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THE ISOLATION OF DNA-MEMBRANE VESICULAR BODIES FROM LYSATES OF BACILLUS CEREUS BY DIFFERENTIAL AND GRADIENT CENTRIFUGATION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Douglas Oldfather Abbott, B.S., M.S.

********

The Ohio State University
1972

Approved by

[Signature]
Adviser
Department of Microbiology
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VITA

Douglas Oldfather Abbott

November 30, 1941 . . . Born, Columbus, Ohio

Education

1955-1959 ....... Columbus Academy, Columbus, Ohio
Graduated, 1959

1960-1962 ............................ The Ohio State University, Columbus, Ohio

B.S. in Zoology, 1964

1966-1970 ............................ The Ohio State University, Columbus, Ohio
M.S. in Microbiology, 1968

Graduate Appointments

1965-66 ................................. Teaching Assistant in Microbiology, Division
of Veterinary Science, University of Wyoming

1966-1967 ............................ Research Assistant in Microbiology, The Ohio
State University

1967-1970 ............................ Teaching Assistant in Microbiology, The Ohio
State University

1970 .....................................National Science Foundation Summer Traineeship

Positions Held

1970- . . . . . . . . . . . . Assistant Professor, Biology Department,
Murray State University, Murray, Kentucky

Publication

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INTRODUCTION

The concept that the bacteria are in a sense 'bags of enzymes' without specific internal compartmentalization has been changing during the last fifteen years. It is now recognized that in some procaryotic organisms, at least, some of the enzymatic activities of the cells are associated with specific internal structures of the cells. In terms of the nucleus, however, it has been usual to think of the bacterial DNA as being free and not arranged in a complex structure as in the eucaryotic chromosome. It now seems to be agreed that there must be some sort of attachment of the DNA to allow the physical separation of the replicating nucleus during the division cycle. Some association of the bacterial DNA with membrane in the cell has been shown in thin sections of the cells in the electron microscope. Previously good isolation procedures for obtaining these attachments have been lacking. It has been shown that, under certain circumstances, DNA of apparently two different densities can be seen in density gradient centrifugations of lysates, but much information about the so-called membrane-bound DNA has been lacking.

The work here involves a procedure developed for the isolation of subcellular vesicular bodies from lysates of Bacillus cereus to isolate vesicles that had DNA attached to them.
LITERATURE REVIEW

The nature and the functional relationships of the internal membranes of bacteria have been of much interest for several years (Salton, 1967; Ryter, 1968; van Iterson, 1965). Much of this interest in internal membranous structures in the bacteria has been stimulated by advances in the study of the ultrastructure of eucaryotic cells. The delineation of various biochemical processes and their structural associations within the highly compartmentalized eucaryotic cell has increased speculation that there might be similar recognizable structures in bacteria with discrete biological functions.

The early electronmicrographs, such as those of Chapman and Hillier in 1955, failed to demonstrate much internal organization or differentiation in bacteria. Indeed, even the presence or absence of a cytoplasmic membrane seemed debatable. With the increase in sophistication of the instruments and techniques for the preparation and visualization of biological material in the electron microscope that has occurred since then, it has been found that the bacteria do often demonstrate internal differentiation.

Most of the early studies on internal membranous structures were done with gram positive organisms and in particular on members of the genus Bacillus. The membranous invaginations found in the Bacillaceae and similar structures in other organisms have been identified as mesosomes after Fitz-James (1960). The presence of mesosomes has now
been demonstrated in a variety of types of organisms, both gram positive and gram negative. It has even been suggested by one reviewer that the bacteria can be said to have two membrane systems, the limiting cytoplasmic membrane and an internal membrane system, the mesosome (Salton, 1967).

The elucidation of the specific functions, if any, of the internal membranes in bacteria has been somewhat complex. In the first place, there has been the problem of what to name the various internal membranous structures. In the gram positive organisms, particularly in the Bacillaceae, identification has not been much of a problem. A mesosome has consisted of an invagination of the cytoplasmic membrane filled with membranous structures, open to the outside of the cell, and demonstrated to maintain an attachment to the nuclear region of the cell.

In a number of organisms, however, the picture of the internal membranes has been shown to be more complicated. In Nitrocytis oceanus for example, Remsen, Valois, and Watson (1967) demonstrated an extremely complex system of internal membranes that the authors stated was reminiscent of the structure of the chloroplast of eucaryotic cells. This highly organized structure in almost no way resembled the structure that has been traditionally called a mesosome. This same kind of membranous internal organization has been found in some other types of bacteria. Proctor, Norris, and Ribbons (1969) showed in the methane-utilizing bacteria internal membranous structures that were quite similar to those seen in Nitrocytis. In this case also there was no evidence of the typical mesosome configurations. Felter et al. (1970) working with
Vibrio marinus have found that the same sort of membranous lamellae were demonstrated in this organism but they were not so tightly packed nor as profuse as in Methanococcus or Nitrocystis. Felter et al. have also shown that there were associated with the myelemma what may have been mesosomes or at least mesosome-like structures. They stated, however, that there had not as yet been mesosomes demonstrated in Vibrio marinus.

In Escherichia coli, which has been shown to demonstrate mesosomes, under certain cultural conditions so-called extra membranes have been found (Weigand, Shively, and Greenwalt, 1970) which in many respects appeared similar to the myelemma found in Vibrio marinus. While these extra membranes in E. coli were temperature dependent, that is their production was related to increased temperature of growth and was therefore not seen under normal conditions, the membranes in Vibrio marinus were also seen first in heat-treated cells. The extra membranes in E. coli also differed from the membranes in Vibrio marinus in that Weigand, Shively, and Greenwalt noted no connection of the extra membrane material with the nuclear region of the cell. Felter et al., however, felt that in the Vibrio the membranes did appear to be associated with the nuclear material.

In Azotobacter vinelandii, Oppenheim and Marcus (1970) have shown a different situation in the arrangement of the internal membranes. In this organism the internal membranes were seen as single intrusions of the cytoplasmic membrane. Ghosh, Lampen, and Remsen (1969) found somewhat similar configurations in certain cultures of Bacillus licheniformis. The tubules in the Bacillus also seemed to be involved with the
extrusion of vesicular elements into the periplasm. In the *Bacillus*
these tubules were present at the same time as were the more typical
septal mesosomes.

