CHAPTER II

MATERIALS AND METHODS
MATERIALS AND METHODS

The strain of *Rhizobium japonicum* 211D, used in the present investigation was kindly supplied by Dr. H. Marekova, Research Institute of Crop Production, Institute of Plant Nutrition, Prague-6, Ruzyne, Czechoslovakia.

The stock cultures of *R. japonicum* 211D were maintained by weekly subculturing them on Mannitol Yeast extract agar (MYA) slants, having following composition (in gr%); mannitol, 1.0; yeast extract (Difco), 0.1, and agar (Difco) 2.0. The cultures were preserved by waxing the slants and storing at 0-4°C.

For the growth, the medium described by Balassa (1) was used having following ingredients in grams/100 ml medium; glucose, 0.5; (NH₄)₂SO₄, 0.1; yeast extract, 0.05; vitamin free casamino acid, 0.05; K₂HPO₄, 0.36; KH₂PO₄, 0.04; MgSO₄, 7H₂O, 0.005 and NaCl, 0.05. The pH of the medium was adjusted to 7.2 and medium was distributed as 200 ml, in 250 ml Erlenmeyer flask. The medium was sterilized by autoclaving at 15 lbs. pressure for 15 min. Glucose and (NH₄)₂SO₄ were sterilized separately and added into the medium aseptically.
DL-β-hydroxybutyric acid (sodium salt) when used into the medium as a carbon source, was replaced on carbon basis, after sterilization by membrane filtration (poresize of membrane filter 0.45 μ) technique. Inoculum was prepared by inoculating a loopful of growth from MYA slants to a 20 ml of Balassa's complex medium in a sugar tube and incubating on a rotary shaker (200 rpm) at 30°C ± 1 for 15 hours. This inoculum was used to inoculate flasks containing 200 ml of medium. The quantity of inoculum used was adjusted in such a way, that each flask contained about 10^8 cells/ml of the medium. The flasks were incubated at 30°C ± 1 on a rotary shaker.

In the later part of the work on ammonia assimilatory enzymes, the medium described by Kurz and LaRue(2) was used having the following ingredients in mgs/1000ml medium; KNO₃, 1000; MgSO₄, 7H₂O 250; NaH₂PO₄ H₂O, 150; CaCl₂ 2H₂O, 150; Fe(330), 28; MnSO₄, 10; H₃BO₃, 3.0; ZnSO₄ 7H₂O, 2.0; Na₂MoO₄ 2H₂O, 0.25; CuSO₄, 0.025; CoCl₂ 6H₂O, 0.025; KI, 0.78; meso-inositol, 100; thiamine-HCl, 10; nicotinic acid, 1.0; pyridoxine-HCl, 1.0 and sucrose, 5,000. The pH of the medium was adjusted to 5.5. Glucose, sucrose, xylose, sodium succinate and KNO₃ were separately prepared and autoclaved. Sodium succinate, glucose and xylose were replaced in the medium on carbon basis.
The semi-anaerobic condition in these experiments were created by incubating the flasks in a glass dessicator under the nitrogen atmosphere. Growth under this condition was harvested after 48 hours of incubation.

Determination of Poly-$\beta$-hydroxybutyrate:

The growth of $R$. japonicum was harvested at the desired period by centrifugation at 5,000 x g for 15 minutes at 0-4°C. The cells were washed twice with cold distilled water. Cells thus obtained were suspended in minimum quantity of distilled water and lyophilized using thermovac lypholizer. PHB was extracted and estimated by following the method of Schlegel et al (3). To 100 to 500 mg dry cells were extracted for 15-16 hrs with 30 ml of chloroform at 30°C. After the extraction, the flask containing cells and chloroform was shaken vigorously by putting in a water bath adjusted at 50°C. The cells were removed by filtration and washed twice with 15 ml. of boiling chloroform. The PHB, from the extract was precipitated by adding four volumes of solvent ether and leaving it undisturbed for 14 hrs. at 15-20°C. The precipitates thus obtained were collected by filtration through Whatman filter paper (No.42) and dried at 40°C. The PHB obtained was measured gravimetrically. The content of PHB was expressed as mg% of dry cell weight.
Extraction and estimation of lipids:

Total lipids were extracted from dried cells with 10 volumes of chloroform:methanol (2:1 v/v) mixture, and were freed from impurities by the method of Folch et al (4). The lipid content was estimated gravimetrically after evaporating solvents to the dryness.

