TRANSPORT OF α-SANTONIN IN *Sphingomonas paucimobilis* STRAIN S ATCC 43388

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CERTIFICATE

The author, hereby, declare that the piece of work presented in this thesis entitled **"Transport of α-Santonin in *S. paucimobilis* Strain S ATCC 43388"** has been carried out by her and that it has not been submitted to any other University or Institution for the award of a degree, a diploma or a certificate.

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DEDICATED

TO

MY HUSBAND

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ABBREVIATIONS

SA	- Santonin agar
GA	- Glucose agar
GM Km	- Glucose kanamycin medium.
h	- Hour(s)
min	- Minutes
sec	- Seconds
RT	- Room temperature
rpm	- Revolutions per minute
M	- Molar
mM	- Milli Molar
mAmps	- Milli amperes
kDa	- Kilo daltons
nm	- Nanometer
g	- Grams
A	- Absorbance
OD	- Optical density
V	- Volts
SDS	- Sodium dodecyl sulfate
PAGE	- Polyacrylamide gel electrophoresis
TEMED	- Tetra methyl ethylene diamine
APS	- Ammonium per sulfate
NADPH	- Nicotinamide adenine di nucleotide phosphate
EDTA	- Ethylene diamine tetra acetic acid
HCl	- Hydrochloric acid
$CHCl_3$	- Chloroform
PNP	- Paranitro phenol phosphate
TE	- Tris ethylenediamine acetic acid buffer

LPS	- Lipopolysaccharide
TLC	- Thin layer chromatography
COSM	- Cold osmotic shock method
CHLM	- Chloroform method
FTM	- Freeze thaw method
G+C	- Guanine + Cytosine
w/v	- Weight / Volume
v/v	- Volume / Volume
UV	- Ultra violet
WCCP	- Whole cell protein profile
NDGE	- Non denaturing gel electrophoresis
ml	- Millilitre
l	- Litre
S cells	- *S. paucimobilis* cells
GHCl	- Glucosamine hydrochloride
OMP	- Outermembrane protein

CHAPTER 1.0

INTRODUCTION

INTRODUCTION

1.1 BIOLOGICAL MEMBRANES

Biological membranes are hydrophobic barriers. Membranes play a central role in both the structural organisation and function of all prokaryotic and eukaryotic cells. Membranes, due to their hydrophobic nature control the essential movement of solute (ions and nutrients) in or out of the cell. This permeability ensures that essential molecules such as glucose, amino acids, and lipids enter the cell, metabolic intermediates remain in the cell and waste compounds leave the cell. The selective permeability of the membrane allows the cell to maintain a constant internal environment. Similarly, organelles within the cell often have a different internal environment from that of the surrounding cytosol and organelle membranes maintain this difference. The transport systems embedded in the membrane consist of hydrophilic phospholipid heads and proteins which help in passage of hydrophilic molecules from the aqueous phase. The passive permeability through the lipid bilayer is very slow for most solutes and mostly protein mediated transport process allow the entry and exit of solutes into or out of the cell. When the integrity of the membrane is lost, the cells are freely permeable to all solutes and finally leading to their death.

In recent years the transport of substances across biological membranes has become an area of increasing importance for our understanding of the metabolism and energy balance of cells. Biological transport includes phenomena such as translocation of substances across intracellular and plasma membranes, transcellular movement through epithelial cells, long distance transport in plants.

1.2 PROKARYOTIC CELL ENVELOPE FUNCTION

Prokaryotic microorganisms offer many advantages as an experimental material for determining mechanisms of transport and their control. They are diverse, occupying

many ecological niches requiring special adaptations of the transport systems. They are especially useful in studying genetic, physiological and metabolic control of transport.

The envelope is the interface between the environment and the cell cytoplasm, and has to perform diverse roles (Poxton, 1993). In general, the functions of the prokaryotic envelope may be enumerated as follows:

1. Shape and rigidity to the cell
2. Protection against osmotic lysis
3. Selective permeability barrier
4. Maintenance of ionic balance between inside and outside
5. Housing of trans-envelope transport machinery
6. Protection against host defence mechanism
7. Protection against antibacterial substances
8. Sensing of the environment
9. Support to the flagellar motors and other appendages, *e.g.,* Pili.
10. Support to fimbriac and other adhesions
11. Involvement in adhesion to surfaces
12. Involvement in penetration/ invasion of eukaryotic cells
13. Matrix for biosynthetic process
14. Involvement in cell division

1.3 STRUCTURE OF A BACTERIAL CELL ENVELOPE

Based on differential staining, bacteria have been categorised as Gram-negative and Gram-positive. The differential staining of these organisms is due to the characteristic structure of their membrane which is given in Fig. 1.1 (Hancock and Poxton, 1988).

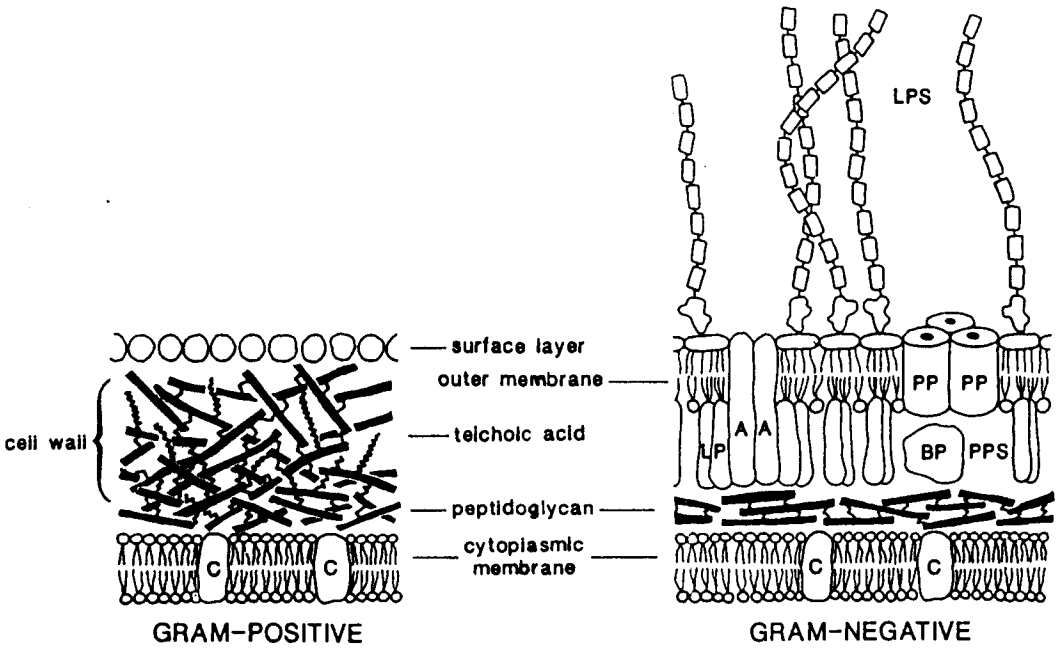


Fig. I·1 Schematic presentation of the cell envelope of Gram-positive and Gram-negative bacteria (Hancock and Poxton, 1988). PP - Porin; C - cytoplasmic membrane-embedded protein carrier; BP - binding protein; PPS - periplasmic space; A - outermembrane protein; LP - lipoprotein.

1.3.1 Structure of Gram-positive Cell Envelope

The cell envelope of Gram-positive bacteria consists of a peptidoglycan wall, underlying which is the cytoplasmic membrane. The peptidoglycan makes up to 40-90% by dry weight of the cell wall. It is 30-80 nm thick. Associated with peptidoglycan are phosphorous containing acidic polymers called teichoic acids or acidic polysaccharides such as teichuronic acids.

1.3.2 Structure of Gram-negative Cell Envelope

Gram-negative bacteria have a complex cell envelope consisting of an outermembrane, the cell wall proper or peptidoglycan and a cytoplasmic membrane. The compartment between the outer and the innermembrane is known as the periplasm.

1.3.2.1 Outermembrane

The outermembrane acts as a barrier for penetration of a wide variety of compounds (Nikaido, 1979) while at the same time permitting the size dependent passage of small molecules (Nikaido and Saier, 1992; Benz, 1994).

1.3.2.1.1 *Isolation*

Several methods have been employed for the isolation of outer membranes of most Gram-negative bacteria, one of them being treatment of cells with Tris EDTA and lysozyme to separate cell walls (Miura and Mizushima, 1968) which on subsequent centrifugation on sucrose density gradient separate into outer and inner membrane with the densities of 1.22 and 1.14 g/cm^3 respectively. Disrupting the cells by pressure at 1,200 to 1,500 kg/cm^2 in a french pressure cell is another frequently used technique. Differential solubilisation of the outer and inner membranes by a nonionic surfactant is easy and time saving method which yields outermembrane rich insoluble material (Schnaitman, 1971; Filip, *et al.*, 1973).

1.3.2.1.2 *Structure and composition*

The outer membrane is constructed asymmetrically and made up of lipopolysaccharide (LPS), phospholipids, proteins in the ratio of 0.3:0.3:1 (Schnaitman, 1970; Smith, *et al.*, 1975), besides the cations like calcium and magnesium. Phospholipids are located in the inner half of the membrane and LPS on the other hand is exposed to the environment.

Molecular sieving action of the outer membrane is determined by certain transmembrane water filled pore forming proteins called porins. They are present in high copy numbers and generally aggregate in the form of trimers and form pores. Their monomer molecular weights are in the range of 33-45 kDa (Hancock, *et al.*, 1979; Benz, *et al.*, 1982). These porins allow the diffusion of hydrophilic compounds not larger than 800 Da from the culture medium into the periplasm (Nikaido and Saier, 1992; Benz, 1994). Depending on their specificity towards substrates porins have been classified into 3 types.

The type I channels are open and do not exhibit any specificity for substrates, although some show preference for anions and cations (Benz, 1994) and porins such as Omp F and Omp C from *E. coli* k12, exhibit little specificity for solutes (Benz, *et al.*, 1985, Nikaido and Rosenberg, 1983) and act as general diffusion pathways (Nakae, 1976; Nikaido and Vaara, 1985) carrying out simple diffusion which occurs when two compartments containing differing concentrations of dissolved substances are separated by membrane to which it is permeable. The movement of molecule is solely due to molecular motion. Diffusion of one substrate is not influenced by others. Diffusion occurs into a compartment with lower concentration until the concentration on both sides is same. The rate of movement of a solute is made more rapid merely by increasing the concentration gradient of the solute. This process cannot continue indefinitely and is limited at very high concentration gradients.

Type II porins, form channels which allow non-specific diffusion of small compounds but they recognise specific substrates. During this transport process the

solute molecule attaches to a membrane bound carrier or porin protein that shuttles back and forth between opposite faces of the membrane, picking up the solute on one side of the membrane and depositing it on the other side, and then returns back for more substrate. In such a model the rate of movement of the solute through the barrier reaches a saturation value as concentration of solute is increased because all the carrier in the system becomes bound to solute. These porins facilitate diffusion of the substrates with a rate higher than the type I porins and are permeable to substrates which are too large for the type I porin channels. lam B displays a binding specificity for malto dextrins (Benz, 1994), Tsx for nucleotides (S) (Benz, 1994), Scr Y for sucrose (Schulein *et al.*, 1991). Expression is influenced by osmolarity and composition of the growth medium. For example, pho E, a general diffusion porin with some specificity for anionic solutes, is induced in membranes of Enterobacteriaceae species under conditions of phosphate starvation (Tommassen and Lugtenberg, 1980) whereas lam B is induced in *E. coli* (Szmelcman and Hofnung, 1975) and *S. typhimurium* (Nikaido and Vaara, 1985) if the cells are grown on maltose or maltodextrin.

The third type of outer membrane proteins are the receptors which are essential for the uptake of substrates that are too large for the channels of the type 1 and II porins, and occur at a very low concentration, for *e.g.*, ferric siderophores, vit B_{12} and colicins.

1.3.2.1.3 *Characteristics of porins of Gram-negative bacteria*

Porins have been studied fairly extensively (Benz *et al.*, 1982; Nikaido and Nakae, 1979). In Enterobacteriaceae sp., they share the following characteristics.

1) Non covalent association with peptidoglycan that is maintained in the presence of SDS.
2) Retention of native oligomeric trimer form in the presence of SDS.
3) The channel diameter of 1.2-1.6 nm, when assayed with liposome swelling or planar lipid bilayer.

The porins from non Enterobacterial species usually do not display the same type of peptidoglycan association as enteric organisms and they often do not form SDS resistant oligomers and also the channels formed from these proteins are much larger in diameter (upto 2.2 nm).

1.3.2.1.4 *Porins from mitochondrial and chloroplast outer membranes*

The outer membrane of mitochondria which acts like a barrier to macromolecules (Nakae, 1985) is freely permeable to small molecules like sucrose, possibly via pores similar to the ones in bacteria. Mitochondrial membranes isolated from various sources like paramecium, rat liver, rat heart, beef, *Neurospora* and yeast suggested the presence of an opened channel which is voltage dependent, anion selective, having a single channel conductance of 4.5 to 5 ns in 1 M KCl forming a large pore with a diameter of 1.5 nm - 2.0 nm. The properties of porins from mitochondria reviewed by Nakae (1985) are summarised in Table 1.1. The chloroplast outer membrane isolated from spinach had a largest pore diameter of 2.5 nm and exclusion limit of 10,000. This is the largest pore so far in outer membranes. Table 1.1 gives the list of porin proteins involved in diffusion process.

1.3.2.1.5 *Energy coupled transport through the outer membrane*

The transport of molecules across the outer membrane was believed to be a simple diffusion process since there is no energy source within this membrane, or within the adjacent periplasmic space. Besides the outer membrane cannot be energised by an electrochemical potential as it contains open protein channels. But recent evidences (Braun, 1995) suggest that the uptake of certain compounds through the outer membrane requires energy. In *Escherichia coli* these compounds include ferric siderophores (ferrichrome, ferric enterochelin, ferric citrate, ferric 2,3 dihydroxybenzoylserine, ferric aerobactin), vit B_{12} and group B colicins and also adsorption of phage T1, ϕ 80 which bind to highly specific receptor proteins in the outer membrane, even in energy depleted cells, but require energy for uptake. The type III porins which act as the receptors for

TABLE 1.1 Porins of Gram-negative bacterial, mitochondrial and chloroplast outermembrane

Source	Nomencl -ature	M.w. kDa	Aggrega -tion	Pore diameter	Solute Selectivity	Exclusion Limit
S. typhimurium	Omp Ds	38	3	1.40	cation	600
	Omp Fs	39	3	1.40	cation	600
	Omp Cs	39	3	1.40	cation	600
N. gonorrhoae	protein 1	32-37	3		anion	
A. salmonicida		42	oligomer	1.20	Relatively non-selective	
A. hydrophida		43	oligomer			
Enterobacter		37		1.20	cation	
		39-40		1.60	non-selective	
Rhodopseudomonas sphaeroides		47	oligomer	1.20		
capsulata		33	oligomer	1.60		
Yersinia pestis		33	oligomer	0.98	weak	
Anabaena variabilis		40-80		1.60	cation	
Chlamydia trachomatis		40				850-2,250
H. influenzae		40				
P. aeruginosa	protein F	35	3	2.20	cation	8,000, 700, 350
	protein P	48	3	0.70	anion phosphate	
	protein D1	50	3		glucose	
proteus		40	oligomer			800
Yersinia enterolitica		43			maltose	
Legionella pneumophila	MOMP	24			cation	

contd. ....

TABLE 1.1 ....Contd.

Source	Nomen-clature	M.w. kDa	Aggre-gation	Pore diameter	Solute Selectivity	Exclusion Limit
E. coli B	Omp F	37.2	3	0.80	cation	400-600
E. coli K-12	Omp C	38	3	1.00	cation	600
E. coli K-12	pho E	36	3	1.10	anion	600
	Nmp C	39.5	3	1.20		
	K	40	oligomer	1.20		
E. coli and S. typhimurium	lam B	47				342 and higher
	Ton A	78				740
	fev B	81				746
	cit	80				245
	cir	74				
	fsx	25				230-290
	bfe	60				1357
Mitochondria						
a) Paramecium		32		1.50	anion	
b) Rat liver		30		1.50	anion	8,000
c) Neurospora		31	3	2.00		
d) Mung beans		35				8,000
Yeast		29				8,000
Chloroplast						
Spinach				2.50		10,000

Source: Nakae (1985)

these compounds are present in very low concentration, which demands the use of specific cell surface receptors for uptake. These receptors bind the Ligands with much higher affinity (km = 0.2 μM) than the Type II porins (km = 0.1 μM).

Evidence for energy coupled transport came from the studies on *E. coli* mutants *Ton* A, *Ton* B resistant to phage T1. The experiments demonstrated that the adsorption of phage T1 to FhuA (the outer membrane receptor) requires cellular energy and *Ton* B, but the uptake of DNA is not *Ton* B dependent and that *Ton* B couples the energised state of cytoplasmic membrane to irreversible adsorption of phage T1 and phage ϕ 80 (Braun, 1995). The experiments suggested that the functional state of the receptor protein is dependent on the energised state of the cytoplasmic membrane and the *Ton* B protein. The receptor assumes an energised and non energised conformation. Uptake of all ferricsiderophores, vit B_{12} and the group B colicins are dependent on *Ton* B activity, and only these receptor mediated uptake processes required *Ton* B. *Ton* B acts like a allosteric regulatory protein which in its energised state regulates a conformational change in outer membrane receptor protein that helps in binding of phages T1 and ϕ 80 and translocation of vitamin B_{12} and ferricsiderophores and colicins into the periplasm. Two outer membrane translocation systems evolved, the Tol and Ton systems. The Ton system constitutes of *Ton* B - Exb B - Exb D which catalyse the ferrichrome and ferro enterobactin uptake. The ferrichrome strongly binds to the outer membrane receptor so that the interaction of *Ton* B - Exb B - Exb D with FhuA dissociates ferrichrome from FhuA, followed by its translocation into the periplasm as seen in Fig. 1.2 A. Tol mutants bind specific colicins but are not killed by them. The tol locus contains four genes *Tol* QRAB where the aminoacid sequence of *Tol* Q is homologous to *Ton* Exb B and *Tol* R to Exb D as shown in Fig. 1.2 B. The tol and ton systems function similarly. No substrates are taken up by tol system. It is believed that the tol system is not just evolved for the uptake of group B colicins and infection F pili-dependent phages, but has other physiological functions because of its leaky outer membrane.

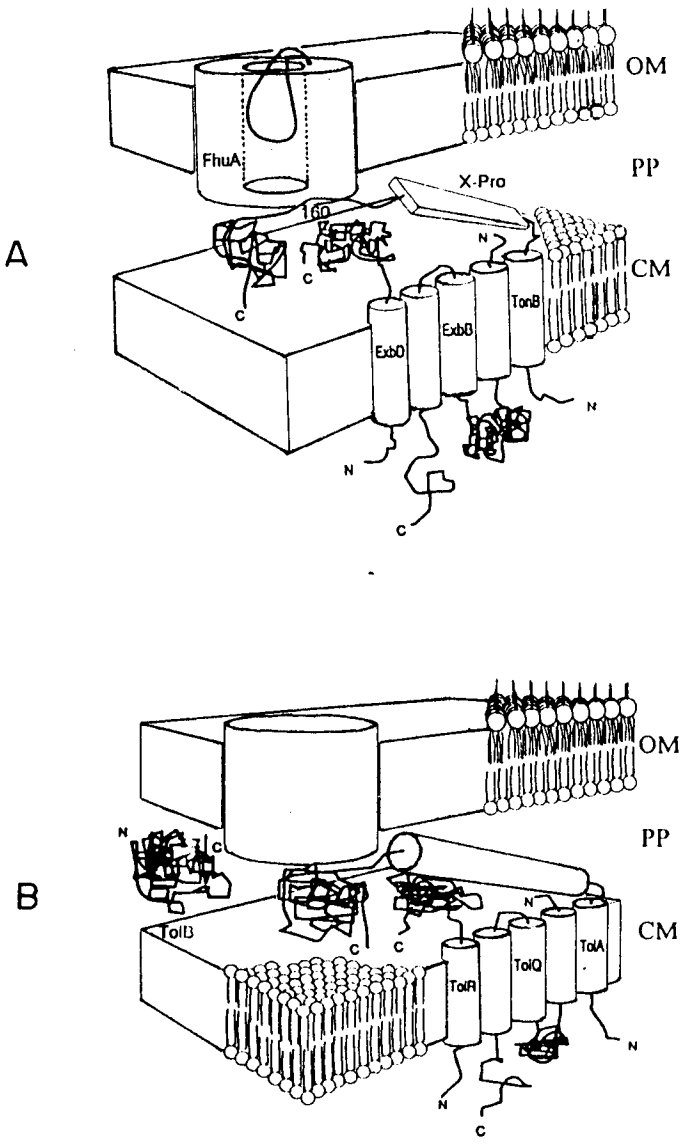


Fig. 1·2 A Location and transmembrane arrangement of the Ton B, Exb B and Exb D proteins in the cytoplasmic membrane (CM). (Braun, 1995)

Fig. 1·2B Membrane topology of the Tol A, Tol R, Tol Q proteins. (Braun, 1995)

1.3.2.2 Lipopolysaccharide

1.3.2.2.1 *Structure and composition of LPS*

LPS are amphipathic molecules found in outer leaflet of the OM in most Gram-negative bacteria and absent in Gram-positive bacteria. Diagrammatic representation of LPS is shown in Fig. 1.3 A. LPS is split by mild hydrolysis into its components like Lipid A and polysaccharide.

Lipid A contains glucosamine, phosphate and fatty acids. The carbohydrate component of Lipid A consists of β-1,6-D glucosamine disaccharide units. All hydroxyl and amino groups of disaccharide are substituted with phosphates, pyrophosphates and fatty acids. The most common fatty acid components are lauric acid (12c), myristic acid (14c), palmitic acid (16c) and 3 molecules of 3-D hydroxy myristic acid (14c). The lipid A part of the molecule is highly conserved, but the substitution pattern of fatty acid and phosphate is variable and results in greater difference in the biological activity of different LPS.

Polyssaccharide chain consists of three components, the inner core, the outer core and the O-antigen side chain. The rough stains (R forms) of Gram-ve bacteria such as *Neisseria sps, Bacteroides roojilis* and *Chlamidies,* lack O-antigen side chain. The O-antigen side chain of smooth strains extends upto 30 nm from the wall surface, has antigenic properties. LPS O-antigen side chain has the same repeating sequence of carbohydrates, and may extend upto 40 units in a chain which vary in length even in the same organism. The core region is much less variable than the O-polysaccharide, with upto 5 core types in *E. coli* and probably one in *Salmonella.* Most variation is in the distal part of the core, the inner part is much more conserved. It consists of 2 regions, the KDO region and the diheptose region. KDO region consists of 3 KDO units and a 8-carbon α-keto sugar acid. The diheptose region consists of 7-carbon heptose sugar called α-glycero-D-mannoheptose. The distinctive ladder like pattern of smooth LPS in SDS-polyacrylamide gels is caused by the separation of mixture of molecules of increasing molecular mass repeating additional O-antigen units added to the basic Lipid

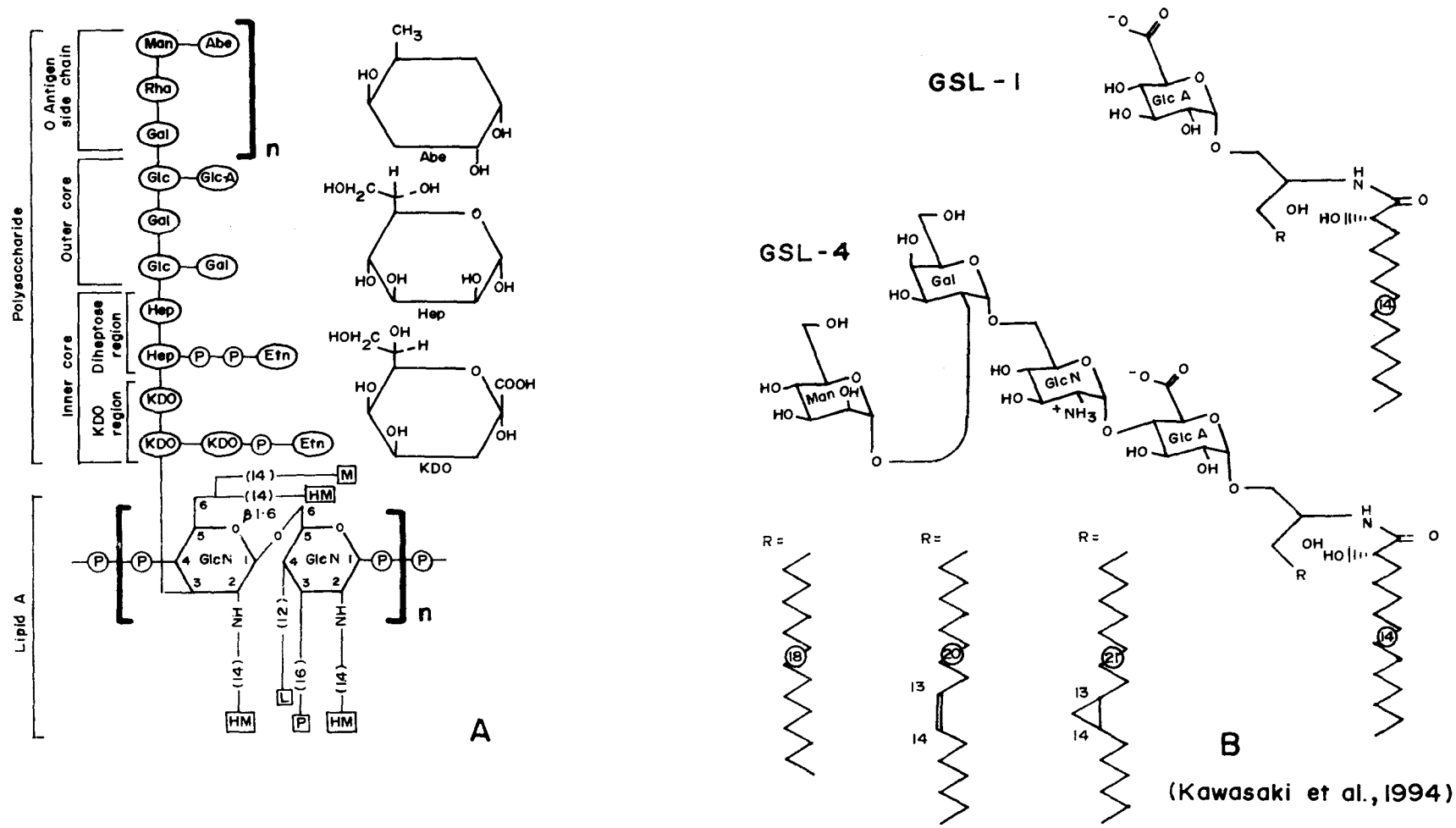


Fig. I.3 A Schematic representation of a unit of a Lipopolysaccharide molecule.

Fig. I.3 B Chemical structures of the GSL of the LPS-free gram-negative bacterium S. paucimobilis. Abe – abequose; Etn – ethanolamine; Gal – galactose; Glc A – glucose acetyl; GlcN – glucosamine; Hep – heptose; KDO – 2-keto-3-deoxyoctonate; HM – β-hydroxymyristic acid; L – lauric acid; M – myristic acid; P – palmitic acid; Ⓟ – phosphate.

A - core oligosaccharide structure (Zahran *et al.*, 1994). Rough LPS, consisting of only Lipid A - core oligosaccharide, does not give rise to the ladder like pattern in SDS polyacrylamide gels, with largest molecules moving slowest and the rough core with no substitutions moving fastest.

1.3.2.2.2 *Isolation*

LPS is most widely isolated by the method of Westphal and Jann (1965) which involves extraction of cells with hot phenol/water mixture followed by purification of LPS by ultra centrifugation. In most cases LPS is isolated from aqueous phase. However, in some cases it is isolated from phenol layers. Because of this undefined behaviour of LPS, the step involving separation of phases is avoided and the combined phenol water layer is dialysed to remove phenol and more recently phenol chloroform pet-ether method (Galanos *et al.*, 1969) is used. LPS of many organisms such as Enterobacteriaceae, *Pseudomonas* (O'Leary *et al.*, 1972) blue-green bacteria (Wecksser *et al.*, 1974) have been extensively studied. LPS may demonstrate a superficial similarity though a great deal of diversity is displayed in composition (Luderitz *et al.*, 1966). Recently environmental conditions during growth have been shown to affect the composition of wall lipopolysaccharide (LPS) of Gram-negative bacteria (Tempest and Ellwood, 1969). Quantitative differences in amounts of amino sugars and unknown reducing components were found in the LPS of *Citrobacter freondii* when cultures were grown in glucose or arabinose and at 5^{o} or 45^{o} C (Korezynki and Wheat, 1970). Compounds like 2KDO (2-keto 3-deoxyoctonate) and β-hydroxy myristic acid which was once considered to be present in LPS are now found to be absent in some species (Rietschel *et al.*, 1975; Stead *et al.*, 1975; Wecksser *et al.*, 1974).

1.3.2.2.3 *LPS-lacking Gram-negative bacteria*

Although LPS is considered to be an important constituent of the outer membrane of Gram-negative bacteria (Rietchel and Brase, 1992), it was found to be lacking in *Sphingomonas paucimobilis* (Khawahara *et al.*, 1991). Very recently *P. paucimobilis* was grouped into a new genus *sphingomonas*. This new name was proposed as earlier studies directed towards isolation of LPS by phenol water (West phal and Jann, 1965) or PCP (Galanos *et al.*, 1969) method were unsuccessful. Instead a glycolipid was obtained. This lipid A type glycolipid has D-glucosamine and 2-hydroxy myristic acid 2-OH (14:0) in an amide linkage.

Quantitatively it is equal to 3-OH myristic acid usually present in lipid A isolated from other organisms (Khawahara *et al.*, 1991). These findings of Khawahara *et al.* (1991) led to the conclusion that this LPS harbours an unusual lipid. The two glycosphingolipids identified were GSL-4A and GSL-1 (Khawahara *et al.*, 1991; Kawasaki *et al.*, 1994), The structure of which are shown in Fig. 1.3 B. Glycosphingolipids are ubiquitous components of the plasma membrane of eukaryotes and absent in prokaryotes, only exemptions being *Flavobacterium* devorans ATCC 10829 (Yamamoto *et al.*, 1978) and *Sphingomonas*. The glycosphingolipids have striking similarities with LPS lipid A of other Gram-negative bacteria.

1) They have similar ratios between negatively charged groups and fatty acids.
2) Computer modelling revealed that they are similar in architecture.
3) They are also capable of forming membranes with similar physico-chemical properties.
4) Like the core-lipid A region (KDO and lipid A) they have α- linked negatively charged pyranosidic glycosyl component adjacent to the lipid portion.
5) Like rough type LPS they also show variation in sugar portion therefore they are considered analogous to R type LPS.