While most of the workers have agreed that the above mentioned
internal membranous structures were not mesosomes, the exact nature of
the mesosome has still not been rigidly defined. There is good agree-
ment that the mesosomes in thin sections have appeared as pockets or
invaginations of the cytoplasmic membrane filled with membranous
structures. The exact nature of the internal configurations have not
been agreed upon and the various reviewers have taken different positions
on the matter. Salton (1967) stated that the mesosome contained internal
membranes that appeared as lamellae, tubes, and in some cases as
vesicles. Ryter (1968) stated that the mesosome was filled with
tubules which were rolled up and that these tubules were also made up
of invaginations of the cytoplasmic membrane. Ryter also admitted as
did Salton that the exact configuration of the mesosomes was not the
same even in the gram positive organisms. This was disregarding the
often obvious structural changes that take place in the mesosome during
the growth cycle of a culture (Kakefuda, Holden, and Utech, 1967;
Stevenson, 1968; Ghosh, Lampen, and Remsen, 1969). It has, however,
been thought that the configuration is the same in the same organism
under the same conditions and that the configuration can differ from
species to species.

It is well to remember at this point that the configuration of
mesosomes or even the presence of internal membranous differentiation
has seemed to some extent to depend on how hard the structures have been
looked for. It has not been uncommon to find that with a given organism, increased interest in the ultrastructure has revealed structures that had not been observed or noted previously (Ghosh, Lampen, and Remsen, 1969).

One of the features that has appeared to be constant in mesosomes from one organism to another has been the reaction of these structures to treatment of the cells in hypertonic solutions. Under these conditions the structural integrity of the mesosome is lost and the mesosome everts (Fitz-James, 1964; Ryter, 1968; Ryter and Landman, 1964; Salton, 1967). This eversion has been shown to take place even in cells that have been pre-fixed before being placed in the hypertonic solution (Fitz-James, 1964). In almost all cases the eversion of the mesosome has resulted in the expulsion of vesicular elements from the cytoplasm into the periplasmic space. The identification of these vesicular elements as mesosomal remnants has been made on the basis that they appeared as the mesosome disappeared and that they appeared in the regions of the cell where mesosomes were common. Ryter, Freheil, and Ferrandes (1967) have shown an interesting variant of the appearance of the extruded vesicles. They have shown that when the mesosomes of *Bacillus subtilis* have been expelled by plasmolysis followed by digestion of the cell wall, the mesosomal remnants have appeared as long strands, up to 20 μm, that may be attached to the cytoplasmic membrane. These strands or tubules displayed a "string of pearls" appearance that by a somewhat different terminology could be called strands of vesicles. Ryter (1968) felt that this structure was not unique to *Bacillus subtilis* and might explain the appearance of the
mesosomes in intact cells that show in thin sections a mesosome full of vesicles. This "string of pearls" structure has been characterized as very fragile and according to Ryter may be easily deformed. It has been essential to pre-fix the cells in gluteraldehyde to maintain this structure.

Another important structural characteristic of the mesosome has been the fact that it has often been found in contact with the nucleus or nuclear region of the cell. According to Ryter (1968) the idea that the nucleus and the membrane were connected came first from genetic data. As techniques for fixation and sectioning specimens for electron microscopy improved, the connection or insertion of the mesosome into the bacterial nucleus has been shown by a number of investigators (Ryter and Jacob, 1963; Kakefuda, 1967; Ellar, Lundgren, and Slepecky, 1968; Fitz-James, 1965; Ryter and Landman, 1964). Studies by Ryter and Jacob (1964) of serial sections in *Bacillus subtilis* and subsequent three-dimensional reconstruction of the pictures showed that the connection between the two structures persisted all throughout the division cycle and was found in all of the cells. Not all mesosomes have demonstrated this connection. Kakefuda (1967) showed that "young" mesosomes newly formed by invaginations of the cytoplasmic membrane failed to show any nuclear attachments. The "mature" mesosomes that were assumed to be functional clearly showed the connection.

Attempts by Ryter and Jacob (1964, 1966) to do quantitative studies on the number of attachments per nuclear regions of *Bacillus subtilis* resulted in somewhat more than one attachment for each nucleus. While the quantitative methods used to get these results were open to error (Ryter, 1968), it appeared that the attachments were at least not
artifacts of preparation. Working with *Escherichia coli*, Altenburg, Brinkley, and Suit (1970) found that the size of the attachments and the numbers of attachments seemed quite variable. Shull et al. (1971), working with conjugally transferred DNA in *Escherichia coli* minicells, found multiple DNA-membrane associations as did Ivarie and Pene (1970) working with *Bacillus subtilis*.

The nature of the insertion of the mesosome into the nuclear region of the cell and the relationship of this attachment to DNA-membrane associations has not been clear in electron micrographs. Work by Ryter and Jacob (1964) with *Bacillus subtilis* showed that the attachment would survive eversion of the mesosome in an hypertonic medium. In sections of these treated cells, the bacterial nucleus was attached directly to the cytoplasmic membrane. Work by Ryter (1968) showed that the same phenomenon could be demonstrated in *Bacillus subtilis* cultured under anaerobic conditions which destroys their mesosomes. She stated that the morphological picture of these cells was similar to that seen in gram negative cells that did not form mesosomes.

The function of the mesosome in the metabolism of the bacterial cell has still not been agreed upon by most researchers. One of the first problems has been that there are organisms that under certain conditions do not contain any mesosomes and yet still are functional cells. Of course the demonstration of the absence of mesosomes can be questioned since various techniques for the visualization of the organelles may give different results as to the presence or absence of the mesosomes in a given culture (Ghosh, Lampen, and Remsen, 1969). Another problem has been, again, the question of what structures are mesosomes.
The fact that the internal membranes in *Azotobacter vinelandii* have been related to the nitrogen source for growth (Oppenheimer and Marcus, 1970) is of dubious interest concerning the function of the quite different internal membranes in *Bacillus*. Even in the genus *Bacillus*, however, other internal membranous structures, besides mesosomes, have been found with specific cellular functions associated with them (Ghosh, Lampen, and Remsen, 1969).

One of the first postulated functions of the mesosome was that they were involved with the development of new cell walls. Chapman and Hillier in 1955 found that the peripheral bodies, assumed now to be mesosomes, were associated with the division plane of *Bacillus cereus*. It has also been shown that the protoplasting of *Bacillus* caused release of mesosomes. The subsequent failure of these organisms to form new cell walls has been related to the loss of the mesosomes (Salton, 1967).

