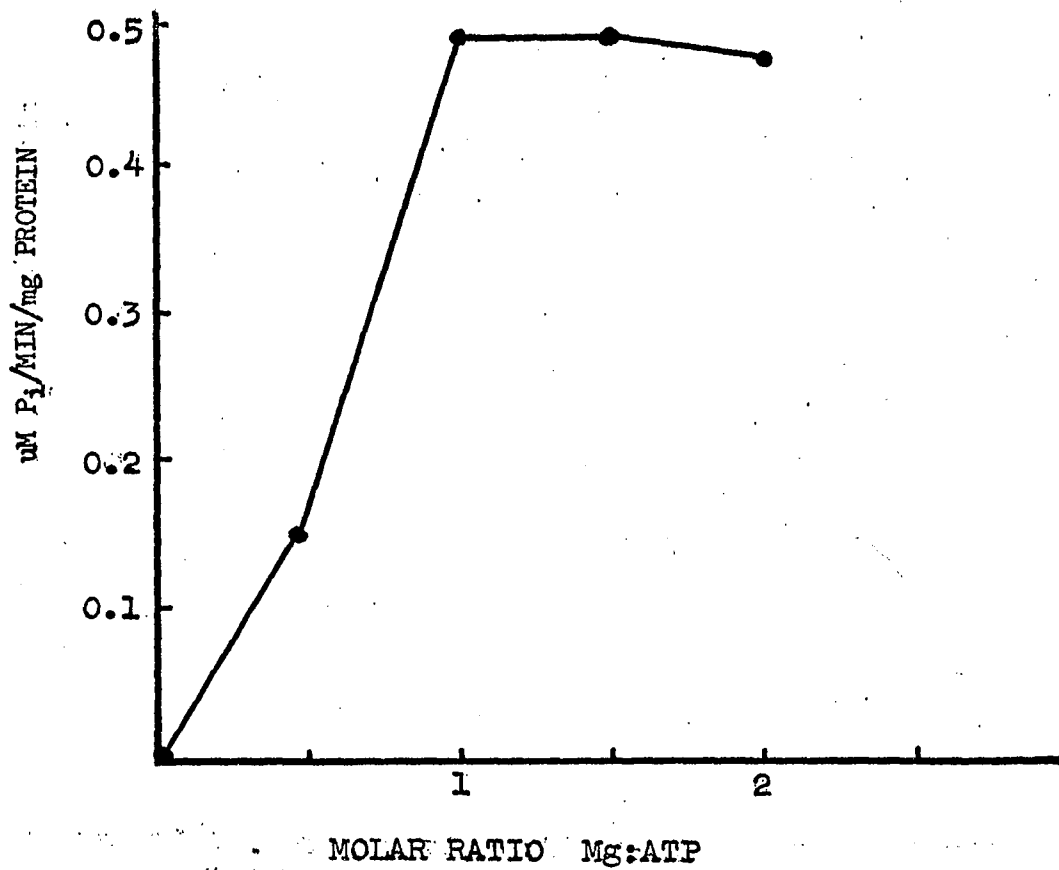


FIGURE 19. The effect of the Mg:ATP ratio on the activity of membrane ATPase.

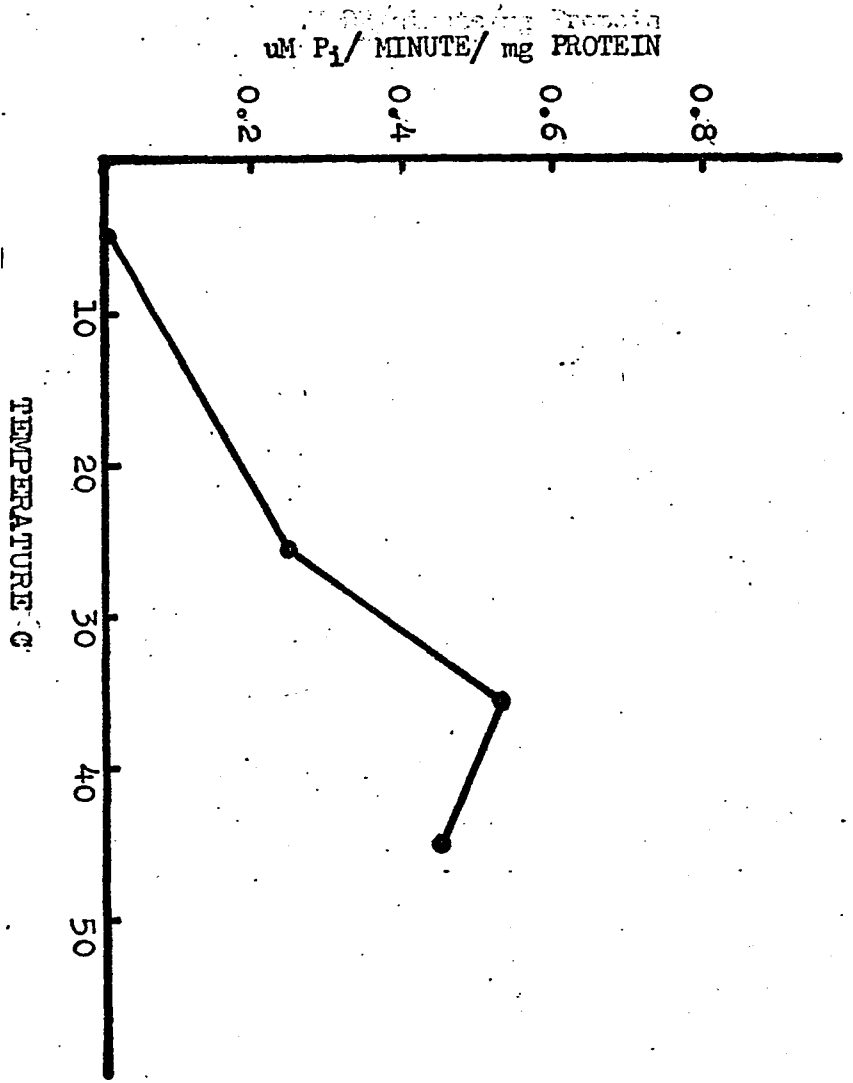


FIGURE 20. Response of membrane ATPase to temperature.

The rate of the reaction was much less profoundly affected by a temperature of 45 C than it was at 25 C, possibly a reflection of the organism's ability to grow at the higher temperature.

Figure 21 shows the effects of pH on the rate of the ATPase. The activity of this enzyme is obviously strongly affected by pH changes. No activity was observed at either pH 5.0 or pH 9.0, although considerable activity was evident in the pH range 6.0-8.0. The maximum rate was found at pH 7.5, but the rate was close to maximum in the range pH 7-8.

