MATERIAL AND METHODS
Live adults of *Trichuris globulosa* were collected from the large intestine of goats (*Capra hircus*), procured from local abattoirs. These were thoroughly washed in physiological saline (0.9% NaCl) in order to remove the adhering materials, and were processed immediately.

All chemicals used for analyses were of highest purity available and procured locally from different commercial establishments.

**RADIOISOTOPES**

U-1\(^{14}\)C-glucose (sp. act. 160 mci/m mole), L-aspartic acid C\(_{14}\) (sp. act. 192/μci/m mole), L-alanine C\(_{14}\) (sp. act. 144 μci/m mole) and L-leucine C\(_{14}\) (sp. act. 288 μci/m mole) were procured from the Bhabha Atomic Research Centre, Bombay, India.

**ANTHELMINTICS**

Two benzimidazole drugs, thiabendazole (2-(4-thiazolyl)-1H-benzimidazole; molecular wt. 201; U.S.P.; powder contains 75% of thiabendazole; Dynamic Pharmaceuticals Pvt. Ltd., Bombay, India) and fenbendazole (methyl 5-phenylthio-1H-benzimidazole-2-yl-carbamate; molecular wt. 299.35; Panacur\(^R\); powder contains 25% drug; Hoechst AG, Frankfurt am Main, Germany)
were used in the present experiment to establish their effects on metabolic pathways of *T. globulosa*.

Preliminary experiments were conducted to find out LD$_{50}$ with thiabendazole and fenbendazole. For this purpose worms were incubated in Kreb's Ringerbicarbonate buffer, pH 6.6 (10.7 g NaCl, 0.2 g KCl, 1.0 g NaHCO$_3$, 0.25 g CaCl$_2$, 2H$_2$O, 0.21 g MgCl$_2$.6H$_2$O, 0.05 g NaH$_2$PO$_4$ and 1.0 g glucose in a volume of 1 litre). Different concentrations of the drugs ranging from 0.2-1 mM were added to the incubation medium in different petri-dishes. Worms were kept at 37°C and were checked for mortality (assessed by loss of movement) by pricking with a sharp needle at different intervals of time.

### ENZYMATIC ANALYSIS

Capability of metabolising carbohydrates by the nematodes was tested by assaying various enzymes of Glycolysis, Kreb's cycle, NAD- and NADP- dependent enzymes, phosphatases and transaminases. A 10% homogenate of the worm in the respective buffers systems of the enzymes or 0.25 M sucrose was centrifuged at 2,000 g for 30 min at 4°C. The supernatant served as the source of enzyme activity.

**Glycogen Phosphorylase (+AMP)**: It was estimated by the method of Niemeyer *et al.* (1961). Reaction mixture contained
0.5 ml of 0.025 M glucose-1-phosphate, 0.1 ml of 0.067 M citrate buffer, pH 6.2, 0.1 ml of 0.075 M Sodium fluoride, 0.1 ml of 0.0025 M AMP, 0.1 ml of 1% glycogen. 0.1 ml of parasite homogenate was added to this reaction mixture. Then the reaction mixture plus parasite homogenate was incubated at 37°C for one hour. 1.0 ml of 10% chilled TCA was added to it and centrifuged for 10 min. 1.0 ml was taken out of it and 0.5 ml of acid ammonium molybdate, 0.2 ml of ANSA and 7.0 ml of distilled water were added. Colour intensity was read at 595 nm.

**Hexokinase**

It was estimated by the method of Crane and Sols (1955). The reaction mixture contained 0.2 ml of 0.075 M ATP- 0.04 M MgCl₂ mixture, pH 7.0, 0.2 ml of 0.1 M Histidine - 0.1 M tris - 0.01 M EDTA buffer (pH 7.4), 0.2 ml of 0.01 M glucose, 3 ml of distilled water and 0.1 ml of 10% nematode homogenate. The reaction mixture was incubated at 30°C for 15 mins and then stopped by adding 1.0 ml of 5% chilled TCA. This was centrifuged and 1.0 ml of supernatant was taken out. To it were added, 0.5 ml of acid ammonium molybdate, 0.2 ml of ANSA and 7.0 ml of distilled water. Colour intensity was read at 595 nm.