Detailed studies of the association of the mesosomes with the process of cell division have been performed by a number of workers. While the exact events of the division process were not the same in different organisms, nor were the configurations of the mesosomes, there was a definite structural association of the mesosomes with the division processes (Ellar, Lundgren, and Slepecky, 1967; Kakefuda, Holden, and Utech, 1967; Salton, 1967). Despite this physical association at the site of cell wall and membrane synthesis, the biochemical or physical function of the mesosome in the process has not been shown. Fitz-James (1964) did suggest that the function of the mesosome might be that of transporting new cell wall material to the site of cell wall
synthesis. Later work (Fitz-James and Hancock, 1965) failed to show any direct involvement of the membrane with the transport of the cell wall precursors. There has also been some negative evidence (Ryter, 1968) indicating that in certain conditions it is possible to revert protoplasts to the bacillary forms under conditions that inhibit the formation of mesosomes.

There has also been a good deal of evidence that the mesosome is involved with the oxidation-reduction reactions of the cell (van Iterson, 1965; van Iterson and Leene, 1964; Ferrandes, Chaix, and Ryter, 1966). Objections have been raised that the work of van Iterson and Leene, showing greater reduction of tellurium in the region of the mesosome, may have demonstrated solely a membrane concentration effect. Even if this were the case, the fact of the mesosome as a compact area of high enzymatic activity of necessity indicated that the mesosome played an important role in the oxidative-reductive reactions of the cell (Ryter, 1968). This would be true even if the rest of the membranes in the cell were to have the same specific activity as the mesosomal membranes. In fact the work of Ferrandes, Chaix, and Ryter (1966) has shown this not to be the case. They separated the everted mesosomal remnants from cytoplasmic membrane fractions of lysates of Bacillus subtilis. The mesosome fraction showed a higher concentration of cytochromes than the cytoplasmic membrane fractions. They also showed that the cytoplasmic membrane fractions that demonstrated cytochrome absorption peaks were contaminated with vesicular bodies that were similar to the mesosome remnants. When cytoplasmic membrane fractions were obtained which did not show the attachment of these vesicular
bodies to the membrane sheets, the fractions failed to show any
cytochrome peaks.

Weibull and Gyllang (1965) working with two stable L forms
found that they lacked the cytochromes that were present in the
parent strains. The assumption was that the lack of the cytochromes
was related to the loss of the mesosomes that took place during proto-
plasting.

Not all mesosomes of course contain cytochromes. There are
a number of anaerobic organisms that display mesosomes and fail to
have any cytochromes. Kakefuda, Holden, and Utech (1967) point out
that this does not preclude the possibility that the energy yielding
reactions of these cells are concentrated in the mesosomes. They
mention that in other systems the glycolytic cycle itself may be
membrane bound.

The relationship of the mesosome to the nuclear region has led
to the idea that the mesosome might be involved in the process of
nuclear division at least, if not with cell division too. There are
essentially three ways that the mesosome has been suspected to be in-
volved with the nuclear division process (Ryter, 1968). The first has
been that it might serve as a structural entity with the DNA, organizing
it so that separation of the strands could take place in an organized
manner. Another has been that the mesosome might act as a sort of
bacterial analogy to the eucaryotic mitotic apparatus. The other pos-
sibility has been that it might serve simply as a pool of enzymatic
activity close to the site of DNA replication.
Unfortunately, purely morphological studies have failed to elucidate the nature of the DNA-membrane attachment or attachments (Ryter, 1968). The necessity of mesosome attachment to the DNA for nuclear division has not been proved since in certain conditions Bacillus subtilis can divide with the nuclear region attached to the cytoplasmic membrane with no mesosomes in the cell (Ryter, 1968). Whether or not attachment to a distinct mesosome is necessary for the process of nuclear division, hypotheses about the mechanism of division rely on the attachment of the nuclear material to membrane in the cell (Ryter, 1968). The concept that the mesosome is an energy source in the nuclear division cycle has not been extensively examined. Howland and Hughes (1969) have worked with Escherichia coli in which the attachment of the nucleus to the membrane is generally not as extensive as in the gram positive organisms. They showed that the addition of uncouplers of oxidative phosphorylation stimulated DNA synthesis. They felt that the association of the respiratory apparatus with the nucleus reflected a need for a proton flux away from the locus of DNA chain unwinding.

 Regardless of the exact biochemical or physical mechanisms involved with the attachment of the mesosome and the bacterial nucleus, it has been shown to be not only a structural but also a functional phenomenon. Higgans and Daneo-Moore (1972) working with Streptococcus faecalis have shown that the size and configuration of the mesosomes were more dependent upon DNA synthesis than on protein synthesis.

Various workers have attempted to isolate membranes from bacteria and separate out the internal membranes from the cytoplasmic membrane.
The methods used have been diverse, including lysis by enzymatic digestion of the cell walls, sonication, shear forces from the use of pressure cells, and combinations of these methods. The lysates have then been separated by differential and gradient centrifugations (Salton, 1967). The results of these attempts have been somewhat varied. DeLey (1963) separated out small vesicular elements that he called oxidosomes. By 1966 Ferrandes, Chaix, and Ryter, by careful lysis and centrifugation, identified their small membrane vesicular fractions as mesosome remnants. The identity of these small vesicular fractions as mesosome remnants has been doubted by some workers (Salton, 1967). It has been shown that the cytoplasmic membrane, when subjected to certain stresses similar to those used in the separation of so-called mesosome remnants, may form small vesicular elements (Wallach, 1967). The separation of biological activity with membrane fractions of uniform size or density has been explained as being the result of breakage of the membrane at pre-existing points of weakness (Gorchein, 1968).

Ghosh and Murray (1968) tried to avoid the complications of possible vesicle formation from the cytoplasmic membrane and worked with an organism that on protoplasting produced extruded mesosomes that could be stabilized on the outside of the protoplasts. They then separated the membranes on Ficoll gradients. An objection to this type of preparation and separation has been the fact that the mesosome itself in the bacterial cell might not be a static organelle and not very stable (Ryter, 1968; Ryter, Frehel, and Ferrandes, 1967). It has been thought to be in a state of dynamic interaction with the cytoplasmic membrane.
Another problem has been that the phenomenon of autolysis, which many have felt essential to normal bacillary growth (Fan, Beckman, and Cunningham, 1972) might in some organisms involve not only the cell wall but also the cell membrane. This has been shown to be the case in *Bacillus cereus* with Mohan et al. (1965) demonstrating the autolysis of the cell wall and Koga and Kusaka (1968) demonstrating the autolysis of the cytoplasmic membrane. In this organism it might be expected that incubation of material under conditions unfavorable to membrane synthesis would result in membrane lysis.

