MATERIAL AND METHODS
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2.1 Organisms

2.1.1 Wild type - Silver ion sensitive strains

Escherichia coli-10407, Staphylococcus aureus-K1015, and Klebsiella pneumoniae, B-5-1, which were sensitive to 5-10 ppm of AgNO₃, were procured from the culture collection of the Department of Microbiology, Panjab University, Chandigarh.

2.1.2 Mutants

(a) Silver ion resistant strains
(b) Silver ion and antibiotic resistant strains
(c) Silver ion and metal ion resistant strains.

These strains were developed from the wild type strain according to the procedure in Sec.2.6.14.

2.1.3 Recipients

E. coli K 12 and S. typhimurium Phage type 36 were procured from Medical College, Rohtak, India.

2.2 Chemicals

All chemicals used in the study were of the analytical grade obtained from manufacturers of repute. Silver nitrate was 99.9% pure.

2.3 Radioisotope

Radioactive silver nitrate (¹¹⁰Ag) was procured from
Shahba Atomic Research Centre, Trombay
BARC, India (Specific activity - 276 Mci/G Ag). For experimental purposes, this solution was diluted with distilled water prior to use. The final dilution factor was achieved in the medium or the suspending solution.

2.4 Buffers

Various buffers used in the study and the methods of their preparation are given in Appendix I. Chloride free glass distilled water was used for preparation of buffers throughout this study.

2.5 Equipment

The name, model, manufacturers and the application of various equipment used in this work is given below:

<table>
<thead>
<tr>
<th>Name</th>
<th>Model</th>
<th>Manufacturer</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Gilford spectrophotometer</td>
<td>Gilford 250</td>
<td>Gilford Laboratories Oberlin, Ohio U.S.A.</td>
<td>For biochemical and enzymatic assays</td>
</tr>
<tr>
<td>3. Gamma counter (Well-type)</td>
<td>MDS 26</td>
<td>ECI, India</td>
<td>For 110Ag counting</td>
</tr>
<tr>
<td>4. Cold centrifuge</td>
<td>K-24</td>
<td>Janetjki B. Germany</td>
<td>Preparation of cell suspensions</td>
</tr>
<tr>
<td>5. Sorvall continuous flow centrifuge</td>
<td>Sorvall RC 5B</td>
<td>Dupont Instruments New Town, CT</td>
<td>Preparation of cell suspensions and aeroplasts</td>
</tr>
</tbody>
</table>
2.6 Microbiological Methods

2.6.1 Media

The media were prepared from the ingredients manufactured by companies of repute. However, in certain cases, dehydrated media (media Laboratories Pvt. Limited, Bombay, India) were used. Detailed composition of various media is given in Appendix-II.

2.6.2 Sterilization

2.6.2.1 Autoclaving was done at 121°C, 15 psi for 20 min.

2.6.2.2 Filter sterilization, wherever necessary was done by using Seitz filter equipment and, for smaller quantities,Millipore membrane filters were used.

2.6.3 Preparation of Inoculum

A loopful of growth from the stock culture on nutrient agar was inoculated into 10 ml of the nutrient broth and incubated at 37°C for 16-18 h. In some cases the inoculum was prepared in 50 ml of nutrient broth.

2.6.4 Measurement of Growth

2.6.4.1 Visual Measurement: The growth in the liquid media was visually graded between - (no growth) to ++ (good growth).

2.6.4.2 Turbidity: The extent of turbidity indirectly quantitating growth, was measured by transferring 3-4 ml of the growth medium into the matched cuvettes. The absorbance was measured at 540 nm.
2.6.4.3 Viable Count: Generally, 0.1 ml of the sample was added to 9.9 ml distilled water blanks in screw-capped tubes. After vigorous shaking, sample was serially transferred to obtain the desired dilution. The viable count in the sample (0.1 ml) was determined by the Pour-Plate procedure (Nottingham and Wyborn, 1975). In some cases, 0.1 ml of the sample was spread evenly on the pre-poured nutrient agar plates, with sterile glass 'spreaders'. In both cases the plates were incubated at 37°C for 24 h. Longer incubation was used, if the size of the colonies was small. The number of colonies were counted, using a colony counter and the plates showing count in the range of 30-300 colonies were used. Averages of duplicates and dilutions were used to determine the viable count.

2.6.4.4 Dry weight: A known culture volume was centrifuged (8000 g, 15 min, RT). The cell pellet was taken in an aluminium dish and placed in an oven at 200°C till the material was dried to a constant weight.

2.6.5 Media used for growth measurement

The following media were used as indicated:

(a) Nutrient Agar: Total viable count.
(b) Nutrient Agar with AgNO₃: Selective viable count of silver ion resistant organisms.
(c) Mac Conkey Agar: Selective medium for conjugation experiments.
2.6.6 Preparation of Cells

(a) Liquid Media: Generally 50 ml of desired liquid media in 250 ml flasks were used. The flasks were kept on shaker (150 rpm) for the desired incubation period at 37 C. The cells were removed by centrifugation (8000 g, 30 min, 4 C). The supernatant unless, specifically indicated, was discarded. The pellet was suspended in a known quantity of the sterile distilled water. After mixing, the cells were separated again by centrifugation (8000 g, 30 min, 4 C). The process was repeated once again. The pellet of washed cells was suspended in sterile distilled water to give a fixed absorbance. In most of the experiments, it was adjusted to 0.07.

(b) Growth in solid media: The cells for chemical analysis were prepared by growing on solid medium. In such experiments, 180 ml of the nutrient agar was autoclaved in Roux bottles and after autoclaving, media were allowed to solidify on one side of the bottle and 3 ml of the inoculum was carefully spread over the entire surface. The bottles were incubated at 37 C for 24 h. The growth was removed by adding 20-30 ml of sterile distilled water and scraping with a sterile glass rod. The cell suspension was centrifuged (8000 g, 30 min, 4 C) to remove the cells. The washing of the pellet was done in the manner described in section 2.6.6(a).

2.6.6.1 Chemical analysis: The washed cell pellets were lyophilized for analysis(Sec 2.8.3-2.8.6).
2.6.6.2 **Immunological studies:** The washed cell pellet was suspended in physiological saline containing 0.5% formaldehyde to give a count of $10^8$ CFU/ml. Suspensions of cells were placed in screw-capped vials and stored in refrigerator. The viability of the cells was tested by streaking on nutrient agar. The preparation was used for raising antisera (Sec 2.11.1.1).

2.6.7 **Preparation of cell free supernatant (CFS)**

The extracellular enzymes and toxins of the wild type and the mutant strains were determined in the CFS. The test organisms were grown in Brain-Heart Infusion broth for 18 h on shaker (150 rpm) at 37°C. The cells were removed by centrifugation (8000 g, 30 min., 4°C) and the CFS was used without any further processing.

2.6.7.1 **C.F.S. for hemolysin of E.coli:** A heavy inoculum ($10^7$ CFU/ml) was inoculated in 10.0 ml of alkaline extract broth (Smith, 1963) (Appendix II) and incubated aerobically at 37°C for 2½ h. The cells were removed by centrifugation (8000 g, 20 min., 4°C). The CFS was collected.

2.6.8 **Intracellular milieu (ICM)**

Cells from 500 ml of the medium were suspended in 20 ml of distilled water in a 50 ml beaker and placed in an ice bucket. The cells were sonicated (at 150 mA for 20 min for *K.pneumoniae* and 45 min for *S.aureus*). The cell sonicate was centrifuged (9000 g, 45 min, 4°C) and the supernatant represented the ICM. The cell debris, representing the cell
wall and the unsonicated material was discarded.

2.6.8.1 Chemical analysis: The whole cell sonicate (Sec 2.6.8) was centrifuged (3500 g, 15 min, 4 C) and the supernatant as such was analysed for various components (sec. 2.8.1 and 2.8.2).

