STUDIES ON THE MEMBRANE OF <u>SARCINA LUTEA</u>

A Thesis Presented by

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ABSTRACT.

Mild nonionic detergents were used to solubilize the pigment and two dehydrogenases from the membranes of S. lutea to study their properties.

Previous studies on the membranes of S. lutea were largely on the function of the carotenoid pigments. It was alleged that the pigment is protein bound. No evidence could be found from gel filtration and electrophoretic experiments for association of the pigment with protein. Analyses showed that the solubilized pigment was phospholipid associated. The pigment was functional in that it protected vitamin K in vitro from visible and near ultra-violet radiation.

L-Malate and NADH dehydrogenases were completely solubilized from the membranes. Properties of the two enzymes in the membrane-bound and the solubilized state were studied. The activity of the bound Malate dehydrogenase was affected by modulators:

(a)   Cl' ions, oxaloacetate, succinate and α-ketoglutarate caused inhibition to a small extent.

(b)   adenosine phosphates inhibited the activity strongly in the order ATP $>$ ADP $>$ AMP.

(c)   Nucleotides containing purine bases (NAD and NADP) in the oxidised state strongly inhibited and in the reduced state activated the enzyme activity. Most of these properties were retained by the solubilized enzyme. It was found that the enzyme required phospholipid for full activity.

NADH dehydrogenase was found to be activated by NAD upto 10^{-5} M and inhibited above this concentration.

A method of purification of the two dehydrogenases has been developed and a scheme for regulation of L-Malate metabolism has been proposed.

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ABBREVIATIONS

AMP, ADP, ATP	Adenosine 5' - mono-, di-, and tri-phosphates.
Bis	N, N', Methylenebisacrylamide.
CTAB	Cetyltrimethylammonium bromide.
DEAE	Diethylaminoethyl
DOC	Deoxycholate.
EDTA	Ethylene diamine tetra-acetic acid.
FAD	Flavin adenine dinucleotide.
MK	Menaquinone.
NAD(H), NADP(H)	Nicotinamide adenine dinucleotide and
	Nicotanamide adenine dinucleotide phosphate (reduced).
NBT	Nitro blue tetrazoleum.
PEG	Polyethylene glycol.
PCMB	Para chloromercuribenzoic acid.
PMS	Phenazine methosulphate.
PPG	Polypropylene glycol.
SDOC	Sodium deoxycholate.
SDS	Sodium dodecyl sulphate.
TCA	Trichloroacetic acid.
TEMED	N,N,N',N', tetramethylethylene diamine.

SECTION 1.   INTRODUCTION

1.   INTRODUCTION

A.   GENERAL

The molecular biology of membranes is a vital and active area of study. To be able to study membrane structure and function it is usually necessary to dissociate the membrane into its components. The techniques for extraction and analysis of the membrane lipids are well worked out and are mainly based on the use of organic solvents. These have also been used to extract the proteins, but the resultant irreversible aggregation and denaturation of many membrane proteins limit their usefulness. Partial and selective protein solubilization can be obtained by a number of methods involving chelating agents, manipulating of ionic strength and pH. Protein perturbants (e.g. urea, guanidine HCl, and chaotropic agents) and enzymatic digestion have also been used. However, these procedures do not lead to solubilization of the proteins which are more strongly bound to lipid matrix of the membrane. For such proteins the use of detergents (synthetic detergents, bile salts and saponins), - appears to provide the most generally useful extraction method presently available. I have attempted to selectively solubilize certain enzymes and carotenoid pigments from the membranes of Sarcina lutea for study, using detergents.

Organic solvents have been used to extract carotenoids, the characteristic yellow pigments from the membranes of Sarcina lutea, a Gram-positive bacterium (Thirkell and Strang, 1967). They also isolated these pigments from another closely related species Sarcina flava and found the major pigment to be a caroteno-glycoprotein (Thirkell and Hunter, 1969). Thirkell and Strang showed that the two organisms synthesized identical carotenoids. They, therefore, speculated that in S. lutea the pigment is complexed to proteins as well. The present study aimed at investigating the associations of carotenoids with proteins and lipids. A nonionic detergent was chosen for this purpose. Carotenoids isolated with this relatively mild treatment were presumed to be in their native state - a carotenoid protein complex may be expected to have remained undisturbed. The detergent also solubilized a number of proteins; three of these were found to be enzymes and were in an active state. Properties of one of these enzymes (malate dehydrogenase) were investigated in membrane bound and solubilized state.

Previous studies on carotenoids of S. lutea have investigated their structure and function. (Jensen et al, 1973; Mathews & Sistrom, 1959). Isolation of these pigments enabled me to study their properties and function in vitro.

B.   MEMBRANES

What one can see of bacteria in a light microscope is the bounding surface of the cytoplasm - the cytoplasmic membrane plus the cell wall. The phenomenon of plasmolysis produced the first visual evidence for a cell wall (Fischer, 1891). Conclusive evidence for a cytoplasmic membrane came with the advent of electron microscopy and the allied thin-section techniques (Kellenberger and Ryter, 1958). Some Gram-negative bacteria e.g. Mycoplasma do not have a cell wall and the cytoplasm is enveloped by the cytoplasmic membrane. Numerous functions are carried out by the cytoplasmic membrane: synthesis of proteins, respiration, transport, and synthesis of cell-wall components (Ellar, 1970). It is therefore the site of numerous entities: enzymes, electron transport chain components, ribosomes etc. Some bacterial membranes also contain brightly coloured carotenoid pigments. Membranes thus provide a varied venue for selective study.

The cells of Gram-positive bacteria are characterized by a well developed system of membranes called mesosomes. These are in the form of organelles as well as simple invaginations of the cytoplasmic membrane.

With proper fixation conditions all biological membranes appear in electron microscopy as two $20A^{o}$ wide layers separated by about $35A^{o}$ unstained space. Robertson (1959) referred to this tram line image as "unit membrane" - a phrase used to emphasize that all three layers are part of one single unit - the membrane.

Studies of membranes have been primarily aimed at an understanding of the arrangement of the components of the membranes, which are mainly proteins and lipids. Human erythrocyte membranes have been generally used as these represent the type membrane and give the basic pattern of biological membranes.

C.   THE FLUID MOSAIC MODEL

Biological membranes are chiefly composed of proteins and lipids. Singer and Nicolson (1972) proposed a mosaic structure of alternating globular proteins and phospholipid bilayer for the membranes. The mosaic appears to be a fluid or dynamic one and, for many purposes, is best thought of as a two dimensional oriented viscous solution.

i.   The Proteins of Membranes

It has been suggested by several workers that the proteins associated with membranes can be classified into two broad categories termed (a) peripheral and (b) integral or structural. The experimental criteria for this classification are given in Table 1. In short, peripheral proteins are those that appear to be only weakly bound to their respective membranes and do not appear to interact with the membrane lipids, whereas the integral proteins are ordinarily more strongly bound to the membrane and exhibit functionally important interactions with the membrane lipids. It is presumed that there is a structural basis for this classification; that peripheral and integral proteins are attached to the membrane in distinctly different ways (Singer, 1974).

By one or more criteria listed in Table 1, about 70 - 80% of membrane proteins are easily recognized as integral (Singer, 1974). These include most membrane associated enzymes, antigenic proteins, transport proteins, drug and hormone receptors. Enzymes that are integral proteins usually require lipid (sometimes specific lipids) for their activities to be expressed (Coleman, 1973). Because of their insolubility in ordinary aqueous media, not many integral proteins have been adequately purified and structurally characterized.

The main points of the Fluid Mosaic Model for membrane structure will be enumerated.

(1)   The ionic and polar heads of the lipid molecules together with all of the ionic side chains of the structural protein, are on the exterior surfaces of the membrane in Van der Waals contact with the bulk aqueous phase.

(2)   Sequences of the structural proteins consisting predominantly of non - polar side chains are in the interior of the membrane together with the hydrocarbon tails of the phospholipids and the relatively non polar lipids.

An integral protein molecule with the appropriate size and structure or a suitable aggregate of integral proteins may traverse the entire membrane: that is they have regions in contact with the aqueous solvent on both sides of the membrane.

Integral (structural) proteins are characterized by unique amino acid sequences which specifically adapt them to interact with the lipid components of the membrane and aqueous environment; the overall conformations of

Table 1. Criteria for distinguishing peripheral and Integral
membrane proteins.

Property	Peripheral Protein	Integral Protein
Requirements for dissociation from membrane.	Mild Treatments sufficient: high ionic strength, metal ion chelating agents.	Hydrophobic bond-breaking agents required : detergents, organic solvents, chaotropic agents.
Association with lipids when solubilized	Usually soluble free of lipids.	Usually associated with lipids when solubilized.
Solubility after dissociation from membrane	Soluble and molecularly dispersed in neutral aqueous buffers.	Usually insoluble or aggregated in neutral aqueous buffers.

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structural proteins are determined by these interactions.

(3)   In addition to integral proteins membranes have peripheral proteins associated with them. These might not be intercalated into the membrane interior, but instead be bound by electrostatic or hydrophobic interactions to the exposed surfaces of integral proteins.

(4)   The phospholipids of the mosaic structure are predominantly arranged as an interrupted bilayer. A small specific portion of the lipid may be more intimately associated with the integral proteins forming lipoproteins.

(5)   The thickness of a mosaic membrane would vary along the surface from that across a phospholipid bilayer region to that across a protein region, with an average value that could be expected to correspond reasonably well to experimentally measured membrane thickness.

(6)   Recent experimental results are consistent with the suggestion that the lipid forms the matrix of the mosaic, and since the membrane lipid is generally fluid rather than crystalline under physiological conditions, the mosaic is expected to be a dynamic one; that is the membrane appears to be a two diamensional viscous solution, with its components able to undergo translational diffusion in the plane of the membrane.

(7)   The integral proteins of membranes and sections of phospholipid bilayer alternate to form a mosaic in the plane of the membrane to a depth that is under thermodynamic control (Pinto Da Silva and Branton, 1970; Tillack and Marchesi, 1970; and Boyer and Remsen, 1970). The depth of intercalation is determined by the size of the protein molecule and its ionic charge distribution. The intercalated protein is free of ionic charges and is bound by hydrophobic interactions to the non-polar interior of the membrane.

ii.   Integral Proteins

It is assumed from their properties that the integral proteins interact directly with at least some of the membrane lipids and are involved with the lipids in making up the structural matrix of the membrane. It was proposed (Leonard and Singer,1966; and Wallach and Zahler,1966) that as a general rule, the molecules of integral proteins were more or less globular and amphipathic; that is, their folded three-dimensional structures were segmented into hydrophilic and hydrophobic ends embedded within the nonpolar interior of the lipid bilayer.

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Lenard and Singer (1966) also recognized the possibility that a molecule of an integral protein might span the membrane protruding from both surfaces if it had two hydrophilic ends separated by an appropriate hydrophobic middle segment. By chemical labelling methods (Bretscher et al.,1971; and Sergest et al.,1973) it has been shown that two major proteins of human erythrocyte membrane span the entire thickness of the membrane. These experiments show that the gross morphological features of certain integral proteins are as predicted: they are globular and deeply embedded in the membrane and, in certain instances span the membrane. Amphipathy of integral proteins has been evidenced in reports (Strittmatter, and Spatz,1971). Using nonionic detergents it has been shown that a particular integral protein may be solubilized with complete retention of its enzymic or antigenic property; indirectly suggesting amphipathy of integral proteins. Deoxycholate shows similar properties. Furthermore, it has also been shown that ordinary soluble proteins bind very little, if any, nonionic detergent (Helenius and Simons,1972). Studies of integral proteins suggest that (1) integral proteins may be generally amphipathic; (2) their exposed hydrophilic ends; whose native conformations determine their respective enzymatic or antigenic activity, do not bind the nonionic detergent and hence are unaffected by it; and (3) their membrane-embedded hydrophobic ends bind the nonionic detergent, but since these hydrophobic ends may have no direct influence on the structure of the hydrophilic ends, the detergent binding does not affect the enzymatic or antigenic activity.

By contrast, ionic detergents such as sodium dodecyl sulphate not only disperse membrane proteins but often inactivate them. This could be attributed to the interaction of such detergents with both the hydrophobic and hydrophilic (active) ends of the amphipathic proteins, since it is well known that ionic detergents bind to, and alter the conformations of ordinary soluble proteins.

iii. Peripheral Proteins:

A few peripheral proteins have been studied and characterized; these include: cytochrome C associated with the inner mitochondrial membrane (Smith et al.,1973) and spectrin associated with erythrocyte membrane that is rapidly released free of lipids from the erythrocyte ghost membranes by the addition of 1mM ethylene diamine tetraacetic acid in water (Singer,1974). α

— lactalbumin of milk is a highly soluble protein and has been characterized

as a peripheral protein of membranes within the mammary gland (Singer, 1974).

In each case studied, the highly soluble peripheral protein appears to become attached to the membrane by specific binding to a particular integral protein of that membrane and furthermore, achieves its function by virture of that binding. This is not to be confused with a situation in which two integral proteins interact while both are embedded in the membrane matrix. Instead, it is proposed that the peripheral protein, when bound, remains completely outside the lipid matrix of the membrane and binds to a region of an integral protein that protrudes into the aqueous phase. Such specific binding occurring in the aqueous phase, presumably involves the same kinds of interactions that hold together the subunits of soluble proteins. Such interactions are often mainly hydrophobic, occurring between specific regions of two homologous polypeptide chains, as between α and β chains of haemoglobin (Perutz, 1969).

Singer (1974) suggests that peripheral proteins will generally be attached to membranes by binding to the exposed hydrophilic ends of specific amphipathic integral proteins of the membrane. To account for this specific binding, in some cases it turned out that a peripheral protein is structurally homologous to the hydrophilic portion of the integral protein to which it specifically binds; for e.g. α-lactalbumin of milk is homologous to the hydrophilic portion of protein A (Singer, 1974). In some cases, the peripheral protein may exist in two conformational states and be preferentially bound to the integral protein when it is in one of these states. Since each integral protein is often localized to certain intracellular organelle or membranes and is likely to be asymmetrically disposed on the surfaces of that membrane, it follows that each peripheral protein attached by the proposed mechanism would therefore be localized to specific membranes and membrane surfaces. An alternative, that peripheral proteins are attached not to integral proteins but to polar heads of lipids is possible but it would not readily account for a high degree of specificity of peripheral protein attachment to membranes nor for functional specificity of the protein. Thus, the special feature of peripheral protein is the relatively weak binding to specific integral proteins in the membrane, which serves to modulate and regulate

specific membrane functions.

The experimental facts that support the Fluid Mosaic Model are summarized:

1. The protein: lipid ratio varies in biological membranes.

2. Proteins are grossly heterogenous in a number of membrane systems No single species is responsible for maintaining the structure of membranes (Kiehn, Holland,1968; Halder et al 1966; Lenard,1970).

3. The largest fraction of membrane proteins is insoluble in neutral aqueous buffers in the absence of membrane lipids (Richardson et al.,1963).

4. The average amino acid composition of membrane proteins is not markedly different from that of simple soluble proteins (Frye Edidin 1970; Rosenberg and Guidotti ,1969).

5. On average, proteins of intact membranes are $\propto$ helical or random coil (Ke,1965; Wallach and Zahler, 1966; and Lenard and Singer, 1966), suggesting that proteins in membranes are globular in shape and do not spread out as monolayers.

6. The presence of lipid was demonstrated by Overton (1967). Majority of lipids is phospholipid. Gorter and Grendel (1925) extracted lipid from erythrocytes and arrived at a lipid area to cell area ratio of 2, suggesting presence of a bilayer.

   Melchoi et al.(1969) and Steim et al.,(1970) showed the structure of lipid in Mycoplasma membranes closely resembles an aqueous lipid dispersion; presumably the latter is in bilayer form. Most investigators appear now to be convinced that in most membranes a substantial part of lipid is in bilayer form.

7. None of the evidence so far obtained permits us to say whether the bilayer is continuous or interrupted (Singer, 1971).

8. Permeability studies on membranes conclusively point out to fluid and dynamic nature of the membrane.

9. Direct evidence of the proposed model is suggested by freeze-etching experiments on a number of membranes, including bacterial and mitochondrial, which show a mosaic-like structure consisting of a smooth matrix interrupted by a large number of particles - later shown to be

protein (Branton,1969; Bayer and Remsen,1970, and Pinto da Silva et al.,
1970).

The fluid mosaic model provides a current working hypothesis
for the study of biological membranes, but it has been under criticism
for the stress on the concept of fluidity that has been implicated.

D.   BACTERIAL MEMBRANES

Bacterial membranes are also 75 - 80A^o thick as in Micrococcus
lysodeikticus (Salton and Chapman,1962).

The membrane system of Gram-positive bacteria represents 16 - 30%
of the cell dry weight (Salton and Freer, 1965). The overall composition of the
membrane in two Gram-positive bacteria, both micrococci is given below:

	Percent Protein	Percent lipid	Principal P-lipid	Reference
M. lysodeikticus.	65 - 68	23 - 26	Diphosphotidyl glycerol	Salton '67b
M. luteus			Phosphotidylglycerol	
(S. lutea)	57	23	-	Salton '67b

Bacterial membranes normally contain no cholestrol; except in some Mycoplasma
species, membranes of which may contain upto 76% of total lipid as cholestrol
(Argaman and Razin,1965). Phosphotidylcholine is usually absent in bacterial
membranes. It has been suggested that cholestrol stabilizes the membranes
other than those of bacteria by the formation of esters with phospholipids (Van
deenen 1964; Van deenen and Van Golde,1966).

Studies on bacterial membrane proteins have been few. We do not know of
an accurate number of proteins in the cytoplasmic membrane. Mirsky (1969)
obtained 12 distinct protein bands from the membranes of B. megaterium on
acrylamide gels, and 15 from those of B. licheniformis.

SDS solubilization of membranes and subsequent electrophoresis on
acrylamide gels containing SDS reduces proteins to their subunits and produces
a number of stained bands. The method results in loss of enzyme activity. 35 - 40
bands on acrylamide gels have been resolved by solubilizing rat erythrocytes,
liver and kidney endoplasmic reticuli (Neville and Glossman,1971).

The carbohydrate content of bacterial membrane is variable; 15 - 20%
(dry weight) in M. lysodeikticus (Gilby, Few and McQuillen,1958) and 10% in
S. lutea (Brown,1961). The large amount of carbohydrate components in the

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membranes of these micrococci is due to the presence of polysaccharide of the mannan type - acidic lipomannan (Powell et al.,1975).

In thickness and overall composition, in particular lipid composition (Korn, 1969), the cytoplasmic membrane of Gram-positive bacteria bears a striking resemblance to the inner mitochondrial membrane.

E.   STUDIES OF BACTERIAL MEMBRANES:

i.   Isolation: Intact cytoplasmic membranes due to their delicate nature may only be produced from organisms whose cell wall may be removed enzymically. The methods employed basically take the form devised by Weibull (1953) for B.megaterium; the cell wall is removed enzymically in hypertonic medium and the resulting protoplast gently lysed by a decrease in tonicity. Weibull (1956) reported that intact cytoplasmic membranes are only obtained when magnesium is present in the medium. This requirement for magnesium ions, though depending absolutely on the organism, has been confirmed many times (Ghosh and Carroll, 1968; Bodman and Welker,1969). Mitchell and Moyle (1956) demonstrated that S. lutea can be converted into protoplasts by incubation with lysozyme in the presence of high concentrations of sucrose or sodium chloride. Unlike intact bacteria, protoplasts are delimited only by fragile membranes which may be ruptured simply by suspension of the organisms in water or dilute salt solutions. The isolated membranes often referred to as protoplast membranes —or "ghosts" represent the plasma-mesosome membrane system of the bacterial cell.

ii.   Membrane Solubilization:

One of the popular techniques to study the membrane has been solubilization by the use of detergents or organic solvents. These procedures dissociate the membrane into its components. Some proteins which are weakly bound to the membrane (peripheral proteins) however, can be obtained by washing procedures using buffers, and effecting the release of the proteins by manipulation of ionic strength and pH e.g. selective release of AT Pase, NADH dehydrogenase and polynucleotide phosphorylase from M. lysodeikticus (Salton, et al.,1971).

Although organic solvents like butanol or pentanol can solubilize membranes completely, these have been shown to denature membrane proteins (Guidotti, 1972;

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and Razin,1972). Most of the recent successful isolations and purifications of integral membrane proteins are based on the solubilization of membranes by mildly ionic and non-ionic detergents under conditions leading to the formation of solutions containing detergent-lipid and different detergent-protein micelles (Gulik-Krzywicki, 1975). Isolation of a membrane protein involves the separation of a given protein - detergent micelle from other protein - detergent and lipid-detergent micelles using density gradient centrifugation, ion-exchange columns or gel-filteration.

Detergents have been used extensively for solubilization, though a delicate balance exists between solubilization and inactivation. In general the in-activation effects follow the pattern non-ionic (Lubrol, Triton x-100, Tween 20) $<$ bile salts (cholate, deoxycholate) $<$ dodecyl sulphate and cetyltrimethylammonium, and are also affected by the relative concentrations of membrane and detergent. Solubilization is generally understood in terms of inability to form a sediment at 100,000 x g for 1 h; to show some retardation on a detergent-equilibrated molecular sieve column; and to be devoid of detectable membrane structures by electron microscopy (Kahane and Razin,1971). The term solubilization is thus operational; it does not imply that a molecular solution or even a homogenous suspension of lipoprotein subunits is obtained but only that a drastic reduction in size has taken place.

Detergents are a special group of lipids. They contain apolar groups of aliphatic or aromatic nature which are hypdrophobic, and polar groups as well which are hydrophilic. Molecules like detergents which are partly hydrophobic and partly hydrophilic are called amphipathic molecules or amphiphiles. When small quantities of an amphiphile are added to water, part of it dissolves as monomers and part forms a monolayer at the air/water interphase. The molecules in the monolayer are in equilibrium with monomers in the bulk solution. When the monomer concentration reaches a critical value, added amphiphile begins to associate to form micelles. Micelles are defined as thermodynamically stable colloidal aggregates, spontaneously formed by detergents above a narrow concentration range (Hutchinson and McBain, 1955). The driving force for the spontaneous aggregation of detergent molecules to form micelles is hydrophobic. The interior of the micelle consists of hydrophobic groups, and the polar groups

cover the surface of the micelle. For most practical purposes micelles can be considered homogeneous in size although in fact their molecular weights are distributed around a mean (Tanford, 1973).

When increasing amounts of detergent are added to a suspension of liposomes prepared from pure phospholipids, following sequence of events occur (Helenius and Simons, 1975):

Stage I: Detergent binding. Detergent is incorporated into the bilayer and causes changes in its physical properties.

Stage II: Lamellar - micellar phase transition. When the bilayers are saturated with detergent, mixed micelles begin to form resulting eventually in complete phase transition.

Stage III: Size-decrease of mixed micelles. After completed phase transition the detergent/phospholipid ratio in the mixed micelles increases and their size decreases.

These general principles of detergent action revealed by studies on model lipid bilayers (liposomes), apply to membranes as well.

There is little detailed information on the structure of the various complexes that are released from membranes as the result of solubilization. The main reason is the complexity of the solubilized mixtures. Enough evidence exists to suggest that at least part of the membrane proteins are originally released from the membranes as lipoprotein - detergent complexes such as from mitochondria (Kagawa,1972) and chlorplasts (Nelson and Neumann, 1972; and Shaw et al,1972) with nonionic detergents.

A very useful phenomenon in the solubilization of membranes is the selectivity with which different components are released from membranes. Like soluble proteins, many membrane proteins possess quaternary structure. Protein-protein interactions in the membrane may be permanent or reversible depending on their functions in the membrane. With mild detergents, the selectivity is partly due to the insolubility of large continuous structures stabilized by permanent non-covalent associations. An increase in detergent concentration leads to a more or less sequential release of different membrane components. Alternatively selective extraction can be obtained by changing the ionic strength.

Selective solubilization has proved extremely valuable in the purification of membrane proteins and active membrane complexes. For example treatment

of mitochondria with appropriate amounts of various detergents results in the separation of a red supernatant rich in cytochromes b, c, and c_1 and a green precipitate rich in cytochromes a, and a_3 (Hall and Crane, 1972; and Kagawa, 1974).

With appropriate detergents in relatively high concentrations complete or almost complete solubilization of most cytoplasmic membranes can be achieved; for example sodium dodecyl sulphate is the most effective detergent for bacilli (Yamaguchi et al.,1967). In solubilizing the membranes of M. lysodeikticus, the non-ionic detergent Nonidet P40 (a polyoxyethylated alkyl phenol), is more effective (95% solubilization) than SDS (75% solubilization) (Salton and Netschey, 1965).

The process of solubilization of components of a membrane is not random. Working with concentrations of SDS (0.05 - 0.10%) insufficient to give complete disaggregation of the membranes of B. subtilis, Bishop et al.(1967b) demonstrated that, at the lower detergent concentration, the greater part of the membrane $NADH_2$ - 2, 6 - dichloro phenolindophenol oxido-reductase is solubilized whereas the succinate dehydrogenase remains insoluble. In the detergent concentration range 0.05% - 0.10%, protein is preferentially solubilized from the membranes to leave a lipid-rich residue. On the contrary, when the membranes of M. lysodeikticus are treated with DOC, a lipid-depleted residue (95 - 97% protein) is produced which accounts for 15% of the membrane (Salton et al; 1968). Virtually all the succinate dehydrogenase and cytochromes of the membranes are present in this residue.

Recently an increased interest has been taken in the cytoplasmic membranes of mycoplasma. There is a great resemblance between the membranes of Gram-positive bacteria and the membranes of mycoplasmas; the phospholipids are very similar and both contain glycolipids of the diglycosyl diglyceride type. The cytoplasmic membrane of Acholeplasma laidlawii (a mycoplasma) is yellow due to carotenoids and flavoproteins (Morowitz and Terry, 1969). Razin et al., (1971) used EDTA, a number of ionic and nonionic detergents for solubilization. Prolonged exposure to EDTA, in low ionic strength caused the release of 11% of the total membrane protein in a water soluble, almost lipid free form. It did not contain any ATPase activity, present in the native membranes. Ionic detergents (SDS and CTAB) though more effective in solubilization caused intensive

denaturation of membrane proteins, and enzyme inactivation. SDOC occupied an intermediate position, in this respect, between the strongly ionic and the nonionic detergents (Razin et al, 1971).

Following phenomena have also been noticed during solubilization studies of membranes:

(a)   Activation or inactivation of the solubilized enzymes. The degree of either activation or inactivation was dependent on the type and concentration of the detergents used (Razin et al., 1965; and Eisenberg et al, 1970).

(b)   Reconstitution of membrane like structures. On dialysis of solubilized membranes in the presence of Mg^{++} irregular aggregation of membrane constituents or partial return to membrane - like structures occurred; as in SDS - solubilized membranes of M. lysodeikticus (Butler et al 1967; and Grula et al, 1967) and in M. laidlawii (Razin et al.,1965).

(c)   Separation of proteins and lipids. When higher concentrations of detergent are added to membranes, separation of the lipids from proteins occurs. The quantities of the detergent needed will depend both on the composition of the membrane and on the detergent itself. Almost complete separation of the membrane lipids from the proteins has been achieved with ionic and nonionic detergents (Helenius and Simons, 1972; and Razin et al.,1972). Instances in which delipidation has been less complete have also been reported (Phillipot, 1971; and Triplett et al.,1972).

iii.   Membrane-associated enzymes in Bacteria:

Membranes of bacteria (mesosomes included) are the sites of numerous enzymes carrying on the metabolic functions of the cell. Some of the enzymes are firmly bound to the membranes. At the present state of our knowledge it is difficult to assign a quantitative parameter to the term "firmly bound". It is in essence an operational term indicating those membrane associations which survive a standard membrane isolation and washing procedure applied to a particular species.

Most of the present knowledge of enzyme localization in cells has come from direct determination of the distribution of enzymes following cell disruption by mechanical means and separation of the particulate membrane fraction (Bendetti and Emmelot, 1968; de-The', 1968; and Racker, 1970). By use of ionic detergents such as cholate various workers have prepared complexes from the

mitochondrial inner membrane capable of catalysing activities of the respiratory chain.

Numerous dehydrogenases, firmly bound to the bacterial membrane and linked to the respiratory chain are known. These include succinate, malate, alcohol and NADH dehydrogenase. These can be readily assayed by using membranes. Solubilization of some of these can be achieved and may be used for purification and study. The presence of two dehydrogenase and ATPase in a detergent solubilized mixture of S. lutea provided me an opportunity to study the properties of these enzymes.

. A membrane-bound malate dehydrogenase which is a flavoprotein has been reported in several microorganisms. A curious feature of many bacteria is the presence of two dehydrogenases utilising the same substrate and producing the same product. For example in S. lutea malate is oxidised by a membrane bound dehydrogenase and also by a soluble enzyme present in the cytoplasm. The product is oxaloacetate. The soluble dehydrogenase is NAD linked while the membrane-bound enzyme is a flavoprotein directly linked to the respiratory chain and therefore NAD independant. It is not known to what extent each pathway functions in vivo, or why two distinct pathways are developed for malate oxidation. The two enzymes may have physiological significance in strictly aerobic micro-organisms by providing a means of oxidising extra particulate NADH. Oxaloacetate formed from the bound NAD independant pathway could be reduced to malate by extra particulate malic dehydrogenase. It has been shown that FAD serves as a cofactor of the bound enzyme. Erickson and Parker (1969) reported the presence of cytochromes a, b and c types and a menaquinone (MK-8) in the electron transport chain of S. lutea. Based on inhibition studies of the membrane-bound respiratory chain, they suggested the following possible scheme for malate oxidation:

$$\text{L MALATE} \longrightarrow \left[\text{Fp} \quad \text{MK-8, Cyt b}_{557}, \quad b_{562} \right] \longrightarrow ? \longrightarrow O_2$$

$$\downarrow$$

$$\left[\text{Cyt. C}_{554}, \text{ Cyt. C}_{549} \right] \rightarrow \left[\text{Cyt. a}_{598}, \text{ Cyt. A}_3 \right] \rightarrow O_2$$
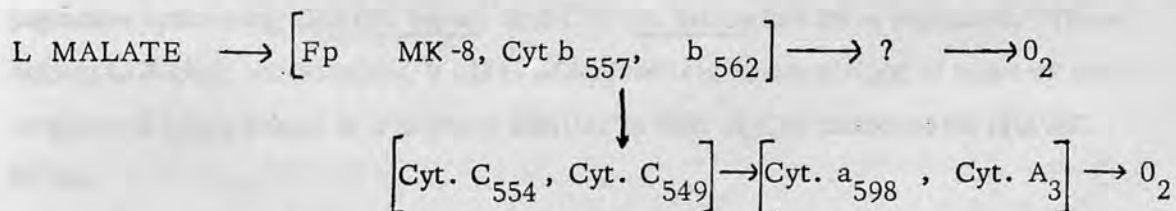
Brodie et al.(1969) reported an inhibitory effect of ATP, ADP and AMP on Mycobacterium phlei malate-vitamin K reductase. The inhibition observed with adenine nucleotides was reversed by addition of FAD. The enzyme requires

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vitamin K_1, phospholipid, and magnesium ions for its full activity. These failed to reverse the inhibition caused by adenine nucleotides.

Eisenberg (1972) reported solubilization of the membrane L-malate and reduced NADH dehydrogenase enzymes from <u>Micrococcus lysodeikticus</u> with DOC. The insoluble residues contained cytochromes of b, c and a type. Nachbar and Salton (1970b) reported the release of a particulate fraction with the $NADH_2$ dehydrogenase activity of the membranes of <u>M. lysodeikticus</u> by treatment with EDTA.

The bacterial membrane AT Pases are readily extractable from the membranes and have received considerable attention. A number of these have been purified to homogeneity and their structure established. Munoz et al.(1969) purified the enzyme from <u>M. lysodeikticus</u> about 50 fold. The purified enzymes do not appear to have lipid requirements. Both membrane bound and soluble forms of bacterial ATPase show maximal activities with the divalent cations Mg^{++} or Ca^{++}. Each ATPase system appears to exhibit its own unique properties and responses to divalent cations, depending on the bacterial species.

F. CAROTENOIDS:

One of the major groups of pigments which are concerned with coloration in microorganisms is carotenoids. <u>S. lutea</u> contains intensely yellow carotenoid pigments in their cytoplasmic membranes.

The bright colour of these lipid-like substances is due to the presence of a chromophore consisting mainly or partly of a chain of conjugated double bonds - the isoprenoid polyene chain. The number of C = C double bonds ranges from 3 to 15; a figure of 10 or 11 is very common. A few carotenoids have a polyene chromophore too short for detection by the human eye. Most carotenoids contain 8 isoprene units, therefore 40 carbon atoms. Occurrence of pigments containing C45 (<u>S. lutea</u>) and C50 (<u>S. lutea</u>) has been reported. These belong to higher carotenoids, a class of hydrocarbons consisting of eight or more isoprenoid units joined in a manner similar to that of C40 carotenoids (IUPAC 1972).