The presumed mesosome fraction of Ghosh and Murray (1969) indicated that there was some DNA attachment to the membranes in this fraction. Ivarie and Pene (1970) using enzymatic lysates of *Bacillus subtilis* demonstrated that all of the DNA would band in one position on Renografin gradients. When the lysates were subjected to shear forces before the density gradient centrifugation, thus breaking the DNA into fragments, two bands appeared in the Renografin gradients, one presumably bound to membranes. The membrane-bound DNA did not appear to be an artifact of preparation since mixing deproteinized DNA with the lysates did not generate any membrane-associated DNA.
MATERIALS AND METHODS

Cultures

The organism used in this study was Bacillus cereus ATCC #4342. The culture medium was the glucose, glycine, glutamic acid salts medium of Lundgren and Beskid (1960) amended with yeast extract. The medium consisted of 10 g glutamic acid, 5 g glucose, 100 mg glycine, 500 mg K₂HPO₄, 500 mg KH₂PO₄, 200 mg MgSO₄·7H₂O, 10 mg FeSO₄·4H₂O, 10 mg MnSO₄·4H₂O, 10 mg NaCl, 13 mg ZnSO₄·7H₂O, 2 ml of a saturated solution of CaHPO₄, 1 g Yeast Extract (Difco), and water to make one liter. The pH was adjusted to pH 6.8 with a 40% solution of NaOH.

Flask cultures were routinely grown in 80 ml of the above medium in 250 ml Erlenmeyer flasks. These were incubated in a reciprocating shaker at 37°C. Spore cultures were produced by inoculating a flask culture with spores from a previous spore culture or with a loopful of cells from an agar slant. The spore cultures were incubated 48 hours until sporulation was completed as determined by observation with a phase contrast microscope.

Eight liter batches of the cells were grown in a New Brunswick Micro-Ferm fermentation device. The medium used was as described above except that the glucose and 250 ml of water were left out until the rest of the medium in the fermentor had been autoclaved and allowed to cool. The glucose, dissolved in 250 ml of water, was filter-sterilized in a Millipore apparatus with sterile HA filters, average pore diameter of 0.45 μm.
To promote uniform results, pre-cultures were used. A flask of 80 ml of medium was inoculated with 2 ml of a spore culture and heat-shocked at 65°C for 15 minutes to speed germination of the spores. The flask was then incubated on the reciprocal shaker for two and one-half hours. Two ml of this first flask were then transferred to the second set of pre-culture flasks. When the optical density at 610 nm reached 0.16, a further preculture was made. When the O.D. again reached 0.16, three of the precultures were aseptically poured into the inoculating port of the fermentor.

The temperature of the fermentor was stabilized at 35°C before inoculation. The agitation was set at 200 r.p.m. and the aeration gauge was set at 4,000 cc of air per minute.

**Isolation of the particulate fraction**

The bacteria were harvested during the logarithmic phase of growth by the use of the Szent-Gyorgyi Blum continuous flow attachment on a Sorvall RC2 B centrifuge. The harvesting was done at 27,000 x g average at 4°C and at a flow rate such that the effluent from the centrifuge was clear.

To obtain smaller samples of the cultures, harvesting was accomplished by sedimenting out the bacteria at 250 ml centrifuge bottles in a GSA head at 4,000 x g in the Sorvall centrifuge.

The bacteria were suspended in a 0.05 M Tris buffer with 0.005 M Mn++ as MnCl at a pH of 7.5 (Koga and Kusaka, 1968). The concentration of the suspension was 10% w/v (wet weight of cells). This mixture was then passed through either a French Pressure cell at
12,000-15,000 lbs/in² or in a Sorvall RM 1 cell fractionator at 12,000-13,000 lbs/in².

The resultant crude lysate was then centrifuged at 12,000 x g average for 20 minutes. The supernate was carefully decanted and centrifuged at 34,000 x g average for one hour. This second supernate was then poured off and the tubes allowed to drain for a few seconds upside down. The resultant pink, gelatinous, translucent pellet was termed the particulate fraction. A flow diagram of the isolation procedure is found in the appendix as Figure 19.

All of the samples were kept at 4° C during the isolation procedure.

Preparation of DNA

For the preparation of purified DNA the method outlined by Marmur (1961) was followed. The cells used were from log phase cultures and prepared by the lysis procedure in exactly the same manner as the material for the particulate fraction isolation.

The first step used from the Marmur procedure was the addition of sodium dodecyl sulfate to the lysate and the subsequent incubation at 65° C for 10 minutes. Then 5 M NaCl was added to the lysate to aid in the disassociation of the proteins and nucleic acids. An equal volume of chloroform-isoamyl alcohol (24-1 v/v) was added and the mixture was shaken for one-half hour. The mixture was then separated into three layers by centrifugation at 5,000 x g for 5 minutes in a Sorvall GLC 1 centrifuge. The upper layer containing the nucleic acid was removed and placed in a beaker. Approximately two volumes of 95% ethyl alcohol were layered on the solution. Precipitated threads of
the nucleic acid were spooled up on a glass stirring rod and then transferred to Marmur's dilute saline-citrate buffer. After the nucleic acids were dissolved, the solution was brought to Marmur's standard saline-citrate concentration by the addition of concentrated buffer solution.

This first deproteinization was followed by deproteinizing with liquified phenol instead of the chloroform-isoamyl alcohol mixture. Subsequent deproteinizations were accomplished with the chloroform-isoamyl alcohol mixture until there was no precipitated protein at the interface following the centrifugation.

The solution was then treated with a 2% ribonuclease (Nutritional Biochemicals) solution prepared according to Marmur at a final concentration of 50 \( \mu g/ml \) at 37\( ^\circ \)C for 30 minutes.

Following this the solution was again deproteinized until there was no protein seen at the interface after centrifugation. The DNA was then spooled out and stored in ethyl alcohol until used.