2.6.8.2 Immunological studies: The ICM (Sec 2.6.8) was filter sterilized using millipore filters and used for raising of antisera (Sec 2.11.1.2).

2.6.8.3 Electrophoretic studies: The intracellular milieu (ICM) was used, after lyophilization, for electrophoretic studies (Sec 2.9.2.1).

2.6.9 Cell walls:

The whole cell sonicate (Sec 2.6.8) was centrifuged (3500 g, 15 min, 4 C). The supernate was recentrifuged (9000 g, 45 min, 4 C). The sediment representing the cell walls was washed several times with distilled water.

2.6.9.1 Chemical Analysis: The cell wall preparation was lyophilized for analysis of various constituents (Sec 2.8.3 - 2.8.9 and 2.6.17-2.6.20).

2.6.9.2 Immunological studies: The cell walls were suspended in distilled water and extracted with butanol (Sec 2.6.16). The protein extract was used for raising of
antisera (Sec. 2.11.1.2).

2.6.9.3 Electrophoretic studies: The washed cell wall preparation was used for electrophoretic studies (Sec. 2.9.2.2) after lyophilization.

2.6.10 Preparation of Spheroplasts

Ref: Lederberg and Clair, 1958.

Procedure

Penicillin sucrose broth (Appendix-II) was used for the preparation of the spheroplasts from K. pneumoniae cells. The medium was prepared in two batches. The batch A (300 ml) did not contain any sucrose, whereas the batch B (700 ml) contained the full complement of 100 grams. The batch A was inoculated with a loopful of the culture from the slant and incubated at 37°C. After 12 h of incubation the culture was mixed with batch B, giving a final sucrose concentration of 10% (w/v). Sterile solutions of penicillin G and Magnesium sulfate were added to give final concentrations of 1000 units/ml and 0.2% w/v respectively. The flasks were incubated at 37°C for 2-3 h and examined for the presence of spheroplasts, under the phase-contrast microscope. The spheroplasts were removed by centrifugation 9000 g, 20 min, 4°C) and suspended in a 10% sucrose solution.

2.6.10.1 Silver uptake studies (Sec 2.12.5.4): The spheroplast suspension was recentrifuged (9000 g, 20 min, 4°C) and suspended in nutrient broth containing 10% sucrose and 0.7 ppm $^{110}$AgNO$_3$. 

2.6.11 Preparation of cell membrane

Ref: Spizizen, (1959)

The spheroplasts (Sec 2.6.10) from 1 litre of broth were suspended in 100 ml of 20% sucrose solution, centrifuged (9000 g, 20 min, 4 C) and suspended in 50 ml, 0.1M NaCl and 0.5 M sodium citrate solution to disrupt the spheroplasts. The suspension was centrifuged (5000 g, 15 min, 4C) and the sediment was discarded. The supernatant was recentrifuged (30,000 g, 20 min, 4 C). The sediment representing the cell membrane was suspended in 0.05 M potassium phosphate buffer, pH 7.2 and centrifuged (30,000 g, 20 min, 4 C).

2.6.11.1 Electrophoretic studies: The sediment representing the washed membranes was used for electrophoretic studies (Sec 2.9.2.2) after lyophilization.

2.6.11.2 Chemical analysis: The membrane preparation was lyophilized for analysis (Sec 2.6.19 & 2.8.9).

2.6.12 Determination of Minimum Inhibitory Concentration (MIC):

The selected concentrations of the test chemical were added to 5.0 ml of nutrient broth and inoculated with a loopful of growth from a 12 h old culture of the test organism. The tubes were incubated at 37 C for 24 h and the growth was determined visually (Section 2.6.4.1). MIC was the lowest concentration at which, no growth was observed.
2.6.13 Determination of Antibiogram
Ref: Bauer, et al. (1966)

Bacterial 'lawns' were prepared on the agar plates by swabbing 0.1 ml of the 12 h old cultures with the help of sterile cotton swabs. Commercially prepared antibiotic discs (Span Diagnostic, India) were placed on the surface of the agar, using sterile forceps. The plates were incubated at 37°C for 24 h and the zone of inhibition was measured in cm.

2.6.14 Development of Resistance

The wild type organism was grown in nutrient broth containing the sub-inhibitory concentration of the test chemical. The organism was grown for a second time in the same concentration of the test chemical and generally, the growth appeared faster on the second passage as compared to the first, at the same concentration. The growth was, then, inoculated into a higher concentration of the test chemical and in general, the increment was 2-3 ppm in the beginning, till growth was observed upto 30 ppm of the test chemical. Further increases in the concentration were made at higher increments (5-10 ppm). This was repeated till the desired resistance levels (70 ppm) were achieved.

2.6.15 Extraction of Capsular material
Ref: Kligman (1948)

Procedure: A known weight of K. pneumoniae cells was suspended in 0.5 N HCl and heated for 35 min at 75°C. The mixture was
cooled and centrifuged (3000 g, 25 min, 4 C). The supernatant was neutralised with equal volumes of 0.5 N NaOH, mixed with 3 volumes of ethyl alcohol, and left for 12-16 h at 4 C. The resulting precipitate was removed by centrifugation (5000 g, 20 min, 4 C), dissolved in distilled water and purified by precipitation with ethanol. The precipitate was separated by centrifugation (5000 g, 20 min, 4 C) and dissolved in distilled water.

2.6.16 Butanol extraction of proteins

Ref: Morton (1953).

Procedure

The aqueous suspension of cell walls was saturated by adding 2 volumes of butanol drop-wise. The dispersion was stirred on a magnetic stirrer for about 30 min. The contents were transferred to a separating funnel to separate the butanol layer (saturated with residue material) from aqueous phase (containing the dissolved proteins). The aqueous layer was collected and centrifuged (80000 g, 15 min, 4 C) to obtain a water clear extract.

The aqueous phase was completely freed from butanol by dialyzing against distilled water. The distilled water was frequently changed and the sample was dialyzed till it was completely free of the characteristic butanol odor. The dialyzed extract was concentrated by dialyzing it against a saturated sucrose solution, so that the final sample contained
at least 0.7-1.0 mg protein per ml. The concentrated protein extract was filter sterilized using millipore filter.

2.6.17 Extraction of the total lipid fraction
Ref: Kates (1972)

Procedure
A known weight (40-50 mg) of the sample was suspended in 1 ml of the distilled water. The suspension was shaken with 3.75 ml of the methanol-chloroform (2:1,V/V) mixture and left at room temperature for about 18-24 h. The mixture was filtered through a Whatman No.1 filter paper. The residue was suspended in 4.75 ml of chloroform-methanol-water (1:2; 0.8,V/V) and filtered. The two filtrates were combined and mixed with 2.5 ml of chloroform and 2.5 ml of water and shaken. The lower chloroform layer was withdrawn and dried under a stream of nitrogen gas. The lipid residue was dissolved in chloroform-methanol (1:1) and stored at 20 °C for analysis.

2.6.18 Preparation of the Phospholipid fraction:
Ref: Kates (1972).

Procedure:
A portion (0.2 ml) of the total lipids (Sec 2.6.17) was mixed with 5.0 ml of chilled acetone and stored in the refrigerator (4-6 °C) for 18 h. The samples were taken out and centrifuged (3000 g, 15 min, 4 °C). The sediment representing
the precipitated phosphatides was freed of excess acetone under a stream of nitrogen. The dried residue was dissolved to a known concentration in chloroform.

2.6.19 Preparation of Methyl esters:
Ref: Prabhudesai (1978)

Procedure:
The phospholipid sample (Sec 2.6.18) containing 100-150 ug of the phosphorous (in chloroform:methanol -1:10) was placed in an ice-water slush. Thionyl chloride at the rate of two ml per 11 ml of the chloroform methanol extract was added drop wise. The samples were removed from the ice-bucket, left at room temperature for 2 h and mixed with 5 ml of chloroform, 5 ml of distilled water. The contents were shaken thoroughly and allowed to separate into two layers. The upper phase (aqueous) was discarded. The lower chloroform layer containing the methyl esters of fatty acids was washed with distilled water to remove water soluble material and water layer was discarded. A total of three or four washings was required. After the final washing, the samples were dried under a stream of nitrogen and stored at -10 C for analysis.