IUPAC defines C40 carotenoids as "a class of compounds built from 8 isoprene units in such a manner that the linking of isoprene units is reversed in the middle of the molecule." (IUPAC 1972, 1975).

i.   Carotenoids of S. lutea

Earliest studies on carotenoids of S. lutea are due to Dieryck (1930).
The pigments were partitioned between 90% methanol and light petroleum.
Epiphase separated a hydrocarbon Sarcinene from a more polar carotenoid
xanthophyll in the hypophase. Chargaff (1932) confirmed the presence of two
types of pigments differing notably in their polarity. From the absorption spectrum
of xanthophyll in visible light; he concluded that an aliphatic nonaene structure
was present. Nakamura (1941) isolated and crystallized a yellow pigment.
This carotenoid had absorption maxima at slightly shorter wavelengths than
sarcinene. Nakamura concluded that the purified pigment was a xanthophyll-
ester. Takeda and Ohta (1941) isolated a xanthophyll - Sarcina xanthin in a
crystalline state, melting point 149 - 150°c, presumably identical with the
xanthophyll described by Chargaff. The only carotene isolated was lycopene
Sobin and Stahly (1942) isolated the pigments from S. lutea and S. flava. He
showed that S. lutea contained two carotenoids and S. flava one; identical
with one of those found in S. lutea. Both carotenoids of S. lutea were
xanthophylls. They did not find any ester or carotenoic acid as expected from
Nakamura's study.

In 1959 Mathews and Sistrom separated a methanol extract of S. lutea into
a carotene (Sarcinene) and a xanthophyll (Sarcinaxanthin). These had identical
absorption spectra and were similar to those described by Chargaff and Dieryck
(1932). Thirkell and Strang (1967) isolated the pigments from S. lutea and
S. flava and resolved both into 7 fractions on silica plates. The polar carotenoid
fractions had the same chromophoric group, and from their absorption maxima,
identical for all fractions, a system of nine conjugated double bonds was suggested.
They concluded that carotenoids in S. flava and S. lutea are very similar. In
1967 Thirkell et al. reported a C_{50} carotenoid in S. flava and suggested the
occurrence of the same pigment in S. lutea. At the same time Jensen and Weeks
(1966) reported a C_{50} carotenoid, Dehydrogenans P_{439} in Flavobacterium
dehydrogenans. In the following years (1970 - 1973) Jensen carried out extensive
study on the structure of the carotenoids in S. lutea, with the use of proton
magnetic resonance and mass spectrometry. She assigned structural formulae
to the carotenoids(Fig. 1):

(1) C_{50} diol:Sarcina xanthin.

(2)   Mono O-glucoside of sarcina xanthin.

(3)   Decaprenoxanthin (F. dehydrogenans P439)

1 and 3 are structural isomers, differing in location of hydroxy groups.

Minor carotenoids reported by Jensen were

a) C_{40} carotene:Lycopene.

b)   three C_{45} mono ols.

No sarcinene was found by Jensen in S. lutea.

ii.   Cellular location of Carotenoids:

Several scattered observations suggest that the carotenoid pigments of chemotrophic bacteria are associated with the cell membrane. Salton (1956) found that the cell walls of the pigmented organisms S. lutea, Micrococcus lysodeikticus, and Staphylococcus aureus are completely devoid of pigments. This was later confirmed by Mathews and Sistrom (1959) for S. lutea. Gilby et al.(1958) isolated membranes of S. lutea, by direct lysis of cells with the use of lysozyme, free of particulate debris. The membrane lipid was extracted by the use of organic solvents, and the absorption maxima (416, 440 and 471 nm) was typical of that obtained for carotenoids.

iii.   In vivo state of carotenoids:

A major draw back in the use of organic solvents such as methanol and acetone for carotenoid extraction is that the in vivo state of the pigments cannot be deciphered since organic solvents have the capacity to separate membrane lipids from proteins resulting in dismemberment of the system.

Possibly the first report on the association of carotenoids with other cellular constituents in non-photosynthetic bacteria is due to Saperstein and Starr (1955) who prepared cell free extracts from the phytopathogenic coryneform bacteria by grinding the cells with alumina. The extract had absorption maxima corresponding to those observed for major carotenoids. Ammonium sulphate (70% saturation) precipitated the protein carotenoid complex. Ethanol denatured the protein and liberated the carotenoids. The original protein extract, and the sedimented protein - carotenoid complex gave positive biuret reactions. Saperstein and Starr concluded that in the coryneform bacteria studied carotenoids were associated with protein particles.

Occurrence of carotenoids in photosynthetic bacteria as protein complexes
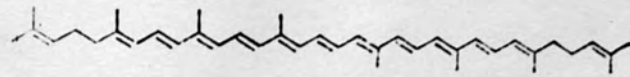
C_{40} - Lycopene
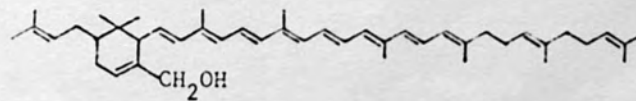
C_{45} - Mono-ol

C_{45} - Mono-ol
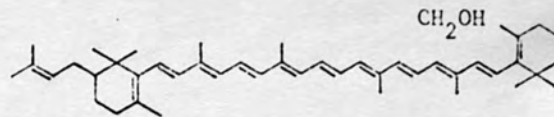
C_{45} - Mono-ol
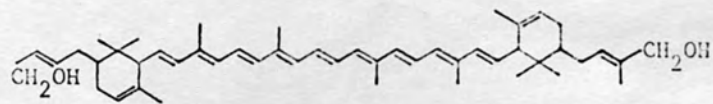
C_{50} - <u>Dehydrogenans</u>-P439 (Decaprenoxanthin)
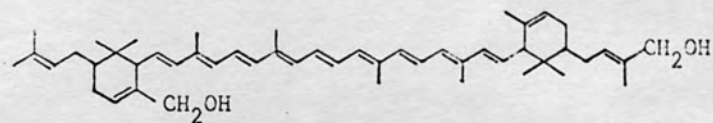
C_{50} - Sarcinaxanthin
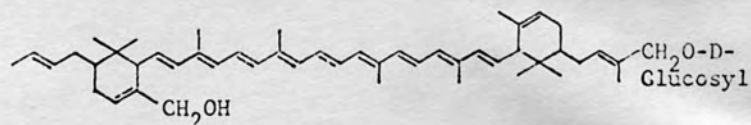
C_{50} - D-Glucoside of Sarcinaxanthin
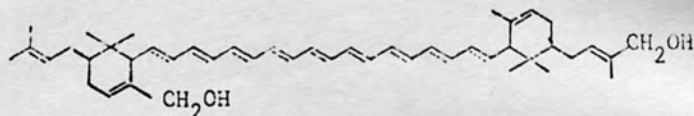
C_{50} - 7, 8 or 7', 8' Dihydrosarcinaxanthin

Fig. 1.   Structure of carotenoids from <u>Sarcina lutea</u>.

is well documented, for example Garcia et al., (1967) obtained a brown
carotenoid-protein complex extracted with Triton X-100 from Rhodospirillum
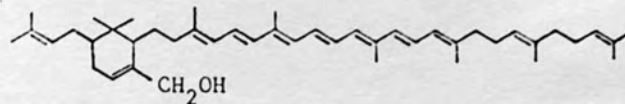rubrum chromatophores. The complex had unusual spectral characteristics
including a peak at 368 nm. Addition of acetone and methanol to the complex
restored the usual carotenoid spectrum. The organic solvents had either
dissociated the complex or altered the binding. The pigment spirolloxanthin
was associated with the single hydrophobic protein of molecular weight of 12000.

Cheesman, Lee and Zagalsky (1967) have reviewed numerous
carotenoprotein complexes in invertebrates. Many had lipoprotein and
glycoprotein associations and were easily extractable. Association of carotenoid
with protein in invertebrates and photosynthetic bacteria appears to be noncovalent
as opposed to that of the bacterial complexes described below. In many cases
the association results in a large shift in their absorption maxima.

The mode of occurrence of carotenoids in membranes of S. lutea were
first looked at by Mathews and Sistrom (1959). They prepared cell-free extracts
containing sub-microscopic pigment-bearing particles from the membranes. They
claimed that the particles bearing carotenoids were the same as those having
succinic dehydrogenase, NADH oxidase, phospholipid, and cytochrome oxidase
activities. They concluded that carotenoids in S. lutea membranes are complexed
with protein.

Thirkell and Strang (1967) extracted and purified the carotenoid pigments
from S. lutea and S. flava. S. lutea synthesizes intensely yellow pigments while
S. flava has red-orange carotenoids when grown on nutrient agar. Thirkell
and Strang suggested that a minute fraction of the pigments in S. lutea could be
complexed with proteins. Thirkell, Strang and Chapman (1967) extracted pigments
from S. flava with methanol and separated them into four fractions. The fourth
fraction which was highly polar and constituted 61% of the total pigment was
further investigated by Thirkell and Hunter (1969). They found a part of this
polar fraction to be a carotenoid associated with a glucose and a peptide. They
considered the carotenoid to be bound to glucose through a glycosidic linkage
involving the reducing group of the hexose, and the amino acids to be linked to
hexose in some way involving one of the remaining primary or secondary OH
groups of the glucose.

Strang (1968) reported that all of the carotenoid in S. flava is localized in the membrane. Attempts were made to dislodge the pigment by use of the synthetic detergents. Hunter and Thirkell (1969) used 1% lubrol to solubilize the pigment. The peaks in the visible region of the spectrum of this solution were similar to those given by free pigment fractions prepared by organic solvents from S. flava (Thirkell Strang and Chapman, 1967). The peaks in the ultra-violet region - at 276nm and at 282nm were due to detergent and aromatic amino acids, and at 226 nm due to peptide bonding. By retension on sephadex G200 columns the solubilized membrane fraction appeared to be a single molecular species. They found that the release of free carotenoid was difficult to achieve indicating bonding was covalent possibly glycosidic.

Another strain of Sarcina - S. morrhuae-has been studied on these lines. Nandy and Sen (1967) isolated free pigments from this bacterium; and their results have been confirmed (Thirkell and Hunter 1970).

iv.   Summary:

Carotenoids are present in all photosynthetic and most non-photosynthetic organisms. The occurrence of these pigments in cell organelles does not follow a definite pattern. Carotenoids have been found free, bound to protein, lipoprotein or glycoprotein. Knowledge about their associations is seriously handicapped by the isolation techniques at present available for these rather inert lipid pigments.

G.   FUNCTIONS OF CAROTENOIDS

There are only two functions which are firmly established for carotenoid pigments: (a) as accessory pigments in photosynthetic organisms transferring radiant energy to the pigments associated with the reaction centre (b) as a protective agent to prevent cells from undergoing damage due to a photodynamic action.

Although the term photodynamic action has been used in the past with somewhat different meanings by different workers, a now generally agreed definition has been given by Blum (1941) as the sensitization of a biological system to light by a substance which serves as a light absorber for photochemical reactions in which molecular oxygen takes part. This requires the simultaneous interaction of three components: visible light, a photosensitizing pigment and oxygen.

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Photosynthetic bacteria are characterized by a high concentration of bacteriochlorophyll which may act as an endogenous photo-sensitizer. Stanier et al.(1955) prepared a blue green mutant of R. spheroides which synthesized only slightly less chlorophyll than the parent, but in which coloured carotenoids were replaced by the colourless phytoene. The cells were rapidly killed with the destruction of chlorophyll by simultaneous exposure to light and oxygen. Exposure to oxygen in the dark had no lethal effect on the mutant. Since the normally pigmented wild type was not affected in this manner, it was concluded that death resulted from photodynamic action, bacterio-chlorophyll being the sensitizer. Carotenoids thus serve a protective function.

The inhibition of carotenoid synthesis by diphenylamine has been observed in many fungi and bacteria, and was first demonstrated in R. rubrum by Goodwin and Osman (1954). Cohen Bazire and Stanier (1958) obtained cells of R. rubrum almost completely devoid of coloured carotenoids, but with a normal content of chlorophyll, by growing them in the presence of diphenylamine. Exposed to light and air such cells display all the symptoms of photo-sensitivity (death and chlorophyll destruction). When diphenylamine is removed, rapid carotenoid synthesis at the expense of accumulated endogenous precursors begins immediately. The restoration of coloured carotenoids is accompanied by a rapid diminution of the induced photo-sensitivity. These experiments led Cohen Bazire and Stanier to suggest that the carotenoid pigments protect the cell from the deleterious effect of chlorophyll catalyzed photo-oxidations.

It has been suggested that the carotenoids found in many non-photosynthetic bacteria may have the same protective function. Here some other compound would be the photo-sensitizer in place of bacteriochlorophyll. The presence of an endogenous photo-sensitizer is not so readily observable and usually special conditions such as the use of exogenous photosensitizers or high light intensities are necessary to demonstrate a photodynamic effect related to carotenoid pigment. Kunisawa and Stanier (1953) demonstrated this with a non-photosynthetic bacterium Corynebacterium poinsettiae, using both the coloured wild type strain and a white mutant which contained only phytoene and traces of phytofluene. In the presence of the photo-sensitizing dye toluidine blue, the white mutant

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strain displayed a lethal aerobic sensitivity whereas the coloured wild type strain was unaffected.

Mathews and Sistrom (1960) reported that carotenoids prevent photo-dynamic killing in S. lutea. The exposure of a cell suspension of the carotenoid-less mutant of S. lutea, in the presence of toluidine blue, to light and air kills more than 99% of the cells within 60 minutes. In the absence of light or of air the cells are not killed. There is no decrease in viability of pigmented cells exposed to light and air in the presence of toluidine blue. In separate experiments without added toluidine blue, they found that colourless mutant of S. lutea is killed when exposed to sunlight in the presence of air; while the carotenoid - containing wild type is not killed under these conditions. They inferred the presence within the cell of substances which can act as photo-sensitizers in the photodynamic killing of carotenoid less cells. The carotenoids prevent the lethal action of these endogenous, as well as of exogenous, photo-sensitizers. Mathews and Sistrom suggested that organisms normally exposed to light and air would contain carotenoid pigments and organisms not exposed to light and air during their life histories would not contain them.

Mathews and Sistrom (1960) suggested that the location of the photo-sensitive site, protected by carotenoids, is close to the pigments themselves and is therefore in the membrane. Recently Prebble and Anwar (1975) reported that the sensitive site in S. lutea is the menaquinone of the respiratory chain.

H.   CONCLUSIONS:

Studies on bacterial membranes are in preliminary stages. Although detailed analysis of the components of the membranes have been known, studies on the structure are few.

Two aspects of bacterial membranes were investigated in the studies to be described:

(a)   Mode of occurrence of carotenoids in S. lutea membranes.

(b)   Properties of some membrane bound enzymes.

Mode of occurrence of carotenoids in bacteria varies. They were reported to be carotenoproteins as in Rhodo spirillum rubrum (Garcia et al., 1967), but free in Sarcina morrhuae (Nandy and Sen, 1967). In S. lutea the pigments have been alleged to be associated to a number of enzymes (Mathews and Sistrom, 1959). With mild nonionic detergents carotenoids were solubilized from the membranes and examined for their associations.

Tightly membrane-bound enzymes in bacteria, classified as integral proteins by Singer (1974), have not been isolated and examined in detail. Using mild, non-ionic detergents it was possible to solubilize some membrane bound enzymes from the membranes of S. lutea.

These studies were aimed to answer the following questions:

1.   Is the major pigment in S. lutea membrane, a carotenoprotein?

2.   Has protein association any bearing on the function of carotenoids?

3.   Is association of the carotenoids with the enzymes necessary for the protection of the respiratory chain?

4.   Can we solubilize the membrane bound dehydrogenases of S. lutea and compare their properties in membrane bound and solubilized state; with a view to study whether some or any of the enzymes is under metabolic regulation?

SECTION 2.   MATERIALS AND METHODS

                A. i. CHEMICALS   ii. BUFFER SYSTEMS
                B.   PREPARATION OF CULTURE
                C.   PREPARATION OF MEMBRANES
                D.   PREPARATION OF DETERGENT EXTRACT
                E.   SPECTROSCOPIC MEASUREMENTS
                F.   ELECTROPHORESES
                G.   STAINING TECHNIQUES
                H.   SPECIFIC ENZYME LOCALIZATION
                I.   SUCROSE DENSITY GRADIENT ELECTROPHORESIS
                J.   PHASE PARTITION
                K.   MOLECULAR SIEVE CHROMATOGRAPHY
                L.   ION-EXCHANGE CHROMATOGRAPHY
                M.   THIN-LAYER CHROMATOGRAPHY
                N.   POLAROGRAPHIC ASSAY METHODS
                O.   ESTIMATION OF VITAMIN K_1 (PHYLLOQUINONE)
                P.   PROTEIN ESTIMATION
                Q.   PHOSPHORUS ESTIMATION

2. MATERIALS AND METHODS.

A. i. CHEMICALS

The following chemicals were obtained for B. D. H. Chemicals Limited (Poole, England):

Acrylamide, Cytochrome 'C', Methylene bis acrylamide, Temed, Amido Schwarz(Naphthalene Black), PMS, Menadione, Sulphosalicylic acid, Coomassie blue G250 and R250, Ammonium per-sulphate, Polyethylene glycol PEG 6000, Polypropyleneglycol, Agarose, L - cysteine Hydrochloride, bromo-phenol Blue, Cetyl trimethylammonium bromide, nonidet P40, nitro blue tetrazolium, ethylene diamine-tetraacetic acid, blue dextran, serum albumin and Triton X-100.

Ficoll, Sephadex - G - series were obtained from Pharmacia (Uppsala, Sweden).

DEAE cellulose and CM cellulose were purchased from Whatman Laboratories England.

Emulphogene BC - 720 was a gift from GAF (Great Britain) limited.

Nutrient broth preparation was obtained from Oxoid, London.

ii. BUFFER SYSTEMS:

a) Borate buffer, pH8.6 : 0.04M in gels, 0.3M in electrode vessels.

b) Formate buffer, pH 3.1 : 0.05M formic acid + 0.01M.
   Na OH, both for gels and electrode vessels.

c) Phosphate buffer, pH8.6 equimolar Na_2HPO4 and KH_2PO_4
   mixed.

d) Tris-citrate-Borate discontinuous systems, pH8.6
   0.005M-Citric acid +0.07M Tris for gels, 0.3M Boric
   acid +0.05M NaOH in electrode vessels.

e) Tris-glycine buffer, pH8.7, 25mM Tris +25mM glycine
   in electrode vessels, 2.5mM Tris +2.5mM glycine in the
   gel and for extraction of the pigments.

f) Tris-Citrate buffer, pH8.6.
   30mM Tris +30mM Citric acid.

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B.   PREPARATION OF CULTURE

Sarcina lutea was maintained on nutrient agar slopes stored in a cold room in the dark. Nutrient broth was used as a medium of growth in which the organism is known to grow luxuriently. A loopful from a nutrient agar slope was used to inoculate 100 ml. of the medium in a 250 ml. conical flask, and the bacteria were grown with shaking in a reciprocating shaker incubator. 5 ml. of this pilot culture was used to inoculate 500 ml. of the medium in one litre Pyrex culture flask, and grown to log phase at $26^{\circ}C$ as above.

C.   PREPARATION OF MEMBRANES

An overnight culture (500 ml.) of S. lutea (pigmented wild type or nonpigmented mutant) was harvested at room temperature by centrifugation at 10,000 xg. for 20 minutes in plastic bottles. Pellets of cells obtained were suspended in 15 ml. 1.5 M. sucrose made in 0.05 M Phosphate buffer pH 6.8. Using a glass Elvehghem homogenizer a uniform suspension of the cells was obtained. A ten ml. solution of lysozyme (1 mgm/ml) in 0.05M Phosphate buffer pH 6.8, was added to the cell suspension and gently homogenized again to attain a thorough mixing of the enzyme. The homogenate was allowed to stand for 15 minutes at room temperature. The homogenate was spun, after incubation, at 40,000 xg for half an hour at $5^{\circ}C$. The protoplast-pellet obtained was suspended in 30 ml. cold phosphate buffer containing 1% (w/v) Na Cl   and homogenized for 10 minutes. The suspension was centrifuged in plastic tubes at 40,000 xg. for 10 minutes. The pellets obtained were resuspended in 30 ml. 1% Na Cl solution, homogenized and the suspension centrifuged at 40,000 xg for 10 minutes. The resulting pellet was termed membrane-pellet.

D.   PREPARATION OF DETERGENT EXTRACT

Membrane pellets were suspended in the detergent solution. After vigourously homogenising the suspension in a glass homogenizer, it was centrifuged in plastic tubes (25 ml. capacity) at 40,000 xg at $5^{\circ}c$ for 45 min. - 1 hour. Resulting supernatant was termed as detergent extract of the membranes according to the concentration of detergent solution used.

E.   SPECTROSCOPIC MEASUREMENTS

Absorption spectra were recorded on a Beckman S.P. 400 recording spectrophotometer, against appropriate detergent blanks; silica cells of 1 cm. light path length and 3.5 ml. capacity were used.

F.   ELECTROPHORESES

i.   Starch gel electrophoresis: Horizontal starch gel electrophoresis was

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performed by the method of Smithies (1955). Gels (13 - 16% Starch) were made in phosphate buffer in perspex trays (9 x 23 x 0.5 cm.) as described by Smith (1968). Samples were applied by inserting pieces of Whatman 3MM filter paper (2 x 0.5 mm) soaked in detergent extracts of the membranes into vertical slits made in the gel. The gels were covered with petroleum ether to prevent evaporation and run at a potential difference of 4 - 7 volts /cm for a period of 2 - 4 hours. After the run gels were sliced horizontally with a slicer and stained for protein.

ii.   Analytical Acrylamide gel electrophoresis:

The technique of disc electrophoresis of Ornstein and Davis (1964) was used. The chemicals used consist of a monomer (acrylamide, $CH_2 = CH \ CONH_2$), co-monomer (methylene bis acrylamide - BIS), an initiator (tetramethylethylenediamine, TEMED) and a catalyst (ammonium per-sulphate). Acrylamide gels were prepared as described by Smith (1968). 7.5% acrylamide gels were made by mixing 3g. acrylamide and 0.08g. Bis. in 40 ml. cold 0.005M Tris citrate buffer pH 8.6 containing 0.1% (w/v) emulphogene. 0.2 ml. TEMED and 0.2 ml 10% (w/v) ammonium per-sulphate were added to the mixture and stirred well. Gels were poured in glass tubes topped up by little buffer and allowed to set in the cold evernight.

0.2 - 0.4 ml. samples of the detergent extract were applied by pipette on top of the gels and allowed to run under a current of 3m A/gel and 180 volts for 3 - 5 hours.

At the end of the run gels were removed from the glass tubes, yellow zones of carotenoids marked with a needle and the gels were stained for protein or carbohydrate.

iii.   Preparative Polyacrylamide gel electrophoresis:
       (Righetti and Secchi, 1972)

       Dimensions of the gel slab: 6 - 8 cm. high., radius : 5 cm.
       7.5% acrylamide gel was made by scaling up the amounts of acrylamide, bis. etc. as used in analytical acrylamide electrophoresis as described above. 2.5mM Tris glycine buffer pH 8.7 containing 0.1% (w/v) emulphogene was used. The gel was poured in a special apparatus made for preparative electrophoresis

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and allowed to set in the cold overnight. A pre-run was given to remove per-sulphate from the gel for an hour. The top of the gel was covered by a 10% (w/v) sucrose layer 2 cm high. 10 ml. detergent extract of the membranes with sucrose added to make it 3% to attain appropriate density, was layered over the sucrose layer. The buffer was then layered over the sample with the use of peristaltic pump. The current through the gel was adjusted to 30 m. amperes with a potential difference of 100 volts. Fractions were drawn with the help of a peristaltic pump attached at the lower end of the gel. 5ml. fractions were collected with the help of a fraction cutter.

G. STAINING TECHNIQUES

(a) Protein stains:

i. Amido schwartz: Initially protein bands were fixed by placing the gels for 1 - 2 hours in 20% w/v sulphosalicylic acid. The gels were left in 7% acetic acid for two hours with two changes to remove sulphosalicylic acid. Fresh 1% (w/v) amido schwartz solution was in glycerol: acetic acid: water in the ratio of 5: 2: 5. Gels were left in the stain for half an hour. Gels were removed from amidoschwartz and destained by placing in 7% acetic acid. Several changes of the acetic acid cleared the background in the gels. The gels may be preserved in the acetic acid solution.

ii. Coomassie Blue G250: Gels were placed in 40 ml.of 12.5% TCA in distilled water, for 5 - 10 min. in order to attain protein fixation. Then 2 ml. of an aqueous 0.25% (w/v) solution of Coomassie Brilliant Blue G250 was added to it and mixed thoroughly. In 12.5% TCA the dye is converted into a colloidal state. This causes a selective binding to the protein zones and prevents the dye's penetration into the network of the gel, thus avoiding a stained background. The excess dye was removed by washing the gels with 7% acetic acid. The gels were stored in 7% acetic acid (Hoffman et al.,1972).

iii. Coomassie Blue R250: Another method used to stain the gels involved fixation of protein in 20% sulphosalicylic acid, removing the acid by 7% acetic acid in water, and staining with a solution of 0.25% Coomassie Blue (CB) in 50% methanol - 7% acetic acid. Complete background destaining was

achieved after renewed soaking in 30% methanol - 7% acetic acid solution over a period of two days.

The method was developed by Lopez and Siekevitz (1973) and was found to stain the protein bands but not the phospholipids in SDS acrylamide gels.

(b)   Carbohydrate Stain

i.   Periodic acid Schiff reagent: The method of Clarke (1964) was followed.

Gels were immersed in 1% (w/v) periodic acid in 3 per cent (w/v) acetic acid for one hour. The gels were taken out and leached in water for one hour and then immersed in commercial schiff's reagent for one hour. The gels were later stored in 1% sodium meta -bisulphate made in water.

H.   SPECIFIC ENZYME LOCALIZATION

i.   L - Malate dehydrogenase:

The method was adapted from Smith (1968). Gels were made in 0.05 M Phosphate buffer containing 0.1% w/v emulphogene and 0.6 g. sodium hydrogen L - malate per litre.

Stain A.   Sodium hydrogen malate   7.8 g.

$0.5 M K_2HPO_4$ 25 ml.

H_2O 65 ml.

Stain B.   Sodium hydrogen malate   15.6 g.

0.5M Phosphate buffer pH 7.0   100 ml.

0.1M Disodium EDTA   10 ml.

NBT   .035 g.

PMS   .004 g.

pH of the mixture B is brought to 7.0 after the addition of NBT. Gels are incubated in solution A at 37°c for 15 minutes and then in B at 37°c for 2 hours, or until dark blue bands appear. Gels were washed with 7% acetic acid and stored in it.

Action of stain can be represented as under: (after Smith, 1968).

(Malate) Substrate ⟶ PMS ⟶ NBT   Dark blue
                                (reduced)   Precipitate.

(oxaloacetate) Substrate ⟶ PMS ⟶ NBT
    (oxidised)   (reduced)   (oxidized)

-38 -
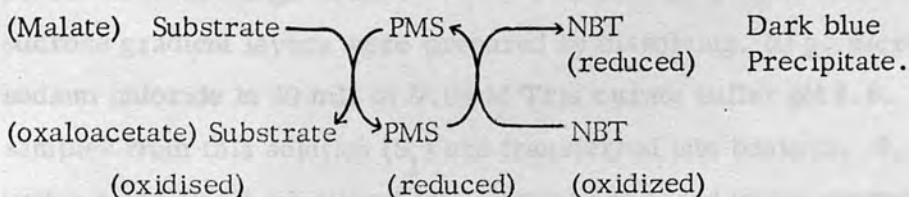
ii.   NADH - dehydrogenase:

Gels were made with 0.05M Phosphate buffer containing 0.1% (w/v) emulphogene and 0.03g. NAD (reduced) per litre. Gels were stained with Stain A and B as in case of malate dehydrogenase.
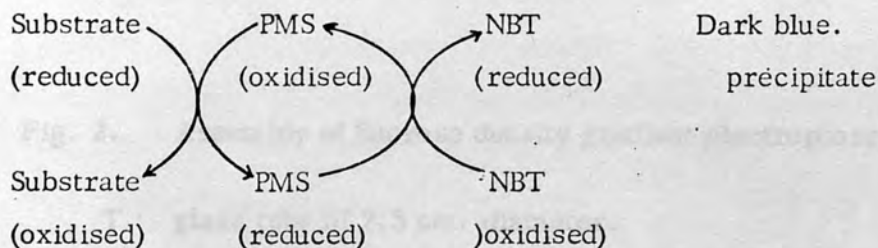
Stain A

NAD (reduced)	0.03 g.
0.5 M K_2HPO_4	25 ml.
H_2O	65 ml.

Stain B

NAD (reduced)	0.06 g
0.5M Phosphate buffer pH 7.0	100 ml
0.1 M Disodium EDTA	10 ml
NBT	0.035 g
PMS	0.004 g

Dehydrogenase stains are based on the transfer of electrons (and hydrogen) according to the series of reactions described by Smith (1968).

Substrate	PMS	NBT	Dark blue.
(reduced)	(oxidised)	(reduced)	precipitate
Substrate	PMS	NBT	
(oxidised)	(reduced)	)oxidised)	

I.   SUCROSE DENSITY GRADIENT ELECTROPHORESIS
(Hjerten, 1971)

The assembly of the electrophoresis is shown in Fig. 2.
A thin dialysis membrane M, moistened with buffer, is fixed, to the lower fire-polished end of the glass tube T by means of a tightly stretched rubber band. The sucrose gradient layers were prepared by dissolving, 60 g. sucrose and 9.3 g. sodium chloride in 40 ml. of 0.04 M Tris citrate buffer pH 8.6. Three 10 ml. samples from this solution (S_1) are transferred into beakers. 5, 10 and 25 ml. buffer samples (1 : 1 diluted tris citrate) are added to the sucrose aliquots.

Fig. 2. Assembly of Sucrose density gradient electrophoresis.

T : glass tube of 2.5 cm. diameter.

S_1 S_2 S_3 & S_4 : sucrose solutions.

b : electrode vessels.

B : beaker containing S_1.

a : side arm.

c : bridge.

M : dialysis membrane

The sucrose solutions are now called S_2 S_3 and S_4 according to the increasing dilution of sucrose. Emulphogene is added to the sucrose solutions to a concentration of 0.1% (w/v). Solution S_1 is pipetted into the tube T to a height of about 5 cm., followed by 2 cm. layers of solution S_2, S_3 and S_4. The gradients thus created in terms of sucrose and Na Cl concentrations are:

	Sucrose % (w/v)	M	Na Cl% (w/v)	M
S_1	75	2.20	12	2.0
S_2	50	1.47	8	1.3
S_3	25	0.73	4	0.67
S_4	21	0.62	3.45	0.56

The increased conductivity provided by the Na Cl further enhances the concentrating effect in the sucrose gradient, consequently increasing the electrophoretic mobility of the migrating moeities. The lower part of tube T is surrounded by solution S_1, using a beaker B kept in the electrode vessel. The membrane-extract sample was then layered on top of solution S_4. The remaining part of tube T, the side arm, the bridge, and buffer baths contain the borate buffer pH 8.6. The discontinuous buffer system is used to create a greater ptoential difference. The overall effect of Na Cl and discontinuous buffer gradient is to give a high potential difference with low current.

J.   PHASE PARTITION WITH POLYMERS:

(Albertson, 1973)

When two sufficiently different polymers are dissolved in water above certain concentrations, two immiscible aqueous phases are formed; one phase is rich in one of the polymers and the other phase is rich in the other. Three polymers: Dextran 600, Ficoll, and Polyethylene glycol formed three phases differing in hydrophobicity on dissolution in the presence of salts like KSCN.

4ml. Emulphogene extract of membranes was partitioned using 440 mg. Dextran, 480 mg. Ficoll, 320 mg. PEG and 760 mg. H_2O. The tube was shaken to dissolve the polymers and achieve equilibrium. The mixture was then centrifuged at 10,000xg. for 10 min. at 20°c to separate the phases. Dextran forms the least hydrophobic bottom phase, polyethylene glycol the most hydrophobic on the top and ficoll having intermediate properties forms the middle
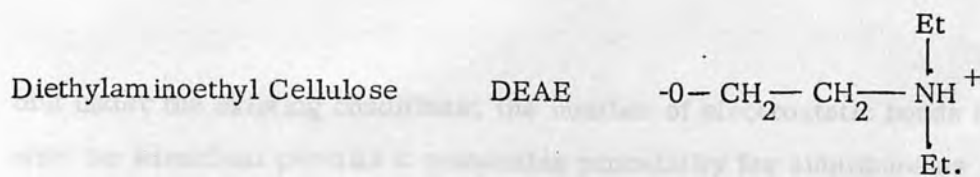
-41-

phase.

