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STUDY OFDEVELOPMENTAL STAGE OF CEPHALOSPORIN β-LACTAMGROUP OF ANTIBIOTICS [BACTERIAL MEMBRANE PROTEINS (BMP)]

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ABSTRACT:

Time is moving for open and difficult challenges of survival process of human being in nature. We think everything about life depends on energy as nothing is beyond energy limits. Man has always lived in an environment that abounds with both living and non-living entities. Micro-organisms can be defined as living creatures so small that individuals cannot be seen without the aid of microscope. Bacteria are the simplest organisms to which the term cell can be applied. They are the smallest form of organic life that is visible under a compound microscope. Because of their ubiquitous nature, a number of studies have been carried out on these organisms. Now in current research or study is showing thatthe molecular orientation of bacterial cell wall is responsible in determining drug resistance. The role of cephalosporins for e.g.: ceftriaxone &cefazolin was studied in relation to its toxicity to *B. subtilis*(Gram positive) &E. coli (Gram negative). In the presence of cefazolin protein release was observed to been enhanced in contrast to ceftriaxone. The protein export in case of intact bacterial cells was higher than membrane vesicles suggesting the involvement of membrane proteins in drug sensitivity/resistance. The extent of protein released was also found to be modulated when both the cells were subjected to temperature treatment. However, maximum protein export was seen when gram positive& gram-negative cells were subjected to EDTA concentrations. From this study it can be concluded that outer membrane orientation determines the therapeutic value of cefazolin& ceftriaxone.

Keywords: Cephalosporins, Ceftriaxone sodium, Cefazolin sodium, EDTA

[I] NTRODUCTION

Gram- positive and gram- negative bacteria are basically classified on the basis of their cell wall architecture. The envelops of different microorganisms differ considerably. A gram negative bacterium has more complex structure as compared to its counterpart, i.e., gram positive bacteria. In bacterial cells the main cellular components has been divided into cytosole,

cytolasmic membrane and cell wall. The cytosole surrounded by cytoplasmic membrane and outerenvelope is known as cell wall [7]. The cell wall surrounds the inner cytoplasmic membrane, maintaining its shape and protecting mechanically fragile cytoplasmic membrane from rupture due to high internal osmotic pressure generated by the cytoplasm.

The cell wall of gram positive bacteria contains peptidoglycan throughout its thickness, in addition it contains 'accessory' or secondary wall polymers, e.g. techoic acid, trichromic acid, polyphosphates, lipocarbohydrates and proteins [3,5]. The glycan strands of the cell wall consist of repeating disaccharide N-acetylenuramic acid (β-1-4)-N-acetylglucosamine (Mur-Nac-GLcNAC) [11]. In most cases, the D-lactyl moiety of each MurNAC is amide linked to the short peptide component of peptidoglycan [4]. Wall peptides are crosslinked with other peptides that are attached to neigbourig glycan strand, thereby generating a three dimensional molecular network that surrounds the cell and provides the desiredexoskeletan function [9].

The outer membrane of the gram-negative cell wall has a bilayer structure (exterior to the peptioglycan) and is composed of lipopolysaccharides (LPs), phospholipids, lipoproteins and proteins. The outer leaflet of the bilayer contains all the LPs which is negatively charged and highly variable in its attached sugar moieties, and also responsible for the antigenic variability. The hydrophilic external polysaccharides of LPs, are covalently linked via a phosphorylated core of the 'Lipid A' moiety which is hydropobically anchored in the lipid layer. The inner leaf contains majority of the phospholipids and the embedded Braun lipoprotein links the outer membrane to the peptidoglcan [1,3]. Traversing both leaves and breaching the hydrophobic barrier posed by the lipid bilayer are the major outer membrane proteins (Omp) or porins, which provides aqueous channels for the access of hydrophilic molecules including antibiotics with a limited size range across the membrane. This size range is generally upto 600 daltons, based on the gross physio-chemical properties of molecular size, hydrophobicity and charge [2, 21]. The cytoplasmic membrane is the final and most serious barrier to entry of macromolecules. In both types of bacteria, it is a lipid bilayer, primarily made up of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, out variations are also found. Embedded in or attached to the membrane are numerous proteins such as the enzymes assembling peptidoglycan, enzymes of the respiratory chain, transport proteins, etc. **[Table-1]**.

