ENZYME LOCALIZATION IN

E. coli var. *mutabilis*

by

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ABSTRACT

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Sphaeroplasts were formed from E. coli by the penicillin method of Lederberg (1956). The sphaeroplasts were lysed and fractionated by differential centrifugation into four fractions; a membrane fraction, a soluble fraction, a small particle fraction and a reconstituted control.

The sphaeroplast fractions were used to study the localization of the following enzymes; aconitase, fumarase, glucose-6-phosphate dehydrogenase, glutamic dehydrogenase, isocitric dehydrogenase, lactic dehydrogenase, malic dehydrogenase, succinic dehydrogenase and the cytochromes.

The capacity for oxidative phosphorylation of the four fractions was also studied using succinate, DPNH, α-ketoglutarate and malate as substrates. The effect of 2,4-dinitrophenol as an uncoupler of bacterial systems was also tested.

Aconitase, glucose-6-phosphate dehydrogenase, glutamic dehydrogenase and isocitric dehydrogenase activities were localized in the soluble fraction. Succinic

dehydrogenase was found in the membrane fraction as were the cytochromes.

Lactic and malic dehydrogenase activities were found in both the membrane and soluble fractions. No conclusion could be drawn as to their localization. Fumarase activity was absent from all four fractions although alumina and sonic extracts showed low activities for this enzyme.

Both the membrane fraction and the reconstituted control displayed equal oxidative phosphorylating capacities using succinate, DPNH, α-ketoglutarate and malate as substrates. Using succinate as substrate the membrane fraction showed no decrease in the P/O ratio in the presence of 2,4-dinitrophenol.

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INTRODUCTION

INTRODUCTION

Studies of enzyme localization in plant and animal tissue have yielded a great wealth of data. This has only been possible due to the rapid advancement in the techniques of isolation of various cell organelles in a relatively undamaged form. Such studies in bacteria, however, have lagged behind. Some serious attempts have been made (Weibull, 1958) using gram positive bacteria, but these methods are not generally applicable to the majority of the gram negative species.

The major part of work on animal and plant tissues has been carried out by using homogenate techniques and isolation of cell constitutents in isotonic media by differential centrifugation. Since the bacteria possess tough walls, harsh treatment is required to disrupt the cells, e.g., maceration with abrasives, or prolonged sonication. This invariably leads to fragmentation not only of cell walls, but also the delicate structures lying within them. Evidence has been obtained (Marr and Cota-Robles, 1957) that submicroscopic particles isolated from

various bacteria by sonication are actually formed by comminution of a larger structure. It is evident that data obtained by various earlier workers, using sonication techniques, gave a false picture regarding the localization of enzymes within the various structures of the bacterial cells.

Weibull (1953 b) introduced the use of lysozyme for the removal of the cell walls of gram positive bacteria and since then a number of workers have used this technique for the isolation of subcellular particles. Although there may be some solubilization of enzymes and comminution of structures using this technique, their occurrence can be minimized.

The following study was undertaken to refine the techniques of cell fraction in a gram negative bacterium, Escherichia coli and to compare the localization of enzymes and cytochromes in gram negative and gram positive bacteria. The enzymes chosen for study were representative of various classes of enzymes found constitutively in the cell.

HISTORICAL

HISTORICAL

Stephenson (1928) initiated research on enzyme localization in bacteria. Since that early beginning a wealth of literature has accumulated on the subject. The earlier workers in this field followed no set pattern of analysis and research along these lines stagnated. The enzymic characterization of animal cells (Hogeboom, Schneider and Striebich, 1953) and plant cells (Goddard and Stafford, 1954) acted as an impetus and provided new methodology adaptable to microbial cells.

The methods utilized by workers in the microbial field followed two different channels. The first -cytochemical methods- utilized substrate indicators which formed insoluble dye complexes when acted on by enzymes in viable bacteria. The second channel- analytical morphology -developed for the greater part from earlier work carried out on animal and plant tissues. The aim of the analytical morphological techniques is the isolation of bacterial cell constituents in bulk in a reasonably purified form and to analyze the isolated fractions.

I. Cytochemical Techniques

In cytochemical procedures a colourless substrate which can be enzymatically reduced by a specific enzyme to form a coloured end product is applied to the cell. After the substrate is absorbed, the cells are microscopically viewed for the appearance of the coloured end product. In other methods a specific enzyme substrate and a colourless compound, capable of reacting with one of the end products of the enzyme reaction to form a coloured complex, are added simultaneously to a viable bacterial culture, and the bacteria are viewed microscopically for the accumulation of the coloured dye-end product complex.

The results of these tests have been demonstrated to be inconclusive due to their lack of specificity and sensitivity. Mudd, Winterschied, DeLamater and Henderson (1951) observed "mitochondria" contained within a limiting membrane in two species of mycobacteria using neo- and triphenyltetrazolium chloride, the Nadi reagent, Janus green B and Baker's stain. All of these stains are capable of non-specific reduction from a colourless state to a

coloured state and when coupled with oxidation-reduction systems are used to detect centres of oxidation-reduction.

In a later work Mudd, Brodie, Winterschied, Hartman, Beutner and McLean (1951) demonstrated mitochondrial particles in Saccharomyces cerevisiae, Escherichia coli, Bacillus megaterium and Micrococcus cryophilus. In 1955, McLean, Mudd and Davis found similar mitochondrial particles in Streptococcus pyogenes and Diplococcus pneumoniae. However, in this later work they observed a diffusion of stain and found only one mitochondrial particle per cell rather than a larger number as was previously observed. Mudd and his co-workers claimed that the centres at which the dye accumulated coincided with the large electron dense regions viewed in the cell by electron microscopy. These large electron dense particles volatized under the electron beam and left the remains of a limiting membrane.

Weibull (1953 a) demonstrated in Bacillus megaterium that the coloured formazan formed by reduction of tetrazolium chloride first appeared in many small particles scattered about the cell. These small particles coalesced

to form large particles, but the latter did not correspond to any electron dense region in the cell. Bacterial membranes and lysates also accumulated the coloured formazan particles after lysis of sphaeroplasts (Weibull, 1953 b). Cota-Robles, Marr and Nilson (1958) reported that particles derived from the hull of Azotobacter agilis also showed formazan accumulation. Glauert and Brieger (1955) and Smith, Wilkinson and Duguid (1954) reported similar formazan accumulation due to a coalescence of small particles in the cell. The formazan accumulation did not correspond to the volutin and metaphosphate particles which volatize under the electron beam.