Sucrose density gradients

Sucrose solutions were made up in the Tris buffer at 10% and 40% sucrose. Linear gradients were constructed from these solutions in a Beckman gradient former. Seven-tenths of a ml portions of the particulate fraction resuspended in buffer were layered gently on top of the gradients. Runs were also made with isolated *Bacillus cereus* and mixtures of isolated DNA and particulate fraction that had been treated for one-half hour with DNase (Nutritional Biochemicals) in a concentration of 0.15 mg/ml.
The gradient tubes were centrifuged in a Beckman model 25.3 swinging bucket rotor in a Beckman 1-2 Ultracentrifuge at 50,000 times g average for one hour.

After removal from the centrifuge, the tubes were harvested by sectioning in a Beckman tube cutter to give seven fractions. The average percentage of sucrose in each fraction was determined by taking the refractive index of each sample in a Bausch and Lomb refractometer and then converting the readings to percent sucrose from the tables in the Handbook of Chemistry and Physics (Hodgman, Weast, and Selby, eds., 1959-1960).

**Sodium iodide gradients**

Sodium iodide gradients were prepared using the Beckman gradient former. Ethidium bromide in a concentration of 100 mg/ml was layered on top of the gradients to give a final concentration of 50 µg/ml after the method of Anet and Strayer (1969). The gradients were then centrifuged in a Beckman SW 41 rotor in a Beckman 1-2 65 B Ultracentrifuge at 125,000 x g average for 69 hours. The particulate fraction material that was layered on top of the gradients was pre-fixed in 3% gluteraldehyde in phosphate buffer before the centrifugation.

Following the centrifugation, the gradients were photographed under ultraviolet light from a Mineralight Black Light Lamp using Ektachrome X film (Kodak) in a Retina Reflex 35 mm camera (Kodak).

**Determination of DNA**

DNA was measured by two different techniques: the diphenylamine method and an ethidium bromide fluorescence method.
Three ml of sample in buffer was mixed with 6 ml of the di-
phenylamine reagent (1 g diphenylamine in 100 ml glacial acetic acid
plus 2.75 ml concentrated H₂SO₄). The solutions were heated in a
boiling water bath for 10 minutes and then cooled to room temperature.
Absorbance of the tubes was determined in a Bausch and Lomb Spectronic
20 at a wavelength of 590 nm.

The standard curves were run using sperm DNA from Nutritional
Biochemicals dissolved in Marmur's saline-citrate buffer. A typical
standard curve follows in the appendix as Figure 20.

DNA was also estimated using the fluorescence technique of
LePecq and Paoletti (1966). In this test 2.5 ml of the sample in
buffer was mixed with 0.3 ml of the ethidium bromide (Calbiochem)
solution (100 µg/ml distilled water). The samples were then placed in
a Farrand Mark 1 Spectrofluorimeter. The excitation wave length was
set at 540 nm and the analysis was accomplished with the setting at
590 nm. A standard curve is found in the appendix as Figure 21.

To eliminate RNA the samples were incubated for one-half hour
with 0.2 ml of Marmur's ribonuclease solution.

Determination of protein concentration

Protein concentration was determined by a modification of the
One-half ml of the sample plus buffer was added to 2 ml of reagent C
which consisted of 50 parts of a 2% Na₂CO₃ solution in 0.1 N NaOH to one
part of a 0.5% CuSO₄ solution in 1% sodium tartrate. This mixture was
allowed to incubate for 10 minutes at which time 0.2 ml of the Folin-
Ciocalteau reagent (prepared by The Ohio State University reagent
laboratory) was added. This was then allowed to incubate for 30 minutes before being read in a Coleman Autoset spectrophotometer set at 500 nm. Standard protein curves were run using Bovine serum Albumin (Sigma) fraction V powder. A standard curve is found in the appendix as Figure 22.

**Visualization of DNA**

Visualization of DNA was accomplished through the microdiffusion technique of Mayor and Jordan (1968) which is a modification of the Kleinschmidt technique (Kleinschmidt, 1968). Samples of the DNA containing material in buffer were placed in drops on a sheet of Teflon. A small drop of a mixture of cytochrome c (Nutritional Biochemicals) in demineralized double distilled water was touched to the surface of the drops. After 15 minutes a 200-mesh copper grid that had been coated with formvar and carbon was gently placed on the surface of the drop. The grid was removed and then dehydrated in absolute ethyl alcohol. The grids were then shadow-cast in a Kinney Vacuum evaporator using platinum wire. The grids were shadowed at two angles normal to each other.

The grids were then examined in an Hitachi HS-8 or a Zeiss EM 9S electron microscope.

**Stability of the fractions**

The structural and biochemical integrity of the particulate and vesicular fractions was checked as suggested by the work of Koga and Kusaka (1968). Samples were incubated at various temperatures in phosphate and Tris buffer. At 15-minute or one-half hour intervals, portions of the samples were centrifuged. Changes in the O.D. in the
visible region of the spectrum and the release of ultraviolet absorbing material into the buffer after centrifugation were followed in a Coleman Autoset or Shimadzu MPS 50 L recording spectrophotometer.

Preparations for electronmicroscopy

Material was prepared for electronmicroscopy by negative staining, shadow casting, and thin sectioning.

Negative stains were accomplished using a 2% aqueous solution of phosphotungstic acid that had been brought to pH 6.1-6.2 with KOH (Ghosh, Lampen, and Remsen, 1969). Material that had dried on a carbon-coated grid was stained for two minutes with the PTA solution which was then removed with filter paper. The grid was placed in a vacuum evaporator for five minutes and then examined in the electron microscope.

Material to be shadow-cast was allowed to dry on the carbon-coated grids. The grids were then shadowed with either gold or platinum wire in a Kinney vacuum evaporator.

Material to be thin-sectioned was fixed by the Ryter-Kellenberger method (Kellenberger, Ryter, and Sechaud, 1958) and then embedded in Epon 812 (Luft, 1961). The polymerized blocks were then sectioned with a Porter-Blum MT-2 ultramicrotome. The grids were then post-stained with lead citrate.
RESULTS

Growth of the cultures

The use of pre-cultures with transfers during the early log phase of growth allowed an abbreviated culture time in the fermentor jars. Cells were harvested from the fermentors in the log phase of growth at an optical density of approximately 0.85 at 610 nm using a water blank to standardize the spectrophotometer. A typical growth curve of the cultures under these conditions follows as Figure 1.