K.   MOLECULAR SIEVE CHROMATOGRAPHY

When a mixture of proteins is passed through a column of Sephadex, fractionation occurs; the components of the mixture are eluted in order of decreasing molecular size. (Porath & Flodin, 1963). The method can be more accurately regarded as providing an estimate of the Stokes radius (Andrews, 1964), since retardation by the gel depends on shape as well as size of the molecules (Ackers, 1967). For globular proteins a correlation between elution volume and molecular wiehgt has been demonstrated, and a graph of elution volume plotted against the log. mol. wt. has been shown to be almost linear over the range of optimal gel function. (Andrews 1962, 1964). The molecular weight of a simple globular protein can therefore be estimated by comparison of its elution volume with those of similar proteins of known molecular weight (Andrews, 1965).

Molecular weight estimations have been performed on cross linked dextrans on the Sephadex series, according to the method of Flodin (1962). Columns were run on 0.1 M - phosphate buffer pH 6.8 containing 0.1% detergent in the cold.

L.   ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange separations are carried out with special materials with an insoluble porous structure containing reactive groups which have associated with them ions which are capable of exchanging with ions in the surrounding medium. Cellulosic adsorbents were shown to be promising anion or cation exchangers (Peterson and Sober, 1956). The basic reactive groups are shown as under:

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Diethylaminoethyl Cellulose   DEAE $-O-CH_2-CH_2-NH^+ \begin{smallmatrix} Et \\ \\ Et \end{smallmatrix}$

Carboxymethyl cellulose   CM $-O-CH_2-C \begin{smallmatrix} O \\ O^- \end{smallmatrix}$

The reaction between the cation-exchange resin $R\ H^+$ and sodium ions would be

$$R\ H^+ + Na^+ \rightleftharpoons R\ Na^+ + H^+$$
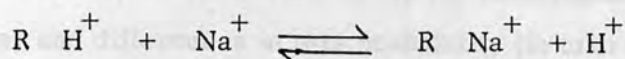
while that between anion exchange resin $R^+\ OH$ and chloride ions would be

$$R^+\ OH + Cl^- \rightleftharpoons R^+\ Cl^- + OH^-$$

Studies on the adsorption of proteins on ion-exchange surfaces show that the formation of multiple electrostatic bonds between the protein and the adsorbent is involved (Peterson and Sober, 1956). Both the protein and the ion-exchanger are polyelectrolytes and they are therefore capable of interacting at several points provided intercharge distances are favorable. In accord with the concept of multiple bonding is the observation that a homologous series of polynucleotides emerges from a cellulosic anion-exchange column in the order of increasing size (Tener et al.,1958; Staehelin et al.,1959).

The conditions required for elution of a given molecule from a given adsorbent depend upon the number of bonds that can be formed between them. Therefore a molecule having the same net surface charge density as another (and hence a similar electrophoretic mobility) but exceeding it significant in size, would require a stronger eluting condition because it would be capable of forming more bonds with the adsorbent.

When a mixture of proteins in an appropriate buffer is applied to an ion-exchange column, some of the proteins become tightly bound to the exchanger through the formation of many electrostatic bonds. The protein will not move so long as the initial conditions are maintained. Other protein components of the mixture, because they lack sufficient charges of appropriate sign to form multiple bonds with the exchanger, move through the column without being retarded.

A third group of proteins may possess charges in   such number and sign

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that under the existing conditions, the number of electrostatic bonds formed with the adsorbent permits a reasonable probability for simultaneous dissociation of all those restraining a given molecule. At the moment of release the molecule moves only to be held again at another site as electrostatic bonds are momentarily re-established. Such molecules are undergoing "true chromatography" which depend upon a series of successive adsorptive and desorptive processes for its high resolving potential. The rate of migration is, then, determined by the instantaneous probability of such release, and differences in this probability (in turn dependent upon the number and sign of the charges as well as their extension in space) are the basis for the differential migration of those proteins under fixed conditions.

Columns were made according to the method of Peterson and Sober (1962). Elution of adsorbed proteins was carried by increasing the ionic strength of the perfusing buffer in stages - stepwise elution.

M.   THIN-LAYER CHROMATOGRAPHY

Thin layer chromatography experiments were performed as described by Truter (1963). The glass plates were coated with 0.25 mm thick layers of alumina with the help of the applicator. The plates were left open in air for fifteen minutes to let alumina set. The plates were transferred to oven at 105°c for 35 minutes. Carotenoid preparations were applied as streaks with a pipette. The plates were developed using 40 : 60 :: acetone : pet. ether solvent system.

Experiments were carried out in air tight glass chambers pre-saturated with the solvent-system.

N.   POLAROGRAPHIC ASSAY METHODS:

Polarographic assays of oxygen were performed using a Fieldlab Beckman oxygen analyser assembly and consists of the analyzer incorporating the amplifier and all system controls, and the sensor. The sensor detects the dissolved oxygen partial pressure and causes a signal that is amplified for and read out on the meter.

In operation the sensor is placed in the sample and a potential of 0.53 volt is applied between the rhodium cathode and silver anode in the sensor. Oxygen in the sample diffuses through a teflon membrane, which separates the

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sample from the cathode - anode assembly. Diffused oxygen is reduced electro-
chemically (consumed) at the cathode. This reduction of oxygen causes a current
flow, proportional to the partial pressure of oxygen. The following reactions
occur:

$$O_2 + 2H_2O + 4\bar{e} \longrightarrow 4OH \quad = \quad \text{Cathode reaction.}$$

$$4\overset{\cdot}{Ag} + 4\overset{-}{Cl} \longrightarrow 4AgCl + 4\bar{e} \quad = \quad \text{Anode reaction.}$$
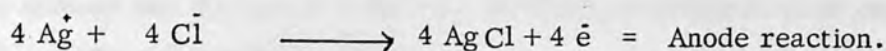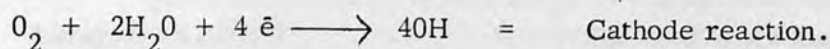
The analyzer was connected to a Bryan's chart recorder, model 27000
with variable chart speeds via a zero suppressor and a scale expander.

Calibration and calculation:

Distilled water at 30°c saturated with oxygen was placed in the
reaction vessel. The scale was calibrated at 7.78 ppm (i.e. 7.78 parts of
oxygen in 1 million parts of fluid) at 30°c, the solubility of oxygen at this
temperature. Full scale deflection on the chart recorder was adjusted so that
0-20 cm was equivalent to 10 ppm. The chart speed was adjusted to 1 cm per
minute. The volume of reaction vessel was 1.46 ml.

Calculation:

If the rate of reaction is = 1·0 ppm. per minute; this is equivalent to an
uptake of 0.08 µg. atoms of oxygen per minute.

i.   Assay of Malate dehydrogenase:

The enzyme activity was assayed on the oxygen electrode by using PMS
or menadione as electron acceptors. Assay mixture contained 0.1 ml. membrane
extract, 0.1 ml. menadione 0.01M, 0.1 ml. sodiumazide 1M., 1 ml. substrate
0.1M. and 0.7 ml. phosphate buffer 0.05M pH 7.0.

It was first brought to 30°c in a constant temperature water bath. This
meant placing the test tubes in the bath for two minutes.

The contents were saturated with oxygen by a thorough aeration on a
whirlimixer. Oxygen uptake was recorded and expressed as µg. atoms of
oxygen per minute per mg. of protein.

ii.   Assay of NADH dehydrogenase:

NADH dehydrogenase assay was carried out using menadione as an
electron acceptor. The reaction mixture consisted of 0.1 ml. membrane extract

in phosphate buffer, 0.5 ml. NADH 2×10^{-4} M in 0.05 M phosphate buffer pH 7.0 and 1.4 ml. of the buffer. Activity was expressed as μg. atoms O_2 taken up per minute per mg. protein.

O.   ESTIMATION OF VITAMIN K_1 (PHYLLO QUINONE):

Assay of vitamin K_1 is based on the method of Kroger and Dadak (1969) who showed that the molar difference absorbance coefficients of vitamin K at 265 and 289 nm upon reduction is

$$(E_{red.}^{-} \; E_{ox.})_{265 \, nm} - (E_{red.}^{-} \; E_{ox.})_{289 \, nm} = -14.7 \, mM^{-1} \, cm^{-1}$$

. The method consisted in reduction of oxidised vitamin K_1 by sodium borohydride (Na BH_4) under acid conditions using acetate buffer pH 5.4.

The method was used to determine the loss of vitamin K_1 in the presence of detergent extract of white and yellow membranes after being illuminated.

P.   PROTEIN ESTIMATION:

The protein in membrane extract was estimated by a method based on the Folin-Ciocalteu phenol reagent as described by Lowry but modified so as to give a higher colour yield and direct proportionality between absorbance at 650 nm and weight of protein within the range 15 - 110μg.

(Folin-Ciocalteu, 1927; Lowry et al, 1951; and Hartree, 1972).

Total nitrogen content and therefore the total protein content was estimated by the Kjeldahl method. The sample is decomposed by digestion in sulphuric acid in the presence of a selenium catalyst and the ammonia determined quantitatively by means of indone trione hydrate (ninhydrate). (Jacobs, 1959).

Q.   PHOSPHORUS ESTIMATION:

The sample is digested with 2 ml. 60% perchloric acid in a Kjeldahl flask. Pyrophosphate formed is converted to inorganic phosphate by hydrolysis at 100°c for 10 min. Colour is developed by amidol in the presence of 8.3% Ammonium molybdate. The intensity of the blue colour read at 600 nm. gives a measure of phosphorus present. (Allen, 1940).

SECTION 3. _____ EXTRACTION OF CAROTENOIDS. _____

A.   INTRODUCTION.

B.   DETERGENT SOLUBILIZATION OF THE PIGMENTS.

C.   EXTRACTION WITH AMMONIACAL ACETONE.

D.   SUMMARY.

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3.   EXTRACTION OF CAROTENOIDS

A.   INTRODUCTION

Various techniques have been used for extraction of carotenoids from bacterial membranes. Mathews and Sistrom (1959) obtained particles of S. lutea membranes bearing the carotenoid pigments by grinding the cells with alumina and by sonication. They also extracted the carotenoids from cells by use of TCA and hot absolute methanol. They suggested that pigments were particulate in nature and associated with proteins in the membrane. Thirkell and Strang (1967) reported that all carotenoids in S. lutea are readily extracted from the membranes by methanol which suggests that they are free pigments or possibly bound to protein by weak linkage. Jensen et al.(1975) resolved the structures of the carotenoid pigments occurring in S. lutea and reported that a major carotenoid is glucose linked. Organic solvents (e.g. methanol) were used for extraction of pigments in these studies. Results obtained about carotenoid association in vivo may be biased by the nature of the agents used for extraction. Lipid solvents such as ethanol, methanol, and acetone were obvious favourites for extraction; but it is known that acetone and alcohols break many lipid-protein and carotenoid-protein linkages. The carotenoid protein link is usually non-covalent, in invertebrates for example (Cheesman et al.,1967) and may be broken by this treatment. Relatively mild agents, detergents, were used in the present studies to determine whether the pigment was associated with protein or any other membrane component. Non-ionic detergents were chosen to solubilize the pigments with a relatively mild action. Any ionic detergent would render the pigments charged even if these were not protein associated. I have developed a gentle, rapid method which leaves the membrane facsimile intact after carotenoids have been extracted.

B.   DETERGENT SOLUBILIZATION OF THE PIGMENTS:

Since Gilby et al.(1958) established that carotenoids in S. lutea are localized in the cytoplasmic membranes, the preparation of membranes by

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enzymic degradation of the cell wall (Weibull, 1956) was adopted as a routine preliminary step. Initially a number of solubilizing agents with varying degrees of solubilizing capacity for biological membranes, and different in action, were tried (Table 2). Two mild, nonionic detergents — Emulphogene BC-720 and Triton x-100 gave the maximum yield of the pigments in the extracts. Membrane-pellets left after detergent treatment were virtually white. Spectra of solubilized pigments in some of the agents used are shown in Fig. 2. These are three peaked in the blue to violet region of the spectrum (500 - 360nm) which is characteristic for carotenoids (Goodwin, 1954). The relative heights of the peaks in each spectrum are different, which may be due to varying amounts of turbidity present, removal of turbidity being made difficult due to the viscous nature of the preparation. The spectrum of the lubrol extract is different from the rest in showing little absorption at shorter wavelengths. Instead of a peak at 418 nm there is a smooth shoulder. A comparison is made between the characteristics of the spectra presented here and those obtained by the use of organic solvents in other laboratories. The absorption maxima of the pigments of S. lutea in Tween 20 (410, 440, and 470 nm) and in Emulphogene and Triton (416, 447, and 476 nm) are close to those obtained for the three major pigments of S. lutea in methanol (415, 439 and 469 nm) by Thirkell and Strang (1967). The shifts in peak positions probably result from the replacement of organic solvents by an aqueous environment. The peak positions in these spectra are identical with those of carotenoids in intact membranes obtained by a difference spectrum of carotenoid containing S. lutea cells against white mutant cells. (Prebble, unpublished observation). This indicates that detergent solubilization did not apparently affect the carotenoid molecules, as judged by the electronic spectrum, and that they are probably extracted in their native state.

Some of the detergent solubilized spectra (Fig. 2) show a hump at 380 nm, which could be due to the presence of flavoproteins. This feature is absent in the spectra of these pigments in solvents such as methanol (Thirkell and Strang 1971), chloroform (Hodgkiss et al., 1954) or light petroleum (Brown, 1961). This suggests that detergents may have solubilized flavoproteins with the pigments. In our initial experiments it was noted that when extracts of the pigments were prepared from frozen cells, which had been stored for some

Table 2   EXTRACTION OF CAROTENOIDS OF S. LUTEA USING DIFFERENT SOLUBILIZING AGENTS.

Solubilizing agent.	Final concentration	Absorption maxima of the pigments solution			Carotenoid content 10^{-4} x mgm/100 mgm cells.
Emulphogene (Nonionic)	1% w/v	418	447	476	40
Triton (Nonionic)	1% w/v	418	447	476	26
Tween 80 (Nonionic)	1% w/v	410	440	470	0.76
Tween 20 (Nonionic)	1% w/v	410	440	470	0.72
EDTA (chelating agent)	0.25mM	418	447	476	7.2
Lubrol (Nonionic)	8 mgm/ml	418	447	476	12.0
Sodium Deoxycholate (Nonionic)	8 mgm/ml	418	447	476	14.0
Sodium Lauryl (anionic) Sulphate	10 mM	418	447	476	12.0

To 2 gm cell-pellet reagent was added to bring the final volume of suspension to 20 ml. The suspension was homogenized in a glass homogenizer for 15 minutes. The content of carotenoids calculated by extinction of the supernatant at 447nm. $E_{447nm.}^{1\%}$ = 2500 (β Carotene) (Goodwin 1954).
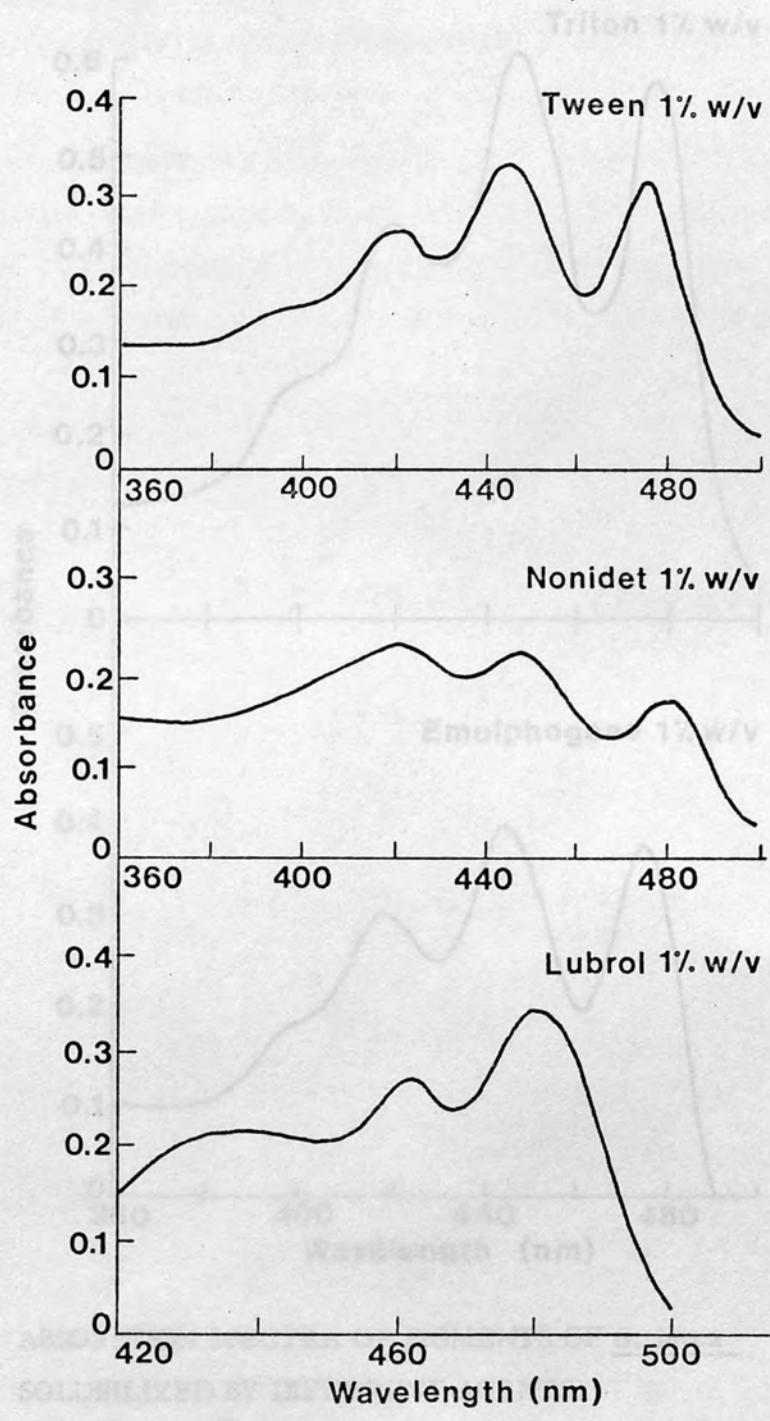
Fig. 2.   ABSORPTION SPECTRA OF PIGMENTS OF S. lutea
           SOLUBILIZED BY DIFFERENT AGENTS.
           Spectra recorded against solubilizing agent blanks.
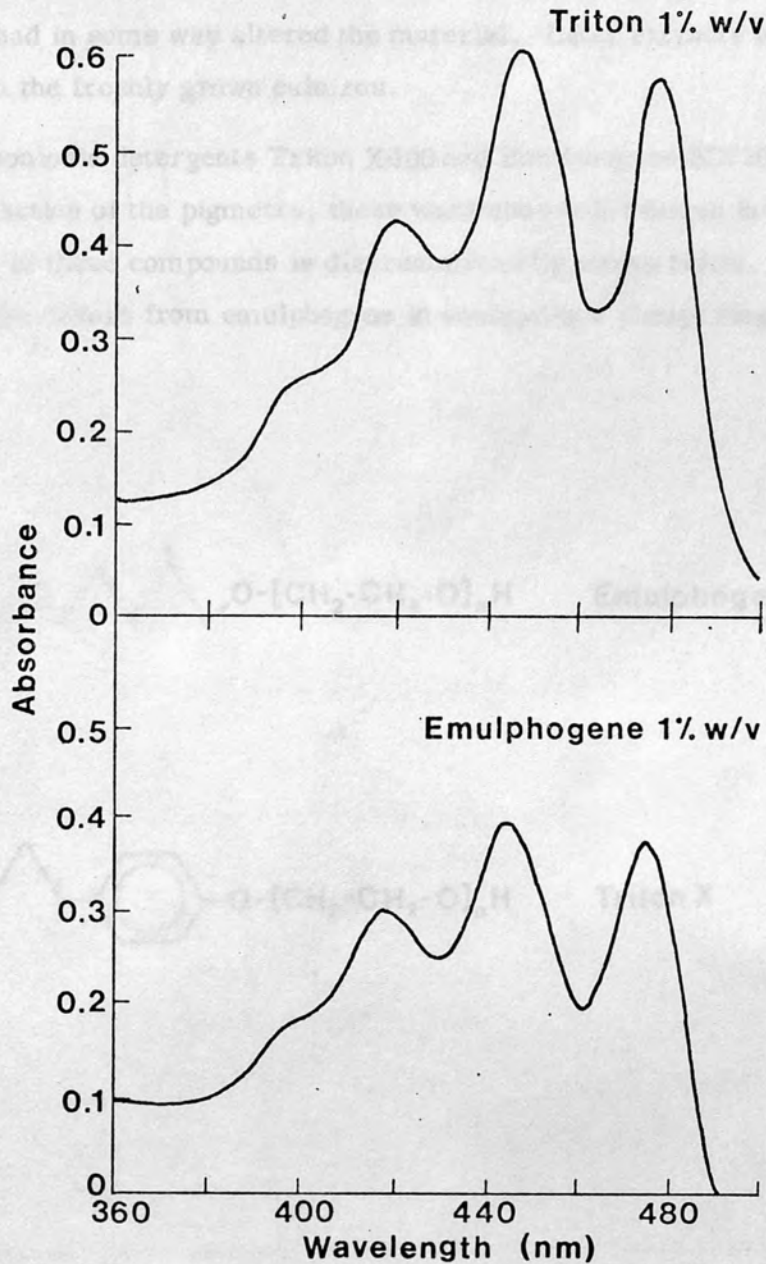
Fig. 2. ABSORPTION SPECTRA OF PIGMENTS OF S. lutea
SOLUBILIZED BY DIFFERENT AGENTS.
Spectra recorded against solubilizing agent blanks.

time, the absorption spectrum did not possess the 380 nm hump. This shows that freezing had in some way altered the material. Later extracts were prepared from the freshly grown cultures.

As the nonionic detergents Triton X-100 and Emulphogene BC-720 gave maximun extraction of the pigments, these were chosen for future investigations. The structure of these compounds is diagrammatically shown below. It can be seen that Triton differs from emulphogene in containing a phenyl ring.

Emulphogene BC

Triton X
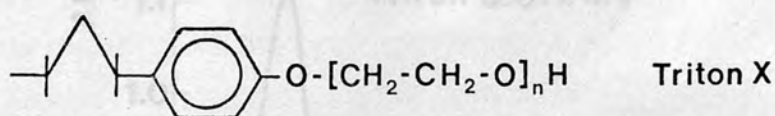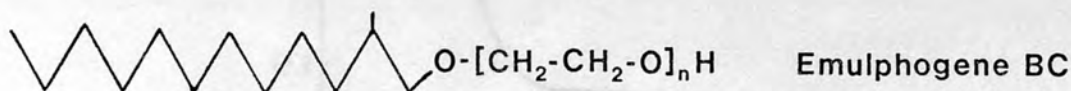
Solutions of solubilized pigments showed strong absorptions in the ultra-violet region around 280 nm. Proteins with aromatic amino acid residues such as Tyrosineand Tryptophan show a peak at about 280 nm. It was necessary to know how much absorbance was due to solubilized proteins and how much due to the detergents in the carotenoid preparations. Absorption spectra of the detergent solutions are shown in Fig. 3 A0.01% solution of Triton shows a peak at 276 nm and one strong peak at 225 nm with extinctions of 0.2 and 0.75 respectively. A0.1% solution of Emulphogene shows a peak at 225 nm and one

-53-
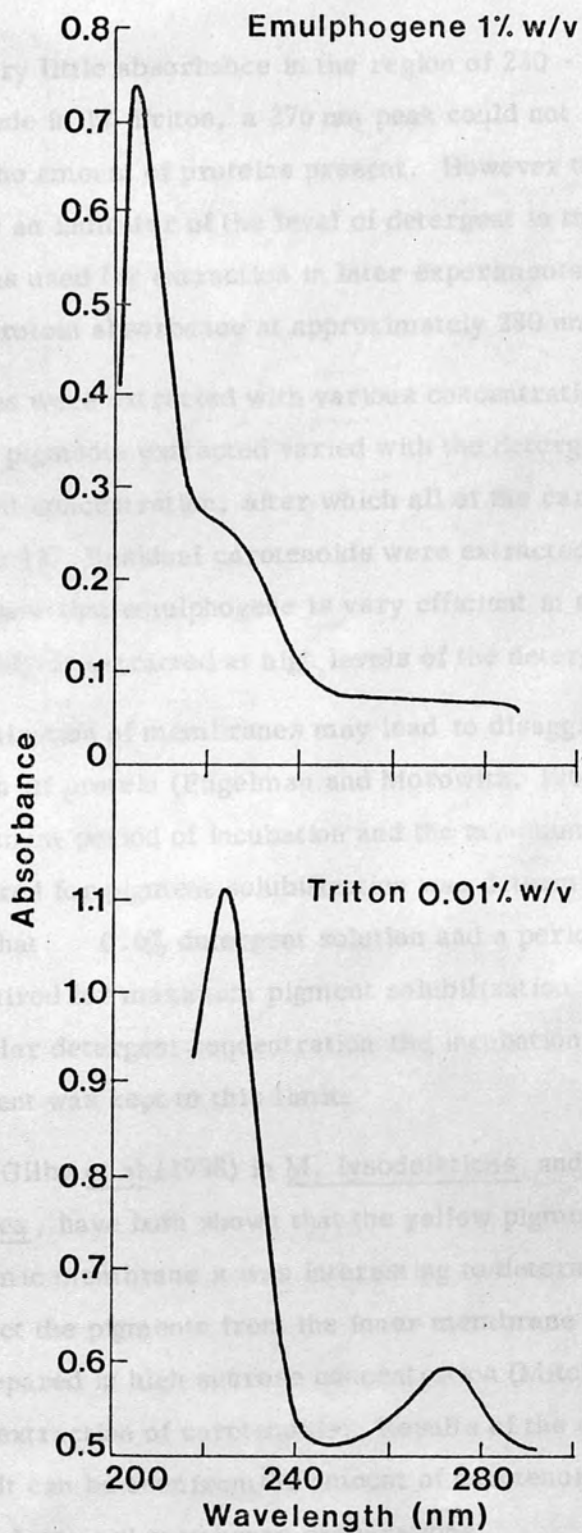
Fig. 3.   SPECTRA OF EMULPHOGENE BC-720 AND TRITON X-100
             SOLUTIONS.
             Spectra recorded against distilled water blanks.

at 205 nm but very little absorbance in the region of 230 - 280 nm. Thus, with extracts made in 1% Triton, a 276 nm peak could not indicate quantitatively, the amount of proteins present. However this absorption band could be used as an indicator of the level of detergent in the preparation. Emulphogene was used for extraction in later experiments because it allowed an estimate of protein absorbance at approximately 280 nm.to be made.

Membranes were extracted with various concentrations of Emulphogene. The yield of the pigments extracted varied with the detergent level until a critical detergent concentration, after which all of the carotenoids are extracted (Table 3). Residual carotenoids were extracted with acetone. These results show that emulphogene is very efficient in solubilizing the pigments and 100% is extracted at high levels of the detergent.

As solubilization of membranes may lead to disaggregation with time and denaturation of protein (Engelman and Morowitz, 1968; and Morowitz & Terry, 1969), the minimum period of incubation and the minimum concentration of detergent required for pigment solubilization was determined (Fig. 4). It can be seen that   0.6% detergent solution and a period of one hour are the optima required for maximum pigment solubilization. In later studies for this particular detergent concentration the incubation period of membranes with the detergent was kept to this limit.

Although Gilby et al.(1958) in M. lysodeikticus and Mathews and Sistrom (1959) in S. lutea , have both shown that the yellow pigments are entirely present in the cytoplasmic membrane it was interesting to determine whether it was easier to extract the pigments from the inner membrane face or not. Protoplasts of the cells prepared in high sucrose concentration (Mitchell and Moyle, 1956) were used for extraction of carotenoids. Results of the experiment are shown in Table 4.   It can be seen from the amount of carotenoid obtained from identical protoplasts and membrane preparations,   that carotenoids are slightly more readily extractable from the protoplasts. For pigment extraction, therefore, preparation of membranes does not seem to be a requirement. It is possible that addition of detergent to the protoplast suspension results in lysis. Later experiments on membranes and protoplasts showed that
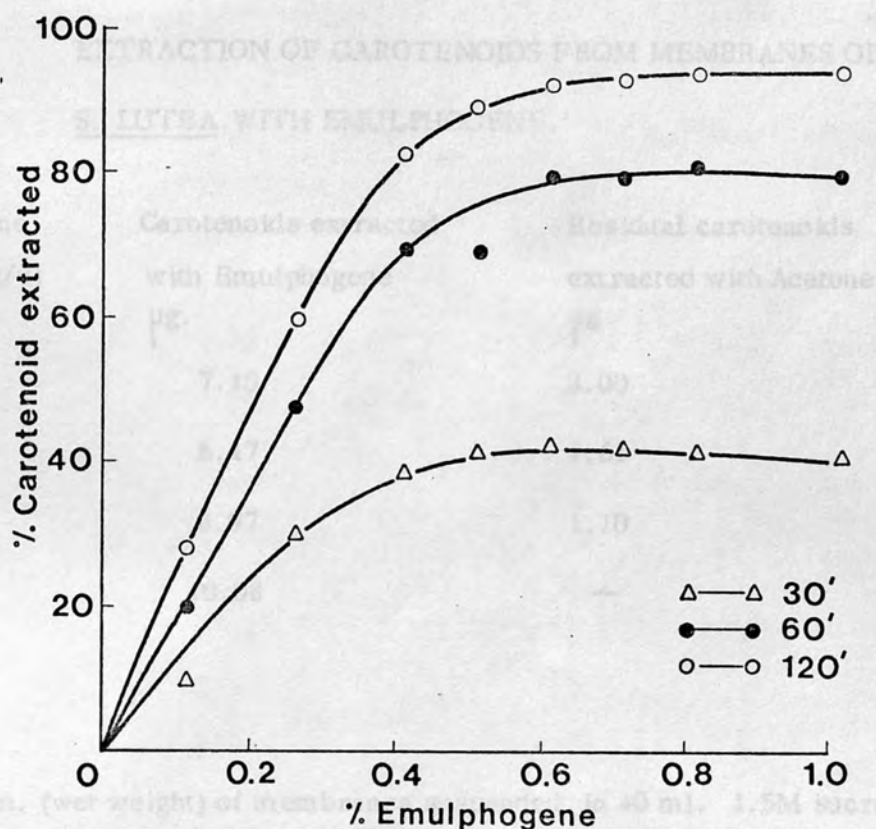
Fig. 4.   EXTRACTION OF CAROTENOIDS FROM S. LUTEA
WITH EMULPHOGENE.

Equal portions of membrane pellets were incubated with
0.1 - 1.0% Emulphogene solutions for 30, 60 and 120 minutes
with occasional shaking on a whirlimixer. The tubes were
then centrifuged for 15 minutes (40,000xg). The supernatant
solutions were read in a spectrophotometer against respective
detergent solutions. The final pellets were re-extracted with
acetone to estimate the residual carotenoids.

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Table 3   EXTRACTION OF CAROTENOIDS FROM MEMBRANES OF

S. LUTEA WITH EMULPHOGENE.

Emulphogene Per cent (w/v)	Carotenoids extracted with Emulphogene µg.	Residual carotenoids extracted with Acetone µg.
0.10	7.10	3.00
0.25	8.47	1.61
0.50	8.97	1.10
1.00	10.08	—

4 gm. (wet weight) of membranes suspended in 40 ml. 1.5M sucrose and divided into 4 equal parts; detergent was added to each to the desired level. Tubes were shaken on a whirlimixer for three minutes and centrifuged at 18,000 r.p.m. (40,000 x g) for 45 minutes. The pellets were then extracted with 4 ml. acetone. Carotenoid content calculated on an arbitrary basis of $E^{1\%}_{447}$ = 2500.

Table 4   EXTRACTION OF CAROTENOIDS FROM PROTOPLASTS AND
                 MEMBRANE PREPARATIONS OF S. LUTEA WITH EMULPHOGENE.