Somewhat similar results were obtained using Caulobacter vibroides (Bowers, Weaver, Grula and Edwards, 1954; Grula, Weaver and Edwards, 1954). The use of cytochemical techniques to localize oxidation-reduction sites has indicated a lack of specificity of the dyes used and tends to throw doubt on the validity of any results obtained cytochemically.

Another histochemical method using the Gomori stain

and glycerophosphate to test for alkaline phosphatase activity has demonstrated a similar lack of specificity in work performed by Bayliss, Glick and Siem (1948) and Wachstein and Pisano (1950). Alexander and Wilson (1955) demonstrated that the localization of alkaline phosphatase by direct assay of cellular fractions does not correspond to the histochemical localization of alkaline phosphatase in A. vinelandii.

The use of cytochemical techniques for enzyme localization studies seems limited because of a lack of specificity and sensitivity of the substrate chromogens. Alexander (1956) proposed three qualifications necessary for a suitable cytochemical indicator; (1) a single enzymic reaction should be responsible for the colour change; (2) dye absorption should be specific, and (3) the accumulation of the coloured dye should take place at the site of enzyme activity.

Marr (1960 b) believes that the enzyme concentration at any one point in the cell is too low to be detected by use of dye substrates and standard optical methods. He maintains that the only method of improving sensitivity is

by use of flourescent substrates. Until development of more specific and sensitive techniques the field of cytochemistry has little to offer to the modern biochemical cytologist.

II. Analytical Morphology

Techniques of analytical morphology include homogenate techniques, derived from previous work on animal and plant tissue, and the more recent techniques of sphaeroplast lysis.

(a) Homogenate Techniques

In homogenate techniques extracts of ruptured bacterial cells are fractionated by differential centrifugation, microscopically examined, and analysed for enzyme activity. Methods of rupture like grinding with abrasives, sonication, osmotic shock, ballistic disintegration, use of grinding mills, and of rapid changes in pressure are not ideal methods to use if subcellular structures are to be preserved. With the use of homogenate techniques it is generally assumed that secondary rupture of granules and

structures, and solubilization of enzymes from particulate matter is kept to a minimum. However, any method as drastic as these may lead to a secondary rupture of particulate matter located either in the cytoplasm of attached to/or part of the cell hull. Soluble constituents located within any particulate material may be released during homogenization, thus producing an erroneous picture of the cellular loci of various enzymes.

Particulate structures separated by differential centrifugation techniques microscopically exhibit a wide range in size and a host of enzyme activities. The use of homogenate techniques does not indicate to the worker the original locale or relation to cellular function of any isolated particles. Electron microscopic studies have shown that the bacterial cell is much simpler than either plant or animal cells and the cytoplasm does not include the wide range of particulate structures found in animal and plant cells but contains only two or three types of particulate structures. These structures have been shown to be ribosomes, lipid inclusions and metaphosphate or volutin granules.

Although homogenate techniques are suitable for enzyme localization studies in animal and plant cells, the size and fragility of bacterial structures limits their use. Repaske (1954) solubilized succinic dehydrogenase activity from A. vinelandii particles by sonication. Hydrogenase activity was also solubilized in a similar manner from Hydromonas facilis by Atkinson and McFadden (1954). Others have also demonstrated the solubilization of enzymes from particulate matter by sonication and other similar methods of cell rupture used in homogenate techniques.

Tissieres, Hovencamp and Slater (1957) demonstrated similar enzyme activities in two distinct particle sizes fractionated from homogenates of A. vinelandii. Many other workers have reached similar conclusions working with a wide variety of bacteria. These examples indicate the limitations and dubious results obtained using homogenate techniques for enzyme localization studies.

Extensive literature has appeared on the isolation of electron transport, cytochrome and other enzymatically

active particles from bacterial cells after homogenization. These topics have been ably reviewed by Schachman, Pardee and Stanier (1952), Mitchell (1955), Alexander (1956), and Marr (1960 a,b).

Weibull (1953 c), Mitchell and Moyle (1956, 1957), Marr and Cota-Robles (1957), Nakada, Nozu and Kondo (1958), Storch and Wachsman (1957 a,b) and DeLey and Dochy (1960 a, b) have reported that these electron transport particles and all other enzymatically active particles prepared from bacteria are probably derived by the comminution of the cytoplasmic membrane. In 1959, Tuttle and Gest demonstrated that chromatophores of Rhodospirillum rubrum do not exist free in the cytoplasm but are part of the cytoplasmic membrane, contradicting earlier results obtained in photosynthetic bacteria.

In 1957 Marr and Cota-Robles introduced a technique of intermittent sonication. Sonic extracts are fractionated and assayed for enzyme activity after various time intervals of sonication. They postulated that if most of the activity of an enzyme is found in the soluble fraction

after short-term sonication and remains soluble after long-term sonication the enzyme is a soluble enzyme of the cytoplasm. Conversely, if most of the activity does not appear in the soluble fraction and is not found in the small particles after short-term sonication and becomes solubilized or associated with the submicroscopic particles after long-term sonication, the enzyme is probably associated with the cell hull. They found that the majority of submicroscopic particles obtained after short-term sonication contained ribose nucleic acid and were devoid of enzyme activity.

Marr and Cota-Robles (1957) using this intermittent sonication technique found glucose-6-phosphate dehydrogenase to be a soluble enzyme in A. vinelandii, while the cytochromes were considered to be derived from the cell hull. Using similar techniques DeLey and Dochy (1960 b) located galactose, gluconate, 2-ketogluconate and d-lactate oxidases (dehydrogenases) in the hulls of Gluconobacter liquifaciens and glucose-6-phosphate dehydrogenase in the soluble cytoplasm. In Acetobacter rancens they demonstrated that glucose oxidase was a hull

enzyme.

The technique of intermittent sonication appears to be the only homogenate technique applicable to bacterial enzyme localization studies. Due to the small size and fragility of bacterial structures other homogenate techniques seem too drastic and may lead to erroneous results and inconclusive findings in enzyme localization studies.

(b) Sphaeroplast Lysis

Weibull (1953 b) first formed and isolated sphaeroplasts of B. megaterium after lysozyme treatment of intact cells in a stabilizing solution of sucrose. In a later work, Weibull (1953 c) osmotically lysed the sphaeroplasts of B. megaterium and fractionated the lysate into six fractions; a ghost fraction derived from the cytoplasmic membrane, four particulate fractions derived from the cytoplasmic particles, and a soluble cytoplasmic fraction. These isolated sphaeroplastic fractions presented a more reliable picture of cellular morphology

than homogenate extracts.