S. paucimobilis is isolated from clinical, non clinical environments and also from rhizospheres of rice plants. The unusual cell surface of the organism could be having some relationship to the environment where the bacterium is living, like for example, for growth and survival of this organism on the plant surface.

1.3.2.3 Periplasm

Proteins present in between outer and inner membrane are called periplasmic proteins. Many of these proteins bind specifically to a substrate and play an important role in their uptake in soluble form. Recent evidence (Tam and Saier, 1993) suggests that in addition to their role in transport and chemoreception, some solute binding proteins function in initiating a sensory transduction. They detect the signals in the external environment and transmit these signals via transmembrane proteins to cytoplasmic constituents. Cytoplasmic proteins then respond usually by altering the rates of specific gene expression. A periplasmic phosphate binding protein of enteric bacteria apparently initiates the signal which results in induction of the phosphate (pho) regulon (Saier, 1993). Extracellular signalling between plant hosts and the bacterial parasite in agrobacterial species and virulence in *Salmonella typhimurium* is dependent on periplasmic binding proteins (Tam and Saier, 1993). In Gram-negative bacteria nutrients are transported across the cytoplasmic membrane by the ABC-type (or ATP-binding cassette type) multicomponent transport systems (Ames *et al.*, 1990). The common protein component of this system include two transmembrane proteins that usually span the membrane about six times each, one or two peripheral-membrane ATP-binding protein(s) localised on the cytoplasmic side of the membrane and a high affinity solute binding protein is periplasmic. The function of this transmembrane component is to channel the solute to the cytoplasm where as the ATP-binding protein provides energy to the system. Ligand-binding proteins confer specificity and affinity. This has been found to be true for many permeases such as histidine (Higgins *et al.*, 1982) maltose (Hengge and Boos, 1983), branched chain amino acids (Landick *et al.*, 1985), oligopeptides (Hiles *et al.*, 1987), ribose (Bell *et al.*, 1986), β-methyl galactoside (Harayama *et al.*, 1983), and phosphate (Surin *et al.*, 1985).

The presence of ABC type transport systems in Gram-positive bacteria has been recently documented, they are lipoproteins anchored to the external surface of the cell membrane with N-terminal glyceride-cysteine. In particular, among the most extensively characterised are the amin system of *Streptococcus pneumoniae*, *Spook* and *app* (Perego *et al.*, 1991; Rudner *et al.*. 1991; Koide and Hoch, 1994) of *Bacillus subtilis* responsible for uptake of oligopeptides. The *rbs* and *dci* A system of *B. subtilis*, (Woodson and Devine, 1994; Marthiopoulos *et al.*, 1991) to transport ribose and dipeptides respectively and the glutamine transport system of *Bacillus stearothermophiles* (Wu and Welker, 1991).

Binding proteins are unique feature of bacterial ABC importers (Fath and Kolter, 1993). These carry out energy dependent transport process and permit uphill transport of a solute against a concentration gradient. Apart from supplying cells with nutrients from their environment, it fulfills a range of physiological functions such as (1) involvement in sustaining osmotic balance between the cell and its environment contributes to the maintenance of the cell volume (2) participates in the transmission of information from muscle cells. (3) transforms free energy derived from electron transport into the chemical form of ATP. (4) plays a role in absorptive activity of the intestinal epithelium and secretary function of the kidneys.

The energy necessary for the accumulation of substrate can be transferred by direct interaction between a carrier and an energy yielding metabolic reaction. Examples of such processes are sodium pump (in animals) and magnesium dependent hydrogen ion translocating ATPase enzyme found in energy stored in membranes such as mitochondria, chloroplasts, and aerobic bacteria. The electrochemical gradient produced by these processes is used to energise secondary active transport which occurs by involving co-transport of substrate and ion. The active absorption of amino acids and sugars across the epithelial cells of small instestine and kidney depends on co-transport of sodium.

Binding proteins, of which there are dozens of different species in *E. coli* can be released into the medium by osmotic shock (Neu and Heppel, 1965), slow freeze thawing (Paoletti *et al.*, 1987), rapid freeze thawing and chloroform method (Ames *et al.*, 1984).

1.3.2.3.1 *Types of transport system*

Transport systems are categorised into two types based on their response to a physical treatment like osmotic shock and nature of mechanism of energy coupling into shock sensitive and shock resistant permeases (Neu and Heppel, 1965; Furlong, 1987). Shock sensitive permeases are also referred to as periplasmic permeases which are inactivated during osmotic shock because of the loss of an essential protein component which is referred to as periplasmic component that binds the transported solute with high affinity. The first periplasmic transport protein to be released by osmotic shock was a sulfate binding protein, part of sulfate permease in *Salmonella typhimurium* (Pardee, 1966). Subsequently, numerous permeases have been studied and characterised (Furlong, 1986; 1987). Shock resistant permeases on the other hand retain all of their activity upon osmotic shock and are powered by proton motive force. A classic example of this class is β-galactoside permease which is extensively studied. This permease is a single protein, which is tightly bound to the membrane.

1.3.2.3.2 *Biochemical properties of periplasmic binding proteins*

They occur in native form, with molecular weights of monomers ranging from 22 kDa to 44 kDa. They are bilobiate and have an assymetric shape with an axial ratio of 4:1, *e.g.*, sulphate binding protein (Arthur *et al.*, 1966). The binding takes place by interaction of substrate with tryptophan residues of the protein in the active site by forming side chain hydrogen bonds which are stable to heat and resistant to -SH reagents (Rosen, 1973; Pardee, 1966; Penrose *et al.*, 1968; Anraku, 1968; Kuzuya *et al.*, 1971; Boos, *et al.*, 1972). Studies indicate one binding site per monomer with dissociation constants ranging from 0.005 μM to 50 μM (Hogg and Englesberg, 1969).

In almost all the cases studied km values are close to kD values indicating that the transport system is largely dependent on the specificity of the protein to its substrate. kD values of some binding proteins isolated from enteric organism reviewed by Tam and Saier (1993) are given in Table 1.2. Conformational change in proteins upon binding to substrates (Weiner and Heppel, 1971; Kuzuya *et al.*, 1971; Parnes and Boos, 1973; Boos *et al.*, 1972) is measured by various methods like fluorescence spectroscopy (Newcomer *et al.*, 1979; 1981a; 1981b), NMR, scanning colorimetry, electrophoretic mobility upon binding. Depending on their sequence similarities the solute binding proteins so far identified and characterised are grouped into eight clusters which are presented in the Table 1.3.

Reconstitution is a simple way of studying the mechanism of action of periplasmic permeases by reconstituting them in membrane vesicles (Hunt and Hong, 1981). Reconstitution means getting back the lost function by the addition of a essential component. Over the past several years a number of methods have been used for this purpose.

Reconstitution in membrane vesicles involves preparation of membrane vesicles from lysozyme-generated spheroplasts from a mutant strain lacking binding protein (Arthur and Hong, 1986; Rotman and Gulzman, 1984) to which a high concentration of binding protein 2mg/ml is added. On providing an energy source the transport of the substrate into the vesicles takes place. Reconstitution in spheroplasts (Masters and Hong, 1981; Gerdes *et al.*, 1977) was shown to be effective in case of pst permease for inorganic phosphate, ribose, glutamine, glutamate and histidine. Reconstitution in whole cells is studied by treating them with calcium phosphate which increases the permeabilisation of outermembrane and allows entry of entire proteins in a manner similar to the entry of DNA during transformation (Brass, 1986; Brass *et al.*, 1981). The procedure is simpler than one involving spheroplasts.

TABLE 1.2 Biochemical properties of periplasmic binding proteins

S. No.	Solute specificity	Molecular mass kDa	Binding affinity
I	***Sugars***		
	1) Maltose	41	1.000
	2) α-Glycerol-P	45	0.200
	3) Xylose	37	0.600
	4) Ribose	30	0.100
	5) Galactose	32	1.000
II	***Amino acids***		
	1) Cystine	28	0.010
	2) Glutamine	26	0.200
	3) Glutamate aspartate	30	1.000
	4) Aspartate orv deurine	37	0.400
III	***Anions***		
	1) Citrate	28	2.000
	2) Phosphate	34	0.800
	3) Sulphate	35	0.020
IV	***Vitamins***		
	1) B_{12}	22	0.005
	2) Thiamine	--	0.100

Source: Tam and Saier (1992)

TABLE 1.3 Clusters of binding proteins, classified based on solute selectivity

Abbreviation[a]	Specificty	Interactive transport system	Organism
Cluster I			
MalEco	Maltose	Mal FGK	*E. coli*
MalSty	Maltose	Mal FGK	*S. typhimurium*
MalKae	Maltose	Mal FGK	*K. aerogenes*
MalSpn	Maltose	Mal MP	*S. pneumoniae*
GlpEco	Glycerol-3-phosphate	UgpACE	*E. coli*
MsmSmu	Multiple sugars	Msm KFG	*S. mutans*
SfuSma	Iron	SfuBC	*S. marcescens*
FbpNme	Iron	--	*N. meningitidis*
Cluster 2			
RibEco	Ribose	RbsACD	*E. coli*
RibSty	Ribose	RbsACD	*S. typhimurium*
AraEco	Arabinose	AraGH	*E. coli*
GalEco	Galactose	MglACE	*E. coli*
GalCfr	Galactose	--	*C. freundii*
ChvAtu	Multiple sugars	None	*tumefaciens*
Cluster 3			
HisSty	Histidine	HisQMP	*S. typhimurium*
LAOSty	Lysine-arginine-ornithine	HisQMP	*S. typhimurium*
GlnEco	Glutamine	GlnPQ	*E. coli*
GlnBst	Glutamine	GlnPQ	*B. stearothermophilus*
OccAtu	Octopine	OccQMP	*A. tumefaciens*
NocAtu	Nopaline	NocQMP	*A. tumefaciens*
BaaNgo	Basic aminoacids	--	*N. gonorrhoeae*
Cluster 4			
LeuEco	Leucine	LivHMGF	*E. coli*
LIVEco	Leucine-isoleucine-valine	LivHMGF	*E. coli*
LIVCfr	Leucine- isoleucine-valine	--	*C. freundii*
LIVPae	Leucine- isoleucine-valine	BraDEFG	*P. aeruginosa*
AmiPae	Amic, transcriptional repressor	None	*P. aeruginosa*

contd. .....

TABLE 1.3 Contd.,

Abbreviation[a]	Specificity	Interactive transport system	Organism
Cluster 5			
OppEco	Oligopeptide	--	*E. coli*
OppSty	Oligopeptide	OppBCDF	*S. typhimurium*
OppSpn	Oligopeptide	AmiCDEF	*S. pneumoniae*
DppBsu	Dipeptide	DciAABCD	*B. subtilis*
OppBsu	Oligopeptide	OppBCDF	*B. subtilis*
DppEco	Dipeptide	--	*E. coli*
NikEco	Nickel	--	*E. coli*
XP55Sli	Not known	--	*S. lividans*
Cluster 6			
SulEco	Sulphate	CysTWA	*E. coli*
SulSty	Sulphate	--	*S. typhimurium*
SulSsp	Sulphate	CysTWA	*Synechococcus sps*
TsuEco	Thiosulphate	CysTWA	*E. coli*
PhoEco	Phosphate	PstCAB	*E. coli*
Cluster 7			
DcaRca	Dicarboxylate	--	*R. capsulatus*
CisStyl	Citrate	--	*S. typhimurium*
Cluster 8			
FecEco	Iron-enterobactin	FecCDE	*E. coli*
FepEco	Iron-dicitrate	FepCDG	*E. coli*
FhuEco	Iron-hydroxamate	FhuBC	*E. coli*
FatVan	Iron-anguibactin	FatCD	*V. anguillarum*
BtuEco	Vitamin B_{12}	BtuCD	*E. coli*

[a] = The abbreviations used for various proteins usually indicate the specificity of the binding protein (first three letters) and the organism from which it is obtained. The genus and species are indicated by the second three letters of the abbreviation.

Source: Tam and Saier (1992)

1.3.2.3.3 *Transport model*

With the information available on periplasmic permeases so far studied and characterised, a model was proposed by Ames *et al.* (1990) for transport of histidine (Fig. 1.4). The substrate (histidine) crosses the outermembrane through a specific or a non specific channel which allows the diffusion of small molecular weight compounds and enters the periplasmic space. Then it is bound by the periplasmic permease (his-*J*) which undergoes a conformational change on binding to the substrate, results in increased affinity of it towards membrane bound complex (composed of proteins His Q, His M, and His P). The membrane bound complex on interaction with periplasmic permease in turn undergoes conformational change which results in release of subtrate from the binding protein and the appearance of binding site(s) on the membrane bound components, allowing the passage of the substrate from one binding site to the other into the cell. Studies with spheroplasts or membrane vesicles deprived of the binding protein or with mutants lacking binding protein showed no transport of the substrate indicating that the membrane components do not have any binding site. The site is formed only on interaction with the binding protein.

An alternative model proposed was opening and closing of a pore through the membrane bound permease components (Ames and Higgins, 1983). In this model, the liganded periplasmic protein component initiates the opening and closing of a pore through which the substrate diffuses. The pore is closed as the free binding protein is released from the membrane. Since a concentration gradient is established, energy must be consumed for transport of the substrate.

1.4 AIM AND SCOPE

α-Santonin a sesquiterpene lactone (structure given in Fig. 1.5) has been isolated from unexpanded buds of several *artemesia sps* belonging to compositae family. It has powerful antihelminthetic action and attained significance as a potential parent compound for antitumour and antimicrobial drugs (Burger, 1960; Fujimoto *et al.*, 1978;

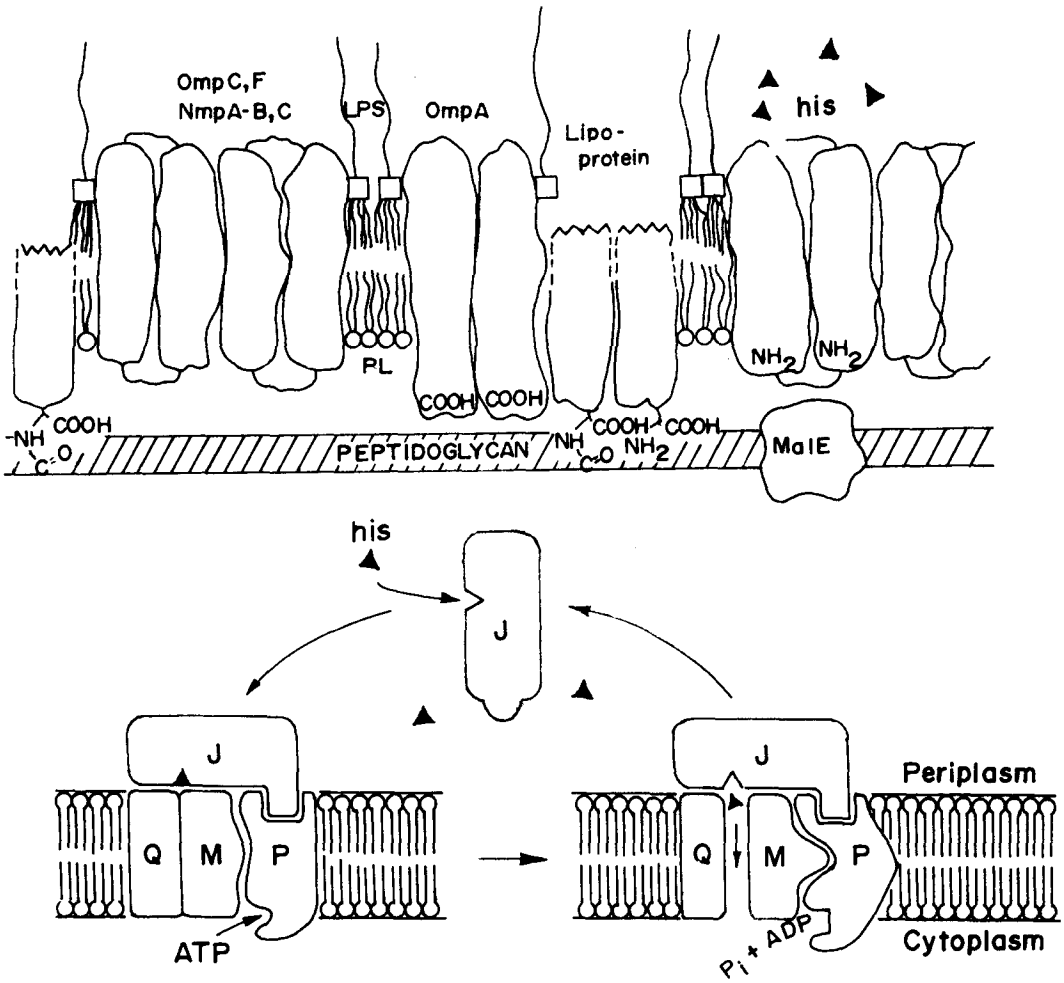


Fig. 1.4 Model suggested for bacterial periplasmic transport system representing the OM Protein, periplasmic protein and inner membrane protein for histidine, as proposed by Ames and Spudich (1976)

Calzada *et al.*, 1980). A number of workers have used microorganisms such as *S. aureofaciens, S. cineriocatus* to transform α-santonin and obtained 1,2 dihydrosantonin and lumisantonin as the products (Hikino *et al.*, 1970; Iida *et al.*, 1981a; Sato *et al.*, 1984; Iida *et al.*, 1981b).

In our laboratory Sangodkar and Mavinkurve (1982; 1984) successfully isolated by enrichment technique, a soil bacterium capable of utilising α-santonin as a sole source of carbon and energy. This culture was assigned to genus *Pseudomonas* based on presence of single polar flagellum, oxidase positive reaction and slow growth on nutrient rich medium led to the tentative identification (Sangodkar and Mavinkurve, 1982) and was referred to as *P. cichorii* initially (Sangodkar and Mavinkurve, 1982, 1984; Naik and Mavinkurve, 1987; Furtado *et al.*, 1987, 1988). However, based on chemotaxonomic tools such as fatty acids, protein and pigment analysis it is now identified as *S. paucimobilis* (Furtado *et al.*, 1994).

S. paucimobilis during the process of utilisation of α-santonin forms a number of transformation products. These products are detected by TLC using benzene : ethyl acetate (80:20) as solvent system and resorcinol in sulfuric acid (1:1) as a spray reagent. The transformation of α-santonin is by two independent pathways. Naik (1986) during his studies on α-santonin degradation pathway isolated and characterised ten intermediate products, six in native form and four in the form of 2,4 DNP derivatives. The products were isolated by using metabolic inhibitors like DCCD and semicarbazide. α-santonin is metabolised via dihydrosantonin as a major energy yielding pathway but in presence of semicarbazide it is utilised via dihydroxysantonin. The enzyme α-santonin 1,2 reductase catalyses the reduction of α-santonin to dihydrosantonin, the first step in the pathway of α-santonin degradation. The enzyme demonstrated in the cell free extract is NADPH dependent, does not require metal ions and has a Km of 0.2 μ mole (Naik and Mavinkurve, 1987). Dihydrosantonin, the key metabolite of α-santonin degradation, undergoes hydroxylation to give 4,5 dihydroxy products which are subsequently utilised. Alternatively, lactone ring is hydrolysed to follow two independent pathways arising from dehydration or dehyrogenation reaction. The

dehydration forms AC_1 (11-dimethyl β-cyperone) via AC_2 (ethyl-3-oxo-eudesm-4 : 6 dione-12-oate=) where as dehydrogenation forms L_3/L_4 via dihydrosantonenic acid. The products ultimately enter the intermediary metabolic pathway via crotonaldehyde (AC) by β-oxidation as seen in Fig. 1.5. The product L_3 (11-dimethyl-eudesm-4-ene-3,6 dione) which was identified and characterised by Furtado *et al.* (1988), appears after about 10-13 h of incubation and persisted for 23 h with its intensity being maximum at 17 h. L_3 being one of the distinct intermediate products is formed from DHS by three consecutive reactions, namely, hydrolytic opening of the lactone ring followed by oxidation of the hydroxyl at C_6 and decarboxylation of C_{12} carboxyl group.

Although DHS was accumulated by using DCCD as a inhibitor at pH 6, recently Colaco *et al.* (1993) identified two more products named as D_1 and D_2 with R*f* 0.20 and 0.22 cm accumulated at pH 8 (Colaco *et al.*, 1993). The compound D_2 was identified as 4,5 dihydroxysantonin (DHXS). The cells incubated with DHS failed to give dihydrodihydroxy derivative, indicating that DHXS is formed via independent pathway and not through DHS.

Simultaneously, study was carried out in our laboratory by Furtado *et al.* (1987) on transport of α-santonin in *S. paucimobilis* cells. This study was intriguing as α-santonin is sparingly soluble, the dissolution of which is 166 μg/ml and forms shiny white crystals in liquid medium and solid medium (Fig. 1.6). The growth of *S. paucimobilis* is noticed in the form of halos or zones around the colonies in the solid media (Fig. 1.6).

The utilisation or disappearance of α-santonin crystals that were distantly situated from a growing colony suggested that

1) for the utilisation, α-santonin is not essential in crystalline form.
2) an extracellular factor for the solubilisation of α-santonin may be involved.

The necessity of direct cell to substrate contact was verified by streaking cells on barriers such as cellophane membranes, millipore filter which were placed on α-santonin agar plates. The growth of *S. paucimobilis* cells on barriers ruled out the necessity of direct contact between cell and the α-santonin crystals. Further observation

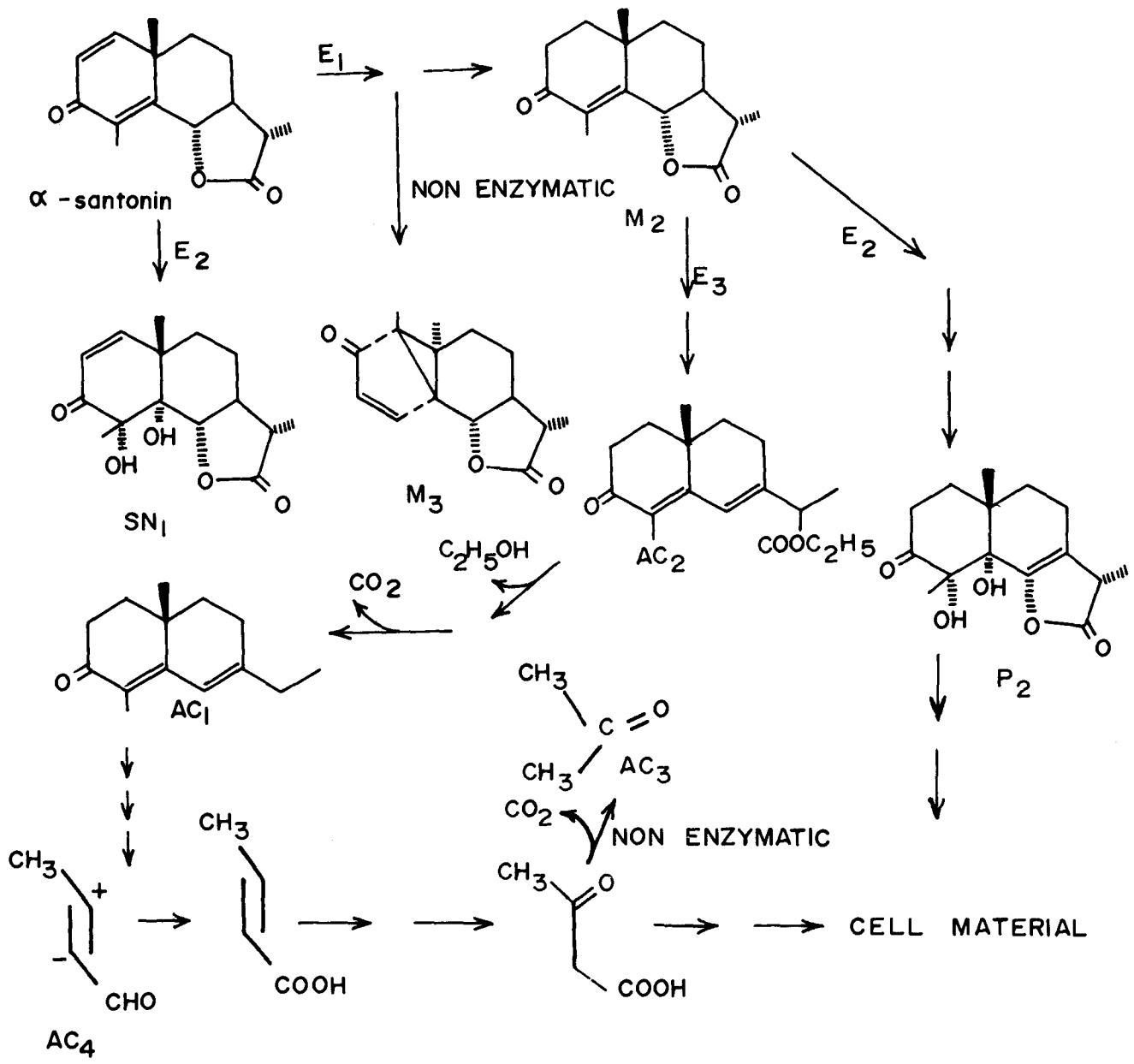


Fig. 1·5 Transformation pathway of α-santonin by Sphingomonas paucimobilis.

E_1 - α-santonin 1,2 reductase

E_2 - Dioxygenase

E_3 - γ-lactone hydrolase

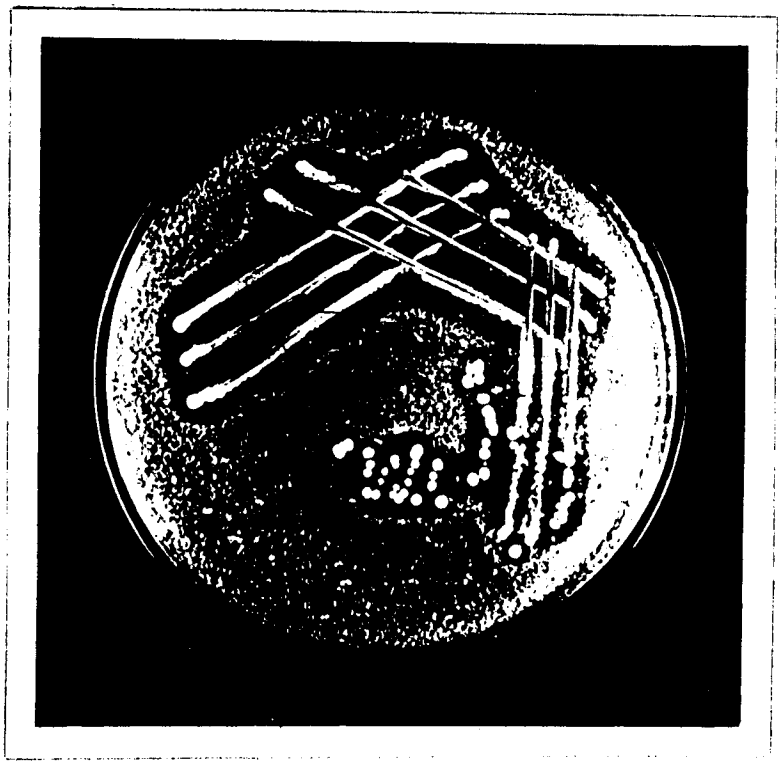


Fig. I·6 Halo due to growth of Sphingomonas paucimobilis strain - S ATCC 43388 on santonin agar.

of halos formed below the barriers, suggested the possibility of secretion of an extracellular solubilising factor.

Studies were carried out to test the presence of solubilsiing factor, by incubating the culture supernatant with the α-santonin in test or on α-santonin agar plates by agar cup method. Dissolution or clearance of crystals was not noticed suggesting that the utilisation is purely by a physical phenomenon and transport of α-santonin in *S. paucimobilis* cells does not require any external biological factor (Furtado *et al.*, 1987).

The study on the mechanism of hydrocarbon uptake is still in infancy, and detailed research is needed to thoroughly out line the process involved. The present work was aimed at studying the molecular mechanism underlining the α-santonin transport by *S. paucimobilis* cells. The work carried out is briefly given in the form of following sections.

1) Effect of substrate (α-santonin) on cell and cell envelope components.
2) Isolation and characterisation of outer membrane protein involved in α-santonin transport.
3) Release of periplasmically located α-santonin binding factor(s).
4) Outline the model mechanism of α-santonin transport pathway.

CHAPTER 2.0

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 MICRO-ORGANISMS

The cultures used in the present work are *Sphingomonas paucimobilis* strain S ATCC 43388 α-santonin degrader (Sangodkar and Mavinkurve, 1982; 1984) and S_{48} a santonin negative transposon mutant (Parmekar and Mavinkurve, 1995). Stock cultures of the wild type *S. paucimobilis* strain S was maintained on mineral salts medium (App. 4.1) with 0.4% α-santonin (SA) slants (Sangodkar and Mavinkurve, 1982; 1984) and subcultured 48 h before use. The mutant S_{48} was maintained in 0.4% glucose (GM Km) mineral salt slants with kanamycin (50 μg/ml). Important characteristics of the cultures are given in Table 2.1.

2.2 GROWTH OF *S. paucimobilis*

2.2.1 Preparation of Liquid Culture

A loop full of *S. paucimobilis* strain S pregrown on α- santonin agar slants was inoculated into a 150 ml flask containing 25 ml of mineral medium with 0.4% (w/v) of α-santonin. The flask was covered with a wet cotton pad to maintain the temperature at 27-30° C, and incubated on a rotary shaker at 200 revolutions per minute (rpm) for 48 h. 5 ml of this culture was transferred into a flask containing 100 ml of 0.4% α-santonin medium and incubated as described earlier. Log phase cells were routinely used for the study.

2.2.2 Preparation of Cell Suspension

S. paucimobilis strain S cells streaked on α-santonin/glucose/nutrient agar slants, S_{48} cells were streaked on glucose kanamycin agar slants and incubated for 48 h at RT. 5 ml of 0.05 M phosphate buffer pH 7.0 (App. 2.1a) was spread on the surface of the plate

TABLE 2.1 Morphological and biochemical characteristics of *S. paucimobilis* (S) and S_{48}

S. No.	Characteristics	S	S_{48}
1	Morphological appearance	Coccobacilli with single polar flagellum	Coccobacilli, smooth, slimy, pale yellow cells
2	Gram stain	negative	negative
3	Oxidase	positive	positive
4	G+C content	63 %	63 %
5	Pigment analysis	yellow pigment in acetone, two peaks at 452 nm and 487 nm	ND
6	Utilisation of compounds as carbon sources		
	a) Sugars	Glucose, maltose, fructose, lactose, sucrose, galactose, arabinose, rhamnose, mannitol, acetate, fumarate and pyruvate	Glucose
	b) Aminoacids	L-alanine	ND
	c) Alcohol	ethanol	ND
	d) Terpenes	α-santonin	-
	e) Aromatic	benzoate, catechol	ND

and the cells dispersed in the buffer with a sterile glass spreader. The cell suspension obtained thus was transferred through a pipette into a centrifuge tube and washed twice at 10,000 rpm at 4° C for 10 min. The cell pellet obtained was resuspended into 0.05 M phosphate buffer pH 7.0 and the volume adjusted to give an optical density of 4.5 using a UV-visible spectrophotometer (Shimadzu) at 450 nm.