The capacity of membranes to hydrolyze substrates other than ATP was investigated. The results, shown in Table VII, indicated a fairly high degree of specificity for the enzyme. Of the substrates tested, only GTP and ITP were attacked to any extent under the assay conditions. ITP, a close structural analog of ATP, was most effective as a substrate, but still represented less than one third of the activity shown against ATP.

Table VIII contains data on the effectiveness of various divalent cations as cofactors of the membrane ATP-ase. Of the ions tested, only three had the ability to act as cofactors: Mg^{++} , Mn^{++} , and Co^{++} . The most effective of the three was Mg^{++} , with Mn^{++} 62% as effective and Co^{++} 58%.

The effects of potential inhibitors and stimulators on the membrane ATPase are shown in Table IX. The most striking effects were produced by ADP and sodium borate, the former inhibiting 75% of the ATPase activity and the latter

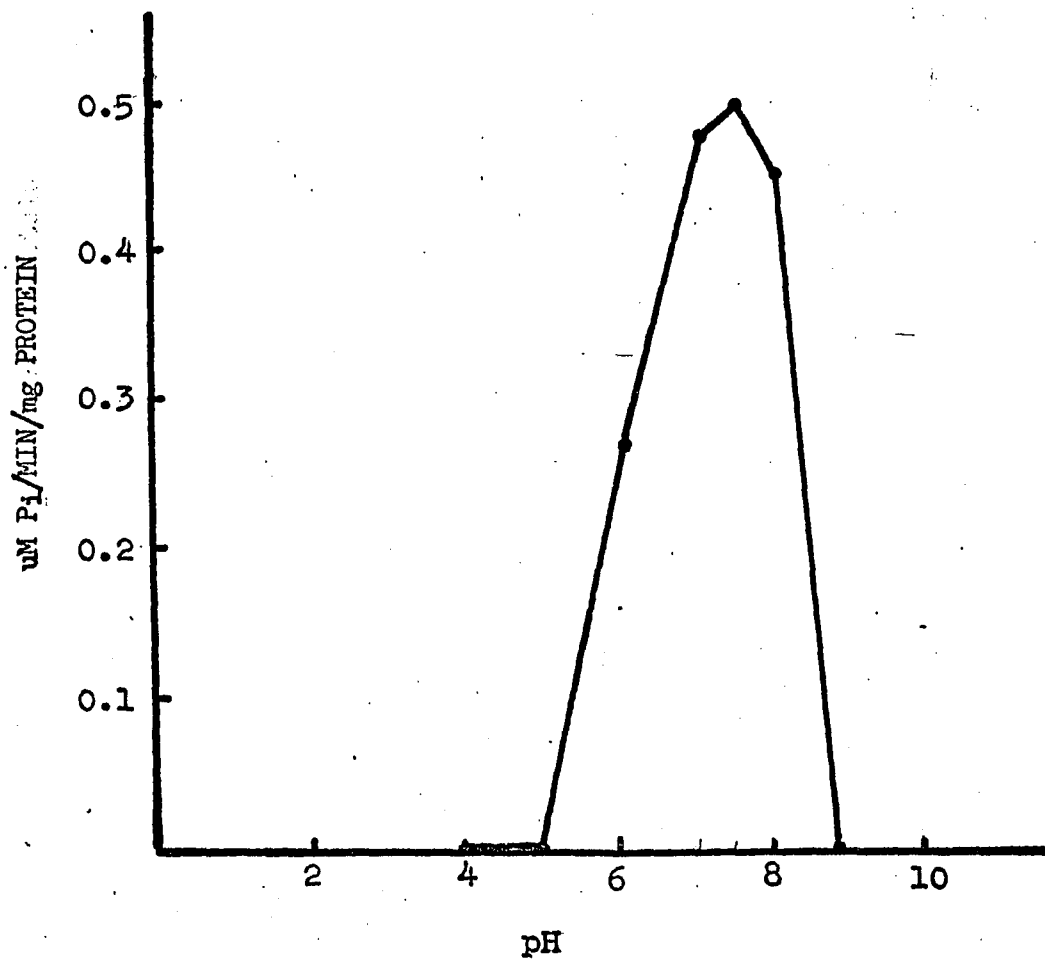


FIGURE 21. Response of membrane ATPase to pH.

TABLE VII
HYDROLYSIS OF SUBSTRATES OTHER THAN ATP BY MEMBRANES

Substrate	Concentration (M/L)	% of ATPase Activity
ADP	0.005	0
AMP	0.005	0
GTP	0.005	5
ITP	0.005	30
P-P*	0.005	0

*pyrophosphate

TABLE VIII
EFFECTIVENESS OF DIVALENT CATIONS AS COFACTORS OF ATPase

Cation	Concentration (M/L)	% Activity
Mg ²⁺	0.005	100
Ca ²⁺	0.005	0
Cu ²⁺	0.005	0
Ba ²⁺	0.005	0
Zn ²⁺	0.005	0
Co ²⁺	0.005	58
Mn ²⁺	0.005	62

TABLE IX
EFFECTS OF VARIOUS AGENTS ON MEMBRANE ATPase ACTIVITY

Addition	Concentration (M/L)	% Activity
None	-	100
IAA*	0.001	78
PCMB**	0.001	85
ADP	0.005	25
NaCl	0.01	90
KCl	0.01	94
NaCl + KCl	0.01 (each)	90
Na ₂ BO ₃	0.001	10

*triiodoacetic acid

**p-chloromercuribenzoate

90%. A considerably smaller degree of inhibition was observed when the system was exposed to the sulfhydryl-reactive agents, triiodoacetic acid and p-chloromercuribenzoate. Treatment with relatively high concentrations of NaCl, KCl or a combination of these salts failed to elicit any stimulation of the reaction, but in fact caused a slight inhibition. This contrasts with the situation found with a number of mammalian ATPases, that are strongly stimulated by the presence of Na^+ and K^+ (79).

Figure 22 shows the effects of trypsin treatment of various cell components on the activities of membrane and cytoplasmic ATPase. It is evident that both membrane and cytoplasmic ATPase activity were diminished by treatment with this enzyme. However, the extent of this diminution was considerably more pronounced in the cytoplasmic ATPase, the activity of which was virtually abolished within 20 minutes of treatment. In contrast, trypsin treated membranes showed a gradual loss of ATPase activity during the first 15 minutes, the process terminating when approximately 50% of the ATPase activity had been destroyed.

Treatment of intact protoplasts with trypsin, followed by lysis and assay of membrane and cytoplasmic ATPase, produced an interesting response. The loss of ATPase activity from the membrane fraction was similar in rate and extent to that found with trypsin treated membranes. The cytoplasmic ATPase activity, however, was unaffected indicating little penetration of trypsin into the protoplast.