In his original work with B. megaterium sphaeroplasts Weibull (1953 c) localized the cytochromes as part of the membrane fraction. Storch and Wachsman (1957 a, b) localized succinate, lactate, fumarate and fifty percent of the malic dehydrogenase activities as well as the cytochromes in the membrane fraction of B. megaterium sphaeroplasts. They also found pyruvate, glucose, citrate, cis-aconitic, the majority of α-ketoglutarate and fifty percent of the malic dehydrogenases in the soluble cytoplasm.

Weibull, Beckman and Bergström (1959) added DPNH oxidase to the list of enzymes localized on the membrane and found isocitric dehydrogenase, catalase, hexokinase and acid phosphatase in the soluble cytoplasm of B. megaterium sphaeroplasts. They again found malic dehydrogenase activity evenly divided between the membrane and soluble cytoplasm fractions.

Beckett and Robinson (1957) located a nitrofurazone reductase on the membranes of B. megaterium sphaeroplasts.

Brenner (1955) lysed B. megaterium sphaeroplasts with ribonuclease and stated that the membrane probably contains ribonucleic acid. Landman and Spiegelman (1955) induced β-galactosidase activity in B. megaterium sphaeroplasts.

Mitchell and Moyle (1956) localized the cytochromes, succinic, formic and malic dehydrogenases, the malic enzyme, acid phosphatase and α-glyceroldehydrogenase activities in the membrane of Staph. aureus sphaeroplasts. In 1957, these same workers calculated the osmotic pressure of the S. aureus sphaeroplasts to be about 20 atmospheres.

Gel'man, Zhukova and Oparin (1959, 1960) found malic acid dehydrogenase and the necessary electron transport system for malic acid oxidation on the membrane of M. lysodeikticus. In 1960 Gel'man, Lukoyanova and Oparin found cytochromes b, c and a, succinic dehydrogenase and cytochrome oxidase in the membranes of M. lysodeikticus sphaeroplasts.

Sphaeroplast lysis of Ps. fluorescens has shown that nicotinic acid hydroxylase activity is associated with the membrane-wall fraction (Hunt, 1959) and mandelic

dehydrogenase activity is also found in the membrane fraction (Nakado and Nozu, 1958).

Tuttle and Gest (1959) found that the chromatophores of Rhodospirillum rubrum were associated with the cytoplasmic membrane of the sphaeroplast. Membrane particles of Al. faecalis derived by sonication have carried out oxidative phosphorylation to a limited extent (Beer, 1960).

DeLey and Dochy (1960 a) have shown that the membrane or hull of lysed Acetobacter suboxydans sphaeroplasts is capable of oxidizing a large number of constitutive hexoses, pentoses, gluconate and 2-ketogluconate. A protease has been localized in both the membrane and soluble cytoplasmic fraction of E. coli sphaeroplasts (Chaloupka, 1961). McQuillen, Roberts and Britten (1959) reported that membrane RNA of E. coli sphaeroplasts is a better protein producer than the free ribosomal RNA found in the cytoplasm.

Sheinin (1959) established a new method for collecting fractions of sphaeroplasts after lysis. The wall fractions, soluble and particulate, were separated

by differential centrifugation from the sphaeroplasts of B. cereus prior to osmotic lysis of the sphaeroplasts. This method was used to localize enzymes associated with the cell wall and the outside of the cytoplasmic membrane. Sheinin (1959) reported that β-penicillinase is a soluble enzyme associated with the wall-outer membrane fraction and γ-penicillinase is found in the membrane fraction of B. cereus.

Using a similar fractionation procedure Murti (1960) localized DPNH cytochrome c reductase, cytochrome oxidase and succinic oxidase activity in the membrane fraction of E. coli and Vibrio cholerae. Isocitric, glucose-6-phosphate and malic dehydrogenases, aspartic acid and adenosine deaminases, aldolase, histidase and isocitritase activity were found in the soluble cytoplasm. The wall fractions were devoid of activity for any of these enzymes.

Malamy and Horecker (1961) found alkaline phosphatase activity in the soluble wall fraction of E. coli after sphaeroplast conversion and fractionation. This enzyme seems to be present in the outermost layer of membrane and is easily released by gentle washing of

membrane preparations.

Abrams, McNamara and Johnson (1961) "metabolically" lysed sphaeroplasts of Streptococcus faecalis by adding glucose to the sphaeroplast suspension. As glycolysis proceeds in the sphaeroplasts they lyse. This lysis may be due to the increased osmotic pressure in the sphaeroplasts. During the course of this work the authors localized ATPase activity in the membrane fraction.

The use of sphaeroplasts has proved to be an invaluable tool in bacterial enzyme localization studies. The fractions isolated after sphaeroplast formation and lysis reproduce anatomical structures far more accurately than any method previously devised. The use of sphaeroplasts has been extended into the fields of electron microscopy, protein synthesis and other similar lines of research where work on intact cells or other types of cellular extracts has proved unrewarding.

Criteria Used in Bacterial Enzyme Localization Studies

Certain criteria must be adhered to in bacterial enzyme localization studies to establish quantitative

validity of results. Alexander and Wilson (1955) refined the qualifications originally proposed by Hogeboom and Schneider (1950) for use in isolated bacterial fractions. These requirements are: (1) the sum of the enzymatic activities of the isolated fractions must be equal to or greater than the amount found in the unfractionated extract; (2) the structure to which the enzyme activity is attributed must have a large percentage of activity and, (3) the fraction to which the activity is attributed must have a concentration (specific activity) equal to or greater than the unfractionated extract.

In 1959, Mathews and Sistrom proposed that two additional criteria should be added for valid results. These criteria are: (1) the activity of the fraction to which the enzyme is attributed should represent a corresponding activity in the intact cell, (2) and if two or more activities are found in one fraction, the ratio of these activities should correspond to a similar ratio found in the intact cell.

In theory these latter two proposals seem valid. In many cases enzyme activities may be quantitatively

assayed in cell fractions but these same assays may be impossible to carry out in intact cells due to the non-permeability of the enzyme substrate, coenzymes or a blockage due to competition by enzyme systems. The first proposal of Mathews and Sistrom is similar to the first proposal of Alexander and Wilson but the latter seems more valid and can be put into practice at all times, whereas the proposal of Mathews and Sistrom is impossible to evaluate in many cases.