Protoplast pellet	Percent Detergent	Total carotenoid extracted $\mu g.$
A	0.10	7.4
B	0.25	9.6
C	0.50	10.1
D	1.00	10.5

Membrane Pellet	Percent Detergent	Total carotenoid extracted $\mu g.$
A	0.1	7.1
B	0.25	8.5
C	0.50	9.0
D	1.0	10.1

Cells harvested from a litre culture were suspended in 30 ml. 1.5M
sucrose and homogenized to give a uniform preparation. 30 ml. 1.5M sucrose
containing 1mg/ml lysozyme were added and the suspension rehomogenized
for even distribution of the enzyme. The mixture was allowed to stand for 15
minutes at room temperature. 25 ml. aliquots were drawn from the mixture
and centrifuged for 30 minutes at 40,000 xg. One of the pellets (A) was washed
in, and resuspended in 30 ml. 0.05M phosphate buffer containing 1% Nacl (w/v),
the other pellet 'B' was resuspended in, 30 ml. sucrose 1.5M solution. Aliquots
of 5 mls. were drawn from suspensions 'A' and 'B' and detergent was added to
concentrations of 0.1, 0.25, 0.5 and 1.0%w/v. After standing for 45 minutes, the
preparations were centrifuged at 40,000 xg for 30 minutes. Carotenoid content
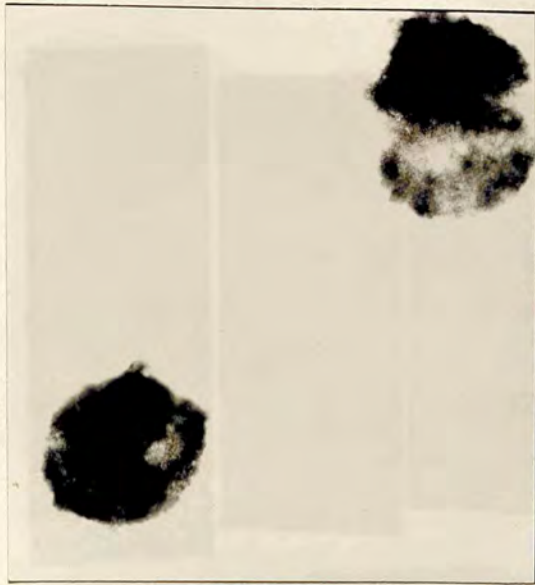in the supernatants were calculated by the arbitrary standard of
$E_{447}^{1\%} = 25,00.$

Final pellets 'A' and 'B' represent membranes and protoplasts
respectively.

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location of malate dehydrogenase (membrane bound), by similar tests gave results which suggested that lower concentrations of detergent did not significantly lyse cells (see Section 6F). On the basis of this experiment it cannot be decided unequivocally that the carotenoid is on the outer face of the cytoplasmic membrane. It is possible, however that the pigments are located in the membranes in such a manner that they are readily extracted from either face of the membrane.

Detergents may solubilize membranes completely or may have a specific action and solubilize a certain number of proteins and lipids. Structural changes in bacterial membranes such as swelling (thickening) due to the detergent, Triton, have been observed (Lengsfeld et al., 1973). Although there was no indication of total solubilization of the membranes, attempts were made to examine the effects of detergent action on the morphology of membranes. Fig. 5 a, b, c and d show electron micrographs of identical membrane preparations — a, b show membranes which were treated with detergent, while c, d were not treated. There is no apparent morphological difference due to extraction. Similar observations were made using phase contrast microscopy. Thus, the detergent action was relatively mild and selective; although a number of proteins including certain enzymes were extracted (See below) and the carotenoids were extracted almost completely.

C.   EXTRACTION WITH AMMONIACAL ACETONE:

A useful method of extraction of carotenoid-complexes is with cold annomiacal acetone. It has been used to extract carotenoid complexes from green leaves of spinach (Nichimura and Takamatsu, 1957) and also for carotenoprotein extraction from various sources (Ke, 1971). S. lutea carotenoids were extracted from the membranes with ammoniacal acetone by the method of Ke (1971). The spectral characteristics showed a shift in the peak positions of the carotenoids but a total absence of absorbance in the ultra-violet region around 280 nm which probably means no extraction of proteins with the pigments. The ammoniacal acetone spectrum of the carotenoids is consistent with the spectrum of the carotenoids in light petroleum obtained by Brown (1961) from S. lutea. Thus, this method does not seem suitable for extraction of carotenoid-

Fig. 5.   Electron Micrographs of S. lutea membranes before and after

treatment with Emulphogene.

Membranes : c, d.

Membranes after treatment with Emulphogene : a, b.

Electron micrographs were obtained by negative staining

after treatment of the samples with tungstic acid.

Magnifications : a : X30,000.

            b : X60,000.

            c : X20,000.

            d : X40,000.

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complexes in S. lutea.

One of the fundamental questions was whether the pigment composition of the detergent solubilized membrane extract was the same as that in the membrane or alternatively whether the action of the detergent was specific and selective. Thin-layer chromatography was performed on alumina plates using an acetone extract of whole cells, a methanol extract of whole cells and an Emulphogene extract of the membranes. 3 pigment fractions in each case were seen when TLC plates were developed in acetone (Table 5). It can be seen that there is one major fraction in each extract and two minor ones. This suggests that the detergent action is not selective.

Strang and Thirkell (1969) reported identification of 4 naturally occurring pigments, on silica gel chromatograms of alcohol extracts of S. lutea. The pigment missing in our preparation of carotenoid extracts could possibly be the minor carotene in S. lutea, lycopene. The fractions obtained on alumina plates are probably the three major pigments of S. lutea: Sarcinaxanthin-glycoside, sarcinaxanthin, and C_{50}-diol having Rf values of 0.3, 0.5, and 0.7 respectively.

D. SUMMARY

Various detergents, EDTA and ammoniacal acetone were used to extract the carotenoids in S. lutea. The pigments were found to be easily and rapidly extracted with the non-ionic detergents Emulphogene and Triton. At high levels 100% extraction of the carotenoids was achieved with these detergents. Thin layer chromatography shows that Emulphogene extracts the three major pigments from the membranes, C_{50}-diol, Sarcinaxanthin, and Sarcinaxanthin-glucoside.

Table 5   CHARACTERIZATION OF CAROTENOIDS IN DETERGENT EXTRACT ON ALUMINA PLATES.

Acetone extract		Methanol extract		Emulphogene extract	
Rf	carotenoid content $\mu g.$	Rf	carotenoid content $\mu g.$	Rf	carotenoid content $\mu g.$
0.3	16	0.3	10	0.3	13
0.5	6	0.5	6	0.5	5
0.7	3	0.7	3	0.7	3

Equivalent weights of cell pellet and membrane pellet were extracted with acetone-methanol and Emulphogene (0.6% in 0.05M phosphate buffer pH7.0) until maximum extraction. To the Emulphogene extract 5 volumes of acetone and 3 volumes of diethyl ether was added in a separating funnel and shaken till the pigments were separated into the acetone layer. The various extracts were reduced to same volume under nitrogen pressure. Solvent used for development: Acetone: petroleum ether (40 : 60 v/v). Pigment fractions were scrapped off the plates and dissolved in 5 mls. acetone. Carotenoid content was calculated on the basis of $E_{447}^{1\%} = 2500$.

SECTION 4. <u>STUDY OF EXTRACTED CAROTENOIDS.</u>

A. INTRODUCTION.

B. PURIFICATION.

i. AMMONIUM SULPHATE FRACTIONATION.

ii. DIALYSIS.

iii. GEL FILTRATION.

iv. STARCH GEL ELECTROPHORESIS.

v. ANALYTICAL ACRYLAMIDE GEL ELECTROPHORESIS.

vi. PHASE PARTITION.

vii. SUCROSE DENSITY GRADIENT ELECTROPHORESIS.

viii. PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS.

C. SUMMARY.

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4.　STUDY OF EXTRACTED CAROTENOIDS

A.　INTRODUCTION

Earlier studies on bacterial carotenoids showed that in photosynthetic bacteria and also in some non-photo synthetic bacteria, the carotenoids were frequently protein-complexed. Saperstein and Starr (1955) obtained pigments from two Coryneform bacteria and from two other non-photosynthetic bacteria: Mycobacterium phlei and Micrococcus agilis, by grinding the cells with alumina and extracting with 0.05M NaCl. Ammonium sulphate at a final concentration of about 70% precipitated all the pigments. They concluded that pigments were portein-associated. A similar study in S. lutea was carried out by Mathews and Sistrom (1959). Extracts of mechanically disrupted cells contained carotenoid particles sedimentable at 23,000 xg. This fraction was analysed and found to contain carotenoids, succinic dehydrogenase and cytochrome 'C' oxidase. Mathews and Sistrom concluded that carotenoids in S. lutea occur in cell free extracts as easily sedimentable particulate protein complexes. Emulphogene and Triton had solubilized the pigments in an aqueous medium without solubilizing the membranes in our studies. It was hoped that if the major pigment is a carotenoprotein this could be readily examined by fractionation of the preparation with ammonium sulphate, electrophoresis and gel filteration. This could result in a pure preparation of carotenoprotein.

B.　PURIFICATION

i.　Ammonium sulphate fractionation:

Initial tests on 1% Triton (w/v in 0.05M phosphate buffer) extracts of carotenoids showed that on adding ammonium sulphate to 10% saturation part of the pigments come out of solution. Further addition of the salt to 30% saturation resulted in the formation of an oily film at the surface of the solution, and a white precipitate that was obtained as a pellet on centrifugation (15,000 xg, 10 min.). The precipitate was found soluble in 0.05M phosphate buffer and absorbed strongly at 260 nm. The formation of a low density phase of solubilized pigments on 30% ammonium sulphate saturation is partly due to the lipid nature of the detergent. This was found when ammonium sulphate was added to 1% (w/v) Triton and 1% (w/v)

Emulphogene solutions. A detergent rich zone (epiphase) and an aqueous zone (hypophase) separated at 3% saturation.

Ammonium sulphate treatment does not alter the carotenoids of S. lutea, as judged by the spectra which are similar to that of untreated material, but increases the detergent concentration as seen from an increased absorbance at 276 nm. These observations are in contrast to those of Saperstein and Starr (1955), and possibly point to a different state of the pigments in this organism.

Techniques were adopted to decrease the level of the detergent in the pigment extract, so that the behaviour of the carotenoids could be studied independently of the detergent.

ii.   Dialysis.

Dialysing Triton and Emulphogene extracts (1% w/v in distilled water) of the membranes over long periods in the cold, resulted in precipitation of some of the solubilized material. The precipitate was extracted with the detergent solution and was found to amount to only 10% of the total pigments. Spectrum of the dialysate showed that neither Emulphogene nor Triton had passed through dialysing sac in appreciable amounts.

In similar experiments when detergent extracts were dialysed against large volumes of acetate buffer (pH4.5) and formate buffer (pH3.1) in the cold, a coloured precipitate was obtained. The pigment was extracted from the coloured pellet with the detergent (1% Triton or Emulphogene). It was found that the pigmented material which precipitated at acidic pH is 5% of the total solubilized material. The final residue was white, soluble in 0.05M phosphate buffer and absorbed strongly at 260 nm but had no absorbance in the visible region.

The difficulty in removing the detergent from the solubilized mixture is probably due to the formation of micelles, which are aggregates of detergent molecules and contain lipid-protein complexes in their structure.

The removal of detergent from detergent-protein complexes derived

from the membrane may lead to protein aggregation and often to the formation of amorphous precipitates. The aggregation tendency is probably due to the hydrophobic domains on the proteins being uncovered as bound detergent disappears (Helenius and Simons, 1974). In our experiments however, the precipitates appear only after very long dialysis.

An alternative technique to dialysis used for the removal of detergent was a diaflo cell with application of nitrogen pressure and continuous washing with buffer. The method quickly reduced the volume but did not appreciably reduce the detergent level. Removal of non-ionic detergents by simple dialysis and ultra filteration has proved difficult for other workers (Razin et al., 1968, and Gaylor and Delwich, 1969).

Attempts were made to fractionate the detergent extract of the membranes on the basis of the size of the molecular species present.

iii.   Gel Filtration:

In gel filtration molecules are separated according to their size. The basis of separation rests in the fact that access to the interior of the bed is governed by size of the solute molecule. The column operates by a diffusional partition of a solute between an interior stationary phase and a mobile exterior phase without the intervention of the specific adsorption of solute molecules in the bed matrix.

Cross linked dextrans of the Sephadex and Sepharose types were used to make columns by the method of Flodin (1962). The columns were run in the cold and dark to avoid protein denaturation and minimize the effects of light on the pigments. The exclusion limits of globular proteins on Sephadex G 75 and Sephadex G200 or Sepharose 4B are approximately 150,000 and 1000,000 daltons respectively.

In initial experiments on gel filtration, pigments migrated on columns as a diffused band, and were obtained as a large zone spread over several

fractions. This trailing was affected by the absence of detergent in the columns and the eluting buffer. Later experiments showed resolution of the pigments as a single fraction when detergents were present in columns and the eluting buffer.

A marked decrease in detergent level of the pigment extract was observed on filtering on Sephadex G75 columns. A 1% Triton extract of membranes concentrated by ammonium sulphate precipitation and dissolved in phosphate buffer was fractionated on Sephadex G75. Results of fractionation are plotted by the absorbance of carotenoids at 447 nm and detergent at 276 nm (Fig. 6). Pigments are obtained as a single fraction in close association with the detergent as shown by the correspondence of the two peaks. It can be seen from this plot that although a high level of detergent is associated with the peak of carotenoids, a significant level of it is eluted in the following fractions. This indicates that the column can reduce the detergent level of carotenoid preparation significantly. The Sephadex column was standardized using cytochrome 'C', serum albumin and Blue Dextran. The pigment was estimated to have a molecular weight of 95,000 daltons. A further test of column filtration was made by using Sephadex G200 and a 1% Triton extract concentrated in a diaflo cell. The sample was layered on a Sephadex G200 column and eluted with the 0.1M Tris HCl pH7.0 buffer containing 0.1% Triton. The pigments are eluted as a single fraction in front of the major detergent peak (Fig.7a) Some detergent was retarded on the column and removed. It is possible that the detergent had formed two different size of micelles the bigger micelles that contained the pigments were eluted first, while the smaller micelles were eluted later and did not contain the pigment. The columns were washed with the buffer and standardized using serum albumin, cytochrome 'C' and Blue Dextran. The pigment was eluted between levels of serum albumin and Blue Dextran. Estimates of molecular size made from the plot of log. molecular weight against elution volume give an average value of 120,000 daltons.

The proteins of the Emulphogene extract of the membranes were partially resolved on a Sephadex G200 column, and showed a separate elution profile. Results are shown in Fig. 7b . It is to be noted that the 276 nm plot

Fig. 6.

FRACTIONATION OF 1% TRITON-SOLUBILIZED PIGMENT
ON SEPHADEX G75.

Sample : 5ml. (19μg. carotenoid/ ml.).

Flow rate : ml/5 min.

Eluting buffer : 0.05M phosphate containing 0.1% Triton.

Size of the column : 40 x 2 cm.

(b)

Absorbance

protein+detergent 276 nm
carotenoid 447 nm

Elution volume (ml)

(a)

Absorbance

276 nm
447 nm

Elution volume (ml)
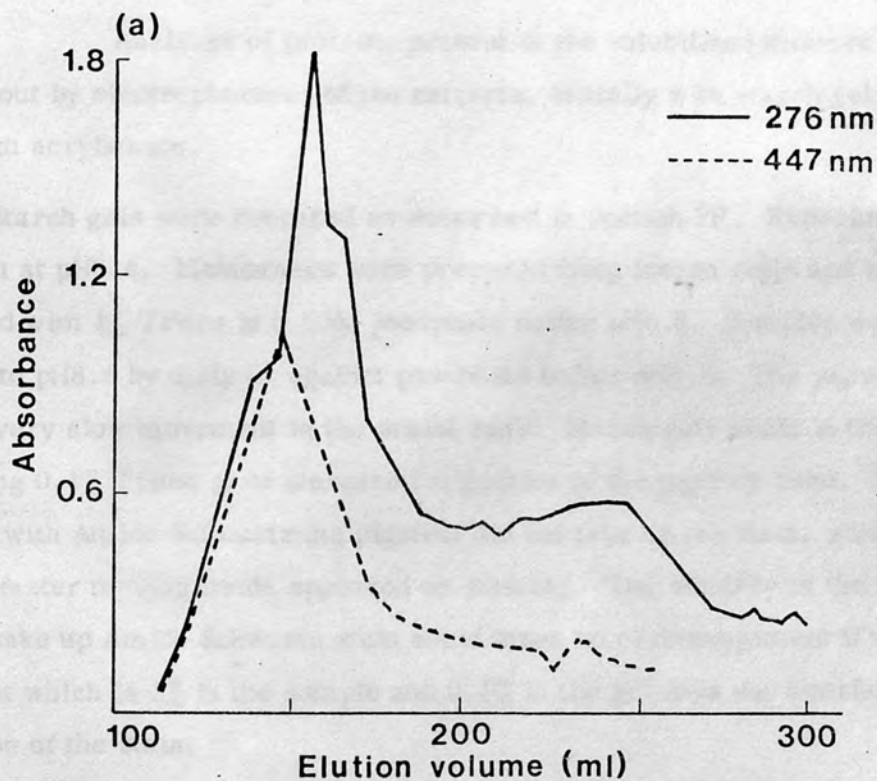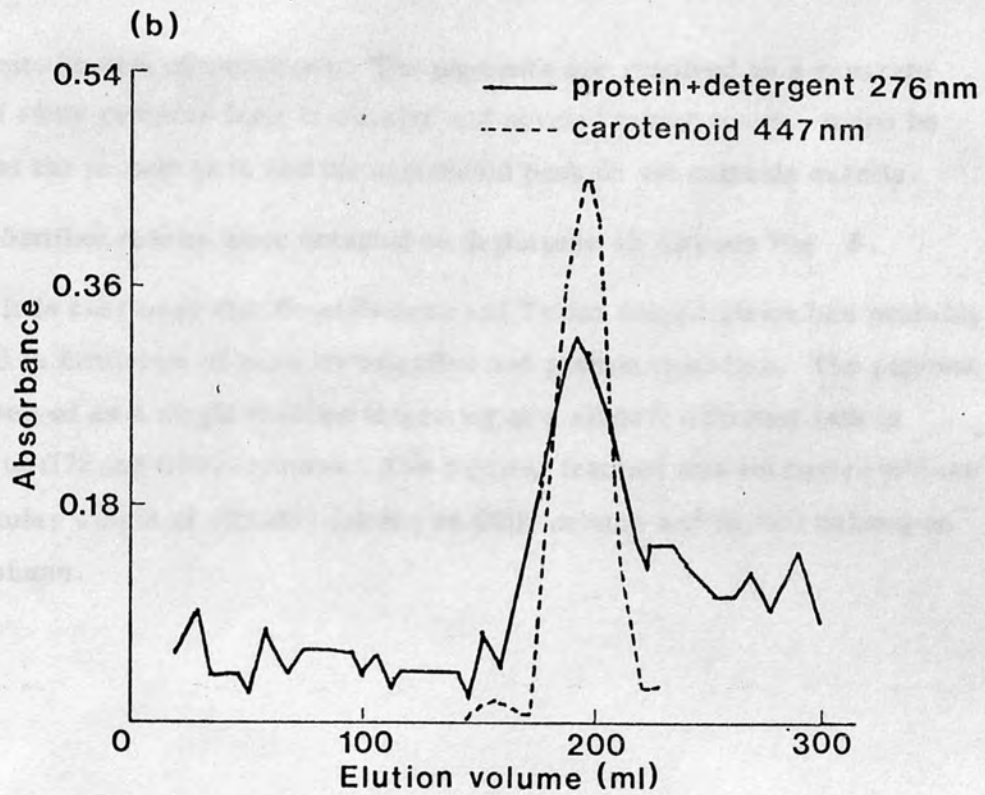
Fig. 7. FRACTIONATION OF SOLUBILIZED PIGMENT ON SEPHADEX G200.

Sample : 5ml (20 μg. carotenoid/ml). Flow rate : ml/5 min.

Size of columns : 40 x 2 cm.

Eluting buffer : 0.1M Tris-HCl, pH7.0 containing 0.1% detergent.

Solubilizing agent : (a) 1% Triton (b) 1% Emulphogene.

represents protein absorbances. The pigments are resolved as a separate fraction while proteins form one major and several minor peaks. It can be seen that the protein peak and the carotenoid peak do not coincide exactly.

Similar results were obtained on Sepharose 4B Column Fig 8.

It is concluded that Emulphogene and Triton solubilization has probably resulted in formation of pigment-micelles and protein-micelles. The pigment was resolved as a single fraction migrating at a slightly different rate to proteins on G75 and G200 columns. The pigment fraction was estimated to have a molecular weight of 120,000 daltons on G200 column and 95,000 daltons on G 75 column.

iv.   Starch Gel Electrophoresis.

Analyses of proteins present in the solubilized mixture were carried out by electrophoreses of the extracts, initially with starch gels but later with acrylamide.

Starch gels were prepared as described in section 2F. Experiments were run at pH8.6. Membranes were prepared from frozen cells and were extracted with 1% Triton in 0.05M phosphate buffer pH6.8. Samples were brought to pH8.6 by dialysis against phosphate buffer pH8.6. The pigment-band showed very slow movement in the initial runs. Starch gels made in the buffer continaing 0.1% Triton gave increased migration of the pigment band. On staining with Amido Schwartz the pigment did not take up the stain, although several faster moving bands appeared on staining. The inability of the pigment band to take up Amido Schwartz stain could mean no carotenoprotein if the detergent which is 1% in the sample and 0.1% in the gel does not interfere in the action of the stain.

Experiments were also performed at pH3.1 with gels made with formate buffer containing 0.1% Triton. It was found that the pigment band migrated in the same direction at pH3.1 and at pH8.6 which shows that the pigment is probably not a protein. The observation that the pigment migrates in the electric field
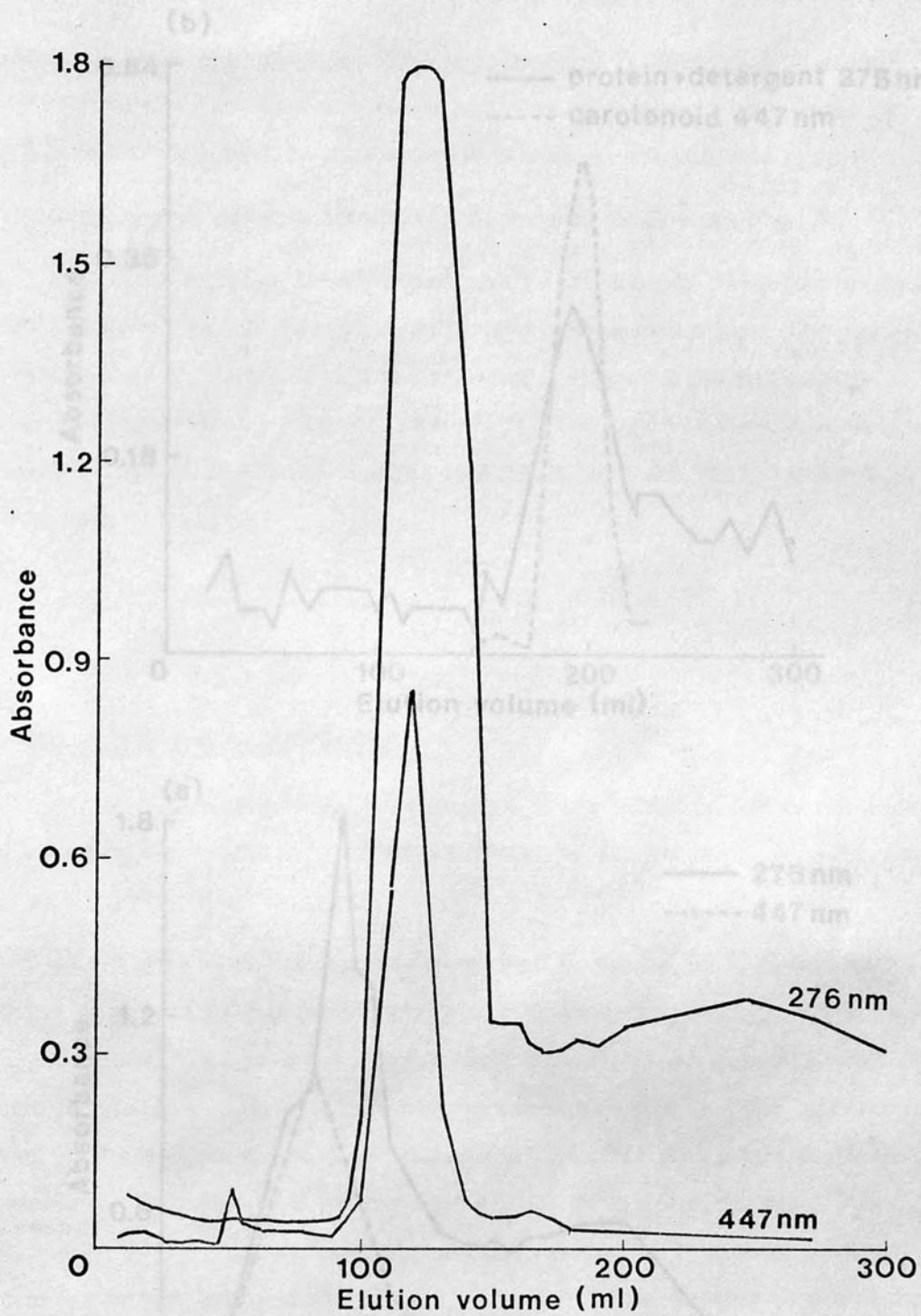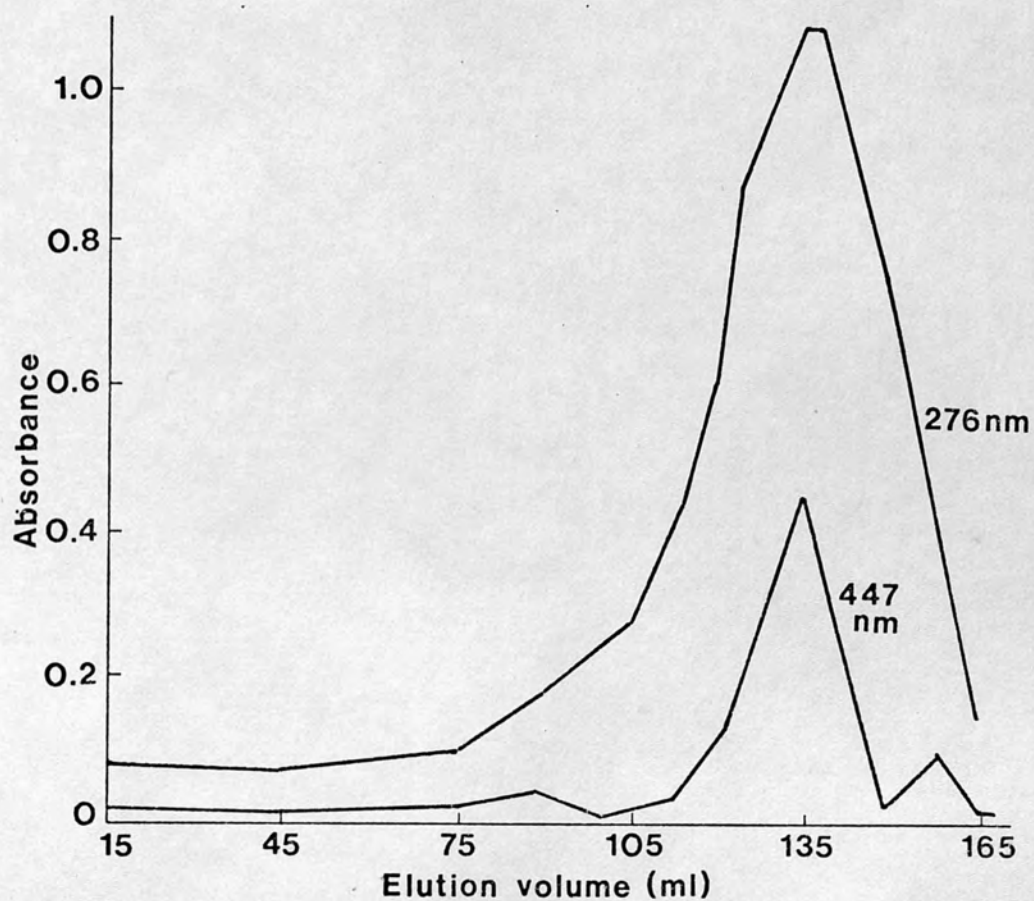
Fig. 8.   FRACTIONATION OF 1% TRITON SOLUBILIZED PIGMENTS
ON SEPHAROSE 4B.

Sample : 5ml (15μg. carotenoid/ml).

Flow rate: ml/5 min.

Eluting buffer : 0.1M Tris-HCl, pH7.0 containing 0.1% Triton.

could be due to association with phospholipids.

v.   Analytical acrylamide gel electrophoresis:

Initially gels of 3, 5, 7.5 and 10% strength (w/v concentration of monomer) were tried. 7.5% gels were found to produce the best results.

One percent Emulphogene extract of the pigments was used for electrophoresis which was performed in the cold at pH8.6 using 0.04M Tris Citrate-Borate buffer system. Carotenoids migrated as a diffused zone which did not stain with Amido Schwartz. About 20 bands were seen after staining for protein (Fig. 9). The pigment does not seem to be a protein or associated to a protein.

The solubilization technique was modified. It was found that Emulphogene at 0.6% (w/v) can solubilize about 80% pigment content (SECTION 3). It was assumed that the detergent action would be selective and limited number of proteins would be solubilized.

0.6% Emulphogene extract of the pigment was prepared from the membranes of fresh cultures of the bacteria. The pigment migrated as a diffused zone on 7.5% acrylamide gels. On staining with Amido Schwarz 3 strong and 6 satellite bands were seen (Fig. 10). Identical results were obtained with coomassie blue staining. The pigment zone remained unstained in each case but two thin bands within the pigment zone were stained for protein.

Emulphogene solubilization can be selective in extracting a limited number of proteins from the membranes of S. lutea. Attempts were made to purify the pigment using a polymer-phase-partition technique.

vi.   Phase Partition:

Carotenoid extracts obtained by detergent solubilization were partitioned using polymers (PEG, Ficol and Dextran). PEG is the more hydrophobic and Dextran the more hydrophilic. Albertsson (1971) reported that

Fig. 9   Electrophoresis of 1% Emulphogene extract on analytical
           acrylamide gels (7.5%).

                        Stain : Amido Schwartz.

                        Buffer : 0.04M Tris Citrate-Borate; pH8.6.

                        Current : 30 m A.

                        Voltage : 250 volts.

                        Duration of run : 3hours.

                        Temperature of surroundings : 8°c.

                        pz : pigment zone.

Fig. 10   Electrophoresis of 0.6% Emulphogene extract on 7.5%

analytical acrylamide gels.

pz : pigment zone.

Buffer system : 0.04M Tris Citrate-Borate; pH8.6.

Current : 3m A/gel.

Voltage : 200 volts.

Temperature of surroundings : 8°c.

when phosphatidylcholine was partitioned in the presence of salts (KCNS etc) in this three-phase system, 94% of the phospho lipid was partitioned in the top PEG phase.

Attempts to partition 1% emulphogene extract of pigments in a mixture of Dextran, Ficoll, and PEG using salts NaCl, NH_4Cl, $(NH4)_2SO_4$ and KCNS showed that only KCNS was effective for complete separation of phases. When solubilized pigments were partitioned in the presence of KCNS, all pigments were separated in the PEG phase. This is consistent with the conclusion above that pigments are phosophlipid associated.

· Solutions of white cakes formed at the interfaces of the polymer system, did not absorb in the ultra-violet or visible region and are thus probably devoid of protein or carotenoid. The top phase containing all the pigments, was subjected to electrophoresis on 7% analytical acrylamide gels at pH8.6 using discontinuous Triscitrate - Borate buffer system. The pigments showed very slow migration. A single protein band was obtained on staining gels with Amido Schwartz, running in front of the carotenoid band (Fig. 11) The carotenoid zone remained totally unstained. It is deduced that the stained band is a hydrophobic protein and that pigment is not carotenoprotein. It is known that KCNS is a hydrogen bond breaker and a carotenoid-protein link may be disrupted in an experiment using this salt; but the pigment did not stain for protein even in the absence of KCNS e.g. starch gel electrophoresis, acrylamide electrophoresis of extracts. PEG phase showed an identical spectrum to that of solubilized pigments in detergents. It contained a high level of detergent. The phase did not contain either NADH dehydrogenase or malate dehydrogenase activity which was originally present in the solubilized pigments (See later).

vii.   Sucrose density gradient electrophoresis:

The principle and assembly of this electrophoresis has been described in SECTION 2.I.   The technique is inherently gentle; sucrose and NaCl are unlikely to cause damage to any constituent of the solubilized mixture.