2.6.20 Extraction of Teichoic acids
Ref: Forrester (1966)

Procedure:
A known weight of the cell walls was stirred with 10 ml of 10% cold TCA at 4 C for 48 h and centrifuged (5000 g, 20 min,
4 C). The supernatant was mixed with two volumes of cold ethanol. The precipitate was removed by centrifugation (5000 g, 20 min, 4 C) and washed twice with cold ethanol. The teichoic acid ppt. was hydrolyzed with 2 N HCl for 3 h at 100 C and the inorganic phosphates were estimated (Sec 2.8.6).

2.6.21 Fractionation of bacterial cells:
Ref: Sutherland & Wilkinson (1971).

Procedure:

The cell pellet was suspended in 3.0 ml of 10 per cent TCA solution, incubated at room temperature (RT) for 30 min and centrifuged (3000 g, 10 min, RT). The pellet representing TCA insoluble material was resuspended in 3 ml of 5 per cent TCA solution and heated at 90 C for 15 min. The sample after cooling, was centrifuged (3000 g, 10 min RT) to separate the nucleic acid fraction(supernatant) and the cell residue (pellet). The pellet was suspended in 3.0 ml of 1 N, NaOH solution and centrifuged (3000 g, 10 min, RT). The supernatant contained the cell proteins and the sediment represented the carbohydrate fraction. Each fraction was analysed for radioactivity and the results were expressed as percentage of 110Ag picked up by whole cells from the total radioactivity added to the system.

2.7 Radioisotope techniques
2.7.1 Counting Procedure
2.7.1.1 Silver (110Ag): Three ml of the test sample in a
tube was placed in the shielded well of the Gamma Counter. The radioactivity was counted for 10 seconds. The background count was subtracted from the sample counts and counts per second (c.p.s.) were calculated.

2.8.0 Analytical Techniques:
2.8.1 Estimation of sulfhydryl groups

Ref: Sedlak and Lindsay (1968).

Reagents:
1. Tris buffer, 0.2 M, containing 0.02 M EDTA, pH 8.2.
2. DTNB (5,5'-dithiobis-2-nitrobenzoic acid), 0.01 M in methanol.
3. Absolute methanol.
4. Standard stock solution: Cysteine hydrochloride $10^{-3}$ M, in 0.02 M EDTA

Sample: Whole cell sonicate (Section 2.6.8), centrifuged (3500 g, 15 min, 4°C)

Procedure:
The sample (0.5 ml) or the standard (0.5 ml) was mixed with 1.5 ml of the Tris buffer and 0.1 ml of the DTNB reagent. The mixture was brought to 10 ml with absolute methanol, allowed to stand for 30 min at room temperature and filtered through a Whatman filter paper. The absorbance of the clear filtrate was measured at 412 nm.

Standard Curve:
Various concentrations of cysteine (1 to $10^{-5}$ M) were used to prepare the standard curve (Appendix III).
The concentration of sulfhydryl groups in the test samples was calculated from the standard curve.

2.8.2 Estimation of Succinate Dehydrogenase

Ref: Kun and Abood (1949)

Reagents:
1. Potassium phosphate buffer 0.1 M, pH 7.4.
2. Sodium succinate, 0.2 M in distilled water
3. Triphenyl Tetrazolium chloride (TTC) 0.1 per cent in distilled water
4. Toluene

Sample: Whole cell sonicate (Sec 2.6.8 centrifuged (3500 g, 15 min, 4 C)

Procedure:

The reaction mixture containing 0.5 ml, phosphate buffer; 0.5 ml, sodium succinate; 1.0 ml, test sample and 1.0 ml, TTC was incubated at 37 C for 30 min. At the end of incubation, 7.0 ml toluene was added to each tube and shaken vigorously. The upper layer was carefully pipetted out and the absorbance was measured at 420 nm.

Quantitation: Absorbance of .001 = 10 units

Specific Enzyme activity: Units of activity protein in sample (mg)

2.8.3 Estimation of Total Carbohydrate:

Ref: Dubois et al. (1956)

Reagents:
1. Phenol sol. in water 5 per cent w/v
2. Conc. sulfuric acid
3. Standard stock solution: glucose 1 mg/ml
Procedure:

One ml of the sample or the standard in a test tube was mixed with one ml of the phenol solution. Five ml of the conc H₂SO₄ was added, using a fast flowing pipette. The tubes were shaken and allowed to stand at room temperature for 30 min. The absorbance was measured at 480 nm.

Quantitation: A standard curve (Appendix IV) was prepared by using various concentrations (20-100 ug/ml) of glucose. The quantitation of the unknown sample was done using the standard curve.

2.8.4 Protein estimation

2.8.4.1 Total protein by Biuret procedure:

Ref: Robinson and Hodgen (1940)

Reagents:

1. Sodium hydroxide 3 N
2. Aqueous copper sulfate solution 2.5 per cent
3. Standard stock solution: 10 mg/ml Bovine serum albumin (BSA)

Procedure:

Two ml of the sample or the standard was taken in a test tube and mixed with 1.0 ml of 3 N NaOH. The tubes were placed in boiling water bath for 5 min and cooled. One ml of 2.5 per cent copper sulfate sol. was added and the contents were shaken thoroughly. The turbidity was removed by centrifugation (3000 g, 5 min, RT) and the absorbance of the supernatant was measured at 540 nm.
Quantitation: A standard curve (Appendix V) was prepared with 1-8 mg/ml of BSA and the quantitation of the unknown sample was done from the standard curve.

2.8.4.2 Protein estimation by Phenol reagent
Ref: Lowry et al. (1951)

Reagents:
1. Alkaline copper sulfate sol: 50 ml of 2% Na₂CO₃ in 0.1 N NaOH and 1.0 ml of 0.5% CuSO₄.5H₂O in 1% sodium potassium tartrate. The two reagents were mixed immediately prior to use.

2. Folin reagent, 2N (BDH): Diluted with distilled water 1:1 to make it 1 N solution.

3. Standard Stock Solution:
Bovine serum albumin (BSA) 1 mg/ml

Procedure:

Five ml of alkaline copper sulfate reagent was added to 1.0 ml of the sample and incubated for 10 min at room temperature. The samples were mixed with 0.5 ml of 1 N Folin reagent. The tubes were incubated for 30 min at 37°C and absorbance was determined at 540 nm.

Quantitation: A standard curve was prepared by using various concentrations (10-100 µg/ml) of BSA. The protein content in the unknown sample was determined using the standard curve (Appendix VI).
2.8.5 Estimation of total lipids:

Ref: Frings and Dunn (1970)

Reagents:
1. Vanillin reagent:
   Vanillin, 0.6% in distilled water - 1 volume
   Orthophosphoric acid - 4 volumes
2. Conc. sulfuric acid
3. Standard stock solution:
   Olive oil in ethanol - 5 mg/ml

Procedure:

The sample (0.1 ml, Sec 2.6.17) or standard was taken in a tube, mixed with 1.9 ml of conc. sulfuric acid and placed in the boiling water for 15 min. After cooling 0.2 ml of the sample was taken out and mixed with 5.0 ml of vanillin reagent. The tubes were allowed to stand at room temperature for 30 min and the absorbance was measured at 540 nm.

Quantitation: A standard curve was prepared by using various concentrations of olive oil (100-500 ug/ml). The quantitation in the test sample was done from the standard curve (Appendix VII).