S.No.	Characteristics	Gram-Positive	Gram-Negative
1.	No. of major layers	1	2
2.	Chemical makeup	Peptidoglycan techoic acid, Lipotechoic acid	Lipopolysaccharides, Lipo proteins peptido Glycan
3.	Overall thickness	Thicker 08-20 nm	Thinner 08-11 nm
4.	Outer membrane	No	Yes
5.	Periplasmic space	Present in some	All
6.	Porin proteins	No	Yes
7.	Permeability to molecules	More permeable	Less permeable

Table: 1.1. Characteristics of Gram +ve and Gram-ve

Many antibiotics exert their action either in or outside the cytoplasmic membrane, and only

those, which have targets within the cytoplasm, must find means of penetration of the cell interior.Whereas, the fungal cell envelope is a complex structure in which the extension zone (hyphal tip) is bound by a wall made up of chitin or cellulose micro-fibrils embedded in the matrix of proteins. Glycans and glycoproteins may also be incorporated. The inner layer of the secondary wall is made up of chitin, overlaid by a proteinaceous layer, outside which is a glycoprotein reticulum, again overlaid by a layer of mixed α and β-glucans [6]. The fungal membrane contains high proportions of carbohydrates in addition to phospholipids and sterols such as cholesterol and ergo sterol. The relative proportions vary according to the growth phase, which in turn affects the response of the organism to antimycotic drugs.

The structure of viruses have also been reviewed which possess bilayer lipid membranes (enveloped viruses), acquired on budding through host cell membrane, reflecting the composition of those membranes, and usually contain one or more surface glycoproteins; others are without membranes [8]. Several bacterial groups lack cell wall structure of typical Gram-positive and Gram-negative bacteria whereas some bacteria have no cell wall at all, inspite of that they appear to be positive and negative in the gram stain for eg. The cells of Mycobacterium and Nocardia contain peptidoglycan and stain gram negative but the bulk of their cell wall is comprised of unique type of lipids, one of them is a very long chain fatty acid called mycholic acid that contributes to pathogenicity and provides resistance to certain chemicals and dyes.

In some bacteria during the life cycle cell can lose these outer walls and deficient forms thus generated are referred to as L-forms or L-Phase variants (L for Lister Institute, where they were discovered). L-forms can be induced artificially by treatment with lysozyme or penicillin that disrupts the cell wall integrity. When gram-positive cell is exposed to either of these two chemicals cell wall synthesis is completely hampered / disrupted and become protoplast. However when a gram-positive cell is exposed to such chemicals it loses its peptidoglycan, but retains its outer membrane and spheroplast is formed.

Since bacterial cells are prokaryotic in their cellular organization therefore the cell membrane provides a site for various functions such as energy reaction, nutrient processing and biosynthesis. Apart from this, the major action of cell membrane is to regulate transport of nutrients, as well as, waste from external environment to cell interior. The cytoplasmic membrane of bacterial cell is selectively permeable with special carrier mechanism for passage to various molecules. The glycocalyx and cell wall act as a barrier for the passage of large molecules but they are not the primary transport apparatus. The cell membrane is also involved in secretion of discharge of metabolic product into extra cellular environment. In addition, bacterial membranes are important site for a number of metabolic activities. In these cells, most of the enzymes of respiration and other energy processing activities are located on the membrane. Enzyme system located on the cell membrane also synthesize structural macromolecules to be incorporated into the cell envelop. These membranes are also responsible for secretion of enzymes and toxins into the environment.

The cytoplasmic membrane lies directly outside the cytoplasm **(Fig 1)**. It acts as a selective permeable barrier between the cytoplasm and the cell environment and is also the site at which may important and in primarily comprises of phopholipids and proteins. The membrane proteins are enzymes and carrier proteins, the latter mediating the specific transport of nutrients and ions. The membranes proteins constitute about 20% of the total bacterial proteins, which are mainly lipophilic on their surface. The electron transport system, the cytochromes, quinones, iron-sulphur proteins and flavin adenine dinucleotides are embedded in the cytoplasmic membrane,

which acts membrane (transmembrane proteins) or they may be exposed on only one face. The cytoplasmic membrane of *Bacillus subtilis* contains predominantly derivative of ethanolamine in the inner layer and glycerol in the outer layer. During metabolism, protons are extruded to the exterior of the bacterial cell, the net result being acidification of the cell exterior, which also becomes positively charged relative to the cell interior. This combined potential, the concentration or osmotic effect of the proton and its electropositivity, is the electrochemical potential of the proton (ΔμH+) which can be quantified and expressed in terms of electrical units (mV). It is the potential of the protein motive force (Δp) which derives ancillary activities [10]. Energy transduction can occur (1) in membranes or (2) in non-membranous components of the cell. In these two processes two different forms of convertible energy currencies are used. These are $\Delta\mu H^+$ and ATP in (1) and (2) respectively.

