MATERIALS AND METHODS

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BACTERIAL STRAINS :

Seventeen strains of Rhizobium and thirteen strains of Bradyrhizobium collected from different sources were used in this investigation. The strains and their sources are listed in Table 2.

CHEMICALS :

The chemicals used were of analytical grade obtained from the following sources :

Sigma Chemical Company (USA) ; E. Merck Chemical Company (Germany) ; Oxoid Limited (London) ; Koch-light (U.K.) ; Difco Laboratories (USA) ; B.D.H. Laboratory Chemicals (England and India) ; Sarabhai M. Chemicals (India) ; S.D.S. Laboratory Chemical Industries (India) ; E. Merck (India) ; Sisco Research Laboratories Private Limited (India) and Loba Chemical Company (India).

MEDIA :

For isolation and maintenance of root-nodule bacteria yeast-extract mannitol (YEM) medium (Vincent 1970) with the

following composition : mannitol, 10 g ; K_2HPO_4, 0.5 g ; $MgSO_4.7H_2O$, 0.2 g ; NaCl, 0.1 g , and yeast-extract, 1.0 g per litre was used. pH of the medium was adjusted to 7.0. Agar-agar at 20 g/litre was added as a solidifying agent. A synthetic SY medium of Sherwood (1970) containing K_2HPO_4, 220 mg ; $MgSO_4.7H_2O$, 100 mg ; $FeCl_3$ in 0.1 (N) HCl, 20 mg ; $CaCl_2$, 40 mg ; NH_4Cl, 500 mg ; thiamine HCl, 100 μg ; calcium panto-thenate, 100 μg and biotin 250 μg per litre and a carbon source was used in certain experiments. Carbon sources used were autoclaved separately at 10 p.s.i. for 20 minutes and then added to the medium to a final concentration of 1% , unless otherwise mentioned. Vitamin solutions, thiamine HCl, Ca-panto-thenate and biotin, were sterilized separately by passage through Millipore filter.

SOLUTIONS :

a) Buffers :

i) Tris-HCl buffer : 0.2 M Tris hydroxymethyl amino methane solutions adjusted to pH values, 7.6, 8.0 and 8.5 respectively, were prepared by the addition of HCl.

ii) Phosphate buffer : 0.2 M phosphate buffer solutions of pH values 6.5, 7.0 and 7.4 were prepared by mixing appropriate volumes of 0.2 M Na_2HPO_4 with 0.2 M NaH_2PO_4. 0.05 M phosphate buffer, pH 7.0 was prepared from 0.02 M stock buffer.

iii) Sodium-acetate buffer : Sodium acetate buffer solutions, pH 5.0, of three different molarities (0.05, 0.1 and 0.2) were prepared by mixing appropriate proportions of 0.2 M acetic acid with 0.2 M sodium acetate and dilution with water.

iv) HEPES buffer : 0.01 M HEPES (N-2-hydroxyethyl piperazine-N'-2 ethane sulphonic acid) buffer, pH 7.0, was prepared by adjusting the pH of 0.2 M solution of HEPES to 7.0 with 0.2 M NaOH and then diluting with water.

b) $HgCl_2$, 0.1% was prepared in 0.1 (N) HCl. Solutions of $MgCl_2$, 0.1 M ; KCl, 1 M ; Na_2HAsO_4, 0.17 M ; NaF, 0.1 M ; cystein HCl, 0.1 M ; ATP, 0.025 M ; $MgSO_4.7H_2O$, 1 M ; 5,5'-dithiobis-2-nitrobenzoic acid, 1 M ; $MnCl_2$, 0.12 M ; potassium ferricyanide, 5% ; oxalic acid, 0.01 M ; phenyl hydrazine hydrochloride, 1% ; trichloro acetic acid (TCA), 10% ; glutathione (reduced), 0.125 M and acetyl CoA, 1 M were prepared in distilled water. β-mercapto ethanol (14.2 M) was diluted to 0.1 M with distilled water.

c) Solutions of NAD^+, 0.004 M ; $NADP^+$, 0.004 M and NADH, 0.002 M were prepared afresh in distilled water and kept in an ice bath until use.