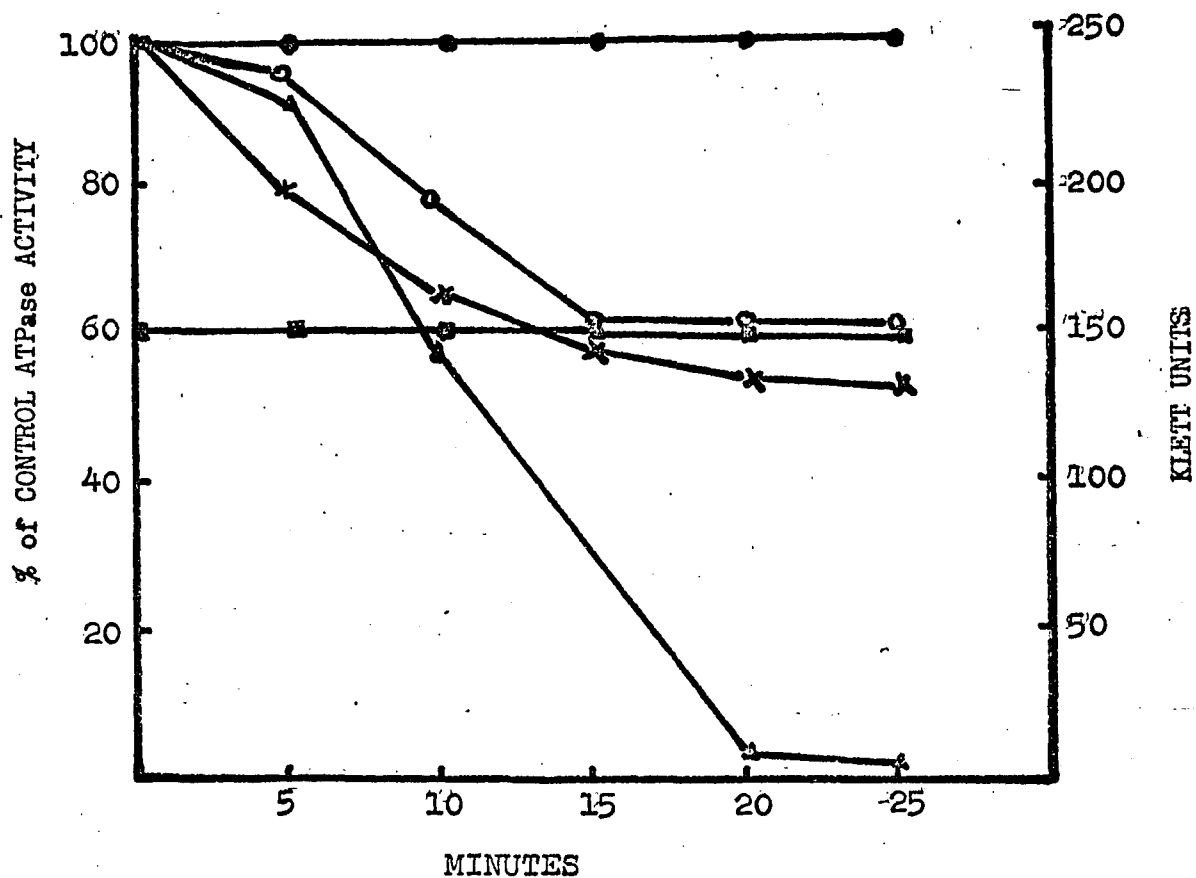


FIGURE 22. Effects of trypsin treatment on the ATPase activity of cell fractions. Symbols: \circ , ATPase level of treated membranes; \times , ATPase level of membranes from treated protoplasts; \blacktriangle , ATPase level of trypsin treated cytoplasm; \bullet , ATPase level of cytoplasm from treated protoplasts; \blacksquare , turbidity of protoplast suspension during treatment.

During the course of trypsin treatment, no change in the turbidity of suspensions of either protoplasts or membranes was detectable. If substantial protoplast lysis had occurred, a measurable drop in turbidity would be expected.

The loss of membrane protein during the course of trypsin treatment of protoplasts and membrane fragments is shown in Figure 23. Almost 50% of the original membrane protein is destroyed by trypsin, in both isolated membranes and membranes derived from treated protoplasts. The similarity of the curves suggest that the majority of trypsin-labile material is present on the outer surface of the protoplast.

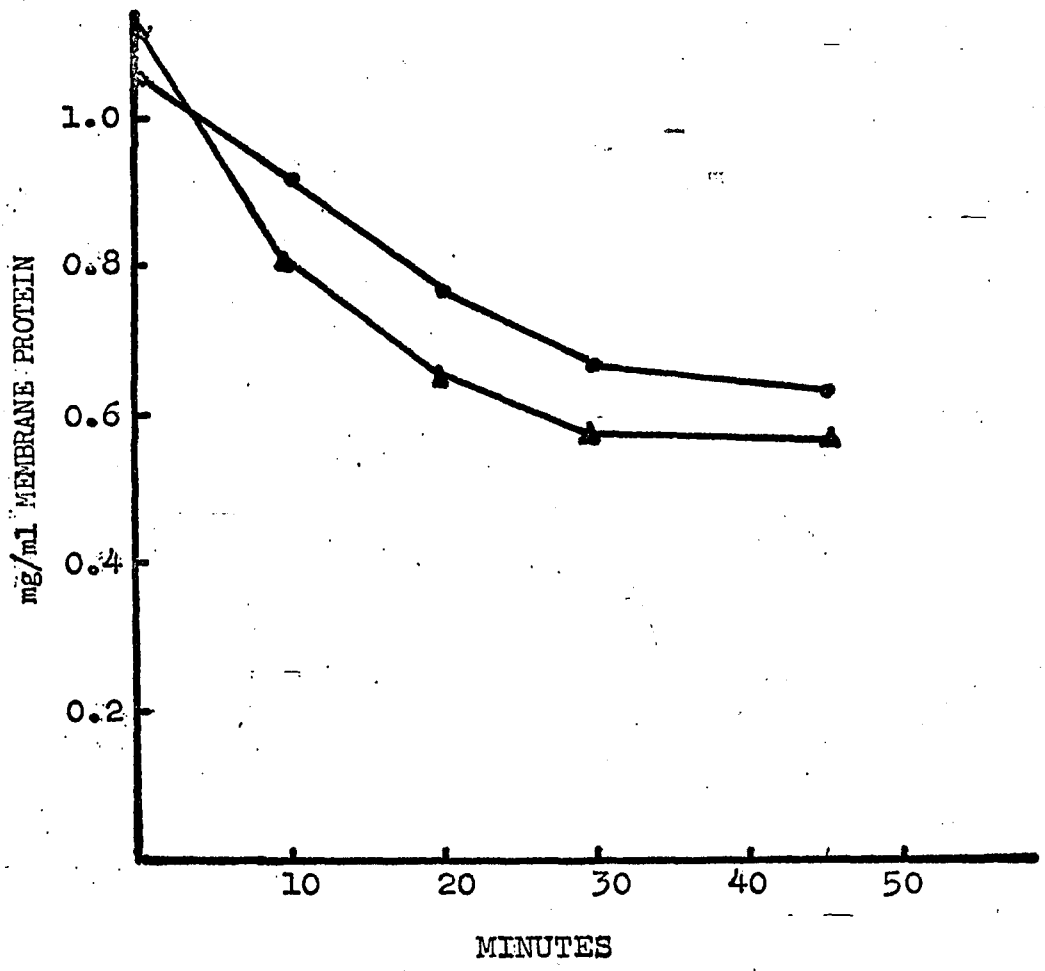


FIGURE 23. Loss of membrane protein during trypsin treatment. Symbols: \blacktriangle , trypsin-treated membranes; \bullet , membranes from protoplasts treated with trypsin.