According to **Fig. 1,** the transmembrane differences in the electrochemical potential of H^+ ions (ΔμH or protonic potential) holds a central position in the energy transduction pattern occurring in 'protonic' membranes. $\Delta\mu H^+$ consists of electrical, chemical and concentrational (osmotic) components, that is, the electric potential difference (ΔΨ) and the pH difference [ΔpH, 35].

Bacillus subtilis is taken as model system and is most widely studied and thoroughly mapped gram-positive microorganism. Studies have focused on many different aspects of this species, including biochemical and morphological changes during sporulation, germination, and outgrowth mechanisms of genetic exchange in transformation and transduction; cell wall synthesis and cell growth and division, enzymatic characterization of many catabolic pathways; and host of others.

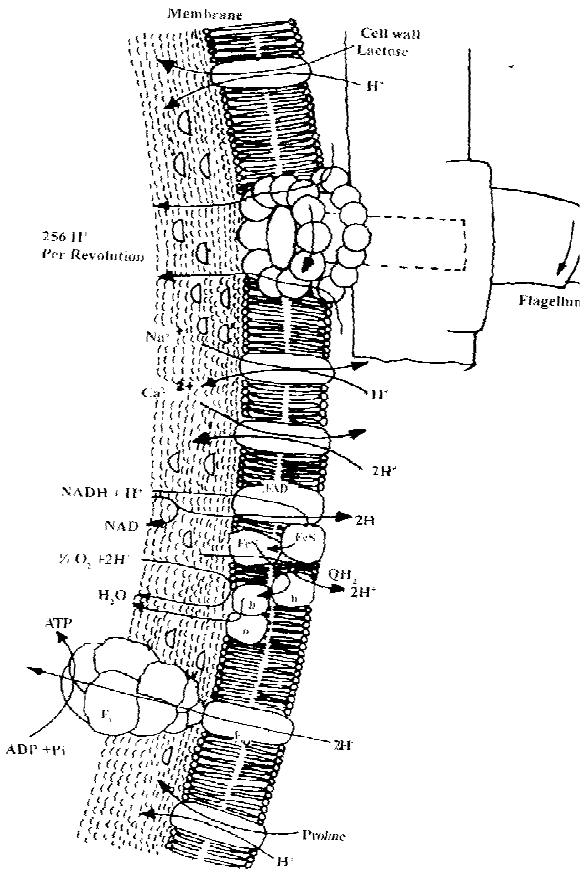


Fig: 1. Cross section of the bacterial outer membrane showing selective permeability of Cytoplasmic membrane (Source: Russell and Chopra., 1990).

The antibiotic penicillins and cephalosporin contain β-lactam ring. Various penicilline and cephalosporin antibiotics contain different substitute groups and exhibit different spectrum of antibacterial activities. Because of these different properties various penicillin and cephalosporin are preferred in the treatment of specific disease .The antibiotic penicillin is produced the filamentous fungus *Penicillumcrysogenum*.Similarly Penicillin N is produced by another fungus known as *Cephalosporiumacrenominum*[13] discovered another type of penicillin having different derivative of β-lactam compound. This penicillin is categorized as cephalosporins. The structure of cephalosporin C was discovered by Abraham and Newter (1961) and X-ray crystallographic analysis confirmed the structure of this antibiotic. [55] The discovery of Cephalosporin C revolutionized the

whole concept of clinical importance of β-lactams. [22]. The mode of action of β-lactam antibiotics involves the inhibition of peptidoglycan biosyntheses. During the inhibition process these β-lactam antibiotics binds to specific proteins located on the cytoplasmic membrane or in the periplasmic space between the inner and the outer membrane of the gram negative bacteria. These specific proteins are popularly known as penicillin binding proteins (PBP's). These proteins are evolved in transpeptidase, transglycocylase, D-D-carboxypeptidases and endopeptidases activities.

On the basis of the chemical structure β-lactam has been classified into five major groups.

Irrespective of their chemical structure the entire β-lactam compounds have four membered β-lactam rings. β-lactam compound are synthesized by variety of microorganisms ranging from prokaryotic to eukaryotic species [25]. The structure of β-lactam ring of penicillin and cephalosporin is in illustrated **(Fig. 1.1)**. The penicillin nucleus of β-lactam ring contains side chain of 6- aminopenicillinic acid and in cephalosporin nucleus of β-lactam ring 7-aminocephalosponic acid is seen. Modification of these side chains generates new species of antibiotics. These newly naturallyoccurring β-lactam class of antibiotics [30].