d) Solutions of 2,6-dichlorophenol indophenol, 1.53×10^{-5} M ; phenazine methosulphate, 0.3 M and potassium cyanide, 0.01 M were prepared afresh in distilled water and kept

away from light until use.

e) Cyclic AMP solution was prepared by dissolving 10 mg of sodium salt of cAMP in 0.05 M sodium acetate buffer, pH 5.0.

f) Substrates for enzyme assays : Solutions of fructose-6-phosphate, 0.05 M, fructose-1,6-biphosphate, 0.025 M and 0.03 M respectively ; glyceraldehyde-3-phosphate*, 0.3 M ; 6-phosphogluconate**, 0.025 M ; sodium succinate, 0.02 M ; sodium malate, 0.03 M ; DL-isocitrate, 0.01 M and 0.04 M ; glyoxylate, 0.03 M and ethanol, 0.1 M were prepared in distilled water.

* DL-glyceraldehyde-3-phosphate was prepared as follows : 10 mg DL-glyceraldehyde-3-phosphate diethyl acetal monobarium salt was dissolved in 0.6 ml of water. To this 150 mg of hydrogen form of a strong cation exchanger resin Dowex-50 (Stock No. 50 x 4 - 200 R) was added and mixed thoroughly. This mixture was placed in a boiling water bath for three minutes with intermittent shaking. It was then chilled quickly and centrifuged. The supernatant containing DL-glyceraldehyde-3-phosphate was decanted. The resin was again washed with water, centrifuged and supernatants were combined and the original volume was restored. The combined supernatant fluids presumed to contain 200 μ moles of DL glyceraldehyde-3-phosphate (100 μ moles of enzymatically active D-isomer).

** 6-phosphogluconate as substrate was prepared as follows : Barium salt of 6-phosphogluconate was dissolved in water after mixing with equivalent quantity of sodium sulphate, when insoluble barium sulphate was formed and precipitated out. The solution was centrifuged and the supernatant containing 6-phosphogluconate (sodium salt), 0.025 M, was used as substrate in enzyme assay.

g) DNSA solution was prepared by dissolving 1 g of 3,5 dinitrosalicyclic acid in 20 ml of 2(N) NaOH to which 50 ml of distilled water was added. To this 30 g of K-Na-tartrate was added and the total volume was made upto 100 ml.

h) Anthrone reagent was prepared by dissolving 200 mg of anthrone reagent in 100 ml of 36(N) H_2SO_4 on ice.

i) Protein reagent was prepared by mixing solutions A, B and C in a ratio of 100:1:1 [Solution A ; 400 mg NaOH + 2 g Na_2CO_3 in 100 ml H_2O ; Solution B ; 2 g K-Na-tartarate in 100 ml H_2O ; Solution C : 1 g $CuSO_4$ in 100 ml H_2O].

j) Glutaraldehyde solution for pre-fixation of samples for electron microscopy was prepared by combining 6 ml of 25% glutaraldehyde with 2.5 ml of 5% dimethylsulfoxide and 41.5 ml of 0.05 M acetate buffer, pH 5.0.

k) 1% osmium tetroxide (OsO_4) was used for post-fixation of samples for electron microscopy and was prepared as follows :

1 g of Osmium tetroxide dissolved in 50 ml of water. Then 10 ml of this solution was added to 10 ml of 0.2 M acetate buffer, pH 5.0 and was kept away from light.

1) Detergent solutions : Sarkosyl NL 91 (1%) was prepared in 0.01 M HEPES-buffer, pH 7.0 and Nonidet P 40 (0.5%) was prepared in water.

ISOLATION OF ROOT-NODULE BACTERIA :

Root-nodule bacteria were isolated from fresh, undamaged, healthy root-nodules of Cicer arietinum L. plants following the procedure described by Vincent (1970) on yeast-extract mannitol agar (YMA) plates. Nodules were cleansed thoroughly and surface sterilized with 0.1% acidified $HgCl_2$ (1-3 minutes) followed by momentary washing in 70% ethyl alcohol. The nodules were washed with several changes in sterile water and then crushed onto a glass slide. The milky fluid from within the nodules was streaked onto YMA plates containing congo red (20 µg/ml). The plates were incubated for 4-5 days. Isolated typical colonies of root-nodule bacteria were picked from this plate and grown in YM broth separately. The cultures after appropriate dilution were plated on YMA medium containing 20 µg congo red per ml. After 5 days single colonies which did not take any stain were picked up and were authenticated by means of host infection test,

cultural characteristics and biochemical properties as per methods described by Vincent (1970). After establishment of purity of the strains, these were labelled as per our laboratory convention with the prefix BICC and were maintained as laboratory stocks.

