PART - II

EFFECT OF HEAVY METALS ON E. COLI AND A. AEROGENES
MATERIALS AND METHODS

ORGANISMS

Escherichia coli and Aerobacter aerogenes isolated from J.N.U. lake water, New Delhi and deposited in the stock culture of this laboratory were used for present studies (Bagde, Khan and Varma, 1981).

MEDIA

The medium used for culturing the E. coli and A. aerogenes was routinely nutrient broth medium. The liquid medium was prepared by adding Beef extract, 3.0 gm and peptone 5.0 gm in one litre of double distilled water. The sterilization was done by autoclaving the medium for 15 minutes at 15 lbs pressure/sq.inch. pH was adjusted to 7.6. While for preparing agar plates for viable counts estimation, 15.0 gm of agar-agar was added to the one litre liquid medium.

CULTURING AND PREPARATION OF INOCULA

The organisms usually grown at 37°C in a nutrient broth medium were maintained at a temperature of 5°C. Subcultures from stock cultures stored at 5°C were incubated for 48 hours at 37°C. These were again subcultured and after incubation for 48 hours used as inocula to inoculate experimental liquid medium. The experiments were carried out in Ehrlenmeyer flasks 100 ml capacity with side arm. The individual flasks containing 44 ml of sterile medium were inoculated by 1 ml of the
inoculum prepared above before incubation. The flasks were incubated for 48 hours at 37°C temperature on a gyrotory shaker.

**ADDITION OF METAL SALTS**

Chromium in form of chromium trioxide, lead as lead nitrate and magnesium as magnesium sulphate used here were of analytical grade. Stock solutions of each salt were made in double distilled water and sterilized. To get the final desired concentrations, appropriate volumes from the stock solution were transferred to separate tubes, sterilized and then introduced aseptically into the experimental flasks containing liquid medium before inoculation with organisms. The final volume of the incubation mixture was 50 ml. The concentrations of chromium during final tests were 0, 5, 8, 12 ppm and 0, 5, 8, 10 ppm for *E. coli* and *A. aerogenes*, respectively. The concentrations of lead tested were 0, 100, 150, 180 ppm and 0, 50, 100, 145 ppm for *E. coli* and *A. aerogenes*, respectively. The concentrations of magnesium (in form of magnesium sulphate) to study the influence of magnesium on the toxicity of Cr and Pb on *E. coli* and *A. aerogenes* were 0, 10, 50, 100, 200 ppm in both cases. Estimations of protein DNA, RNA and oxygen uptake were made after 0, 12, 24, 36 and 48 hours incubation. While cell density of the bacterial suspension was measured at 530 nm using 1 cm light path, at 6 hour intervals for 48 hours in a Lumichem-20 spectrophotometer (Scientific Instruments Service, India).
ESTIMATION OF VIABLE COUNTS OF BACTERIA

Counts of both test organisms were made by standard dilution plate counter procedure by inoculating 0.1 ml of serial ten fold dilution of culture on surfaces of nutrient agar petri plates, distributing inoculum evenly by means of broad platinum loop. After incubating at 37°C for 48 hours, bacterial colonies formed by viable cells were counted.

PROTEIN ESTIMATION

Protein was estimated by Folin-Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951). The method was based on the principle that protein reacts with the Folin's reagent to give coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein as in the biuret test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Reagents:
1. Alkaline sodium carbonate solution - 2.0 gm (2%) of \( \text{Na}_2\text{CO}_3 \) was dissolved in 0.1N NaOH and made to 100 ml.
2. Copper sulphate-sodium potassium tartarate solution - 0.5 gm of CuSO₄ (0.5%) and 1 gm (1%) of Na, K, tartarate were dissolved in distilled water and made to 100 ml. This solution was prepared fresh.
3. Alkaline solution - This was prepared by mixing 50 ml of alkaline sodium carbonate solution and 1 ml of copper sulphate - Na, K, tartarate solution on the day of use.
4. **Folin's reagent** - This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid. The commercial Folin - Lowry reagent obtained from Patel Chest Clinic, (CSIR, laboratory, Delhi University, Delhi) was diluted with equal volume of distilled water, prior to use.

5. **Standard protein** (Bovine Serum Albumin solution 0.2 mg/ml).

**PROCEDURE**

To 1 ml of the test solution, 5 ml of alkaline solution was added. Mixed thoroughly and allowed to stand at room temperature for about 10 to 15 minutes. Then 0.5 ml of diluted Folin - Lowry reagent was added rapidly with immediate mixing. After 30 minutes, extinction was read on Bausch and Lamb spectronic-20 against blank at 750 nm and 1 cm light path. A standard curve was prepared in the same way using standard solution in the range of 10 to 200 μg/ml and protein concentration of the test solution was determined from standard curve.