In the present studies the method was chosen for following reasons:

Fig. 11   Electrophoresis of the pigment from the polyme phase-
              partition system.

              pz : pigment zone.
              Buffer system : 0.04M Tris Citrate-Borate; pH8.6.
              Current : 3m A/gel.
              Voltage : 240 volts.
              Duration of the run : 2 hours.
              Temperature of the surroundings : 8°c.

(a)   to purify the pigments by removing those proteins that were migrating
       in front of the carotenoid zone.

(b)   to remove detergent from the solubilized pigment.

(c)   to obtain a sharp,concentrated carotenoid band that can be further
       analysed.

Initial runs were performed using 1% Emulphogene (w/v) extract
of membranes. It was found that the migration of the solubilized pigment was
very slow. The method was modified: 0.6% Emulphogene extracts were
prepared and discontinuous buffer system was used so as to purify the pigment
with quicker runs on sucrose density gradient column. This would presumably
cause minimum secondary dissociation by detergent.

Membranes obtained from fresh cultures of cells were washed with
distilled water and 0.6% Emulphogene extract of the pigment (in 0.04M Tris
Citrate buffer pH8.6) was obtained. A sample of 15 mls. of the extract was
subjected to sucrose density gradient electrophoresis for 8 hours. The position
of the pigment band at the end of the run is shown in Fig. 12. The pigment is
concentrated to a thin layer. Samples were drawn from the pigment zone, and
used for analytical acrylamide electrophoresis on 7.5% gels for two hours.
The gels were stained for protein (with Amido Schwarz, Coomassie blue R250
and G250) and for carbohydrate (with Periodic acid Schiff's base). The carotenoid
zone   stained for carbohydrate (Fig 13). In the gels stained for protein
the carotenoid zone did not take up any dye. A single protein band was seen,
and the carotenoid zone was located behind and separate from the protein (Fig 13).

Emulphogene solubilized pigment (0.6%) can be partially purified on
sucrose density gradient electrophoresis. This is shown by subsequent
electrophoresis of the pigment on 7.5% acrylamide gel, where only one protein
band is localized. The pigment fraction stains for carbohydrate probably due
to the sarcinaxanthine-glycoside. This is consistent with our previous report
(SECTION 3   ).

In a similar experiment 0.6% Emulphogene extract sample (15ml) of
pigment (in 0.04M Tris citrate pH8.6 buffer) was run on sucrose density
gradient column using a discontinuous buffer system for 20 hours. Position of
the pigment band at the end of the run is shown   in Fig. 14.   The gradients

Fig. 12.　Electrophoresis of 0.6% Emulphogene extract on sucrose density gradients.

S1, S2, S3 and S4 are sucrose solutions: 75, 50, 25 and 21% (w/v)

Buffer: 0.04M Tris Citrate-Borate, pH8.6.

Current: 10mA.

Voltage: 240 volts.

Duration of run: 8 hours.
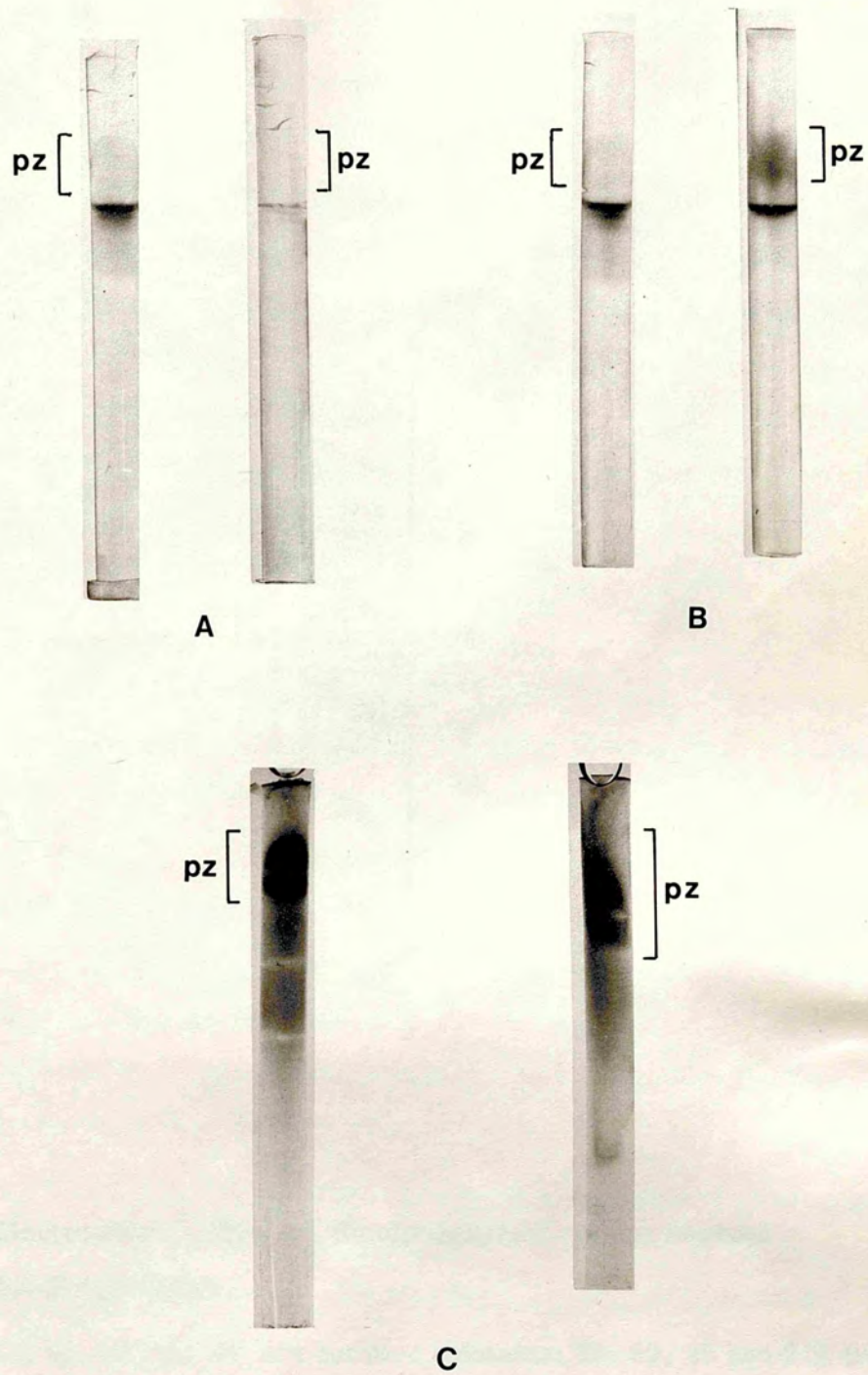
Fig. 13   Staining for proteins and carbohydrates after electrophoresis
            of 0.6% Emulphogene extract on 7.5% Acrylamide gels.

            pz : pigment zone.

            A : Stained with Coomassie Blue.

            B : Stained with Amido Schwartz.

            C : Stained with Periodic Acid Schiff Reagent.

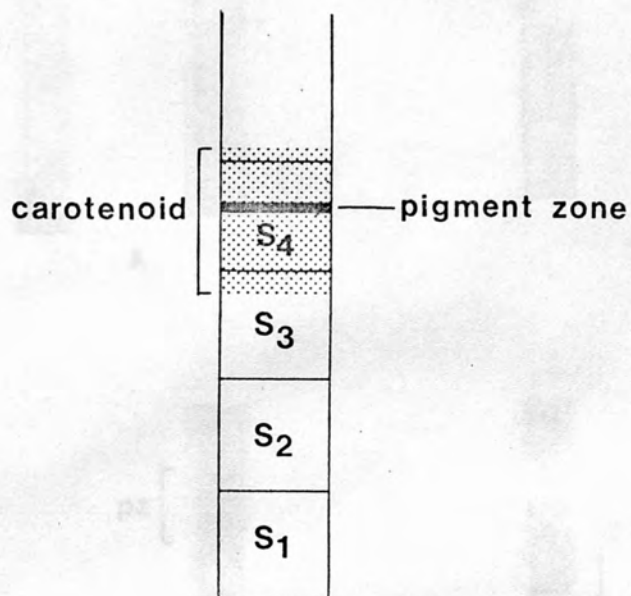Fig. 14.   Electrophoresis of 0.6% Emulphogene extract on sucrose
            density gradients.

            S1, S2, S3 and S4 are sucrose solutions: 75, 50, 25 and 21% (w/v).
            I - VI : Fractions drawn from the pigment zone.
            Buffer : 0.04M Tris Citrate-Borate, pH8.6.
            Current : 13mA.
            Voltage : 240 volts.
            Duration of run : 20 hours.

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were divided into Fractions (Fig. 14) and analysed for carotenoid, protein and phospholipid content (Table 6). These were used to find Minimum Molecular Weight; which is the weight of protein associated with 1 carotenoid mole.
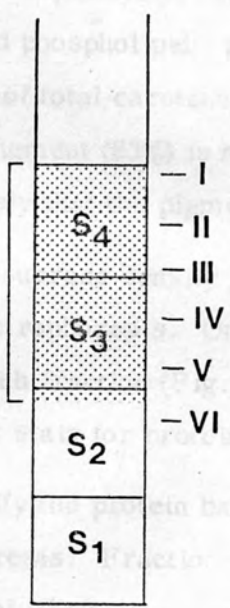
Fractions I and II (Table 6) contain 65% of total carotenoids. The minimum molecular weight calculated as 3,000 and 4,000, respectively, are those of a small peptide. The phospholipid : protein ratio (mg. phospholipid/mg. protein) is 1: 0.04 and 1 : 0.072 respectively. Fractions III and IV have minimum molecular weights 4,500 each and phospholipid : protein ratios of 1 : 0.03. These four fractions contain 83% of total carotenoids in the fractions. Analyses of these fractions show that the pigment (83%) is not protein bound but phospholipid associated. It is therefore unlikely that the pigment is a carotenoprotein.

Fractions I - IV from the sucrose density gradient column were used for analytical acrylamide gel electrophoresis. One strong protein band was stained with Amido Schwarz in each fraction (Fig.15). The pigment zone spread to 2/3 length of the gel but did not stain for protein.

It was interesting to identify the protein band running in close association to the pigment band in electrophoresis. Fractions I to VI were analysed for their ability to oxidise a number of substrates (e.g. L-malate, succinate and NADH) using polarographic assay techniques (See SECTION 2). Strong malate and NADH dehydrogenase activities, but no succinate dehydrogenase activity could be found (Table 7).

Fractions II and III which showed strong malate and NADH dehydrogenase activities were pooled and subjected to analytical acrylamide gel electrophoresis and specifically stained for Malate and NADH dehydrogenase using Nitro blue tetrazoleum dye (See SECTION 2). Thin bands of the enzyme were seen in acrylamide gels (Fig. 16). The protein band is thus composed of at least the two dehydrogenases, malate and NADH.

viii. <u>Preparative Polyacrylamide gel electrophoresis:</u>

0.6% emulphogene extract in 2.5mM Tris glycine buffer was subjected to preparative polyacrylamide gel electrophoresis (For details see SECTION 3.) The sample was layered over a 10% sucrose

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Table 6.   ANALYSES OF PIGMENT FRACTIONS FROM

SUCROSE DENSITY GRADIENT ELECTROPHORESIS.

Fraction number	Protein mg/ml.	Phospholipid mg/ml.	Carotenoid µg/ml.	P-lipid: Protein	Minimum molecular weight $x10^3$.
I	0.02	0.456	3.5	1:0.044	3.0
II	0.03	0.413	3.4	1:0.072	4.4
III	0.01	0.381	1.1	1:0.026	4.5
IV	0.01	0.375	1.0	1:0.027	4.5
V	0.24	0.313	1.0	1:0.767	120.0
VI	0.50	0.312	0.6	1:1.603	417.0
VII	0.18	0.219	0.2	1:0.822	450.0
VIII	0.17	0.200	0.1	1:0.850	850.0
IX	0.17	0.063	0.0	1:2.698	-

Minimum molecular weights are calculated from the carotenoid and protein content of the fraction assuming one molecule of carotenoid is associated to one mole of protein and the molecular weight of a carotenoid is 500 (β Carotene). Carotenoid content of the fraction based on

$$E_{447}^{1\%} = 2500 \text{ for } \beta \text{ carotene.}$$

Protein estimation based on Micro Kjeldahl method of estimation of Nitrogen (SECTION 2)

Phospholipid estimation based on Phosphorus estimation (Allen, 1940).

Phospholipid content = 25x Phosphorus content.
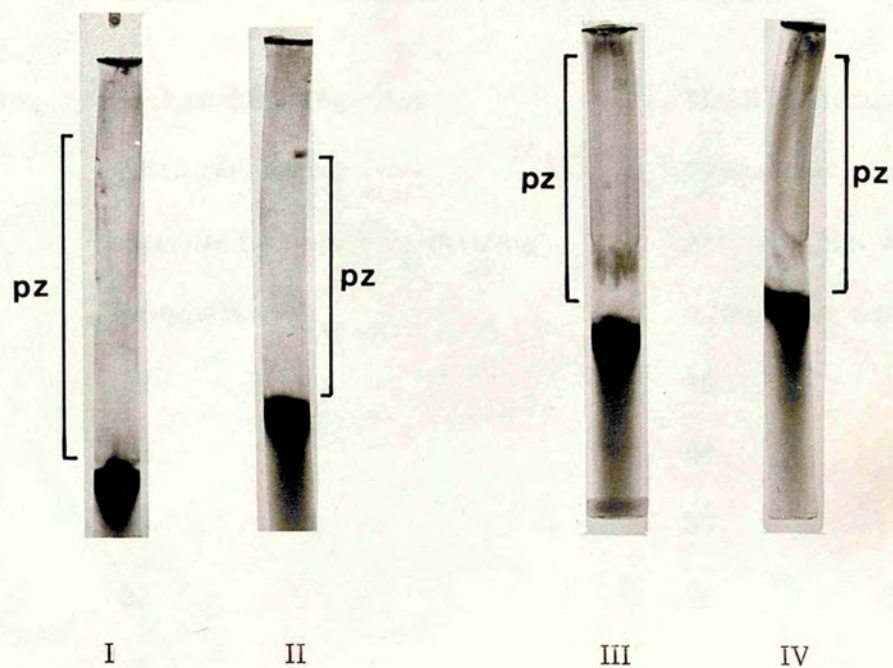
Fig. 15   Fractions I - IV from sucrose density gradient column stained
          for proteins on acrylamide gels.
          pz : pigment zone.
          Buffer system : 0.04M Tris Citrate-Borate, pH8.6.
          Duration of run : 4 hours.
          Current : 3m A/gel.
          Voltage : 200 volts.
          Stain : Amido Schwartz.

Table 7.   ASSAY OF DEHYDROGENASE ACTIVITIES IN FRACTIONS

FROM SUCROSE DENSITY GRADIENT COLUMN

Fraction number	Malate dehydrogenase (PMS reductose) Activity (µg atom O_2/min/mg protein)$\times 10^{-2}$.	NADH dehydrogenase (Menadione reductase) Activity (µg. atom O_2/min/mg protein)$\times 10^{-2}$.
I	20	15
II	50	66
III	68	87
IV	32	54
V	22	30
VI	12	12

Volume of the fractions used for assay : 0.1 ml.

Substrates used 0.1M malate; 0.001M NADH.

For details of assay methods see Section 2.E.3.
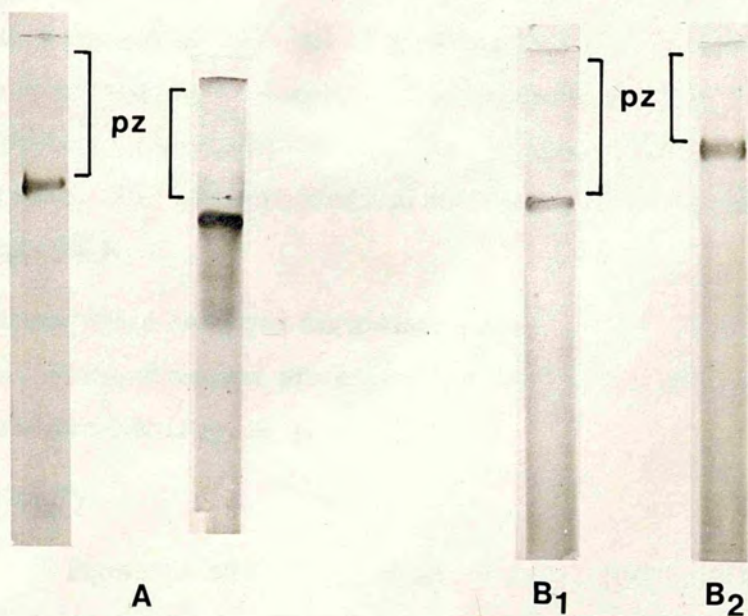
Fig. 16   Fractions II and III from sucrose density gradient column stained
on acrylamide gels for:

A : Protein

B : Specific dehydrogenase

pz : pigment zone

Stain : Amido Schwartz.

B_1 : Stained for NADH dehydrogenase.

B_2 : Stained for Malate dehydrogenase.

Buffer system for electrophoresis : 0.04M Tris Citrate-Borate; pH8.6.

Duration of run : 1 hour.

Current : 3m A/gel.

Voltage : 240 volts.

Temperature of surroundings : 8°c.

For details of protein and specific enzyme staining procedure
see Section 2.

layer (in the buffer) placed on top of the gel. The run was conducted for 20 hours. Migration of the pigment was very slow; this was eluted as a broad peak along with but incompletely separated from a protein peak. The elution profile shows 6 protein peaks (Fig. 17). Fractions 49 - 55 were pooled and subjected to electrophoresis on analytical acrylamide gels. A single band stained for protein with Amido Schwarz. It was close to but separate from the pigment zone. The pigment zone did not stain for protein with Amido Schwarz (Fig. 18).

Fractions were analysed for malate dehydrogenase activity. It was found that one of the strongest protein peak in the profile had malate dehydrogenase activity (Fig. 18).

C.   SUMMARY

Pigments of S. lutea do not occur as carotenoproteins in the detergent solubilized state. They are probably phospholipid associated. The claim is based on following observations:

1.   Pigments do not migrate as compact bands on acrylamide gels.

2.   Pigments can not be stained for proteins.

3.   Sucrose density gradient electrophoresis of solubilized pigments shows a low protein but high phospholipid content   associated with the carotenoid fractions.

4.   A reproducible minimum molecular weight based on carotenoid content is not obtained.

5.   Pigment   is not sedimentable at any concentration of Ammonium sulphate.

-86-
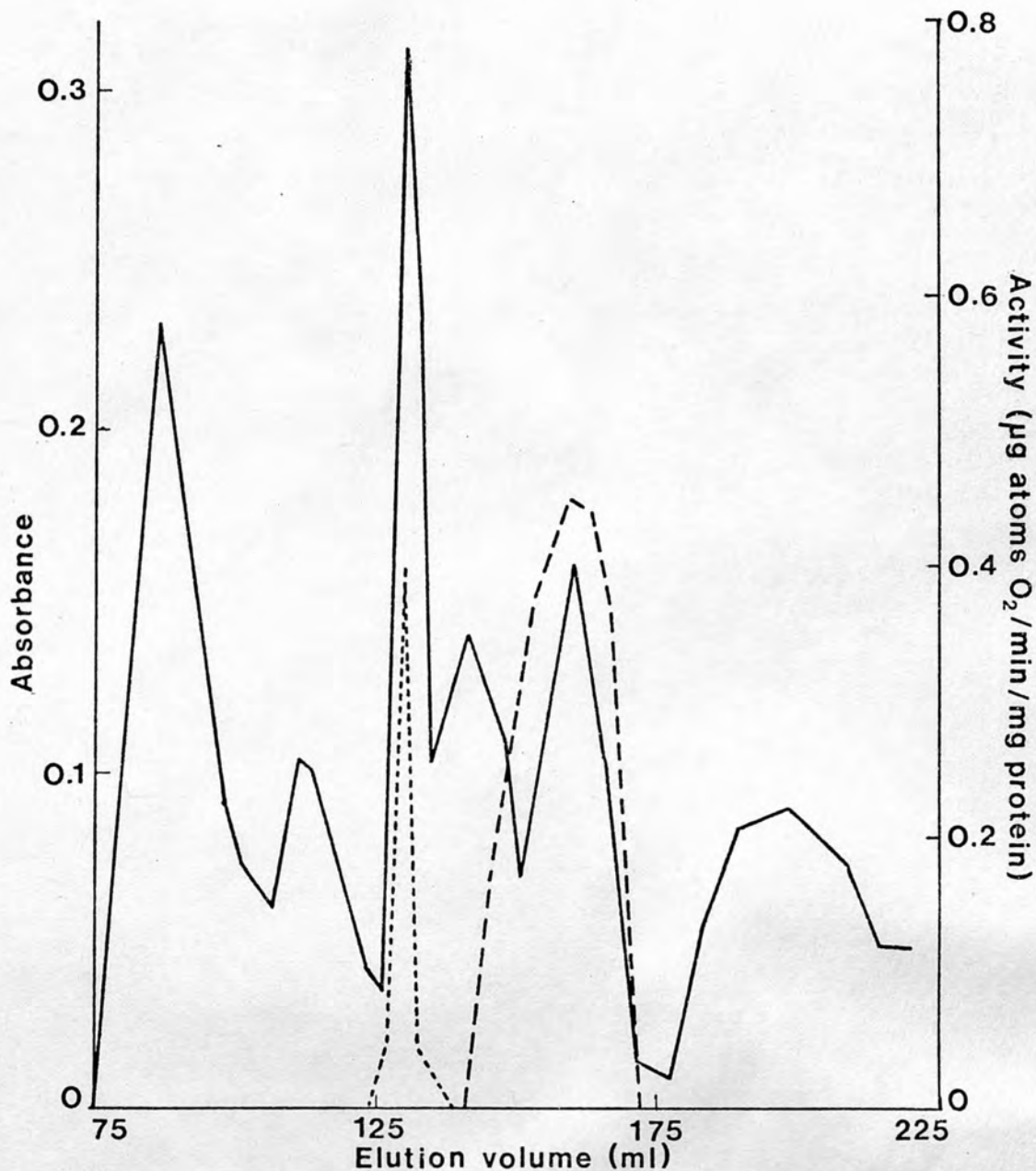
Fig. 17.   PREPARATIVE POLYACRYLAMIDE ELECTROPHORESIS OF THE SOLUBILIZED PIGMENT.

Gel strength : 7.5% Dimensions : 5 cm. high, 5.5 cm diameter.

Sample : 15ml. (7 µg. carotenoid/ml) in 2.5mM Tris-glycine containing 0.6% Emulphogene.

Eluting buffer : 25mM Tris-glycine, pH8.6.

Elution rate : ml/4min. Each fraction : 4ml.

Current : 20 m A. Voltage : 500 volts.

---------- Malate dehydrgoenase activity, ------ Pigment (447 nm absorbance), ————protein absorbance.
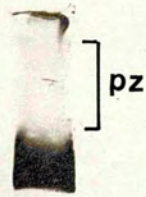
Fig. 18   Pigment fractions 49 - 53, from preparative acrylamide
           gel electrophoresis, stained for protein on analytical
           acrylamide gels.

           pz : pigment zone.

           Buffer system : 0.04M Tris Citrate-Borate; pH8.6.

           Current : 3m A/gel.

           Voltage : 240 volts.

           Duration of run : 1 hour.

           Stain : Amido Schwartz.

           Temperature of surroundings : 8°c.

SECTION 5.   PROTECTION BY CAROTENOID (IN VITRO STUDY)

It is known that carotenoids protect microorganisms against lethal effects of visible light. In *S. lutea* carotenoids protect vitamin K (toconaquinone) in the respiratory chain of malate oxidase (Anwar, 1975). It was worth investigating whether detergent-solubilized carotenoids from *S. lutea* membranes could protect vitamin K_1 (phylloquinone, the commercially available K vitamin) in vitro. Results of illuminating vitamin K_1 solution with 462 nm light in the presence and absence of detergent solubilized carotenoid pigments from the membranes are shown in Table 8. The estimations of vitamin K are based on Kröger and Dadak (1969)'s borohydride reduction method (See Section 3.0). The destruction of vitamin K_1 was 9% when carotenoids were present and 80% when absent. This adds support to the carotenoid protection of vitamin K in cell membranes.

A test of the protective capacity of carotenoids on vitamin K was made by investigating the effect of solubilized extract from yellow as well as white membranes, on vitamin K_1 under illumination. The results are shown in Table 9. It can be seen that in the presence of the yellow extract from pigmented *S. lutea* the vitamin K_1 loss was 6% and in the presence of white extract from the non-pigmented membranes it was about 23%.

It has been reported that carotenoids do not offer protection to vitamin K_1 against near ultra-violet light (Brodie and Ballantine, 1960). An experiment in which vitamin K_1 was irradiated with 360 nm light (10 minutes) in the presence of white and yellow extracts from non-pigmented and pigmented membranes of *S. lutea* simultaneously, showed that loss of vitamin K_1 in white membrane extracts was 69% and in yellow membrane extracts 53 % (Table 10). This protection offered by carotenoids around 360 nm was reproducible.

Solubilization by detergents renders the extracted components into micelles. These are probably aggregates of molecules of the detergent forming a sphere with hydrophilic portion of the detergent outside and hydrophobic tails inside the sphere. When vitamin K solution and detergent solubilized pigments are mixed together micelles containing carotenoids and vitamin K are presumably formed. It is thus possible that vitamin K and carotenoids are brought much

5.   PROTECTION BY CAROTENOID (IN VITRO STUDY):

It is known that carotenoids protect microorganisms against lethal effects of visible light. In S. lutea carotenoids protect vitamin K_2 (menaquinone) in the respiratory chain of malate oxidase (Anwar, 1975). It was worth investigating whether detergent-solubilized carotenoids from S. lutea membranes could protect vitamin K_1 (phylloquinone, the commercially available K vitamin) in vitro. Results of illuminating vitamin K_1 solution with 462 nm light in the presence and absence of detergent solubilized carotenoid pigments from the membranes are shown in Table 8. The estimations of vitamin K are based on Kroger and Dadak (1969)'s borohydride reduction method (See Section 3.0). The destruction of vitamin K_1 was 9% when carotenoids were present and 34% when absent. This adds support to the carotenoid protection of vitamin K_1 in cell membranes.

A test of the protective capacity of carotenoids on vitamin K was made by investigating the effect of solubilized extract from yellow as well as white membranes, on vitamin K_1 under illumination. The results are shown in Table 9. It can be seen that in the presence of the yellow extract from pigmented S. lutea the vitamin K_1 loss was 4% and in the presence of white extract from the non-pigmented membranes it was about 23%.

It has been reported that carotenoids do not offer protection to vitamin K_1 against near ultra-violet light (Brodie and Ballantine, 1960). An experiment in which vitamin K_1 was irradiated with 360 nm light (16 minutes) in the presence of white and yellow extracts from non-pigmented and pigmented membranes of S. lutea simultaneously, showed that loss of vitamin K_1 in white membrane extracts was 69% and in yellow membrane extracts 53 % (Table 10). This protection offered by carotenoids around 360 nm was reproducible.

Solubilization by detergents renders the extracted components into micelles. These are probably aggregates of molecules of the detergent forming a sphere with hydrophilic portion of the detergent outside and hydrophobic tails inside the sphere. When vitamin K solution and detergent solubilized pigments are mixed together, micelles containing carotenoids and vitamin K are presumably formed. It is thus possible that vitamin K and carotenoids are brought much

Table 8.   EFFECT OF ADDITION OF DETERGENT SOLUBILIZED
CAROTENOIDS TO PURE VITAMIN K_1 AND ILLUMINATION
AT 462 nm FOR 16 MINUTES.

Without detergent
solubilized pigments.   Control   Illuminated

$(E_{red.} - E_{ox.})_{265 nm} - (E_{red.} - E_{ox.})_{289 nm} =$ - 0.48   - 0.32

Concentration of K_1 (µg/ml) =   14.6   9.7

With detergent solubilized
pigments added.

$(E_{red.} - E_{ox.})_{265 nm} - (E_{red.} - E_{ox.})_{289 nm} =$ - 0.41   -0.38

Concentration of K_1 (µg/ml) =   12.6   11.5

One ml. detergent solubilized pigment solution added to
4 ml. of 1mM solution of vitamin K_1 and sonicated before
illumination. For details see Materials and Methods. (Section 2).

Table 9. EFFECT OF ILLUMINATION OF VITAMIN K_1 IN PRESENCE
OF YELLOW AND WHITE MEMBRANE EXTRACTS WITH
462 nm.

Detergent extract from non-pigmented membranes.	control	illuminated
$(E_{red.} - E_{ox.})_{265nm} - (E_{red.} - E_{ox.})_{289\ nm}$ =	- 0.58	- 0.45
Concentration of K_1 ($\mu g/ml$) =	17.6	13.6

Detergent extract from pigmented membranes.		
$(E_{red.} - E_{ox.})_{265\ nm} - (E_{red.} - E_{ox.})_{289\ nm}$ =	-0.57	-0.55
Concentration of K_1 ($\mu g/ml$) =	17.5	16.9

1ml. detergent extract from white or yellow membranes
was sonicated with 4ml. of about 1.2mM vitamin K_1
sonicated solution.

Time of illumination: 12 minutes.

Table 10.   EFFECT OF ILLUMINATION OF NEAR ULTRA-VIOLET LIGHT (360nm) ON VITAMIN K_1 WITH ADDITION OF YELLOW AND WHITE MEMBRANE EXTRACTS.

Detergent extract from non-pigmented (white) membranes		Control	Illuminated
$(E_{red.} - E_{ox.})_{265nm} - (E_{red.} - E_{ox.})_{289nm}$	=	-0.23	-0.19
Concentration of K_1 (μg/ml)	=	16.0	5.0

Detergent extract from pigmented membranes			
$(E_{red.} - E_{ox.})_{265nm} - (E_{red.} - E_{ox.})_{289nm}$	=	-0.45	-0.54
Concentration of K_1 (μg/ml)	=	12.0	5.6

4ml. vitamin K_1 (1mM) was added separately to 2ml. of yellow and white membrane extracts and sonicated for 3 minutes. A 2ml. aliquot was used for illumination and assay of vitamin K_1.

Time of illumination: 16 minutes.

closer together in this artificial system than in membranes, thus giving protection.

SECTION 6.   STUDY OF DEHYDROGENASES OF S. LUTEA.

A.   <u>INTRODUCTION</u>

         Bacterial cells contain numerous dehydrogenases which take part in anaerobic transformations (glycolysis, various fermentations), in the tricarboxylic acid cycle and in other processes leading to the generation of NADH or NADPH. Many of these enzymes are dissolved in the cytoplasm of the bacterial cells and are connected with the respiratory chain only through NAD. The oxidation of NADH involves the flavin enzyme NADH dehydrogenase which is insoluble, firmly bound with the cytoplasmic membrane and directly linked to the respiratory chain. Similarly, succinate dehydrogenase is membrane associated but the strength of its connection with the respiratory chain differs in different bacteria (Gelman <u>et al,</u> 1967). Bacterial dehydrogenases, thus fall into two categories - membrane associated and therefore directly linked to the respiratory chain and soluble but linked to the respiratory chain through NAD. Whereas the latter can be easily obtained and purified by usual purification methods, the former require varying degrees of perturbation of the membrane for their release, depending on the strength of their binding, and have therefore not been studied in great detail. In <u>S. lutea</u> the binding of these enzymes to the membrane has not been investigated so far. Mild perturbing agents (such as nonionic detergents) used to extract the pigments in our studies also solubilized some enzymes. The properties of these have been studied and attempts have been made to purify one of them, malate dehydrogenase.

B.   <u>PRELIMINARY INVESTIGATIONS</u>

         The distribution of some dehydrogenases in fractions of cytoplasmic and particulate (membrane) material obtained during membrane preparation has been examined. The procedure involves degradation of the cell wall in sucrose by the muralytic enzyme - lysozyme and osmotic rupture and washing (See Section 2C). This gave rise to (a) the sucrose fraction which presumably

contains degradation products of the cell wall - and was therefore not examined for enzyme activities (b) buffer (containing 1% NaCl) washings of the membrane (c) the membranes (d) the extract of the membranes prepared by Emulphogene BC-720 (e) the membranes left after extraction. Dehydrogenase activities in these different fractions for three substrates (Succinate,lactate,and malate) were estimated (Table 11). It can be seen that the lactate dehydrogenase and succinate dehydrogenase enzymes were released from the membrane during the preparation procedure. These therefore appear to be loosely bound with S. lutea membranes. Loss of succinic oxidase activity in lysozyme - prepared membranes of M. lysodeikticus has been reported previously (Shah and King,1965).