Antibiotics are anti-microbial substances produced by microorganisms. Specifically they are the secondary metabolites produced by microorganisms. About 15,000 antibiotics have been discovered and of these, about 200 have been approved for human use [24]. The availability of these clinically effective anti-microbial agents has regularly been followed by the rapid emergence of strains of resistant to them. This has seriously reduced the therapeutic value of many important antibiotics and has posed fresh challenges demanding new strategies in trying to circumvent this problem. Consequently it is a major stimulus to the pharmaceutical industry in its constant search for newer and more effective anti-microbial drugs [39]. This calls for a better understanding of bacterial resistance in different fields of biology, medicine and evolution. Besides problems with the antibiotic resistance, the world market for antibiotics have increased from 28 billion US $ in 1996 to 45 billion US $ in 2004 and is expected to grow further by 10% in terms of sales by coming few years [44].

There is a deep relation between structure and activities of cephalosporins and penicillins [26, 51]. However, the analysis is difficult to get strong view on relationship between structure and activity as inhibition of the target enzyme(s) since the measured value (MIC) depends upon at least three processes: penetration of the antibiotics into the cell, its sensitivity to the β-lactamase(s) which the cell might produce and finally, its interaction with the target enzyme(s).

Irrespective of their complex structure all the β-lactam compounds have four membered β-lactam rings. The basic structure of cephalosporins (7-amino cephalosporanic acid) contains three components: dihydrothiazine ring (6-membered), an attached β-lactam ring (4-membered) and a side chain ofCephalosporinswere brokendownpreferably by three types of bacterial enzymes (i) β-lactamases (ii) amidases,and (iii) esterases.

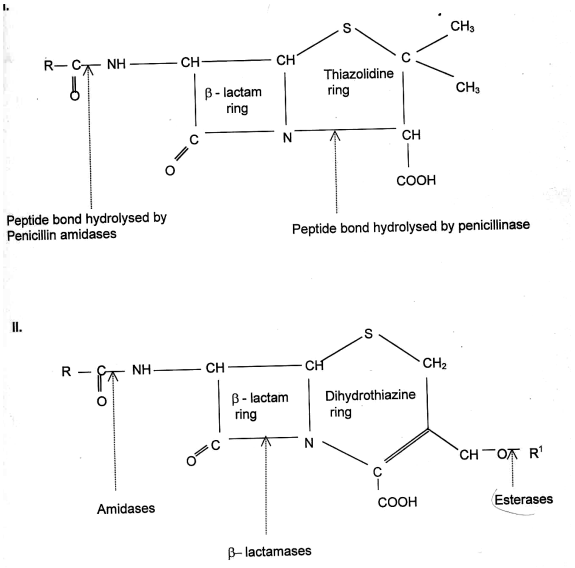


Fig.1.1: General structure of Penicillin &Cephalosporins showing sites of action of enzymes.

In cases (i) and (ii) peptide bond is hydrolysed to yield peniciollic acid and 7-aminocephalosporanic acid (7-ACA) in cephalosporins. These enzymes decrease biological activities of the antibiotic but they do not alter its characteristic bicyclic structure. **(Fig. 1.1)**

Cephalosporins on the basis of their time of introduction as well as biochemical and biological properties can be grouped in to five generations:

First Generation cephalosoporins:

As soon as 7-ACA became available, numerous novel cephalosoporins were prepared by reacylation process [27] using methodologies developed in penicillin field. This provided cephalothin, the first semi synthetic cephalosporin to reach the therapeutic market. It has moderate range of activity and has been extensively used.

Many variations were prepared and tested on 3- and 7- substituents but few of them reached to the final efficacy. The most successful were cefazolin and cefamandole [32], which are two to four times more active than cephalothin against susceptible gram-negative bacteria. They are still the leading injectable cephalosporins.

Second Generation Cephalosporins:

Following the wide spread use of Penicillins and Cephalosporins, bacteria (notably, Enterobacteria) resistant to them become increasingly frequent in clinical specimen and as pathogens. They include cefuroxime, cefamandole and cefoxitin, active against *staphylococci* and a wide range of gram-negative bacteria. Cefoxitin in particular is active against anaerobes.

Third Generation Cephalosporin:

This group of β-lactam has increased antibacterial potency against wide range of organisms. Resistance to β-lactamases is the characteristic feature of this group of cephalosporins. Cefotoxine, ceftizoxine, ceftriaxone are some names, which comes under this generation. They are up to 100 fold more active against many species that were regarded as sensitive to their predecessors.

Fourth Generation Cephalosporins:

This generation of cephalosporins is with high potentials against wide range of microorganisms. They are also been denoted as broad-spectrum cephalosporins with enhanced activity against gram-positive bacteria and do posses β-lactamase stability. Cefepine, cefprione comes under this generation of cephalosporins. [40].