The cells at this stage of growth as seen in the phase contrast microscope were short rods without large internal structures such as the poly-β-hydroxybutyric acid granules that are common in these organisms. These granules did not appear under these growth conditions until the O.D. reached approximately 1.5 at 610 nm. Autolysis of the cells was checked by incubating the cells in phosphate buffer and then examining the organisms under a phase contrast microscope. Cells harvested from the fermentor at an O.D. of 0.85 showed no autolysis with 10 minutes incubation time while those taken from the fermentor at O.D. 1.3 or 1.4 routinely showed a tendency to lyse in 10 minutes.

While optical microscopy of the bacteria harvested at 0.85 O.D. gave no indication of internal structures or inclusions, negative stains of these cells viewed in the electron microscope show internal differentiation typical of organisms containing mesosomes. Figure 2 is an Electronmicrograph of a negatively stained Bacillus cereus harvested at O.D. 0.85.
Fig. 1. Growth curve of *Bacillus cereus* in the fermentor jar after inoculation with precultures.
Fig. 2. Negatively stained *Bacillus cereus* after harvesting at 0.85 O.D. at 610 nm. Magnification 72,500.
Isolation of the particulate fraction

The crude lysate from the pressure cell was viewed in the phase contrast microscope to check on the breakage. The isolation of the particulate fraction by differential centrifugation was routinely done with unfixed cells and cell lysates. Attempts were made to use cells and lysates fixed in gluteraldehyde. While the fixation in gluteraldehyde has been postulated to maintain everted vesicles in a more natural configuration (Ryter, 1968) and stop autolysis (Higgins, Pooley, and Shockman, 1970), it also resulted in the absence of the pink, gelatinous, translucent particulate fraction. The material that normally sedimented during the second centrifugation was removed from suspension during the first centrifugation when gluteraldehyde fixed specimens were used. The DNA/protein ratio of the various fractions involved in the isolation of the particulate fraction is found in Table 1.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>mg DNA/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>First precipitate</td>
<td>0.60</td>
</tr>
<tr>
<td>First supernate</td>
<td>1.70</td>
</tr>
<tr>
<td>Second supernate</td>
<td>0.92</td>
</tr>
<tr>
<td>Particulate fraction</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Sucrose density gradients

The sucrose density gradients that were run with the particulate fraction were harvested in seven fractions with the material from the top of the gradient labeled fraction 1 and the material from the bottom labeled fraction 7. The calculated percentages of sucrose in each fraction follows in Table 2.
TABLE 2

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Avg. Percentage Sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.7%</td>
</tr>
<tr>
<td>2</td>
<td>20.2%</td>
</tr>
<tr>
<td>3</td>
<td>23.3%</td>
</tr>
<tr>
<td>4</td>
<td>26.4%</td>
</tr>
<tr>
<td>5</td>
<td>29.5%</td>
</tr>
<tr>
<td>6</td>
<td>31.9%</td>
</tr>
<tr>
<td>7</td>
<td>33.6%</td>
</tr>
</tbody>
</table>

The protein concentration found in each of the fractions follows in Figure 3. There were two peaks of protein concentration in the gradient. The first was in the region of fraction 4 and the second, a very sharp peak, was in the region of fraction 6.

The DNA content of the various fractions is shown in Figure 4. As with the protein concentrations, there was a sharp peak in fraction 6. Fraction 7 was the only other sucrose gradient fraction to contain appreciable quantities of DNA. It should be noted, though, that the DNA concentration of fraction 7 was only approximately one-half of that found in fraction 6.

Gradients were also run with the particulate fraction that had been treated with DNase prior to being layered on the gradients. Aliquots of purified Bacillus cereus DNA were added to the DNA-free particulate fraction on the gradients before centrifugation. The distribution of DNA in these gradients follows in Figure 5. No sharp peak of DNA was found associated with any fraction and the greatest concentration of DNA was found in the bottom of the gradient rather than in fraction 6.
Fig. 3. Protein concentration of the sucrose density gradient fractions as determined by Lowry's technique.
Fig. 4. DNA concentrations of the density gradient fractions as determined by the fluorescence technique of LePecq and Paoletti.
Fig. 5. DNA concentrations of the density gradient fractions that had been treated with DNase and then centrifuged with purified *B. cereus* DNA. Fractions 1 and 2 demonstrated no DNA.
mg of DNA/ml

fraction number
That this sucrose density gradient procedure separates vesicular elements has been shown previously (Abbott and Pfister, 1969). Figure 6 is an electronmicrograph of a negatively stained preparation of the vesicles found in fraction 6. Figure 7 is an electronmicrograph of a shadow-cast preparation of this fraction. The majority of the vesicles in this fraction were found to be about 100 nm in diameter though there were some that were smaller, with diameters down to about 50 nm. Figure 8 is a thin section of the particulate fraction showing the 3-tracked membrane profile of the vesicles.

The fractions were also used in a microdiffusion technique to visualize the DNA. Figure 9 is an electron micrograph of a preparation of fraction 6 that had been used in the diffusion technique. The strands of DNA were found to be associated with vesicles that also passed over into the cytochrome layer during diffusion. These vesicles were in the same size range as the majority of those seen in the negative-stained and shadow-cast preparations of the same fraction.

Figure 10 is another print from the same negative as in Figure 9. This shows the vesicle-strand association more clearly. The strands as they pass along the vesicle appeared even more beaded than they do when they were free on the grid. Also the diameter of the strand appeared to increase, though it was still in the size range that Kleinschmidt (1968) indicated uncomplexed DNA demonstrates in this type of technique. Figure 11 is another high magnification print showing what may have been two vesicles clumped together with a strand running across the top. Figure 12 is a lower magnification electronmicrograph
Fig. 6. A negatively stained preparation of material in fraction 6. V indicates vesicle. Magnification 70,000 x.
Fig. 7. Shadow-cast preparation of vesicles in the 6th fraction. Magnification 40,000 x.
Fig. 8. Thin section of vesicles from the particulate fraction. M indicates the typical 3-tracked membrane configuration. Magnification 72,000 x.
Fig. 9. Platinum shadow cast of fraction 6 DNA and vesicles from DNA diffusion technique. V indicates vesicle, D indicates DNA. Magnification 87,000 x.
Fig. 10. Platinum shadow cast material from fraction 6 that had been treated in the DNA diffusion technique. C indicates vesicle-strand associations. Magnification 100,000 x.
Fig. 11. Platinum shadow cast material from fraction 6 that had been treated in the DNA diffusion technique. C indicates vesicle-strand associations. Magnification 90,000 x.
Fig. 12. Platinum shadow cast material from fraction 6 that had been treated in the DNA diffusion technique. C indicates vesicle-strand associations. Magnification 72,000 x.
showing the vesicle association and some extension of the strand onto the grid. Again the portion of the strand on the vesicle appeared larger, about 15 nm, while as it left the vesicle, it appeared to be somewhat smaller than 10 nm.