**Glucose-6-phosphatase**

It was assayed by the method of Swanson (1955). The reaction mixture consisted of 0.1 ml of 10% tissue homogenate,
0.3 ml of 0.1 M citrate buffer, pH 6.2, 0.5 ml, 0.1 M glucose-6-phosphate and 1.0 ml distilled water. The mixture was incubated at 37°C for one hour and then to it was added 1.0 ml of 10% TCA to stop the reaction. It was centrifuged and 1.0 ml of the supernatant was taken out. 0.5 ml of acid ammonium molybdate, 0.2 ml ANSA and 7.0 ml distilled water were added. Colour intensity was read at 595 nm.

Fructose-1, 6-diphosphatase

This enzyme was assayed by the method of Pogeel and McGilvery (1955). The reaction mixture consisted of 0.5 ml 10% homogenate, 0.1 ml 0.05 M fructose-1, 6-diphosphate, 0.4 ml 0.05 M boric acid - NaOH buffer, pH 9.5, 0.1 ml 0.905 M MnCl	extsubscript{2} and 0.1 ml 0.05 M MgSO\textsubscript{4}. The mixture was incubated for one hour at 37°C and the reaction was stopped by adding 1.0 ml of 10% TCA. This was then centrifuged and to 1.0 ml of the supernatant were added 0.5 ml acid ammonium molybdate, 0.2 ml ANSA and 7.0 ml distilled water. Colour intensity was read at 595 nm.

Glucose-6-phosphate isomerase

It was estimated by the method of Slein (1954). The reaction mixture contained 0.5 ml of 4 times diluted 10% nematode homogenate, 0.1 ml 0.1 M glucose-6-phosphate and 0.4 ml 0.1 M tris buffer, pH 9.0; this mixture was incubated at 37°C for
30 min. Then 3.5 ml 30% HCl and 1.0 ml 0.1% alcoholic resorcinol were added and the mixture was heated at 80°C for 10 min. This was cooled and the colour intensity read at 520 nm.

**Lactate dehydrogenase**

It was estimated by the method of King (1959). The reaction mixture consisted of 1.0 ml buffered substrate (125 ml glycine buffer 0.1 M + 75 ml 0.1 N NaOH + 5 ml 70% sodium lactate). 0.2 ml 5% homogenate in 0.25 M sucrose solution was added to this reaction mixture. This was incubated at 37°C for 10 min. To it was added 0.2 ml NAD (10 mg/2 ml distilled water) and then kept it exactly for 15 min. Then 1.0 ml dinitrophenyl hydrazine reagent was added and the mixture was incubated for 15 min at 37°C. 1.0 ml of 0.4 N NaOH was added to it and the intensity of the colour developed was read at 440 nm.

**Succinate dehydrogenase (SDH)**

It was estimated by the method of Kun and Aboud (1949). The reaction mixture contained 0.5 ml of 0.2 M sodium succinate, 0.5 ml of 0.1 M phosphate buffer, pH 7.4, 1.0 ml of 10% homogenate in 0.25 M sucrose and 0.5 ml of 0.1% triphenyltetrazolium chloride. The mixture was incubated at 37°C for one hour. 3.5 ml of acetone was added to it; the mixture then centrifuged. Colour was read at 420 nm.
Malate dehydrogenase (MDH)

It was estimated by the method of Davidson and Cortner (1967). Reaction mixture contained 15 mM oxaloacetic acid, 12 mM NADH, 0.1 M phosphate buffer, pH 7.5 and 0.02 ml of the homogenate preparation. Enzyme activity was measured at the rate of oxidation of NADH at 340 nm in a double beam spectrophotometer recorder (Uvikon 860, Kontron Instrument, Zurich, Switzerland), using the kinetic mode.

Glucose-6-phosphate dehydrogenase (G-6-PDH)

It was estimated by the method of Jullian and Reithal (1975). Reaction mixture contained 35 μM glucose-6-P, 0.1 M MgCl₂, 11 mM NADP, 0.1 M triethanolamine buffer, pH 7.6 and 0.1 ml of the homogenate preparation. Enzyme activity was measured at the rate of reduction of NADP at 340 nm in double beam spectrophotometer recorder using kinetic mode, as above.