2.8.6 Estimation of Phospholipids: (Total phosphorous)

Ref: Bartlett (1959)

Reagents:
1. Ammonium Molybdate - 5% w/v
2. Amidol in 20% Sod.metabsulfite - 1% w/v
3. Standard stock solution:
   Potassium dihydrogen phosphate \( (KH_2PO_4) \) - 4.4 mg/ml
**Procedure:**

A known quantity (0.1 to 1.0 ml) of the sample (Sec 2.6.18) or the standard was mixed with 1.0 ml perchloric acid and heated in a sand-bath, till the disappearance of the yellowish brown tinge. The tubes were removed from the sand bath and cooled. The digested sample was mixed with 5.2 ml of distilled water, 0.2 ml of Ammonium molybdate solution, and 0.2 ml of amidol solution. The tubes were placed in boiling water for 15 min, cooled and the absorbance was determined at 820 nm.

**Quantitation:** A standard curve was prepared using various concentrations of phosphorous (1-10 µg/ml). The quantitation in the test sample was done from the standard curve (Appendix VIII).

\[
\text{Phospholipid} = 25 \times \text{Total phosphorous in the sample}
\]

2.8.7 Hexosamines:

Ref: Rondle and Morgan (1955).

**Reagents:**

1. **Buffer A**
   - Sodium bicarbonate: 4.2 g
   - Sodium carbonate: 5.3 g
   - Distilled water: 100 ml

2. **Acetyl Acetone Sol**
   - Acetyl Acetone: 2.5 ml
   - Buffer A: 50.0 ml

3. **Ehrlich's reagent**
   - p DABA (p-Dimethyl amino benzoic acid): 0.8 g
   - Ethanol: 30 ml
   - HCl: 30 ml
4. Standard stock solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl glucosamine</td>
<td>15 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Sodium hydroxide</td>
<td>3 N</td>
</tr>
</tbody>
</table>

Procedure

One ml of the sample was mixed with 3.0 ml of 3 N NaOH and boiled in a boiling water bath for 3 min. After cooling, the extract was centrifuged (500 g, 10 min, RT) and the sediment discarded.

Two ml of the sample or the standard (made alkaline with NaOH) was taken in a tube and mixed with 1.0 ml of freshly prepared acetyl acetone. The tubes were placed in boiling water for 20 min. After cooling 5 ml of ethanol was added to each. The contents were shaken and mixed with 1.0 ml of pDABA. The tubes were heated at 70°C for 10 min, cooled and the absorbance was measured at 530 nm.

Quantitation: A standard curve was prepared using various concentrations of N-acetyl glucosamine (10-100 ug/ml) and the hexosamine content was calculated from the standard curve (Appendix IX).

2.8.8 Teichoic acids:

Ref: Forrester (1966)

Procedure

The teichoic acid content of *S. aureus* cells (Sec 2.6.20) was determined on the basis of the phosphate content by the
micro-Bartlett procedure (Sec 2.8.6).

2.8.9 Analysis of the Methyl esters of fatty acids:

Procedure

The GLC analysis was carried out with 'Pye Unichem' gas chromatograph using a column packed with diethyl glycol succinate (DEGS), 100-200 mesh; diatomite C-AW). An exact quantity of one μl of methyl esters in chloroform (Sec 2.6.19) was used for analysis. The column and detector temperatures were 180 C and 210 C respectively. The flow rate of carrier gas (nitrogen) was 40 ml/min.

Identification of fatty acid peaks: Fatty acid peaks were identified by chromatographing standard mixture of methyl esters of fatty acids. The retention times were used for identification of the fatty acids in the unknown sample. The area under the peaks was measured by triangulation method (Area = 1/2 Base x Height). It was assumed that the area of the peak was directly proportional to the concentration of the fatty acids. The percentage of each fatty acid was calculated by dividing the quantity of the desired fatty acid by total quantity of all fatty acids.

2.8.10 Hemolysins of S. aureus and E. coli:

Ref: Linkish and Vogt (1972)

Procedure:

Erythrocytes were prepared from rabbit blood collected
in 1.5 per cent sodium citrate solution. The cells were removed by centrifugation (500 g, 5 min, RT) and suspended in physiological saline to give a 2% suspension. One of the RBC suspension was mixed with 1.0 ml of the test sample, incubated at 37 C for 2 h and centrifuged (500 g, 5 min, RT). The absorbance of supernatants was determined at 545 nm. Protein estimation in the samples was done as described in Sec 2.8.4.1.

Quantitation: Absorbance of 0.001 = 1 unit

\[ \text{hemolysin specific activity} = \frac{\text{Units}}{\text{protein in sample (mg)}} \]

2.8.1 Beta and Delta hemolysins:

The procedure described in section 2.8.10 was followed, however the rabbit blood was replaced by sheep and human blood for Beta and Delta hemolysins respectively.

2.8.12 Coagulase:

Ref: Attenbern (1966)

Reagents:
1. Phosphate buffered saline (PBS), pH 7.2
2. Rabbit plasma

Procedure:

The test sample (0.5 ml) was serially diluted two-fold in PBS. Diluted (1:10 with PBS) rabbit plasma (0.5 ml) was added to each tube. The tubes were shaken to mix the contents, incubated at 37 C for 2 h and examined for clot formation.
Quantitation: The reciprocal of the highest dilution showing 1+ clot was expressed as the coagulase titre.

2.8.13 Deoxyribonuclease:
Ref: Lachica et al. (1971)

Reagents:
Toluidine Blue - O-DNA Agar:
1. DNA (Calfthymus) - 0.3 g
2. Calcium chloride, . - 0.01M, 1.0 ml
3. Sodium chloride - 10 g
4. Agar - 10 g
5. Tris-HCl buffer - 0.05M, pH 9.0, 1 litre

Procedure:
Agar was dissolved in this buffer by heating and mixed with 3.0 ml of 0.1M toludine-O dye. Twenty ml of agar was poured in each petriplate and allowed to solidify at room temperature. Sterile steel wells were placed aseptically on the agar plates. 0.1 ml of the sample was applied to the wells and the plates were incubated at 37 C for 12 h. The zones of clearance were measured in cm.

Quantitation: Zone diameter of 0.01 cm = 1 unit
Units of DNA /mg protein = \frac{\text{Units}}{\text{protein in sample (mg)}}

2.8.14 Thermonuclease
Ref: Lachica et al (1969)

Procedure:
The sample was heated in a boiling water bath for 10 min. The thermonuclease activity in the heat treated
sample was estimated by the procedure described in Sec 2.8.13.

2.8.15 Phospholipase:

Ref: Haberman and Hardet (1972)

Reagents

Egg yolk agar

1. Agar - 0.25 g
2. Potassium phosphate buffer, 0.05 M, pH 7.5 - 25 ml
3. Diluted Egg yolk (1 part egg yolk + 3 parts saline) - 0.25 ml
4. Calcium chloride solution, 0.01 mM - 0.25 ml
5. Sodium azide - 0.01 per cent

Procedure:

Agar was dissolved in phosphate buffer by heating and mixed with diluted egg yolk at 50°C. Sterile steel wells were placed aseptically on the surface of the agar and 0.1 ml of the sample was applied to the wells. The plates were incubated at 37°C and the zone of clearance was measured in cm.

Quantitation: Zone diameter of 0.01 cm = 1 unit

Phospholipase units/mg protein = \[ \frac{\text{Units}}{\text{protein in sample (mg)}} \]

2.8.16 Protease:

Ref: Drapeau (1976)

Reagents

1. Casein solution - 1% w/v
2. TCA solution in water - 10% w/v.
Procedure:

One gm casein was dissolved in 50 ml of 0.01M, NaOH by gentle heating and stirring. Dissolved casein was diluted with 40 ml of distilled water and 5.0 ml of 1 M Tris base. The pH was adjusted to 7.8 with phosphoric acid and the volume was made to 100 ml with distilled water.