MAINTENANCE OF CULTURES :

Cultures of Rhizobium and Bradyrhizobium were maintained on slopes of yeast-extract mannitol agar (YMA) at 4°C. The cultures were transferred to fresh slants at intervals of 2 months.

PREPARATION OF INOCULUM :

Initially all the strains of rhizobia and bradyrhizobia were grown in 25 ml of YM broth under shake conditions (120 r.p.m.) at 28°C. From this an aliquot was transferred to SY medium of Sherwood (1970) containing the carbon source to be used in experimental medium and allowed to grow till early stationary phase. This was used as inoculum. Generally, 2% inoculum (approximately 6.8×10^9 CFU/ml) was used to start experimental cultures.

PROPAGATION OF CULTURES :

Rhizobium and Bradyrhizobium strains were propagated in 250 ml flasks containing 100 ml SY medium of Sherwood under shake condition on a rotary shaker (120 r.p.m.) at 28°C.

MEASUREMENT OF BACTERIAL GROWTH :

Cultures were grown in 100 ml nephalometric flasks containing 40 ml SY medium of Sherwood with appropriate carbon sources on a rotary shaker (120 r.p.m.) at 28°C starting from 2% inoculum. Growth was followed by measuring the turbidity of the cultures at regular intervals in a Klett Summerson Photoelectric Colorimeter (Model 900-3, USA) using a red filter (660 nm).

ANAEROBIC GROWTH OF RHIZOBIUM :

For anaerobic growth of Rhizobium sp. (Cicer arietinum) BICC 620 a medium of the following composition was used : K_2HPO_4, 500 mg ; $MgSO_4.7H_2O$, 200 mg ; NaCl, 100 mg ; yeast-extract, 300 mg ; mannitol, 10 g and KNO_3 at 6 mM in a litre, pH 7.0. After inoculation anaerobic condition was created following the recommendations of Daniel and Appleby (1972). The thick walled flasks were filled with medium and rubber stoppered leaving no dead space. The cells were harvested after 6 days of growth at 28°C.

NUTRITIONAL CHARACTERISTICS :

Carbon nutrition was determined by auxanographic method (Parke and Ornston 1984) using agar plates with a basal SY medium of sherwood containing yeast-extract (0.1%, w/v) but no other carbon source. Concentrated solutions (2%) of appropriate carbon sources were put in the wells of the plates which were made with the help of a cork-borer. Growth on control plates, where the major carbon source was omitted, was attributed to the utilization of yeast-extract as the carbon source. Yeast-extract was provided since many strains do not grow in the total absence of yeast-extract (Lindström et al. 1983). Growth around the well of a particular carbon source was determined after 5 days and by comparison with the level of growth in the control plate.

CARBON STARVATION :

Carbon starvation of Rhizobium was performed as follows : Rhizobium sp. (Cicer arietinum) BICC 620 was initially grown for 24 hours in SY minimal medium of Sherwood containing 1% glucose as carbon source. The cells were harvested, washed with and then transferred to sterile minimal medium without carbon and allowed to incubate. At various time periods of starvation the cells were used for enzyme assays.

In another set of experiment, cells were harvested after 24 hours of starvation, washed and transferred to a medium with

1% glucose At different time periods the levels of carbohydrate metabolic enzymes were measured in these cells.