The same argument concerning intact cells holds true for the second proposal of Mathews and Sistrom. Another point must also be taken into consideration conerning the second proposal of Mathews and Sistrom. Enzymes released from the cell may undergo different rates of inactivation depending on the extraction conditions. If two enzymes have different rates of inactivation with a given method of fractionation, the ratios of fractionated enzymes and the enzymes in the intact cells, if amenable to assay, may differ greatly although the activities have been properly localized.

If the second proposal of Mathews and Sistrom stated that the ratio of enzyme activites found in any fraction was equal to a similar ratio of an unfractionated extract prepared in the same manner as the fractionated extract, the proposal would be more valid.

The first workers in the field of enzyme localization followed no set code of nomenclature for centrifugal fractionation studies and have left no clear record of the procedures they utilized. Alexander and Wilson (1955) proposed a nomenclature to standardize centrifugal fractionation studies. Instead of using speed and duration of centrifugation, which is meaningless unless the author reports the instrument utilized, they proposed that a correct nomenclature would utilize gravitational force (g) and the duration of centrifugation. They also proposed that the supernatant solution after centrifugation should be termed "s" and the precipitate or centrifugal pellet obtained be designated as "p". Thus 100s120 would mean a supernatant solution obtained after 120 minutes centrifugation (suffix of the fraction symbol in minutes) at 100×10^3 g

(prefix of the fraction symbol x 10^3 g) and 20p60 would mean a precipitate obtained after 60 minutes centrifugation at 20 x 10^3 g. Many workers have started to use this nomenclature and their reports hold more meaning for future workers in this field.

MATERIALS AND METHODS

MATERIALS AND METHODS

Growth Conditions and Preparation of Sphaeroplasts

Escherichia coli var. *mutabilis* (ATCC #9980) was used in all experiments described here. The culture was routinely maintained on nutrient agar slants. Sphaeroplasts were prepared by a slight modification of the penicillin method first described by Lederberg (1956).

A 0.01 ml standard loop of a 24 hour nutrient broth culture of the cells was transferred into 170 ml of sterile Penassay broth (Difco) in a 500 ml Erlenmeyer flask. The flask was shaken on a rotary shaker for 12 hours at room temperature. The contents of the flask were then transferred into a 1 l Erlenmeyer flask containing 340 ml of sterile Penassay broth supplemented with sufficient sucrose, $MgSO_4$ and penicillin (potassium salt) to give final concentrations of 0.5 M sucrose, 0.01 M $MgSO_4$, and 1,000 I.U. of penicillin per ml. The flask was then incubated at room temperature on a rotary shaker and at various time intervals hanging drop suspensions were viewed under the Phase

Contrast microscope. After 1-3 hours sphaeroplast formation appeared to be complete resulting in 90-100% conversion.

Purification of Sphaeroplasts

All operations were carried out at -5° to +5° C. In each experiment four 500 ml batches of sphaeroplast suspensions were pooled. The sphaeroplasts were sedimented by centrifugation at 3,000 x g for 15 minutes and the resultant pellets were carefully resuspended in 400 ml of cold 0.5 M sucrose. The suspensions were again centrifuged at 1,000 x g for 15 minutes and the sediment containing intact cells was discarded. The sphaeroplasts remaining in the supernatant fluid were washed twice by repeated centrifugation at 3,000 x g for 15 minutes and resuspended each time in 400 ml of cold 0.5 M sucrose.

After the second wash, the sphaeroplasts were suspended in 50 ml of cold 0.5 M sucrose and centrifuged at 10,000 x g for 15 minutes to form a compact pellet. This pellet was then weighed out and used for further experimentation.

In the manometric assays of succinic acid dehydrogenase

activity and oxygen uptake during oxidative phosphorylation the endogenous respiration was found to be extremely high. Sphaeroplasts used in these assays were purified at room temperature in the same manner as previously described. After the second wash the sphaeroplasts were resuspended in 1 l 0.5 M sucrose and allowed to respire for 4 hours at room temperature. After respiration the sphaeroplasts were sedimented at 3,000 x g, cooled in 50 ml of 0.5 M sucrose and treated in the usual manner.

Fractionation of Sphaeroplasts for Localization Studies

All fractionations were carried out at 4° C. One g wet weight of purified, washed sphaeroplasts was lysed in 30 ml of a buffer containing 0.001 M cysteine, 0.01 M $MgSO_4$, and 0.001 M Tris-HCl, pH 7.5. After lysis, 1 mg of crystalline desoxyribonuclease was added to reduce the viscosity. The suspension was gently stirred for 30 minutes prior to fractionation. The fractionation procedure used is outlined in figure 1 (page 26).

The various fractions were stored at 0°-4° C during enzyme assays and prior to oxidative phosphorylation studies.