Strand-vesicle associations were not demonstrated in any of the other fractions, particularly from fraction 7 which did contain appreciable quantities of DNA.

DNase-treatment of the samples prior to the microdiffusion technique resulted in no strands visible in the preparations. Also, when the fractions were not fixed after harvesting from the density gradients, incubation of the fractions at room temperature for 15 minutes was sufficient to render the visualization of the strands impossible.

Sodium iodide gradients

Sodium iodide gradients were run using purified \textit{Bacillus cereus} DNA mixed with ethidium bromide, fixed particulate fraction with ethidium bromide, and DNase-treated particulate fraction with added \textit{Bacillus cereus} DNA mixed with ethidium bromide.

After centrifugation, the tubes were photographed under ultra violet light to place the sites of the nucleic acids by the fluorescence of the ethidium bromide. The relative distances of the fluorescence bands are given in Table 3.

Figure 13 is a negative print of the particulate fraction gradient showing the three bands. Figure 14 is of the gradient with the purified DNA and Figure 15 is of the DNase-treated fraction with added DNA.
Stability of the vesicular fractions

The stability of the particulate fraction was checked by incubation in a phosphate buffer. The decrease in the optical density of the fraction was followed and the released soluble material was determined by centrifuging out the particulates, then reading the change in absorbence of the buffer. It was found that there was a decrease in the optical density at 610 nm which was related to a rise in the absorbence of the supernate as read at 280 nm. Figure 16 shows the changes that took place at 35°C. Even when the particulate fraction was incubated at 0 to 2°C there was a release of ultra violet absorbing material as is shown in Figure 17. While the increase in the absorbence of the supernate is not as dramatic at 0 to 2°C as it was at 35°C, it was still readily detectable.

The effects of both temperature and composition of the suspending buffer on the stability of the vesicular elements in the particulate fraction is shown in Figure 18. This is a composite graph of the protein content in the sucrose density gradient fractions that had been prepared three different ways. The first is the protein concentrations of the gradient fractions using particulate fraction suspended in the Tris buffer with added Mn²⁺ as was normally done. Here, however, the resuspended particulate fraction was allowed to incubate.
Fig. 13. Negative print of sodium iodide gradient of the particulate fraction and ethidium bromide. Photographed with ultra violet light. B indicates bands.
Fig. 14. Negative print of sodium iodide gradient of *Bacillus cereus* DNA and ethidium bromide. Photographed with ultra violet light. B indicates bands.
Fig. 15. Negative print of sodium iodide gradient of DNase-treated particulate fraction with added *Bacillus cereus* DNA and ethidium bromide. Photographed with ultra violet light. B indicates bands.
Fig. 16. Decrease in optical density of the particulate fraction and related increase in absorbence in the ultra violet of the supernate under incubation at 35° C.
35°C

Particulate Fraction

O.D. at 610 nm

30 60 90

Time in Minutes

Supernate

A at 280 nm

30 60 90

Time in Minutes
Fig. 17. Increase in absorbence of the supernate with the particulate fraction at 0 to 2°C plotted against time.
0-2°C

Supernate

A at 280 nm

time in minutes
Fig. 18. Protein concentrations of sucrose density gradients prepared with three different samples: particulate fraction in tris-Mn$^{++}$ buffer incubated one-half hour, particulate fraction prepared in phosphate buffer, particulate fraction in tris-Mn$^{++}$ buffer kept in the cold.
Incubated 1/2 hour

mg of protein/mg

tris-Mn^2+ buffer
phosphate
buffer
tris-Mn^2+ buffer

fraction number
at room temperature for one-half hour before being layered on the
gradient. The next is the protein concentration of the gradient
fractions prepared with particulate fraction handled in the usual
manner with all the material kept at 0 to 4°C from the lysis of the
cells through the density gradient centrifugation. The difference
here was that the buffer used was 0.1 M phosphate buffer at pH 7.2
instead of the Tris-Mn
t buffer. The last is the protein concen-
tration of gradients using material prepared in the tris-Mn
t buffer
and kept at 0 to 4°C until after the sucrose density gradient
centrifugation. Both the phosphate buffer sample and the incubated
sample showed the same pattern of protein concentration which was
quite different from the sample prepared in the tris-Mn
t buffer and
kept under refrigeration.
DISCUSSION

The problem of isolating membranous fractions from bacteria involves a certain amount of risk. The question often is asked whether the isolated elements can still reveal anything about the actual relationships inside the cells. The isolation of subcellular units from viable organisms almost by definition induces artifacts in the material isolated. The living system is in a dynamic state and most of the structural-functional units are probably in a constant state of flux in the system. It is important, therefore, that in the isolation of units from a cell some thought be given to possible effects the isolation procedure will have on the induction of artifacts in the system being studied.

The membranous elements in the particulate fraction used in this work are quite susceptible to autolysis even with the buffer system used in this work. Previous work with this material (Abbott and Pfister, 1969) had led to the finding that the material in the middle of the gradients would, if isolated as rapidly as possible, yield high specific activity for succinic dehydrogenase. This portion of the gradient also exhibited a higher number of vesicular elements in the electron microscope than would be predicted by the relative protein concentrations of the gradient fractions. It is now evident that the low protein concentration in the middle of the gradient was an artifact of the isolation system in that much of the protein in the
particulate fraction solubilized in the initially used phosphate buffer and did not get centrifuged down in the gradient. This is borne out not only by the very altered protein profiles of the gradient fractions when the tris-Mn\textsuperscript{++} buffer was used but also by the observation that the light scattering by particulates at 650 nm in the particulate fraction decreased with incubation in the phosphate buffer.