2.3 PREPARATION AND EXTRACTION OF CELL AND CELL ENVELOPE COMPONENTS

2.3.1 Extraction of Whole Cell Proteins

Whole cell proteins were extracted from *S. paucimobilis* cells by the method of Jinchichiro *et al.* (1991). The cells grown on different growth substrates were suspended to an absorbance 1.0 at 450 nm in 0.05 M phosphate buffer pH 7.0. 10 ml of the above suspension centrifuged and pelleted at 4,500 rpm for 10 min. To the pellet one ml of 1% w/v solution of SDS was added and stirred on a vortex mixer and left for 30 min at RT. To 10 µl of supernatant obtained by centrifugation, equal volume of sample buffer (App. 3.3.5) was added and boiled at 100° C for five min. The samples were subjected to discontinuous SDS polyacrylamide gel electrophoresis.

2.3.2 Preparation of Outermembrane Proteins

Outer membrane proteins were obtained by sarkosyl extraction method (Filip. *et al.*, 1973). Log phase cells 200 ml were harvested and washed twice and resuspended in the 20 mM Tris HCl buffer pH 7.4 (App. 2.2b) to an absorbance of 4.5 and sonicated on ice, for one minute (4 bursts with a pulse of 30 sec). Efficiency of sonication was monitored by streaking the culture on the plate after every burst. After 4 bursts of total sonication time of 4 min., there was a considerable cell damage and a very few colonies appeared. Unbroken cells were removed by centrifugation at 10,000 g for 10 min at 4°C and supernatant containing the envelope protein fraction was centrifuged at 36,000 rpm (1,00,000 g) for 1 h at 4° C on a 80 Ti rotor to pellet the envelope proteins. The envelope

proteins suspended in two volumes of 20 mM Tris/HCl pH 7.2 containing 0.5% w/v sarcosine anhydride for 30 min at RT to solubilise the cytoplasmic membrane fraction. The suspension was then centrifuged at 36,000 rpm for 1 h at 4° C. The pellet was washed and resuspended in 20 mM Tris/HCl pH 7.2 to a concentration of 20 mg of protein/ml and stored at -70° C until further use. The suspension was then used for preparation of liposomes, electrophoresis and purification.

2.3.3 Release of Periplasmic Proteins

Periplasmic proteins were extracted from the cells by three methods namely cold osmotic shock (Neu and Heppel, 1965), chloroform (Ames *et al.*, 1984) and by freezing and thawing (Paoletti *et al.*, 1987). Log phase cells suspended to an absorbance (A) of 4.5 at 450 nm in 0.05 M phosphate buffer pH 7.0 were used for all the methods unless otherwise specified.

a) Cold osmotic shock method (COSM)

20 ml of cells suspended in 20 ml of cold 0.2 M magnesium chloride in 0.05 M Tris/HCl buffer pH 8.0, were incubated at 22° C for 1 h with constant stirring. Cells were then centrifuged at 14,000 rpm at 4° C for 10 min. The supernatant containing periplasmic proteins was passed through millipore filter 0.45 μm and dialysed against 1 mM magnesium chloride in 10 mM Tris buffer pH 7.4 at 4° C.

b) Chloroform method (CHLM)

Cell pellet of 20 ml cells was vortexed with 200 μl of chloroform and maintained at RT for 15 min., to which 2 ml of 20 mM Tris/HCl pH 8.0 was added. The supernatant, separated by centrifuging at 6,000 g for 20 min and filtered through 0.45 μm millipore.

c) Freeze thaw method (FTM)

20 ml of cells were frozen at -20° C for 2 h followed by thawing at RT for 1 h. The cycle is repeated twice and the cells were left overnight at -20°C. The thawed cells were separated by centrifugation at 6,000 g for 20 min. The supernatant was filtered through 0.45 μm millipore to remove any residual cells.

2.3.4 Extraction of Lipopolysaccharide (LPS)

LPS was isolated from *S. paucimobilis* cells by the method of Hitchcock and Brown (1983). 1.5 ml of cells washed and suspended in phosphate buffered saline pH 7.0 and centrifuged at 9000 g for 15 min to pellet down the cells. The cell pellet is then solubilised in 100 μl of sample buffer containing 2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris/HCl pH 6.8 and bromophenol blue at 100° C for 10 min. The lysate is cooled and treated with 50 μg of proteinase K at 60°C in a water bath for 60 min. The samples were then electrophoresed at a constant current of 35 mA on a 15% SDS polyacrylamide gel and then stained with silver nitrate according to the method of Tsai and Frasch (1982).

2.3.4.1 Staining of the lipopolysaccharide

Step 1 **Fixing:** The gel was fixed overnight in 25% v/v isopropanol and 7% v/v acetic acid.

Step 2 **Oxidation:** The fixing solution is poured off and the gel is oxidised for 5-10 min in 150 ml distilled water containing 1.05 g of per-iodic acid, 4 ml of 25% v/v isopropanol in 7% v/v acetic acid. This solution is made just before use.

Step 3 **Washing:** The gel is then washed eight times with 200 ml of tripled distilled water after incubating for 30 min for each wash.

Step 4 **Staining:** The gel is stained for 10 min in a solution containing 0.1 N sodium hydroxide (28 ml), 1 ml concentrated ammonium hydroxide (29.4%), 5 ml of 20% w/v silver nitrate and 115 ml of distilled water. The solution is prepared just before use by adding silver nitrate solution drop wise with constant stirring, otherwise a brown precipitate is formed which causes a lot of background staining.

Step 5 **Washing:** Gel is washed four times with 200 ml of distilled water over the period of 40 minutes.

Step 6 **Developing:** The gel is then transferred to a freshly prepared developing solution containing 0.005% citric acid and 0.019% formaldehyde (dilute 38% in 200 ml of water at 25^{0} C).

Step 7 When desired staining is achieved, the gel is repeatedly washed with large volumes of water.

NB: Water used for silver nitrate staining is millipore filtered water. Staining should be done in glass containers and not in plastic containers.

2.4 ELECTROPHORETIC TECHNIQUES

Proteins were separated on a native polyacrylamide gel (NPG) and discontinuous SDS-PAGE as described by Laemmli (1970). A NPG is used if the activity of the protein or structure of protein complex under study is to be maintained. The separation is based on the charge of the protein molecule, whereas in SDS preparations, migration is determined not by its intrinsic electrical charge of polypeptides but by molecular weight. Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by wrapping around the polypeptide backbone. SDS confers a net negative charge to the polypeptide in proportion to the length of the protein, , so that the proteins move according to equal charge density and the separation is based on its molecular weight.

2.4.1 Polyacrylamide Gel Electrophoresis.

Native polyacrylamide gel is prepared and run according to the procedure given below for SDS polyacrylamide gel except for omitting SDS from all the solutions.

2.4.2 Sodium Dodecyl Polyacrylamide Gel Electrophoresis

a) Preparation of the gel

Separating gel mixture is prepared according to Table 2.2 TEMED and ammonium per sulphate (dry) are added last and the flask is gently swirled to mix the contents. Solution is poured into the glass plate sandwich to a level of about 4 cm from top and overlaid with water saturated butanol, after about half an hour the plates are tilted to pour off the water layer. Stacking gel solution is prepared according to the proportions given in Table 2.2. The comb is inserted into the sandwich and the stacking gel mixture is poured.

b) Preparation of the sample

Equal parts of protein containing 100 μg of protein/ml sample and sample buffer mixed in a microfuge tube are boiled for 10 min.

c) Loading of the sample

Slowly the comb is removed from the gel, the sample is loaded into each well and the lower and upper tanks are filled with the running buffer (App. 3.3.6), power supply is set to a constant current of 25 mAmps or voltage (130 v). When the dye reached the bottom in about 5 h the power supply is turned off.

TABLE 2.2 Composition for preparation of the gel

S. No.	Constituents	Separating gel 10% T 0.27% C	Stacking gel 4% T 0.21% C
1	30% T 0.8% C	8.4 ml	1.6 ml
2	Buffer (lower Tris)	6.3 ml	--
3	Buffer (upper Tris)	--	2.0 ml
4	Distilled water	10.4 ml	4.8 ml
5	APS (10%)	250.0 µl	100.0 µl
6	TEMED	20.0 µl	10.0 µl

APS = Ammonium per sulphate (dried)
T = Acrylamide concentration expressed in terms of % T
C = bis acrylamide concentration expressed in terms of % C
TEMED = Tetra ethylene methyl ethylene diamine

d) Staining and destaining

Gel is stained with coomassie blue (App. 3.3.8) and destained in destaining solution 1 (App. 3.3.7) for 1h and then transferred into destaining solution 2 (App. 3.3.7).

2.5 ANALYTICAL ASSAYS

2.5.1 Transport Assay

Uptake of α-santonin by *S. paucimobilis* cells is measured according to the method of Furtado *et al.* (1987). 5 ml of cells suspended to absorbance of 4.5 with 0.05 M phosphate buffer at pH 7.0 were incubated with 2.5 ml of saturated α-santonin solution (App. 1.2) on a rotary shaker for varying time intervals. The entire contents were filtered through 0.45 μm membrane filter to separate the cells. The filtrate was assayed for residual α-santonin colorimetrically (Furtado and Mavinkurve, 1981).

2.5.2 α-Santonin Assay

Filtrate obtained from transport was extracted twice with 1:1 (v/v) of distilled chloroform using a separating funnel. The organic layer was carefully drained into fresh tubes and evaporated to dryness by heating. The residue was dissolved in 3 ml of 18 N sulphuric acid (App. 3.2a) and boiled with 0.5 ml of freshly prepared 0.8% ferric chloride (App. 3.2a) for 10 min in a boiling water bath. The reaction mixture was then cooled, diluted with 3 ml of absolute alcohol and the resulting colour complex measured at 485 nm using Schimadzu UV-240 Spectrophotometer.

2.5.3 Dehydrogenase Assay

α-Santonin 1,2 reductase is an NADPH dependent enzyme (Naik and Mavinkurve, 1987). The oxidation of NADPH in a reaction mixture was measured to assay the enzyme activity in shock fluid. Decrease in absorbance at 340 nm in reaction

mixture containing 0.5 ml of shock fluid (150 μg/ml), 0.4 ml of saturated α-santonin solution and 0.1 ml of NADPH (2 mM) prepared in TE buffer (App. 2.3) were monitored for 10 min in a time scan mode on Schimadzu UV-240 Spectrophotometer.

2.5.4 Equilibrium Dialysis Binding Assay

Binding of α-santonin by shock fluid was studied by equilibrium dialysis according to the method of Stinson *et al.* (1977). One ml of shock fluid eluted out from cells by subjecting to freezing and thawing was taken in a pretreated dialysis bag (App. 1.3) and dialysed overnight against saturated santonin solution in 0.05 M phosphate buffer with constant stirring. After overnight incubation the contents were extracted with chloroform and analysed for α-santonin colorimetrically (Furtado and Mavinkurve, 1981).

2.5.5 Filtration Assay

Filtration assay was performed as described by Brozosted *et al.* (1993; Lever, 1972). To 5 ml of shock fluid containing 100 mg of protein/ml, 1 ml of saturated santonin solution is added and incubated at refrigeration temperature for 1 h. The proteins were precipitated by adding slowly 5 ml of saturated solution of ammonium sulphate. The solution was filtered through 0.45 μm millipore filter, washed twice with ammonium sulphate solution and the filter paper was then suspended in 0.05 M phosphate buffer, pH 7.0, so that the precipitated proteins are transferred into the buffer. The extracted solution was then checked for the presence of α-santonin spectrophotometrically by scanning at 242 nm which is the absorption wave length of α-santonin. The absorbance value at 242 nm gives the concentration of α-santonin bound by the proteins. The proteins precipitated without prior incubation to α-santonin were also checked for absorbance at 242 nm which is used as a control.

2.5.6 Protein Assay

Protein content was estimated by Lowry method (Lowry *et al.*, 1951). To 1 ml of sample containing 0.1-1 mg of protein, 1 ml of reagent ABC is added (App. 3.2b). After 10 min., 3 ml of Folin Ciocalteau reagent (1:10 dil.) is added and was left for 30 min. Absorbance values read at 640 nm on a UV Spectrophotometer. Bovine serum albumin (1 mg/ml) was used as a standard. The unknown protein concentration is calculated from the standard graph.

2.5.7 Oxygen Analysis

Cells washed and suspended in 0.05 M phosphate buffer pH 7.0 to an absorbance of 4.5 at 450 nm were checked for oxygen consumption in the presence of saturated santonin solution (166 μg/ml) in buffer using Gilson oxygen analyser (Sangodkar and Mavinkurve, 1991). The reaction mixture contained 1.5 ml of 0.05 M phosphate buffer pH 7.0 (oxygen saturated), 100 ml of washed cells of *S. paucimobilis* grown on α-santonin or freeze thawed cells or freeze thawed cells resuspended in shock fluid and 200 μl of substrate. Percent oxygen uptake of cells per minute was calculated.

2.5.8 Transformation of α-Santonin

a) Preparation of extracts

S. paucimobilis strain S cells utilise α-santonin as a sole source of carbon and form various intermediary products (Sangodkar and Mavinkurve, 1982, 1984; Naik and Mavinkurve, 1987; Furtado *et al.*, 1988; Colaco *et al.*, 1993). Thin layer chromatography is used as a tool to identify transformation products of α-santonin. 5 ml of log phase cells adjusted to an absorbance 4.5 at 450 nm were incubated with 0.025 % w/v α-santonin for varying time intervals and extracted with distilled chloroform (1:3 ratio) thrice. The chloroform extracts were concentrated by evaporating chloroform and subjected to thin layer chromatography.

2.5.9 Thin Layer Chromatography

a) Preparation of plates

Plates are prepared with 40% solution of silica gel in water and dried in oven at 120° C for 30 min. The concentrated chloroform extracts are spotted on an activated silica gel plate with a fine capillary tube.

b) Developing solvent

Chromatograms are developed in a pre-saturated chamber with solvent system ethyl acetate : benzene (20 : 80) and visualised using a lactone spray reagent (App. 3.1). The sprayed plates were kept in a preheated oven at 100° C for 15 min. The transformation products appear as coloured spots. The colour of the compound is noted and R*f* values calculated.

2.5.10 Liposome Swelling Assay

Liposome swelling is the technique which determines the rate of change in the optical density of the liposome suspension upon dilution with 20 to 30 volumes of test solute of which the osmolarity is adjusted to that of the impermeant solute (Luckey and Nikaido, 1980a). The permeant solute enters the liposome and increases the intravesicular osmolarity and the consequent influx of water into the vesicles results in swelling of the liposome membranes. The rate of swelling is followed optically at 500 nm.

a) Preparation of liposomes

5.3 μmol of phosphotidyl choline isolated from egg yolk (App. 1) (Pangborn, 1980) dried as a thin film at the bottom of a tube and then suspended in 1.0 ml of aqueous solution of crude outermembrane proteins (40 μg) isolated from cells grown on glucose or α-santonin by the method of Filip *et al.* (1973). The sample in 5 ml screw

cap tube is sonicated for 15 seconds with a pulse of 30 seconds and the cycle is repeated 40 times until the total sonication time was 10 min., later it is dried in a evacuated dessicator for 2 h. The dried tube was finally resuspended by vortex mixing in 1.5 ml of 17 % (w/v) dextran (Himedia) containing 5 mM Tris HCl pH 7.5. The control vesicles are made similarly without the addition of the outermembrane fraction.

b) Assay

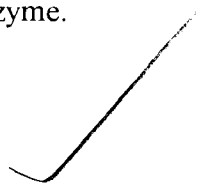
30 µl of liposomes prepared as described above was diluted with 0.63 ml of 0.04 M solution of sugar containing 5 mM Tris/HCl pH 7.5 in a 1 ml cuvette. The contents are mixed rapidly and optical density noted at 500 nm on a time scan mode for 5-8 min on a UV spectrophotometer. The sugars used for the assay were arabinose, ribose, glucose, galactosamine HCl, sucrose and x-gal and hydrocarbon α-santonin. It was not possible to prepare a 0.04 M α-santonin solution as the solubility of α-santonin is 166 µg/ml. Hence the crystalline unfiltered solution of α-santonin was used and the method was slightly modified. After mixing the contents, the crystals were allowed to settle down for 1 min and the change in absorbance noted for 8 min.

2.5.11 Fluorescence Spectroscopy

Periplasmic proteins were eluted by freezing and thawing *S. paucimobilis* strain S cells grown on α-santonin. 2 ml of shock fluid concentrated by ammonium sulphate precipitation containing 500 µg/ml was diluted with 0.05 M phosphate buffer pH 7.0 to make the volume to 3 ml, was checked for excitation and emission maximum on Hitachi Fluorescence Spectrophotometer. To see the effect of binding of the substrate on to the protein, 2 ml of concentrated shock fluid was diluted to 3 ml with different substrate concentrations of the substrate α-santonin (0.13, 0.26, 0.39, 0.52 and 0.65 µM) and checked for excitation and fluorescence intensity noted at emission wavelength (340 nm). The total volume of the reaction mixture was maintained to 3 ml.

2.5.12 Alkaline Phosphatase Assay

Phosphatase activity of shock fluid was measured by the method of Belsare *et al.* (1972). To 1 ml of shock fluid, 4 ml of 0.5 M Tris/HCl buffer pH 8.0 and 1 ml of 100 μM p-nitro phenyl phosphate were added and flasks were incubated on the shaker for 60 min in the dark at RT. Then 1 ml of 0.5 M calcium chloride and 4 ml of 0.5 M sodium hydroxide were added. The mixture was centrifuged at 4000 rpm for 15 min and the absorbance values of the yellow colour developed was read at 400 nm. Activity of the protein was expressed as μg of PNP/mg of enzyme.



CHAPTER 3.0

RESULTS

RESULTS

3.1 EFFECT OF GROWTH SUBSTRATE ON CELL AND CELL ENVELOPE COMPONENTS

Cell adapts to various environmental conditions such as temperature, pressure, nutritional stress, and salt concentration by changing its cell surface composition. In response to changes in growth conditions many organisms regulate their fatty acid, lipid and protein composition, which reflects on membrane fluidity that is necessary for the effective functioning of the biological membrane. The effect of temperature and pressure on fatty acid composition of bacteria is widely investigated (Kamimura *et al.*, 1993; Nordstrom, 1993). Effect of cations and anions on membrane composition has also been reported (Stanlotter *et al.*, 1979).

3.1.1 Effect of Growth Substrate on Uptake of α-Santonin

S. paucimobilis is capable of utilising glucose and α- santonin as a sole source of carbon. *S. paucimobilis* cells grown on 0.4% glucose and 0.4% santonin mineral medium show different pattern of uptake of α-santonin (Fig. 3.1). The cells grown on glucose or α-santonin showed similar initial uptake of 32.6 μg/ml/min of α-santonin at 3 min., but differed in the later uptake. α-santonin was depleted at a rate of 6 μg/ml/min by cells grown on α-santonin at 10 min., leading to complete depletion by 20 min. But cells grown on glucose showed effusion of α-santonin at a rate of 3 μg/ml/min for 10 min and with no further effusion.

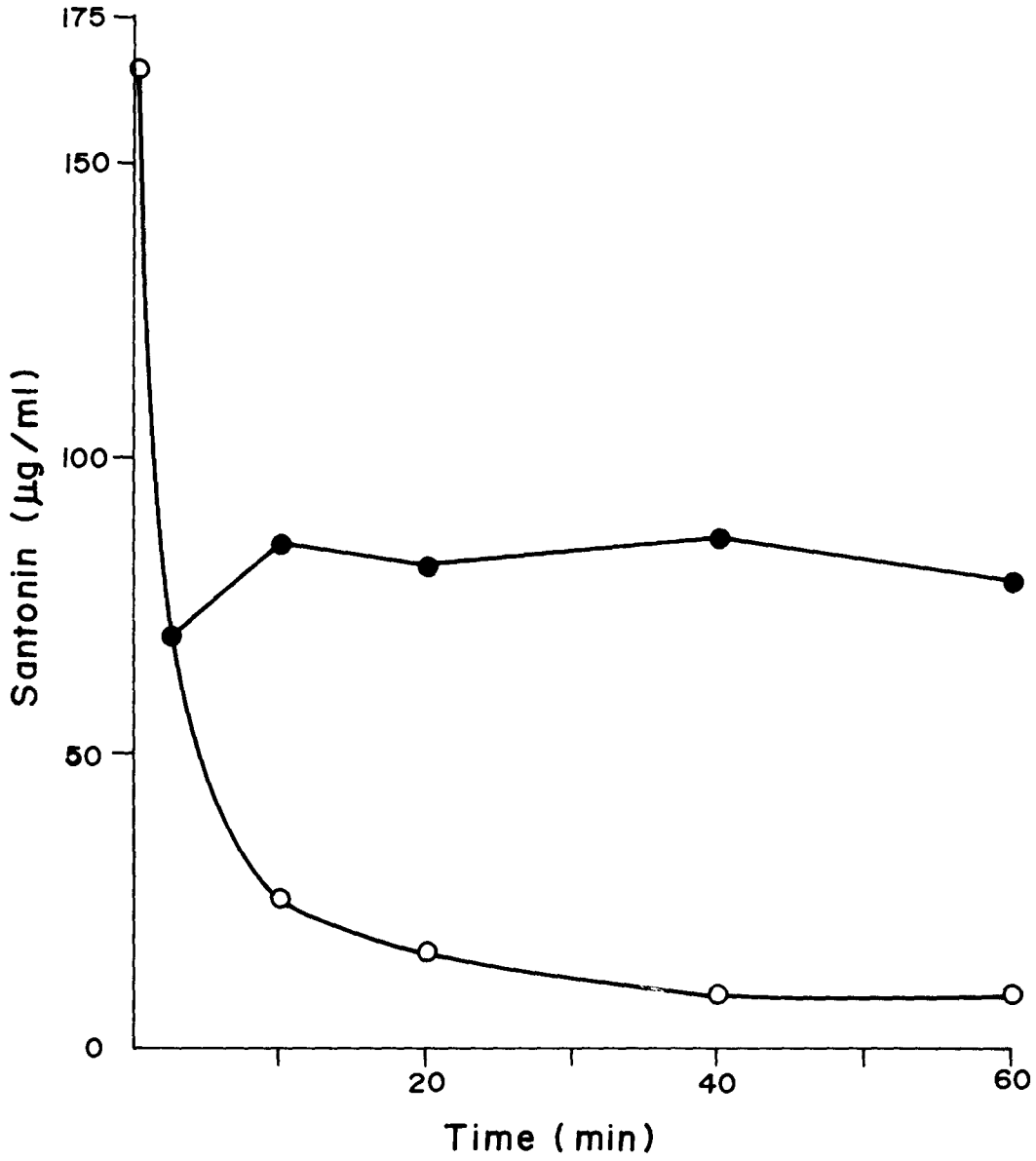


Fig. 3·1 Depletion of α-santonin by S. paucimobilis strain S cells grown on glucose (●) and santonin (○). Amount of santonin in the filtrate plotted.

3.1.2 Effect of Growth Substrate on Whole Cell Protein Profile

The SDS-PAGE profile of whole cell protein extracts, shows induction and repression of some proteins in presence of substrates (Fig. 3.2). Lane 2, which gives the profile of cells grown on rich medium (nutrient), shows the absence of 94, 80, 68, 64, 34 kDa and repression of 70 kDa. *S. paucimobilis* cells grown on glucose (lane 3) show a significant similarity to cells grown on 0.4% α-santonin (lane 4) except for a few dissimilarities like absence of 80, 68 kDa (indicated by arrows) and presence of more intense 46 kDa. Distinct band of 64 kDa in cells grown on glucose is significantly repressed in cells grown on α-santonin. The 80, 68, 46 kDa proteins in lane 4 specifically appeared in presence of α-santonin. The densitographs of the PAGE profile (Fig. 3.3) confirm the above visual observations as indicated by arrows.

3.1.3 Effect of Growth Substrate on Outermembrane Protein Profile

The SDS-PAGE profile of outermembrane proteins isolated from *S. paucimobilis* cells grown on glucose and α-santonin show majority of the proteins being common (Fig.3.4), except for α-santonin inducible 68 kDa and α-santonin repressible 64 kDa protein. The similar induction and repression of these proteins were seen in whole cell protein profile (Fig. 3.2).

3.1.4 Effect of Growth Substrate on LPS

S. paucimobilis cells grown on glucose, α-santonin and nutrient show a rough type of LPS on SDS-PAGE due to the absence of O-specific side chains (Fig. 3.5) in comparison to *P. aeruginosa.* The major differences noticed are in the low molecular mass region. In lane 1, the LPS of *S. paucimobilis* cells grown on α-santonin show the induction of the first component (core region) and the absence of the second component (lipid A) indicated by arrows (Fig. 3.5). The cells grown on glucose (lane 2) show the presence of both the components (lipid A and core). The absence of the first component is clearly noticed in the cells grown on nutrient (lane 3).

FIG. 3.2 SDS-PAGE profile of whole cell proteins extracted from *S. paucimobilis* strain S cells grown on nutrient (lane 2), glucose (lane 3) and santonin (lane 4). Molecular weight markers are in lane 1. Arrows indicate the change in protein profile under the condition assayed.

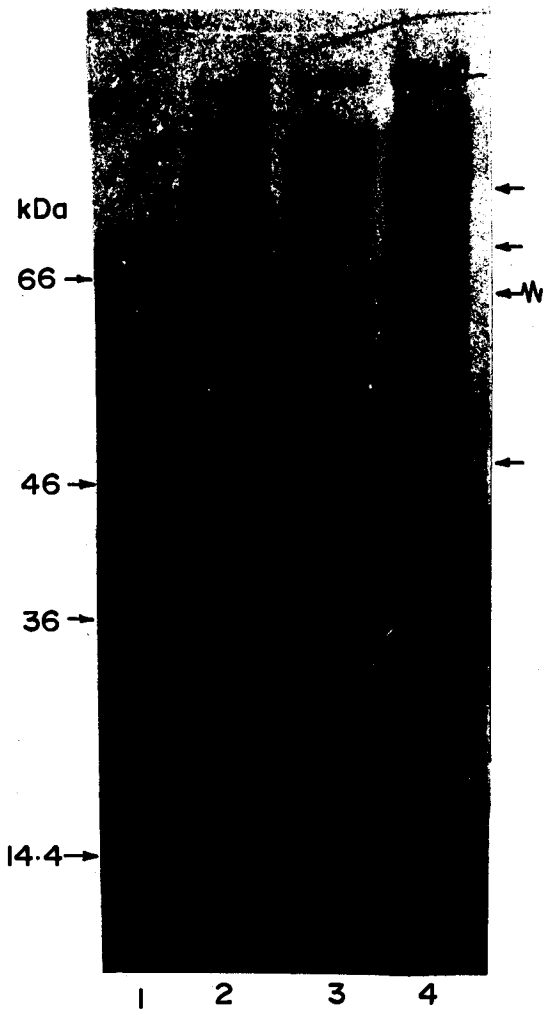


Fig. 3·2

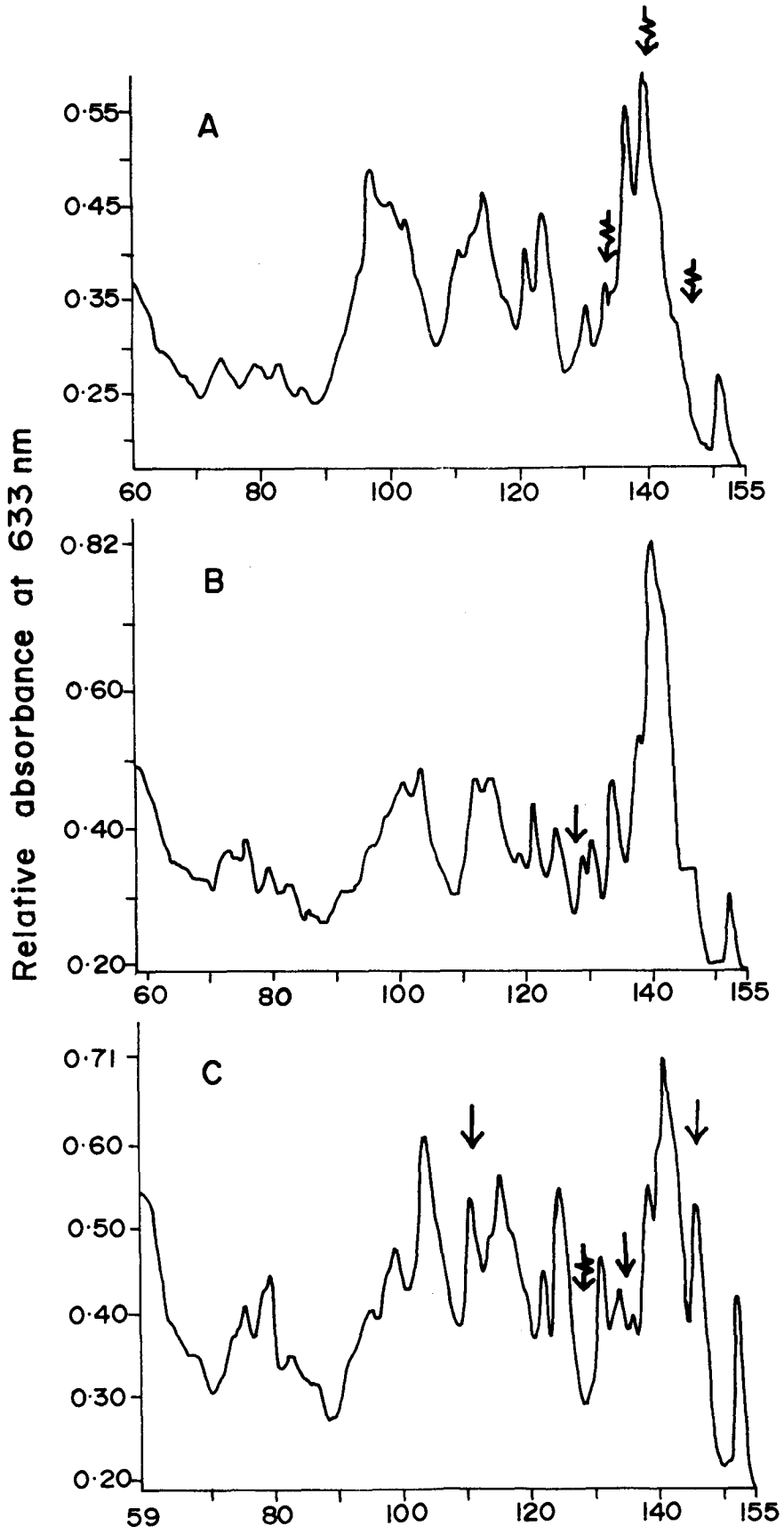


Fig.3·3 Scanning densitogram of SDS-PAGE profile of whole cell proteins extracted from S.paucimobilis cells grown on nutrient (A), glucose (B), and α-santonin (C).