Recently several observations of pharmaceutical applications are being made to emphasize role of newly synthesized anti-microbials suitable for selective drug delivery. However, not much importance is projected in relation to requirement of newly identified effective drugs. β-lactams offers greater advantage in clinical purposes, as it appears to have wider effective sites in host/pathogen. Looking into the necessity of affective treatment procedures it becomes essential to explore the behavior of causative organism in response to treatment of drugs. Since, every biological metabolic reaction is being governed by existing exterior stimulus there by modifying the effectiveness of drugs. In order to study and explore the probability of involvement of bacterial outer-membrane in determining drug sensitivity. Cefazoline and Ceftriaxone are selected in present study. Although involvement of several membrane factors have been reported in other microbes. The study includes the elaborated concept of involvement of bacterial outer membrane in confirming resistance /susceptibility to CT and CZ, when exposed under laboratory conditions.

Presently, Gram positive, *Bacillus subtilis* bacterial species is selected for carrying out the observations. The results exploring the physiological modification involved in development of drug sensitivity under laboratory conditions. In view to understand the role of cellular enzymes (functional proteins) in drug (CT and CZ) sensitivity / susceptibility effect of both the drugs is seen in the growing culture of *Bacillus subtilis*.

Lastly characterization of outer membrane proteins is done confirm the probable role of cellular protein in determining drug sensitivity.

[II] MATERIALS AND METHODS

2.1Organism and Culture Conditions:

Bacillus subtilis NCIM 2063 strain used for the present study is a non-pathogenic Gram- positive rod, and obligate aerobe (Plate 01: a; b). It is known to form protective end spore thereby providing tolerance to extreme environmental conditions. It was obtained from National Chemical Laboratory (NCL) Pune, India. The bacterial culture was routinely maintained on presterilised NAM at 04^0C. The culture was routinely monitored by single colony isolation method on Nutrient Agar plates for confirmation of purity of culture.

Chemical as well as reagents used in the present experiments were of reagent grade. However, antibiotics preferred for the experimental purpose for eg. Ceftriaxone (CT) sodium and Cefazolin (CZ) sodium were procured from Lupin Laboratories Ltd. M.P., India under the common generic names.

2.2 Assay of protein profile using HPLC:

Confirmation of stress proteins as evident by the presence of β-lactams , CT and CZ was done by the HPLC (Shimadzu model LC-10AT) for the study of molecular weight determination of proteins found in the sample Bovine serum albumin (67 kDa), Ovalalbumin (45 kDa), Chymotrypsinogen (25 kDa), Cytochrome (12.5 kDa) and Insulin (0.57 kDa) was used in pure form. The migration of protein in the column was observed when 25 µl of protein samples were injected. The proteins show different rate of movement due to their different molecular size for every sample 4000 psi pressure was maintained and flow rate was adjusted to 1.0 ml min-1 the solid phase was protein-PAK column (Shimadzu) and mobile phase was 0.1 M phosphate buffer (pH 6.8). The proteins were measured at absorbance of 280 nm after carrying out elution process for 30 minutes. Protein –PAK column (7.8 mmx30 cm, total permeation volume of 12 ml) provide rapid separation, purification and characterization of proteins using gel filtration. These columns are packed with a rigid hydrophobic porous silica gel and are manufactured with exclusive bonding processes that improve column stability and minimize non-specific adsorption. HPLC technique offers advantages of speed and specificity and uses equipment that is versatile in its operation. Column is the essential feature of HPLC. The column allows high resolution at speedy flow rate. HPLC offers advantages in speed and ease of sample recovery. Therefore, it offers greater advantage over electrophoresis and open column chromatography. The column Chromatographic analysis helps in separation and identification of biological samples. Allowing the sample to move in a column containing partitioning material and eluting the mixture by pumping the solvent through the column do this.

2.3 Effect of various factors:

The concentrations used for CT and CZ were 1.5 ppm and 18 ppm respectively. The membrane vesicles of *Bacillus subtilis* thus prepared are treated in buffer solution and samples are separated by centrifugation at 10,000 rpm for 10 minutes (Remi, C-24 refrigerated centrifuge). The detection of protein was done at 0, 30 minutes time intervals. Using HPLC at 280nm pH, temperature and EDTA are used as stress conditions similar to growth studies in the presence and absence of β-lactam antibiotics (CT and CZ). Total protein content of *Bacillus subtilis* was measured and the membrane vesicles are also being considered for detection of membrane protein. Similar to detection of stress proteins the data is supported by estimating cellular enzymes in the presence of varying physiological condition of pH, Temp, and EDTA etc.