The presence of malate dehydrogenase on the membranes as well as in the emulphogene extract provided an opportunity to study the properties of the enzyme. The oxidation of L-malate is a key step in the tricarboxylic acid cycle but may be effected in different micro-organisms in different ways. In S. lutea as in M. lysodeikticus malate is oxidised by two enzymes; one of which is soluble in the cytoplasm (Cohn, 1958). The soluble enzyme is NAD-linked. The membrane-bound enzyme is a flavoprotein linked directly to the respiratory chain in the membrane. It is not known why two enzymes are present for the oxidation of L-malate. The properties of the flavoprotein enzymes were investigated with the hope that such studies would assist in understanding the role of malate dehydrogenases in malate metabolism.

The electron transport chain of S. lutea has been suggested by Erickson and Parker (1965) as:

Substrate $\longrightarrow$ Flavoprotein dehydrogenase $\longrightarrow$ MENAQUINONE $\longrightarrow$ cyt. + b $\longrightarrow$ O$_2$

X ⋮ PMS Y -SH ⋮ MENADIONE MK8

-97-
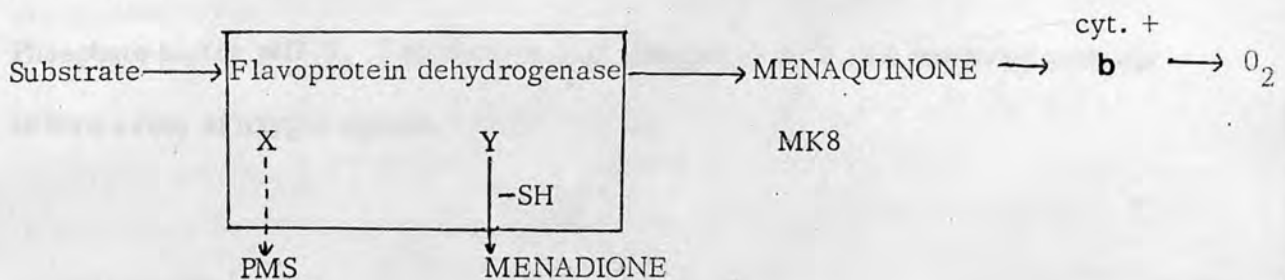
Table 11. MALATE,LACTATE,AND SUCCINATE DEHYDROGENASES IN FRACTIONS OBTAINED DURING MEMBRANE PREPARATION AND THEIR SOLUBILIZATION (PMS REDUCTASE).

ACTIVITIES (μ g. atoms O_2/min/mg. protein)$\times 10^{-2}$.

Substrate	Membranes	Washings(Buffer)	2% Emulphogene Extract	Membranes after extraction.
L-Malate	8.3	Not measured	7.1	1.5
Lactate	6.7	4.4	No activity	1.8
Succinate	6.3	5.8	No activity	1.5

Reaction mixture contains 0.2ml. of the fraction, one ml. 0.1M substrate, 0.1ml. Azide (1M), 0.1ml(0.01M) PMS, final volume brought to 2.0 mls. with 0.05M Phosphate buffer pH7.0. The mixture was brought to 30°c and saturated with air before assay of oxygen uptake.

In our investigations, the malate dehydrogenase system was studied by blocking the terminal oxidase with azide and using artificial electron acceptors: PMS which taps electrons at the flavoprotein level, and menadione which accepts electrons at a remote site from the first one on the flavoprotein dehydrogenase complex. It is to be noted that the electron acceptors are auto-oxidisable and the block by azide at the terminal O_2 inhibits the path of electrons by 98%. (Bernath and Singer, 1962).

The properties of the membrane bound malate dehydrogenase were initially investigated. The progress of the dehydrogenation reaction was followed by artificial electron acceptors PMS and Menadione. Reduction of PMS in the reaction shows that the enzyme is a flavoprotein. D-malate as well as L-malate were tried and it was found that membranes could only oxidise L-malate. The membranes therefore do not possess any enzyme for D-malate oxidation. The oxygen uptake was very low in PMS reductase system as compared to menadione reductase system (Fig. 19). It can be seen that the activity of the dehydrogenase was inhibited at high substrate concentrations. Reciprocal plots of the velocity of the reaction against substrate concentration gave a higher K_m with PMS reductase system (Table 12). It can be seen that the enzyme has a K_m 1.25mM on the membrane and in solubilized state (studies to be described later).

The Malate dehydrogenase was most active at pH 6.9 at 30^oc.

Various factors: ions, intermediates of tricarboxylic acid cycle, nucleotides and adenine nucleotides were used to study their effects on malate dehydrogenase activity All but reduced NAD and NADP inhibited the enzyme activity. Measurements were made using PMS as well as Menadione as electron acceptors (Tables 13 and 14). The inhibitory effects of factors were pronounced in the malate-menadione reductase system. Reduced NAD and NADP activated the enzyme. This effect could only be detected with the malate-menadione-reductase system. In malate - PMS - reductase system the PMS was found to be spontaneously reduced by the adenine nucleotides. Inhibition of malate dehydrogenase by ions, TCA cycle intermediates, nucleotides (ATP, ADP, AMP, NAD, NADP) but activation by reduced NAD and NADP suggests that the flavoprotein dehydrogenase is a regulatory enzyme in the citric acid cycle.
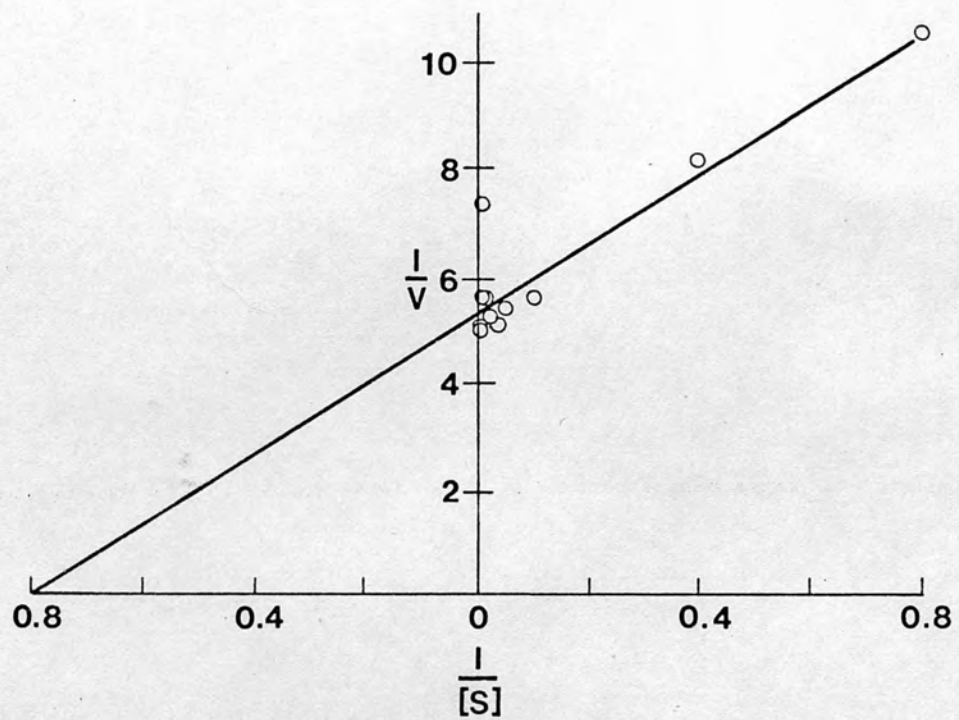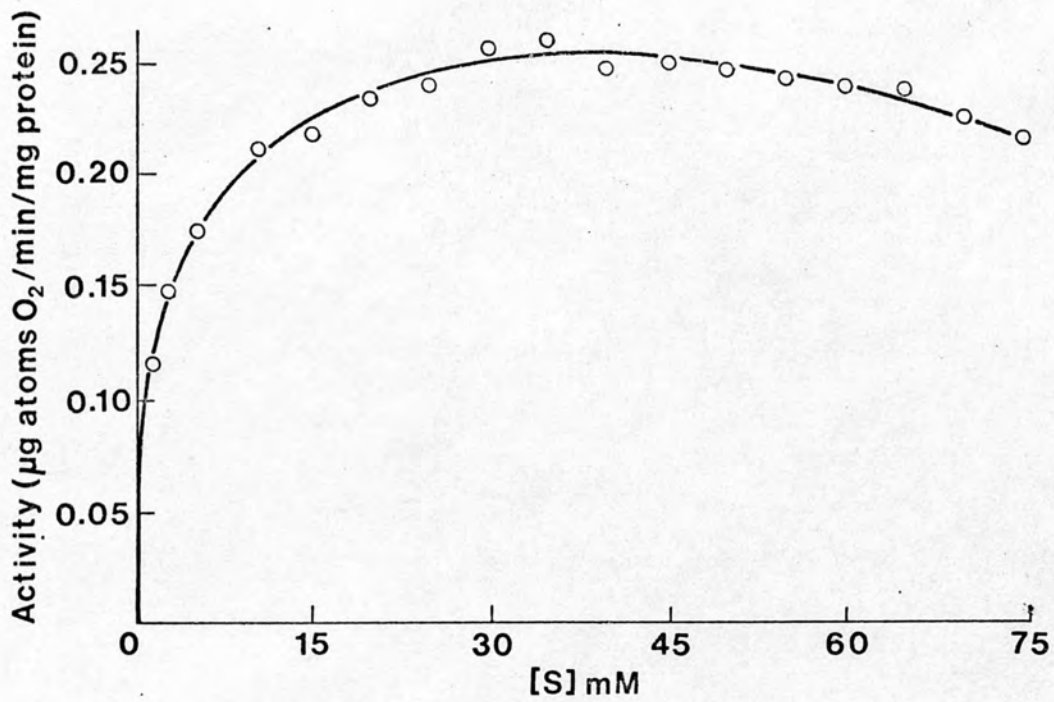
Fig. 19.

ASSAY OF L-MALATE DEHYDROGENASE

(PMS - REDUCTASE) ON MEMBRANES.

[S] = Substrate concentration.
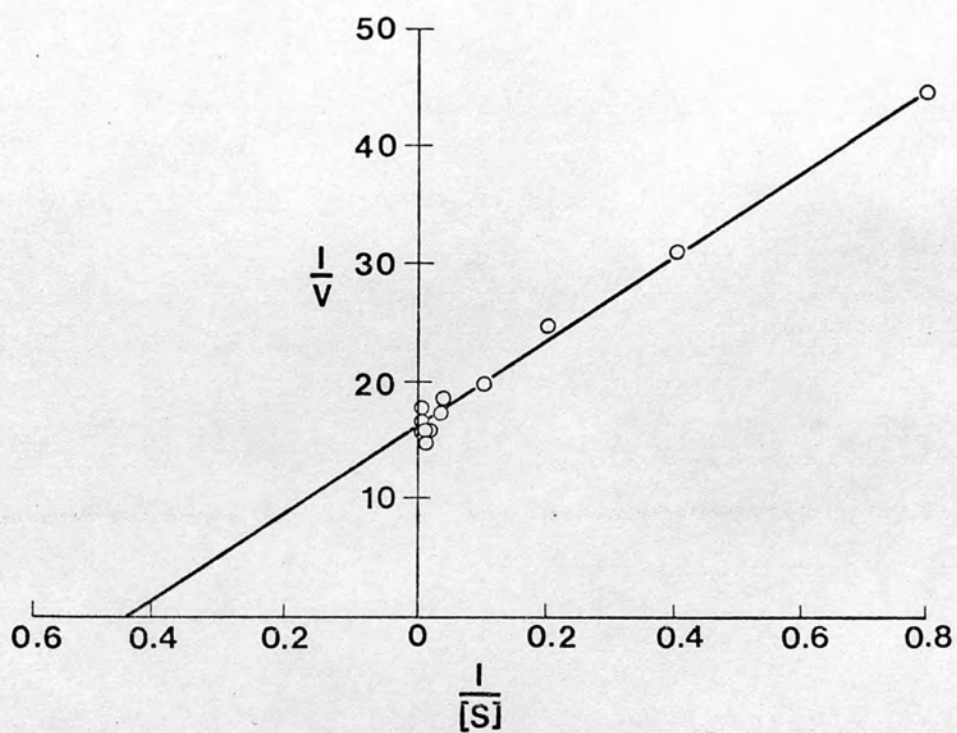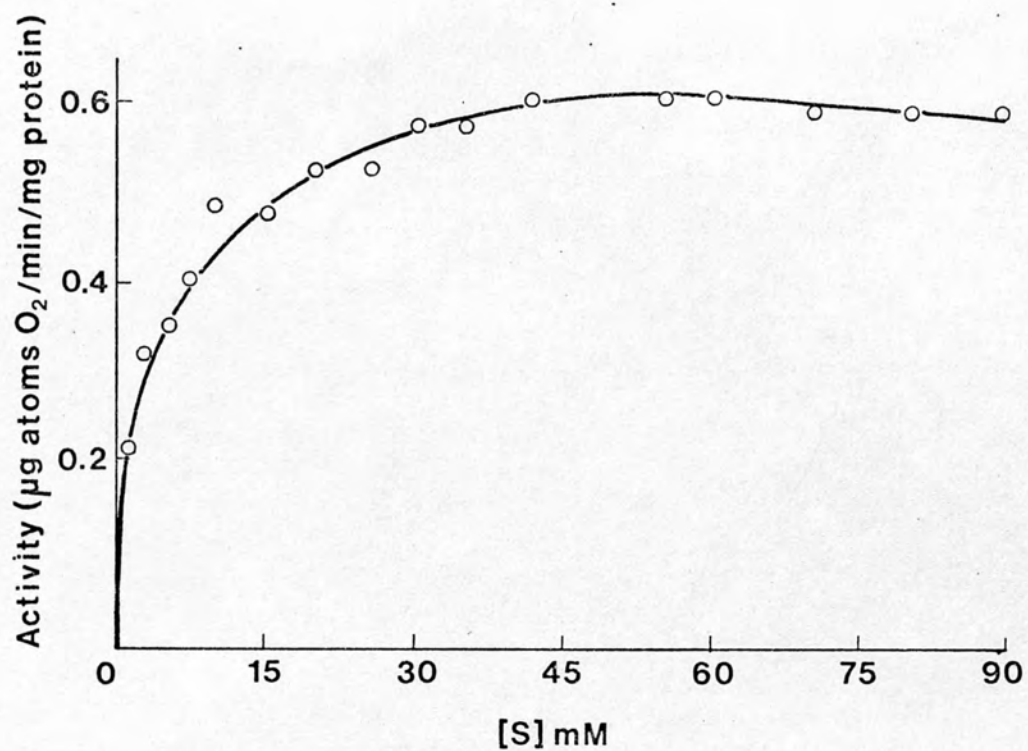
$\frac{I}{V}$ = minutes.

Fig. 19. ASSAY OF L-MALATE DEHYDRGOENASE (Menadione reductase) ON MEMBRANES.

[S] = Substrate concentration.

$\frac{1}{V}$ = minutes.

Table 12.   Km   FOR MALATE DEHYDROGENASE IN MEMBRANE—

ASSOCIATED AND SOLUBILIZED STATE.

(a)   Studies on the membrane.

$Km_{(S.D.)}$	Vmax		Electron Acceptor.
mM	(μg. atoms 0_2/min/mg. protein)$\times 10^{-2}$		
1.25 $\pm$ 0.053	24.7		MENADIONE
1.32 $\pm$ 0.049	24.1		MENADIONE
2.23 $\pm$ 0.083	6.3		PMS

(b)   Study on the enzyme in solubilized state (Emulphogene solubilized)

1.25 $\pm$ 0.083	26.3		MENADIONE.

Table 13. STUDY OF EFFECT OF FACTORS ON MALATE DEHYDROGENASE (MEMBRANE ASSOCIATED STATE) - MALATE PMS REDUCTASE.

Factor	Concentration M	Activity (μ g. atoms O_2/min/mg. Protein)$\times 10^{-2}$.	% inhibition
Malate dehydrogenase		43.0	
+ATP	2×10^{-4}	40.0	7
+ATP	2×10^{3}	30.0	30
+ADP	0.5×10^{-7}	38.0	11
+ADP	2×10^{-4}	34.0	21
+ADP	2×10^{3}	32.0	26
+AMP	2×10^{-4}	40.0	7
+AMP	2×10^{-3}	38.5	10
+NAD	2×10^{-4}	36.5	5
+NAD	2×10^{3}	31.0	28
+NaCl	2×10^{-3}	40.0	7
+NaCl	2×10^{-2}	27.2	37
+NaCl	0.2	27.0	37
+NADP	2×10^{-5}	35.5	17
+NADP	2×10^{3}	30.1	30
Oxaloacetate	2×10^{-3}	38.7	10
∝-ketoglutarate	2×10^{-3}	40.0	7
Succinate	2×10^{-3}	39.0	9

Concentration of azide 0.1 ml. (1M), PMS 0.1 ml. (.01M), L-Malate 0.1M.
Volume of membrane used for assays: 0.3 ml. see Section 2.N. for experimental details.

Table 14.   STUDY OF EFFECT OF FACTORS ON MALATE DEHYDROGENASE
(MEMBRANE-ASSOCIATED STATE) - MALATE MENADIONE
REDUCTASE.

Factor	Concentration M	Activity (μg. atoms O_2/min/mg. Protein)$\times 10^{-2}$	% Inhibition
Malate dehydrogenase		66.7	
+ATP	2×10^{-5}	55.0	17.5
+ATP	2×10^{-3}	35.0	47.0
+ADP	2×10^{-4}	53.0	20.5
+ADP	2×10^{-3}	33.0	50.5
+AMP	2×10^{-4}	62.0	7.0
+AMP	2×10^{-3}	59.0	12.0
+NAD	2×10^{-4}	53.4	20.0
+NAD	2×10^{-3}	40.8	39.0
+NADP	2×10^{-4}	45.8	30.0
+NADPH	2×10^{-4}	101.0	50(Activation)
+NADH	2×10^{-4}	111.0	60(Activation)
Oxaloacetate	2×10^{-3}	60.0	10.0
$\propto$-ketoglutarate	2×10^{-3}	61.4	8.0
Succinate	2×10^{-3}	58.7	12.0
NADH dehydrogenase		22.0	

Assay of NADH dehydrogenase (menadione reductase) was carried out with 0.2mM NADH

as substrate and of malate dehydrogenase with 0.1M L-Malate. Membranes used:

0.3ml; containing 2mg. Protein/ml. Assay method described in Section 2.N.

Reduced NAD was found to be oxidized by the membranes showing the presence of an NADH dehydrogenase enzyme. Although oxidised by the membranes, it was found that NAD (reduced) had positively promoted the oxidation of malate (Table 14); oxidation of malate in the presence of NADH was much greater than the combined oxidations of NADH and malate by the membranes.

Effects of ATP and NAD on malate dehydrogenase activity were studied in detail. It was found that inhibition increased with increase in the concentration of the effector at fixed substrate and enzyme concentrations. Kinetics of the enzyme in the presence of these effectors were studied. Plots of the enzyme activities at different substrate concentrations and their corresponding regression lines of reciprocal plots (Fig. 20) show that the inhibition by ATP and NAD is of competitive type.

C.   ILLUMINATION STUDIES:

Sensitivity of the membrane-bound malate dehydrogenase enzyme of S. lutea, to radiation in the blue region of the spectrum in absence of an external photo sensitizer has been demonstrated (Prebble and Huda,1972; and Anwar, 1975). It was also reported that the lesion was caused at two different sites in the flavoprotein. The first can be assayed with PMS, the second can be assayed with menadione as an electron acceptor. Experiments to study these sites of inactivation of the enzyme were carried out. Investigations were also undertaken to see whether the effectors inhibited the enzyme even after light inactivation.

An interference filter of band width 460 $\pm$ 5nm was selected and membranes were illuminated at an intensity of 15 w/m^2. In the malate - PMS reductase system (Table 15), 15 minutes irradiation caused 24% inactivation. It can be seen that the inhibition caused by effectors is suppressed after the membranes have been illuminated. This suggests that the anions act at the flavoprotein dehydrogenase complex probably at the same site as the blue light. Similar

-105-
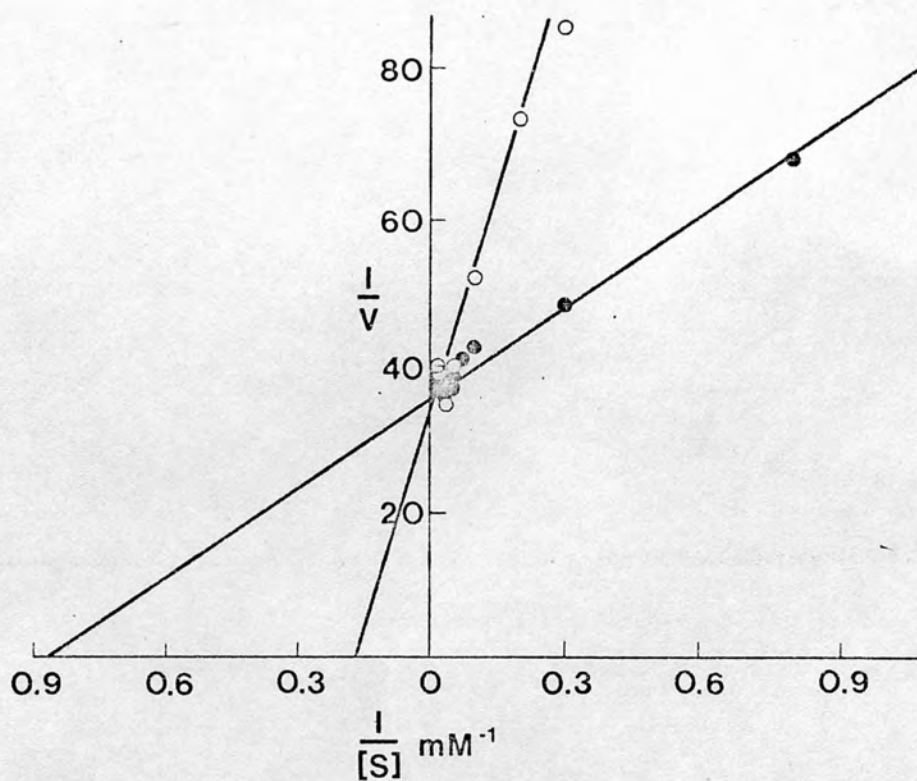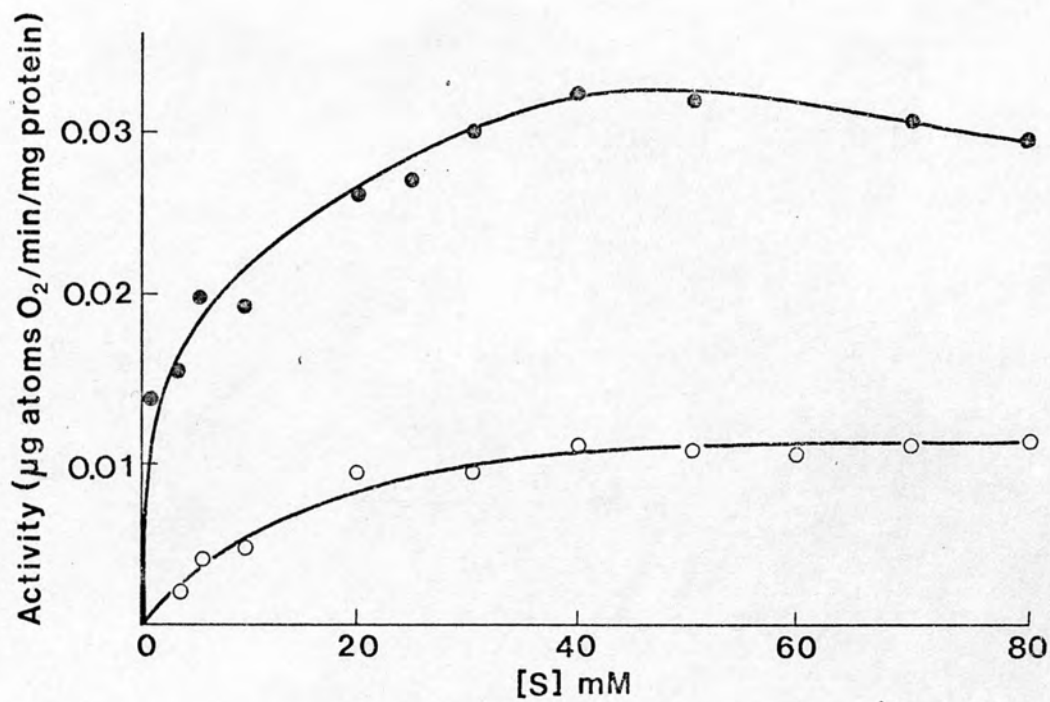
Fig. 20.

EFFECT OF ATP ON L-MALATE DEHYDROGENASE
(MENADIONE REDUCTASE).

[S] = Substrate concentration.

$\frac{1}{V}$ = minutes.

•———• Malate dehydrogenase, o———o Malate dehydrogenase +ATP
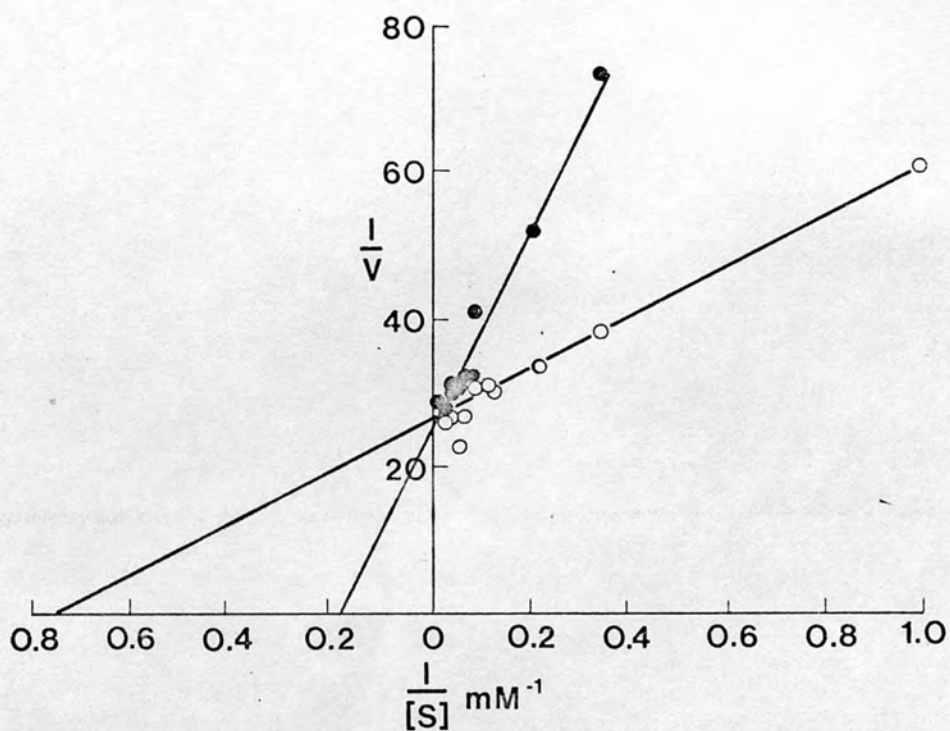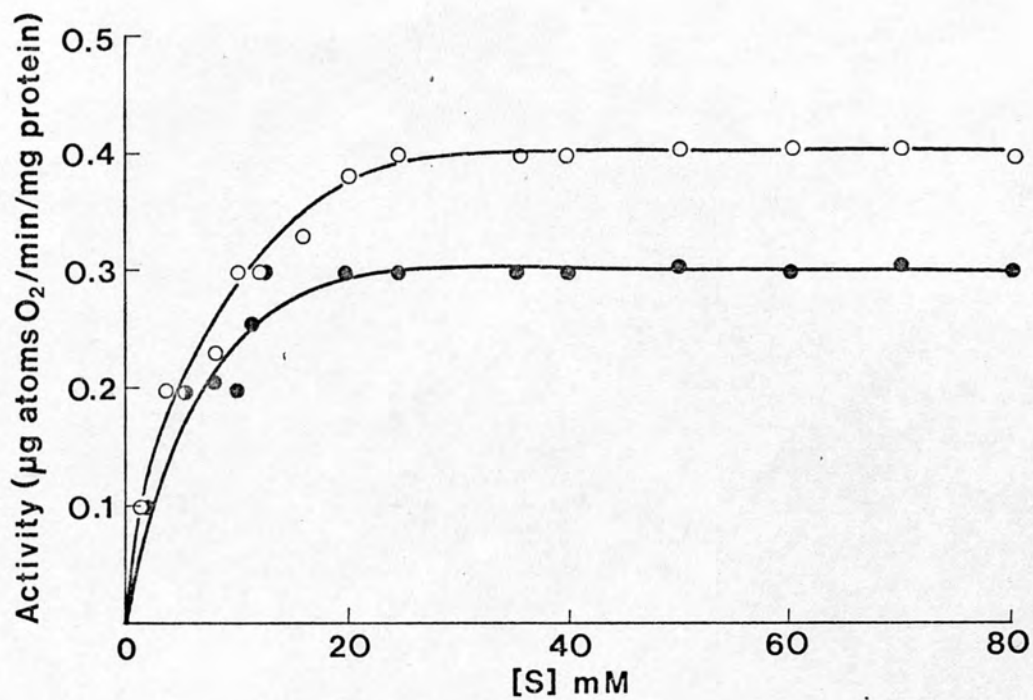
Fig. 20.   EFFECT OF NAD ON L-MALATE DEHYDROGENASE
            (MENADIONE REDUCTASE) ACTIVITY

[S] = Substrate concentration

$\frac{i}{V}$ = minutes.

o———o Malate dehydrogenase, ●———● Malate dehydrogenase +NAD

Table 15. STUDY OF EFFECT OF 460 nm ILLUMINATION ON MEMBRANES OF S. LUTEA WITH REFERENCE TO INHIBITION CAUSED BY EFFECTORS ON MALATE DEHYDROGENASE (PMS-REDUCTASE).

EFFECTORS	CONCENTRATION M	ACTIVITIES (μ g. atoms 0_2/min/ mg. Protein)X10^{-2}		% INHIBITION	
		CONTROL	ILLUMINATED	by Light	by Effector
Malate dehydrogenase		10.6	8.1	24	-
+ATP	2×10^{-3}	9.0			15
+ATP	2×10^{-3}		7.3	31	10
+ADP	2×10^{-3}	9.4		-	12
+ADP	2×10^{-3}		7.5	29	7
+AMP	2×10^{-3}	9.3		-	12
+AMP	2×10^{-3}		7.5	29	7
+NAD	2×10^{-3}	7.8			25
+NAD	2×10^{-3}		6.6	38	17

For high light intensity a filter of band width 460 $\pm$ 5nm was used. (15 w/m^2).

Time for illumination: 15 minutes.   Substrate: 0.1M L-Malate.   Membranes used: 0.5ml. Assay method is described in Section 2.N.

results were obtained with menadione reductase system (Table 16). It can be seen that activation of the enzyme by NADH is increased after the membranes have been illuminated for 15 minutes. The NADH dehydrogenase activity decreases after illumination but the activation of malate activity is increased, suggesting that NADH has a real effect on the flavoprotein enzyme activity.

It has been demonstrated that there are sulphydryl groups, in some bacterial respiratory chains which probably are attached to non-haem iron protein (Brodie and Kurup, 1967; Kaback, 1972). The presence of -SH in the malate flavoprotein dehydrogenase complex was tested by use of a -SH poison: parachloro-mercuribenzoic acid. The enzyme activity was reduced by 32% (Table 17). The second site of light inactivation which could be studied only with menadione was found partially repairable by cysteine (Table 18) showing that the lesion caused by 460 nm. light involved a -SH in the flavoprotein. It can be seen that in the illuminated system inhibitions caused by ATP, and NAD are greater in the presence of cysteine than in its absence; suggesting that the site of action of effectors and 460 nm. light is the same and repairable by -SH. This site is beyond the flavoprotein - PMS reductase point was shown by another experiment (Table 19). It can be seen that light inactivation is not repairable by cysteine in the PMS reductase system. Also NAD and ATP inhibit the illuminated malate dehydrogenase to the same extent irrespective of the presence of cysteine. In both cases light blocks the action of effector.

Table 16.   STUDY OF 460 nm ILLUMINATION ON MEMBRANES OF S. LUTEA WITH REFERENCE TO INHIBITION CAUSED BY EFFECTORS ON MALATE DEHYDROGENASE - MENADIONE REDUCTASE.