↓-induction ↕-repression

FIG. 3.4 SDS-PAGE profile of outermembrane proteins isolated from cells grown on glucose (lane 1) and santonin (lane 2) ↑ indicates the induction of 68 kDa and ↟ repression of 64 kDa.

FIG. 3.5 SDS-PAGE profile of lipopolysaccharide isolated from *S. paucimobilis* cells grown on santonin (lane 1), glucose (lane 2), nutrient (lane 3) and LPS of *P. aeruginosa* (lane 4). Arrows indicate the changes.

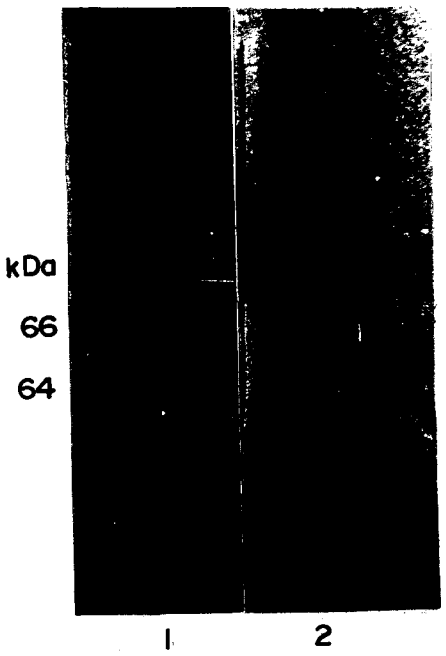


Fig. 3·4

Fig. 3·5

3.2 CHARACTERISATION OF OUTERMEMBRANE PROTEINS OF *S. paucimobilis*

As described earlier in section 3.1.3 the culture conditions influenced the outermembrane protein profile and there was an appearance of 68 kDa protein in presence of α-santonin with a simultaneous repression of 64 kDa protein. The study carried out to characterise the 68 kDa protein is described below.

3.2.1 Substrate Specific Induction

Outermembrane proteins were isolated from *S. paucimobilis* cells grown on glucose incubated with the 0.025% substrate α-santonin for varying time intervals (0, 5, 30, 60 min and 24 h). SDS-PAGE profile of OMP isolated is given in Fig. 3.6. Glucose grown cells in lane a, do not show the presence of 68 kDa protein, where as the same cells incubated with santonin lane b-f show the presence of 68 kDa with increasing intensity as incubation time progresses, finally corresponding similar to the cells grown on α-santonin (Lane g). Densitographs of the same shows quantitative induction and repression indicated by arrows in Fig. 3.7 of protein expression and incubation time.

3.2.2 Pore Formation

In several Gram-negative bacteria that have been investigated, the outer membrane contains major proteins called porins which form channels in planar lipid bilayers (Benz *et al.*, 1985, 1986; Hancock and Carey, 1980; Hancock *et al.*, 1979, 1982; Yashimoro *et al.*, 1983; Vachon *et al.*, 1986). Functional role of 68 kDa protein as a santonin specific porin pore was studied.

FIG. 3.6 SDS-PAGE profile of outermembrane proteins isolated from *S. paucimobilis* cells grown on glucose (lane a), and cells grown on glucose incubated with α-santonin for varied time intervals 0, 5, 30, 60 min and 24 h (lanes b-f), and cells grown on santonin (lane g)↑indicates the induction of 68 kDa in cells grown on α-santonin and↑indicates the repression of 64 kDa in S cells grown on α-santonin.

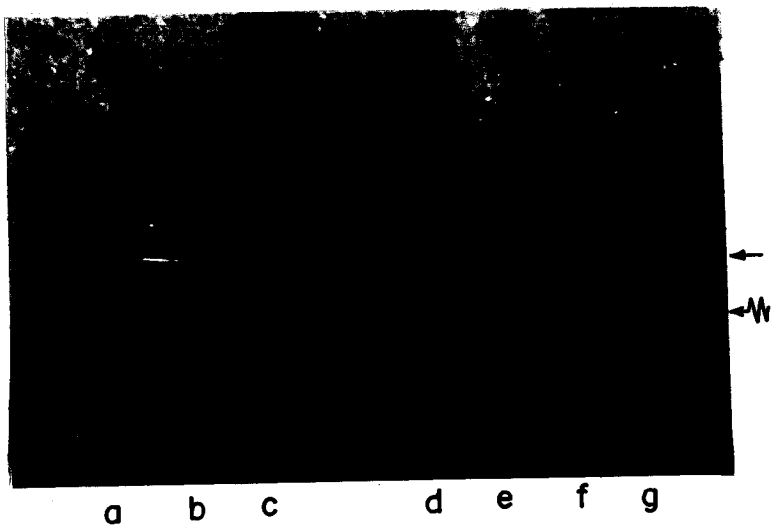


Fig. 3·6

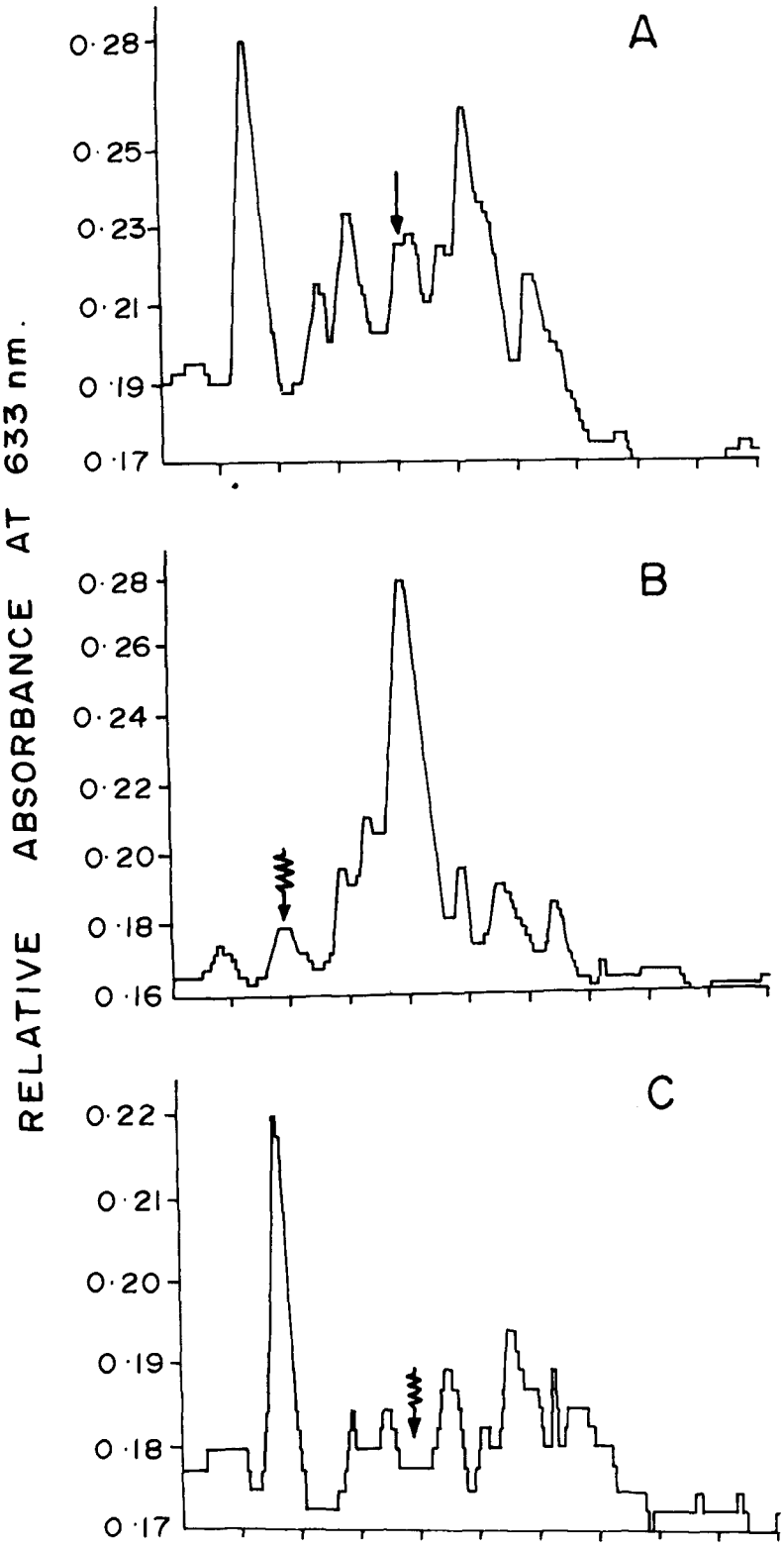


Fig. 3·7 Densitograph of SDS-PAGE profile of outermembrane proteins extracted from S. paucimobilis strain S cells grown on glucose incubated with α-santonin for 30 min (A), cells grown on santonin (B), glucose (C).

↓ - induction of 68 kDa protein in (A)

↯ - repression of 64 kDa protein in (B) and 68 kDa in (C)

3.2.2.1 Solute selectivity

The pore function of the OMP was studied by liposome swelling assay. Liposome membranes were reconstituted with phosphotidyl choline and outermembrane proteins isolated from *S. paucimobilis* strain S cells grown on α-santonin and glucose. The diffusion of solutes of molecular weights ranging from 90-360 Da into the liposomes was read as function of change in the optical density of the liposomes . The concentration of 0.04 M required for the assay was very difficult to be obtained with α-santonin because of its poor solubility (166 μg/ml). The problem was sorted out by adding extra crystalline α-santonin in the photometric cuvette, which was allowed to settle down before the start of recording. This was standardised and it took one minute for the crystals to settle down. From the results obtained outer membrane proteins containing (68 kDa) functioned as a porin pore for α-santonin and was not permeable to other solutes like glucose, sucrose, arabinose and rhamnose (Fig. 3.8 A). OMP isolated from glucose on reconstitution showed permeability to the substrates like glucose, arabinose and failed to show any distinct swelling with α-santonin as seen in Fig. 3.8 B. Effect of substrate concentration on rate of swelling was studied by diluting liposomes with substrate concentrations ranging from 0.01 M to 0.08 M..The results indicated that the rate of swelling increased with the increase in substrate concentration (Fig. 3.9).

3.2.3 Fluorescence Spectroscopy of OMP

The protein ligand interaction was monitored on a fluorescence spectrophotometer. The protein has excitation and emission wave lengths of 280 nm and 340 nm. The protein incubated with the substrate although showed same excitation and emission wave lengths there was a quench in fluorescence. The substrate α-

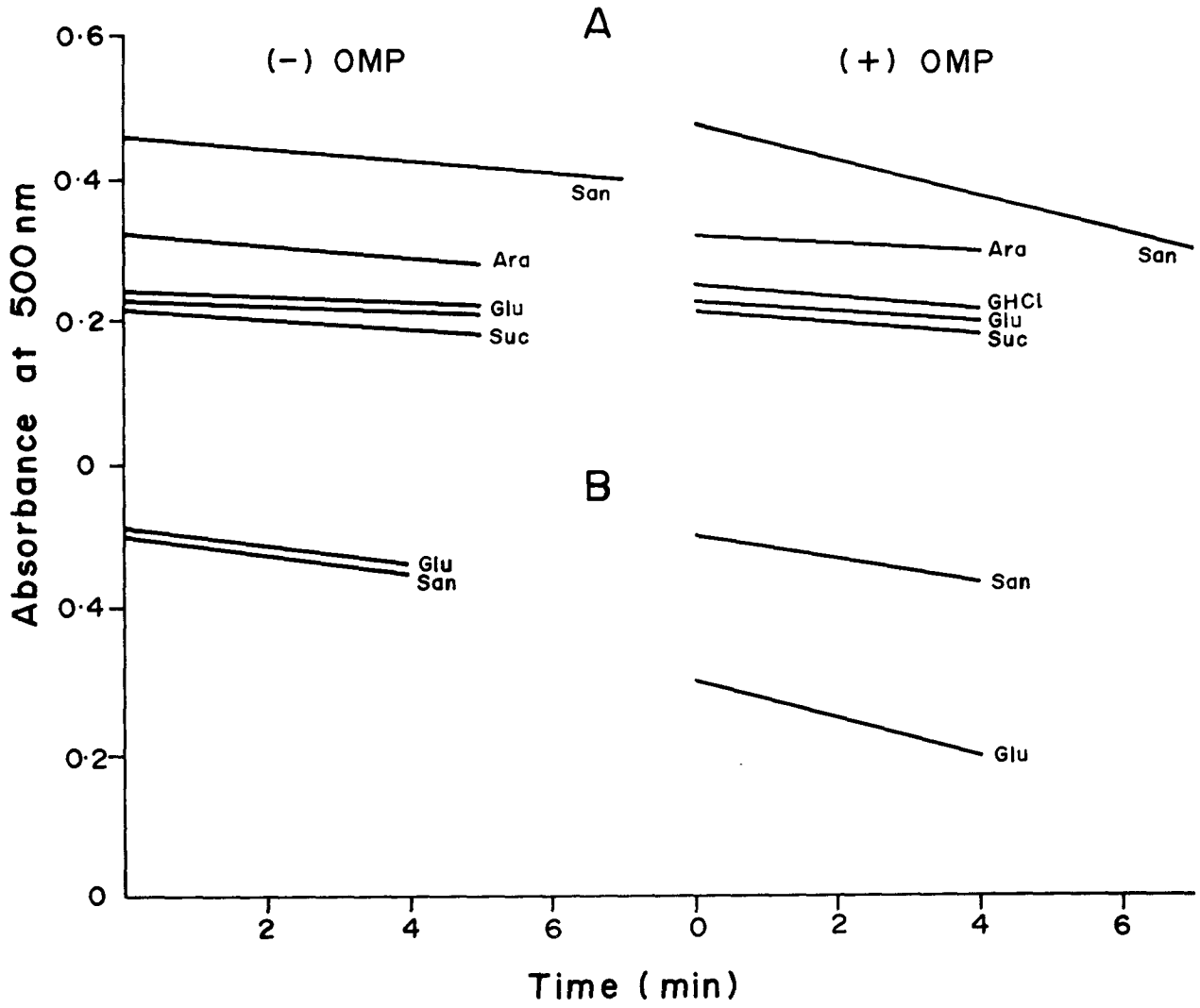


Fig. 3·8 Liposome swelling assay with outermembrane proteins isolated from S. paucimobilis cells grown on Santonin (A), Glucose (B). Liposomes diluted with 0·04 M saccharide solutions viz., Glucose (Glu), Sucrose (Suc), Glucosamine HCL (GHCL), Arabinose (Ara) and Lactone, α-Santonin (San)

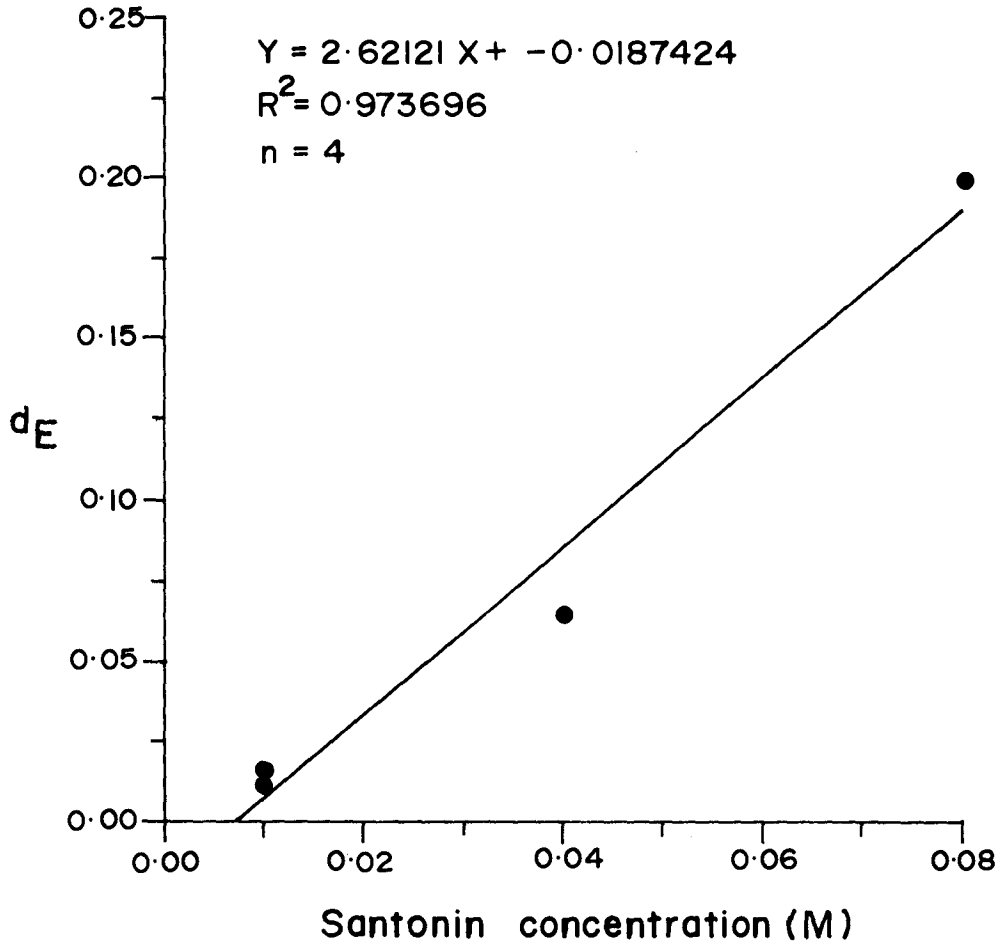


Fig. 3·9 Effect of substrate concentration on the rate of liposome swelling. As the substrate concentration increases, there is linear increase in decrease of absorbance (d_E) for 5 min.

santonin does not show excitation or emission at these wavelengths. The outermembrane proteins incubated with increasing concentrations of substrate (0.66 - 12.7 x 10^{-4} M) shows corresponding linear increase in fluorescence quench (results tabulated in Table 3.1). Fig 3.10 gives the linear regression curve.

3.2.3.1 Binding constant

Quench as a function of substrate concentration was analysed in terms of binding of the substrate by protein using established procedures (Appu Rao and Cann, 1981; Lehrer and Fasman, 1966). It is assured that binding of each substrate molecule to the protein causes the same degree of quench and the binding is statistical. A double reciprocal plot was drawn against 1/Q vs 1/C and the Q_{max} is calculated by extrapolating the line to 1/C = 0 as seen in Fig. 3.11. The intrinsic drug binding constant K was calculated from the mass action plots $\beta/1-\beta$ vs C_f. The slope of the curve gives K = 2.5 μM in Fig. 3.12.

3.2.4 Sensitivity to Heat and Reducing Conditions

Outer membrane proteins are previously described as heat modifiable on SDS-PAGE, and the structures of major outer membrane proteins of several bacteria are altered by heat and reducing agents (Hindahl and Iglewski, 1984; Newhall and Jones,1983). The effect of heat and 2-mercaptoethanol treatment on the migration of OMP in SDS-PAGE is given in Fig. 3.13. At RT with or without mercaptoethanol the 68 kDa migrates as one single band. At 60° C the 68 kDa protein dissociates into a very closely associated bands. At 100° C besides dissociation of 68 kDa, a new band is seen at 80 kDa. At 60° C and 100° C with mercaptoethanol 68 kDa is partially broken, and the band at a higher molecular mass of 80 kDa is distinct. The protein was found sensitive to trypsin digestion as the band corresponding to 68 kDa is missing (Fig. 3.13, lane g).

TABLE 3.1 Fluorescence studies of outermembrane proteins

S.No.	Intensity	$1/Cx10^4$	1/Q	β	β/(1-β)	C_f
1	625	1.50	0.093	0.107	0.119	6.6
2	543	0.76	0.044	0.221	0.283	13.0
3	504	0.50	0.035	0.280	0.388	20.0
4	477	0.38	0.031	0.318	0.466	26.0
5	416	0.30	0.024	0.405	0.686	33.0
6	293	0.15	0.017	0.581	1.386	66.0
7	146	0.07	0.012	0.791	3.780	127.0

$\beta = Q/Q_{max}$,
$C_f = C_o - \beta X C_e$,
C_o = Molar constituent concentration of the substrate,
C_e = Molar constituent concentration of the protein

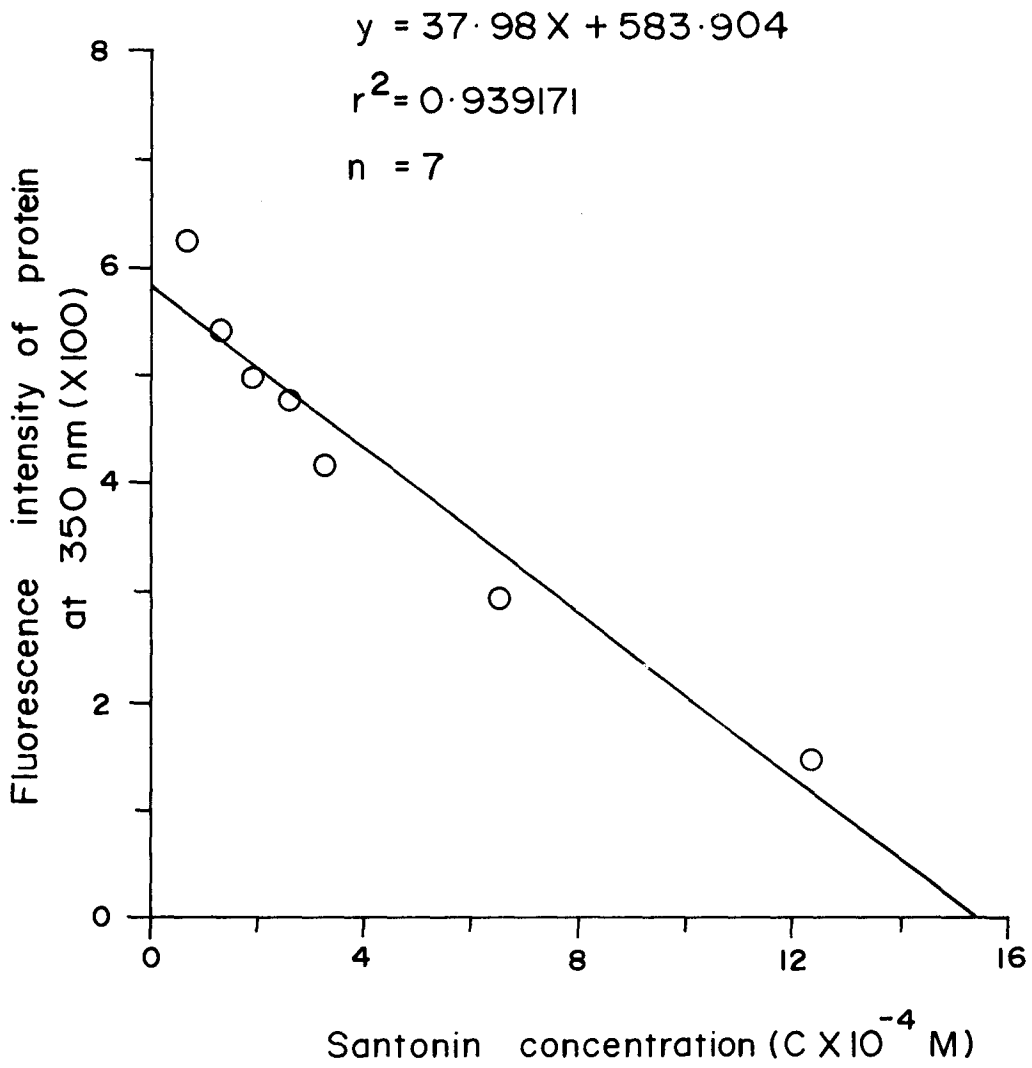


Fig. 3·10. Linear regression curve of substrate concentration vs. quench in fluorescence of outermembrane proteins.

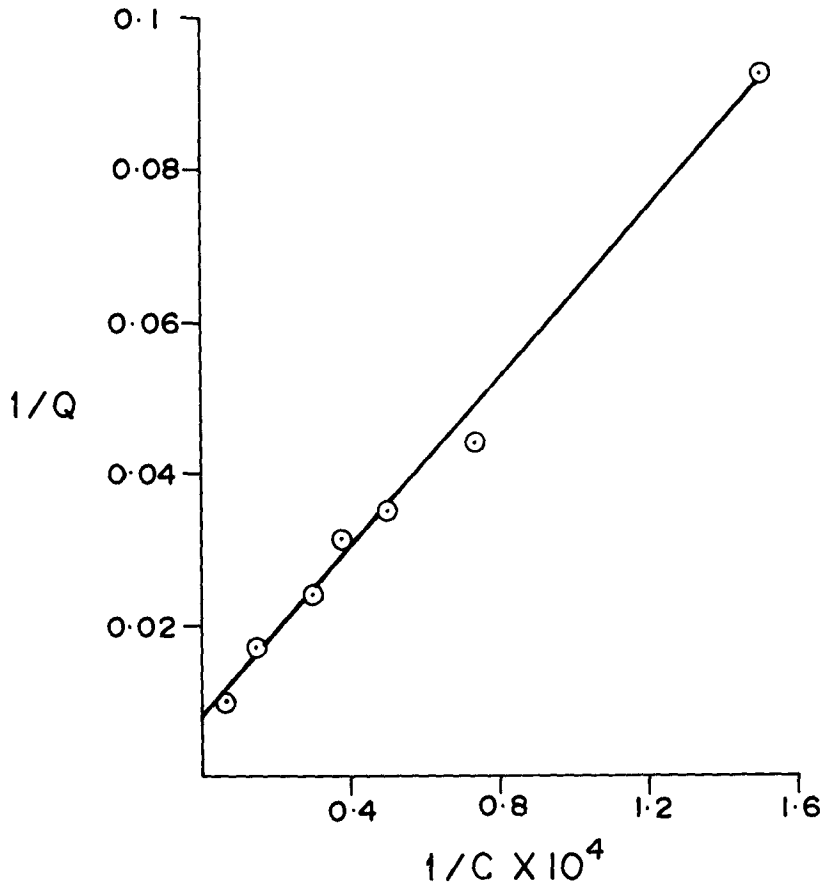


Fig. 3·11 Double reciprocal plot of 1/Q vs 1/C. Intercept on y axis gives the Q_{max}.

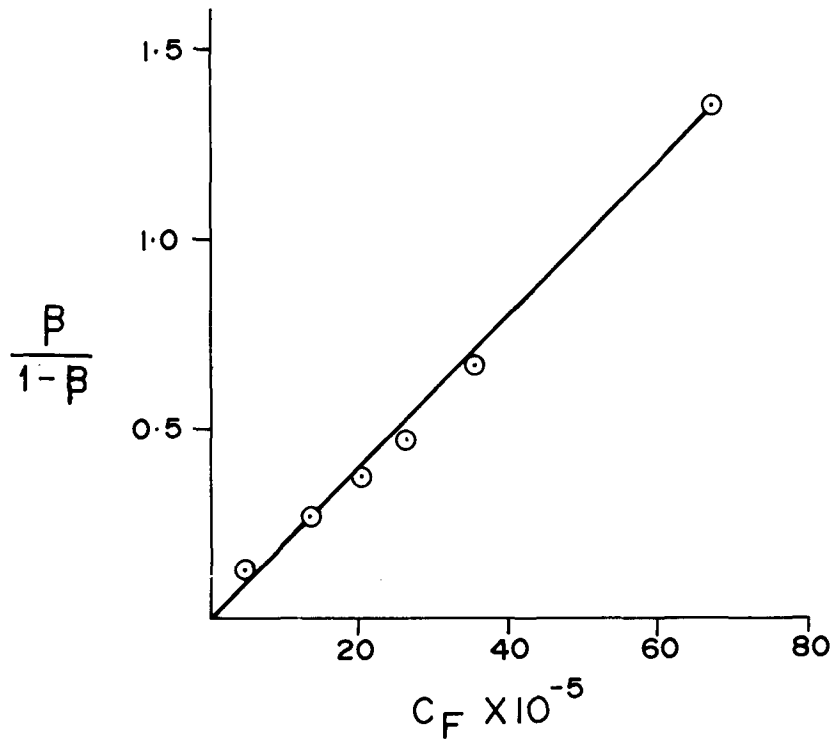


Fig. 3·12 Mass-action plots of β/1-β against C_f for determination of drug binding constant K, being given by the slope of the plot.

FIG. 3.13 SDS-PAGE profile of outermembrane proteins isolated from *S. paucimobilis* cells grown on α-santonin, treated with mercaptoethanol (lanes a-c), and without mercaptoethanol (lanes d-f) at RT (lanes a and d), at 60^0 C (lanes b and e), at 100^0 C (lanes c and f). Treated with trypsin (lane g).