[III] RESULTS

The Gram-positive bacteria, *Bacillus subtilis* when subjected to antibiotic stress the profile of protein as studied in the presence of Ceftriaxone (CT) and Cefazolin (CZ) β-lactam antibiotic stress reflects

variation in protein profile by HPLC studies. Similarly *Listeria monocytogenes* Gram-positive bacteria cells has been reported to have acid shock with HCL had significantly greater heat resistance when compared with non-acid-shocked cells [33]. Studies with Gram-negative *Escherichia coli* have shown that a shift to a lower pH induces the synthesis of at least four heat shock proteins [49].It has been shown that acid induced death is the direct result of lowered pH_i [28]. Severe acidic pH creates a situation whereby protons leak across the membrane faster than housekeeping pH homeostasis (the ability of an organism to maintain its cytoplasmic pH at a value close to neutrality, despite fluctuations in the external pH) systems can remove them [34]. The result is an intracellular acidification to levels that damage or disrupt key biochemical processes [20]. Weak acids in their unprotonated form can diffuse into the cell and dissociate thereby lowering the intracellular pH (pH_{in}) and resulting in the inhibition of various essential metabolic and anabolic processes. In response to encounters with acids, microorganisms have evolved complex inducible acid survival strategies [15].

The gradual susceptibility of *Bacillus subtilis* to CT and MIC_{50} (1.5 ppm) in contrast to CZ MIC_{50} (18 ppm) appears to be differential selectivity of bacterial membrane to both the β-lactams even at neutral pH / the sensitivity to drugs was more pronounced at alkaline pH. The integrity of membrane proteins in the influence of CT reflects valuable amount of release of protein **(Fig. 3.3)**, after 30 mins of run shows reason for susceptibility to β-lactam. In the present study the availability of proteins under stress was seen to be modified under various physiological conditions. The appearance of drug resistance in therapeutic purposes under treatment conditions might be responsible for induction of variable protein species under clinical procedures. The appearance of proteins in the medium cannot be confirmed by present investigation that they were genetically induced by β-lactam antibiotics.

Fig:3.1: The Protein released from membrane vesicles of *B.subtils* suspended in Tris-HCl , when grown in varying concentrations of EDTA (0.25 and 0.75 mM) and Mg^{2+}ion (1 and 15mM) in presence and absence of CT and CZ after 30 mins of incubation.

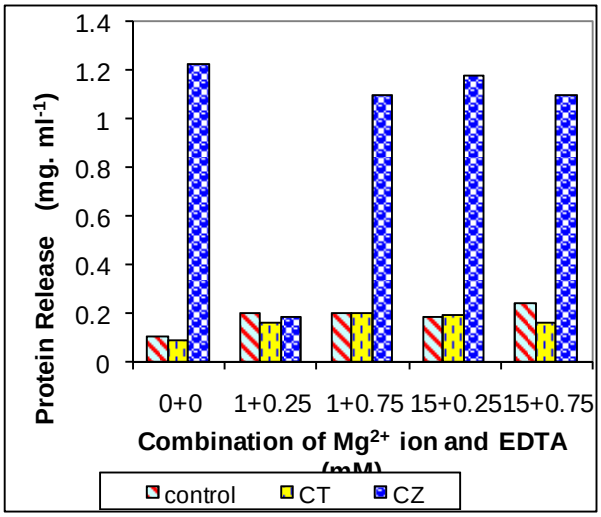


Fig: 3.2 : Molecular Weight determination of standard protein mixture containing Bovine serum albumin 67KD, Ovalalbumin 45 KD, Chymotrypsinogen 25 KD, Cytochrome 12.5 KD, and Insulin 5.7 KD as monitored by HPLC.

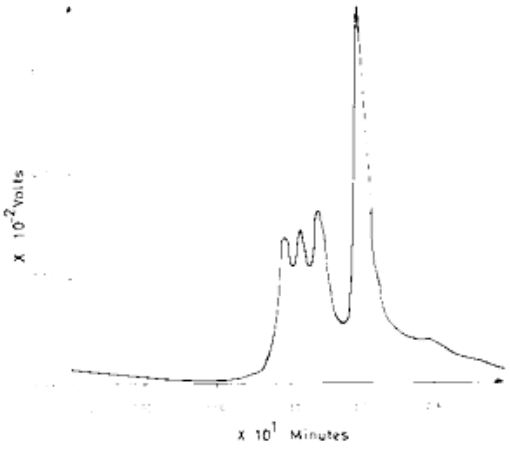
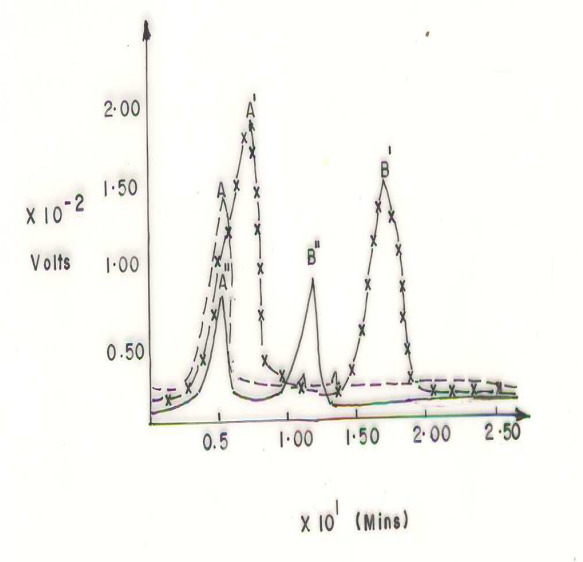