EFFECTOR	CONCENTRATION mM.	ACTIVITIES (μ g atoms O_2/min/ mg Protein)$\times 10^{-2}$.		% INHIBITION	
		CONTROL	ILLUMINATED	by Light	by Effector
Malate dehydrogenase		80	50	38	
+NAD	2	57		-	2
+NAD	2		45	44	10.0
+ATP	2	35		-	45
+ATP	2		38	53	25
+NADH	0.2	128			60 (Activation)
+NADH	0.2		100		100 (Activation)
NADH dehydrogenase		23	20		

NADH dehydrogenase assay done with 0.2mM NADH as substrate - menadione reductase. Malate dehydrogenase assay performed with 0.1M L-Malate.

Conditions of irradiation: 20 minutes illumination with filter giving 460 $\pm$ 5 nm light. Assay methods are described in Section 2.N.

Table 17.   INHIBITION OF MALATE DEHYDROGENASE BY PARACHLORO-

MERCURIBENZOATE - MENADIONE REDUCTASE.

	CONCENTRATION	ACTIVITY (μg. atom O_2/min/mg. Protein)$\times 10^{-2}$.
Malate dehydrogenase		86
+PCMB	0.1mM.	58

Substrate: 0.1M L-Malate. Assay method is described in Section 2.N.

Table 18. EFFECT OF ADDITION OF CYSTEINE ON ACTIVITY OF MALATE DEHYDROGENASE IMPAIRED BY ILLUMINATION AT 460 nm MALATE MENADIONE REDUCTASE.

FACTOR	CONCENTRATION	ACTIVITY (μ g. atoms O_2/min/mg. Protein)$\times 10^{-2}$		% INHIBITION	
	mM	CONTROL	ILLUMINATED	by light	by effector
Malate Dehydrogenase		80	50	38.0	-
+NAD	2	57		-	2
+NAD	2		45	44	10.0
+ cysteine		78			
+ cysteine			74		7.3
cysteine +NAD			65	17	19.0
+ATP	2	35			47.0
+ATP	2		138	51 3	25.0
cysteine +ATP			50	-	35.0

Concentration of cysteine used throughout the experiment was 2×10^{-5} M.

Substrate: 0.1M L-Malate. Membranes used: 0.5ml.

Period of illumination: 15 minutes.

Table 19. EFFECT OF ADDITION OF CYSTEINE ON 460 nm ILLUMINATED

IMPAIRED ACTIVITY OF MALATE DEHYDROGENASE - MALATE

PMS REDUCTASE.

FACTOR		CONCENTRATION	ACTIVITY (μ g. atom O_2/min/mg. Protein)X10^{-2}		% INHIBITION	
		M	Control	Illuminated	by light	by effector
Malate dehydrogenase			20.8	15	28.0	-
	+NAD	2×10^{-3}	15.6		.	25.0
	+NAD	2×10^{-3}		12.7	18.0	15
	+ cysteine	2×10^{-5}	24.8			
	+ cysteine	2×10^{-5}		15.0		
+NAD	+ cysteine		22.6		-	15.0
+NAD	+ cysteine			13.0		15.0
	+ATP	2×10^{-3}	17.2		-	17.5
	+ATP	2×10^{-3}		13.8	19.8	8.3
+ATP	+ cysteine			13.5	8.8	8.5
+ATP	+ cysteine		21.7		-	20.0

Concentration of cysteine used in the experiment is 2×10^{-5}M. Substrate: 0.1M

L-Malate. Membranes used: 0.3ml.

Time of illumination: 15 min.

D.   STUDY OF EMULPHOGENE EXTRACT OF THE MEMBRANES:

The detergent extract of the membranes showed very strong activities for malate dehydrogenase. It was desirable to study the properties of the flavoprotein enzyme off the membrane in order to distinguish it from the bound enzyme.

Kinetic studies of the extracted enzyme showed K_m 1.25 and V_{max} 0.244 which is the same as of the membrane bound enzyme (Table 12).

The action of effectors on the properties of extracted malate dehydrogenase was studied in the menadione reductase system (Table 20). It can be seen that the inhibitory effect of anions on the enzyme activity was pronounced in the extracted state. Also the activation by NADH and NADPH was increased by 16%. However the tricarboxylic acid cycle intermediates (succinate, and $\propto$-ketoglutarate) showed no change in their degree of inhibition. It is to be noted that chloride ions and oxaloacetate appear to have no effect on the extracted enzyme.

E.   ILLUMINATION STUDY OF EXTRACTED

MALATE DEHYDROGENASE:

Illumination of the extracted malate dehydrogenase resulted in loss of activity; but the lesion could not be repaired by cysteine (Table 21). It is deduced that the solubilization of the enzyme from the membranes resulted in the loss of a light labile -SH from the flavoprotein or masking of the group due to conformational changes arising from detergent action. This finding is also substantiated by failure of PCMB to inhibit the enzyme activity in the extracted state (Table 22). It can be seen from Table 21 that after illumination, activation of the malate dehydrogenase activity by NADH is almost doubled. This necessitated an investigation of other dehydrogenases and enzyme activities in

Table 20.   STUDY OF EFFECT OF FACTORS ON EMULPHOGENE EXTRACTED

MALATE DEHYDROGENASE ACTIVITY - MALATE MENADIONE

REDUCTASE.

EFFECTORS	CONCENTRATION mM	ACTIVITY (μ g. atoms O_2/min/mg. Protein)$\times 10^{-2}$	% INHIBITION
Malate dehydrogenase		15.8	
+ATP	2	5.0	54
+ADP	2	4.8	61
+AMP	2	13.3	16
+NAD	2	4.7	70
+NADP	2	6.3	60
+NADH	0.2	27.9	77 Activation
+NADPH	0.2	26.2	66 Activation
Oxaloacetate	2	15.5	2
∝-ketoglutarate	2	13.1	17
Succinate	2	13.9	12
NADH dehydrogenase		5.4	

Substrates: 0.1M L-Malate; 0.2mM NADH. Membrane extract used for assays: 0.1 ml.

Table 21.   STUDY OF EFFECT OF FACTORS ON 460 nm LIGHT IMPAIRED ACTIVITY OF MALATE DEHYDROGENASE IN PRESENCE AND ABSENCE OF CYSTEINE -(MALATE MENADIONE REDUCTASE)

Factor	Concentration mM	Activities (μg. atoms 0_2/min/mg. protein) $\times 10^{-2}$		% Inhibition	
		Control	Illuminated	By light	By effector
Malate dehydrogenase		80	50	38.0	
+NAD	2	57		-	28.8
+NAD	2		35	21.0	30.0
+NADH	0.2	138			72.5 (activation)
+NADH	0.2		117		134 (activation)
+ cysteine	0.02	50			37.5
+ cysteine	0.02		74		36.7
+ NAD + cysteine		35.7			28.6
+ NAD + cysteine			6.45		29.0
+ NADH + cysteine		110			120.0 (activation)
+ NADH + cysteine			1.66		124.0 (activation)
NADH dehydrogenase		20			

Substrates: 0.1M L-Malate, 0.2mM NADH. Concentration of cysteine used: 0.02mM.

Table 22. EFFECT OF PARACHLOROMERCURIBENZOIC ACID ON

EMULPHOGENE EXTRACTED MALATE DEHYDROGENASE

(MALATE MENADIONE REDUCTASE)

	CONCENTRATION	ACTIVITY
		(μg. atoms O_2/min/mg. protein)$X10^{-2}$.
Malate dehydrogenase		66
+PCMB	0.2mM	63

The experiment was carried out three times and similar results obtained.

Substrate: 0.1M L-Malate.

the extract. It was found that the extract possessed strong NADH dehydrogenase and a weak ATPase activities (Table 23). The observed activation of malate dehydrogenase could have resulted from the presence of NADH dehydrogenase. Presence of ATPase and these two dehydrogenases with all the pigments in the emulphogene extracts may suggest that these are attached to particles formed as a result of comminution of the membrane during detergent extraction. When the detergent extract was centrifuged at 169000 xg over a 20% sucrose gradient no sedimentation of the material occurred. It therefore seemed unlikely that these emzymes or pigment were particulate.

F.   STUDY OF EXTRACTED NADH DEHYDROGENASE:

Release of the malate and NADH dehydrogenases from the membrane varied with the concentration of emulphogene used. Membranes and protoplasts were extracted for the same time at various concentrations of the detergent. In Fig. 21 , it can be seen that at low detergent levels the release of the two dehydrogenases is poorer from protoplasts than from membranes. This may suggest that at higher detergent concentrations protoplasts lyse. From the curves obtained for the release of the two dehydrogenases from the protoplasts it can be seen that while the NADH dehydrogenase level rises sharply upto 0.5% detergent the malate dehydrogenase level changes only slightly. It is concluded that as the extraction of the two dehydrogenases is slow at low detergent concentrations, both are inside the protoplasts but not on one and the same particle because their release does not parallalize each other.

Davis et al.,(1975) prepared electron transport particles from M. phlei containing strong NADH dehydrogenase activity. The activity of the NADH dehydrogenase was found to undergo inhibition and activation at different concentrations of NAD at the same pH and ionic conditions. The effect was pronounced in aged preparations of the membranes of the bacteria. It was interesting to investigate this property in the solubilized enzyme from S. lutea. It was found that NADH dehydrogenase was activated below 10^{-5}M NAD and

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Table 23. EXAMINATION OF 0.6% EMULPHOGENE-EXTRACTED ENZYME ACTIVITIES.

Substrate	Concentration XM	Electron Acceptor	Activity
L-Malate	0.1	Menadione	86 (μg. atoms O_2/min/ mg. protein)X10^{-2}.
NADH	2×10^{-4}	Menadione	36 (μg. atoms O_2/min/ mg. protein)X10^{-2}.
ATP	2×10^{-2}	-	86(μg. P/mg. protein)

The method of assay of Malate and NADH dehydrogenase are described in Section 2'N'.

The Emulphogene extract was prepared in 2.5mM Tris-citrate buffer pH7.0. For ATPase assay, 0.025 ml. of the extract and 0.1 ml. of buffer substrate (30mM Tris citrate pH8.6 + 2mM Mg cl$_2$ containing 20m MATP/ml) were mixed, incubated at 38° c for 30 minutes, and 0.02 ml. 30% TCA added. The preparation was centrifuged for 10 minutes at 10,000 r.p.m. To 0.1 ml. of the supernatant 0.9 ml. distilled water and 1 ml. 60% HCl O$_3$ was added and mixed. 1 ml. Amidol reagent (1% Amidol in 20% sodium metabisulphate in distilled water) was added and the contents were mixed. To this 0.5 ml. 8.3% Ammonium molybdate was added. Tubes were allowed to stand for 10 minutes and the colour developed was read at 600 nm. Portein content was estimated as described in Section 2.P.

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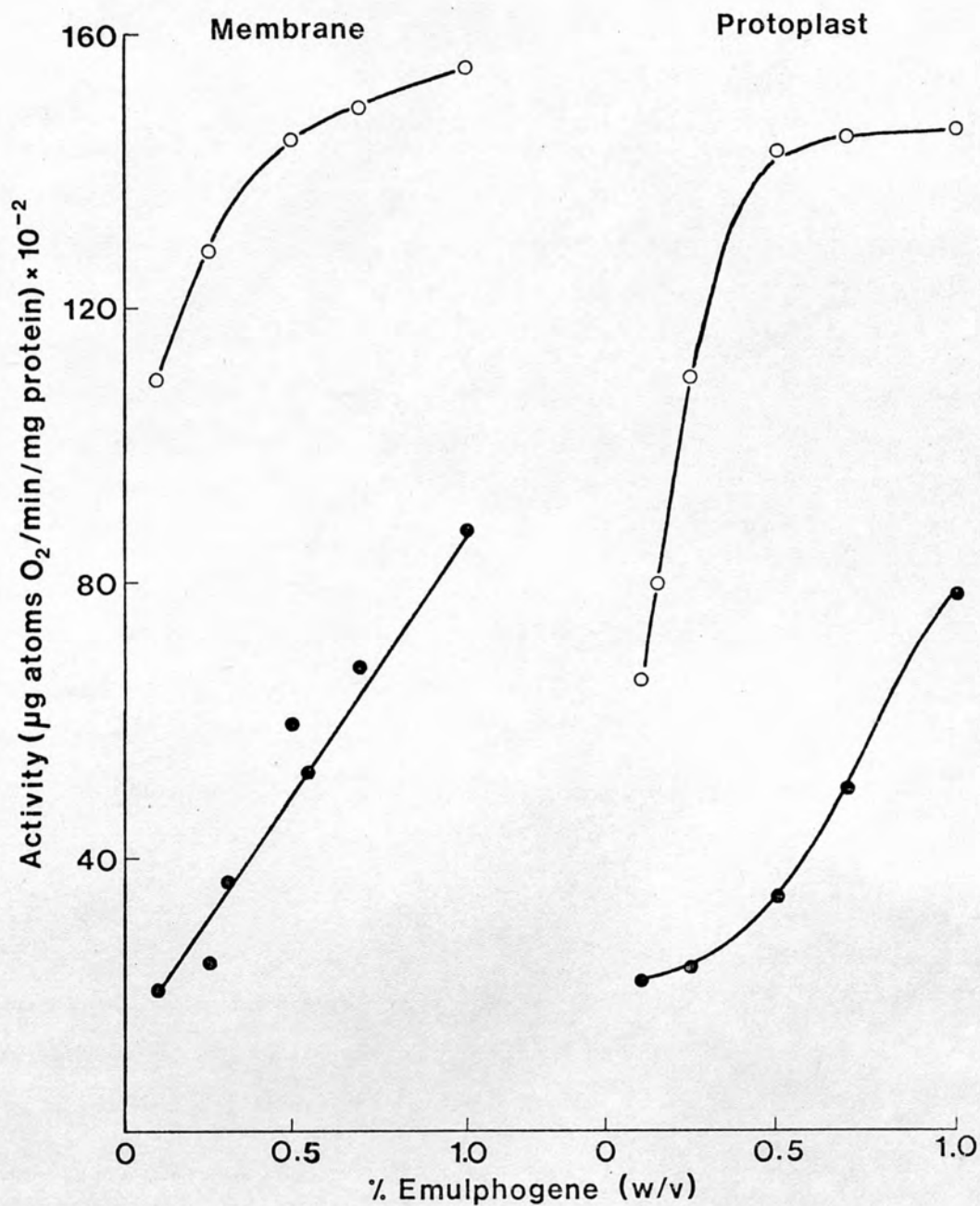
Fig. 21.

EXTRACTION OF L-MALATE AND NADH DEHYDROGENASE
(MENADIONE REDUCTASE) FROM MEMBRANES AND PROTOPLASTS
OF S. lutea.

o————o NADH Dehydrogenase

●————● L-Malate Dehydrgoenase

Substrates : 0.1M L-Malate, 2×10^{-4}M NADH.

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inhibited by levels of NAD above 10^{-5}M (Fig. 22).

G.   PURIFICATION OF THE DEHYDROGENASES:

DEAE - cellulose column chromatography with a step-wise elution was used to fractionate the two dehydrogenases solubilized from the membrane. A 6ml. sample of 0.6% Emulphogene extract in 0.005M phosphate buffer pH 7.0 was adsorbed on the column (30 x 1.5cm) and washed with twice the bed volume of the same buffer. Pigment was eluted with 0.05M phosphate buffer. Further etution was carried out with 0.15M phosphate buffer (Fig. 23). It can be seen that the carotenoids were obtained as a separate fraction eluted ahead of the two dehydrogenases. The pigment fraction was analysed for Phosphorus and Nitrogen and was found to be mostly phospholipid (Table 24). The fraction contains quinones as shown by an absorption spectrum taken before and after borohydride reduction. (Fig. 24). Malate dehydrogenase is less strongly adsorbed on to DEAE-cellulose column and is eluted before NADH dehydrogenase. It is contaminated by only traces of NADH dehydrogenase. This therefore entirely excludes the possibility of NADH dehydrogenase and malate dehydrogenase being associated with the same particle. It also affirms our previous findings that carotenoids in the solubilized state are not associated with protein.

The recovery of malate dehydrogenase from the malate dehydrogenase was ca. 65%. The inactivation was partly accounted for by the removal of phospholipid. The malate dehydrogenase obtained from the cellulose column was activated on the addition of the phospholipid fraction. (Table 25). Thus membrane bound malate dehydrogenase in S. lutea is a phospholipid requiring enzyme. When phospholecithin was added to malate dehydrogenase fraction off the cellulose column, activation of the enzyme to 200% was obtained (Table 26). NADH dehydrogenase required no phospholipid and was obtained from the column in an active state. Recovery was 87%. NADH dehydrogenase was eluted as two peaks close together which may suggest that the enzyme is a family of proteins rather than a single molecular species. The nature of the heterogeneity of NADH dehydrogenase was not investigated.

NADH dehydrogenase and malate dehydrogenase, partially purified by adsorption chromatography on the DEAE column, were reexamined for their properties in the solubilized state. NAD activated NADH dehydrogenase at
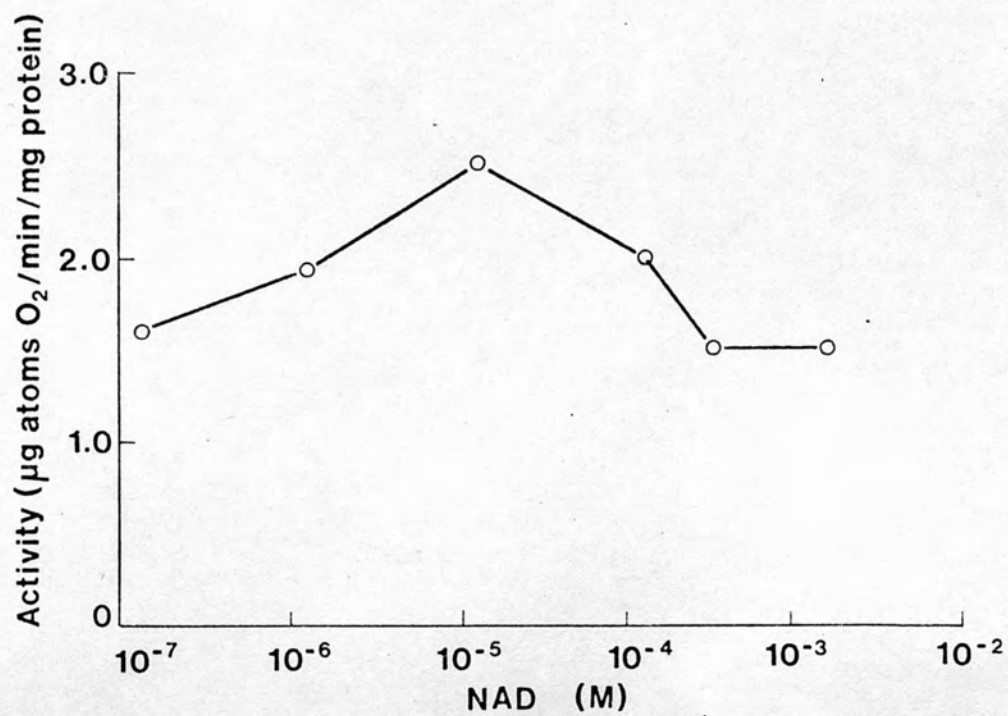
Fig. 22.   EFFECT OF NAD ON SOLUBILIZED NADH

DEHYDROGENASE (Menadione Reductase).

Substrate: 1.8×10^{-4} M NADH
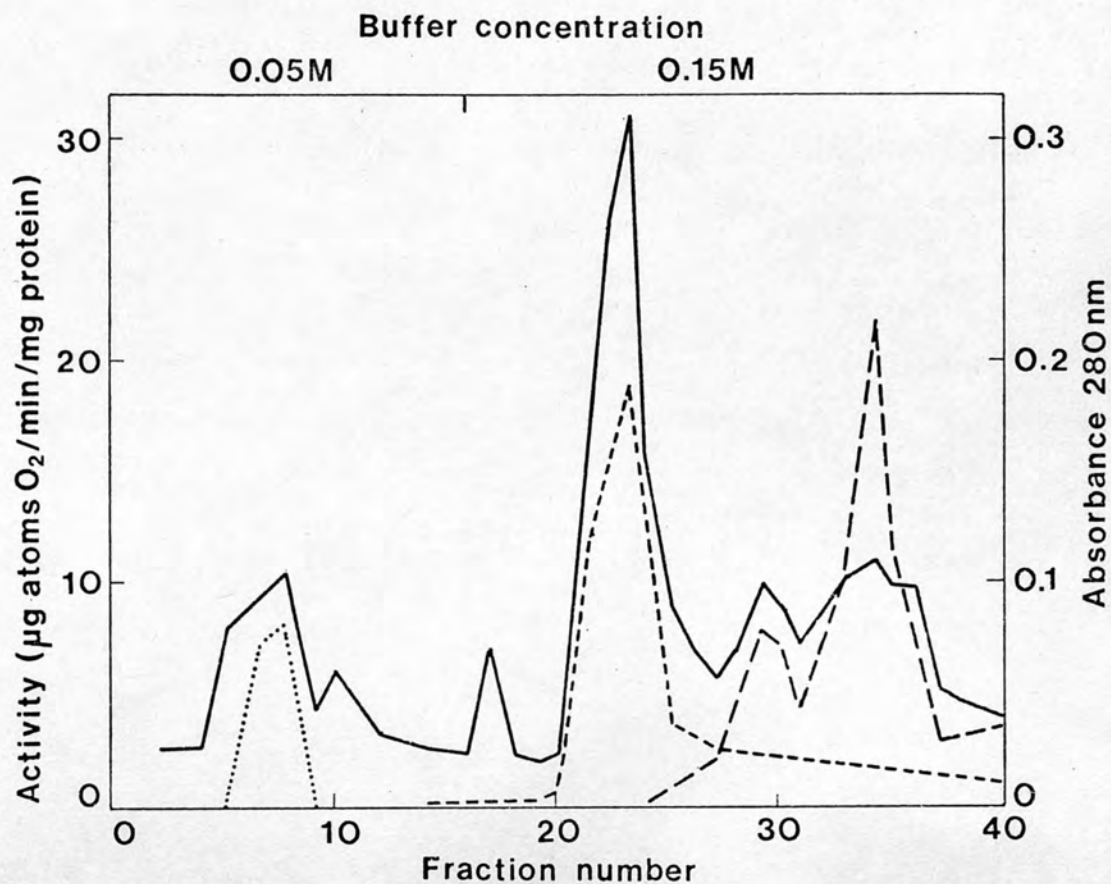
Fig. 23.   ELUTION PROFILE OF
            FRACTIONATION OF 0.6% EMULPHOGENE
            EXTRACT ON DEAE - CELLULOSE COLUMN

············   ABSORBANCE AT 447 nm (CAROTENOID)
————————   ABSORBANCE AT 280 nm (PROTEIN)
— — — — —   MALATE DEHYDROGENASE ACTIVITY.
— — — — —   NADH DEHYDROGENASE ACTIVITY.

Dimensions of Column = 30 x 1.5 cm.

Eluting buffer = Phosphate pH8.6.

Elution rate = ml/5min.

Fraction volume = 5 mls.

Table 24. ANALYSIS OF CAROTENOID FRACTION FROM

DEAE - CELLULOSE COLUMN.

	Carotenoids	Nitrogen	Protein	Phosphorus	Phospholipid
	µg.	µg/ml	mg/ml	µg./ml.	mg/ml.
Expt. 1.	60	3.8	0.03	6.0	0.15
Expt. 2.	80	2.4	0.02	8.8	0.22

Carotenoid content was estimated from absorbance at 447 nm. (for β carotene $E_{447}^{1\%} = 25,00.$).

Nitrogen was estimated by Micro Kjeldahl's method (See SECTION 2).

Phosphorus was estimated by Allen's method (1940)
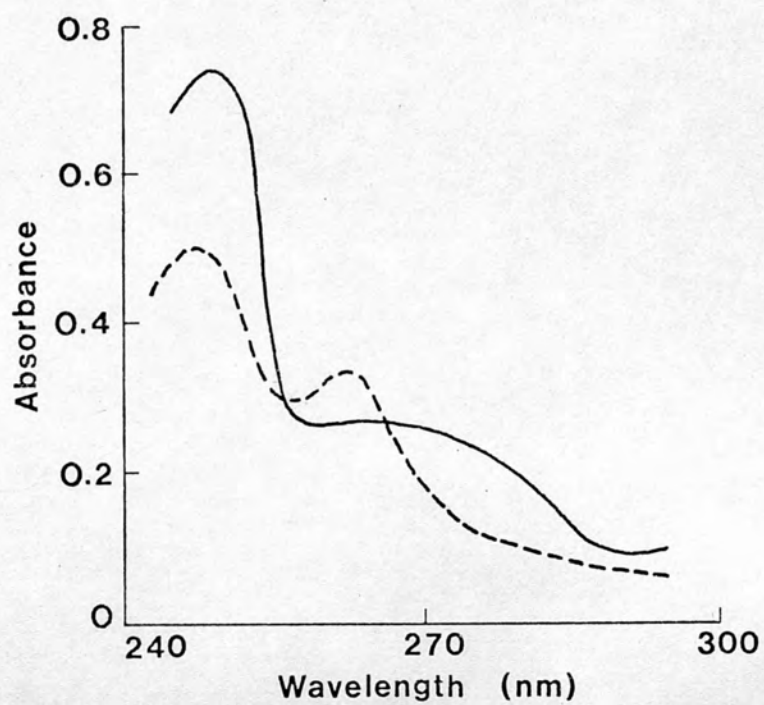
Fig. 24.

BOROHYDRIDE REDUCTION OF QUINONES
IN THE CAROTENOID FRACTION ISOLATED
USING DEAE - CELLULOSE COLUMN

--------- Quinones (oxidised)

—————————— + Sodium Borohydride
Quinones (reduced)

Table 25.   ACTIVATION OF INACTIVATED MALATE DEHYDROGENASE BY

MEMBRANE PHOSPHOLIPID

(DEAE-CELLULOSE COLUMN).

	Phospholipid added mg	Activity (µg. atoms O_2/min/mg. Protein)$\times 10^{-2}$.	% Activation
Malate dehydrogenase	0.00	50	
	0.05	75	50
	0.075	80.5	61
	0.120	80.5	61

Electron acceptor used: Menadione. Substrate: 0.1M L-Malate.

Table 26.   ACTIVATION OF INACTIVATED MALATE DEHYDROGENASE BY

ADDED PHOSPHOLECITHIN.

Phospholecithin added mg.	Malate dehydrogenase activities (µg. atom O_2/min/mg. Protein)$\times 10^{-2}$.		% Activation
	Before adding phospholipid	after adding phospholipid	
0.05	12	18	50
0.075	12	25	108
0.100	17	50	194
0.150	17	55	223

Activities were measured with menadione as an electron acceptor.

Substrate: 0.1M L-Malate.

10^{-5}M and at lower concentrations, while it was inhibited strongly at higher concentrations (Fig. 25).

H.   STUDY OF PURIFIED MALATE DEHYDROGENASE:

Initially an absorption spectrum of the enzyme obtained from the DEAE cellulose column was obtained (Fig. 26). In contrast to the spectra of mammalian succinate dehydrogenase which show two peaks (Hatefi and Davis, 1971), the purified bacterial malate dehydrogenase in our studies has one peak around 415nm. This suggests that the absorbing species is probably a part of the total flavoprotein dehydrogenase originally present in the membranes. This is substantiated by our previous findings that the solubilized enzyme does not exhibit some of its properties which it does while being membrane bound viz: inhibition by Cl' ions and PCMB.

The kinetics of the purified enzyme were the same in the membrane bound and in the solubilized state: K_m : 1.25 V_{max} : 0.245.

Inhibitory action of anions on the malate dehydrogenase activity was reinvestigated with the purified malate dehydrogenase in its activated form (Phospholipid fraction added). Inhibitions caused by ATP, ADP, AMP and NAD at different concentrations are plotted in Fig. 27 . It can be seen that ATP and NAD inhibit the activity most strongly.

NADH was found to activate the malate dehydrogenase in the membrane bound and detergent solubilized state to 60%. Since the membranes and the detergent extract contained NADH dehydrogenase activity a quantitative effect of NADH on the malate dehydrogenase activity was not reliable. The purified malate dehydrogenase which had negligible NADH dehydrogenase activity was used to test the NADH effect. It was found that malate dehydrogenase was activated by 30% by NADH (Fig. 28). This effect reaffirms the conclusion that the malate dehydrogenase is a regulatory enzyme, inhibited by NAD and activated by NADH.

-127-
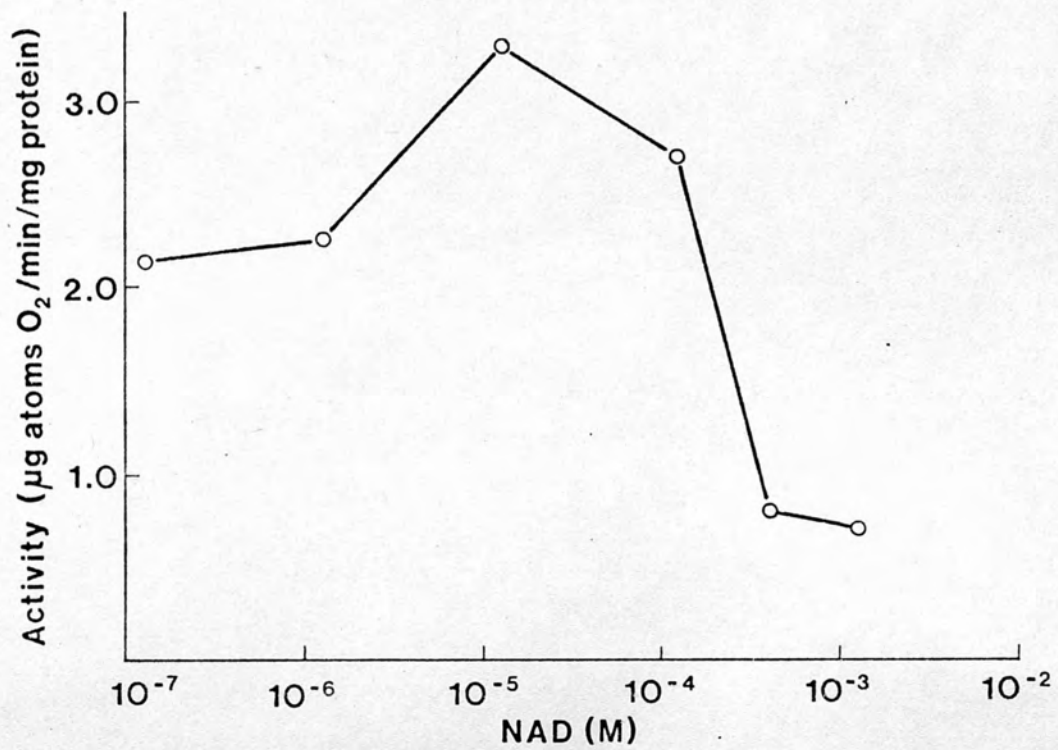
Fig. 25.   EFFECT OF NAD ON EMULPHOGENE SOLUBILIZED
NADH DEHYDROGENASE AFTER FRACTIONATION
ON DEAE - CELLULOSE COLUMN

Substrate: 2×10^{-4} M NADH
Electron acceptor = Menadione
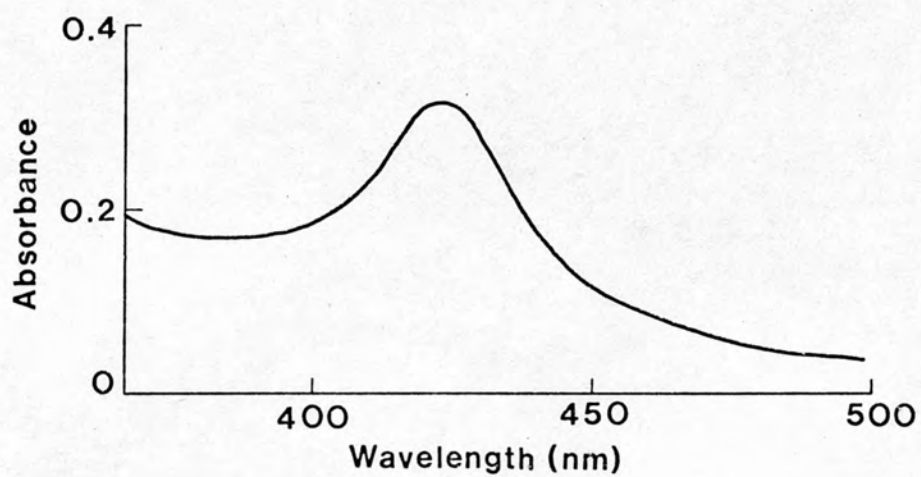
Fig. 26.   ABSORPTION SPECTRUM OF SOLUBILIZED MALATE
DEHYDROGENASE AFTER PURIFICATION ON
DEAE - CELLULOSE COLUMN.
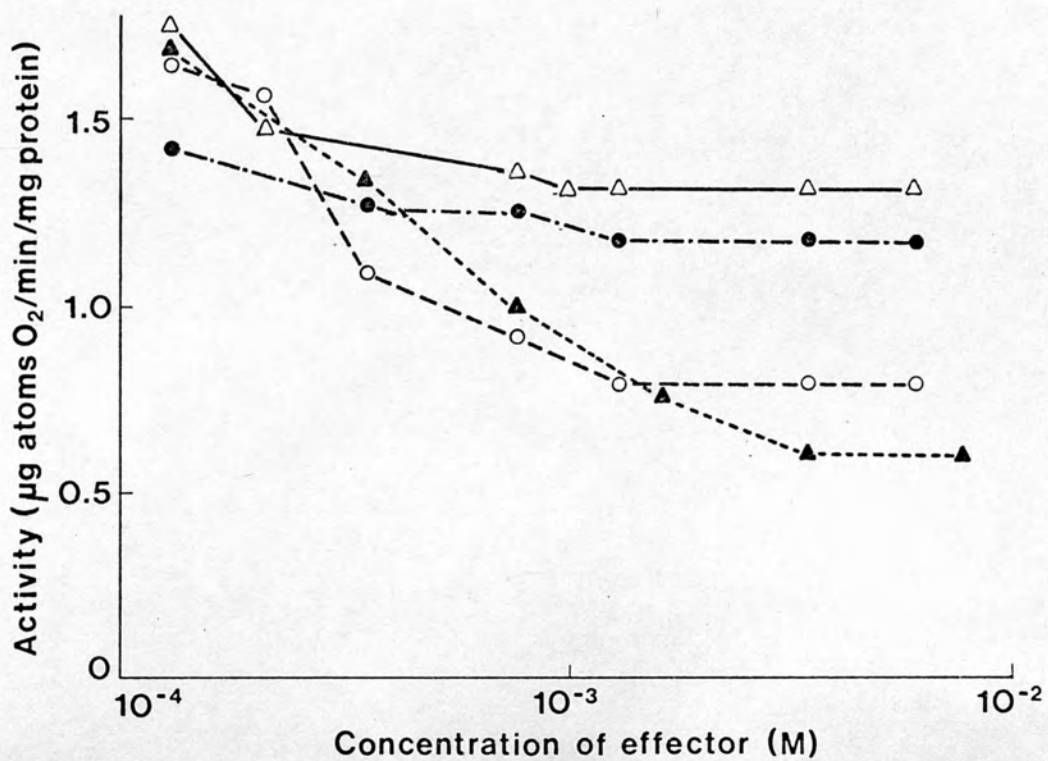
Fig. 27.