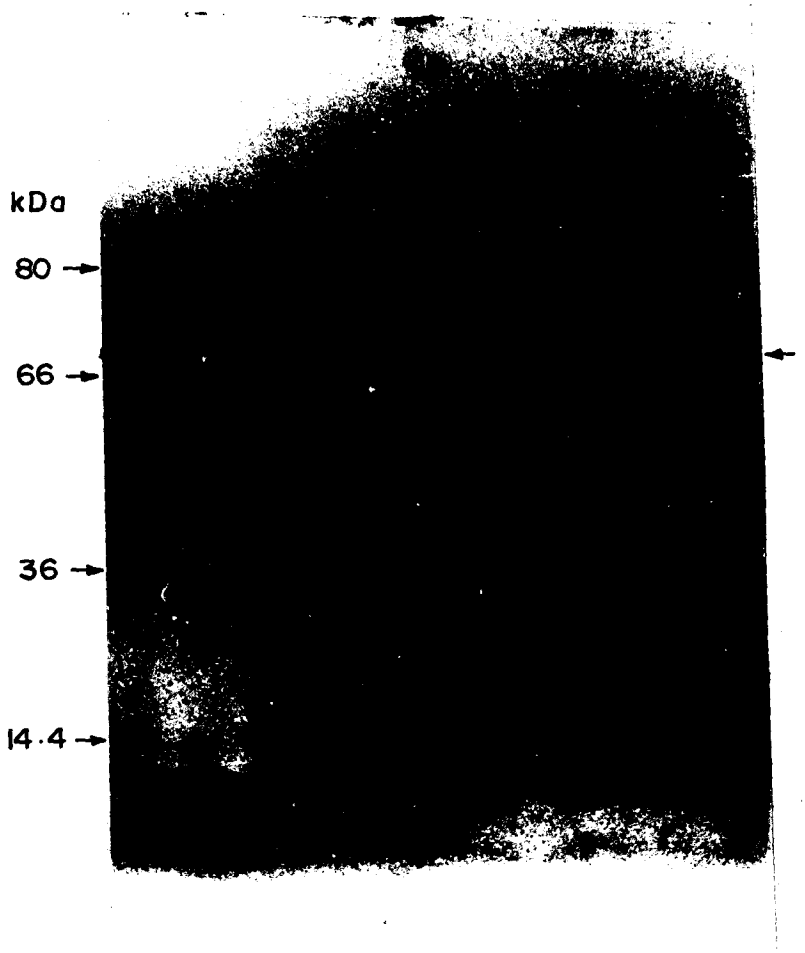


Fig. 3·13

3.3 RELEASE OF PERIPLASMIC PROTEINS FROM CELL ENVELOPE OF *S. paucimobilis*

Periplasmic proteins were extracted from *S. paucimobilis* cells by three methods *viz.*, cold osmotic shock (COSM) by Neu and Heppel (1965), chloroform extraction (CHLM) by Ames *et al.* (1984) and multiple cycles of freezing and thawing (FTM) by Paoletti *et al.* (1987). The shock fluid obtained by each of the above methods was filtered through 0.45 μm millipore filter and analysed for protein content (Lowry *et al.*, 1951), Alkaline phosphatase activity (Burns, 1978; Belsare *et al.*, 1992) and α-santonin binding activity by equilibrium dialysis binding assay (Stinson *et al.*, 1971; Shailaja Raj *et al.*, 1996). The work carried out to optimise the conditions required for maximum release of periplasmic proteins is described below.

3.3.1 Comparison of Methods on Release of Periplasmic Proteins

3.3.1.1 Protein assay

The three methods differed in the amount of protein released as given in Table 3.2. The freeze thaw method released the maximum amount of protein (110 μg) without any effect on the viability of the cells.

3.3.1.2 Alkaline phosphatase activity

Alkaline phosphatase because of its location, is often used to monitor the release of periplasmic proteins. The shock fluid obtained by freezing and thawing showed 10.5 μmoles/ml of supernatant as compared to COSM (5.0) and CHLM (1.3). But the specific activity of the enzyme in all the three methods remained the same as seen in Table 3.2.

TABLE 3.2 Analysis* of periplasmic proteins from *S. paucimobilis* cells released by three methods

		Method used		
		FTM	COSM	CHLM
A. Analysis of released proteins				
1	Total protein released (mg/g of wet cell weight)	8.0	3.80	0.89
2	Alkaline phosphatase[1] (μmole/ ml of supernatant)	10.5	5.00	1.30
3	Alkaline phosphatase[1] (μmole/ mg of protein)	26.2	26.30	29.20
4	α-santonin binding activity (μ mole santonin / mg of protein)	2.4	1.61	0.30
B. Transport activity of shocked cells				
1	Rate of uptake[2] (μg α-santonin/ml /min)	0.7	1.50	ND
2	% reduction in transport	90.0	70.00	ND

* = mean values of observations in triplicates
1 = expressed in terms of Paranitrophenol (PNP) released from PNP phosphate
2 = Rate of uptake of α-santonin by untreated wild type cells is 7.3 μg / min.

3.3.1.3 α-Santonin binding activity

Among the methods employed the maximum α-santonin binding activity was found in the shock fluid released from freeze thawing as given in Table 3.2.

3.3.1.4 Transport activity

The uptake of α-santonin is an energy dependent process involving a binding protein. The transport activity of the cells devoid of periplasmic proteins is used as a measure to check the total removal of periplasmic proteins. The uptake is reduced to 90% in FTM and 70% in COSM and rate of uptake being only 0.7 μg/ml/min as compared to 1.5 μg/ml/min in COSM as given in Table 3.2.

3.3.2 Effect of Physico-Chemical Parameters on Release of Periplasmic Proteins by Freeze Thaw Method

3.3.2.1 Cell density

Amount of periplasmic proteins released by freeze thaw method varied significantly with the increase in cell density as seen in Fig. 3.14 A. The cells suspended to an absorbance of 1.0, 4.5, 10 and 20 have shown a release of 75, 110, 130, 150 μg of protein/ml of shock fluid. The amount of protein released increased from the cell densities ranging from 2.6 to 12.5 mg/ml of wet cell weight corresponding to 1.0 and 4.5 absorbance and there after the increase is very marginal which is clearly noticed by the plateauing of the curve in Fig. 3.14 A.

3.3.2.2 Freeze thaw cycles

The cells were subjected to three freeze thaw cycles and the eluted protein varied according to number of cycles the cells were subjected. The cells subjected to first freeze thaw cycle released 75 μg of protein/ml, 110 μg/ml for two freeze thaw cycles and with no further increase with subsequent cycles as seen in Fig. 3.14 B. An over

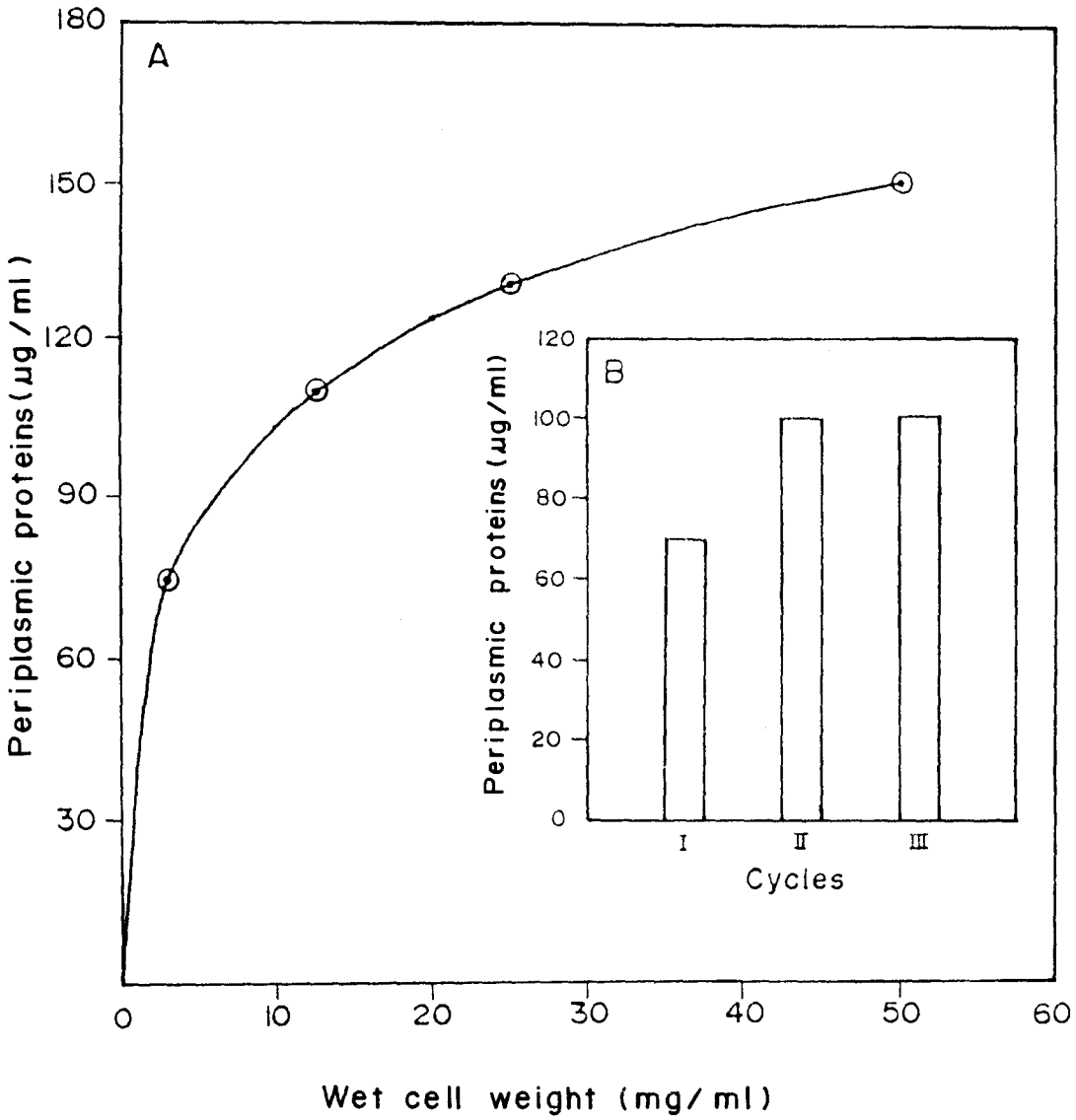


Fig. 3·14 Standardisation of Freeze thaw method.

A. Effect of cell concentration on release of periplasmic proteins.

B. The amount of proteins released after each cycle of freeze thawing from the cells suspended to an absorbance 4·5 at 450 nm

night frozen cells release 88 μg of protein /ml of shock fluid not shown in figure. The shock fluid of over night frozen cells showed 10 μmoles/ml of alkaline phosphatase activity and 280 μg/ml of α-santonin binding activity.

3.3.2.3 Temperature

The cells were frozen at temperatures -4° C, -20° C and -70° C to note the effect of freezing temperature on release of periplasmic proteins. The cells frozen at -4° C have released only 70 μg/ml and 110 μg/ml at -20° C with no further increase with increase in freezing temperature as seen in Fig. 3.15 C.

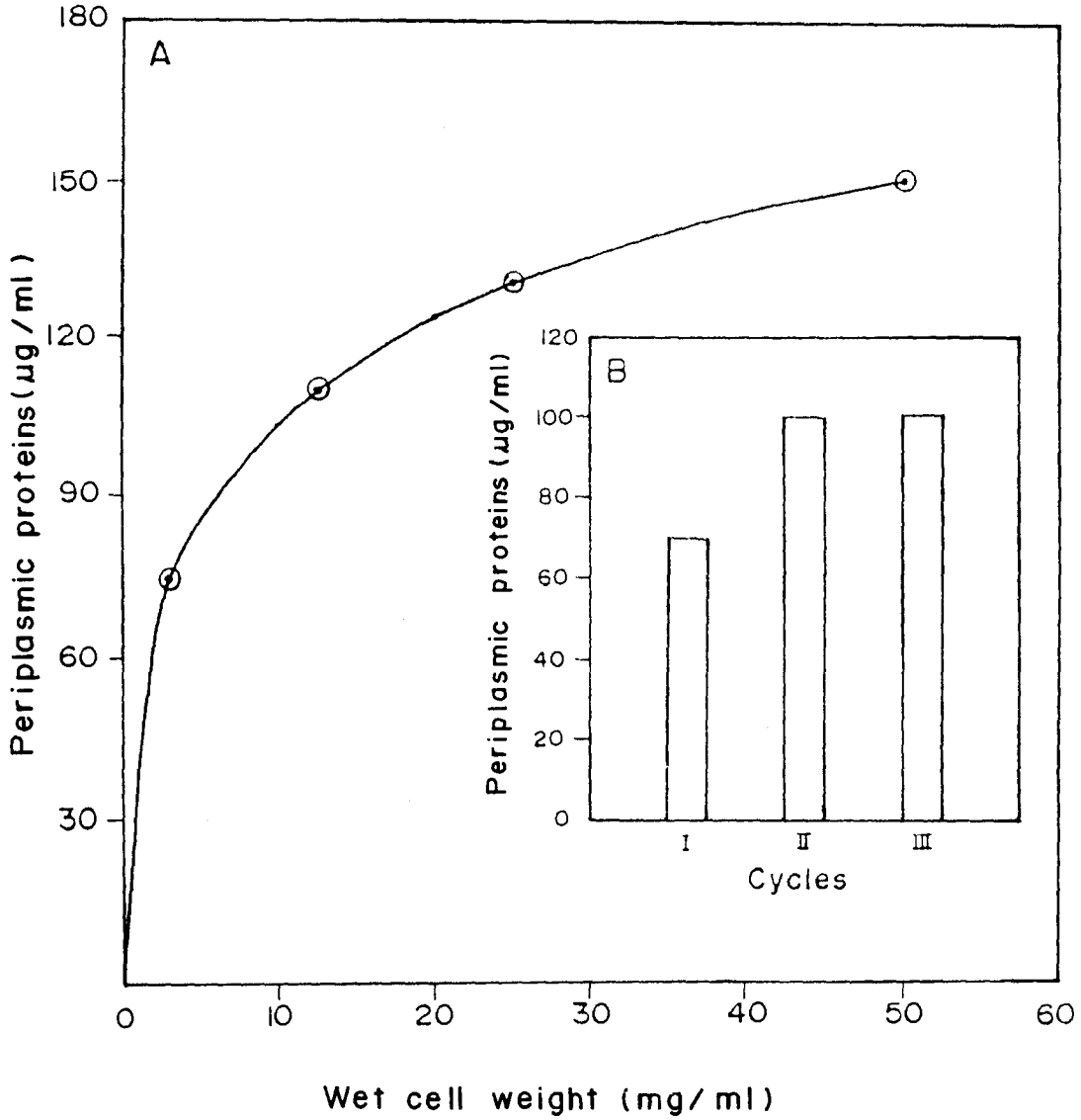
3.3.2.4 Buffer and pH

The cells suspended in phosphate buffer released 110 μg of protein/ml as compared to cells in Tris HCl buffer, 55 μg/ml as seen in Fig. 3.15 A. The variation and amount of proteins released was noticed. The cells suspended in acidic pH released only 45 μg of protein/ml as compared to alkaline pH seen in Fig. 3.15 B.

3.4 PERIPLASMICALLY LOCATED α-SANTONIN BINDING PROTEIN

3.4.1 SDS-PAGE of Periplasmic Proteins

Periplasmic proteins released from *S. paucimobilis* strain S cells by repeated freezing and thawing were subjected to SDS-PAGE. The SDS-PAGE profile of shocked cells (Fig. 3.16, lane 2) on comparison with the *S. paucimobilis* strain S cells grown α-santonin (Fig. 3.16, lane 1) show a diminished band at 46 kDa and several other minor proteins. Apparently, the same protein is preferentially released into the shock fluid which is a major band as indicated by arrows in Fig. 3.16, lane 3. Densitographs of SDS-PAGE profile of *S. paucimobilis* strain S cells (Fig. 3.17 A) the thawed cells (Fig. 3.17 B) and shock fluid (Fig. 3.17 C) also confirm the findings. The shock fluid was also checked for the presence of carbohydrate and lipid components which were found to be absent.



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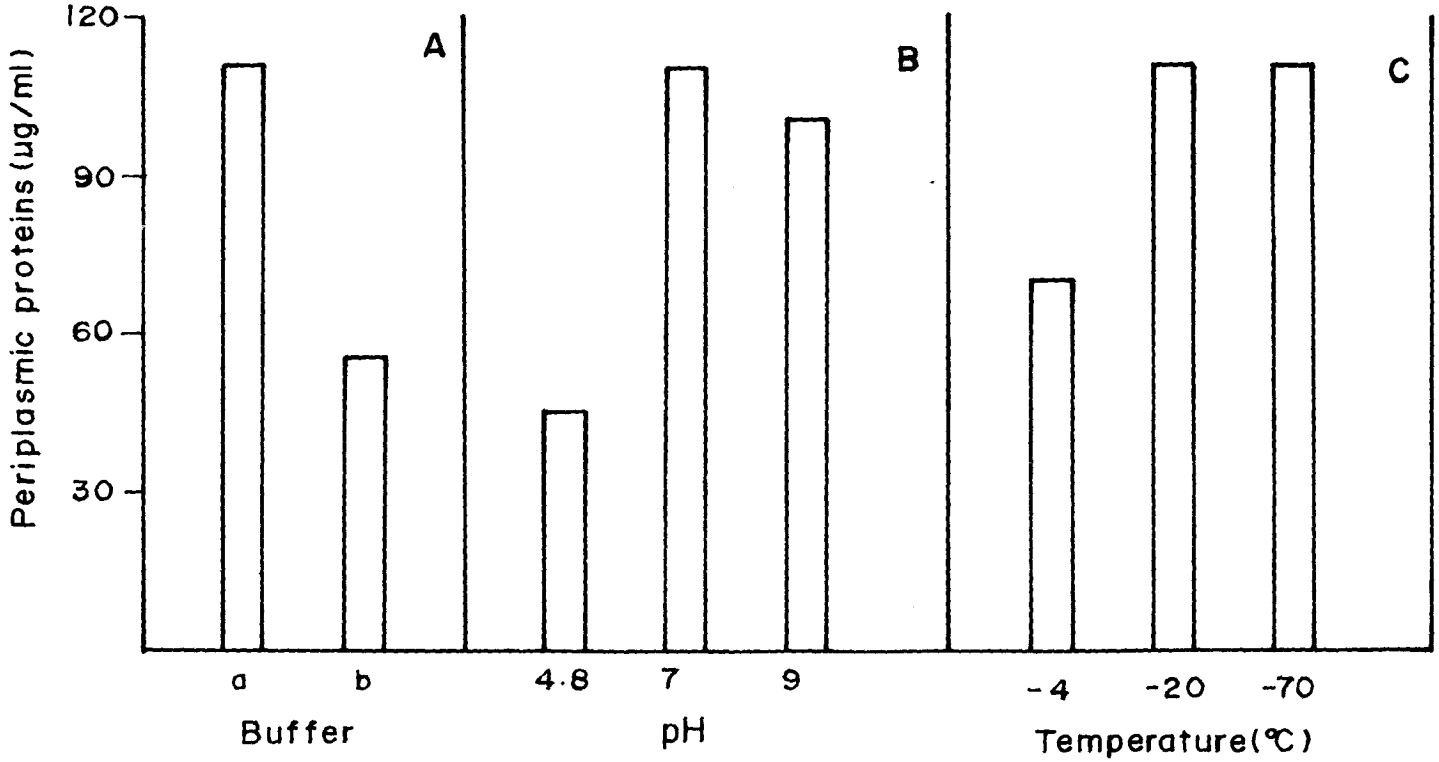

Fig. 3·15 Effect of A. Buffer : (a) phosphate, pH 7·0, 0·05 M, (b) Tris Hcl ; B. pH ; C. Temperature, on amount of periplasmic proteins released by freeze thaw method.

FIG. 3.16 SDS-PAGE profile of whole cell protein extracted from α-santonin grown cells (lane 1), freeze thawed cells (lane 2) and shock fluid (lane 3).

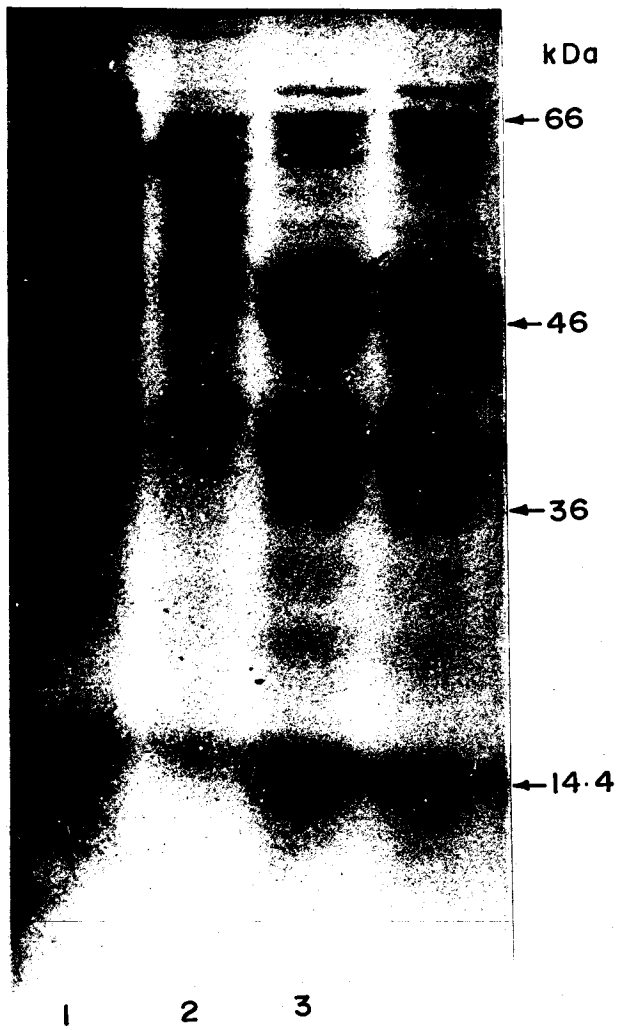

Fig. 3·16

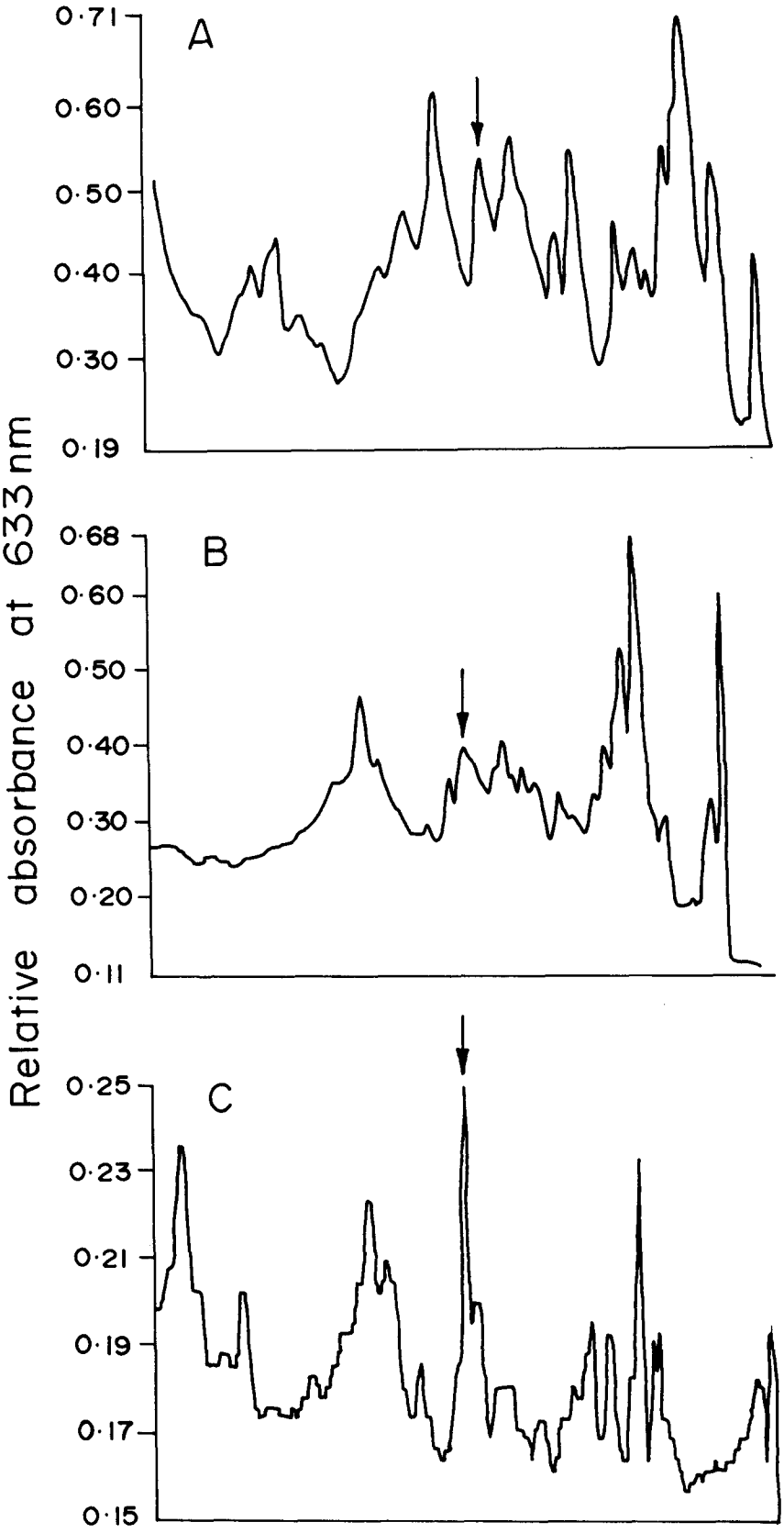

Fig. 3·17 Scanning densitogram of SDS-PAGE profile of whole cell proteins extracted from α-santonin grown S. paucimobilis (A), freeze thawed cells (B) and shock fluid (C).

3.4.2 α-Santonin Binding Activity of Shock Fluid

The shock fluid released by freeze thawing of *S. paucimobilis* strain S cells shows an affinity for α-santonin. The α-santonin binding capacity of the protein was tested by three methods *viz.*, equilibrium dialysis described earlier in section 3, filtration and fluorescence studies.

3.4.2.1 Filtration assay

Filtered ammonium sulfate precipitates of shock fluid and shock fluid incubated with α-santonin were dissolved in 0.05 M phosphate buffer pH 7.0 and scanned for the presence of α-santonin. As seen in Fig. 3.18, the shock fluid not incubated with α-santonin used as a control does not show a peak at 242 nm which is the absorption maxima for α-santonin. Whereas the shock fluid incubated with α-santonin shows a peak at 242 nm. Phosphate buffer and BSA (1 mg/ml) used as controls on incubation with α-santonin did not show any peak at 242 nm.

3.4.2.1.1 *Enzyme assay*

The cell free extract of *S. paucimobilis* strain S cells show dehydrogenase activity which is monitored by decrease in absorbance at 340 nm using NADPH as a coenzyme (Naik and Mavinkurve, 1987). An assay procedure was developed using precipitated solution having bound α-santonin. This precipitated protein solution having bound α-santonin was used as a substrate, and when cell free extract and NADPH were provided, the activity of the enzyme when monitored for 7 min. at 340 nm showed a decrease in absorbance (Fig. 3.19).

3.4.2.2 Fluorescence spectroscopy

The protein ligand interaction was monitored on a fluorescence spectrophotometer. The protein has excitation and emission wave lengths of 280 nm and

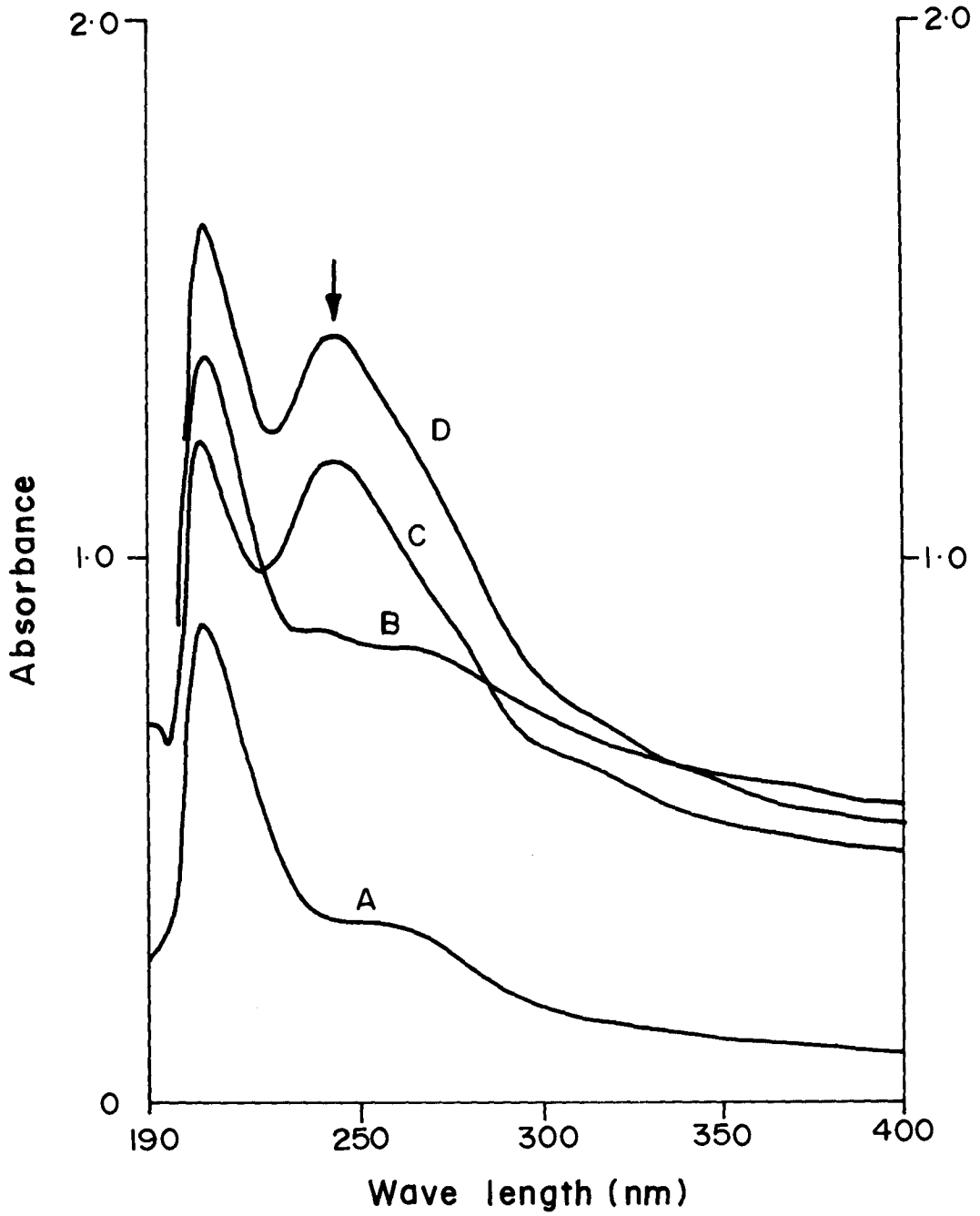

Fig. 3·18 Spectrophotometric scan of shock fluid, A; ammonium sulphate precipitated periplasmic proteins, B; proteins incubated with α-santonin isolated from cells grown on glucose, C; santonin, D. The peak at 242 nm. (↓) indicates the presence of bound α-santonin.