Fig: 3.3: Molecular weight determination of protein released in supernatant of intact cells of *B.subtilis* in the presence of antibiotic stress at pH 5.0 as monitored by HPLC. (-------- without antibiotic,-x-x- presence of CT and presence of CZ



[IV] DISCUSSION

Further on attempt has been made to identify the role of membrane proteins in determining drug sensitivity in Pharmaceutical implications. The transport intake of antibiotics including β-lactams across the bacterial outer surface to cell interior plays an important role with respect to susceptibility of *Bacillus subtilis*. In order to examine, apart from protein quantification observations, in the presence of various stress conditions, the type of protein was examined and compared with standard of known protein molecules **(Fig. 3.2)**. The molecular weight marker proteins selected for the present investigation involves combination of Bovine serum albumin (67 kDa), ovalalbumin (45 kDa), Chymotrypsinogen (25 kDa), Cytochrome (12.5 kDa) and Insulin (5.7 kDa). The growth inhibition of *Bacillus subtilis* as observed at pH 5.0was found to be 86% (maximum) in contrast to CZ **(24%, Fig. 3.1)**. The amount of protein released relates the pattern of growth inhibition in the presence of β-lactam antibiotics as the amount of protein estimated in the medium, outside the cell was fairly enhanced in the presence of CT in contrast to CZ. When HPLC studies were performed under such experimental conditions molecular property of bacterial cell wall reflects the size of proteins present in the supernatant at pH 5.0, 7.0 and 8.0 **(Fig. 3.2 & 3.3)**. With reference to the standard curve of pattern of separation of protein mixture through HPLC **(Fig. 3.2)** the molecular size of protein observed at pH 5.0 in the absence of β-lactam at MIC_{50}. Only one species of protein was seen **(Fig. 3.3)**. As far as, speciation of protein leakage is concerned CT, caused release of protein (A) after 5 mins which falls under category of protein smaller than 5.7 kDa, the known mol. Wt. Of Insulin indicated in standard **(Fig. 3.3)**. With CT the two peaks A' and B' were visible after 6.0 and 17 mins approximately and both fall below 67 kDa, the known mol. wt. of Bovine serum albumin indicated in standard (**Fig. 3.3**). At pH 7.0 in the presence of CZ two species of proteins were visible A" and B" and all appear before 20.0 mins of flow peak C was seen after 15 mins with reasonably low resolution therefore cannot be predicted as a component of *Bacillus subtilis* associated with membranes and thus available in washed form in the supernatant. At highly alkaline pH of 8.0 the toxicity was reduced when *B.subtilis*cells were grown in the presence of β-lactam stress. In the presence of CZ the first peak appeared after 20 mins, whereas CT caused leakage of membrane proteins even after 12 mins of exposure peak A'. The toxicity / sensitivity to β-lactams specially CT was more pronounced and is confirmed by leakage / release of protein (peak A') after 13 mins of running time.

The EDTA chelator was capable of causing growth reduction upto 82%, 84%, 82% in the absence of β-lactam antibiotics, CT (MIC_{50}), CZ (MIC_{50}) respectively **(Fig. 3.1)**. In case of intact cells of *Bacillus subtilis* the protein released in the medium when cells were subjected to EDTA stress was found to be more in the presence CZ (MIC_{50}). However the pattern of protein released from membrane vesicles prepared from *B.subtilis* showed maximum protein in the suspended medium in the absence of EDTA (0.25mM). The washing of proteins from vesicles might be due to presence of Tris ions in the suspension buffer

causing release / washing of inside out membrane vesicles [57, 58] as illustrated in the previous observation. The type of proteins when determined by HPLC shows no remarkable variation in appearance of peaks after withdrawal of sample from 30 mins of incubation. The only reason of similar pattern of appearance of protein peaks might be due to involvement of EDTA only at outer surface not at molecular level. The proteins released occupies molecular ratio below 25 kDa, although peak appearing at 2 minsapprox shows molecular size greater than selected in standard curve i.e. 67 kDa**(Fig. 3.1)**.

