Slides were smeared with Mayer's albumen and a drop containing spores was put on them. The surplus water was soaked out with a micropipette and when a very thin film of material was left on the slides, the smears were fixed in various fixatives, viz. Carnoy, Schaudin, Bouin and Zenker. Although several fixing fluids were used during the course of these investigations, Carnoy's chloroform-alcohol proved to be the best fixative for cytochemical studies on myxosporean parasites. The cytochemical techniques employed for study of carbohydrates, proteins and nucleic acid and various enzymes are as follows:

(a) CARBOHYDRATES

Periodic acid-Schiff (PAS) test (McManus, 1946, 1948; Pearse, 1968)

Hydrated smears were oxidized for 10 minutes in 1% aqueous periodic acid and washed in distilled water for five minutes. This was followed by the treatment with Schiff's reagent and washing with water. The periodic acid breaks up the C-C bond in 1:2 glycol groups (CHOH-CHOH) converting them into dialdehydes to give a substituted dye which is pink in colour.

Acetylation control and KOH reversal

Two sets of similar slides were simultaneously downgraded to water and treated in a mixture of acetic anhydride (16 ml)
and dry pyridine (24 ml) for 18 hours at room temperature. One set of slides was further treated with 0.1 N KOH for 15 minutes. Both sets of slides were washed and proceeded as for normal PAS technique. The acetylation blocks the reaction of 1:2 glycol groups with periodic acid, converting them to esters but this effect is reversible in the alkaline media such as dilute KOH. This confirms the presence of 1:2 glycol group.

**Toluidine blue method for metachromasia** (Kramer and Windrum, 1954 and Pearse, 1968)

Carnoy's fixed smears were brought to water and stained with 0.1% toluidine blue in 30% ethanol, dehydrated, cleared and mounted in DPX. Mucopolysaccharides showing γ-metachromasia stain red or pink, β-metachromasia gives purple colouration, whereas α-metachromasia gives blue colour.

(b) **PROTEINS**

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

The stain was prepared by dissolving 1% mercuric chloride and 0.05% bromophenol blue in 20% aqueous acetic acid. Carnoy's fixed slides were downgraded before putting in the above solution and kept in the same for about 10-30 minutes at room temperature. The slides were differentiated in 0.5% aqueous acetic acid, dehydrated in two changes in tertiary butyl alcohol, cleared and mounted in QEX. The sites of proteins stain blue.
(c) **NUCLEIC ACIDS**

**Feulgen reaction for DNA (Feulgen and Rossenbeck, 1924)**

Carnoy's fixed smears were hydrolyzed in N HCl at 60°C for an optimum time of eight minutes by which aldehyde groups from deoxyribose sugar of DNA are released. The slides were immersed in Schiff's reagent for thirty minutes; the reagent reacts with the free aldehyde groups to produce a purple dye in the nuclear chromatin alone.

**Methyl green/pyronin G method (MG/PG) for DNA and RNA (Kurnick, 1955 and Pearse, 1968)**

This method is specific for DNA and RNA. MG/PG is a mixture of two dyes, one of which (methyl green) is selective for "polymerized" DNA and the other (pyronin G) has an affinity for RNA in both nucleolus and cytoplasm. The mixture was prepared after separate extractions of the stains with chloroform as recommended by Kurnick (1955). The smears were treated with MG/PG solutions and blot dried with filter paper. The slides were then passed through two changes of n-buty alcohol (5 minutes each) and cleared in xylene. The sites of DNA stain green and of RNA pink.

**Controls**

For the extraction of DNA and RNA, both the slides were treated with 5% perchloric acid at 60°C for 3 hrs. RNA was extracted with 10% perchloric acid at 40°C for 18 hrs. (Erickson et al., 1949). Both slides were then proceeded as for normal methyl green-pyronin G staining.
ENZYMES

For the localization of enzymes, living spores were smeared on the slides and dried at room temperature for two minutes.

Lead nitrate method for acid phosphatase (Gomori, 1950)

Slides were incubated in the substrate mixture for 6-8 hours at 37°C. Substrate was prepared by dissolving 60 mg of lead nitrate in 50 ml of acetate buffer (pH 4.8 to 5.0) and then 5 ml of 3% sodium-β-glycerophosphate was added. Turbid solution was filtered and filtrate was used. After incubation, slides were rinsed and immersed in 1% yellow ammonium sulphide, washed again with water and mounted in glycerine jelly. Areas with acid phosphatase activity turn blackish brown. The control sections were incubated in solution without β-glycerophosphate.

Calcium cobalt method for alkaline phosphatase (Gomori, 1952)

Freshly prepared slides were incubated in the substrate mixture for 6 hours at 37°C. The incubating medium for alkaline phosphatase consisted of 3% sodium-β-glycerophosphate, 20 ml; 2% sodium barbitone, 30 ml; 2% CaCl₂, 4 ml; 2% MgSO₄, 2 ml and distilled water 30 ml; pH of the solution was adjusted at 9-9.4. Slides were washed and treated with 2% cobalt nitrate and finally treated with 1% yellow ammonium sulphide for 1-2 minutes. The alkaline phosphatase sites turn black or brownish black. For control, incubation medium without β-glycerophosphate was used.
Method for Adenosine triphosphatase (Wachstein and Meisel, 1960)

Preparation of substrate mixture:

- 125 mg % ATP (dissodium salt) 20 ml
- 0.2 M Tris buffer (pH 7.2) 20 ml
- 2% Lead nitrate 3 ml
- Distilled water 2 ml

Slides were incubated for 1-2 hours at 37°C in substrate mixture. After incubation sections were rinsed in water and developed in 5% yellow ammonium sulphide for one minute. Slides were again rinsed in water, fixed in 10% formalin and mounted in glycerine jelly. Control substrate mixture lacked ATP. Black deposits of lead sulphide indicate the enzyme activity.

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

Preparation of substrate mixture:

- 125 mg % Potassium glucose-6-phosphate 20 ml
- 0.2 M Tris buffer (pH 6.7) 20 ml
- 2% Lead nitrate 3 ml
- Distilled water 7 ml

Slides were incubated in the substrate mixture for 2-3 hrs at 32°C and then washed in distilled water. Smears 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. In control, substrate
lacked glucose 6-phosphate.

**Method for succinic 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

Slides were incubated in substrate for 45 minutes at 37°C, then washed in running water and post fixed in 10% formalin. After fixation smears were again rinsed in water and mounted in glycerine jelly. Blue coloured deposits indicate the presence of SDH. For control sections the medium lacked sodium succinate.