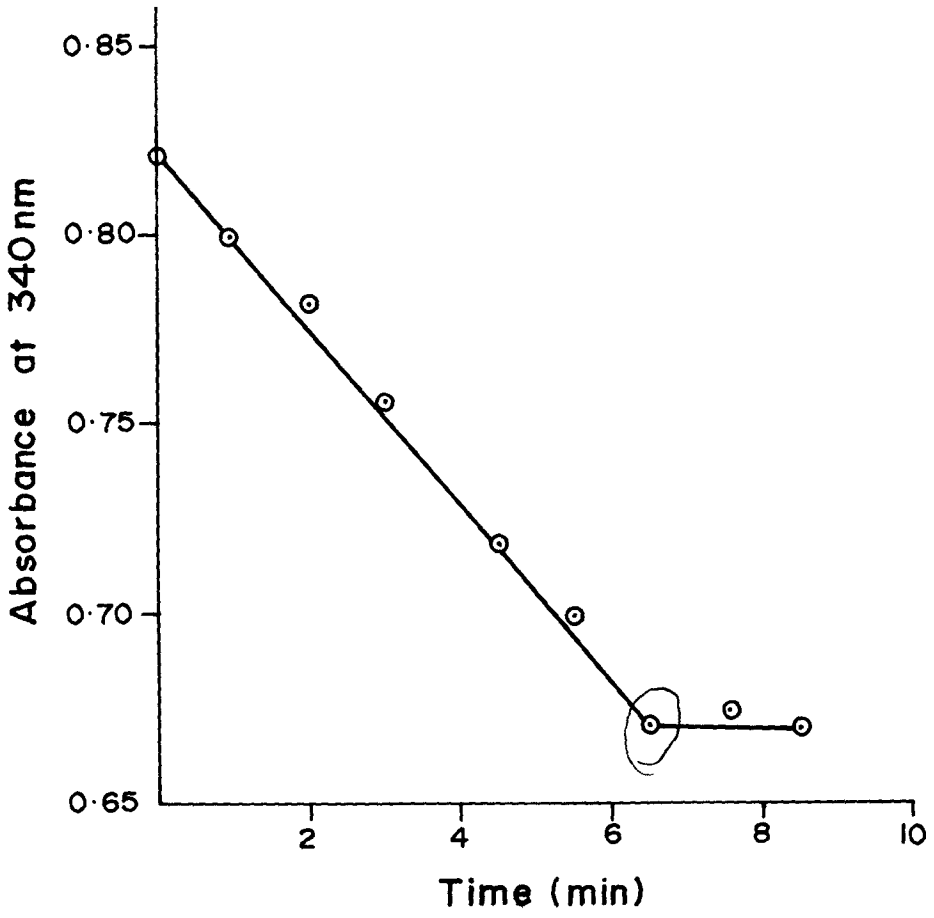

Fig. 3·19 Oxidation of NADPH due to reduction of α-santonin bound to periplasmic protein in presence of the santonin 1,2 reductase in the cell free extract.

340 nm. The protein incubated with the substrate although showed same excitation and emission wave lengths there was a quench in fluorescence. The substrate α-santonin does not show excitation or emission at these wavelengths.

3.4.2.2.1 *Effect of substrate concentration*

The shock fluid incubated with increasing concentrations of substrate (1.3 - 6.5 $x10^{-4}$ M) shows corresponding linear increase in fluorescence quench as given in Fig. 3.20 A and shows a linear regression in Fig. 3.20 B. Quench values obtained are tabulated in Table 3.3.

3.4.2.2.2 *Effect of temperature*

When the shock fluid is incubated with the substrate the decrease in fluorescence was noticed for 5-12 min. When effect of temperature was studied the refrigerated sample (16 - 20° C) shows the decrease in quench faster than the room temperature sample (Fig. 3.21). The refrigerated sample took 5 min. for the completion of reaction, where as the RT sample took 8-12 min.

3.4.2.2.3 *Binding constant*

Quench as a function of substrate concentration was analysed in terms of binding of the substrate by protein using established procedures (Appu Rao and Cann, 1981; Lehrer *et al.,* 1966). It is assumed that binding of each substrate molecule to the protein causes the same degree of quench and the binding is statistical. Quench values obtained by varying substrate (α-santonin) concentration are tabulated in Table 3.3. A double reciprocal plot was drawn against 1/Q vs 1/C and the Q_{max} is calculated by extrapolating the line to 1/C = 0 as seen in Fig 3.22. The intrinsic drug binding constant K was calculated from the mass action plots β/1-β vs C_f. The slope of the curve in Fig. 3.23, the drug binding constant obtained was K = 0.016 μM or 0.16 x 10^5 M^{-1}

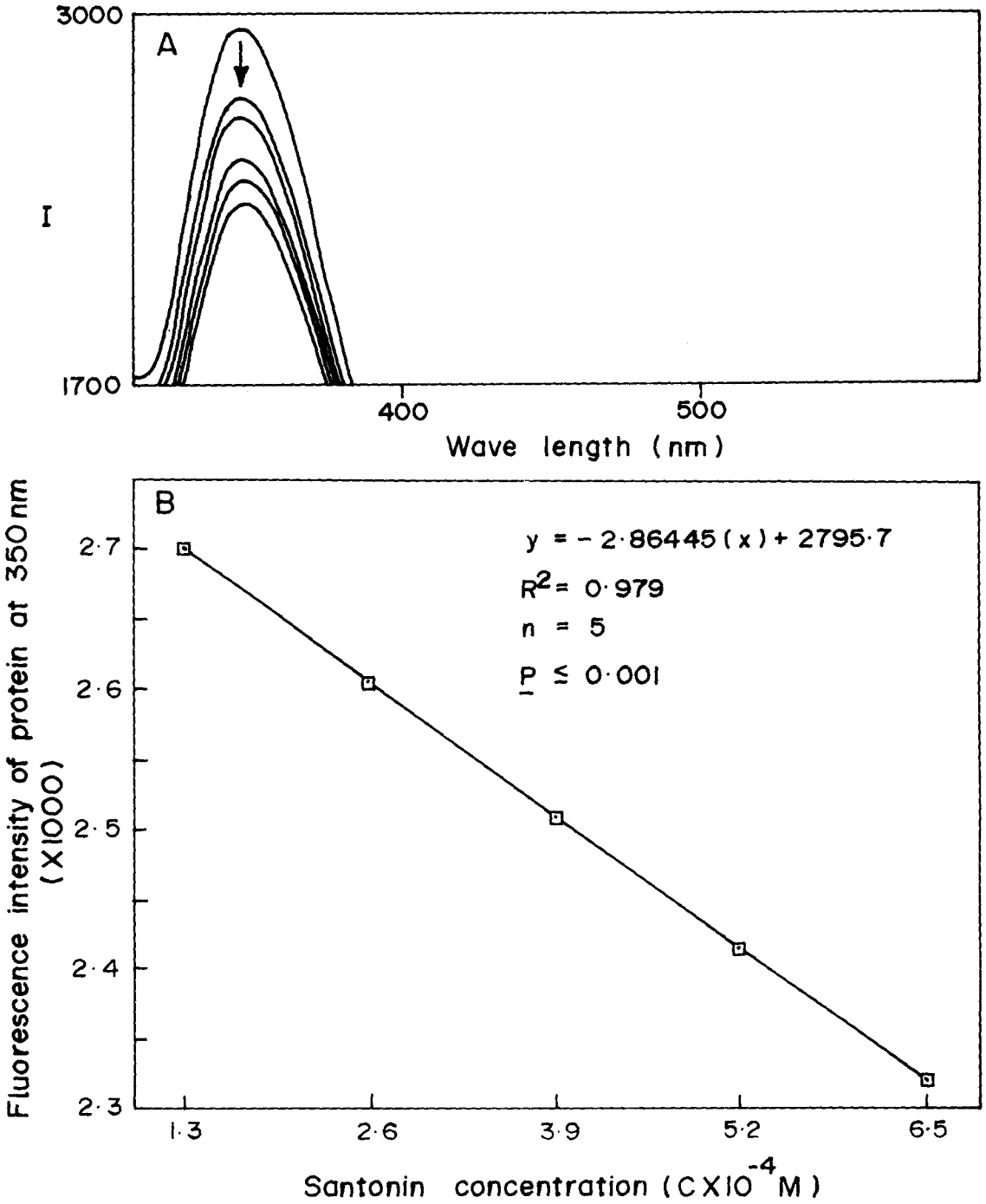

Fig. 3·20 A. Emission scan of shock fluid incubated with varying concentration of α-santonin (1·3-6·5 X10^{-4}M), arrow indicates quench in fluorescence.

B. Linear regression curve of substrate concentration vs. fluorescence intensity of periplasmic proteins.

TABLE 3.3 Fluorescence studies of periplasmic proteins

S.No.	Intensity	$1/C \times 10^4$	1/Q	β	β/(1-β)	C_f
1	2291	0.769	0.392	0.035	0.036	12.98
2	2130.	0.384	0.106	0.131	0.151	25.88
3	2031	0.256	0.073	0.190	0.235	38.82
4	1954	0.192	0.059	0.235	0.309	51.78
5	1866	0.153	0.048	0.288	0.405	64.74

$\beta = Q/Q_{max}$
$C_f = C_o - \beta \times C_e$
C_o = Molar constituent concentration of the substrate
C_e = Molar constituent concentration of the protein

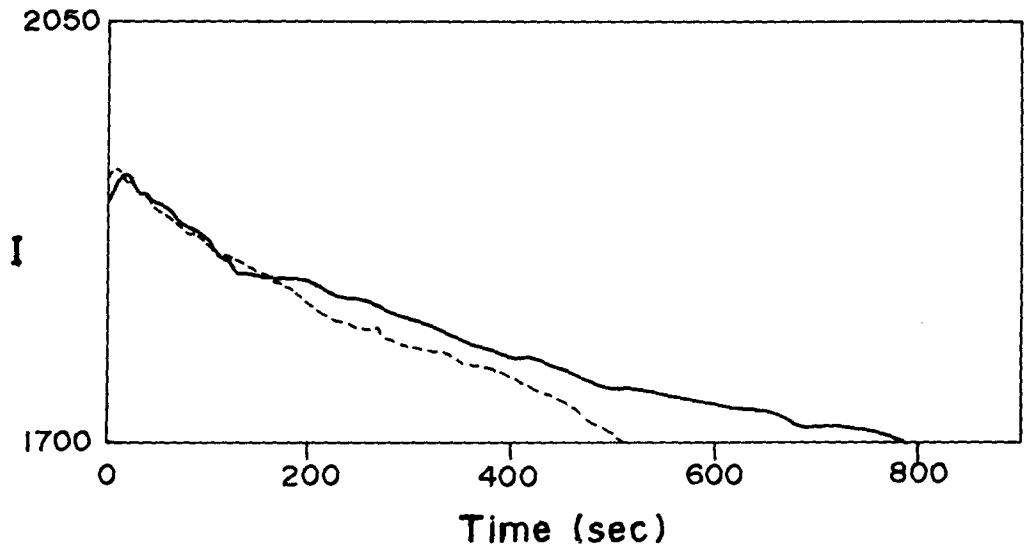

Fig. 3·21 Time scan of quench in fluorescence intensity of periplasmic proteins in presence of substrate α-santonin, at room temperature (—) and at 16°C (----)

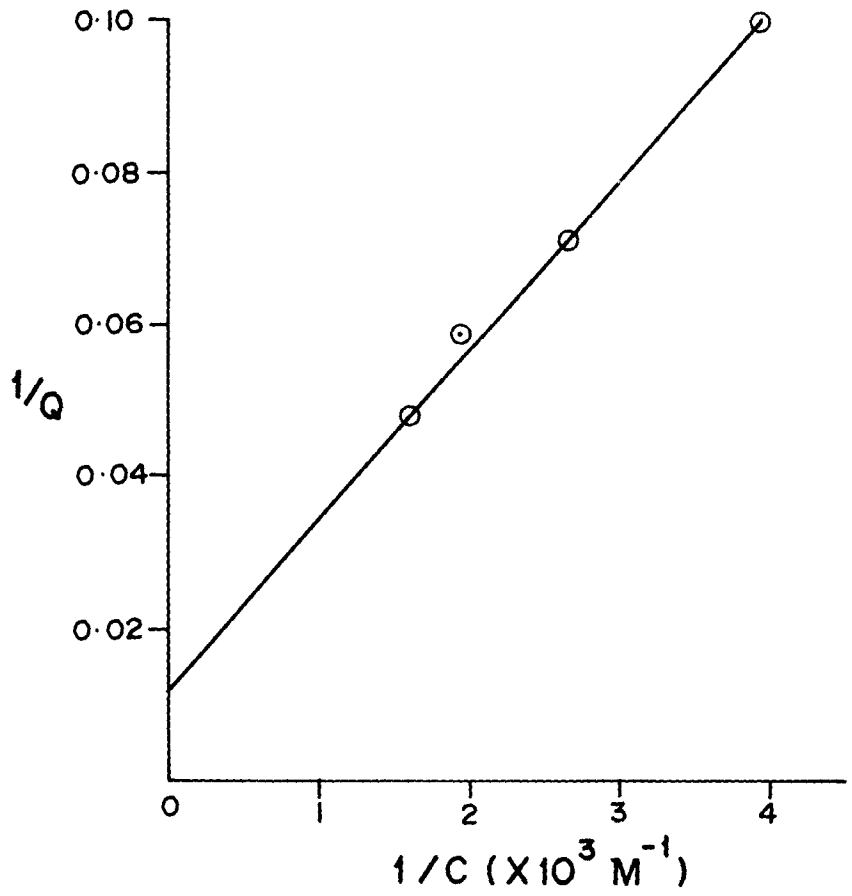

Fig. 3·22 Double reciprocal plot of 1/Q vs 1/C. Intercept on y axis gives the Q_{max}

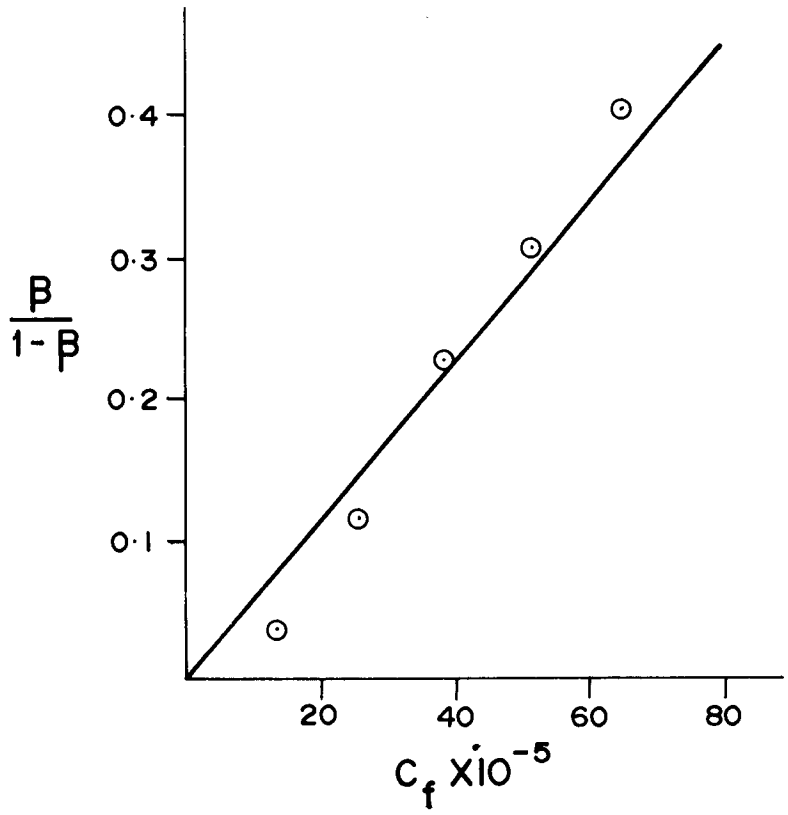

Fig. 3·23 Mass action plot of $\beta/1-\beta$ vs C_f. $\beta = Q/Q$ max, C_f = molar constituent concentration of free substrate. The slope of the curve gives intrinsic drug binding constant (K).

3.4.3 Dehydrogenase Activity of Shock Fluid

Shock fluid was assayed for α-santonin dehydrogenase activity in terms of oxidation of NADPH at 340 nm for 10 min. The decrease in absorbance was not noticed in the shock fluid extracted from *S. paucimobilis* cells grown on α-santonin or glucose. The cell free extracts of the same has given an enzyme activity of 0.22 units of decrease in absorbance/min/mg/ml of cell free extract.

3.4.4 α-Santonin Transforming Ability of Shock Fluid

In order to check whether it is a mere binding or there is any enzymic conversion of α-santonin into an other product, the shock fluid was incubated with α-santonin for varying time intervals. TLC of the chloroform extracts of the shock fluid incubated with α-santonin showed only the presence of α-santonin at R*f* 0.45 cm at 0 min, 1 h and 24 h.

3.4.5 Reconstitution

3.4.5.1 Transport of α-santonin

The pattern of uptake of α-santonin in *S. paucimobilis* strain S cells appears to be biphasic (Fig. 3.24). The initial uptake referred to as type 1 occurs in 2-3 min. of exposure of cells to substrate α-santonin with rate of uptake being 25 μg/ml/min. The later uptake called as type 2 occurring after 3 min. is slow, gradual and energy dependent. The rates of uptake for 15 min. and 30 min. incubation time are 9.1 and 5 μg/ml/min Surprisingly, the freeze thawed cells show similar type 1 uptake and impaired type 2 uptake (Fig. 3.24). The restoration of the impaired type 2 was seen in freeze thawed cells on reconstitution with the shock fluid (Fig. 3.24).

3.4.5.2 Transformation of α-santonin

S. paucimobilis strain S cells incubated with α-santonin form an array of transformation products (Sangodkar and Mavinkurve, 1982, 1984; Naik and

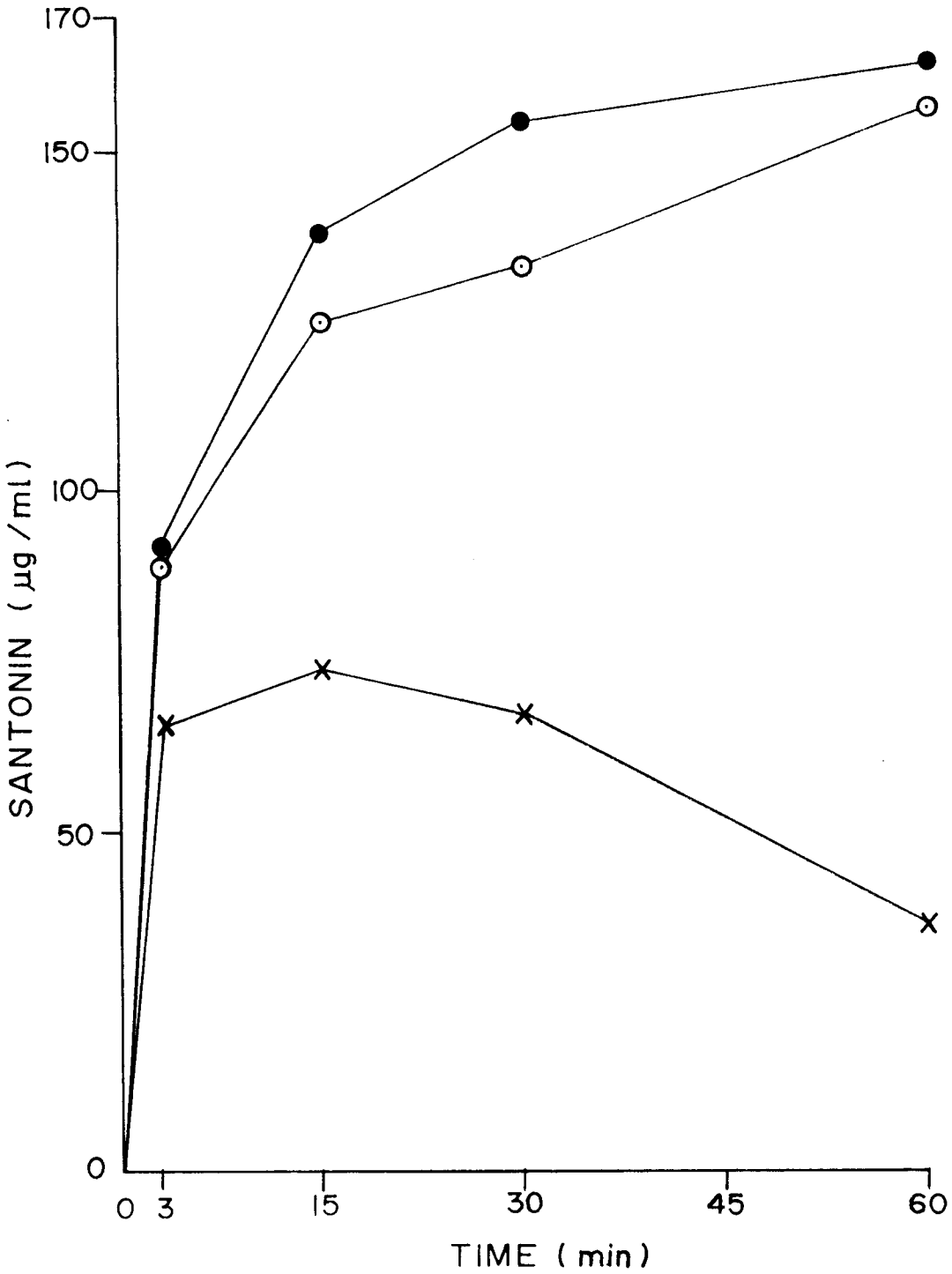

Fig. 3·24 Transport of α-santonin by S. paucimobilis cells. The amount of α-santonin taken up by the cells is estimated from the residual α-santonin in the filtrate. X – freeze thawed cells; ⊙ – freeze thawed cells suspended in shock fluid; ● – S cells.

Mavinkurve, 1987; Furtado *et al.*, 1988; Colaco *et al.*, 1993) which are detectable by TLC (Naik and Mavinkurve, 1987). The α-santonin transformation pattern of *S. paucimobilis* strain S cells show eight products with R*f* values ranging from 0.1 to 0.7 cm (Fig. 3.25). α-santonin (R*f* 0.45 cm) is used as a control. Freeze thawed cells show only one transformation product with R*f* 0.5 cm besides α-santonin. Whereas the reconstituted cells show 95% recovery of all transformation products except for one with R*f* value 0.7 cm. (Fig. 3.25).

3.4.6 Transport Related Conformational Change

Earlier results indicated a quench in fluorescence on incubating the shock fluid with the substrate α-santonin. Fluorescence changes of binding protein, upon binding with substrates, are observed and interpreted as indications of conformational change. A non denaturing gel electrophoresis was performed after incubating the shock fluid with α-santonin at 16° C for 10 min. Lane 1 in Fig. 3.26 shows the crude extract of the shock fluid, and lane 2 shows the profile of shock fluid incubated with substrate α-santonin. Shock fluid (lane 1) and shock fluid incubated with substrate (lane 2) although show the same profile, there is an extra protein band at third position from below in lane 2. The second band from below in lane 1 which is very intense is seen as a very faint band in lane 2.

3.4.7 Identification of α-Santonin Binding Protein

To identify the protein which moved at a different mobility in presence of substrate α-santonin, a preparative non denaturing gel electrophoresis was performed and the protein which moved at a lower mobility was eluted from the gel in Hoefer electroeluter and was checked for its molecular weight on a SDS-PAGE. The lane 2 shows the purified protein with molecular weight of 46 kDa and two minor bands indicated by arrows (Fig. 3.27). Crude shock fluid also shows the corresponding protein in lane 1.

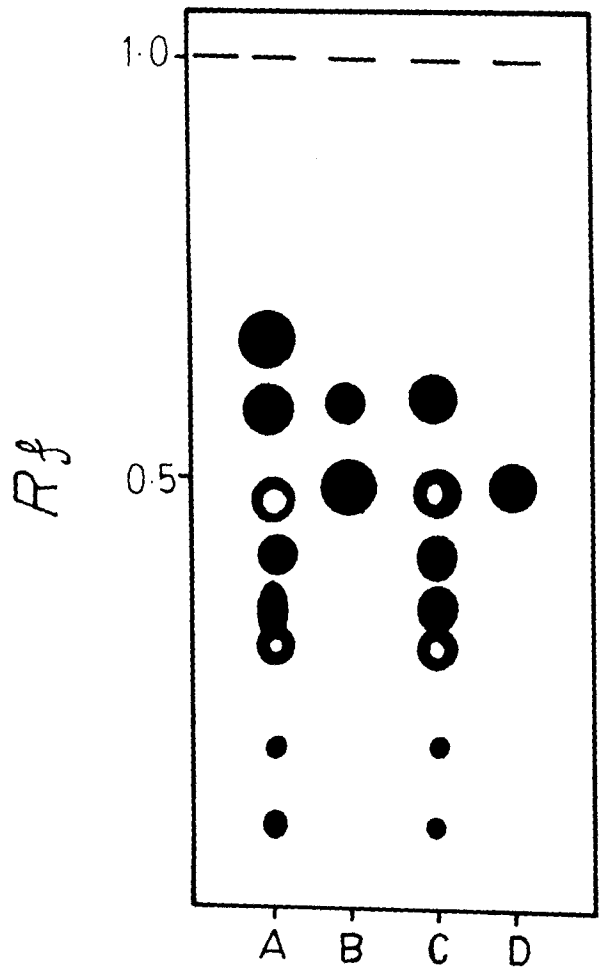

Fig.3·25 Thin layer chromatogram of chloroform extracts of S.paucimobilis cells (A), freeze thawed cells (B), freeze thawed cells suspended in shock fluid (C), incubated with α-santonin for 24 h. α-santonin control (D), •-o-● spots with increasing intensity.

FIG. 3.26 NDGE profile of shock fluid (lane 1), shock fluid incubated with substrate α-santonin (lane 2) and molecular weight standards for nondenaturing gel (lane 3).

FIG. 3.27 SDS-PAGE protein profile of the shock fluid containing 110 μg of protein/ml (lane 1), purified α-santonin binding protein (lane 2) and molecular weight markers (lane 3).

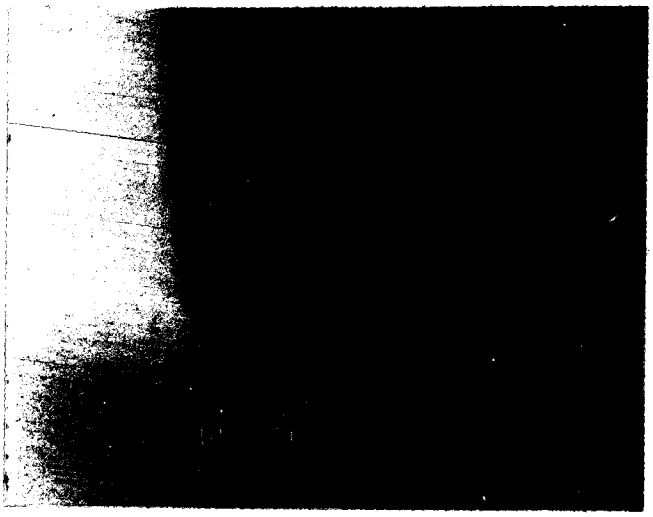

Fig. 3·26

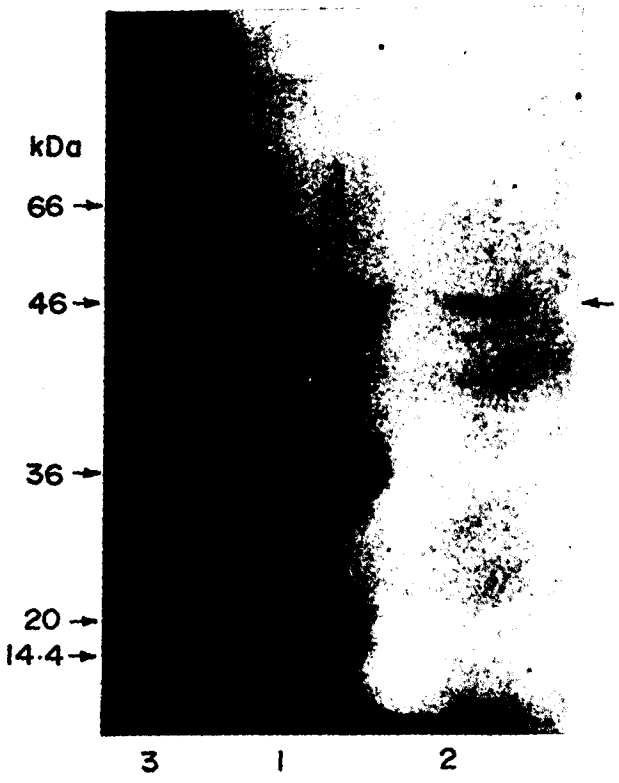

Fig. 3·27

3.5 COMPARATIVE STUDIES OF *S. paucimobilis* AND α-SANTONIN NEGATIVE MUTANT S_{48}

S_{48}, a santonin negative mutant was isolated by Tn 5 mutagenesis of *S. paucimobilis* cells in our laboratory. This transposon mutant S_{48} is identical to parent cells morphologically and culturally. It, however, fails to utilise α-santonin as a sole source of carbon. It is an ideal negative control to study the transport mechanism of α-santonin.

3.5.1 SDS-PAGE Profile of Whole Cell and Cell Envelope Proteins of S and S_{48}

3.5.1.1 Whole cell protein profile

Whole cell proteins were isolated from S_{48} cells grown on glucose kanamycin medium. The SDS-PAGE profile of whole cell protein extracts shows absence of two low molecular weight proteins in the range of 20-40 kDa (Fig. 3.28). The pattern otherwise is very similar to *S. paucimobilis* cells grown on glucose. The induction of 68 kDa protein which is the distinct feature of α-santonin grown cells is not seen as indicated by arrows in Fig. 3.28.

3.5.1.2 Cell envelopes of S_{48}

The SDS-PAGE profile of cell envelope proteins of *S. paucimobilis* cells shows the presence of 68 kDa protein which is absent in S_{48} and does not appear even on incubation of S_{48} in presence of α-santonin as seen in Fig. 3.29. Besides, S_{48} shows absence of two low molecular weight proteins in the envelope preparation which were also found to be absent in whole cell protein profile of S_{48} cells as indicated by arrows in Fig. 3.29.

FIG. 3.28 SDS-PAGE profile of whole cell proteins extracted from santonin negative mutant S_{48} strain grown on glucose (lane 2) and *S. paucimobilis* cells grown on glucose (lane 1). Arrows indicate the missing proteins under the conditions assayed.

FIG. 3.29 SDS-PAGE profile of envelope proteins isolated from *S. paucimobilis* cells grown on α-santonin (lane 1), santonin negative mutant S_{48} (lane 2), and S_{48} cells incubated with α-santonin (lane 3). Molecular weight markers are in lane 4.