Adenosine triphosphatase (Mg²⁺-dependent) (ATPase)

This was estimated by the method of Kielley (1955). The reaction mixture contained 0.2 ml of 0.2 M tris buffer, pH 7.6, 0.2 ml of 5 mM MgCl₂, 0.2 ml of 10% nematode homogenate prepared in 0.25 M sucrose solution and 0.2 ml of 5 mM ATP. The mixture was incubated for 30 min at 37°C. 2.0 ml of 10% chilled TCA was added to stop the reaction. It was then
centrifuged. 1.0 ml of the supernatant was taken and 0.5 ml of acid ammonium molybdate, 0.2 ml of ANSA and 7.0 ml of distilled water was added. Colour intensity was read at 595 nm.

Acid phosphatase

It was estimated by the method of Natelson (1963). Acid Phosphatase Reagent - 425 mg of sodium diethyl barbiturate was dissolved in 20 ml of distilled water and to it 500 mg of sodium $\beta$-glycerophosphate and 5 ml of 1 N acetic acid was added. Total volume was made up to 100 ml and its pH was adjusted to 5.2 with 0.1 N acetic acid.

To 0.5 ml of the homogenate was added 1.0 ml of acid phosphatase reagent. The reaction mixture was incubated for 1 hr at 37°C. 1 ml of 10% TCA was added to it and it was centrifuged. 1 ml of the supernatant was taken and 0.5 ml of acid ammonium molybdate, 0.2 ml of ANSA and 7.0 ml of distilled water were added. Colour intensity was measured at 595 nm.

Alkaline phosphatase (ALP)

It was estimated by the method of Natelson (1963). Alkaline phosphate reagent - 500 mg of sodium-$\beta$-glycerophosphate and 425 mg of Sodium diethyl barbiturate was dissolved in 100 ml of distilled water. Its pH was adjusted to 8.5 with 0.1 N acetic acid or 0.1 N NaOH and stored in refigerator.
It was estimated in the same manner as described for ACP.

Alanine oxoglutarate and aspartate oxoglutarate (GPT and GOT)

Transaminases were estimated according to the method of Reitman and Frankel (1957). The reaction mixture contained 0.1 ml of enzyme source and 0.5 ml of buffered substrate (buffered substrate for alanine oxoglutarate: 900 mg alanine in 9.0 ml of distilled water, 0.25 ml 1 N NaOH, pH 7.4, 14 mg \( \alpha \)-ketoglutaric acid in 0.2 ml 1 N NaOH, pH 7.4, volume was made up to 50 ml with phosphate buffer, pH 7.4; buffered substrate for aspartate oxoglutarate: 1.330 gm of Dl-aspartic acid in 9.8 ml of 1 N NaOH (pH 7.4), 14 mg of \( \alpha \)-ketoglutaric acid in 0.2 ml of 1 N NaOH (pH 7.4), the volume was made up to 50 ml with phosphate buffer). The mixture was incubated at 37°C for 60 min and 30 min for aspartate oxoglutarate and alanine oxoglutarate respectively. 0.5 ml dinitrophenyl hydrazine was added to it and the mixture again incubated at 37°C for 20 min. Then 5.0 ml of 0.4 N NaOH was added. Colour was read on 510 nm.

The effects of thiabendazole and fenbendazole on enzymes were determined by the direct addition of these drugs (100 \( \mu \) moles each) to the assay system.
**Protein estimation**

Enzyme protein content was quantitated by modified sodium dodecyl sulphate- Lowry procedure of Lees and Paxman (1972).

**Reagents used**

a) $5\%$ sodium dodecyl sulphate (SDS) in 0.5 $\text{N} \ \text{NaOH}$.

b) Copper tartarate solution - 2.1 g sodium tartarate - 50 ml, 
   $1\% \ \text{CuSO}_4 \cdot 5\text{H}_2\text{O} - 50 \text{ ml}, 1 \ \text{N} \ \text{NaOH} - 2.5 \text{ ml}$.

c) 2 $\text{N}$ Folin's phenol reagent diluted with distilled water in the ratio of 1:1.

d) $2\% \ \text{Na}_2\text{CO}_3$

e) BSA standard prepared in distilled water.

**Procedure** : To the sample and standard was added 0.5 ml of SDS solution. It was mixed in a vortex mixture. 2.5 ml of copper tartarate solution was added to it and kept for 45 min at room temperature. The colour, thus developed, was read at 750 nm.