Glucose, Pyruvate and Acetate determination:

Glucose was estimated by using the glucose oxidase reagent as described by Dahlqvist (5). The methods of Rose (6), and Friedmann (7) were used to determine the acetate and pyruvate concentrations respectively.

Extraction of haemoglobin:

For the extraction of haemoglobin, cells were collected by centrifugation at 0-4°C at desired period of growth. The cells were washed with distilled water and resuspended in 0.2M tris-HCl buffer (pH 8.8) to get 30% (w/v) cell suspension. The cell suspension was saturated with nitrogen by passing nitrogen gas through the capillary. The cell suspension was then placed in a homogenising bottle containing glass beads (0.1 mm diameter B Brown and Co., West Germany). The glass beads to the wet cell ratio was kept 1:5 (w/w). The cells were then homogenized using Brown homogenizer, Model MSK (B. Brown and
Co., West Germany) at 4000 rpm. for 3 min. The temperature during this process was maintained at 0-4°C by passing liquid CO₂ through the side capillary. The glass beads were removed by decantation and homogenate was then centrifuged at 12,000 x g for 20 min. at 0-4°C. The supernatant thus obtained was used for the estimation of haemoglobin, as well as for the determination of the activity of δ-aminolevulinic acid synthetase.

Haemoglobin estimation:

The haemoglobin was assayed from the supernatant by the pyridine haemochrome method, described by Appleby (8) as follows:

To a suitable dilution of supernatant equal amount of 4.4 M pyridine in 0.2M NaOH was added. After about 5 min. the absorbance was read at 410 nm. From the standard value obtained by using pure haemoglobin preparation (from Bovine), the amount of haemoglobin present in cell free extract was calculated.

Preparation of cell free extract:

The cells after desired period of growth were harvested in cold (0-4°C) by centrifugation at 5,000 x g for 10 minutes, washed twice with 0.01M tris-HCl buffer
(pH 7.0). The cells were ruptured using glass powder (2:1 w/w) and 10% (w/v) cell homogenate was prepared in 0.05 M tris-HCl buffer (pH 7.0). The cell homogenate thus obtained was centrifuged for 15 min. at 12,000 x g at 0-4°C. The supernatant obtained was used for the enzyme assay.

The protein in the crude cell free extract was estimated by the method of Lowry et al (9) using bovine serum albumin as the reference.

**Enzyme assays:**

The measurements of optical density at 340 nm were carried out using a Carl-Zeiss (VSU2) Spectrophotometer. Unless specified, the unit of enzyme was defined as the amount of enzyme which brought about 0.01 change in O.D. per minute at 30°C.

**B-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30):**

(D-β-hydroxybutyrate NAD Oxidoreductases)

The enzyme activity was estimated as described by Bergmeyer et al (10). The assay system in a total volume of 1.5 ml. contained; 0.5 ml of 0.2 M tris-HCl buffer, pH 8.0; 0.05 ml. of 0.1M MgCl₂; 0.05 ml of 0.005 M NAD; 0.1 ml. of 0.5M β-hydroxybutyrate and an appropriate concentration of the enzyme. The change in O.D. was followed for 3 minutes.
Acetoacetyl CoA reductase (E.C. 1.1.1.36):

Acetoacetyl CoA reductase was assayed by the method of Senior and Dawes (11). The assay system, in a total volume of 1.5 ml., contained 0.75 ml. of 0.2 M potassium phosphate buffer, pH 5.5; 0.05 ml of 0.1 M MgCl₂; 0.01 ml of 0.02 M dithiothreitol; 0.025 ml of 0.005 M NADPH; 0.06 ml. of 0.002 M acetoacetyl CoA and suitable quantity of the enzyme. The change in O.D. was followed for 3 minutes. NADPH was destroyed at pH 5.5 at a rate (ΔE₃₄₀/min) varying between 0.005 to 0.01, and assay had to be corrected for this destruction. The reactants minus substrates, were preincubated at 30°C for 3 minutes before addition of NADPH. The acid destruction of NADPH was recorded for 2 min. before addition of acetoacetyl CoA and measurement of the initial rate of NADPH oxidation.