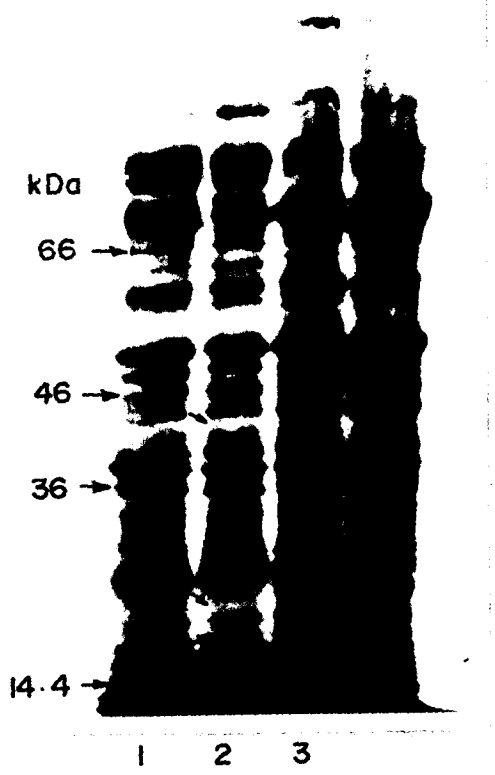

Fig. 3·28

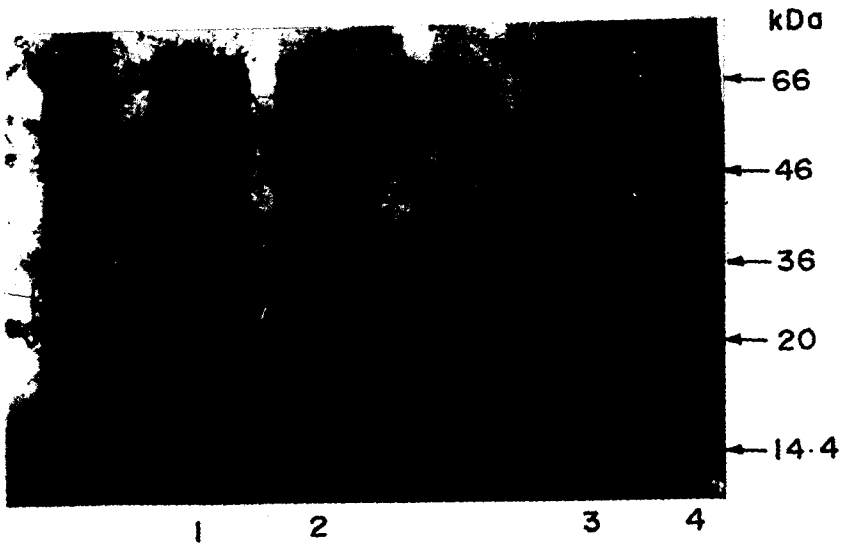

Fig. 3·29

3.5.2 Binding Activity of Shock Fluid Eluted from S_{48}

Periplasmic proteins were released from S_{48} by repeated freezing and thawing. The shock fluid when checked for α-santonin binding activity by equilibrium dialysis binding assay showed 230 μg/ml against control 166 μg/ml of 0.05 M phosphate buffer. Phosphate buffer was used as a control as the proteins were suspended in it.

3.5.3 Transport and Transformation of α-Santonin by S_{48} Cells

3.5.3.1 Transport assay

The pattern of α-santonin depletion in S_{48} is different from S. paucimobilis grown on α-santonin. The initial depletion at 3 min. is similar in both giving a rapid depletion of 23 μg /ml/min. A further uptake, that is type II is seen upto 10 min. with a depletion rate of 3 μg/ml/min for S_{48} and 10.1 μg/ml/min for *S. paucimobilis.* In *S. paucimobilis* santonin seems to be completely depleted by 20-30 min. whereas in S_{48}, at 15 min., the santonin that is taken in is effused out as seen in Fig. 3.30. The ratio of uptake of α-santonin at 10 min. by *S. paucimobilis* and S_{48} seems to be 3:1.

3.5.3.2 Transformation of α-santonin by S_{48}

S. paucimobilis strain S cells incubated with α-santonin form an array of transformation products (Sangodkar and Mavinkurve, 1982, 1984; Naik and Mavinkurve, 1987; Furtado *et al.,* 1988; Colaco *et al.,* 1993) which are detectable by TLC (Naik and Mavinkurve, 1987). The α-santonin transformation pattern of *S. paucimobilis* strain S cells show eight products with R*f* values ranging from 0.1 to 0.7 cm (Fig. 3.25). α-santonin (R*f* 0.45 cm) is used as a control. S_{48} cells show only one feeble product as yellowish brown spot at R*f* 0.65 cm (Fig. 3.31 B), whereas the cell free extracts of the same cells on incubation with α-santonin gave rise to transformation pattern similar to *S. paucimobilis* cells as seen in Fig. 3.31.

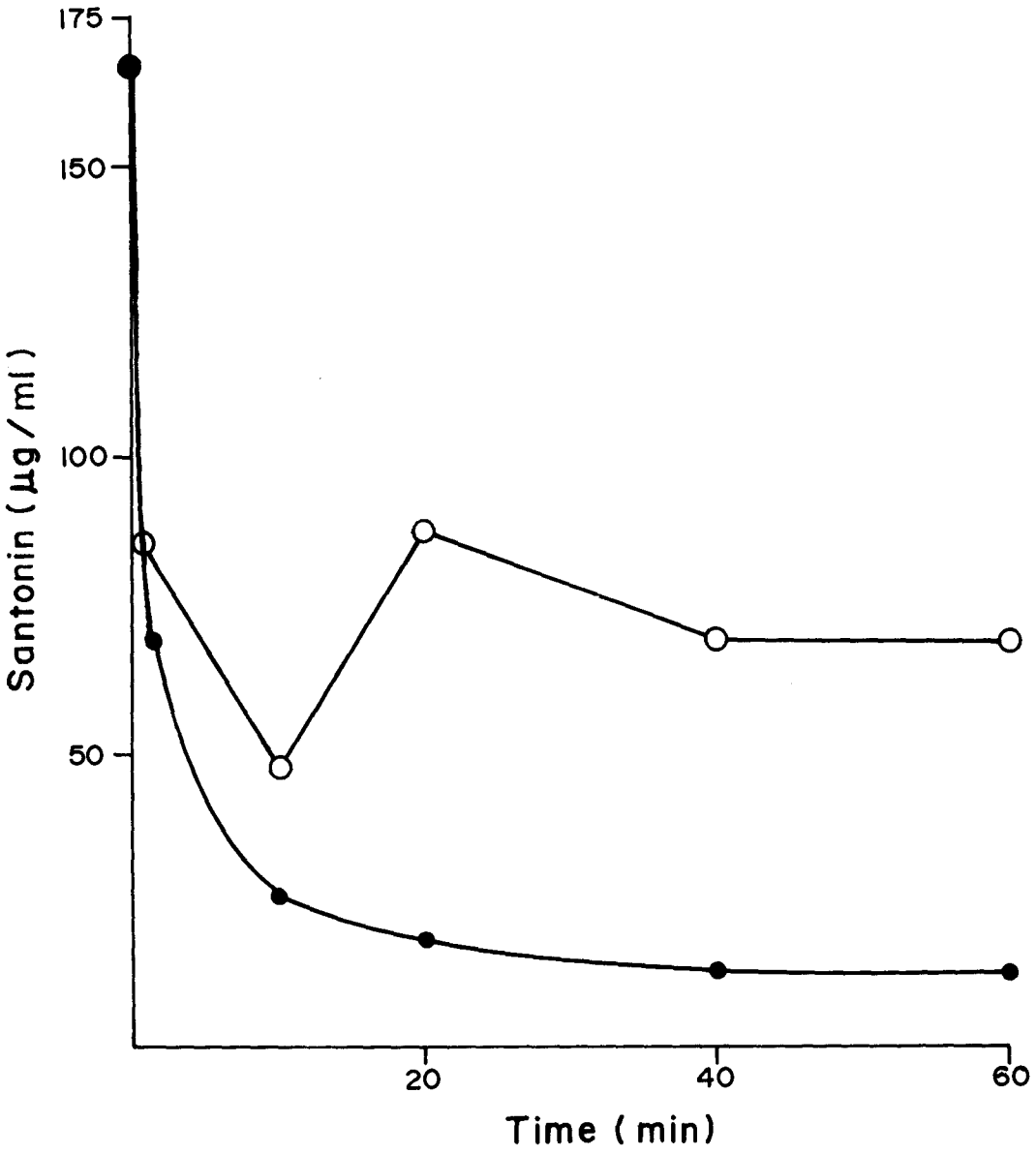

Fig. 3·30. Depletion of α-santonin by S. paucimobilis cells grown on santonin (●), and S_{48} cells grown on glucose kanamycin (○). Amount of α-santonin in the filtrate plotted.

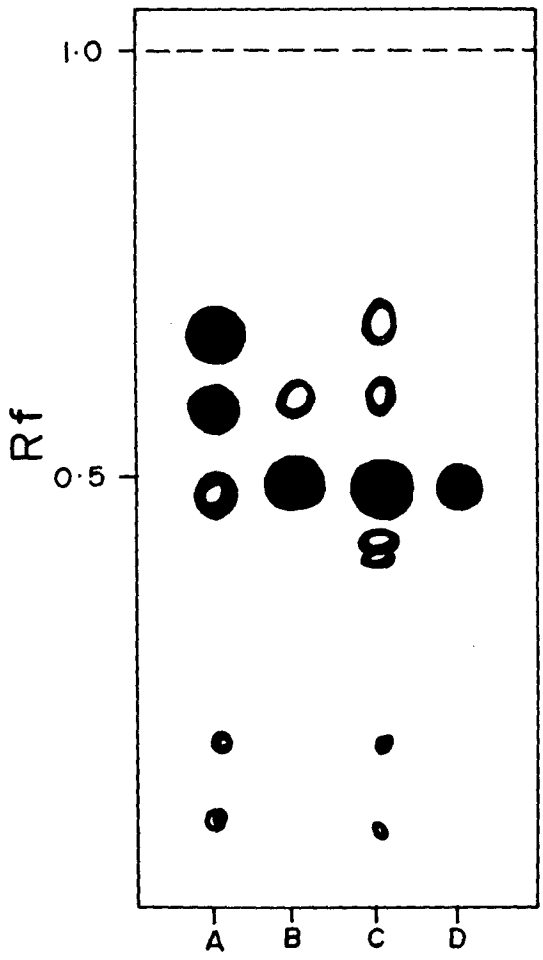

Fig. 3·31 Transformation of α-santonin with cell free extracts of S. paucimobilis cells (A), S_{48} cells (C) incubated with α-santonin for 16 h at RT and S_{48} whole cells incubated with α-santonin for 24 h (B). α-santonin as control (D).

CHAPTER 4.0

DISCUSSION

DISCUSSION

Sphingomonas paucimobilis strain S grows on α-santonin agar plates and forms clear, crystal free zones around the colonies caused by solubilisation and utilisation of α-santonin. Earlier studies (Furtado *et al.*, 1987) indicated that halos created by dissolution of substrate do not involve any extracellular factor. The concentration of dissolved santonin always remained 166 μg/ml in solution. Similar reports of substrate being replenished by a physical phenomenon of dissolution has been reported in the utilisation of steroids, solid paraffins and alkanes (Jones and Baskevitch, 1973). The above observations that cells utilise α-santonin available in dissolved form, prompted the study of the specific factors involved in transport of α-santonin .

4.1 EFFECT OF GROWTH SUBSTRATE ON CELL AND CELL ENVELOPE COMPONENTS

The transport assay system, involved incubation of *S. paucimobilis* cells with saturated aqueous santonin solution followed by filtration of reaction mixture at specific time intervals, to monitor the depletion of α-santonin in the reaction mixture. The method was effective, as seen in Fig. 3.1, helped to assay uptake of α-santonin by cells. *S. paucimobilis* cells grown on 0.4% α-santonin or 0.4% glucose, when checked for α-santonin transport activity showed varied response. The pattern of uptake can be phased into type I, nonspecific and type II, specific (Fig. 3.1). The cells grown on α-santonin show complete depletion of α-santonin by 20 min., whereas the cells grown on glucose show only the type I uptake at 3 min. This indicates that the α-santonin transport is governed by substrate inducible system. The type I transport is unaffected by sodium azide and DCCD unlike type II which is linked to ETC or PMF (Furtado *et al.*, 1987; De Costa, 1991). Studies done in our laboratory confirmed the nonspecific nature of type I transport by incubating *E. coli* cells grown on nutrient agar with saturated santonin solution (166 μg/ml) which show uptake of 55 μg per ml at 3 min, with no further

transport (De Costa, 1991). This led us to conclude that the nonspecific adsorption of the substrate on to cell gave rise to the depletion at 3 min. In type II uptake the rate of depletion is much more and faster in santonin grown cells as compared to glucose or nutrient grown cells . Earlier studies indicated that when cells are preincubated with santonin for 30 min., showed an increase in depletion as against non incubated cells (De Costa, 1991).

Chemical analysis of *S. paucimobilis* showed that lipid content and the type of lipid varies with pre growth substrate (De Costa, 1991). For example, santonin grown cells fail to show the presence of myristic acid, oleic acid and stearic acid. Similarly, cells grown on glucose and nutrient fail to show the presence of 4 methyl hexadeconoic acid (De Costa, 1991). Our results are consistent with these as we see the change in LPS composition (Fig. 3.5) in cells grown on different carbon sources. The results indicate that the substrate plays an important role in altering the membrane fluidity or by inducing the components essential for transport.

The effect of growth substrate on whole cell and cell envelope components are delineated in Fig. 3.2 - 3.5. The SDS-PAGE profile of whole cell proteins shows distinct substrate induced differences in the pattern (Fig. 3.2). The 80, 68 kDa protein induced and 46 kDa protein which is more intense in presence of α-santonin may have an important role to play either in α-santonin transport or degradation.

Outer membrane, the first component which comes in contact with the substrate, is known to be altered by culture conditions (Lugtenberg *et al.*, 1976; Hancock *et al.*, 1983; Poole and Hancock, 1986). Protein composition of the outer membrane of Gram-negative bacteria is relatively simple. SDS polyacrylamide gel electrophoretogram of outermembrane from *S. paucimobilis* cells shows only a limited number of bands that correspond to major proteins (Fig. 3.4). The SDS-PAGE profile of OMP from α-santonin grown cells indicates the presence of a specific protein 68 kDa which is also seen in whole cell protein profile. Besides, glucose grown cells incubated with 0.025% α-santonin for various time intervals show the substrate specific induction of 68 kDa

protein, its intensity increasing linearly with incubation time as seen in Fig. 3.6. The other substrate inducible proteins of Gram-negative are the lam B proteins of *E. coli* in presence of maltose (Benz *et al.*, 1987) and protein D_1 of *P. aeruginosa* (Hancock and carey, 1980) in presence of glucose. A similar effect is seen in *S. paucimobilis* strain S cells in presence of α-santonin.

4.2 CHARACTERISATION OF OMP OF *S. paucimobilis*

4.2.1 Pore Forming Ability of *S. paucimobilis* OMP

Our results indicate that santonin is taken up in the dissolved form by *S. paucimobilis* cells (Furtado *et al.*, 1987). Normally, the transport of such compounds across the outermembrane of Gram-negative bacteria is governed by simple diffusion process through the pores made by pore forming proteins called porins (Benz, 1994).

The presence of santonin inducible 68 kDa protein in the outer membrane (Fig. 3.4), suggests its role in transport of α-santonin possibly in the form of santonin specific receptor. Many of the porin pores of Gram-negative bacteria have been successfully reconstituted into liposomes, vesicles or lipid bilayer membranes (Benz *et al.*, 1986, 1987; Schulein and Benz, 1990; Maier *et al.*, 1987). Such reconstitution helps in establishing the integrity and the pore function of the porin pore though it is possible that several artifacts and absence of essential components may not conclusively establish the activity.

Nikaido and coworkers (Nikaido and Rosenberg, 1981, 1983; Nikaido, 1983) introduced the liposome swelling method for studying the permeability properties of porin pores. This method has allowed a meaningful comparison of different porin pores from *E. coli* outer membrane (Luckey and Nikaido, 1980a, 1980b). Further it was used to demonstrate the greater specificity of the lam B channnel for maltose and maltodextrans than for other saccharides. The properties of a variety of porin pores from different Gram-negative bacteria were also investigated using this method (Douglas *et*

al., 1981, 1984; Flammann and Weckesser, 1984; Vachon *et al.*, 1986; Yashimura *et al.*, 1983).

During our present work in an attempt to characterise the 68 kDa protein and to study its role in transport, liposomes were prepared with or without outermembrane proteins. The liposome swelling with saturated santonin solution gave a very little activity, the increase in substrate in crystalline form allowed us to monitor the swelling more appropriately. The presence of crystalline α-santonin at the bottom of the cuvette helped in faster rate of dissolution of α-santonin into solution as it is depleted or taken in by liposomes as seen in Fig. 3.9. As the substrate concentration increased the rate of swelling also increased. This type of assay system can be used for feebly soluble compounds. The results show that the proteins forming specific pore for α-santonin are present in α-santonin grown cells (Fig. 3.8 A) but not in cells grown on glucose (Fig. 3.8 B). OMP containing the 68 kDa protein which is inducible only in the presence of substrate α-santonin, formed pore which allowed the passage of only α-santonin, indicating that the importance of 68 kDa possibly as a substrate specific porin pore as seen in Fig. 3.8 A. We do not see transport of any other sugar molecules in liposomes prepared from *S. paucimobilis* cells grown on α-santonin. As seen earlier with the induction of santonin specific porin, there is also a simultaneous repression of 64 kDa protein (Fig. 3.4). 64 kDa protein could be a porin pore for sugar compounds. Porins are isolated and characterised from Gram-negative bacteria such as *E. coli, S. typhimurium, P. aeruginosa, H. influenzae, N. gonorrheae, P. mirabilis, R. capsulatus,* and *P. denitrificans*. One interesting class of porins are those outermembrane proteins which are involved in high affinity substrate transport and contain channels with saturable binding sites for specific substrates. Few examples of such porins are Lam B (Benz, 1988) and protein D (Hancock and Carey, 1980) and nucleotide specific porin (Maier *et al.*, 1987). The mechanism of solute specificity is not known. In comparison with lamB protein in *E. coli* which is specific to maltose and maltodextrins, this 68 kDa follows the same specificity and inducibility to the substrate. It would be interesting to study whether this also allows the passage of santonin related compounds.

The specificity of the porin pore may be due to specific binding of the substrate to some residues on the porin pore. The binding was thus investigated by fluorescence spectroscopy as it is a good tool for monitoring protein ligand interaction.

4.2.2 Fluorescence Spectroscopy of OMP

On addition of increasing concentrations of α-santonin (0.66 - 12.6 x 10^{-4} M) to outermembrane proteins a linear quench in fluorescence as a function of substrate concentration is observed indicates the binding of the substrate to the protein (Fig. 3.10). Substrate on binding to the protein causes either change in conformation of the protein or blocks the residues on the protein which contribute to the fluorescence of the protein molecules. Tryptophan and tyrosine are the residues that usually contribute to the fluorescence of the protein (Miller *et al.*, 1983; Yashihiro *et al.*, 1992). Emission at 340 nm noticed is due to tryptophan residues. Binding is calculated based on the relative percentage of 68 kDa protein obtained from the densitograph of the SDS-PAGE of outermembrane preparation. Affinity constant of the 68 kDa protein as calculated from slope of the mass action plots (Fig. 3.12), for α-santonin was found to be 2.5 μM.

4.2.3 Heat Modifiability and Mercapto-Ethanol Sensitivity of OMP

The migration of proteins in SDS-Polyacrylamide gel electrophoresis is directly related to the amount of SDS bound. Other factors such as the change in the conformation of the SDS protein complexes leading to a frictional drag can also influence the mobility (Hancock and Carey, 1980).

The heat modifiablility of OMP was observed in both the presence and absence of 2-mercaptoethanol (Fig. 3.13). In absence of mercaptoethanol at 60° C or 100° C, the protein dissociates into monomer closely associated with 68 kDa. But at 100° C there is an appearance of 80 kDa protein. This effect of appearance of 80 kDa protein is seen at 60° C in presence of mercaptoethanol indicating that mercaptoethanol enhances the heat modifiability at lower temperature (60° C). The appearance of a new band 80 kDa is

caused due to frictional drag in presence of heat. It may be of interest to recall that 80 kDa protein is seen in whole cell protein profile of cells grown exclusively on α-santonin (Fig. 3.2). The simplest explanation for the effect of 2-mercapto ethanol is that it is affecting one or possibly two inter chain cystine disulfide bonds. This would cause an alteration in the conformation of the protein and thus increase the binding of SDS and increase the frictional drag of the molecule.

The presence of inter chain disulfide bonds are also reported from porins of *L. pneumophila* (Hindahl and Iglewski, 1984) and *chlamydiae* (Newhall and Jones, 1983). Heat modifiability is attributed to the high β sheet content of outermembrane proteins (Hancock and Carey, 1979). It has been demonstrated that the heat modifiable OMP of *E. coli* contain β-structure sequences which are stable in SDS solution, but are destroyed upon heating. Our results are consistent with results for protein D_1, D_2, G and H_1 in that heating in SDS at temperatures around 70 to 100° C caused a decrease in the mobility of the protein (*i.e.,* an increase in the apparent molecular weight). Since porins exist as trimers and the appearance of closely associated monomer in presence of heat and 2-mercaptoethanol treatment only suggest a possibility that the heat modifiable protein (80 kDa) is a part of 68 kDa protein. The appearance of 80 kDa in presence of α-santonin in whole cell protein extracts, further strengthens the conclusion drawn.

4.3 RELEASE OF PERIPLASMIC PROTEINS FROM CELL ENVELOPE OF *S. paucimobilis*

4.3.1 Comparison of Methods on Release of Periplasmic Proteins

Substrate binding proteins situated in periplasmic space play an important role in nutrient transport, demonstrated by their specific binding to the substrates and lack of transport of substrate in the cells devoid of such transport proteins. Efficient release of periplasmic proteins from the organisms in entirety is desirable. Several techniques have been developed which selectively and quantitatively release a group of enzymes from organisms. Frequently used techniques are osmotic shocking (Neu and Heppel, 1965)

freeze thawing (Paoletti *et al.*, 1987) and chloroform methods (Ames *et al.*, 1984). When *E. coli* cells are converted into spheroplasts in a hyper osmolar solution containing Tris EDTA and lysozyme a group of degradative enzymes were liberated. Similar class of enzymes and other factors involved in active transport are released when cells treated with Tris EDTA are subjected to an abrupt osmotic transition (Neu and Heppel, 1965). Treatment of *E. coli* with Tris-EDTA without osmotic transition does not result in significant release of periplasmic enzymes, but liberates 30-50 % of LPS and increases the nonspecific permeability. However, the studies done with *S. itersonii*, release of periplasmic proteins required only Tris EDTA treatment without any osmotic transition in contrast to *E. coli* which requires two steps. The basic difference between periplasmic enzyme release in Enterobacteriaceae and *S. itersonii* is the porosity of the cell wall after the EDTA treatment. Effective release of periplasmic proteins from an organism depends on the cell wall, composition and the suitability of the method employed. Chloroform is reported to release maximum periplasmic proteins from *Pseudomonas* (Lall *et al.*, 1989). However in the present work with *S. paucimobilis* strain S, FTM was more efficient and released more periplasmic proteins than COSM and CHLM (Table 3.2).

Alkaline phosphatase, a periplasmic enzyme, is often used to monitor the release of periplasmic proteins (Lall *et al.*, 1989). Eluant of FTM showed release of 10.5 µmoles of p-nitro phenol/ml as compared to COSM and CHLM, the trend corroborating the total proteins in the shock fluid, released by each of the three methods (Table 3. 2). Invariant specific activity of the enzyme in shock fluids indicated that the methods differed in their efficiency to release periplasmic proteins only quantitatively. In this regard, our results corroborate with the work done by Kier *et al.* (1977) on Salmonella phosphatases. Although the phosphatase enzymes of Salmonella and other organisms like *E. coli* behave similar in activity in intact cells, they are not efficiently released by osmotic shock procedures. Whereas the same procedure has effectively released other proteins like the *J* histidine binding protein (Kier *et al.*, 1977). The reason could be due to the damage of the enzymes during osmotic shock procedure. With the results obtained, we could infer that the phosphatase could be best released by freezing and

thawing without causing any damage to their activity (Shailaja Raj *et al.*, 1996). High α-santonin binding activity (2.40 μmoles/mg of protein) in the periplasmic proteins released by FTM reduced the α-santonin transport in the concerned shocked cells by 90%, on comparison to only 70% by COSM (Table 3.2). This confirms the periplasmic location of α-santonin binding factor and the efficiency of the FTM in eluting them. This method also has selective advantages over other conventional methods, as it does not involve any chemical treatment such as lysozyme, EDTA, chloroform or toluene and releases selectively periplasmic proteins without loss of apparent cell damage.

4.3.2 Effect of Physico-Chemical Parameters on Release of Periplasmic Proteins by Freeze Thaw Method

4.3.2.1 Cell density

Release of periplasmic proteins by FTM increased significantly with increase in the cell density (Fig. 3.14 A) from 2.6 - 12.5 mg/ml and plateaued thereafter, possibly due to the decrease in inter cellular solute space and cell to cell surface contact. Cell density with absorbance of 4.5 optimally elutes periplasmic proteins.

4.3.2.2 Freezethaw cycles

The proteins were released in the shock fluid partially after the first freeze thaw (FT) cycle and to a maximum extent after the second FT cycle and with no further increase on subsequent FT cycles (Fig. 3.14 B). A single overnight freezing and thawing (Paoletti *et al.*, 1987) of cells at -20°C released proteins almost equal to two freeze thaw cycles. The shock fluid showed 10 μmoles of alkaline phosphatase activity and 280 μg per ml of α-santonin binding activity. This indicates that over night freezing and thawing has an advantage considering the less damage caused to the proteins, cell viability and the convenience. Although for quicker release of proteins the cells can be frozen twice and thawed to get the same activity.

4.3.2.3 Temperature

Cells freezed more effectively at -20° or -70°C and released 110 μg/ml of periplasmic proteins. This may be due to rapid ice-crystal formation resulting in alteration of membrane permeability (Peter and Robert, 1974), as against release of only 70 μg/ml proteins at -4°C (Fig. 3. 15 C).

4.3.2.4 Buffer and pH

The amount of proteins released may vary with the solutes in the suspending medium (Peter and Robert, 1975). The cells suspended in Tris HCl buffer (pH 7.0) eluted only 55 μg/ml as compared to 110 μg/ml released by cells suspended in phosphate buffer, pH 7.0 (Fig. 3.15 A). At acidic pH (4.8), proteins released were reduced significantly to 45 μg/ml (Fig. 3.15 B).

Responses of Gram-negative cells depend upon the cell genotype, menstrum in which they are suspended and the freeze thaw rates (Peter and Robert, 1974), as the cooling rates differ with the solute in which the cells are suspended. When the rate of cooling is low the damage caused to permeability barrier is high, for *e.g.,* saline freezing causes more permeability damage than the water freezing, which is due to internal ice formation. It can be deduced that the conditions which favor the intra cellular freezing also favor an increase in permeability damage. The same may be true with the cells suspended in phosphate buffer as the phosphates favor more intracellular freezing than the Tris leading to release of more amount of proteins. The extra damage caused to the barrier in presence of phosphates seems to be a general action of salt over the cooling rate range and due to action of concentrated solute which accumulates when the suspending fluid freezes (Peter and Robert, 1975).

4.4 LOCALISATION OF α-SANTONIN BINDING PROTEIN

On freeze thawing, major 46 kDa protein and some minor bands are released from *S. paucimobilis* cells into the medium in which they are suspended (Fig. 3.16). The simultaneous appearance of the 46 kDa protein in the shock fluid seen in Fig. 3.16 and 3.17 shows that it is a periplasmic protein which is released.

4.4.1 α-Santonin Binding Activity of Shock Fluid

The shock fluid released by freeze thawing of *S. paucimobilis* cells shows an affinity for α-santonin. When dialysed against saturated α-santonin solution, it binds 158 μg of α-santonin/ml as against only 98 μg in corresponding buffer control. Concentrated shock fluid (150 μg of protein/ml) binds α-santonin to super saturation level, 198 μg/ml, indicating that with increase in protein content, there is a proportional increase in the α-santonin binding capacity of the shock fluid. However, the control 0.05 M phosphate buffer was showing only 98 μg/ml of binding, when solubility of α-santonin in solution is 166 μg/ml. It was logical to explain the phenomena as binding only if the control had shown 166 μg/ml, concentration equal to α-santonin solubility in solution. The feeble solubility of α-santonin and the absence of extra santonin to replenish were thought to be causing problem. Hence excess α-santonin in crystalline form was added to the saturated santonin solution. Phosphate buffer 0.05 M, pH 7.0, when dialysed against this solution yielded 166 μg/ml of α-santonin and the shock fluid in contrast bound 275 μg/ml reaching a supersaturation level.

4.4.1.1 Filtration assay of shock fluid

Filtration assay results confirm the binding of the substrate α-santonin to the protein as the ammonium sulfate precipitated protein shows a peak at 242 nm (Fig. 3.18) as compared to non incubated protein solution. The peak at 242 nm indicates the presence of α-santonin bound to the protein. The controls used were phosphate buffer and BSA to verify any non specific interaction with α-santonin. The results showed that

α-santonin does not have any interaction with BSA or phosphate buffer indicating that this method can be used to study the specific binding interaction of α-santonin with the protein. The assay procedure developed clearly shows that the decrease in absorbance (oxidation of NADPH) monitored at 340 nm was due to the availability of the bound santonin for conversion to dihydrosantonin by the enzyme santonin 1,2 reductase present in the cell free extract (Fig. 3.19). The enzyme activity of cell free extract in presence of shock fluid with the bound substrate shows 0.22 units of decrease in absorbance/mg of protein/ml/min., which matches with the activity in presence of substrate (α-santonin).

Although all these methods confirmed the release of periplasmic proteins and binding of α-santonin by them, a more sensitive tool, like fluorescence spectroscopy was used to study this interaction.

4.4.1.2 Fluorescence spectroscopy of periplasmic proteins

Quench in fluorescence at 350nm is observed on addition of increasing concentrations of α-santonin (0.66 - 6.6 x 10^{-4} M) to the shock fluid (Fig. 3.20 A and B). Linear quench in fluorescence as a function of substrate concentration indicates the binding of the substrate to the protein (Fig. 3.20 B) and is usually contributed by the tryptophan residues (Miller *et al.*, 1983; Yashihiro *et al.*, 1992). Substrate on binding to the protein causes either change in conformation of the protein or blocks the residues on the protein which contribute to the fluorescence of the protein molecules (Zukin *et al.*, 1979). Tryptophan and tyrosine are the residues that usually contribute to the fluorescence of the protein. Emission at 340 nm noticed is due to tryptophan residues. On addition of α-santonin to shock fluid, decrease in fluorescence was noticed for about 12 min at RT (28° C) and within 5 min at lower temperatures (16° C) indicating the effect of temperature on α-santonin binding (Fig. 3.21). Binding is calculated based on the relative percentage of 46 kDa protein obtained from the densitograph of the SDS-PAGE of shock fluid. Affinity constant of the 46 kDa protein as calculated from slope of the mass action plots (Fig. 3.23), for α-santonin was found to be 0.016 μM per

monomer which is similar to binding affinity of some amino acid residues such as cysteine (0.01 μM) indicating a high affinity transport system.