INHIBITION BE EFFECTORS ON L-MALATE DEHYDROGENASE
(Menadione reductase) IN THE PRESENCE OF ADDED
PHOSPHOLIPID FROM DEAE - CELLULOSE COLUMN.

△——△——△   +AMP

▲---▲---▲   +ATP

o--o--o   +NAD

●--●--●   +ADP

Substrate = 0.1M L-Malate
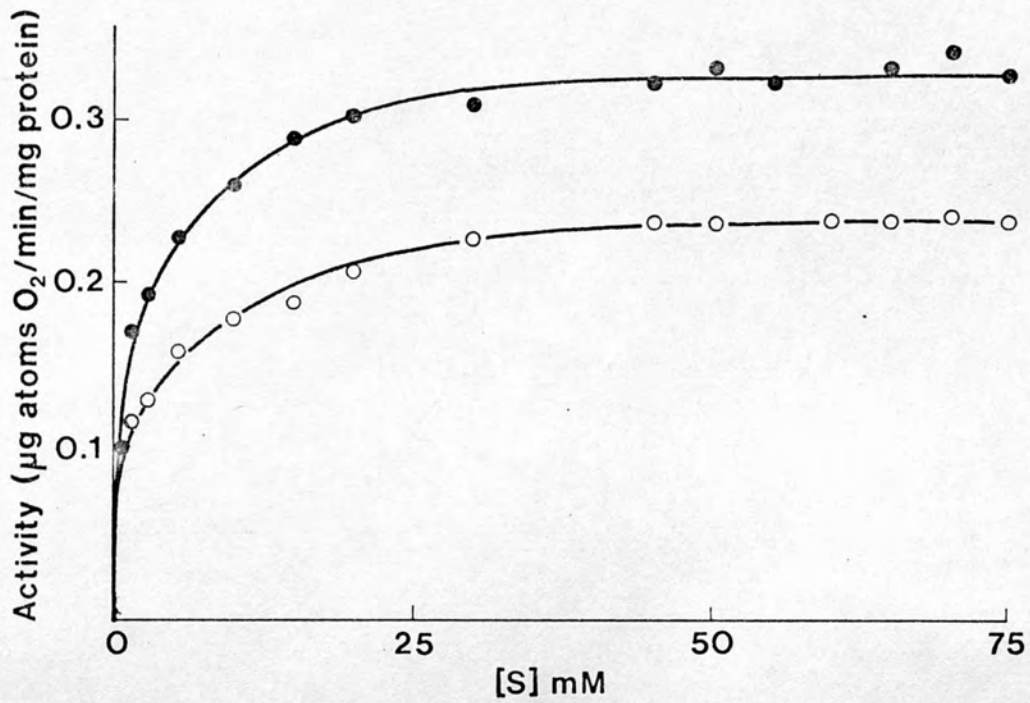
Fig. 28.

ACTIVATION OF PURIFIED MALATE DEHYDROGENASE

FROM DEAE - CELLULOSE COLUMN BY NADH.

[S] = Substrate L-Malate

NADH = 2mM

Electron acceptor = menadione

o———o Malate dehydrogenase activity

●———● Malate dehydrogenase activity with

added NADH.

The malate dehydrogenase and NADH dehydrogenase fractions obtained from DEAE cellulose column chromatography, showed one major and one minor band. Using acrylamide electrophoresis specific dehydrogenase staining techniques showed that each enzyme was heterogenous. (Fig. 29).

I.   SUMMARY:

Two membrane bound dehydrogenases of S. lutea - malate dehydrogenase and NADH dehydrogenase - can be solubilized from the membranes with emulphogene and purified on DEAE cellulose column.

The malate dehydrogenase is a phospholipid requiring enzyme and is under regulation.Reduced NAD activated the enzyme strongly while NAD, ATP, and ADP inhibited the enzyme activity largely. Intermediates of TCA cycle also inhibited the enzyme activity. K_m is 1.25mM. with menadione as an electron acceptor.

The NADH dehydrogenase in the solubilized state by detergent, was found to be activated by NAD at 10^{-5}M or lower concentrations and inhibited by concentrations higher than 10^{-5}M NAD. The enzyme was shown to be inactivated by light.
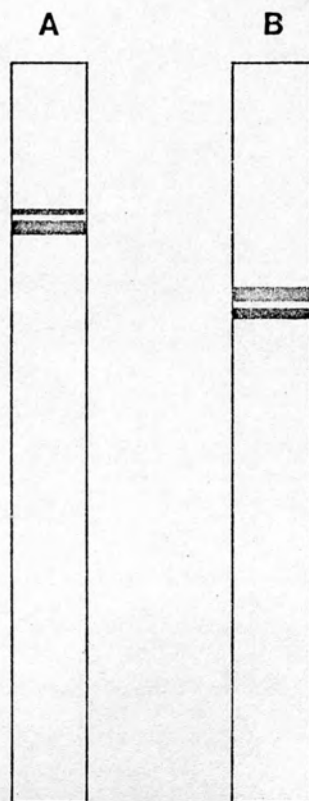
Fig. 29.　　　　　　　SPECIFIC ENZYME STAINING FOR

(A)　MALATE DEHYDROGENASE

(B)　NADH DEHYDROGENASE

For staining technique see Section 2.H.

Section 7.   <u>DISCUSSION</u>

7.   DISCUSSION

In certain cases in nature carotenoids are combined with proteins.
But in no way is this a prerequisite. It was for instance noted quite early
that the pigments (carotenoids and chlorophylls) from photosynthetic bacteria
had different absorption spectra when extracted with organic solvents than when
bacteria were physically disrupted to produce an aqueous extract. The free
pigments would not, of course be soluble in aqueous media. This was first
interpreted by Lubimenko (1922) as indicating that they were complexed to
protein, without giving any specific reasons.

The work was extended by Katz and Wassink (1939) and by French (1940)
who found that they could isolate water-soluble pigment complexes which had
absorption spectra identical to the pigments in undisrupted cells. French further
found that the pigment complexes could be precipitated with ammonium sulphate
taken to indicate that protein was involved.

As to a specific complex of carotenoid with protein which may be
separated from the non-photosynthetic bacteria it was reported by Saperstein
and Starr (1955) that cells ground with alumina gave rise to particles of
sedimentation value of 35S upon extraction with NaCl. These particles were
sedimentable at high (70%) ammonium sulphate concentration. Precipitation
altered the protein, since the reconstituted protein-carotenoid complex showed
a much higher sedimentation value following this treatment. Based largely on
ultracentrifugation data they concluded that the carotenoids were associated with
a specific protein in these particles. They also speculated that the pigment may
be required for protein synthesis! Physical methods of cell rupture would give
rise to large particles such as ribosomes having a 35 - 40S value. Obtaining
carotenoids as particulate material is therefore inevitable. Ultracentrifugation
data does not unequivocally establish protein association. Homogeneity of the
particles was not examined and no stoichiometric data has been advanced to
support the claim.

A similar approach was made by Mathews and Sistrom (1959) for the
pigments of Sarcina lutea. They suggested that the particles which were formed
by sonication and grinding of the cells had carotenoid pigments and succinic

dehydrogenase, NADH oxidase, and cytochrome oxidase activities. These, presumably fragments of the membrane, sedimented at 23ooooxg. It was suggested that the carotenoids were complexed with proteins in the membrane. Association with protein would require a direct proof rather than showing the coexistence of the carotenoids and certain enzymes on one and the same particle. The paper gives no real evidence for this claim.

Brown (1961) extracted the pigments from S. lutea using 1. methanol 2. petroleum spirit and 3. SDOC . SDOC is a much stronger detergent than Triton or Emulphogene. Absorption maxima of the pigments in these solutions were identical. Carotenoproteins studied in invertebrates are reported to be associated with electrostatic links (Cheesman et al.,1967). These are likely to be broken by acetone, alcohol and petroleum spirit. This reasoning suggests that pigment in S. lutea is not carotenoprotein.

Thirkell and Strang (1967) extracted the pigments from S. lutea and S. flava, which are very closely related species, by refluxing with methanol. The extracts from the two organisms were developed by thin-layer chromatography and the pigments of both organisms were found to be identical. They suggested that the apparent difference in the colour of the two colonies may be due to differences in the concentration of the individual pigments or to the way in which they exist in vivo.

Further investigation into the carotenoids of S. flava revealed a very polar fraction that was about 61% of the total pigments (Thirkell,Strang and Chapman,1967). This fraction was found to be a carotenoid associated with glucose and a peptide (Thirkell and Hunter,1969). The carotenoid-glycoprotein was isolated from the membranes by the use of non-ionic detergent Lubrol-L. (Hunter and Thrikell,1969). They also reported that the bonding between carotenoid and protein is covalent possibly glycosidic. The non-ionic detergent isolated the complex in an intact form. It was purifiable by $(NH4)_2 SO_4$ precipitation and identified as a single molecular species on Sephadex columns and by sedimentation at 60,000 rpm.(120,000xg).

These studies provided a background to the pigment associations that might be expected in S. lutea. Use of nonionic and ionic detergents as well as other agents to extract the pigments showed that nonionic detergents were most

effective. Since the detergent, - Emulphogene, was found to have a mild action
on the membrane, the solubilized pigments are expected to be in their native
state. Numerous observations arising at different stages of the study suggest
that the pigments are lipid associated. These will be cited as evidence for the
above claim.

(a)   Carotenoproteins isolated from invertabrates in dilute salt
solutions (Cheesman et al.,1967) and from bacteria in non-ionic (Hunter and
Thirkell, 1969) and ionic detergents (Ursula Schwenker and Gabriel Gingras,
1973) were all precipitated with ammonium sulphate. Detergent solubilized
pigments in our studies could not be brought down at any concentration of the
salt. Solubilized pigments of S. lutea   separated as an oily film on
additon of the salt due to the presence of detergent as well as low density lipids.

(b)   Detergent solubilized carotenoid pigments of S. lutea were not
particulate. The release of pigments was easily achieved by suspending the
membranes in a detergent solution and homogenizing the mixture for $\frac{1}{2}$ hour.
The solubilized material could not be sedimented even at 170,000xg on a sucrose
gradient. The carotenoproteins isolated from bacteria so far reported were
shown to have specific sedimentation value and this has been used as a criterion
for their characterization. The solubilized pigments in our studies bear no
similarity to carotenoproteins that have been reported (Thirkell and Strang, 1967;
and Mathews and Sistrom,1959).

(c)   Resolution of solubilized pigment on Sephadex and Sepharose
columns showed that the pigment fraction has a molecular weight of the order of
10^5. Since a normal carotenoid β carotene has a molecular weight of 500, it is
presumed that the solubilization procedure has formed pigment micelles of a
homogeneous size. The pigment fraction shows high absorbance around 280 nm
with Emulphogene as solubilizing agent indicating presence of proteins in these
micelles.

(d)   Solubilized pigments were   subjected to
starch gel electrophoresis. Pigments migrated only in those starch gels that
contained a low detergent level. The migration was very slow, which possibly
resulted from osmotic flow. Runs conducted at acidic and alkaline pH did not
reverse the direction of migration. Association of the pigments with phospholipid
may explain the electrophoretic behaviour.

(e)   Analytical acrylamide gel electrophoresis provided a vivid picture
of the electrophoretic mobility of the pigment complex. It was noticed that the
pigments migrated down the gel as a diffused band. 20 other bands were stained
that ran infront of the pigment band. The pigment band stained very poorly.
Pigments extracted with low detergent levels showed very few bands on acrylamide.
In this case two protein bands were seen running infront of the pigment band.
The pigment band did not stain as a protein. This shows pigments are not protein
bound. The pigments might be phospholipid linked. This would enable the
pigments to migrate on acrylamide gels but make the development of a properly
stained protein band impossible. It is also possible that the pigments are in a
detergent rich zone that might prevent the penetration of the dye in the gel.
Proteins which are comparatively mobile are not in the detergent rich zone and
therefore are stained well. Although this is a possibility, it is not likely because
the acrylamide gels are made in buffers containing a low level of detergent.
These experiments show clearly that pigments are not behaving as proteins on
acrylamide gels.

(f)   Sucrose density gradient electrophoresis.

The sucrose density gradient electrophoresis was originally
designed by Hjerten (1971) for concentration of charged species ranging from
ions to cells. The gradients contain sodium chloride and give a very high

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potential difference with minimum current. Solubilized pigment samples were subjected to sucrose density gradient electrophoresis. 1% Emulphogene solubilized pigment showed very slow migration and formation of a diffused band. 0.6% Emulphogene solubilized pigment migrated faster and formed thin concentrated band in short runs but, a less diffused band in long runs. Fractions drawn from the pigment band on sucrose electrophoresis column show a phospholipid to protein ratio of 1:0.04. This shows that pigment is phospholipid associated. The minimum molecular weight based on carotenoid content for this fraction is 3,000 which can only be a peptide.

The pigment fractions from the sucrose electrophoresis column were subjected to analytical acrylamide gel electrophoresis. The pigment did not stain for protein but a thin protein band was seen very near to the pigment band. I conclude that sucrose density gradient electrophoresis gently removes protein from solubilized pigment; crude extract shows 20 protein bands on acrylamide gels but one after it has been subjected to sucrose electrophoresis. In neither case did the pigment stain for protein.

Since a high phospholipid content is always associated with the pigment and it migrates in electric field but does not stain for protein, I conclude that carotenoids are phospholipid associated.

It is possible that pigments have been dissociated from the proteins, in the alleged pre-existing carotenoprotein complex in the membrane, by the prolonged exposure to detergent. Dissociation by detergents depends on their nature and concentration, type of the lipoprotein complex, and the type of membrane being solubilized. At moderate concentrations non-ionic detergents are reported to leave protein aggregates and even lipoprotein particles intact (Razin et al.,1972, and Hunter and Thirkell,1969). I selected Emulphogene that causes minimum dissociation of the lipo-protein complexes. It was found that the membranes were intact even after solubilization of pigments; as shown by electron micrographs and phase contrast microscopy of membranes before and after extraction. Moreover enzymes solubilized from the membranes were found in an active state in spite of long exposure to the detergent. I, therefore, think that the speculation about dissociation action of detergent could conveniently be disregarded in the present case.

(g)   Polymers of different properties when mixed in aqueous medium
form phases of different hydrophobic and hydrophilic properties. A mixture
of lipid and proteins can be partitioned into phases of hydrophobic and hydrophilic
properties. It was reported that 94% of the phospholipid from a phospholipid -
protein mixture could be obtained in hydrophobic top phase by the partition method.
(Albertsson, 1973). Solubilized pigments were partitioned and found to be
separated in the phospholipid rich zone. Hydrophobic substances (proteins)
formed cakes at the interface of the phases. Partition of all pigments in the
extract in the top phase of the system that separates 94% of phospholipids suggests
that pigments are phospholipid associated. The cakes formed at the interface were
colourless. The phase containing the pigments was extracted and subjected to
analytical acrylamide gel electrophoresis. It showed poor mobility, migrated
as a very diffused band but did not stain for protein. One protein band in close
proximity of the pigment band was seen and is presumably a hydrophobic protein.
Migration of the pigments even after partition without development of a compact
stained protein band suggests that pigments are not protein bound. It should be
noted that protein association with pigments mainly by hydrogen bonds would be
broken by KCNS and would render the pigments free and uncharged and therefore
electrophoretically immobile.

(h)   The Elution profile of detergent solubilized pigments from
DEAE-cellulose column showed that the pigments are adsorbed on the column.
These are eluted first, followed by malate dehydrogenase, NADH dehydrogenase
and other proteins. The pigment fraction obtained from the DEAE-cellulose
column showed high phospholipid but very low protein content. Mathews and
Sistrom (1959) claimed pigments of S. lutea and NADH dehydrogenase are
one and the same particle. Our findings on the contrary show that the two
are separate molecules in the solubilized state.

(i)   Detergent solutions of varying concentrations were used for solubilization of the pigments. Analyses of the extracts obtained shows that the release of the pigments did not correspond with that of the malate and NADH dehydrogenase. This shows that pigments and enzymes are not one and the same particle.

Thirkell and Strang (1967) reported extraction of pigments from S. lutea. These were identified as seven fractions on thin layer chromatography. Similar experiments performed with detergent solubilized pigments showed three fractions which are possibly the major carotenoids in the organism. On this basis it is argued that most of the carotenoids in S. lutea are phospholipid associated.

(j)   Model:

Orientation of carotenoids in the membrane of S. lutea has been the theme of my studies. Various experimental facts that enable me to suggest a possible model of the membrane are as under:

(i)   Solubilization with various concentrations of Emulphogene show that after sucrose density gradient electrophoresis the pigment contains a very high lipid content. The pigment migrates in an electric field but unlike proteins cannot be stained with respective dyes. This indicates that it is lipid associated.

(ii)   Emulphogene could solubilize as much pigment from the protoplasts as from the membranes. This suggests that the pigment can be readily extracted from the outer face of the membrane.

(iii) The pigment is very easily extracted with Emulphogene. This suggests a loose association with other components of the membrane.

(iv)   The carotenoids of S. lutea bear polar terminals. An association with phospholipids would involve links between the polar groups of carotenoids

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and phospholipids. I visualize that the molecule would span the membrane, and the length of the carotenoid molecule would be sufficient for this requirement (Dingle and Lucy, 1965).

A model of the membrane is shown in Fig. 30.

Mathews and Sistrom (1960) showed that carotenoids in S. lutea protected various enzyme activities against lethal photosensitization. The sites effected include quinones of the respiratory chain. Involvement of quinones (benzo-quinone or naphthoquinone) in the respiratory chain in microorganisms has been established (Brodie et al., 1970) by a number of techniques including destruction at 360 nm. Erickson and Parker (1969) showed that the naphthoquinone MK-8 in S. lutea respiratory chain is located between the flavoprotein dehydrogenase complex and cytochrome 'b'. Prebble and Huda (1970) and recently Anwar (1975) showed that carotenoids protected MK-8 from photo-inactivation. Carotenoids solubilized with detergent were examined for their protective function. In vitro experiments carried out with synthetic vitamin K showed less destruction by 460 nm light in the presence of detergent extract from yellow membranes than in the presence of an extract obtained from white mutant cell membranes. The extracts were phase partitioned before use in the experiments. This shows that carotenoids were functional in the solubilized state and even after drastic treatment of phase separation in the presence of KCNS. As the salt is known to break hydrogen bonds - the type of associations proteins might be having with carotenoids; a carotenoprotein is likely to undergo change in configuration. Since the pigments retain their function it is logical to conclude that these are solubilized in a state that is similar to their original state in membrane probably phospholipid associated as we have shown that these are phospholipid associated in the solubilized state.

Solubilized pigments of S. lutea, in our studies, showed protection of vitamin K from near ultra-violet radiation as well. Theoretically there is no reason as to why this phenomenon cannot occur. In the artificial system we have brought the pigments and vitamin K in close association that makes protection possible in near ultraviolet region.

Studies on the function of carotenoids in vitro are limited in number. On the basis of studies discussed so far, I am skeptical about the trend among

STUDY OF MALATE DEHYDROGENASE

Oxidation of malate is a step in the tricarboxylic acid cycle in mitochondria and the protons... cytoplasm... The reaction requires NAD which is thereby obtained in a reduced state. NADH can then be utilized in synthesis of lipids and proteins. Membrane-bound malate dehydrogenase from S. lutea was extracted in membrane solubilizers. The enzyme was studied in its natural state, as well as in a detergent-solubilized state. It was found that most of the properties of the enzyme were retained in the solubilized state.

L-malate was oxidized by the membrane-bound enzyme as well as detergent-solubilized enzyme with ... as an electron acceptor, indicating that the enzyme is a flavoprotein. The solubilized enzyme was found to be whole or part of the dehydrogenase complex. D-malate was not oxidized either with membranes or with the solubilized enzyme. This shows the enzyme is stereospecific.

EFFECT OF NUCLEOTIDES

Membrane bound as well as solubilized malate dehydrogenases were inhibited by adenosine phosphates, adenine nucleotides, chloride ions, oxaloacetate, α-ketoglutarate and succinate.

Inhibition caused by adenosine phosphates were in the order ATP> ADP> AMP. In mitochondrial oxidation of malate by a membrane bound enzyme does not require NAD and is coupled to oxidative phosphorylation (Asano and Brodie, 1963).

Sarcinaxanthin-glycoside

R = H
R' = D-Glucose

○ polar lipid head

⌇ non polar lipid tail

◯ polar protein region

Fig.30 Membrane Model

scientists to see that protein association of carotenoids is a general phenomenon and has some bearing on the functions of these pigments.

STUDY OF MALATE DEHYDROGENASE:

Oxidation of malate is a step in the tricarboxylic acid cycle in mitochondria and the product is oxaloacetate. The reaction requires NAD which is thereby obtained in the reduced state. Reduced NAD could then be utilized in synthesis of lipids and proteins. Membrane-bound malate dehydrogenase from S. lutea was present in membrane solubilizates. The enzyme was studied in its natural state, as well as in a detergent solubilized state. It was found that most of the properties of the enzyme are retained in the solubilized state.

L-malate was oxidised by the membrane bound enzyme as well as detergent solubilized enzyme with PMS as an electron acceptor, indicating that the enzyme is a flavoprotein. Detergent might have solubilized whole or part of the dehydrogenase complex. D-malate was not oxidised either with membranes or with the solubilized enzyme. This shows the enzyme is stereospecific.

EFFECT OF MODULATORS:

Membrane bound as well as solubilized malate dehydrogenases were inhibited by adenosine phosphates, adenine nucleotides, chloride ions, oxaloacetate, $\propto$-ketoglutarate and succinate.

Inhibition caused by adenosine phosphates were in the order ATP$>$ ADP$>$ AMP. In micrococci oxidation of malate by a membrane bound enzyme does not require NAD and is coupled to oxidative phosphorylation (Asano and Brodie, 1963).

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In intact cells oxidation of L-malate would lead to depletion of ADP and increase of the ATP level. This would result in increased inhibition by ATP.

ATP, ADP and AMP all inhibit the activity of L-malate dehydrogenase in both the membrane-bound as well as the solubilized state. These are presumably allosteric effects.

Oxaloacetate is the product in most cases of dehydrogenation of malate, and therefore we expect that accumulation of the product would inhibit the enzyme activity by feedback mechanism.

Succinate is an intermediate metabolite in the tricarboxylic acid cycle and differs in chemical structure from malate in not having an hydroxyl group. It is therefore possible that this ion may have inhibited the enzyme competetively being a substrate analog.

Adenine nucleotides acted as modulators. The malate oxidation was inhibited by NAD and NADP. It was activated by NADH and NADPH. The effects were observed both in membrane bound enzyme as well as membrane solubilized enzyme. Since the membrane bound enzyme does not require NAD for the reaction, the observed effect of modulator was investigated. The solubilized enzyme was purified on a diethyl aminoethyl cellulose column. The effects were confirmed. Since NAD is found to inhibit the reaction and NADH activates, it is suggested that the NAD/NADH ratio regulates the metabolic oxidation of malate in bacterial cells of S. lutea.

It is known that malate could be oxidised by another enzyme in S. lutea. This enzyme is soluble and cytoplasmic. This enzyme requires NAD for its action so that oxidation of malate is coupled with reduction of NAD. Reduced NAD could then be oxidised to NAD by NADH dehydrogenase enzyme present in the membrane, in S. lutea. These two pathways for malate oxidation have been found in M. lysodeikticus (Cohn, 1956) as well as in M. phlei (Asano and Brodie,1963; and Kaneshiro and Brodie,1965).

In bacterial cells of S. lutea the oxidation of malate to oxaloacetate would depend on NAD: NADH ratio of the cell; hence the pathway followed. When malate is fed into the cell system with a high level of NAD already present

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in cell, oxidation of the substrate through membrane bound enzyme will be inhibited and cytoplasmic enzyme pathway would start functioning, as a result producing oxaloacetate and NADH. Reduced NAD will be used in metabolic synthesis. Conversely at high levels of NADH in the cell, oxidation of malate by cytoplasmic enzyme will be reduced by feed back inhibition thereby indirectly promoting the activity of the membrane bound malate dehydrogenase.

The two pathways serve different purposes in cell metabolism. The membrane bound malate oxidising enzyme couples phosphorylation to oxidation. This enzyme, therefore, serves to build the energy of the system in the form of ATP. The cytoplasmic enzyme couples oxidation of malate to the reduction of NAD, which could be used in biosynthetic processes like synthesis of amino acid, proteins and lipids. NAD : NADH ratio in intact cell therefore serves a switch on -off mechanism of ATP synthesis (energy producing) to NAD reduction for biosynthesis of metabolites. The ratio of NAD : NADH would indirectly be regulated by the metabolic needs of the cell. The mechanism suggested above is diagrammatically represented in Fig. 31.

Solubilized pigment preparation contained malate as well as NADH dehydrogenase. Fractionation on diethylamino-ethyl cellulose column, showed that:

(1) NADH dehydrogenase is a separate enzyme and is modulated by the NAD concentration. Its activity was promoted by NAD at 2×10^{-5} to 5×10^{-5} M concentration and inhibited above 5×10^{-5} M concentration.

(2) Malate dehydrogenase loses part of its activity on fractionation due to separation of the lipids from the protein. When bacterial or synthetic phospholipid is added to the inactive form of the enzyme, full activity is restored. The activated purified enzyme also shows modulation by NAD, NADH, ATP, ADP, AMP, oxaloacetate, succinate, and $\propto$-ketoglutarate.

Requirement of phospholipid for activity has been shown for malate vitamin -K -reductase of M. phlei (Imai and Brodie, 1973). The absorption spectrum of the purified malate dehydrogenase from S. lutea with added phospholipid was obtained.

Malate dehydrogenase in a solubilized form did not show inhibition by
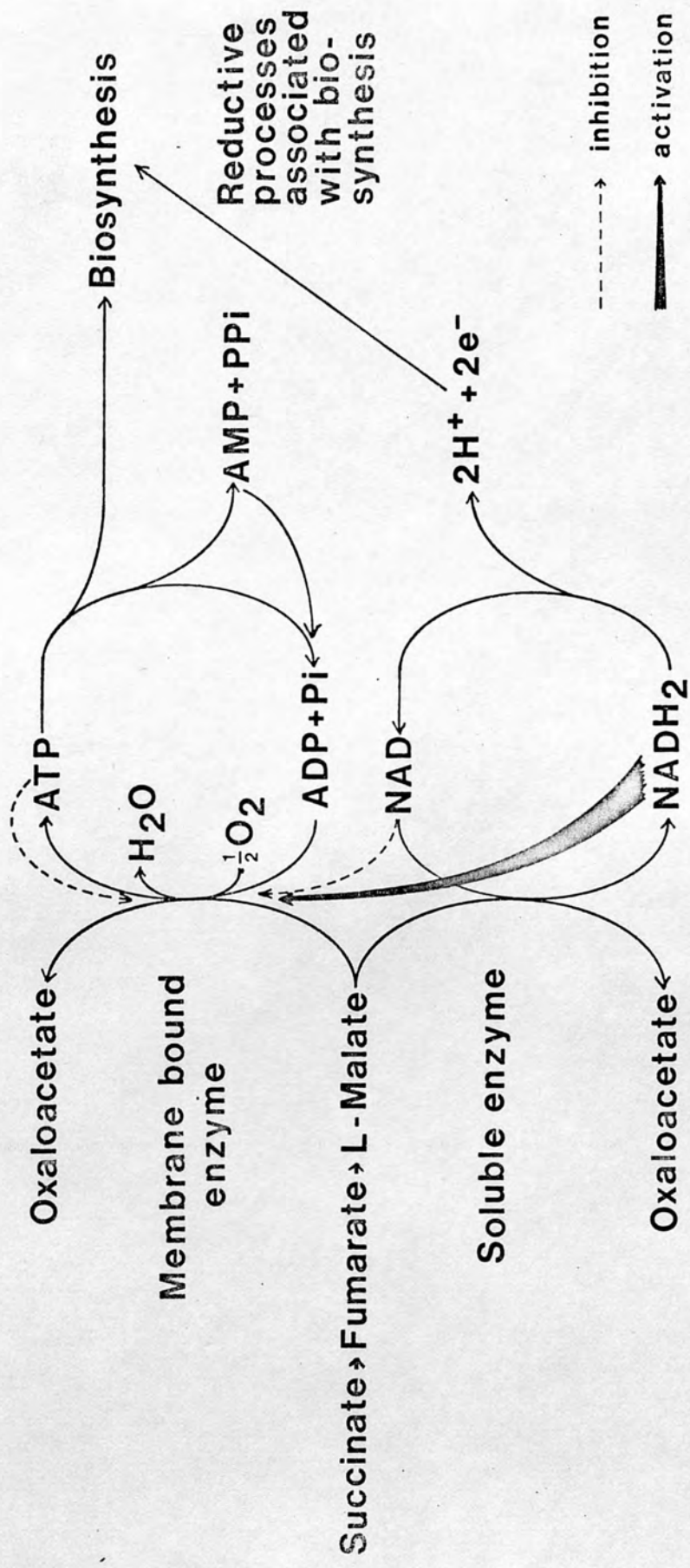
Fig.31 Scheme for Metabolic Regulation of Malate Oxidation in Sarcina lutea

chloride ions. This might have resulted from conformational changes in enzyme structure due to solubilization; detergents may unfold certain groups on enzyme or mask others.

The malate dehydrogenase flavoprotein complex has been found to be sensitive to 460 nm radiation (Prebble and Huda, 1972). The lesion was at TWO sites; ONE was assayable with phenazine methosulphate and the second with menadione as an electron acceptor. The second site involved an -SH group. The presence of -SH group on the flavoprotein complex was demonstrated by the inhibitory action of PCMB on malate dehydrogenase activity in membrane-bound state. The same inhibitory action could not be demonstrated on the activity of detergent-solubilized malate dehydrogenase. It is possible that detergent has solubilized only a part of the flavoprotein complex, the part of the molecule that does not contain -SH group. Illumination of membranes of S. lutea at 460 nm showed a loss of malate dehydrogenase activity. Also the effect of modulators on the enzyme, after illumination, were reduced indicating that sites of effectors were on the flavoprotein complex.

Solubilization of three enzymes - malate dehydrogenase, NADH dehydrogenase, and ATPase only with detergents, permits us to classify these, according to the criteria of Singer and Nicolson (1972), as integral bacterial-membrane proteins.

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