DISCUSSION

Effects of Serum Fractions on Protoplasts

This work developed from an attempt to apply the anti-serum labelling techniques of Cole (17) to the protoplast membrane. This worker made use of the fact that homologous antiserum, in the absence of complement, has no effect on the viability of bacterial cells. Thus, a bacterial cell coated with FITC-labelled antiserum would continue to grow and from the change in the labelling pattern, the mode of wall growth could be deduced.

On the basis of these facts, it was surprising that not only membrane antiserum, but also normal serum, both in the absence of complement, demonstrated such profound effects. There is almost no literature on this topic. The Treponeme Immobilization Test may be an analogous phenomenon, but too little is known either of its mechanism, or of the surface structure of T. pallidum to make a definite statement. The eucaryon, Ameba proteus, is totally unaffected by homologous antiserum; a fact used to study the dynamics of its movements (80).

The lytic effect of normal serum on protoplasts merits some comment. If this should prove to be a general property of normal sera, it will have some significance as a defense mechanism against bacterial invasion. It may act in conjunction with serum lysozyme, causing rupture of protoplasts induced by this enzyme. Beyond the facts that it is heat-stable

and dialyzable, nothing is known of the nature of this substance. It may be a concerted effect of a number of agents, rather than a single compound. The turbidity changes produced by the factor(s) are similar to those produced by glucose, as reported by Abrams (3). However, the concentration of glucose required for protoplast lysis is quite high (0.01 M/L) and such massive amounts would not be found in a healthy animal. The blood platelet factor 'plakin' may be the responsible agent, since it is known to be active against gram-positive bacteria and seems to exert its effect on the membrane (51).

The inhibition of glucose transport by membrane anti-serum is the first reported example of an agent that is specifically anti-permease. As such, it provides a new class of tools for the study of transport phenomena. With the aid of purified anti-permease antibodies, it should be possible to isolate and characterize the responsible components of the membrane, thus gaining insight into the mechanism of the process. The means by which the antibody exerts this inhibition is unclear. It may be that, by attaching itself to the permease, the antibody molecule prevents the change in orientation of the permease necessary to transfer its substrate to the interior of the cell. The postulation of some change in molecular conformation is a prominent feature of most theories concerned with transport mechanisms (79), although there is no direct evidence for it. A second possible mechanism involves mechanical occlusion of the receptor site of the permease, preventing the access of glucose. A third possibility involves action at an allosteric site on the permease,

preventing derepression of the permease activity. The existence of such allosteric components of transport systems has been suggested for the galactoside permease of E. coli (44). There is no present basis for a choice between these alternatives, but further work may provide one.

The different degrees of inhibition of transport observed with amino acids, suggests the presence of a variety of antipermease antibodies in the membrane antiserum, rather than a single, general antibody. Differing degrees of inhibition can then be explained by differences in the amounts of specific antibodies present. On this basis it can be postulated that definite structural differences exist between permeases of differing specificity; that each permease is a specific antigen. Then, the results obtained by gel-diffusion analysis of the membrane antigens greatly underestimate the actual number of antigens present. With more sensitive methods of analysis, it should be possible to detect these antigens.

Analysis of Membrane Antigens

The mechanisms by which sonication and detergents disrupt bacterial membranes are not clearly understood. It is known that ultracentrifugally homogeneous particles are produced by these agents, although the particle sizes differ (66). Since it is extremely unlikely that covalent bonds are broken under the conditions used, the linkage between these particles in the native membrane may be ionic. Some evidence for this possibility exists (8).

The results clearly show, however, that the solubilized membrane is not antigenically homogeneous. This may indicate that the membrane is composed of subunits of approximately equal size, but of differing composition. Thus, each subunit would be composed of some basal unit and a complement of some enzyme or permease. In view of the indicated mechanism of membrane growth in this organism, insertion of such specialized subunits would be an efficient means of response to environmental change, e.g. the induction of a permease system in response to its exogenous substrate. The application of sensitive separative techniques to solubilized membranes and the analysis of the purified particles should clarify this matter.

The estimates of the number of antigens present in the membrane should be considered as a minimum. Application of more sensitive techniques, such as immunoelectrophoresis, may multiply the number of detectable antigens in these preparations.

Since essentially the same results were obtained by sonication, sodium lauryl sulfate treatment and Triton-X treatment, it may be concluded that all three agents act at the same point on the membrane. In view of this, sonication must be considered the most desirable method for preparing soluble membrane antigens, since, as a purely mechanical process, it does not introduce alien materials into the preparation.

As was mentioned in the presentation of the results, the major line of precipitation is most likely glycerol teichoic acid. This antigen, characteristic of the Group D

streptococci, has been found to be associated with the membrane (69).

Growth of the Protoplast Membrane

The maintenance of viability of protoplasts labelled with FITC is surprising, since the process involves the formation of covalent bonds between FITC and membrane components. The most likely site of reaction is an amine group, such as the free ϵ -amine group of lysine. Analysis of labelled proteins has indicated this preference (12). The fact that such reaction does not cause gross damage indicates that the reacted groups are not essential to any membrane activity. Recent work indicates that, under the proper conditions, cells of Proteus mirabilis and S. aureus may be stained with FITC and still maintain viability (35).

The explanation for the loss of viability when FITC penetrates into the cytoplasm is, as yet, unknown. It is obvious that some vital function, probably involving amine groups, is inhibited. For the purposes of this investigation, this inhibition is fortuitous, since it provides a built-in control, limiting vital staining to the membrane.

Since the labelling process is statistical, a certain number of protoplasts will remain unlabelled, or only lightly labelled, while another group will be heavily labelled. This presents the possibility that only unlabelled protoplasts are growing and that all labelled protoplasts are inhibited. However, the congruence of the growth curve of labelled protoplasts with that of unlabelled protoplasts indicates that almost equal numbers of protoplasts are growing in both cases.

In addition, if this were the case, it would be expected that photographs of samples of FITC-labelled protoplasts would contain large numbers of fluorescent 'ghosts', the number remaining constant throughout the growth process. Examination of the photographs indicates that this is not the case.