NADPH-NAD Transhydrogenase (E.C.1.6.1.1):

The method of Stein et al (12) was used to determine NAD(P)H-NAD transhydrogenase activity. The standard assay mixture contained in u moles in 3 ml; potassium phosphate buffer, (pH 7.4) 100; NADP, 0.1; NAD, 0.5; isocitrate (trisodium salt), 10; KCN, 10 and enzyme concentration ranging from 150 to 300 ugs. NAD was added after the system had stabilized with respect to endogenous activity. Change in O.D. was measured at 340 nm.
NADH Oxidase (E.C. 1.6.99.3):

The reduced NAD oxidoreductase activity was measured by the method of Green and Ziegler (13). The standard assay mixture contained in μmoles, potassium phosphate buffer, pH 7.6, 100 μmole; EDTA, 1.0; NADH, 0.5 and an appropriate concentration of enzyme (100-200 μgs). The assay system was made to 3.0 ml with distilled water and change in O.D. at 340 nm was measured.

Glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49):

The method suggested by Kornberg and Horecker (14) was used for the estimation of glucose-6-phosphate dehydrogenase. The assay system composed of 100 μ moles of tris-HCl buffer, (pH 7.5); 10 μ moles of MgCl₂; 0.25 μ mole of NADP, and suitable concentration of enzyme (10-80 μgs). The assay system was made to 3.0 ml with distilled water and change in O.D. at 340 nm was recorded.

6-phosphogluconate dehydrogenase (E.C. 1.1.1.44):

The enzyme was assayed by the method of Kornberg and Horecker (14) as mentioned for glucose-6-phosphate dehydrogenase except that glucose-6-phosphate was replaced by 6-phosphogluconate.
Isocitrate dehydrogenase (E.C. 1.1.1.42):

The enzyme activity was assayed by the method of Severo Ochoa (15). The assay system in a total volume of 3.0 ml contained, 100 μ moles of tris-HCl buffer, pH 7.4; 10 μ moles of MgCl₂, 0.25 μ mole of NADP; 0.1 μ mole of sodium isocitrate and an appropriate concentration of enzyme (10-80 μgs). The change in O.D. at 340 nm was recorded.

Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.13):

The enzyme was assayed by the method of Senior and Dawes (16). The assay system contained, 100 μ moles of sodium pyrophosphate buffer, pH 7.8, containing 4.0 mM cysteine; 20 μ moles of Na₂H₂AsO₄, pH 7.8; 0.25 μ mole of NADP; 5.0 μ moles of fructose-1,6-diphosphate in 30 mM pyrophosphate buffer, pH 7.8, containing 4.0 mM cysteine; 0.25 ml aldolase solution (750 μg of protein/ml); 20 to 100 μg of enzyme protein, and distilled water to make final volume 3.0 ml. The increase in O.D. at 340nm was followed.

Malic enzyme (E.C. 1.1.1.40):

The method of Severo Ochoa (17) was used to determine malic enzyme activity. The assay system in a total volume of 3.0 ml contained 1.0 ml of 0.1 M tris-HCl, (pH 7.4);
0.05ml of 0.05M malate; 0.1 ml of 0.1 M MnCl₂, 5H₂O; 0.05 ml of 0.005M NADP and enzyme extract containing 100-200 µg of protein. The change in absorption was recorded at 340 nm.

Glutamate dehydrogenase (E.C. 1.4.1.2):

NAD glutamate dehydrogenase was measured by following the method of Thomulka and Moat (18). The reaction mixture consisted of 100 µ moles of tris-HCl buffer, pH 7.3; 15 µ moles of α-ketoglutarate (pH 7.0); 0.25 µ mole of NADH; 15 µ moles of ammonium sulphate and an appropriate concentration of enzyme in a total volume of 3.0 ml. Ammonium sulphate was omitted from blanks and the decrease in absorbance at 340 nm was recorded.