Five ml of the casein solution was equilibrated at 37 C for 5 min. Ten ml of the enzyme sample was added, the contents were mixed and incubated at 37 C for 10 min. The reaction was stopped by adding 5 ml of TCA solution. The tubes were allowed to stand at 37 C for 15 min and the contents filtered through a Whatman No.1 filter paper. The absorbance of the filtrate was measured at 280 nm.

Quantitation: One unit of enzyme activity is defined as the amount of enzyme, which liberates acid soluble fragments equivalent to 0.001 A_{280} per minute at 37 C and pH 7.8 under defined conditions.

Units/mg = \frac{A_{280} \text{ (test)} - A_{280} \text{ (reagent blank)}}{10 \text{ min} \times \text{protein in reaction (mg)}} \times 1000

2.8.17 Acid and Alkaline phosphatases:

Ref: Bessey et al (1946)

Reagents:

1. Acetate buffer 0.1 M, pH 5.2 (See Appendix I, page 183)
2. Tris buffer 0.1 M pH 9.1 (See Appendix I, page 183)
3. p-nitrophenyl phosphate disodium 0.4% w/v
4. Sodium hydroxide, 1M
Procedure

2.8.17.1 **Acid phosphatase:** The reaction mixture containing 1.5 ml, acetate buffer; 1.0 ml sample and 0.5 ml, p-nitrophenyl phosphate disodium was incubated at 37°C for 30 min. The reaction was stopped by the addition of 1.0 ml of 1 M, NaOH and the absorbance was determined at 400 nm.

Quantitation: 

1 unit = Absorbance of 0.362 at 400 nm.

Enzyme activity = $A_{400} \times 2.76$

2.8.17.2 **Alkaline phosphatase:** The procedure described for acid phosphatase (Sec 2.8.17.1) was used. However, Tris buffer, 0.1M, pH 9.1 was substituted for the acetate buffer.

Quantitation: 

1 unit = Absorbance of 0.084 at 400 nm.

Enzyme activity = $A_{400} \times 11.82$

2.8.18 **Protein A:**

Ref: Sjoquist and Stalenheim (1969)

Reagents:

1. Sheep RBC suspension in saline, 5 per cent
2. Antisheep hemolysin (ASH) in saline diluted 1:400.

Procedure:

*Staphylococcus aureus* was grown for 12 h at 37°C in BHI Broth. The cells were killed by placing in boiling water for 10 min. Equal volumes of the 5 per cent RBC suspension and ASH were mixed and incubated at 37°C for 30 min. At the end of the incubation, the cells were centrifuged (500 g, 5 min, RT) and washed with saline twice. The washed cells were suspended in
saline to give a 2 per cent RBC suspension.

The sample (0.05 ml) was serially diluted two-fold in saline in micro titration plates, and mixed with 0.05 ml of the 2 per cent RBC suspension. The plates were gently shaken to mix the contents, incubated at 37°C for 2 h and observed for agglutination.

Quantitation: The reciprocal of the highest dilution showing agglutination was expressed as the units of protein A.

2.9.1 Chromatography Procedures:

2.9.1.1 Gel permeation Chromatography: Sephadex G-10 and G-75 were swollen in distilled water for 24 h and degassed in a boiling water bath for 2 h. The water was decanted. The sephadex was resuspended in several volumes of buffer and washed twice before packing. The columns were packed according to the recommended procedure (Kumar and Vadehra, 1980). The packed material was equilibrated with elution buffer by passing several bed volumes of the buffer. The column material, size and buffer used in this study is given below:

<table>
<thead>
<tr>
<th>Column material</th>
<th>Size</th>
<th>low rate (ml/hr)</th>
<th>Elution Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sephadex G-10</td>
<td>22 cm x 1.5 cm</td>
<td>15</td>
<td>Tris buffer, 0.5M, pH 7.2</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>22 cm x 1.5 cm, 45 cm x 1.8</td>
<td>18</td>
<td>Tris buffer, 0.5M, pH 7.2</td>
</tr>
</tbody>
</table>

The sample was gently applied on top of the packed material.
and allowed to enter the column. Small amount of buffer was carefully added on the top without disturbing the column material. to wash the sides of the column. The column was connected with a buffer reservoir and 3.0 ml fractions were collected. Almost 2 bed volumes of the eluate were collected and the protein content of each fraction was determined (Sec 2.8.4.2).

2.9.2 Electrophoretic techniques

2.9.2.1 Polyacrylamide gel electrophoresis:

Ref: Davis (1964)

Reagents:

1. Solution A
   - Acrylamide - 28.0 g
   - Bis-acrylamide - 0.735 g
   - Distilled water - 100 ml

2. Solution B, pH 8.9
   - Tris - 36.3 g
   - 1N HCl - 48 ml
   - TEMED - 0.23 ml
   - Distilled water - 100 ml

3. Solution C
   - Ammonium persulfate - 140 mg
   - distilled water - 100 ml

4. Tris-glycine buffer, pH 8.0
   - Tris - 0.6 g
   - Glycine - 2.8 g
   - Distilled water - 1000 ml
5. Staining solution:

- Commassie blue: 100 mg
- Acetic acid: 10 ml
- Methanol: 30 ml
- Distilled water: 60 ml

6. Destaining solution:

- Methanol: 30 ml
- Acetic acid: 70 ml
- Distilled water: 1000 ml

Preparation of gels:

7.5 per cent polyacrylamide gels

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ml</td>
<td>10 ml</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

Procedure:

Glass tubes (10x0.8 cm) were thoroughly cleaned and dried in an oven prior to use. The tubes were plugged at one end with rubber stoppers and held vertically in a gel casting apparatus. The gel solution was added carefully to each tube with the help of a clean pasteur pipette and layered with a few drops of distilled water. Tubes were left for 1 h at room temperature for polymerisation.

Electrophoresis: The tubes, containing polymerized gels were attached to the upper buffer tank and dipped into lower tank. Both the tanks were filled with the Tris-glycine buffer. Samples (50 μl) were mixed with bromophenol blue (5 μl) and
applied to each gel. A constant current of 3 mA per tube was applied, till the dye front reached 1 cm from the lower end.

**Staining/Destaining of Gels:** The gels were carefully removed from the tubes and stained for 1 h at 37 C. For destaining of gels, these were thoroughly washed in water and destained with destaining solution with repeated changes every 4-6 h.

### 2.9.2.2 SDS gel electrophoresis:
Ref: Weber and Osborn(1969)

**Reagents:**

#### A. Sample buffer, pH 7.0

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monobasic sodium dihydrogen phosphate (NaH₂PO₄, anhydrous)</td>
<td>0.34g</td>
</tr>
<tr>
<td>Dibasic sodium hydrogen phosphate (Na₂HPO₄, anhydrous)</td>
<td>1.02g</td>
</tr>
<tr>
<td>SDS</td>
<td>1.0 g</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>0.015g</td>
</tr>
<tr>
<td>Urea</td>
<td>36g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

#### B. Gel Buffer, pH 7.5

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄</td>
<td>6.8g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>20.45g</td>
</tr>
<tr>
<td>SDS</td>
<td>2.0g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
C. Acrylamide
   - 22.20 g
Bis-acrylamide
   - 0.6 g
Distilled water
   - 100 ml

D. TRIMED

E. Ammonium persulfate
   - 100 mg
Distilled water
   - 15 ml

F. Fixative:
   Methanol
   - 400 ml
   Acetic acid
   - 70 ml
   Distilled water
   - 530 ml

G. Staining solution:
   Methanol (50%)
   - 454 ml
   Acetic acid
   - 46 ml
   Commasie brilliant blue
   R-250
   - 1.25 g

H. Destaining solution:
   Methanol
   - 50 ml
   Acetic acid
   - 75 ml
   Distilled water
   - 100 ml

Preparation of Gels: Reagent B (15 ml) was mixed with 13.5 ml of reagent C and degassed. Freshly prepared reagent E was also degassed separately and 1.5 ml was added to the above solution followed by addition of 0.05 ml of reagent D.

