CHAPTER - II

GENERAL METHODS AND MATERIALS

Culture and maintainance of the organism :-

The organism used during this piece of work was Leishmania donovani, Strain 81. The strain was maintained at 22°C on slants of slightly modified Ray's medium (214). The medium consisted of the following materials.

Glucose	-	1 gm
Beef-heart extract	-	50 ml
'Oxoid' peptone	-	1 gm
Sodium chloride	-	0.6 gm
Agar	-	2.5 gm
Rabbit blood	-	10 ml

The pH was adjusted to 7.2 and the volume was made upto 100 ml. Slants were made with about 10 ml of this media in test tubes (6'' x 3/4'').

Luxuriant growth of this organism was found within three days after subculture. The organisms were maintained by subcultures made with an interval of 2 days.

After long sub-culturing on this medium, the colonies became rough and the growth rate gradually became sluggish. Thus, at the end of each month it was subcultured into the condensation fluid of Nicole, Novy, Macneal (N.N.N.) medium. This medium had the following composition.

Agar	-	14 gm.
Sodium chloride	-	6 gm.
Distilled water	-	900 ml.

After tubing and sterilizing in the autoclave, the medium was cooled to about 50°C and one third volume of sterile whole rabbit blood was added. After mixing well the tubes were set in slants. On slanting this medium at room temperature, a condensation fluid accumulated at the bottom. Subcultures were made in this fluid with the cells grown on our stock medium, when necessary. The tubes were kept in a B.O.D. incubator at 22°C ± 1°C for 15 days. Sufficient growth took place at the end of this period in the fluid. Subcultures were made with this fluid on the stock medium, the composition of which has already been described.

Preparation of Liquid medium :-

The liquid medium had the same composition as that of the solid medium, excepting the agar and whole blood. The blood drawn from the ventricles of rabbit heart was hemolysed with sterile distilled water (1:4) in a large sterile centrifuge tube and kept overnight in a refrigerator. Next day it was centrifuged at 750 x g for half an hour in a cold centrifuge. The supernatant was collected with a sterile capillary tube and 20 ml of this was added to 80 ml of the medium.

Preparation of cell-suspension :

The cells of L. donovani after 72 hours of growth on blood-agar medium were scraped carefully from the surface with a sterile platinum wire loop and suspended in normal saline (0.15 M, pH 7.4) maintained at 22°C and centrifuged quickly in a cold -- centrifuge (14 ± 1°C) at 1200 x g. The cells were washed twice with normal saline in the same way and collected. The cell pellet thus obtained was finally suspended in normal saline (0.15 M, pH 7.4) and was used in different experiments as 'cell suspension'.

Saline-phosphate buffer :

The solution used in some of the experiments under the name 'saline-phosphate buffer' was prepared by mixing measured volumes of 0.1 M saline and 0.2 M Na-phosphate buffer (pH 7.4). This solution contained 85 μmoles of NaCl and 100 μmoles of Na-phosphate buffer per ml.

Antibiotic solutions :

Mycobacillin :- Solution of mycobacillin (5 mg per ml) was prepared by dissolving pure crystalline mycobacillin in sodium bicarbonate solution (0.1 M) in water.

As mycobacillin solution was routinely prepared by dissolving the antibiotic in 0.1 M $NaHCO_3$ solution, the

vehicle (0.1 M sodium-bi-carbonate solution), was added in required quantity in the controls in all the different experiments whenever necessary.

Solutions of other antibiotics :

Solutions of streptomycin sulphate, Na-penicillin G., tetracycline hydrochloride and neomycin sulphate, (all of concentration 5 mg/ml) were prepared by dissolving the antibiotics in pure glass distilled water.

O_2-uptake studies :

Oxygen uptake studies were done by the conventional manometric techniques (215) in a Warburg apparatus.

Determination of Protein :

Protein was estimated in different experiments by biuret method (216).

Determination of 260 nm and 280 nm absorbing materials in the cell-free supernatant :

5 ml of the incubation mixture obtained after incubation with antibiotic was centrifuged in a cold centrifuge ($10^{\circ} \pm 1^{\circ}$C) at 2000 x g for 5 minutes to separate out all the cells. The clear supernatant (cell-free under the microscope) was taken out with a capillary and a known volume of it was treated with

an equal volume of cold perchloric acid solution (0.5 M) for 20 minutes. The precipitate, if any, was separated by centrifugation at 1200 x g for 30 minutes. The clear supernatant was taken out and its absorbancy at 260 nm and 280 nm was measured by use of a Beckman DU spectrophotometer.

Preincubation of cells with mycobacillin :

A known volume of cell suspension prepared with cold normal saline (0.15 M, pH 7.4) was incubated with a known concentration of mycobacillin for the desired period at a definite temperature. The incubation mixture was then quickly centrifuged in a high speed centrifuge at 20°C, and the cell-free supernatant was rejected. The cells were then washed once with cold normal saline (0.15M, pH 7.4). The cell pellet thus obtained was resuspended in the normal saline and used in the experiment.

In each of the experiments performed with such cells preincubated with mycobacillin, a control was run side by side. As mycobacillin was routinely dissolved in 0.1 M $NaHCO_3$ solution, the cell suspension used in control was prepared by incubating an equal volume of the original cell suspension with required amount of 0.1 M $NaHCO_3$ solution under identical condition.

Incubation with ^{14}C-labelled compounds and measurement of uptake of such labelled compounds :

In all such experiments uniformly labelled ^{14}C-glucose or [2-^{14}C] glycine, etc. were dissolved in glass distilled water and the radioactivity of such solutions was determined from dried 1 ml solution by a Nuclear Chicago Model 202 gas flow counter. 0.5 ml of a cell suspension of L.donovani (containing 4-5 mg of protein per ml) was added to one ml of the incubation mixture containing known amount of sugar or amino acid with known radioactivity. All incubations were made at 30°C. Suitable controls were incubated for zero-time under identical conditions for non-specific absorption of radioactivity. All the experimental data were corrected by this value in each case. The incubation in each case was stopped by adding to the incubation mixture four volumes of isotonic diluting solution containing 100 μmoles of the unlabelled compound and Na-phosphate buffer (pH 7.4) previously cooled to 4°C. Immediately after the addition of the diluting solution the tubes were placed in an ice-bath. The mixture was then centrifuged at 1500 x g for 15 minutes in a cold centrifuge (4° ± 1°C). The cell free supernatant was discarded. The cell pellet was then washed thrice with the cold diluting solution by centrifugation at 1500 x g at low temperature in a cold centrifuge. The washed cell-pellet thus obtained was extracted with 70% ethanol. The radioactivity of the alcohol extract was determined on dried aliquots in a Nuclear Chicago gas flow counter.

Materials and reagents :

The bacteriological peptone, mentioned as 'Oxoid' peptone was purchased from Oxoid division of Oxo Ltd, London. All other chemical reagents were of analytical reagent grade.

All the radio-chemicals used in course of this work were obtained from the isotope division of Bhaba Atomic Research Centre, India. Mycobacillin was obtained as a gift from Prof. S.K. Bose, Biochemistry Department of Calcutta University.

Na penicillin-G, streptomycin sulphate were obtained as gifts from Glaxo Laboratories (India). Tetracycline hydrochloride and neomycin sulphate were obtained as gifts from Pfizer Private Ltd. and Prof. S.K. Majumdar of the Department of Food Technology and Biochemical Engineering of Jadavpur University respectively.