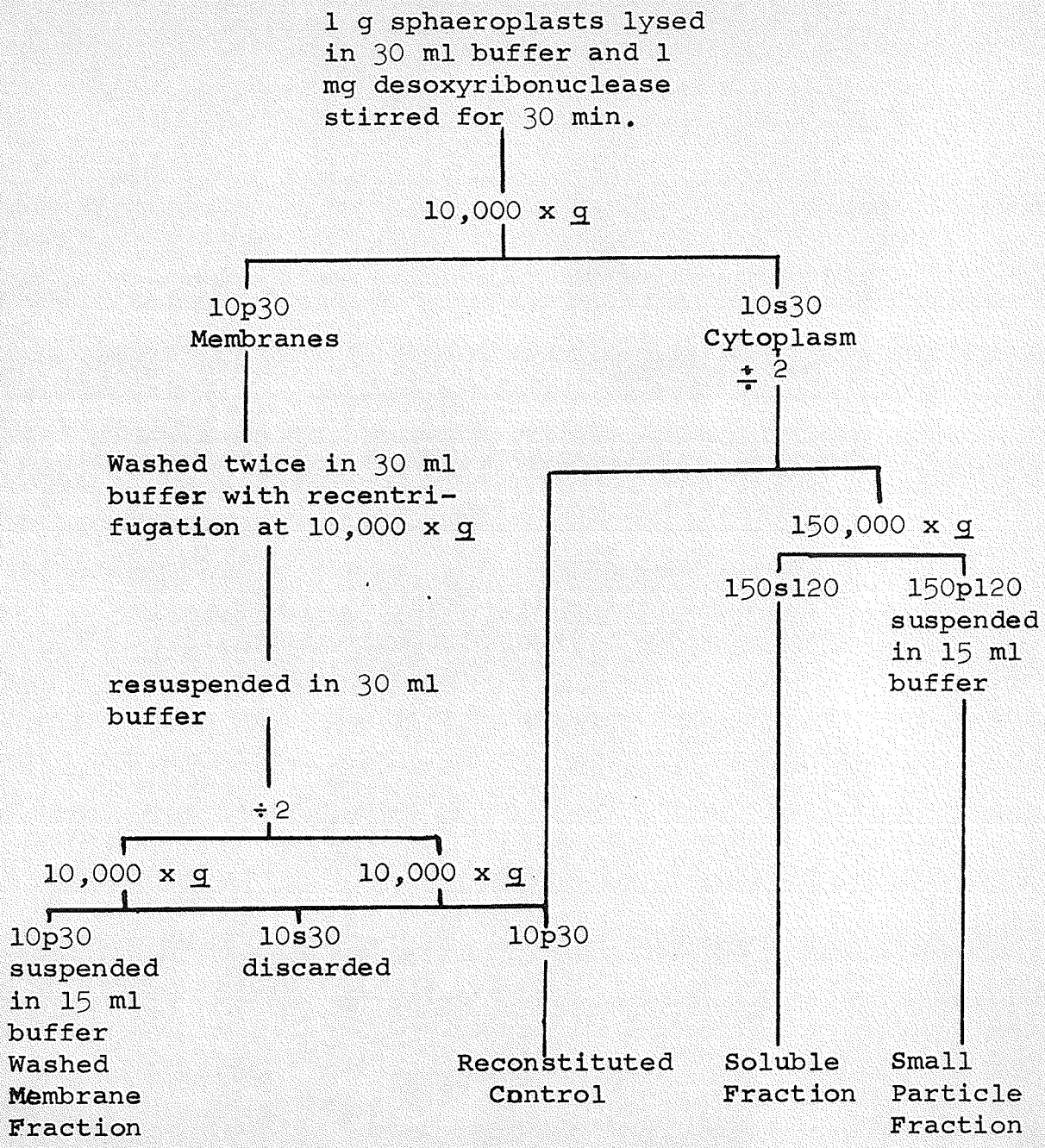

Figure 1. Summary of fractionation procedure used for the isolation of various components of the cells. Centrifugal symbols are similar to those used by Alexander and Wilson (1955; see 'Historical')

Protein and nucleic acid content of the various fractions was determined either immediately or after storage at -20° C for a few days.

Enzyme Assays

Aconitase:

Aconitase activity was measured spectrophotometrically at 240 mμ by following the amount of cis-aconitic acid formed from citrate after a method described by Anfinsen (1955). The reaction mixture contained: 2.9 ml of 0.03 M citrate in 0.05 M phosphate buffer, pH 7.4, and 0.1 ml of a suitably diluted enzyme preparation in quartz cuvettes of 1 cm light path. The reference cuvette contained 3.0 ml of 0.1 M phosphate buffer without the enzyme. One unit of the enzyme is defined as the amount causing an increase in optical density of 0.001 per minute.

Fumarase:

Fumarase was assayed by following the disappearance of malate at 290 mμ according to the method of Racker (1950). The reaction mixture contained: 2.8 ml of 0.05 M phosphate

buffer, pH 7.4; 0.1 ml of 0.05 M sodium l-malate, and 0.1 ml of enzyme in quartz cuvettes of 1 cm light path. The reference cuvette lacked the substrate. One unit of enzyme is defined as the amount causing an optical density increase of 0.001 per minute under the above conditions.

Glucose-6-phosphate dehydrogenase:

Glucose-6-phosphate dehydrogenase activity was measured by following the reduction of TPN spectrophotometrically at 340 mμ after an assay procedure described by Waygood and Rohringer (1962). The reaction mixture contained in a final volume of 2.0 ml: 0.1 ml of 0.5 M Tris-HCl buffer, pH 7.5; 0.2 ml of 0.1 M $MgCl_2$; 0.1 ml of 3×10^{-3} M TPN; 0.1 ml of 0.02 M glucose-6-phosphate; enzyme, and water. The reference cuvette lacked TPN. A unit of enzyme is defined as the amount which causes an initial optical density increase of 1.0 per minute.

Glutamic acid dehydrogenase:

Glutamic acid dehydrogenase activity was assayed spectrophotometrically at 340 mμ by following the oxidation of TPNH (Sanwal and Lata, 1961). The assay mixture

contained: 20 μmoles α-ketoglutarate; 120 μmoles ammonium sulphate; 0.40 μmoles TPNH; 273 μmoles Tris-HCl buffer, pH 7.5, and enzyme in a final volume of 3.0 ml. The reference cuvette lacked α-ketoglutarate. One unit is defined as the amount of enzyme causing an optical density decrease of 0.001 per minute.

Isocitric dehydrogenase:

Isocitric dehydrogenase activity was assayed by measuring the reduction of TPN spectrophotometrically at 340 mμ following the method of Kornberg (1955 b). The incubation mixture contained: 0.2 ml of 0.5 M phosphate buffer, pH 7.0; 0.1 ml of 0.1 M $MgCl_2$; 0.02 ml of .025 M TPN; 0.1 ml of 0.005 M d,l-isocitrate; water, and enzyme to a final volume of 3.0 ml. The reference cuvette lacked d,l-isocitrate. One unit of the enzyme is defined as the amount causing the reduction of 1 μmole of TPN in 20 minutes.

Lactic dehydrogenase:

Lactic dehydrogenase was assayed spectrophotometrically by following the oxidation of DPNH at 340 mμ after the method of Kornberg (1955 a). The assay system

contained in a final volume of 3.0 ml: 1.5 ml 0.1 M phosphate buffer, pH 7.4, 0.1 ml of 0.002 M DPNH, 0.1 ml of 0.01 M sodium pyruvate, water and enzyme. The reference cuvette lacked pyruvate. One unit of activity is defined as that amount of enzyme which causes a decrease in optical density of 1 per minute.