Procedure: Sec 2.9.2.1.
Electrophoresis: The gel tubes were attached to the buffer tank and both the upper and lower tanks were filled with gel buffer B. The samples were dialyzed against the buffer (Sol.A) for 2 h prior to use and mixed with a few drops of glycerol before application. Generally, 50 µl of the sample was applied. A constant current of 8 mA per gel tube was applied, till the dye front reached 1 cm of the lower end. The gels were carefully removed from the tubes using a syringe.

Staining/destaining of gels: The gels were immersed in solution F for 10 h and stained in solution G for 2 h at 37°C. Destaining was done with solution H. The solution was changed several times.

2.10. Genetic techniques

2.10.1 Curing of the Resistance:

Ref: Miller (1972)

Reagents:

1. Acridine orange/Acriflavine - 25-100 µg/ml
2. Ethidium bromide - 2-400 µg/ml
3. Sodium dodecyl sulfate - 0.1-100 mg/ml

Procedure:

Various concentrations of the curing agents were tested for determination of MIC (See 2.6.12). The test organism was incubated in the highest concentration of the curing agent, which allowed growth, for 48-96 h at 37°C. An inoculum from the test medium and the control (without curing agent) was plated on nutrient Agar. The isolated colonies were tested
for resistance to half or one third (20-30 ppm) of the original level of resistance (70 ppm) by the procedure described in Sec. 2.6.12.

**Calculations:**

\[
\text{Curing rate} = \frac{\text{Number of colonies cured}}{\text{Number of colonies tested}} \times 100
\]

2.10.2 **Transfer of the Resistance:**

Ref: Miller (1972)

**Test Organisms:**

(i) BM 21 E. coli K-12 F^- Lac^+ NA^-

(ii) S. typhimurium phage type 36 NA^-

The donor and the recipient cultures were incubated in L Broth (Appendix II) for 24 h at 37 C. The cultures were diluted, after incubation, in the ratio of 1:40 for the donor and 1:20 for the recipient, in fresh LB medium and incubated at 37 C to achieve a population of \(10^8\) CFU/ml. Mating mixtures were prepared by mixing 5 ml each of the donor and the recipient in a 250 ml flask. The mixtures were incubated at 37 C for 2-12 h. At the end of the desired incubation period, 0.2 ml of the mixture was plated on to MacConkey agar plates containing 50 ppm of nalidixic acid (NA) and 40 ppm of silver nitrate (Sec 2.6.5). The nalidixic acid was used to selectively prevent the growth of the donor cells. The silver ion resistant colonies were counted after 72 h of incubation.
Calculations:

Frequency of transfer = \frac{\text{Number of transconjugants}}{\text{number of donor cells}}

2.11.0 Immunological techniques

2.11.1 Raising of Antisera:

Ref: Campbell et al. (1963).

2.11.1.1 Antisera to Bacterial O-Antigen

(a) Preparation of Antigen - Sec 2.6.6.2

(b) Immunisation schedule - The following injection schedule in rabbits was followed:

<table>
<thead>
<tr>
<th>Day No.</th>
<th>Dose</th>
<th>Route of Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2 ml</td>
<td>Intramuscular (I/M)</td>
</tr>
<tr>
<td>3</td>
<td>0.5 ml</td>
<td>I/M</td>
</tr>
<tr>
<td>6</td>
<td>0.5 ml</td>
<td>I/M</td>
</tr>
<tr>
<td>9</td>
<td>0.5 ml</td>
<td>Intravenous (I/V)</td>
</tr>
<tr>
<td>12</td>
<td>0.5 ml</td>
<td>I/V</td>
</tr>
<tr>
<td>15</td>
<td>1.0 ml</td>
<td>I/V</td>
</tr>
<tr>
<td>20</td>
<td>1.0 ml</td>
<td>I/V</td>
</tr>
<tr>
<td>25</td>
<td>bleed through marginal vein</td>
<td></td>
</tr>
</tbody>
</table>

2.11.1.2 Antisera to Cytoplasmic Antigen and Cell-wall Antigen:

(a) Preparation of Antigens:

1. Cytoplasmic antigen Sec 2.6.8.2
2. Cell-wall antigen Sec 2.6.9.2; 2.6.16

(b) Immunisation schedule: The following injection schedule in rabbits was followed:
### Table: Preparation and Injection Schedule

<table>
<thead>
<tr>
<th>Day No.</th>
<th>Antigen</th>
<th>Adjuvant</th>
<th>Route of Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>Intraperitoneal (I/P)</td>
</tr>
<tr>
<td>7</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
<td>I/P</td>
</tr>
<tr>
<td>14</td>
<td>1.0 ml</td>
<td>-</td>
<td>Intramuscular (I/M)</td>
</tr>
<tr>
<td>21</td>
<td>1.0 ml</td>
<td>-</td>
<td>I/M</td>
</tr>
<tr>
<td>28</td>
<td>1.0 ml</td>
<td>-</td>
<td>I/M</td>
</tr>
<tr>
<td>35</td>
<td>1.0 ml</td>
<td>-</td>
<td>Intravenous (I/V)</td>
</tr>
<tr>
<td>38</td>
<td>bleed through marginal vein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.11.2 Preparation of antisera:

Ten ml of blood collected through marginal vein of the ear was allowed to coagulate by incubating at 37 C for 1 h and 24 h at 4 C. The clotted material was centrifuged (500 g, 5 min, RT) to separate the serum. Sodium azide (0.01%) was added as a preservative and the serum was stored at -10 C.

2.11.3 Detection of Antibodies

2.11.3.1 Agglutination titres: The Agglutination test was performed with O-Antigen preparations. Serum (0.05 ml) was serially diluted two fold in physiological saline in the wells. The O-antigen preparation (0.05 ml) was added to each well. The plates were gently shaken to mix the contents and incubated at 37 C for 2 h and, 6 h at 4 C.

Quantitation: The reciprocal of the highest dilution showing Agglutination was expressed as the Agglutination titre.

2.11.3.2 Ouchterlony's double gel-diffusion technique:

Ref: Ouchterlony (1953)
Reagents:

1. Sodium barbitone buffer, 0.025 M, pH 8.2
2. Agarose

Procedure:

Agarose (1.0 g) was dissolved in 100 ml of sodium barbitone buffer in a boiling water bath. Three ml of the molten agarose solution was poured on glass slides to form a uniform layer. The agarose was allowed to solidify at room temperature. A central and two peripheral wells were cut in the Agarose with the help of a well cutter. The central well was filled with the antiserum and the peripheral wells were filled with antigens. The slides were kept in a moisture chamber for 1-2 days and were observed for the appearance of lines of precipitation.

2.12.0 Experimental Design
2.12.1 Incidence of metal ion and antibiotic resistance

Escherichia and Salmonella cultures obtained from CRI, Kasauli, were tested for silver, mercury and cadmium ion resistance (Sec. 2.6.12). The antibiotic resistance pattern of silver resistant isolates was also determined (2.6.13).

2.12.2 Development of Resistance:

Three test organisms, K.pneumoniae, S.aureus and E.coli were used for development of resistance to silver ions by the procedure given in Sec.2.6.14. Nalidixic acid (NA) and Mercuric chloride (Hg) resistance was also developed in Ag+ strains
by similar procedure (Sec 2.6.14). A battery of Ag<sup>r</sup>, Ag<sup>r</sup>NA<sup>r</sup> and Ag<sup>r</sup>Hg<sup>r</sup> strains was developed.

2.12.3 Concomitant development of resistance

The Ag<sup>r</sup>, Ag<sup>r</sup>NA<sup>r</sup> and Ag<sup>r</sup>Hg<sup>r</sup> strains were tested for co-development of resistance to other metal ions or antibiotics (Sec. 2.6.12 and 2.6.13).