ISOLATION OF BACTEROIDS :

Isolation of bacteroids was carried out according to the method of Sutton et al. (1977) with slight modification. 2.5 g of freshly harvested nodules were homogenized with 10 ml of 5.0 mM potassium phosphate buffer, pH 7.5 in a prechilled mortar and pestle and passed through three layers of cheese cloth. Cell debris were removed by centrifugation at 750 x g for 10 minutes at 4°C. The pellet was discarded and the supernatant was recentrifuged at 10,000 x g for 10 minutes at 4°C to sediment bacteroids. The supernatant, this time, was discarded and the pellet was gently resuspended in normal saline and then centrifuged through a cushion of 20% sucrose at 30,000 x g for 10 minutes (Legocki and Verma 1980). The ratio of bacterial suspension to sucrose cushion was 1:5. To obtain pure bacteroids, the plant membrane envelope contaminations in the pellet was removed by suspending it in 10 ml of 0.5% Nonidet P-40 and centrifugation at 10,000 x g for 10 minutes (Verma et al. 1978).

PREPARATION OF BACTERIAL CELL-FREE EXTRACTS :

Cultures of rhizobia and bradyrhizobia were harvested by centrifugation at 10,000 x g for 10 minutes at 4°C. The cells were washed thrice by centrifugation each time after resuspending the cell pellet in fresh 0.05 M phosphate buffer, pH 7.0. The washed cells were finally resuspended at 20% in 0.05 M phosphate buffer, pH 7.0, containing 5 mM β-mercaptoethanol. Washed cell suspension thus obtained was subjected to sonication at 4°C with an ultrasonic needle probe at 100 W for a total of 5 minutes in a Braun Sonicator (Model 1510, USA), allowing a probe rest for one minute intermittently after every 30 second pulse. Cell debris and whole cells were removed from the crude extract by centrifugation at 15,000 x g for 10 minutes. The supernatant, which is the crude cell-free extract (CFE), thus obtained was kept in an ice bath and used as source of enzymes.

ENZYME ASSAYS :

Enzyme assays were carried out at room temperature (25 - 27°C) and optical densities of the assay mixtures were recorded in a Shimadzu UV-240 double beam recording spectrophotometer (Graphicord, Japan). The key enzymes (Table 3) involved in central carbohydrate metabolic pathways were assayed.

The assays of all the dehydrogenase linked enzymes and dehydrogenase enzymes, with the exception of succinate dehydrogenase, were carried out in quartz cuvettes of 1 cm light path in a UV- visible double beam spectrophotometer and the optical densities were recorded at 340 nm in time scan mode. Each set of assay had two controls in which CFE or substrate was omitted respectively. Conversion of $NAD^+/NADP^+$ to NADH/NADPH or vice versa was recorded as the increase or decrease in O.D. The change in O.D. per minute was calculated from the linear portion of the enzyme activity curve and expressed as Δ O.D. / min. The molar extinction coefficient value of reduced $NAD^+/NADP^+$ is known to be 6.22×10^3. Activities of the enzymes were calculated from the amount of NADH/NADPH produced or consumed during the reaction as measured from Δ O.D. / min and taking the molar extinction coefficient value of the reduced co-factors into consideration. Specific activities were subsequently expressed as n moles of substrate consumed per minute per mg of protein. The amounts of protein added to the reaction mixtures were measured separately for the calculation of specific activities.

Reaction mixtures for assay of the enzyme were as follows :

(i) Phosphofructokinase (EC. 2.7.1.11) was assayed according to the procedure of Heath and Gaudy (1978) by linking it to glyceraldehyde-3-phosphate dehydrogenase system. The reaction mixture contained :

Tris-HCl buffer, pH 8.0	67 μ mol
KCl	50 μ mol
Na_2HAsO_4	17 μ mol
$MgCl_2$	5 μ mol
ATP	2.5 μ mol
NAD^+	0.25 μ mol
Fructose-6-phosphate	2.5 μ mol
Fructose biphosphate aldolase	5 units
Glyceraldehyde-3-phosphate dehydrogenase	20 units
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

(ii) Fructose biphosphate aldolase (EC 4.1.2.13) was assayed following the procedure of Heath & Gaudy (1978) with the following reaction mixture :

Tris-HCl buffer, pH 7.5	67 μ mol
KCl	100 μ mol
Na_2HAsO_4	17 μ mol
Cystein HCl (freshly prepared)	10 μ mol
NAD^+	0.25 μ mol
Glyceraldehyde-3-phosphate dehydrogenase	10 units
Fructose-1,6-biphosphate	2.5 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

[7] Two moles of NAD^+ were assumed to be reduced per mole of fructose-1,6-biphosphate cleaved because the excess triose-phosphate isomerase (approximately 0.5 U) of the extract would convert dihydroxyacetone phosphate to glyceraldehyde-3-phosphate [7].