Malic acid dehydrogenase:

Malic acid dehydrogenase activity was assayed by following the oxidation of DPNH spectrophotometrically at 340 mμ (Ochoa, 1955). The assay system contained: 0.3 ml of 0.25 M glycine buffer, pH 7.4; 0.1 ml of 0.0015 M DPNH; 0.1 ml of 0.0075 M oxalacetate, pH 7.4; water, and enzyme in a final volume of 3.0 ml. The reference cuvette lacked DPNH. One unit of activity is defined as that amount of enzyme which causes a decrease in optical density of 0.01 per minute.

Succinic acid dehydrogenase:

The manometric method of Singer and Kearny (1957) was followed to assay succinic acid dehydrogenase. The main compartment of the Warburg vessel contained: 0.5 ml

of 0.3 M phosphate buffer, pH 7.6; enzyme and water to give a final volume of 1.9 ml. The side arm contained: 0.3 ml of 0.2 M succinate, and 0.2 ml of 1% phenazine methosulphate. Three tenths ml of 0.01 M HCN was added to the main compartment just prior to assay. The control contained all of the above reagents with the exception of succinate. One unit of the enzyme is defined as the amount causing an oxygen uptake of 1 µl per hour.

Cytochromes:

Prior to assay the various fractions were concentrated to 5 ml by freeze drying. Two cuvettes of 1 cm light path were filled with 2 ml of the same fraction and the difference in optical density was scanned over the range of 350-700 mµ using a spectrophotometer. A small amount of sodium dithionite was added to one of the cuvettes and the same range was scanned again. The peaks of the reduced cytochromes were used for identification of the cytochromes present in the fraction.

Determination of Protein and Nucleic Acid

Protein:

Quantitative protein determinations were carried out colorimetrically using Folin-Ciocalteu Reagent after the method of Lowry, Rosebrough, Farr and Randall (1951). A standard curve was made using crystalline bovine albumen.

Nucleic acid:

The quantity of nucleic acid was determined spectrophotometrically by measuring the ratio of optical density at 280 and 260 mμ after the method of Warburg and Christian (1942).

Oxidative Phosphorylation Studies

In the oxidative phosphorylation experiments, phosphate esterification was measured by trapping the esterified phosphate in the form of glucose-6-phosphate as described by Aleem and Nason (1960). If catalytic amounts of ADP (0.2 μmoles) are used and the system contains glucose, hexokinase and Mg^{++} ions, the esterified phosphate will be trapped as glucose-6-phosphate and ADP will be released for further

phosphorylation as described in the following equations:

$$P_i + ADP \xrightarrow[\text{phosphorylation}]{\text{oxidative}} ATP$$

$$\text{glucose} + ATP \xrightarrow[\text{hexokinase}]{Mg^{++}} \text{glucose-6-}PO_4 + ADP$$

Oxygen uptake was measured manometrically in Warburg vessels containing: 10 μmoles K_2HPO_4/ml reaction mixture, 0.2 μmoles ADP, 15 μmoles $MgCl_2$, $P^{32}O_4$ (100,000 cpm), 0.5 mg hexokinase, 50 μmoles glucose, 0.3 M Tris-HCl buffer, pH 7.6, and enzyme in the main flask. The centre well contained 0.2 ml 20% KOH. Twenty μmoles of the various substrates tested were placed in the side arm. The final volume was 2.7 ml. The flasks were equilibriated for 5 minutes at 25° C, then the stopcocks were closed and the flasks were equilibriated for a further 5 minutes. After equilibriation the contents of the side arms were tipped into the flasks and readings were taken at 15 minute intervals for 1 hour.

After calculation of oxygen uptake, the inorganic

$P^{32}O_4$ was extracted according to a modified method of Rose and Ochoa (1956). The reaction mixture was deproteinized by addition of cold 10% trichloracetic acid. The $P^{32}O_4$ of the supernate of the trichloracetic acid precipitation was extracted by successively extracting the phospho-molybdate complex with a 50/50 v/v mixture of isobutanol/benzene and anhydrous diethyl ether was used for the final extraction. When ascorbate, DPN, or DPNH were used in the reaction mixtures, isobutanol instead of the 50/50 v/v mixture of isobutanol/benzene was used to extract the phospho-molybdate complex. Isobutanol is used to extract the phospho-molybdate complex from reaction mixtures containing pyridine nucleotides and/or ascorbate and has proved superior to the isobutanol/benzene mixture for phosphate extraction in these cases.

Total counts directly from the 10% trichloracetic acid supernate and counts of the various samples in which the $P^{32}O_4$ was extracted were carried out on a Model D-47 gas flow counter (Nuclear Chicago).

RESULTS

RESULTS

Sphaeroplast Formation and Lysis

Although many attempts were made to induce sphaeroplast formation by following published procedures, the only method yielding sphaeroplasts that could be easily lysed was that of Lederberg (1956). Studies on growth of *E. coli* var. *mutabilis* in Penassay broth showed that the cells reached their maximum stationary phase in 12 hours. In succeeding experiments therefore the cells were grown for 12 hours prior to sphaeroplast formation. There was an appreciable lag in growth when pregrown cells were inoculated in the penicillin-sucrose fortified Penassay broth.

Owing to this lag, perhaps, the cells required 1-3 hours for sphaeroplast formation. By trying different centrifugal force-time combinations it was found that the sphaeroplasts could be sedimented by centrifugation at 3,000 x *g* for 15 minutes and that intact cells could be sedimented down from the sphaeroplast suspension by

centrifugation at 1,000 x g for 15 minutes. These steps were incorporated in the procedure used to purify the sphaeroplasts for enzyme studies.

The osmotic pressure of the sphaeroplasts was calculated by resuspending fixed amounts of sphaeroplasts in gradually increasing concentrations of sucrose and measuring the optical density against a 0.1 M sucrose blank at 660 mμ. The highest optical density was found in the 0.3 M sucrose tube and the optical density on either side of the peak fell off sharply. This result corresponds to an osmotic pressure of about 6-7 atmospheres.

Complete lysis of the sphaeroplasts was achieved by resuspending 1 g of sphaeroplasts in 25 ml of distilled water. When the sphaeroplasts lysed desoxyribonucleic acid was released in a highly polymerized form and tended to make the lysate viscous. The addition of 1 mg of desoxyribonuclease completely depolymerized the desoxy-ribonucleic acid and reduced the viscosity.