**Effect of anthelmintics on the enzymes**

The effect of anthelmintics, thiabendazole and fenbendazole were studied by adding the drug at an amount of 100 $\mu$ moles to the standard incubation system. This concentration was determined on the basis of laboratory experiments when there was loss of movement in the worms.
Enzyme kinetic studies

$V_{\text{max}}$ (maximum of apparent initial enzyme velocity) and $K_m$ (substrate affinity constant) of the above mentioned enzymes were determined by assaying the enzymes at different substrate concentrations (Lineweaver and Burk, 1934). Similarly, $V_{\text{max}}$ and $K_m$ of the enzyme after in vitro addition of thiabendazole and fenbendazole were also studied. Thiabendazole and fenbendazole were added directly to the enzyme system (100 μ moles each) in aqueous suspension of 0.1% DMSO (dimethyl sulphoxide). Control incubations received 0.1% DMSO solution.

GLUCOSE UPTAKE STUDIES

The nematodes were incubated in 4 ml volume of the incubation medium at 37°C under aerobic conditions in the presence of 1 μci of U-$^{14}$C-glucose. Incubation medium were made in Kreb's-Ringer saline containing 25 mM tris (hydroxymethylamine methane)-HCl buffer at pH 7.4 (KRT of Read et al., 1963). The buffer was identical to KRT except that maleate had been replaced by HCl since maleate was shown to be toxic in metabolic studies (Webb, 1966). In experiments where effects of thiabendazole and fenbendazole were studied, the drugs dissolved in 0.1% DMSO were added to the incubations media at a final concentration of 100 μ moles of the total volume of incubating medium. Control incubations received equal volume of 0.1% DMSO.
In order to minimise the effects of diurnal variations, experiments were performed on each day at 10 AM. The nematodes were randomly allocated into groups of 3-4 worms, each group constituting a single sample. After 2 min incubation, the incubation medium was rapidly removed by aspiration. Worms were rinsed thoroughly in 3x10 ml changes of cold KRT, blotted on coarse filter paper and radioactivity extracted overnight in 2.0 ml of 70% ethanol. The extracted carcasses were dried overnighed at 80°C, in pre-weighed aluminium foil pans and ethanol extracted dry weights were determined. Aliquots of incubation medium and worm-ethanol-extracts were counted in a liquid scintillation spectrometer (Beckman Instrument) using methanolic toluene and phosphors (4 g PPO + 500 mg POPOP/700 ml toluene + 300 ml methanol) as the scintillant.

Absorption velocities were calculated from specific activity of the medium and the total activity in the worm extracts. The activity has been expressed as μ mole substrate absorbed/mg ethanol extracted dry weight/2 min. For this the CPM/sample were converted to specific activity by comparison with appropriate standards and employing appropriate calculations (Starling and Fisher, 1975).

To determine the effect of Na⁺ deletion on glucose uptake, Na⁺ concentration of KRT was altered by replacing NaCl isosomatically with choline chloride, LiCl or KCl.
The data from the uptake of glucose has been expressed as a function of uptake velocities (V) and substrate concentration (S) and three linear transformations of Michaelis-Menten equation, the 1/V Versus 1/S plot of Lineweaver and Burk (1934), the V versus V/S plot of Eadie and the S/V versus S plot of Woolf (Siegel, 1976) were constructed into graphical plots. The uptake of $^{14}$C-glucose at different time intervals was also studied.

Effect of pH and temperature on $^{14}$C-glucose uptake by the worms in the standard assay system was studied using buffers, 0.1 M citric acid - sodium citrate (pH 4.0-5.2), 0.1 M phosphate (pH 6.2-7.9) and 0.1 M tris-HCl buffer (pH 7.9-9.5) and a temperature ranging from 4-60°C.

INCORPORATION OF RADIOACTIVITY OF $^{14}$C-GLUCOSE INTO DIFFERENT MACROMOLECULAR FRACTIONS

Metabolism was studied by measuring the CO$_2$ evolved to study the $^{14}$C-incorporated CO$_2$. Warburg type flask was used. 10% KOH was placed in the central well and the nematodes and incubation medium containing radioisotope in the flask. The flask was tightly stoppered and kept in shaking water bath for 90 min. KOH which absorbed CO$_2$ formed by metabolism was removed with a filter paper piece, from the central well. The filter paper piece was put to scintillation vial and after adding
liquid scintillant which extracted all the radio-carbon from filter paper, counts were taken.