(iii) Glyceraldehyde-3-phosphate dehydrogenase (EC. 1.2.1.12) was assayed according to the procedure supplied by Sigma Chemical Company (USA) with the purchase of DL-glyceraldehyde-3-phosphate diethyl acetal (monobarium) salt. The reaction mixture contained :

Tris-HCl buffer, pH 8.5	300 μ mol
Na_2HAsO_4	510 μ mol
Cystein HCl (freshly neutralized to pH 7.0)	10 μ mol
Sodium fluoride	600 μ mol
β-NAD^+ (Sigma Product No. N7004)	10 μ mol
CFE (300 - 500 μg protein approx.)	0.10 ml.

To the blank cuvette, 0.40 ml H_2O was added and to the test cuvette 0.30 ml H_2O + 0.1 ml of substrate solution containing 0.3 μ mol of glyceraldehyde-3-phosphate was added. The final volume of the reaction mixture was 3.0 ml.

(iv) The Entner-Doudoroff (ED) enzymes were assayed as the combined activities of 6-phosphogluconate dehydratase (EC. 4.2.1.12) and 2-keto-3 deoxy-6-phosphogluconate aldolase (EC. 4.1.2.14), according to the procedure described by Lessie and Vander Wyk (1972). Composition of the reaction mixture was as follows :

Tris-HCl buffer, pH 8.5	100 μ mol
β-Mercaptoethanol	10 μ mol
NADH	0.4 μ mol
6-phosphogluconate, sodium salt	5 μ mol
Lactate dehydrogenase	100 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

(v) 6-phosphogluconate dehydrogenase (EC. 1.1.1.44) was assayed according to the method described by Tigerstrom and Campbell (1966) with the following reaction mixture :

Tris-HCl buffer, pH 7.6	100 μ mol
$MgCl_2$	2 μ mol
6-phosphogluconate, sodium salt	5 μ mol
$NADP^+$	0.4 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

(vi) Isocitrate dehydrogenase (EC. 1.1.1.42) was assayed by the method of Khouw and Mc Curdy (1969). Composition of the reaction mixture was as follows :

Tris-HCl buffer, pH 7.6	300 μ mol
$MgCl_2$	10 μ mol
$NADP^+$	0.2 μ mol
DL-isocitrate	10 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

(vii) Succinate dehydrogenase (EC 1.3.99.1) was assayed as described by Ells (1959) with the following reaction mixture :

Phosphate buffer , pH 7.6	50 μ mol
Potassium cyanide	0.1 μ mol
2-6,dichlorophenol indophenol	1.53×10^{-5} μ mol
Phenazine methosulphate	0.3 μ mol
Sodium succinate	2.0 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml.	

The O.D. of the mixture was recorded at 600 nm following the addition of phenazine methosulphate last.

(viii) Malate dehydrogenase (EC 1.1.1.37) was assayed by following the method of Englard and Siegel (1969) with slight modification. The reaction mixture was as follows :

Phosphate buffer, pH 8.5	100 μ mol
L-malate, sodium salt (freshly prepared)	8.5 μ mol
NAD^+	0.37 μ mol
CFE to a total volume of 1.0 ml	

(ix) Isocitrate lyase (EC 4.1.3.1) was assayed after Mc Fadden (1969). The procedure is as follows : A mixture containing the following ingredients was preincubated in test tubes at 30°C for 10 minutes :

Tris-Mg^{++} buffer (0.1 M), pH 7.6	1.5 ml
Glutathione (reduced) (0.125 M)	0.2 ml
CFE (300 - 500 μg protein approx.)	0.1 ml