The substitution of the tris-Mn\textsuperscript{++} buffer and the handling of materials at temperatures below 4\textdegree C reduced the tendency of the proteins to go into solution.

This procedure, however, is not claimed to achieve isolation of membranes in their exact configuration as found in the bacterial cell. The suggestion of Ryter (1968) that the everted mesosome vesicles are attached to each other appears to have much merit. Unfortunately, if the attachment is not broken, separation of the attached vesicles becomes a great practical problem. The procedure for maintaining the attachments, pre-fixation in gluteraldehyde, was tried with the result that all of the vesicles did indeed sediment together.

While using this pre-fixation technique would, on a purely structural basis, allow the examination of the membranes in a state closer to that of these membranes in the cell, it has one great limitation. This is that the purpose of these studies is to develop techniques for studying and to establish structural-functional relationships. Once the membranes have been treated with fixatives, biological activity in terms of enzymatic activity at least is destroyed.

The phenomenon of membrane associated DNA has been reported previously but whether the relationship was casual or real has still
been open to debate. The use of shear forces in the isolation of membrane fractions has the effect of breaking the DNA strands. If the DNA is membrane-bound at some point or points, breakage would tend to separate the DNA into that which is still membrane-bound and that which is now free (Ivarie and Pene, 1970). Another idea is that the broken strands of the DNA might more readily attach to membrane elements than the entire DNA would.

In the sucrose density gradients run with the particulate fraction there were two fractions of DNA which sedimented at different rates through the sucrose. The deproteinized DNA mixed with DNA-free particulate fraction did not separate out into a slow and fast sedimenting type but was of the more rapidly sedimenting type presumed to be unattached to membrane.

The sodium iodide gradients run with the purified DNA were a check on the possibility that the DNA from these organisms might consist of two species of different densities. Sodium iodide was chosen since Anet and Strayer (1969) showed that a greater separation of DNAs of different densities was obtained in these gradients than in cesium chloride gradients. The results clearly indicated that the isolated DNA banded out as a single band. Also the mixture of the deproteinized DNA and DNA-free particulate fraction failed to result in two bands. The result with the particulate fraction on the sodium iodide giving three bands is explained by the fact that the particulate fraction had some residual RNA present which also would give a fluorescence band in the gradients. The weaker middle band is in the portion of the gradient that the isolated DNA was found in.
An objection can still be raised about the origin of the DNA membrane attachment since incubation of the particulate fraction necessary to remove DNA results in a lessening of the proteinaceous material in the sixth fraction. If the slow sedimenting DNA were the result of a casual association between the readily lysed vesicles in this fraction, the association would not take place between purified DNA and pre-incubated particulate fraction. This still, however, supposes the association of the DNA with a particular type of vesicle.

The nature of the actual physical association between the DNA and the membranous vesicles is not easy to discern in the electron microscope. The shadowing by a metal is necessary to increase the contrast around the DNA strands and make them visible (Kleinschmidt, 1968). To a certain extent the more the shadow the more the contrast. The problem is that the more the contrast and shadow, the grainier the background becomes. High magnification then gives little but interesting patterns of dots that do not lend themselves to any serious interpretation. In those cases where the DNA is wrapped around a vesicle it does appear that the strands become more beaded and somewhat thickened.

The presence of the vesicles in the diffusion preparation for electron microscopy does not depend entirely on the attachment of the DNA to them. The diffusion technique depends on the diffusion of the DNA to a monolayer of denatured cytochrome c on the surface of the drop. The diffusion of the DNA is of course then dependent on the time used and the concentration of DNA in the sample. Also the shorter lengths of DNA diffuse more quickly than the longer. The vesicles in the suspension also diffuse into the cytochrome c layer and they do so even in cases where there is no DNA in the sample.
The fact that there were no clear indications of DNA strands from the seventh sucrose density gradient fraction was of some problem. It appears, however, that the DNA from that fraction was lost during the centrifugations and washing procedures that the fractions were subjected to before being run in the diffusion technique. It would be expected that if the DNA in this fraction were not membrane-associated the centrifugation used would not pellet it out readily.

The fact that the majority of the DNA in the sucrose density gradients appeared in the membrane-associated fraction should not be surprising since the isolation technique was developed to separate vesicular elements. Looking at Table 1 gives ratios of DNA to protein with the lowest ratio in the particulate fraction. When this is combined with the fact that most of the protein has been discarded in the first precipitation and the second supernate, it is clear that the DNA in the particulate fraction has been enriched for that which was vesicle-associated.

The question still remains as to the nature and origin of the vesicles separated out on the sucrose density gradients. The membranous nature of the vesicles is shown in the typical 3-tracked appearance of the vesicles in Figure 8. The identification of these membranous vesicles as mesosomal in origin has been challenged on the basis that isolation procedures can cause some of the cytoplasmic membrane to form vesicular elements. It should be noted that repeated washing and incubation of membranes that will accomplish this transformation were avoided since the particular vesicles being separated were very susceptible themselves to conversion and lysis. Also the fact
remains that as these cells are broken up the internal membranes evert as vesicles and a fraction containing the vesicles from a lysate must contain these everted mesosomal remnants. Combining all this with the evidence from thin sections of whole cells indicating a mesosome DNA attachment leads to the conclusion that the vesicular elements enriched for and isolated in this procedure were of mesosomal origin.
SUMMARY

Lysates of cultures of the bacterium *Bacillus cereus* were separated by differential and gradient centrifugation to isolate DNA-vesicular attachments.

The DNA-vesicular fractions were examined to determine that the attachment was not an artifact of preparation.

The problem of autolysis of subcellular vesicular elements was examined. The autolysis was lessened by the use of a Tris buffer with added Mn<sup>++</sup> and low temperatures throughout the isolation procedures.

The DNA-vesicular elements were also viewed under the electron-microscope as a physical characterization of the attachment.
APPENDIX
Fig. 19. Flow diagram of the isolation procedure for the particulate fraction.
Crude Lysate

Centrifuge 12,000 X g 20 min.

Supernate

Precipitate (Particulate Fraction)

Centrifuge 34,000 X g 1 hr.

Supernate

Precipitate (Cells, cell walls, large membranes)

Density Gradient
Fig. 20. Diphenylamine standard curve.
DNA dihydroxyamine standard curve
Fig. 21. Ethidium bromide standard curve. Fluorescence is given in fluorescence units.
Fig. 22. Protein standard curve.


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