It is also necessary to consider the possible contribution of membrane 'stretching' to the observed diminution of fluorescence. This would occur if the rate of increase of cytoplasmic volume were greater than the rate of growth of the membrane. Since it is known that the membrane has some elasticity, the result would be a thinning of the membrane over the entire surface of the protoplast and diminution of the intensity of fluorescence. Calculating the increase in surface area of the protoplasts during the growth process, using the apparent diameters of the photomicrographs, a figure of 2.3 is arrived at. In order to avoid stretching of the membrane, the amount of membrane protein should show a similar increase. Inspection of Figure 12 shows that, during that interval, the amount of membrane protein increased by a factor of 2.1. Thus, membrane stretching does not make any great contribution to the observed results.

It has been observed that the cell wall of Group A streptococci reproduces along an equatorial band (14). If the situation is the same for Group D streptococci, it is necessary to reconcile the difference between wall and membrane biosynthesis. Actually, no great difficulty exists to

prevent such a reconciliation. The membrane-bound enzymes concerned with wall synthesis may occupy a thin, equatorial line. As the size of the cell increases, additional membrane subunits, containing the enzymes of wall synthesis, may be inserted all along the line. At the same time, insertion of other membrane components can proceed in other parts of the cell.

In addition, growth by intercallation makes it easier to explain the formation of mesosomes and other intracellular membranes. It has been shown (63) that mesosomes are not always simple intrusions of membrane, caused by excess membrane growth in a volume limited by the cell wall, but are finger-like projections that do not merge with the cell membrane during protoplast formation, but separate from the membrane to form internal vesicles, or evaginate, forming projections on the protoplast surface. The formation of these structures cannot be explained if the membrane reproduces equatorially, but can be if the membrane reproduces by intercallation.

It was recently suggested that newly formed chromatin may be separated into two masses through the mediation of the membrane (28,32). In the proposed model, the two strands of chromatin are attached to separate mesosomes, which, as the cell expands, pull the strands apart. In comparing the efficiency of equatorial growth and intercallated growth in separating the chromatin, it can be seen that if the two mesosomes are immediately adjacent to the equator of the cell,

then equatorial growth is more effective, since the amount of growth between the mesosomes is greater in this case. However, as the distance between the mesosomes increases, the superiority of equatorial growth diminishes until, when the mesosomes are directly opposite, the efficiencies of the two mechanisms are equal, because the determining factor in this case is the rate of increase in cell diameter, which is the same in both cases.

The obvious change in refractility of the protoplasts during the first hour of growth merits some comment. The most obvious explanation is that, as growth proceeds, the density of the protoplast, and hence its refractive index, decreases. This would be the case if the synthesis of cytoplasmic material did not keep pace with the increase in protoplast volume. It is also during this period that the turbidity and the membrane protein decrease. The fate of cytoplasmic protein and nucleic acid during this interval is not known. The three phenomena are probably related, the loss of membrane density accounting for the change in refractive index reflected in the other two.

The initial loss of membrane protein, apparently a general characteristic of protoplast growth, produces, in FITC-labelled protoplasts, some diminution of fluorescence. This diminution, like the subsequent growth, proceeds by intercallation.

ATP Pool-driven Transport of Amino Acids

The significance of this work is primarily methodological. It represents the first instance of active transport

occurring in an otherwise non-metabolizing cell. Streptococcus faecium was a particularly good organism to use for this purpose, because it can produce ATP from glucose only by means of the E-M-P pathway and has a very low level of endogenous metabolism (19). This means that once glycolysis has been inhibited, no further ATP can be produced and that the ATP thus produced is not rapidly consumed by endogenous metabolism.

The choice of the amino acid, alanine, was made because it is transported in amounts large enough to be easily detectable. On the other hand, the choice was unfortunate, because alanine also is transported passively. In order to establish a stoichiometric relationship between the amount of alanine transported and the amount of ATP consumed in the process, it is necessary to assume either that passive transport continues in the presence of active transport, or that it does not continue. There is no definite evidence to support either assumption; the observation that passive transport continues undiminished when active transport has been inhibited by iodoacetic acid only demonstrates that the two modes of transport do not share one component of a permease system — they may share, and compete for, other components. This question will remain undecided until it becomes possible to specifically inhibit the passive transport of this amino acid.

On the assumption that passive transport does not continue, a relationship between ATP consumption and alanine

transport can be established. Data from Figures 17 and 19 show that 10 $\mu\text{g}/\text{mg}$ cells of ATP were consumed, in excess of endogenous utilization, and 25 $\text{m}\mu\text{M}/\text{mg}$ cells of alanine was transported. On a molar basis, 20 $\text{m}\mu\text{M}$ caused the transport of 25 $\text{m}\mu\text{M}$ of alanine, per mg of cells. The ratio, alanine transported: ATP consumed, is 1.25:1. This is virtually an equimolar relationship. The same ratio has been established for the transport of thiogalactosides in E. coli (44).

With the available data, it is not possible to make a meaningful calculation, if it is assumed that passive transport continues. Under these circumstances it would be necessary to know precisely when the ATP pool was exhausted, and how much alanine had been transported, passively and actively, up to that time. The data only indicate that ATP was exhausted at some point in a ten minute interval. Since passive transport would continue after active transport had ceased, a false, low value for active transport would result from the subtraction of the passive transport from the figure obtained with preincubated cells.

From the effect of triiodoacetic acid on the transport system, it may be tentatively deduced that the active transport process involves sulfhydryl groups, while passive transport, at least that of alanine, does not. A similar dependency has been found in E. coli galactoside transport (30). This result lends some support to the postulation of a contractile carrier molecule, although the entire hypothesis is based on an admittedly wispy analogy with the sulfhydryl-dependence of actomyosin contraction.

Although these results are of a preliminary nature, it is evident that the technique can be of great use in the study of transport. In combination with other approaches, such as antiserum inhibition, substantial progress can be made.

Properties of the Membrane ATPase of Streptococcus faecium

The enzyme found in the membrane of Streptococcus faecium appears to be a specific ATPase. The only other nucleoside triphosphate attacked to a significant extent by membrane fragments was inosine triphosphate, a close structural analog of ATP, differing only in the substitution of a hydroxyl group for an amine group on the purine ring. Even this slight modification, however, is sufficient to decrease the rate of hydrolysis about 70%, when compared to ATP. This suggests the possibility that the l-amino group may be involved in the catalytic process. It has been suggested (79) that the interaction of ATP with divalent cations results in the formation of a chelate, involving the terminal phosphate residue and the l-amino of the purine ring. The fact that, in this system, the optimal Mg:ATP ratio is 1 also lends support to the possibility of chelate formation of the type described.