To determine the amount of radioactivity contained in glycogen after incubation of *T. globulosa* in the $^{14}$C-labelled glucose, the following procedure was used: 5 mg non-radioactive glycogen (to serve as carrier) 0.3 ml 10% Na$_2$SO$_4$ and 2 volume of 100% ethanol were added to 3 ml of total TCA soluble fraction. The contents were thoroughly shaked and on flocculations centrifuged at 1,000 x g for 10 min. The supernatant was decanted into a separate test tube and the pellet was washed with 1 ml of 100% ethanol and recentrifuged. The supernatant was decanted and added to the previous one and the pellet was dried with gentle heat. The pellet contained all the glycogen and was then hydrolysed with 1 ml of 2 N H$_2$SO$_4$ at 100ºC for one hour. 0.2 ml of aliquot of hydrolysate was pipetted out into scintillation vial and neutralized by 0.2 ml hyamine 10X and dissolved in 10 ml of liquid scintillation counting mixture and radioactivity determined.

The procedure of Crabtree *et al.* (1977) was followed to extract cold trichloroacetic acid (TCA) - soluble fraction, lipids, RNA, DNA and proteins in the incubated parasites. The complete sequence of isolation is as follows:
To 1 ml of 10% tissue homogenate, 2.5 ml of 10% TCA was added and centrifuged for 10 min at 3,000 x g at 4°C. Supernatant-I was removed and 2.5 ml of 10% TCA was added to the pellet again and centrifuged at the same rate. The supernatant-II was added to supernatant-I, these constituting the TCA-soluble fraction. Then 5.0 ml of 95% ethanol was added to the pellet and centrifuged at 3,000 x g for 10 min. The supernatant-III contained lipids. The step was repeated by adding 5.0 ml of ethanol : ether (3:1) to the pellet and the supernatant-IV was added to supernatant-III. Then 2.0 ml KOH was added to the pellet which was incubated at 37°C for 20 hrs. It was then neutralized with 6N HCl and 2.0 ml of 5% TCA was added and centrifuged at 3,000 x g for 10 min. Supernatant-V contained RNA while the pellet constituted proteins and DNA. To the pellet, 2.0 ml of 5% TCA was added and incubated for 15 min at 90°C and then centrifuged at 3000 x g for 10 min. The supernatant-IV contained DNA. The step was repeated once again. Supernatant-VI and VII were pooled and assayed for DNA. The pellet was dissolved in 1N KOH, incubated at 85°C for 15 min and brought up to 10 ml with distilled water which was accepted as the protein fraction. A definite amount of each fraction was taken into scintillation vial for counting.

AMINO ACID UPTAKE STUDIES

The nematodes were incubated in 4 ml of incubation
medium at 37°C under aerobic conditions in the presence of 1 μCi of L-aspartic acid C14, L-alanine C14 and L-leucine C14. Incubation, incubation medium, extraction of radioisotope in 70% ethanol, scintillant, counting calculations and data expression, were done in the same manner as described in uptake studies on 14C-glucose.

The effects of Na⁺ deletion, pH and temperature was also studied by following the same procedure as described above under carbohydrate metabolism.

INCORPORATION OF RADIOACTIVITY OF 14C-AMINO ACIDS INTO DIFFERENT MACROMOLECULAR FRACTIONS

To determine the 14C incorporated CO₂, glycogen, TCA soluble fraction, lipids, RNA, DNA and proteins, the methods followed were the same as in the case of 14C-glucose uptake studies, described above.

EFFECT OF METABOLIC INHIBITORS ON THE UPTAKE OF 14C-GLUCOSE AND 14C-AMINO ACIDS.

Several compounds commonly regarded as metabolic inhibitors were studied to determine whether they also affected the nutrient uptake processes by the worm. The effect of different compounds, viz. glucose, phlorizin, ouabain, maltose, sucrose, lactose, glucose-6-phosphate, iodoacetate, p-nitrophenyl phosphate, glucose-1-phosphate, fructose-1-6-diphosphate and
fructose-1-phosphate was examined on $^{14}$C-glucose uptake. Influx of $^{14}$C-glucose was seen in the presence of increasing concentrations of unlabelled glucose, phlorizin and ouabain at different substrate concentrations of $^{14}$C-glucose (5, 10, 20, 40 mM) and the data were transformed into Dixon plots (Siegel, 1976).