The TLC pattern of shock fluid incubated with α-santonin for varied time intervals depicted that the α-santonin is not transformed nor enzymatically converted into another form. Therefore, the interaction between the protein and α-santonin that is occurring is due to mere binding.

4.4.2 Reconstitution

Reconstitution was studied in terms of restoration of transport, transformation and oxygen uptake of *S. paucimobilis* freeze thawed cells. The oxygen uptake of *S. paucimobilis* strain S cells grown on α-santonin, when monitored on Gilsons Oxygraph was 5.448 nmoles/l. On freezing and thawing the same cells, showed reduced uptake (4.576 nmoles/l) which was restored to original level, on reconstitution with the released shock fluid.

4.4.2.1 Transport of α-santonin

Freezing and thawing of *S. paucimobilis* cells does not affect Type I uptake of the substrate as the freeze thawed cells show Type I uptake nearly equal to the control cells. In contrast, the later slow uptake, Type II, occurring after 3 min is seen to be totally impaired in freeze thawed cells (Fig. 3.24). This observation indicates the two independent sequential events involving uptake of α-santonin during the Type I and Type II phases. Such a biphasic uptake Kinetics has been reported previously by Watanabe and Watanabe (1974).

Restoration of such shock sensitive, Type II transport activity of the freeze thawed cells to 95% by the shock fluids implies the involvement of periplasmically located transport factor or substrate binding proteins, as has been reported earlier in the transport of phosphate (Gerdes *et al.*, 1977), ribose (Galloway and Furlong, 1979),

glutamine (Masters and Hong, 1981), β-galactoside (Kundig *et al.*, 1966), maltose (Johann, *et al.*, 1986) and sugars and amino acids (Anraku *et al.*, 1973).

4.4.2.2 Transformation of α-santonin

Sphingomonas paucimobilis strain S cells incubated with α-santonin form an array of products, detectable by TLC (Colaco *et al.*, 1994; Furtado *et al.*, 1988; Naik and Mavinkurve, 1987; Sangodkar and Mavinkurve, 1982). Reduction of α-santonin, to 1,2 dihydrosantonin by saturation of 1,2 double bond is the first reaction catalyzed by NADPH dependent dehydrogenase, 1-2 α-santonin reductase (Naik and Mavinkurve, 1987). Binding proteins often possess or are associated with dehydrogenases in the periplasm (Fasses *et al.*, 1992). The shock fluid however failed to show α-santonin dehydrogenase activity, as monitored by the decrease in absorbance of NADPH at 340 nm and also to show any transformation products. The freeze thawed cells formed a single feeble product, less polar than α-santonin, at R*f* 0.57, corresponding to lumisantonin (Naik and Mavinkurve, 1987). The same cells however, when suspended in shock fluid, showed complete transformation of α-santonin, as with normal cells (Fig. 3.25).

4.4.3 Identification of α-Santonin Binding Protein

Binding proteins undergo a chemical change in its chemical environment upon binding with substrate which causes a quench or enhancement in fluorescence intensity. As we have seen earlier fluorescence spectroscopy results have demonstrated the same indicating that the protein is undergoing a conformational change. The demonstration that a substrate induced conformational change occurs in all binding proteins analysed indicates that it is an essential aspect of their mechanism of action. Variety of methods are used for measuring substrate induced conformational change. One of the them being change in electrophoretic mobility. When a crude shock fluid incubated with substrate α-santonin was subjected to nondenaturing gel electrophoresis a protein at band no. 3 position moved at a different mobility. This indicated that the protein is undergoing a

conformational change on binding. On observing the results we find that the protein exists in two forms, one is bound with altered conformation and the other in nonbound form. The reasons for this type of existence of the protein could be (1) due to the effect of pH (8.3) of the electrophoresis buffer on the protein with bound substrate. At this pH, santonin loses its lactone ring. The substrate which is not altered is still bound to the protein. (2) May be due to nonavailability of the substrate to bind all protein molecules in the sample. Similar reports are observed with galactose binding protein which exists in two forms, the ratio of which is determined by the substrate concentration. The altered protein eluted from the gel on subjecting to SDS-PAGE confirmed the presence of the 46 kDa protein which is released into the medium on freeze thawing *S. paucimobilis* cells. The α-santonin binding protein is tripeptide protein with a major band at 46 kDa and 2 minor bands corresponding to 44 and 42 kDa respectively.

4.5 COMPARITIVE STUDIES OF *S. paucimobilis* AND S_{48} CELLS

S_{48} a santonin negative mutant was isolated (Borkar *et al.*, 1991) in our laboratory. This was used as a negative control during our studies on α-santonin transport. The whole cell protein profile of S_{48} shows the absence of two low molecular weight proteins in the range of 14-20 kDa as seen in Fig. 3.28. The role of these proteins in *S. paucimobilis* cells was particularly of interest especially to know whether the absence of these proteins led to the non-utilisation of α-santonin by this organism due to defect in transport or catabolic degradation of α-santonin.

4.5.1 Transformation of α-Santonin by S_{48} Cells

Results of the transformation pattern indicate that a feeble product (DHS) is formed in 4 h of incubation with α-santonin. But when sonicated cell free extacts were incubated with α-santonin at RT for 24 h, the transformation pattern was similar to *S. paucimobilis* cells (Fig. 3.31). This shows that it has all the enzyme machinery required for α-santonin degradation.

4.5.2 Transport Activity of S_{48} Cells

A simple transport assay of α-santonin was performed with S_{48} cells. There is a rapid instantaneous depletion during first 3 min on exposure of *S. paucimobilis* cells as well as S_{48} to santonin as seen in Fig. 3.30. The rate of uptake being 23 μg/ml/min. As described in the results, there is transport for about 10 min and further uptake was stopped. The transport that occurred for the first 10 min also was reduced by 50% as compared to uptake by *S. paucimobilis* cells (10.1 μg/ml/min). These results show that although uptake takes place for 10 min, it is not as effectively transported as in case of *S. paucimobilis* cells. Further santonin uptake is completely stopped, and whatever is taken up is also effused out at 15 min to 60 min. This indicates that there is a slight initial binding to substrate which is not transported further, and the binding seems to be reversible as the santonin later on is effused out.

As the above results suggest the possibility of S_{48} being a transport mutant, each envelope component of S_{48} was being isolated to study the site of mutation. Periplasmic protein were isolated by repeated freezing and thawing of S_{48} cells. The shock fluid thus eluted out when checked for α-santonin binding showed similar binding activity as *S. paucimobilis* cells grown on either santonin or glucose. This indicates that the binding protein is constitutively present in the cell. This also further proves the evidence that the little substrate that trickles down the outermembrane is carried by the protein upto to level of inner membrane and gives rise to the formation of a feeble product dihydrosantonin (DHS). On freeze thawing S_{48} cells lost the uptake which other wise occurred in S_{48} cells. This indicates that the uptake that occurred for 3-10 min in S_{48} was due to binding protein located in the periplasmic space. This binding being reversible, and as no further transport of the compound occurred, α-santonin was released into the medium extracellularly which accounted for the effusion of α-santonin at 15-30 min. These results gave a clue to the involvement of membrane proteins in transport which is supported by the results of the thawed cells not showing any uptake or transformation of α-santonin.

4.5.3 Cell Envelopes of S_{48} Cells

To localise the site of mutation each membrane component was isolated from S_{48} cells and analysed. Comparative studies of cell walls and cell membranes isolated from S and S_{48} cells analysed on SDS-PAGE profile indicated that the cell walls of S_{48} lack two membrane components (Fig. 3.29) and S_{48} on incubation with α-santonin does not synthesize the 68 kDa protein. Simultaneously there is induction of a 38 kDa protein indicated by arrows in Fig. 3.29.

The biochemical study of the membrane bound components has lagged far behind than that of the binding proteins, since they are more difficult to study. The number of membrane components involved ranges between two to four was determined for several permeases by genetic or recombinant DNA techniques (Ames, 1986). Genetic data have been obtained which indicate the existence of direct interaction between the binding protein and the membrane bound components of histidine transport system (Ames and Spudich, 1976).

The above results only show a possibility of the proposed mechanism or the site of mutation, but a lot of genetic studies should be done to prove the proposed theory. Only then a complete detailed process of Tn 5 mutagenesis in S_{48} leading to santonin negative mutation can be explained.

4.6 TRANSPORT MODEL

From the results obtained a specific model can be drawn for α-santonin transport in *S. paucimobilis* strain S. The crystalline hydrophobic α-santonin dissolves in the liquid by merely a physical phenomena. The dissolved α-santonin crosses the outermembrane through a specific channel formed by a santonin specific 68 kDa OMP. This protein is santonin inducible having specific sites for α-santonin with a binding affinity of 2.5 μM and is sensitive to trypsin digestion. α-Santonin after passing through the porin pore encounters the 46 kDa periplasmically located α-santonin binding

protein, which is constitutively present in the cell. This protein undergoes conformational change on binding and has affinity (k = 0.016 μM) for α-santonin. The santonin bound protein in its altered conformation possibly interacts with membrane bound components from where it is released into cytoplasm for degradation.

SUMMARY

SUMMARY

S. paucimobilis utilises α-santonin as a sole source of carbon. In aqueous medium α-santonin has a saturation maxima of 166 μg ml^{-1} and is utilised by *S. paucimobilis* in dissolved form. α-santonin transport across the cell envelope of *S. paucimobilis* is governed by a biphasic pathway. Initial type I santonin depletion observed within 3 min is instantaneous and is due to substrate cell surface contact. Further type II uptake was with complete depletion of α-santonin by 30 min of incubation. This uptake was effected by metabolic inhibitors such as DCCD, sodium azide suggesting the role of energy linked transport process.

Growth substrate markedly effected the cell and cell envelope components. A 68 kDa outermembrane protein is seen induced in presence of α-santonin forms substrate specific porin pore and allows the passage of only α-santonin. The 68 kDa protein is heat modifiable and sensitive to trypsin digestion. with an affinity of 2.5 μM for α-santonin. The dissolved α-santonin which enters the periplasm through the outer membranes is bound by periplasmic proteins in the periplasm. The shock fluid eluted from *S. paucimobilis* suspended in 0.05 M phosphate buffer by two freeze thaw cycles or overnight freezing showed high periplasmic protein content, alkaline phosphatase activity and α-santonin binding activity. This method is therefore suitable for extraction of periplasmic proteins especially α-santonin binding proteins from *S. paucimobilis*.

Repeated freezing and thawing released a major 46 kDa protein into the shock fluid which is capable of binding α-santonin as demonstrated by equilibrium dialysis binding assay, filtration assay and fluorescence spectroscopy. This protein, which has an affinity constant K = 0.016 μM for α-santonin, restores transport and transformation and oxygen uptake of freeze thawed cells suspended in shock fluid indicating the periplasmic location of α-santonin binding protein which undergoes conformational change on binding to substrate. Based on this, it is pertinent to deduce that α-santonin transport is governed by a substrate specific outer membrane protein and a 46 kDa α-

santonin binding protein located in the periplasmic space of the cell. Comparative studies with S_{48} indicated that it is a transport mutant as it lacks membrane components essential for α-santonin transport. In addition, it also does not show induction of 68 kDa OMP.

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APPENDIX

APPENDIX - 1

1.1 Preparation and Purification of Crude Lecithin (Pangborn, 1980)

1.1.1 Preparation of crude lecithin

Egg yolks of two fresh hen eggs were separated from whites and collected in a beaker. To this 8.2 ml of chloroform : methanol (2:1 v/v) was added and homogenised for 30 seconds using a homogeniser followed by addition of equal volumes (41 ml) of chloroform and distilled water and homogenised again. The mixture was centrifuged at 3000 g for 15 min at RT. The upper aqueous layer is discarded and the chloroform layer is removed through a hole in the interfacial protein layer. .The organic solution is then passed through sintered glass funnel and dried in a rotary evaporator. The yellow oil is dissolved in 130 ml of ethanol. The mixture was centrifuged at 3000 g for 15 min at 4° C to remove any undissolved material. To this ethanolic solution 5 ml of 50% w/v aqueous cadmium chloride solution is added and allowed to stand for 60 min at 4° C. The precipitate was separated from solution by centrifugation at 3000 g for 15 min at 4° C. The precipitate was redissolved in 17 ml of chloroform and then added to 117 ml of ethanol which contains 0.85 ml of 50% (w/v) aqueous cadmiun chloride solution. This is again allowed to stand for 60 min. at 4° C and removed by centrifugation. The above two precipitation steps repeated until precipitate is white and the supernatant is colourless. The final precipitate obtained was redissolved in 25 ml of chloroform. The chloroform solution is then mixed with 25 ml of 30% v/v ethanol in water thoroughly in a separating funnel. The lower chloroform layer is removed and the upper aqueous layer is discarded. The above step is repeated until cadmium chloride is shown to be completely removed from the system by absence of precipitate when a drop of silver nitrate is added to 1 ml of upper aqueous phase. The solution was evaporated to dryness in pre weighed round bottomed flask. The residual lipid is weighed and dissolved to a final concentration of 50 mg/ml.

Weight of the flask	: 74.8300 g
Weight of the flask + lipid	: 75.3056 g
Weight of the lipid	: 475.60 mg

Amount of crude lecithin obtained from two eggs = 475.6 mg is dissolved in 9.5 ml of chloroform to give a concentration of 50 mg/ml

1.1.2 Purification of crude lecithin

Thirty grams of neutral alumina is suspended in 40 ml of chloroform and the slurry poured into a glass column plugged at the bottom with a glass wool. After the bed of the column is settled down, excess chloroform is removed and 100 ml of fresh chloroform is passed into the column, without disturbing the bed, it is allowed to run through a flow rate of about 10 ml/min. After the chloroform is passed 10 ml of 5 % solution of crude lecithin is loaded onto the column. After it is run into the column, it is washed with another 8 ml of chloroform. And then 100 ml of mixture of chloroform : methanol at a ratio of (9:1) is run into the column at the same rate. The first 10 ml fraction is collected and discarded. 30 of 5 ml fractions were collected and each fraction was assayed by TLC to ascertain for the presence of phospholipid. The fractions were pooled, evaporated to dryness on a rotary evaporator. Pure lecithin obtained was redissolved in chloroform to a final concentration of 50 mg/ml.

1.1.3 Thin layer chromatography

Crude phospholipid preparation isolated from egg yolk and the purity of the fractions obtained by column chromatography were ascertained by thin layer chromatography. The fractions spotted on a silica gel plate and developed in a solvent system chloroform : methanol : water in the ratio of 65:25:4 and the spots visualised on exposure of the plate to iodine vapours. Mixture in crude extract had three compounds with R*f* values 0.098, 0.50, 0.40 cm and the purified fraction showed the presence of pure lecithin. The purified lecithin obtained was used for the preparation of liposomes.

1.2 Saturated santonin solution

0.2 g of α- santonin is added to 100 ml of 0.05 M phosphate buffer pH 7.0 and kept on the shaker for 10 min. The solution is then filtered through Whatman no.1 filter paper to remove undissolved crystals.

1.3 Treatment of dialysis bag

The dialysis bags are treated by boiling in 200 ml of 0.05 M EDTA solution for 30 min. The bags are then transferred to distilled water or buffer and boiled again for 30 min. The treated bags are stored in buffer at refrigerated temperature.

APPENDIX - 2

2.0 BUFFERS

2.1 Phosphate buffer

a) 0.5 M phosphate buffer

Potassium dihydrogen phosphate	: 13 g
Dipotassium hydrogen phosphate	: 26 g
Distilled water	: 500 ml

0.05 M phosphate buffer is prepared by diluting the stock solution.

b) Phosphate buffered saline

Sodium chloride	: 8 g/l
Potassium chloride	: 0.2 g/l
Potassium dihydrogen phosphate	: 0.2 g/l
Disodium hydrogen phosphate	: 2.9 g/l

pH 7.4 stored at 4^{o} C.

2.2 Tris (hydroxy methyl) amino methane HCl (Tris HCl) buffer

Stock solutions

A - Tris base (0.2 M)	: 24.2 g
Distilled water	: 1000 ml
B - HCl	: 4 ml
Distilled water	: 49 ml

Mix 90 ml of A and 26.8 ml B to get a buffer of pH 8.0

50 ml of solution A + x ml of solution B diluted to 200 ml

pH	X
9.0	5.0
8.8	8.1
8.6	12.2
8.4	16.5
8.2	21.9
8.0	26.8
7.8	32.5
7.6	38.4
7.4	41.4
7.2	44.2

2.3 Tris EDTA buffer (TE buffer)

	Stock solution	**volume**
Tris	60.570 g/ml	250 ml
EDTA	37.22 g/ml	5 ml
2-mercaptoethanol	0.75 ml	0.75 ml

pH is adjusted to 7.2 with 5 N HCl at RT.

Distilled water is added to make the volume to 500 ml

APPENDIX - 3

3.0 REAGENTS

3.1 Visualising Agents

Resorcinol in methanolic sulphuric acid

Resorcinol : 2 g

Methanol : 100 ml

Dissolve and make volume to 500 ml to 2% sulphuric acid

3.2 Colorimetric Reagents

a) For santonin assay

i) 18 N sulphuric acid

36 N sulphuric acid : 50 ml

Distilled water : 50 ml

ii) 0.8% ferric chloride

ferric chloride : 800 mg

Distilled water : 100 ml

b) For protein estimation (Lowry's reagents)

Reagent A:

Sodium carbonate : 100 g

0.5 N sodium hydroxide : 1000 ml

Reagent B:

Copper sulphate : 1 g

Distilled water : 100 ml

Reagent C:

Potassium tartaurate : 2 g

Distilled water : 100 ml

Reagents A, B, C are mixed in the ratio of 0.75 : 0.15 : 0.15 to make reagent ABC.

c) EDTA solution 0.5M

Disodium EDTA : 186.1 g

Distilled water : 800 ml

Few sodium hydroxide pellets are added to adjust the pH of the solution to 8.0 to dissolve EDTA.

3.3 Reagents for Polyacrylamide Gel Electrophoresis

3.3.1 Acrylamide solution

Acrylamide : 29.2 g

Bis acrylamide : 0.8 g

Distilled water : 40 ml

Final volume with distilled water made upto 100 ml and filtered through Whatman no.1 filter paper.

3.3.2 Upper Tris (0.05 M)

Tris base : 3.03 g

SDS : 0.2 g

Distilled water : 50 ml

pH is adjusted to 6.8 with concentrated hydrochloric acid.

3.3.3 Lower Tris (1.5 M)

Tris base : 18.16 g

SDS : 0.4 g

Distilled water : 100 ml

pH is adjusted to 8.5 with concentrated hydrochloric acid.

3.3.4 Ammonium persulphate solution (10%)

Ammonium per sulphate	: 0.1 g
Distilled water	: 1.0 ml

3.3.5 Sample buffer (Laemmli, 1970)

Upper Tris buffer	: 1.25 ml
10% SDS	: 3.0 ml
Glycerol	: 1.0 ml
Distilled water	: 4.75 ml
Mercaptoethanol	: 0.5 ml

3.3.6 Running buffer (pH 8.3)

Tris base	: 3.03 g
SDS	: 1 g
Glycine	: 14.4 g
Distilled water	: 1000 ml

3.3.7 Destaining solution

	I	II
Methanol	500 ml	70 ml
Acetic acid	100 ml	50 ml
Distilled water	400 ml	880 ml

3.3.8 Staining solution

Coomassie blue	: 1g
Methanol	: 500 ml
Acetic acid	: 100 ml
distilled water	: 400 ml

solution is filtered through Whatman no.1 filter paper.

3.3.9 Dye solution

Bromophenol blue	: 0.1g
50% sucrose solution	: 100 ml

APPENDIX - 4

4.0 PREPARATION OF MEDIA

4.1 Composition of mineral medium

Constituents	Final concentration in the medium (g/100 ml)	Stock solution (g/100 ml)	Stock solution in medium (ml)
Distilled water	100 ml	-	91.224
*$FeSO_4.7H_2O$	0.006 g	-	0.006 g
K H_2PO_4	0.63	12.6	5
KH_2PO_4	0.182	18.2	1
NH_4No_3	0.1	10.0	1
$MgSO_4.7H_2O$	0.01	1.0	1
$Na(MO_4)_2$	0.00006	0.6	0.01
$MnSO_4$	0.00006	0.6	0.01
$CaCl_2.2H_2O$	0.0075	1.0	0.75

*added as solid

Chemicals are added in the order given in the table

4.2 Preparation of liquid and solid media

Media	DS Mineral medium (ml)	Distilled water (ml)	Glucose (g/100 ml)	Santonin (g/100 ml)	Agar (g/100 ml)	Km ml
Santonin (L)	50	50	-	0.4	-	-
Santonin (S)	50	50	-	0.4	2	-
Glucose (L)	50	50	0.4	-	-	-
Glucose (S)	50	50	0.4	-	2	-
Glucose Km (L)	50	50	0.4	-	-	0.2
Glucose Km (S)	50	50	0.4	-	2	0.2

Km = Kanamycin stock solution (2.5 mg/ml) filter sterilised and added separately.

Ds = double strength; (L)= liquid; (S)= solid.

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Release of periplasmic proteins from *Sphingomonas paucimobilis* strain S ATCC 43388

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Alkaline phosphotase and α-santonin binding proteins were released in the periplasmic shock fluid from *S. paucimobilis* strain S ATCC 43388, on treatment with freeze thaw (FTM), cold osmotic shocking (COSM) and chloroform (CHLM) method. Such cells devoid of periplasmic proteins showed corresponding reduction in the transport of α-santonin. Amongst the methods employed FTM was found to be most effective,

Sphingomonas paucimobilis strain S ATCC 43388 initially identified as *Pseudomonas* sp. is capable of utilizing α-santonin, a hydrophobic and feebly soluble sesquiterpene, as a sole source of carbon[1-5]. Initial interaction of these cells occurs with α-santonin available in soluble form[3]. Periplasmically located substrate binding proteins of gram negative bacteria play an important role in the initial interaction and uptake of various compounds in soluble form[6-9]. Several methods are used for elution of periplasmic proteins to study their role in the transport[7-9]. Organisms are likely to respond differently to individual method used for release of periplasmic proteins, depending on the cell surface composition[10]. The present study has been undertaken to compare different methods employed to elute out periplasmic proteins from *S. paucimobilis*.

Microorganisms and media—*S. paucimobilis* strain S ATCC 43388 was maintained on 0.4% santonin agar slants[1]. The cells grown in 0.4% santonin agar plates at room temperature for 24 hr were suspended to a desired cell density of 4.5 OD at 450nm (12.5 mg wet weight). Wet weight of the cells was determined after decanting the suspending fluid following centrifugation.

Chemicals—α-santonin was obtained from Alchem International Pvt. Ltd., New Delhi. All the chemicals used for media preparations, buffers and reagents were of analytical grade. Tris hydroxy methyl amino methane, potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate were obtained from Qualigens.

Extraction of periplasmic proteins—Periplasmic proteins were extracted from the cells by three methods viz cold osmotic shock (COSM)[7], chloroform extraction (CHLM)[8] and multiple cycles of freezing (−20°C) and thawing (FTM)[9].

Analysis—The shock fluid obtained by each of the above methods was passed through 0.45μm Millipore filter and analyzed for protein content[11], alkaline phosphatase activity[12].

α-santonin binding activity of shock fluid was determined by equilibrium dialysis binding[13]. Shock fluid was dialyzed overnight against saturated α-santonin solution in 0.05 *M* phosphate buffer (*p*H 7) at room temperature, with constant stirring. The contents from the bag were extracted with chloroform and analyzed for α-santonin colorimetrically[3,14].

Rate of uptake of α-santonin by treated and untreated cells was measured by estimating the amount of undepleted α-santonin in the reaction mixture, by the method reported earlier[3,14].

Substrate binding proteins situated in periplasmic space play important role in nutrient transport, demonstrated by their specific binding to the substrates and lack of transport of substrate in the cells devoid of such transport proteins. Efficient release of periplasmic proteins from the organisms in entirety/is desirable. Depending on cell surface characteristics, each organism responds differently to the physicochemical treatments and the release of periplasmic proteins thus varies with the method employed[10]. Chloroform is reported to release maximum periplasmic proteins from *Pseudomonas*[10]. However in the present work with *S. paucimobilis strain S* FTM was more efficient and released more periplasmic proteins than COSM and CHLM (Table 1).

Alkaline phosphatase, a periplasmic enzyme is often used to monitor the release of periplasmic proteins[10]. Eluant of FTM showed release of 10.5 μmoles of *p*-nitro phenol, PNP/ml as compared to COSM and CHLM, the trend corraborating the total proteins in the shock fluid, released by each of the three methods (Table 1). Invariant specific activity of

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the enzyme in shock fluids indicated that the methods differed in their efficiency to release periplasmic proteins only quantitatively. High α-santonin binding activity (2.40μmole/mg of protein) in the periplasmic proteins released by FTM reduced the α-santonin transport in the concerned shocked cells by 90%, on comparison to only 70% by COSM (Table 1). This confirms the periplasmic location of α-santonin binding factor and the efficiency of the FTM in eluting them. This method also has selective advantages over other conventional methods, as it does not involve any chemical treatment such as lysozyme, EDTA, chloroform or toluene and releases selectively periplasmic proteins without loss of apparent cell damage.

Effect of physicochemical parameters on release of periplasmic proteins by FTM

Cell density—Amount of protein released by FTM increased significantly with increase in the cell density (Fig. 1A) from 2.6-12.5 mg/ml and plateaued thereafter, possibly due to the decrease in inter cellular solute space and cell to cell surface contact. Cell density with absorbance of 4.5 optimally elutes periplasmic proteins.

Table 1—Analysis* of periplasmic proteins from *S. paucimobilis* strain S ATCC 43388 cells released by three methods

	Method used		
	FTM	COSM	CHCM
A. Analysis of Released Proteins			
1. Total Protein released (mg/g of wet cell)	8.0	3.8	0.89
2. Alkaline Phosphotase[a] (μmole/ml supernatant)	10.5	5.0	1.3
3. Alkaline phosphotase[a] (μmole/mg of protein)	26.2	26.3	29.2
4. α-santonin binding activity (μmole santonin/mg protein)	2.40	1.61	0.30
B. Transport activity of the shocked cells			
1. Rate of uptake[b] (μg α-santonin/ml/min)	0.7	1.5	ND
2. % Reduction in transport	90	70	ND

*mean values of observations in triplicates.
[a]expressed in terms of Paranitrophenol (PNP) released from PNP phosphate.
[b]Rate of uptake of α-santonin by untreated wild type cells is 7.3 μg/min.

Freezethaw cycles—The proteins were released in the shock fluid partially after the first and to a maximum extent after the second FT cycle and with no further increase on subsequent FT cycles (Fig. 1B). A single overnight freezing and thawing[9] of cells at −20°C released less proteins than the two freeze thaw cycles.

Temperature—Cells freezed more effectively at −20 or −70°C and released 110μg/ml of periplasmic proteins, possibly due to rapid ice-crystal formation resulting in alteration of membrane permeability[15], as against release of only 70μg/ml proteins at −4°C (Fig. 2C).

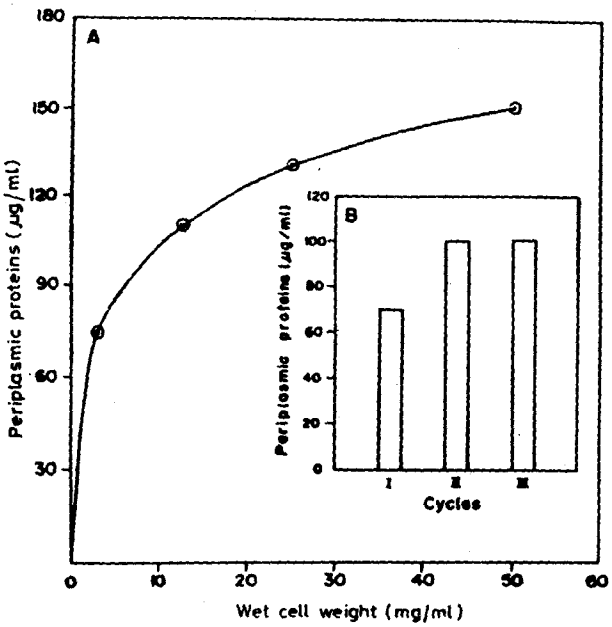

Fig. 1—Standardization of freeze thaw method. (A) Effect of cell concentration on release of periplasmic proteins. (B) Amount of proteins released after each cycle of freeze-thawing from the cells suspended to an absorbance 4.5 at 450nm.

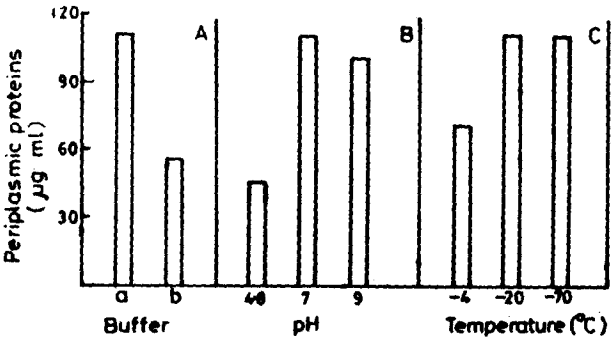

Fig. 2—Effect of A: buffer: (a) phosphate, *p*H 7.0, 0.05*M*, (b) Tris HCl; B: *p*H; and C: temperature, on the amount of periplasmic proteins released by FTM.

Buffer and pH—The amount of proteins released may vary with the solutes in the suspending medium[16]. The cells suspended in Tris HCl *p*H (7.0) eluted only 55μg/ml as compared to 110 μg/ml released by cells suspended in phosphate buffer, *p*H (7.0) (Fig. 2A). At acidic *p*H (4.8), proteins release was reduced significantly to 45μg/ml (Fig. 2B).

The shock fluid eluted from *Sphingomonas paucimobilis* strain S ATCC 43388, suspended in 0.05*M* phosphate buffer by FTM two cycles showed, high periplasmic protein content, alkaline phosphatase activity and α-santonin binding activity. This method is therefore suitable for extraction of periplasmic proteins especially α-santonin binding proteins from *S. paucimobilis* strain S ATCC 43388.

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