The requirement for Mg^{++} is also fairly specific in this system, as Table VIII shows. That Mn^{++} and Co^{++} can substitute for Mg^{++} to a substantial degree in the reaction is unusual, especially in view of the fact that the more closely related Ca^{++} and Ba^{++} ions are inactive. The ability of Mn^{++} to substitute for Mg^{++} in reactions of this type has been reported (4), but a similar capacity for Co^{++} has not. However,

since Co^{++} is relatively rare as a required cofactor, few investigators have studied its ability to substitute for other ions.

The response of the enzyme at various temperatures is not unusual. The optimum temperature for ATPase activity, 37 C, is the same as the optimum growth temperature of the organism. That the enzyme maintains considerable activity at 45 C is consistent with the ability of the organism to grow at this temperature (19).

The membrant ATPase has a strong response to changes in pH. Especially interesting is the change from a high level of activity at pH 8.0 to virtual abolition of activity at pH 9.0. Since the organism itself is quite resistant to high pH (19), it is likely that the pH sensitive portion of the enzyme is not exposed to the external environment. The fact that intact cells and protoplasts do not hydrolyze ATP, as shown in Table VI, supports this view.

Data presented in Table IX indicate the response of the membrane ATPase to potential inhibitors and stimulators of enzyme activity. There is a limited inhibition produced by the sulfhydryl reactive agents p-chloromercuribenzoate and triiodoacetate. However, the extent of inhibition, considering the fairly high concentrations used, does not indicate a pronounced sulfhydryl dependence in the enzyme. On the other hand, it is possible that sensitive groups are relatively inaccessible to the inhibitors, since the enzyme is embedded in the membrane structure. The results of Abrams (7)

concerning the effects of sulfhydryl inhibitors on a bacterial membrane ATPase that had been released from the membrane structure, indicate that the enzyme remains insensitive to these agents.

The strong inhibition produced by ADP is not unusual. Its close structural analogy to ATP, combined with the fact that it is not hydrolyzed by the membranes, strongly indicates competitive inhibition. Indeed, this has been established by the kinetic data of Abrams (7). In view of this activity, it is probable that in the intact cell the activity of this enzyme is regulated by retroinhibition by ADP. It is also probable that, in the isolated membranes, the change in rate is due not only to the decrease in ATP concentration, but also to the accumulation of its product, ADP.

Since no stimulation of the enzyme was observed in the presence of Na^+ , K^+ or both these ions, the enzyme does not appear to be of the class of membrane ATPases found in mammalian systems. Thus, a simple analogy between the functions of Na^+ , K^+ -stimulated ATPase, which has been implicated in ion transport, and the ATPase of Streptococcus faecium is precluded. It remains possible that this enzyme is implicated in active transport processes, but the nature of this involvement cannot be indicated by a simple extrapolation from the systems found in higher organisms.

The profound inhibitory effect of borate on the system provides additional insight into the nature of ATPase catalysis. The most likely explanation of this effect lies in the ability

of the borate ion to complex with cis vicinal hydroxyl groups, an activity long used in the study of carbohydrate structure. The 2',3' hydroxyl groups of the ribose moiety of ATP have the appropriate configuration needed for complex formation with borate. The inhibition of ATP hydrolysis in the presence of small amounts of borate ion indicates that one or both of these hydroxyl groups may be involved in the enzymic process.

Some indication of the position of the membrane ATPase in the structure of the membrane has been gained by the study of the effects of trypsin treatment of cell fractions on the activity of the membrane and cytoplasmic ATPases. The fact that when protoplasts were treated with trypsin, a significant loss of membrane ATPase activity was noted, indicates that some portion of the enzyme is accessible at the outer surface of the membrane. That significant amounts of trypsin did not penetrate the protoplast membrane is indicated by the fact that during the course of the treatment the levels of cytoplasmic ATPase remained constant, although this enzyme is quite sensitive to degradation by trypsin. It is possible that trypsin penetrated only as far as the inner surface of the membrane, leaving cytoplasmic components unaffected, but this could not be tested. When the rate and extent of loss of ATPase activity from membranes and protoplasts is compared, in Figure 20, the loss of activity is more rapid in the case of protoplasts. If penetration of the protoplast were necessary prior to degradation of ATPase, the opposite would be the case.

The maintenance of the initial turbidity during the entire course of trypsin treatment, is inconsistent with the postulation of substantial lysis of protoplasts, leading to penetration of trypsin into the cytoplasm.

It is evident, from Figure 21, that there is a substantial loss of protein during the course of trypsin treatment from both membrane fragments and the membrane of the treated protoplast. The amount of material lost from the membranes is somewhat greater than from the protoplast. This is to be expected, since more protein would be exposed to proteolysis in the isolated membrane fragments than in the unlysed protoplast. It is surprising that the protoplast membrane could be degraded to this extent without substantial lysis resulting. A possible explanation is that the majority of the majority of the material lost could be lysozyme, adhering to the membrane. Alternatively, the trypsin-labile proteins of the membrane may not be intimately involved in the maintenance of structural integrity. Neither possibility is obviated by the data.

The picture of the membrane ATPase that emerges from this investigation is as follows. It is a membrane-bound enzyme showing a strong specificity for Mg:ATP. Three likely points of enzyme-substrate interaction are: The γ -phosphate group; the 1-amino residue of the purine ring; and the 2', or 3' hydroxyl of ribose. The enzyme is not strongly sulfhydryl dependent, is subject to retroinhibition by ADP, and has a component, other than the active site, susceptible to trypsin

degradation and exposed to the outer surface of the proto-plast membrane.

At present, no function can be ascribed to this enzyme. It will be necessary to develop a fairly specific method for inhibiting the enzyme, in vivo, before a definitive study of its function can be undertaken.

LITERATURE CITED

1. Abram, D. 1964. Electron microscope observations on the fine structure of bacterial cytoplasmic membranes. *J. Cell Biol.* 23:3A.
2. Abram, D. 1965. Electron microscope observations on intact cells, protoplasts and the cytoplasmic membrane of Bacillus stearothermophilus. *J. Bacteriol.* 89: 855-873.
3. Abrams, A. 1959. Reversible metabolic swelling of bacterial protoplasts. *J. Biol. Chem.* 234:355:388.
4. Abrams, A., P. McNamara, and F. B. Johnson. 1960. Adenosine triphosphatase in bacterial cell membranes. *J. Biol. Chem.* 235:3659-3662.
5. Abrams, A., and P. McNamara. 1962. Polynucleotide phosphorylase in isolated bacterial cell membranes. *J. Biol. Chem.* 237:170-175.
6. Abrams, A., L. Nielsen, and J. Thaemert. 1964. Rapidly synthesized ribonucleic acid in membrane ghosts from Streptococcus faecalis protoplasts. *Biochim. Biophys. Acta* 80:325-337.
7. Abrams, A. 1965. The release of bound adenosinetriphosphatase from isolated bacterial membranes and the properties of the solubilized enzyme. *J. Biol. Chem.* 240:3675-3681.
8. Brown, J. W. 1965. Evidence for a magnesium-dependent dissociation of bacterial cytoplasmic membrane particles. *Biochim. Biophys. Acta* 94:97-101.
9. Brenner, S., F. A. Dark, P. Gerhardt, M. H. Jaynes, O. Kandler, E. Kellenberger-Nobel, K. McQuillen, M. Rubio-Huentos, M. R. J. Salton, R. E. Strange, J. Toncsik, and C. Weibull. 1958. Bacterial Protoplasts. *Nature* 181:1713-1715.
10. Burnham, J. C., and G. J. Hogeage, Jr. 1967. Adenosine phosphate hydrolases in cell fractions of *Vitreoscilla*. *J. Bacteriol.* 93:191-198.
11. Chapman, G. B., and J. Hillier. 1953. Electron microscopy of ultra thin sections of bacteria. I. Cellular division in Bacillus cereus. *J. Bacteriol.* 66:362-373.