2.12.4 Comparison of the Ag<sup>s</sup> and Ag<sup>r</sup> strains:

2.12.4.1 Colony characteristics:

The colony morphology of the Ag<sup>s</sup> and Ag<sup>r</sup> strains was studied on nutrient agar and Mac Conkey agar.

2.12.4.2 Growth Curves: Growth profiles of the Ag<sup>s</sup> and Ag<sup>r</sup> strains were studied by turbidimetric (Sec. 2.6.4.2) and viable count procedures (Sec. 2.6.4.3). Dry weight of the separated cell mass, wherever indicated was determined by procedure in Sec. 2.6.4.4.

2.12.4.3 Chemical Analysis: The cells and cell walls were analysed for various chemical constituents as given in the following sections:

2.12.4.3.1 Whole Cells: The whole cells were analysed for:

(a) Capsular material: Carbohydrate content of capsular material (Sec. 2.8.3).
(b) Total carbohydrate (Sec.2.8.3).
(c) Total protein (Sec. 2.8.4.1)
(d) Total lipid (Sec. 2.8.5)
(e) Phospholipid (Sec. 2.8.6).
2.12.4.3.2 **Cell Walls**: In addition to the parameters described in Sec. 2.12.4.3.1, the cell walls were analyzed for:

(a) Hexosamines (Sec. 2.8.7)
(b) Teichoic acids (Sec. 2.8.8).

2.12.4.3.3 **Intracellular milieu (ICM)**: Whole cell sonicate (Sec. 2.6.8) was centrifuged (3500 g, 15 min, 4 c) and analysed for:

(a) Sulfhydryal group (Sec. 2.8.1).
(b) Succinate dehydrogenase (Sec. 2.8.2).

2.12.4.4 **Enzyme Profiles**:

2.12.4.4.1 **Enzyme Preparation** (Sec. 2.6.7 and 2.6.7.1).

The qualitative and quantitative differences in the enzyme profiles of Ag$^+$ and Ag$^-$ *K.pneumoniae* and *S. aureus* cells was determined for the following enzymes:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Procedure described in Sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Alpha-hemolysin</td>
<td>2.8.10</td>
</tr>
<tr>
<td>b) Beta and Delta hemolysin</td>
<td>2.8.11</td>
</tr>
<tr>
<td>c) Coagulase</td>
<td>2.8.12</td>
</tr>
<tr>
<td>d) Deoxyribonuclease</td>
<td>2.8.13</td>
</tr>
<tr>
<td>e) Thermonuclease</td>
<td>2.8.14</td>
</tr>
<tr>
<td>f) Phospholipase</td>
<td>2.8.15</td>
</tr>
<tr>
<td>g) Protease</td>
<td>2.8.16</td>
</tr>
<tr>
<td>h) Acid and Alkaline Phosphatase</td>
<td>2.8.17</td>
</tr>
<tr>
<td>i) Protein A</td>
<td>2.8.18</td>
</tr>
</tbody>
</table>
2.12.4.5 **Fatty acid profile:** GLC analysis (Sec. 2.8.9) of the methyl esters of fatty acids of the phospholipid fraction isolated from cell walls (Sec. 2.6.9.1) and cell membranes (Sec. 2.6.11) was carried out.

2.12.4.6 **Chromatographic Analysis:** The ICM was chromatographed on Sephadex G-10 and G-75 by the procedures in Sec 2.9.1.1.

2.12.4.7 **Electrophoretic analysis:** The ICM (Sec. 2.6.8) was analysed by the Polyacrylamide gel electrophoresis (PAGE) (Sec. 2.9.2.1), while cell membranes (Sec. 2.6.11) and cell walls (Sec. 2.6.9.1) were analysed by SDS PAGE (Sec. 2.9.2.2).

2.12.4.8 **Genetic Studies:** The exact location of the genetic determinant for silver ion resistance was studied by the following experiments:

(a) **Curing of the Resistance:** Curing of the silver ion resistance in *K. pneumoniae* and *S. aureus* was attempted by the procedure described in Sec. 2.10.1. Attempts were made to cure cells of Hg and NA resistance in Ag\(^R\)Hg\(^R\) and Ag\(^R\)NA\(^R\) strains by the same procedure.

(b) **Conjugation experiments:** The transfer of silver ion resistance to sensitive strains was attempted by the procedure described in Sec. 2.10.2.

2.12.4.9 **Immunological differences:** The Ag\(^s\) and Ag\(^r\) strains of *K. pneumoniae* were used for raising antisera against O-Antigen (Sec. 2.11.1.1), the cell-wall (Sec. 2.11.1.2) and ICM (Sec. 2.11.1.2). The antisera prepared were tested for
the specificity and cross-reactions against the antigens according to the procedures described in Sec. 2.11.3.1 and 2.11.3.2.

2.12.5 Uptake of Silver

2.12.5.1 Uptake vs Growth

Sterile nutrient broth (45 ml in 250 ml Erlenmeyer flasks) was mixed with enough $^{110}\text{AgNO}_3$ to give a concentration of 10 ppm. The mixture was inoculated with 5.0 ml of the 18 h old culture of the test organism and incubated at 37°C on a shaker (150 rpm). At 3 h intervals, 3 ml of the growth medium was aseptically withdrawn and centrifuged (3000 g, 10 min, RT). The cells were washed twice in distilled water and finally suspended in 3.0 ml of distilled water. The $^{110}\text{AgNO}_3$ in cells and supernatants was measured (Sec. 2.7.0). The cell growth was followed by optical density measurement (Sec.2.6.4.2) viable cell count (Sec.2.6.4.3) as well as by the dry weight determination, of the separated cell mass (2.6.4.4).

2.12.5.2 Binding of $^{110}\text{Ag}$: One ml of the ICM (Sec.2.6.8) or the cell wall suspension (Sec.2.6.9) was incubated with 10 ppm of $^{110}\text{AgNO}_3$ for 30 min at 37°C. At the end of the incubation, 2.5 ml of 10 per cent (TCA) was added and the TCA soluble and insoluble fractions were separated by centrifugation (3000 g, 10 min, RT). The radioactivity was measured (Sec.2.7.0).
2.12.5.3 Kinetics of $^{110}\text{Ag}$ uptake by cellular fractions:

Nutrient broth (150 ml in 500 ml Erlenmeyer flasks), containing $^{110}\text{AgNO}_3$ was inoculated with 50 ml of the 18 h old culture of the test organism. The flasks were incubated at 37°C and at every 3 h intervals, 25 ml of the culture was aseptically withdrawn and centrifuged (8000 g, 20 min, 4°C). The cells were washed twice with distilled water and fractionated (Sec. 2.6.21).

2.12.5.4 Uptake of $^{110}\text{Ag}$ by Spheroplasts:

Spheroplasts (Sec. 2.6.10) and cells (Sec. 2.8.6) were suspended in nutrient broth, containing 10% (w/v) sucrose, to an optical density of 0.07. The suspension was mixed with enough $^{110}\text{AgNO}_3$ to give a concentration of 0.7 ppm. Tubes were incubated at 37°C and 3.0 ml sample was drawn immediately and at 30 min intervals. The cells and spheroplasts were centrifuged (8000 g, 15 min, 4°C) and washed with 10 per cent sucrose solution. The radioactivity of the pellets and the supernatants was measured.

2.12.6 Factors affecting silver uptake:

The following factors were studied for their effect on the uptake of $^{110}\text{Ag}$ by bacterial cells. In general, the cells (Sec. 2.6.6) were suspended in the desired medium, to give an absorbance of 0.07 and enough $^{110}\text{AgNO}_3$ was added to give a concentration of 0.7 ppm, or the indicated concentration.
2.12.6.1 **Suspension medium:**

The cells were suspended in distilled water or nutrient broth, incubated at 37°C for 30 min, centrifuged (3000 g, 10 min, RT) and the radioactivity in sediment and supernatant was measured.