Likewise inhibitions of absorption of L-$^{14}$C-aspartic acid, L-$^{14}$C-alanine and L-$^{14}$C-leucine caused by the presence of unlabelled amino acids and some other compounds like glucose, iodoacetate, ATP and ouabain, were studied. Inhibitors at concentrations of 1 mM (Read et al., 1963) were tested at different substrate concentrations (1.0 - 20 mM) of $^{14}$C-amino acid and the data were analyzed by the method of Lineweaver and Burk (1934). An inhibition constant ($K_i$) was calculated for the inhibition, as described by Read et al., (1963) by the following formula.

$$\text{Slope of inhibited curve} = \frac{K_t}{V} (1 + \frac{[I]}{K})$$

where $K_t$ = transport constant, equivalent to apparent Michaelis constant, $V$ = maximum velocity by extrapolation of the inhibited curve and $[I]$ concentration of inhibitory amino acid.

**TRANSCUTICULAR UPTAKE**

Experiment was designed to examine whether absorption
occurred through the cuticle of *T. globulosa*. For this two batches of worms, ligated and non-ligated were taken. The two extreme ends of worms were ligated by dipping them in colloid in. This is a nitrocellular compound with the empirical formula of $\text{C}_{12}\text{H}_{16}\text{N}_4\text{O}_8$. This compound contracts on drying, forming a tight seal, which makes it an ideal compound to occlude the oral and anal openings of the parasite. The worms were incubated in KRT buffer contains 50 mM glucose and 5 µCi $^{14}$C-glucose for different intervals of time. Worms were transferred to chilled KRT solution and washed repeatedly with the same solution to remove any adhered radioactivity. Radioactivity was measured as described before. The difference of uptake between ligated and non-ligated worms was taken as a measure of transcuticular absorption.

HISTOLOGICAL TECHNIQUES

Tissues were fixed for histology in Bouin 24 hrs and Zenker 24 hrs and 6 hrs (Pearse, 1968). Bouin fixed material was transferred to 70% alcohol for 2 hrs and was given several change to remove excess picric acid. Mercury from Zenker fixed material was removed by treating the sections in 70% iodized alcohol. Excess of iodine was removed by treating the sections with 5% sodium thiosulphate (Lillie, 1965) Bouin and Zenker fixed materials were processed further through graded alcohol series
embedded in paraffin wax (58-60°C) and sectioned 5-7 µ thick. The section were then stained as under.

**Delafield's haematoxylin/eosin**

Zenker fixed sections were own graded from alcohol series to water and stained in Delafield's haematoxylin for 10 min. A 1.0% solution of aqueous HCl was used for differentiation of the sections. The sections were upgraded up to 90% alcohol, counterstained in eosin for 30 seconds and then differentiated in 90% alcohol. Stained sections were dehydrated in absolute alcohol, cleared in xylene and mounted in D.P.X.

**HISTOCHEMICAL TECHNIQUES**

The following histochemical techniques were employed for the localization of carbohydrates, proteins and various enzymes:

**Carbohydrates**

**Periodic acid - Schiff technique (PAS)** (Mc Manus, 1946, 1948; Pearse, 1968).

The test was performed on 7 µ thick paraffin sections of the worms mainly fixed in Carnoy. Hydrated sections were oxidized for 10 min in 1% aqueous periodic acid and washed in distilled water for 5 min. This was followed by the
treatment with Schiff's reagent for 30 min and washing in running water for 30 min. The periodic acid breaks up the C-C bond in 1:2 glycol groups (CHOH-CHOH) converting them into dialdehydes (CHO-CHO) which combine with Schiff's reagent to give substituted dye which is pink in colour.

**Best's carmine test for glycogen** (Best, 1906)

Sections of Carnoy fixed, alcohol stretched material were deparaffinized and brought to absolute alcohol. To avoid any loss of glycogen, sections were coated with 1% celloidin prepared in 50:50 absolute alcohol and diethyl ether. Slides were down graded and stained in carmine solution for 30 min, followed by differentiation in Best's differentiator. The sites of glycogen stain red.

**Toluidine blue method for metachromasia** (Kramer and Windrum, 1955; and Pearse, 1968).

The Bouin-fixed sections were brought to water and stained with 0.1% toluidine blue in 30% ethanol, dehydrated, cleared and mounted in DPX. Mucopolysaccharides showing α-metachromasia stained red or pink, β metachromasia gave blue colour.

**Proteins**

**Mercury bromophenol blue (Hg-BPB) method** (Bonhag, 1955)

For localization of proteins in general, Carnoy and Zenker
fixed paraffin sections were stained in Hg-BPB solution (1% HgCl₂ and 0.05% bromophenol blue in 2% aqueous acetic acid and again washed in water, dehydrated in two changes in tertiary butyl alcohol, cleared in xylene and mounted in D.P.X. The proteins stain deep blue.