12. Cherry, W. B., M. Goldman, and T. R. Carski. 1960. Fluorescent antibody techniques in the diagnosis of communicable diseases. U. S. Public Health Service Publication 729.
13. Chesbro, W. R. 1961. Lysozyme and the production of osmotic fragility in enterococci. *Can. J. Microbiol.* 7:952-955.
14. Chesbro, W. R., and J. B. Evans. 1962. Uptake and transamination of amino acids by Streptococcus faecium. *Biochim. Biophys. Acta* 58:538-549.
15. Cohen-Bazire, G. 1963. Some observations on the organization of the photosynthetic apparatus in purple and green bacteria. pp. 89-110. In Gest, H. and L. P. Vernon (eds.) *Bacterial Photosynthesis*. The Antioch Press, 1963.
16. Cole, H. A., and D. E. Hughes. 1965. The enzymatic activity of the outer shell of Lactobacillus arabinosus. *J. Gen. Microbiol.* 40:81-90.
17. Cole, R. M., and J. J. Hahn. 1962. Cell wall replication in Streptococcus pyogenes. *Science* 135:722-725.
18. Cronenwett, C. S., and R. P. Wagner. 1965. Over-all synthesis of isoleucine by membrane fractions of Salmonella typhimurium. *Proc. Natl. Acad. Sci. U. S.* 54:1643-1650.
19. Deibel, R. H. 1964. The group D streptococci. *Bacteriol. Revs.* 28:330-366.
20. DeMoss, R. D., and R. C. Bard. 1957. Physiological and biochemical techniques. pp. 169-198. In Conn, H. J. (ed.) *Manual of Microbiological Methods*. McGraw Hill, 1957.
21. Drapeau, G. R., and R. A. MacLead. 1963. Nutrition and metabolism of marine bacteria. XII. Ion activation of adenosine triphosphatase in membranes of marine bacterial cells. *J. Bacteriol.* 85:1413-1419.
22. Fernandez-Moran, H., T. Oda, P. V. Blair, and D. E. Green. 1964. A macromolecular repeating unit of mitochondrial structure and function. *J. Cell Biol.* 22:63-100.
23. Few, A. V., A. R. Gilbey, and G. U. F. Seaman. 1960. An electrophoretic study on structural components of Micrococcus lysodeikticus. *Biochim. Biophys. Acta* 38:130-136.

24. Finean, J. B. 1961. Chemical ultrastructure in living tissues. *J. Cell Biol.* 19:271-279.
25. Fischer, A. 1903. *Vorlesungen uber bacterien.* Jena. 1903. Cited by Mitchell, P. 1959.
26. Fitz-James, P. C. 1958. Cytological and chemical studies of the growth of protoplasts of B. megaterium. *J. Biophys. Biochem. Cytol.* 4:257-266.
27. Fitz-James, P. C. 1960. Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. *J. Biophys. Biochem. Cytol.* 8:507-528.
28. Fitz-James, P. C. 1965. Discussion. *Bacteriol. Revs.* 29:293-298.
29. Forrest, W. W. 1965. Adenosine triphosphate pool during the growth cycle in Streptococcus faecalis. *J. Bacteriol.* 90:1013-1018.
30. Fox, F. F., and E. P. Kennedy. 1965. Specific labelling and partial purification of the M protein, a component of the β -galactoside transport system of Escherichia coli. *Proc. Natl. Acad. Sci. U. S.* 54:891-899.
31. Freimer, E. H. 1963. Studies of L forms and protoplasts of group A streptococci. II. Chemical and immunological properties of the cell membrane. *J. Exptl. Med.* 117:377-399.
32. Ganesan, A. T. and J. Lederberg. 1965. A cell membrane bound fraction of bacterial DNA. *Biochem. Biophys. Res. Commun.* 18:824-835.
33. Gilby, A. R., A. V. Few, and K. McQuillen. 1958. The chemical composition of the protoplast membrane of Micrococcus lysodeikticus. *Biochim. Biophys. Acta* 29:21-29.
34. Green, D. E., E. Murer, H. C. Hultin, S. H. Richardson, B. Salmon, G. P. Brierly, and H. Baum. 1965. Association of integrated metabolic pathways with membranes. I. Glycolytic enzymes of the red blood corpuscle and yeast. *Biochim. Biophys. Acta* 112:635:647.
35. Grunberg, E., and R. Cleeland. 1966. Fluorescence and viability of Proteus mirabilis stained directly with fluorescein isothiocyanate. *J. Bacteriol.* 92:23-27.
36. Hayashi, M., and R. Vchida. 1965. A cation-activated adenosine triphosphatase in cell membranes of halophilic Vibrio parahaemolyticus. *Biochim. Biophys. Acta.* 110:207-209.