2.12.6.2 **$^{110}$AgNO$_3$ Concentration:** The cell suspensions prepared in nutrient broth were mixed with different concentrations of $^{110}$AgNO$_3$ (0.7, 2.4 and 5 ppm), incubated at 37°C for 30 min and centrifuged (3000 g, 10 min, RT). The radioactivity was measured.

2.12.6.3 **Metabolic Inhibitors:** Three ml of cell suspension in distilled water was mixed with the following chemicals and the controls had appropriate amounts of distilled water.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Sodium cyanide</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>2. 2,4 Dinitrophenol</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>3. Sodium azide</td>
<td>10.0 mM</td>
</tr>
</tbody>
</table>

The samples were incubated at 37°C for 30 min and centrifuged (3000 g, 10 min, RT). The fractions were analysed for radioactivity.

2.12.6.4 **Toluene:** Three ml of the cell suspension in distilled water was mixed with toluene (0.3%, v/v) and incubated at 37°C for 30 min. At the end of the incubation period, the cells were centrifuged (3000 g, 10 min, RT) and the radioactivity was measured (Sec. 2.7.0).
2.12.6.5: **Chelators**: Sterile nutrient broth (50 ml in 250 ml Erlenmeyer flasks) was mixed with sub-inhibitory concentrations of the isotope (10 ppm) and EDTA (100 ppm). The mixture was inoculated with 5.0 ml of the 18 h old culture of the test organism. The flasks were incubated at 37°C on a rotary shaker (150 rpm) and 3.0 ml of the culture was withdrawn at intervals of 1.5, 3.0, 4.5, 6.0 and 24 h and centrifuged (3000 g, 10 min, RT). The cells were washed twice with 3.0 ml of distilled water. The radioactivity in the cells (resuspended in 3.0 ml of d.w) and the supernatants was measured (Sec 2.7).

2.12.7 **Effect of AgNO₃ on selected parameters**

2.12.7.1 **Binding of Silver to -SH groups**;

2.12.7.1.1 **Binding of silver by -SH groups in cysteine**;

Procedure:

The cysteine solution (0.25 ml, 5 x 10⁻⁵ M) was mixed with 0.1 to 0.25 ml of the stock AgNO₃ solution to give concentrations ranging from 1x10⁻⁵ to 5x10⁻⁵ M. Final volume was made to 0.5 ml with 0.02M EDTA. The tubes were incubated at 37°C for 15 min. The -SH content was determined (Sec.2.8.1). The concentration of AgNO₃ resulting in complete disappearance of the characteristic 'yellow' color was used for calculating the molar ratio of binding.

Molar ratio of binding: \[
\frac{\text{SH content (control)} - \text{SH content (test)}}{\text{Conc. AgNO₃}}
\]
2.12.7.1.2 Binding of silver to -SH groups in ICM: The ability of the -SH groups in intracellular milieu to bind silver was determined by estimation of -SH groups prior to and after treatment with AgNO₃.

Procedure:

A known quantity (0.5 ml) of the milieu was mixed with 0.05 ml of Ag NO₃ solution giving a concentration of 1x10⁻⁵ M. The mixture was incubated at 37°C for 15 min and assayed for SH groups (Sec 2.8.1). The molar binding ratio was calculated as given in Sec. 2.12.7.1.1.

2.12.7.2 Inhibition of Succinate dehydrogenase (SDH): SDH activity (Sec. 2.8.2) of whole cell sonicate (Sec. 2.6.8) was determined. Effect of silver ions on the SDH activity was estimated by mixing one ml of the test sample with 0.025 ml of stock AgNO₃ solution, giving concentrations of 20 or 40 ppm of AgNO₃. The mixture was incubated at 37°C for 15 min and assayed for SDH. Per cent decrease in SDH activity with respect to the control was calculated.

2.12.7.3 Leakage of Cell contents: Three ml of the washed cell suspension (Sec. 2.6.6) was made 10 and 40 ppm with respect to silver nitrate. The tubes were incubated at 37°C for 90 min. The samples were removed and centrifuged (3000g, 15 min). Absorbance of the cell free supernate was measured at 260 and 280 nm.
Quantitation: The protein and nucleic acid content was calculated from the following relationships:

1. Protein (mg/ml) = 1.55 $A_{280}$ - 0.76 $A_{260}$
2. Nucleic acids (mg/ml) = 0.063 $A_{260}$ - 0.036 $A_{280}$

2.12.8 Factors affecting silver ion toxicity

2.12.8.1 Inoculum size: Sterile nutrient broth (50 ml in 250 ml Erlenmeyer flasks) containing 10 ppm of $^{110}$AgNO$_3$ was mixed with enough inoculum to give initial counts of $10^2$, $10^6$, and $10^8$ CFU/ml. The flasks were incubated at 37°C on a rotary shaker (150 rpm). The viable count was determined at every 3 h intervals (Sec. 2.6.4.3).

2.12.8.2 Growth medium: The test media (nutrient broth, peptone water, brain heart infusion and trypticase soya broth) were mixed with 0.5 ppm of AgNO$_3$, inoculated with a loopful of the test organism and incubated at 37°C. Growth was determined by measurement of absorbance (Sec 2.6.4.2) at 4 h intervals.

2.12.8.3 Mercaptoethanol: Nutrient broth containing 50 ppm of AgNO$_3$ was mixed with 50 and 250 ppm of mercaptoethanol. The mixtures were inoculated with a loopful of the 18 h old culture of the test organism and incubated at 37°C for 24 h. Presence of turbidity indicated that the inhibitory properties of AgNO$_3$ were neutralized.

2.12.8.4 Antibiotics: Minimum inhibitory concentrations of the various antibiotics for K. pneumoniae Ag$^+$ were determined
(Sec. 2.6.13). Nutrient agar (20 ml) was mixed with subinhibitory concentration of the test antibiotic and 40 ppm of AgNO₃. The agar was inoculated with 0.2 ml of the 8 h old K. pneumoniae Ag culture, mixed and poured into petri plates. The plates were incubated at 37°C for 18 h and the CFU/ml were determined.

2.12.8.5 Chelators: The following chelating agents at the indicated concentrations were used:

1. EDTA 10-100 ppm.
2. Pencillamine - 100 ppm.

Nutrient broth was mixed with 100 ppm of chelating agent and 10-50 ppm of AgNO₃ and inoculated with a loopful of the 18 h old culture of K. pneumoniae Ag⁺ or S. aureus Ag⁺. The samples were incubated at 37°C for 24 h. Visual turbidity was graded (Sec 2.6.4.1) at the end of the incubation period.

2.12.8.5.1 Neutralization of 'EDTA effect' by Magnesium sulfate

Effect of Magnesium sulfate on the growth of K. pneumoniae Ag⁺ in the presence of EDTA was studied. Nutrient broth (50 ml) containing 100 ppm of EDTA and 10 ppm of AgNO₃ was mixed with 200 ppm of MgSO₄, inoculated with a loopful of the 18 h old culture and incubated at 37°C. Growth was followed by measurement of absorbance (Sec. 2.6.4.2) at 3 h intervals.

2.12.8.5.2 Neutralization of silver ion toxicity by Pencillamine

The MIC of Pencillamine for K. pneumoniae Ag⁺ was determined (Sec. 2.6.12). Nutrient broth (50 ml in 250 ml Erlenmeyer flasks) was mixed with 5 or 10 ppm of AgNO₃ and a sub-inhibitory concentration of Pencillamine (100 ppm) inoculated with a
loopful of the 18 h old culture and incubated at 37 C. Growth was followed by measurement of absorbance (Sec. 2.6.4.2) at 3 h intervals.