A more appropriate lysing solution was tested in order to preserve ribosomal particle size and to protect thiol enzymes. A buffer solution containing 0.001 M

cysteine, 0.01 M $MgCl_2$ and 0.001 M Tris-HCl buffer, pH 7.5, was tried and was just as effective as the water. In further experiments 1 g of sphaeroplasts was lysed with 30 ml of 0.001 M cysteine-0.01 M $MgCl_2$-0.001 M Tris-HCl buffer, pH 7.5.

Enzyme Localization Studies

A representative group of enzymes previously localized in bacteria were chosen for study. Enzyme assays were first carried out on alumina and sonic extracts to see if the enzymes were present and to perfect the assay procedures. The results of the localization studies in the sphaeroplast fractions are presented in Tables I and II. Linearity, both with regard to enzyme concentration and activity, was established in all assays.

Fumarase, a thiol enzyme, was found to be lacking in all fractions although both alumina and sonic extracts contained small amounts of the enzyme. If fumarase is an unstable enzyme it might have undergone denaturation in the 6 hour period between sphaeroplast lysis and enzyme assay of the fractions.

Aconitase, glucose-6-phosphate dehydrogenase,

glutamic dehydrogenase and isocitric dehydrogenase were found mainly in the soluble fraction. Lactic and succinic dehydrogenases were found mainly in the membrane fraction. Malic dehydrogenase was found in high concentration in both the membrane and soluble fractions.

When the cytochromes were assayed the concentrations were extremely low in both the membrane fraction and the reconstituted control. The soluble and small particle fractions were devoid of cytochromes. Freeze-drying was used to concentrate the various fractions. Both the membrane fraction and the reconstituted control exhibited peaks at 415-430 mμ, 480 mμ, 550 mμ, 565 mμ and 610 mμ. These peaks correspond to the following substances; the soret band of cytochromes a, b and c; a reduced flavin; cytochrome c; cytochrome b; and cytochrome a_4.

Protein and Nucleic Acid Content

The results of the protein and nucleic acid determinations are found in Table III. The largest quantity of the protein is found in the soluble fraction and the major part of the nucleic acid is found in the

small particle fraction. The ratio of protein:nucleic acid in the small particle or ribosomal fraction is 1.6 which compares favourably with the ratio of 1 which other workers have reported.

Oxidative Phosphorylation Studies

Using succinate as substrate for oxidative phosphorylation studies, the first attempts showed no difference in oxygen uptake between the control flasks and the test flasks. The same results were obtained previously when succinic dehydrogenase was assayed. The oxygen uptake of both the control and test flasks was high indicating a high endogenous succinate concentration. Only the reconstituted control and the membrane fraction showed this oxygen uptake.

To reduce this endogenous substrate, intact sphaeroplasts were allowed to respire 1-18 hours at room temperature in 0.5 M sucrose. After 4 hours there was an appreciable difference in the oxygen uptake between the control and test flasks. This difference in oxygen uptake did not increase if the time of endogenous respiration

was increased.

Using succinate, DPNH, α-ketoglutarate and malate as substrates, the reconstituted control and washed membrane fraction gave almost similar values of oxidative phosphorylation. No oxygen uptake or phosphate esterification was observed using the soluble or small particle fractions. 2,4-dinitrophenol was tested as an uncoupler with succinate as substrate. DPN, ascorbate and cytochrome c were added as accessory factors. The results using the washed membrane fraction are shown on Table IV.

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TABLE I

Relative amounts[1] of enzymes found in the isolated fractions of *E. coli* sphaeroplasts

Enzyme	Reconstituted Control	Washed Membranes	Soluble Fraction	Small Particle Fraction
Aconitase	1500.00	45.00	1650.00	0.00
Fumarase[2]	0.00	0.00	0.00	0.00
Glucose-6-phosphate dehydrogenase	1.57	0.08	1.88	0.00
Glutamic dehydrogenase	2250.00	150.00	3000.00	0.00
Isocitric dehydrogenase	15.50	0.30	13.10	0.00
Lactic dehydrogenase	15.90	12.00	3.00	0.00
Malic dehydrogenase	540.00	540.00	720.00	0.00

Table I (cont'd)

Succinic dehydrogenase	750.00	825.00	0.00	0.00

[1]These values are expressed as the total number of enzyme units found in each fraction.

[2]Fumarase activity was absent from all fractions isolated although alumina and sonic extracts of intact cells contained the enzyme in low concentration.

TABLE II

Relative enzyme concentration[1] of fractions isolated from sphaeroplasts of *E. coli*

Enzyme	Reconstituted Control*	Washed Membranes	Soluble Fraction	Small Particle Fraction
Aconitase	1.00	0.17	1.40	0.00
Fumarase[2]	0.00	0.00	0.00	0.00
Glucose-6-phosphate dehydrogenase	1.00	0.29	1.65	0.00
Glutamic dehydrogenase	1.00	0.40	1.65	0.00
Isocitric dehydrogenase	1.00	0.11	1.18	0.00
Lactic dehydrogenase	1.00	4.34	0.24	0.00
Malic dehydrogenase	1.00	5.76	1.29	0.00

Table II (cont'd)

Succinic dehydrogenase	1.00	6.33	0.00	0.00

[1]Concentration refers to enzyme units/mg protein.

*The concentration of each enzyme in the reconstituted control is made equal to 1.00.

[2]Fumarase activity was absent from all fractions isolated although alumina and sonic extracts of intact cells contained the enzyme in low concentration.

TABLE III

Protein and nucleic acid content of the isolated fractions of *E. coli* sphaeroplasts

Fraction	Protein		Nucleic acid	
	Amount*	% of total	Amount*	% of total
Washed Membranes	98 mg	17.3	1.2 mg	5.9
Soluble Fraction	440 mg	77.7	1.2 mg	5.9
Small Particle Fraction	28 mg	5.0	18.0	88.2
	566 mg		20.4 mg	
Reconstituted Control	564 mg		18.4 mg	

*These values are the total amount (mg) found in each fraction.

TABLE IV

Oxidative phosphorylation by the washed membrane fraction

Substrate	Accessory Factors	μmoles $P^{32}O_4$ esterified per hour[1]	μatoms O_2 uptake per hour[2]	P/O ratio
Succinate	-	0.48	9.6	0.05
Succinate	10^{-3} M DNP	0.47	9.5	0.05
Succinate	10^{-4} M DNP	0.44	9.0	0.05
Succinate	.33 mg DPN per vessel	0.42	10.4	0.04
Succinate	1 mg/ml cyt. c 10^{-2} M ascorbate	0.75	12.4	0.06
DPNH	-	1.33	22.2	0.06
α-ketoglutarate	.33 mg DPN per vessel	0.62	7.7	0.08

Table IV (cont'd)

Malate	.33 mg DPN per vessel	0.81	4.5	0.18

[1]These values have been corrected for both background count and phosphate esterification of the control.