**ESTIMATION OF DEOXYRIBOSE NUCLEIC ACID**

DNA estimation was done following diphenyl-amine reaction method (Plummer, 1971).
Principle - It is based on the principle that when DNA is treated with diphenylamine under acidic conditions a blue compound is formed with a absorption maxima at 595 nm. This reaction is given by 2-deoxypentoses in general and is not specific for DNA. In acid solution, the straight chain form of the deoxypentose is converted to the highly reactive - hydroxylevulinaldehyde which reacts with diphenylamine to give a blue complex. In DNA only the deoxyribose of the purine nucleotides reacts, so that the value obtained represents half of the total deoxyribose present.

Reagents:
1. Deoxyribose - Nucleic Acid (commercial sample).
2. Trichloroacetic acid (10%) - 10.0 gm of trichloroacetic acid was dissolved in distilled water and made it to 100 ml.
3. Diphenylamine reagent - 1.0 gm of pure diphenylamine was dissolved in 100 ml of glacial acetic acid and 2.5 ml of concentrated sulphuric acid was added to it. This solution was prepared fresh each time.

PROCEDURE
To 2 ml of test solution, 2 ml of 10% TCA was added and heated the solution at 95°C for 15 minutes, cooled and 1 ml was removed in the test tube. To this 2 ml of water
and 5 ml of diphenylamine reagent was added and heated on a boiling water bath for 10 minutes, cooled and extinction was read against blank at 595 nm and 1 cm light path by Spectronic-20 (Bausch and Lamb). A standard curve was prepared using standard DNA (commercial sample) in the range of 25 to 500 μg/ml for ready reference. DNA concentration of test solution was calculated referring standard curve and noted as μg/ml.

ESTIMATION OF RIBOSE NUCLEIC ACID

**Principle** - The orcinol reaction method followed here for the estimation of RNA (Plummer, 1971) is based on the principle that the reaction is in general for pentoses and depends on the formation of furfural when the pentose is heated with concentrated HCl. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour. Only the purine nucleotides give significant reaction.

**Reagents:**

1. **Ribonucleic acid** (50 μg/ml).
2. **Orcinol reagent** - 100 mg of ferric chloride (FeCl₃·6H₂O) was dissolved in 100 ml concentrated HCl, and 3.5 ml of 6% orcinol in alcohol was added to it.
PROCEDURE

2 ml of test solution was mixed with 3 ml of orcinol reagent. It was heated on boiling water bath for 20 minutes, cooled and extinction was measured at 665 nm and 1 cm light path by spectronic-20 (Bausch and Lamb) against an orcinol blank. A standard curve was prepared in the same way taking standard RNA (commercial sample) in the range of 10 to 100 μg/ml. RNA in test solution was calculated referring to standard curve.

OXYGEN UPTAKE MEASUREMENT

Oxygen uptake by the organisms in the test solution was determined using oxygen electrode (Yellow Springs Instruments Co., Ohio, U.S.A.). Values correspond to the oxygen utilized in 5 minutes period and it is expressed as microlitre of oxygen utilized per 3 ml/5 minutes. Oxygen uptake was determined at 0, 12, 24, 36 and 48 hours incubation period taking 3 ml of test solution. The calculations of the actual oxygen consumption were made as follows:

1. Assume a change from 82 to 64 per cent of saturation in 5 minutes this means that 82-64 = 18% of the solution was consumed in 5 minutes.

2. Assume the solution was 3 ml of Ringers solution at 37°C and ambient barometric pressure contains 5.0 μl O₂/ml of
solution then a 3 ml sample contained

5 x 3 = 15 ml O₂ saturated.

3. A change of 18% in saturation means 15 x 18% =

2.7 ml O₂ was consumed in 5 minutes or on an hour
basis; 2.7 x \frac{60}{5} = 32.4 ml.
RESULTS

EFFECT OF CHROMIUM ON THE GROWTH AND VIABILITY OF E. COLI

The sensitivity of chromium towards the growth of pure cultures of E. coli as determined by growth measurement is given in Fig. 1. At 5 and 10 ppm chromium concentrations, there was observed a long lag phase followed by retarded growth. An increasing metal concentrations progressively reduced the optical density of bacteria which in turn reflects the bacterial growth. At 5 and 10 ppm metal concentrations, the bacterial growth was recorded only 67.2 and 32.8 per cent, respectively as compared to 100 per cent in control treatment (Table I). At 12 ppm concentration, the growth was completely inhibited.

Fig. 2 shows the viable bacterial counts of E. coli on treatment with chromium metal. There was observed a very rapid loss of viable colonies with the increasing chromium addition in the growth medium. Percent loss of viable counts was 34.5 and 73.4% at 5 and 10 ppm of chromium metal, respectively. No viable bacterial colony was observed at 12 ppm (Table I).

EFFECT OF CHROMIUM METAL ON THE GROWTH AND VIABILITY OF A. AEROGENES

Similar to Escherichia coli, chromium strongly inhibited the growth and viability of Aerobacter aerogenes (Fig. 3).