The reaction was initiated with the addition of 0.2 ml of 40 mM isocitrate solution followed by thorough mixing. After incubation for 10 minutes at 30°C the reaction was stopped with the addition of 1 ml 10% TCA. At this stage the reaction mixture was stored overnight at 2°C, if required. From the

reaction mixture, 1 ml was transferred to a 50 ml beaker. To this 6 ml of oxalic acid- phenyl hydrazine hydrochloride mixture (5 parts of 10 mM oxalic acid + 1 part freshly prepared 1% phenyl hydrazine HCl) was added and heated until just boiling on a preheated hot plate. The beaker was removed immediately from the hot plate, cooled at room temperature for 5 minutes and then chilled on ice for 2 minutes. 4 ml of concentrated HCl was added to it followed by 1 ml of 5% potassium ferricyanide and the preparation was mixed thoroughly. When a large number of assays were made the ferricyanide solution was added in a timed sequence. Seven minutes after the addition of ferricyanide, O.D. of the mixture was recorded at 520 nm against a blank without any CFE in a Shimadzu double beam spectrophotometer (model UV 240, Graphicard, Japan).

When the length of the light path of the cuvette is 1 cm the yield of glyoxylate in μ moles per reaction vessel (i.e., 2.0 ml of original incubation mixture) is given by O.D. at 520 nm divided by a factor of 1.15. 1 unit of enzyme is that amount which catalyzes the disappearance of 1 n mole of isocitrate per minute at 28°C under conditions of assay. The amount of isocitrate that disappears is equivalent to the amount of glyoxylate produced. Specific activity is defined as units/mg of protein.

(x) Malate synthase (EC 4.1.3.2) was assayed following the procedure described by Cooper and Beevers (1969). The reaction mixture contained the following ingredients :

Phosphate buffer, pH 6.5	100 μ mol
Acetyl CoA	0.5 μ mol
Glyoxylate	3.0 μ mol
$MgCl_2$	10 μ mol
5,5' dithiobis-2-nitrobenzoic acid (DTNB)	0.1 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

The reaction was initiated by the addition of glyoxylate and continued at 35°C for 10 minutes in a cuvette when O.D. of the mixture at 412 nm was measured spectrophotometrically against a reagent blank. Molar extinction co-efficient value of DTNB is known to be 1.36×10^4. Molar activity was calculated from the glyoxylate consumed during the reaction as measured from ΔO.D./min and taking the molar extinction co-efficient value of the DTNB into consideration. Specific activity was subsequently expressed as n moles of substrate consumed per minute per mg of protein.

(xi) Malic enzyme (EC 1.1.1.40) was assayed following the method of Hsu and Lardy (1969). The reaction mixture contained the following reagents :

Tris-HCl buffer, pH 7.4	40 μ mol
L-malate, sodium salt	3 μ mol
$MnCl_2$, $4H_2O$	12 μ mol
$NADP^+$	0.34 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

(xii) Fructose biphosphatase (EC. 3.1.3.11) was assayed as described by Stowers and Elkan (1983) with slight modification. The reaction mixture was as follows :

Tris-HCl buffer, pH 8.0	40 μ mol
$MgSO_4.7H_2O$	12 μ mol
Fructose-1,6-biphosphate	3 μ mol
Glucose-6-phosphate dehydrogenase	0.5 units
$NADP^+$	0.5 units
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

(xiii) Alcohol dehydrogenase (EC. 1.1.1.1) was assayed by the procedure described by Obradors et al. (1988). The reaction mixture was as follows :

Ethanol	100 μ mol
NAD^+	1 μ mol
Sodium phosphate buffer, pH 8.0	4.8 μ mol
CFE (300 - 500 μg protein approx.) to a total volume of 1.0 ml	

ESTIMATION OF PROTEINS :

Protein in the CFE was estimated according the method of Lowry et al. (1951). To a 0.5 ml aliquot of appropriately diluted protein sample, 5.0 ml of the protein reagent was added and left for 10 minutes at room temperature (25-27°C). Then 0.5 ml of Folin-phenol reagent(after dilution with equal volume of H_2O) was added to it and the solution was allowed to stand at 37°C for 30 minutes. Optical density of the solution at 660 nm was read against a corresponding blank and the amount of protein was calculated from a standard curve prepared using bovine serum albumin (fraction V, Sigma Chemical Co., USA) as standard.