The unit of enzyme was defined as the amount of enzyme that brought about oxidation of 1.0 µmole of NADH per minute at 30°C.

Alanine dehydrogenase (E.C. 1.4.1.1):

The method described by Thomulka and Moat (18) was followed for the estimation of alanine dehydrogenase. The assay system contained in µ moles; tris-HCl buffer, pH 7.5, 100; sodium pyruvate, 5; ammonium sulphate, 10; NADH, 0.25 and 100-200 µg of enzyme protein in a total volume of 3.0 ml. The reaction was started by the
substrate addition and the decrease in O.D. was noted at 340 nm.

The unit of enzyme was defined as the amount of enzyme that brought about oxidation of 1.0 nmole of NADH per minute at 30°C.

**Glutamate Synthase (E.C. 2.6.1.53):**

The method described by Roon et al. (19) was used to assay glutamate synthase activity. Assay system contained, 100 μ moles of potassium phosphate buffer, (pH 7.8); 15 μ moles of α-ketoglutarate (pH 7.0); 10 μ moles of glutamine (freshly prepared); 0.5 μ mole of NADH and a suitable concentration of enzyme in a total volume of 1.5 ml. The reaction was started by the addition of glutamine and decrease in absorbance at 340 nm was recorded.

The unit of enzyme was defined as the amount of enzyme that brought about oxidation of 1.0 nmole of NADH per minute at 30°C.

**Glutamine Synthetase (E.C. 6.3.1.2):**

The enzyme was assayed by the method of Elliot(20). The estimation is based on the fact that the enzyme is capable of forming γ-glutamyl hydroxamic acid with hydroxylamine as the amino donor. The assay system in
a total volume of 3.0 ml contained, 100 μ moles of tris-HCl buffer; (pH 7.5); 50 μ moles of sodium glutamate; 20 μ moles of hydroxylamine; 10 μ moles of MgSO₄, 7H₂O; 10 μ moles of ATP and an appropriate concentration of the enzyme. ATP was omitted from the blanks. The hydroxamic acid formed in the reaction was estimated by the method described by Lipmann and Tuttle (21).

The unit of enzyme was defined as the amount of enzyme that brought about formation of 1.0 nmole of hydroxamic acid per 20 minutes at 30°C.

Glutamate-oxaloacetate transaminase (E.C. 2.6.1.1):

The enzyme activity was estimated by measuring the disappearance or formation of oxaloacetate, as indicated by the decrease or increase in O.D. at 265 nm as described by Dixon and Severin (22). The standard assay mixture contained in μ moles, tris-HCl buffer (pH 7.5), 100; sodium glutamate, 10; oxaloacetate (sodium salt freshly prepared), 10; pyridoxal phosphate, 0.1 and 300-400 μg of enzyme protein. In all blank tubes glutamate was added after stopping the reaction with 0.1 ml of 10% TCA. The change in O.D. at 265 nm was measured after removing the precipitated enzyme protein by centrifugation, as the enzyme protein interferes while reading at 265 nm.
Glutamate pyruvate transaminase (E.C. 2.6.1.2):

The enzyme activity was estimated by measuring the disappearance and formation of glutamate and alanine by following the method of Giri et al (23). The assay system in a total volume of 1.0 ml contained in μ moles; tris-HCl buffer, pH 7.5, 100; sodium glutamate, 10; sodium pyruvate, 10; pyridoxal phosphate, 0.1 and an appropriate quantity of the enzyme protein (150-200 μgs).

Assay of δ-aminolevulinate synthetase (E.C. 2.3.1.37):

The assay of δ-aminolevulinate synthetase was made from the cell free extract prepared as described for haemoglobin estimation. The activity of this enzyme was determined immediately after the preparation of cell free extract.

δ-ALA synthetase was assayed by method of Tait (24). The assay system contained following ingredients in μ moles in a total volume of 5.0 ml tris-HCl buffer (pH 7.0), 50; glycine, 25; sodium succinate, 20; pyridoxal phosphate, 2; ATP, 2; MgCl₂, 7H₂O, 3; MnSO₄, H₂O, 20; CoA, 40 μg and an appropriate concentration of enzyme preparation. CoA was omitted from the blanks. After 20 minutes of incubation at 37°C, the reaction was stopped by the addition of 0.5 ml of 10% TCA. The precipitates were removed by centrifugation, and δ-aminolevulinate
formed, was measured by the method of Mauzerall and Granick (25). The unit of enzyme was defined as the amount of enzyme required for the formation of 1.0 μmole of δ-aminolevunic acid per 20 minutes at 30°C.