[2]These values have been corrected for endogenous oxygen uptake (control).

DISCUSSION

DISCUSSION

Keeping the original aim of the work, enzyme localization, in mind one must evaluate the methods and techniques employed. The use of sphaeroplast lysis seems superior to both homogenate and cytochemical techniques for enzyme localization studies. The fractionation of the sphaeroplast lysate into various fractions must also be evaluated.

Using enzyme markers, glucose-6-phosphate dehydrogenase as the soluble marker and succinic dehydrogenase as the particulate or membrane marker, one can ascertain the purity of the fractions obtained.

The results presented in Table I indicate that the washed membrane fraction is contaminated with four percent (glucose-6-phosphate dehydrogenase) of the soluble fraction. However, the soluble and small particle fractions are not contaminated by membrane or particulate enzymes derived from the membrane as indicated by the succinic dehydrogenase marker.

Adhering to the modified criteria of Alexander and

Wilson (1955, see 'Historical') originally proposed by Hogeboom and Schneider (1950) the results found in Tables I and II can be evaluated. Aconitase activity was confined for the greater part to the soluble fraction. The small amount of activity found in the membrane fraction lies within the four percent contamination of the membrane fraction by the soluble fraction. This enzyme has not been localized previously in bacterial sphaeroplasts.

Fumarase activity was absent from all fractions isolated although alumina and sonic extracts contained small amounts of the enzyme. This result could be due to one of two causes: (1) the enzyme is located between the cell wall and the outer membrane similar to alkaline phosphatase (Malamy and Horecker, 1960) and β-penicillinase (Sheinin, 1959) or (2) the enzyme is unstable and was denatured in the six hour period between sphaeroplast lysis and enzyme assay.

It is doubtful that the enzyme is located on the outer surface of the sphaeroplast. Both Weibull,

Beckman and Bergström (1959) and Alexander (1956) reported that fumarase is found mainly as a soluble enzyme. It is known that fumarase is a thiol enzyme and is not too stable. Although 0.001 M cysteine was added to the lysing buffer solution, the enzyme may have been inactivated during the six hour period of sphaeroplast fractionation.

Glucose-6-phosphate dehydrogenase activity was confined mainly to the soluble fraction and was used as an enzyme marker to determine contamination of the membrane and small particle fractions by the soluble fraction. This finding is in agreement with previous results obtained by Murti (1960) working with E. coli and Vibrio cholerae sphaeroplasts.

The soluble fraction also contained the majority of the glutamic dehydrogenase activity. The small amount of activity residing in the membrane fraction may be due to contamination of this fraction by the soluble enzyme. This enzyme, also, has not previously been localized in bacterial sphaeroplasts.

Isocitric dehydrogenase activity was found in high concentration in the soluble fraction. This enzyme was previously localized in the soluble fraction of B. megaterium sphaeroplasts by Weibull, Beckman and Bergström (1959).

Lactic dehydrogenase activity was found mainly in the membrane fraction although twenty per cent of the activity was localized in the soluble fraction. This result could be due to three different causes; (1) that there are two distinct lactic dehydrogenases, one located on the membrane the other localized in the solube fraction; (2) lactic dehydrogenase is easily solubilized off the membrane, and (3) that there has been a comminution of the membrane during lysis.

The results of Storch and Wachsman (1957 a, b) obtained with B. megaterium sphaeroplasts indicate that the enzyme is localized on the membrane while those of Weibull, Beckman and Bergström (1959) show that the enzyme was present in both the membrane and soluble fractions. These results may lead to the conclusion that the enzyme is located on the membrane and may be easily

solubilized although it does not rule out the possibility of two distinct lactic dehydrogenases.

The third possibility may be rejected as indicated by the lack of succinic dehydrogenase, the membrane marker, in the soluble fraction. Although no final conclusion can be drawn the results show a high concentration (specific activity) in the membrane fraction.

Similarly the malic dehydrogenase activity was divided between the membrane and soluble fractions. These results are much the same as those obtained in B. megaterium sphaeroplasts (Storch and Wachsman, 1957 a, b; Weibull, Beckman and Bergström, 1959). Mitchell and Moyle (1956) localized malic dehydrogenase in the membranes of Staph. aureus sphaeroplasts while Murti (1960) localized malic dehydrogenase in the soluble fraction of E. coli and Vibrio cholerae sphaeroplasts. The high concentration of malic dehydrogenase activity in both the membrane and soluble fractions points to the possibility of two distinct enzymes although the possibility of a

membrane enzyme that is easily solubilized may not be excluded.

Succinic dehydrogenase activity was localized solely in the membrane fraction. Similar results have been reported in sphaeroplasts of B. megaterium (Storch and Wachsman, 1957 a, b; Weibull, Beckman and Bergström, 1959), Staph. aureus (Mitchell and Moyle, 1956), M. lysodeikticus (Gel'man, Lukoyanova and Oparin, 1960), E. coli, and Vibrio cholerae (Murti, 1960).

The cytochromes, the terminal oxidase system and the electron transport system are located on the membrane as indicated by the cytochrome assay and oxidative phosphorylation studies. The cytochromes have been previously localized on the sphaeroplast membranes of B. megaterium (Weibull, 1953 c), Staph. aureus (Mitchell and Moyle, 1956), and M. lysodeikticus (Gel'man, Zhukova and Oparin, 1959, 1960; Gel'man, Lukoyanova and Oparin, 1960).

Oxidative phosphorylation studiesshow that the terminal oxidase system is located on the membrane (see

Table IV) and although the P/O ratios are quite low the only fraction capable of carrying out oxidative phosphorylation is the membrane fraction. Beer (1960) reported that the membranes of Alcaligenes faecalis sphaeroplasts carried out oxidative phosphorylation to a limited extent.

No uncoupling of oxidative phosphorylation was observed with 2,4-dinitrophenol. Aleem and Nason (1960) reported similar findings with Nitrobacter agilis particles. Ascorbate and cytochrome c together raised the P/O ratio with succinate as substrate. This may have been due to a reduction of the electron transport chain and perhaps also an increased availability of cytochrome c. Ascorbate or cytochrome c separately had no effect on the P/O ratio.

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