When cells were grown in the presence of 1 mM Mg^{2+} ion concentration in combination with chelator 0.25 mM the pattern of protein as observed by HPLC studies in illustrated in **(Fig. 3.2)**. No much effect on pattern of inhibition of growth by chelation (EDTA, 0.25 mM) contrary to, that the protein released from the intact cells when *Bacillus subtilis* was grown in the presence of EDTA (0.25 mM) protein leakage in the exterior of the cell was potentiated by CZ. Therefore, from the present study it could be inferred that the toxicity of CT as observed greater than CZ was not due to washing of membrane proteins but alteration of intake (uptake) pattern regulated by cellular transport mechanism. Similar to EDTA, Mg^{2+} ion alone was found to enhance protein release even at 5mM concentration up to 15 mM in the presence of CZ not in the absence, of β-lactam. However, the absence of Mg^{2+} ions in the growth medium caused similar pattern of protein release into the exterior of the cells. The observation pattern thus confirms that Mg^{2+} does not pay any role in making availability of rather structured, as well as functional protein of *B.subtilis* cells. The resolution analysis of proteins in the presence of 1.0 mM $Mg^{2+}$and 0.25mM EDTA shows appearance of protein peak after 20 mins of sample run in the column showing presence of different molecular configuration of protein greater than 67 kDa when referred with standard curve.

[V] CONCLUSION

The selective nature of organisms to prevailing stress– β-lactam antibiotics is obviously concerned with the architecture of outer membrane. *Bacillus subtilis* Gram-positive bacteria advocates variation in achieving cellular morphogenetic dimensions. It is known to disrupt the barrier of stress applicable in the growth condition by undergoing morphological as well as physiological modifications. The induction of spore formation is an established fact with *Bacillus subtilis*, which offers obstructions in laboratory appearance as well as positive diagnosis due to this property. The therapeutic application of cephalosporins therefore faces a challenging situation to successful, susceptibility for drug treatment and application.

Modifications of compositional structure by strengthening ionic density with reference to cephalosporin administration in clinical trials have become a routine task for therapy. In the present study more protonated species of β-lactam i.e. Ceftriaxone proved to be more effective in contrast to cefazolin as far as sensitivity of *Bacillus subtilis* is concerned. The MIC_{50} for ceftriaxone (CT) was observed to be 1.5 ppm in contrast to first generation β-lactam antibiotic i.e. cefazolin with MIC_{50} 18 ppm concentration. The relative sensitivity of bacterial cells to both the cephalosporins was more positively inclined to third generation species. The role of modified cephalosporin was observed under various physiological situations under laboratory conditions such as pH, Temp, chelating agent and Mg^{2+} ion concentrations. The sensitivity of *Bacillus subtilis* to β-lactam was more potentiated at acidic pH than at alkaline pH. At alkaline pH only 16% inhibition of growth of *Bacillus subtilis* in Dye's minimal media at 37 ± 1^0C was seen in contrast to 60% inhibition of growth in the presence of CZ. The growth was enhanced/potentiated in the presence of chelating agent – EDTA at 0.25 mM concentration in combination with MIC_{50} of CT and CZ 84% inhibition of growth was recorded in the presence

of CT (MIC_{50}) in contrast to 82% inhibition in the presence of CZ (MIC_{50}). The growth inhibition with 0.25 mM EDTA alone was also seen to be 82%. It could be concluded from the present observation that EDTA alone has negative role in bacterial survival. The chelation of ions by EDTA is known to disrupt the orientation and integrity of cell wall thereby causing leakage of cellular substances thus resulting into retardation of *Bacillus subtilis* cell growth. Mg^{2+} at a concentration of 15mM with 0.75mM EDTA was highly effective in all the condition of control as well as with CT and CZ antibiotics. However ATPase activity in the presence of 15mM Mg^{2+} ion + EDTA (0.75mM) was seen to be 56, 18 and 22 $\mu gPi.mg.protein^{-1}.min^{-1}$ in the absence of antibiotics and in the presence of CT and CZ respectively. As far as effect of temperature is observed at 25^0C and 50^0C in relation to drug sensitivity the level of inhibition in absence of antibiotic and in their presence was more pronounced at 25^0C and 50^0C. This might be because of conformational change of *Bacillus subtilis* due to temperature stress. However, at routine temperature of 37^0C no inhibition of growth was recorded in control conditions whereas, both the cephalosporins selected for the present study were found to have similar level of inhibition of growth of *Bacillus subtilis* i.e. 62% and 60% the presence of CT and CZ respectively.

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