37. Hazen, P. O., H. Goldfine, and P. J. Williams. 1966. Phospholipids of bacteria with extensive intracytoplasmic membranes. *Science* 151:1543-1544.
38. Hill, P. B. 1962. Incorporation of orthophosphate into phospholipid by isolated membrane fractions of bacteria. *Biochim. Biophys. Acta* 57:386-389.
39. Hughes, D. E. 1962. The bacterial cytoplasmic membrane. *J. Gen. Microbiol.* 29:39-46.
40. Ibbot, F. A., and A. Abrams. 1964. The phospholipids in membrane ghosts from Streptococcus faecalis protoplasts. *Biochem.* 3:2008-2012.
41. Isquith, A. J., and W. R. Chesbro. 1963. Pools, confluxes and transport of amino acids in Streptococcus faecium. *Biochim. Biophys. Acta.* 74:642-658.
42. Kekwick, R. A. 1940. The serum proteins of multiple myelomatosis. *Biochem. J.* 34:1248-1257.
43. Kellenberger, E., and A. Ryter. 1958. The cell wall and cytoplasmic membrane of Escherichia coli. *J. Biophys. Biochem. Cytol.* 4:323-325.
44. Kepes, A. 1963. Permeases: identification and mechanism. pp. 38-48. In N. E. Gibbons (ed.) *Recent progress in Microbiology. Symposia held at the VIII International Congress for Microbiology, Montreal, 1962.* University of Toronto Press, 1963.
45. Kepes, A. The place of permease in cellular organization. pp. 155-169. In J. F. Hofman (ed.) *The Cellular Functions of Membrane Transport.* Prentice Hall, 1964.
46. Keston, A. S. 1956. Specific colorimetric enzymatic reagents for glucose. *Abst. of Papers, 129th Meeting A.C.S.* 31 C.
47. Kodicek, E. 1963. Aspects of the constitution of bacterial membranes. pp. 23-37. In N. E. Gibbons (ed.) *Recent Progress in Microbiology. Symposia held at the VIII International Congress for Microbiology, Montreal, 1962.* University of Toronto Press, 1963.
48. Markovitz, A., and A. J. Dorfman. 1962. Synthesis of capsular polysaccharide (hyaluronic acid) by protoplast membranes of group A streptococci. *J. Biol. Chem.* 237:273-279.

49. Marr, A. G. 1960. Localization of enzymes in bacteria. Vol. I, pp. 443-468. In I. C. Gunsalus, and R. Y. Stanier (eds.) The Bacteria: A Treatise on Structure and Function. Academic Press, 1960.
50. Marsh, C., and W. Militzer. 1956. Thermal enzymes. VII. Further data on an adenosine triphosphatase. Arch. Biochem. Biophys. 60:433-438.
51. McQuillen, K. 1960. Bacterial protoplasts. Vol. 1, pp. 249-359. In I. C. Gunsalus, and R. Y. Stanier (eds.). The Bacteria: A Treatise on Structure and Function. Academic Press, 1960.
52. Mitchell, P. 1959. The biochemical cytology of microorganisms. Ann. Rev. Microbiol. 13:407-440.
53. Moore, L. D., and D. J. O'Kane. 1964. A membrane associated enzyme particle of Streptococcus faecalis. Bacteriol. Proc. p. 96.
54. Moore, L. D., and W. W. Umbreit. 1965. Membrane-associated protein synthesis in Streptococcus faecalis. Biochem. Biophys. Acta 103:460-477.
55. Murray, R. G. E., and S. W. Watson. 1965. Structure of Nitrocystis oceanus and comparison with Nitrosomonas and Nitrobacter. J. Bacteriol. 89:1594-1609.
56. Nielson, L., and A. Abrams. 1964. Rapidly labelled ribosomal RNA associated with membrane ghosts of Streptococcus faecalis. Biochem. Biophys. Res. Commun. 17:680-684.
57. Ouchterlong, O. 1962. Diffusion-in-gel methods for immunological analysis, II. Progr. Allergy 6:30-154.
58. Porter, R. R. 1959. The hydrolysis of rabbit V-globulin and antibodies with crystalline papain. Biochem. J. 73:119-127.
59. Potter, V. R. 1947. The assay of animal tissues for respiratory enzymes. J. Biol. Chem. 169:17-37.
60. Pardee, A. B., and L. S. Prestidge. 1966. Cell free activity of a sulfate binding site involved in active transport. Proc. Natl. Acad. Sci. U.S. 55:189-191.
61. Robertson, J. D. 1959. The ultrastructure of cell membranes and their derivatives. Biochem. Symp. 16: 3-43.
62. Ryter, A., and F. Jacob. 1963. Etude au microscope electronique des relations entre mesosomes et noyaux chez Bacillus subtilis. Compt. Rend. Aca. Sci. Paris 257:3060-3063.

63. Salton, M. R. J., and J. A. Chapman. 1962. Isolation of membrane-mesosome structures from Micrococcus lysodeikticus. J. Ultrastructure Res. 6:489-498.
64. Salton, M. R. J. 1964. The Bacterial Cell Wall. American Elsevier, 1964.
65. Salton, M. R. J., and J. H. Freer. 1965. Composition of the membranes isolated from several gram-positive bacteria. Biochim. Biophys. Acta 107:531-538.
66. Salton, M. R. J., and A. Netschey. 1965. Physical chemistry of isolated bacterial membranes. Biochim. Biophys. Acta 107:538-545.
67. Shockman, G. D., and J. O. Lampen. 1962. Inhibition by antibiotics of the growth of bacterial and yeast protoplasts. J. Bacteriol. 84:508-512.
68. Shockman, G. D. 1963. Protoplast membrane of Streptococcus faecalis. J. Bacteriol. 85:168-175.
69. Shockman, G. D., and H. D. Slade. 1964. The cellular location of the streptococcal group D antigen. J. Gen. Microbiol. 37:297-305.
70. Strehler, R. L., and J. R. Totter. 1952. Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination. Arch. Biochem. Biophys. 40:28-41.
71. Teller, J. D. 1956. Direct quantitative colorimetric determination of serum or plasma glucose. Abstr. Papers 130th Ann. Meeting A.C.S., p. 69C.
72. Van Iterson, W. 1962. Membranous structures in microorganisms. pp. 14-22. In N. E. Gibbons (ed.) Recent Progress in Microbiology. Symposia held at the VIII International Congress for Microbiology, Montreal, 1962. University of Toronto Press, 1963.
73. Vorbeck, M. L., and G. V. Marinette. 1965. Intracellular distribution and characterization of the lipids of Streptococcus faecalis (ATCC 9790). Biochem. 4:296-304.
74. Weibull, C. 1956. Bacterial protoplasts: their formation and characteristics. pp. 111-126. In Bacterial Anatomy, Sixth Symposium of the Society for General Microbiology. Cambridge University Press, 1956.

75. Weibull, C., B. Zacharias, and H. Beckman. 1959. Affinity of lysozyme to structural elements of the bacterial cell as studied with labelled enzyme. *Nature* 184:1744-1745.
76. Weibull, C., H. Beckman, and L. Bergstrom. 1959. Localization of enzymes in Bacillus megaterium strain M. *J. Gen. Microbiol.* 20:519-531.
77. Weibull, C., and H. Beckman. 1960. Growth of bacterial L forms and bacterial protoplasts. *J. Bacteriol.* 79:638-649.
78. Weibull, C., J. W. Greenawalt, and H. Law. 1961. The hydrolysis of adenosine triphosphate by cell fractions of Bacillus megaterium I. Localization and general characteristics of the enzyme activities. *J. Bio. Chem.* 237:847-852.
79. Wolfe, L. S. 1964. Cell membrane constituents concerned with transport mechanisms. *Can. J. Biochem.* 42:971-988.
80. Wolpert, L., and C. H. O'Neill. 1962. Dynamics of the membrane of Amoeba proteus, studied with labelled, specific antibody. *Nature* 196:1261-1266.