**Ninhydrin-Schiff method for protein bound NH₂ groups** (Yasuma and Itchikawa, 1953).

Paraffin sections of Zenker and Carnoy fixed material, after hydration, were treated first with 0.5% ninhydrin solution in absolute alcohol for 16-20 hours at 37°C and then with Schiff's reagent for 30 min. This was followed by washing in running water for 10-30 min, dehydration in alcohol series, clearing in xylene and mounting in D.P.X. NH₂ groups of proteins, if present in sufficient numbers, produce magenta colour. Ninhydrin reacts with free amino groups of ε-amino acids to produce an aldehyde containing one less carbon atom than the original amino acid. The resulting tissue aldehydes are then demonstrated by Schiff's reagent; the tissue turns magenta.

**Enzymes**

**Lead nitrate method for acid phosphatase** (Gomori, 1950)

Cryostat sections were incubated in the substrate mixture for 6-8 hrs at 37°C. Substrate for acid phosphatase was prepared by dissolving 60 mg of lead nitrate in 50 ml of acetate
buffer (pH 4.8-5.0) and than 5 ml of 3% sodium \( \beta \)-glycerophosphate was added. Turbid solution was filtered and the filtrate was used. After incubation, sections were rinsed and immersed in 1% yellow ammonium sulphide, washed again and mounted in glycerine jelly. Areas with acid phosphatase activity turned blackish brown.

**Calcium cobalt method for alkaline phosphatase** (Gomori, 1952)

Cryostat sections were incubated in the substrate mixture for 6 hrs at 37°C. The incubating medium for alkaline phosphatase consisted of 3% sodium \( \beta \)-glycerophosphate, 20 ml; 2% sodium barbitone, 30 ml; 2% \( \text{CaCl}_2 \), 4 ml; 2% \( \text{MgSO}_4 \), 2 ml and distilled water 30 ml. pH of the solution was adjusted at 9-9.4. Sections were washed, and treated with 2% cobalt nitrate and finally treated with 1% yellow ammonium sulphide for 1-2 min. The alkaline phosphatase sites turned black or brownish black.

**Calcium method for adenosine triphosphatase** (Padykula and Herman, 1955).

Preparation of incubating medium:

- 0.1 M sodium barbiturate (2.06 g/100 ml) 20 ml
- 0.18 M \( \text{CaCl}_2 \) (1.998 g/100 ml) 10 ml
- Distilled water 30 ml
- Adenosine triphosphate (disodium salt) 152 mg

Solution was freshly prepared.
As soon as the ATP was dissolved, pH was adjusted to 9.4 with 0.1 M NaOH and final volume made 100 ml with distilled water. Cryostat sections of 10-15 μ thickness were incubated in the incubating medium for 3-4 hrs at 37°C and then washed in three changes of 1% CaCl₂. Sections were transferred to 2% CaCl₂ for 3 min, washed in distilled water and developed in dilute yellow ammonium sulphide and washed again. Sections were mounted in glycerine jelly.

Method for glucose-6-phosphatase (Wachstein and Meisel, 1956)

Preparation of substrate mixture:

- 125 mg glucose-6-phosphate (disodium salt) 20 ml
- 0.2 M tris-buffer (pH 6.7) 20 ml
- 2% lead nitrate 3 ml
- Distilled water 7 ml

Cryostat sections of 10-15 μ thickness were incubated in substrate mixture for 2-3 hrs at 32°C and then washed in distilled water. Sections were developed in dilute yellow ammonium sulphide and again washed in water, post fixed in 6% neutral formaldehyde and mounted in glycerine jelly. Brownish black deposits indicate the sites of glucose-6-phosphatase activity.
Method for succinate dehydrogenase (Nachlas et al., 1957)

Preparation of substrate mixture:

- 0.2 M phosphate buffer (pH 7.6) 10 ml
- 0.2 M sodium succinate 10 ml
- 0.1% nitroblue tetrazolium 20 ml

Cryostat sections on slides were incubated in substrate mixture for 45 min at 37°C, then washed in running water and post fixed in 10% formalin. After fixation, sections were again rinsed in water and mounted in glycerine jelly. Blue colour deposits indicate the presence of SDH.