Protein reagent was prepared by mixing reagent A (2% Na_2CO_3 + 0.4% NaOH), reagent B (2% Na-K-tartrate) and reagent C (1% $CuSO_4$) in a ratio of 100:1:1).

ESTIMATION OF SUGARS :

Glucose was estimated by anthrone-method (Plummer, 1971) or by dinitrosalicylic acid (DNSA) method (Bernfeld, 1955). Following the protocol of anthrone method 4 ml of anthrone reagent was added to 1 ml of appropriately diluted test sample and mixed rapidly. Tubes were covered with glass balls to prevent loss of water due to evaporation and were placed in

a boiling water bath. After 10 minutes, the contents were cooled and the optical densities of the samples were read at 520 nm against a reagent blank. The amount of glucose was estimated from a standard curve prepared with glucose.

During measurement of glucose using DNSA method an equal volume of DNSA solution was added to a test sample and boiled in a water bath for 5 minutes. The reaction mixture was cooled and read at 520 nm against a reagent blank. The amount of glucose was estimated from a standard curve prepared with glucose.

ELECTRON MICROSCOPY :

Scanning electron microscopy was used to study the morphology of bacterial cells. The specimens were prepared as follows :

Cells were harvested by centrifugation at 10,000 x g for 10 minutes. Bacterial pellets were washed thrice with normal saline and prefixed with a mixture of 3% glutaraldehyde and 5% DMSO in 0.05 M acetate buffer, pH 5.0, for 30 minutes. Cells were then harvested by centrifugation at 10,000 x g for 10 minutes and the pellets were washed thrice with 0.1 M sodium acetate buffer, pH 5.0. The pellets were then post-fixed with osmium tetroxide solution

for 30 minutes. Cells were collected by centrifugation at 10,000 x g for 10 minutes and were dehydrated with a series of ethyl alcohol starting with 30% via 40, 50, 60, 70, 80, 85, 90 and 95% and finally with 100% with 10 minutes of dehydration in each grade. The cells were then spread on a clear glass slide (1 sq.cm.). The slide was mounted on a stub with double side adhesive tape and silver dag and coated slowly with a very thin (2-5 nm) layer of gold (Boyd and Brorers 1971) in a sputtering unit prior to examination under scanning electron microscope (Philips, Model PSEM-500, Holland).

ISOLATION OF OUTER MEMBRANE PROTEIN :

Outer membrane proteins (OMP) were isolated from bacteroids and from free-living Rhizobium sp. (Cicer arietinum) BICC 620 grown in different carbon sources following the method described by Fillip et al. (1973). Cells were harvested from their mid log phase of growth by centrifugation at 10,000 x g for 10 minutes at 4 °C and washed several times with 10 mM HEPES buffer, pH 7.0. The pellets obtained were resuspended in the same buffer and were subjected to sonication until the turbidity of the suspension decreased by about 80% . The materials were centrifuged at 10,000 x g for 10 minutes to sediment the intact cells and cell debris. The pellets were discarded and the supernatants containing the membrane fraction

were collected. It was diluted with HEPES buffer, pH 7.0 and then centrifuged at 105,000 x g for an hour at 4°C in an ultracentrifuge (Hitachi model 55P-72, Japan). The pellet containing both OMP + inner membrane protein was treated with 1% Sarkosyl NL-91 in 10 mM HEPES buffer, pH 7.0 for 30 minutes at room temperature. The pellet containing OMP fraction was recovered by centrifugation at 105,000 x g for an hour at 4°C and was washed with cold distilled water to remove Sarkosyl by centrifugation at 105,000 x g for an hour at 4°C.

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) :

SDS-PAGE analysis of OMP of Rhizobium grown on different carbon sources as well as that of bacteroids isolated from Cicer arietinum were performed by the method of Laemmli (1970), using separating gels at a concentration of 12.5% (w/v). SDS-protein standards were also run in a separate lane in the same gel. Gels were stained with Coomassie brilliant blue after electrophoresis and destained with a solution of 5% methanol and 7.5% acetic acid.