Purification of B-hydroxybutyrate dehydrogenase:

A 30% (w/v) crude cell-free extract in 0.05 M sodium phosphate buffer pH 7.6, containing 10 mM B-mercaptoethanol was made 0.01% (w/v) with respect to protamine sulphate, allowed to stand for 30 minutes, centrifuged at 5000 x g for 15 minutes and the precipitate discarded. Sufficient ammonium sulphate was added to the supernatant fluid to bring the final concentration to 20% saturation. After 90 min. precipitates were collected by centrifuging at 15,000 x g for 15 min. and dissolved in 2.0 ml of 0.05 M sodium phosphate buffer, pH 7.6 containing 10 mM B-mercaptoethanol. To the supernatant fluid ammonium sulphate was added over a period of 2 hours to bring the final concentration to 80% saturation and allowed to stand for an additional 2 hrs. at 0°C. The protein precipitate formed was collected by centrifuging at 15,000 x g for 15 min. and then dissolved in 10.0 ml of 0.05 M sodium phosphate buffer, pH 7.6, containing 10 mM B-mercaptoethanol, and dialysed against 10 mM sodium phosphate buffer, pH 7.6, containing 10 mM
β-mercaptoethanol 0.001 mM NAD and 1.0 mM β-hydroxybutyrate for 2 hours. Ammonium sulphate fraction (20-80%) was further mixed with calcium phosphate gel (1.0 mg protein to 15 mg gel) and allowed to stand for 5 minutes. Thereafter it was centrifuged at 5000 x g for 5 minutes and the supernatant was checked for the enzyme activity. The precipitated gel was mixed with 10.0 ml of 0.5 M sodium phosphate buffer, pH 7.6 containing 10 mM β-mercaptoethanol and allowed to stand for one hour with intermittent shaking. It was then centrifuged at 5000 x g for 10 minutes and the above procedure was repeated. The activity was recovered in the second eluate while the first eluate was discarded.

Purification of acetoacetyl CoA reductase:

A 30% (w/v) crude cell free extract was prepared in 0.05 M potassium phosphate buffer, pH 7.2, containing 10 mM β-mercaptoethanol. It was then made 0.01% (w/v) with respect to protamine sulphate, allowed to stand for 30 minutes, centrifuged at 5000 x g for 15 minutes and the precipitates discarded. Sufficient ammonium sulphate was added to the supernatant fluid to bring the final concentration to 10%. After 90 min. precipitates were collected by centrifugation at 15,000 x g for 15 minutes and dissolved in 2.0 ml of 0.05 M potassium
phosphate buffer, pH 7.2 containing 10 mM β-mercapto-ethanol. To the supernatant fluid ammonium sulphate was added over a period of two hours to bring the final concentration to 60% saturation and allowed to stand for an additional two hours at 0°C. The protein precipitated was collected by centrifugation at 15,000 x g for 15 min. and then dissolved in 10.0 ml of 0.05 M potassium phosphate buffer, pH 7.2, containing 10 mM β-mercapto-ethanol and dialysed against 10mM potassium phosphate buffer, pH 7.2, containing 10mM β-mercaptoethanol for two hours. Ammonium sulphate fraction (10-60%) was further mixed with calcium phosphate gel (1.0 mg protein to 15 mg gel) and allowed to stand for 5 minutes. Thereafter it was centrifuged at 5000 x g for 5 min. and the supernatant was checked for the enzyme activity. The precipitated gel was mixed with 10.0 ml of 0.5 M potassium phosphate buffer, pH 7.2 containing 10 mM β-mercaptoethanol and allowed to stand for one hour with intermittent shaking. It was then centrifuged at 5000 x g for 10 min, and the above procedure was repeated. The enzyme activity was recovered in the third eluate while the first two eluates were discarded.
REFERENCES


