MATERIALS AND METHODS

*Limnophyton obtusifolium* (L.) Miq. belongs to Alismataceae and its descriptive embryological details are studied by Johri (1935) and Murty (1935). Alismatales are best attracted by various authors for their morphophysiological studies (Charlton, 1978a, b; Lieu, 1978a, b). Our interest to choose this plant is that 1. The inflorescence contains both male and bisexual flowers ranging in development from early bud primordia to open flowers, which facilitate the study of sequential stages and the associated physiological processes at the cell, tissue and organ level. 2. The cells have high morphometric potentials and precise cellular dimensions. 3. Microsporogenesis in this plant is ideally suitable like other classic instances viz. *Tradescantia, Lilium, Allium, Trillium* and *Triticum* because: a. microsporocyte development is highly synchronous, we can survey a huge population to analyse essential chemical make up in the cells. b. Tapetum is periplasmodial where the developing microsporocytes are embedded in the tapetal tissue. c. Pollen is trinucleate thus analysis can be extended upto male gametes. *Paspalidium geminatum* Stapf. belongs to Poaceae (Cook, 1974) and is quite suitable to study the cytochemical turnovers during the reproductive differentiation because of its dome shaped shoot apex.

*Limnophyton obtusifolium* (L.) Miq. and *Paspalidium geminatum* Stapf. were collected (August, 1977) over its flowering season.
(July-November) from its natural habitat. *Lipophyton* was spotted near Chandola lake which is 8 km away from our institute. *Propalidium* was collected from our botanical garden. The flower buds and dissected shoots were fixed immediately after collection in different fixatives according to the metabolite to be studied. The following fixatives were used in this study. 1. Carnoy's fluid: Ethanol 95% and glacial acetic acid in 3:1 (v/v). It is customary to use absolute ethanol in Carnoy's fixative (Jensen, 1962). Since absolute ethanol showed a shrinkage of protoplast of young tissues, 95% ethanol in Carnoy's fluid proved beneficial. Material was kept for 1 hour in this fixative. 2. FAA: 90 ml 70% ethanol mixed with 5 ml glacial acetic acid and 5 ml 40% formaldehyde (BDH, India). Material was kept overnight. 3. Neutral buffered formaldehyde (NBF): 200 gms of sodium acetate (BDH, India) was dissolved in one litre of 10% formaldehyde and diluted 10:1 with tap water. pH of the solution was 7.0 ± 0.1. 4. TCA: 10 gms of trichloroacetic acid (E. Merck, Germany) was dissolved in 100 ml of 80% ethanol. Material was fixed for 1 hour. After proper fixation materials were preserved in 70% ethanol and dehydrated by passing through ethanol-tertiary butyl alcohol series, infiltrated at 57°C and embedded in paraplast (Arthur. E. Thomas, Philadelphia, U.S.A.) sectioned uniformly at 10 microns thickness using a Leitz Wetzlar Germany Microtome.
Ribbons containing sectioned material were affixed on albuminized Blue star (1.15 mm thickness) glass slides.

DNA

(Feulgen and Rossenbeck, 1924; McLeish and Sunderland, 1961)

Ever since Feulgen reaction to detect DNA in a cell developed a large number of publications are devoted to it by leaps and bounds. Nevertheless, chemical significance of the technique has given rise to much debate. However, the aldehyde groups give a typical magenta colour with Schiff's reagent (Clark and Meischen, 1978; Brenpreisa and Freivalds, 1979). In Feulgen reaction the period of hydrolysis is a critical step. It releases the aldehyde groups of deoxyribose sugar from the DNA helix. The optimum time is judged by trial and error with different timings. The time of hydrolysis varies from plant to plant. It was found 10 minutes in Paspalidium and 12 minutes in Limnophyton at 60°C with 1 N HCl (Fig. 1).

Period of hydrolysis: In both the plants during hydrolysis the stain intensity suddenly decreased just before reaching the optimum time (Fig. 1). In other words, there are two peak periods of hydrolysis (in Paspalidium 6th and 10th minutes and in Limnophyton 6th and 12th minutes) where intensive reaction was obtained. In our further study on the acid lability of Feulgen nucleus three types of
nuclei with different features in orientation of chromatin, quality and quantity of nuclear constituents were chosen. For such analysis anthersacs of Limnophyton at 3MC and at binucleate (vegetative and generative) phases seemed suitable. Chromatin in premeiotic microspore mother cell nuclei are diploid. The binucleate pollen contained two nuclei with quite contrasting characters. Vegetative nucleus contains dense chromatin with great sparsity in a relatively bigger nucleus. Whereas in generative nucleus the chromatin is highly condensed in a smaller nuclear volume and DNA is bound with different histones from that of the vegetative one. Inspite of vast differences in the nuclear material, the hydrolytic curves (60°C 1M HCl) for these three types of nuclei showed a trend similar to that of the first experiment as in shoot apical meristem of Limnophyton. There is a strong consistency in the results showing a peak period of hydrolysis at the 12th minute. Just before attaining this period, there is a steep fall in the extinction values at the 10th minutes of hydrolysis in vegetative and microspore mother cell nuclei (Fig. 1 B, C) but the generative nuclei did not show any decrease in stain intensity during this period.
Fig. 1. Relative Feulgen absorbance (extinction values) at different periods of hydrolysis (10 M HCl at 60°). The shoot apical tissue of *Paspalidium* showed peak absorption at 10th minute and 12th minute in case of *Limnophyton*. The three histograms represent the acid (10 M HCl 60°C) lability of the generative, vegetative and megaspore mother cell nuclei of *Limnophyton*. All three nuclei in spite of their different chromatin structure, and ploidy levels showed a uniform peak extinction value at 12th minute of hydrolysis.
FIG. 1

LIMNOPYTON
Generative nucleus

Vegetative nucleus

Megaspore mother cell nucleus

Time of Hydrolysis (IN HCL. 60°C)

Relative Feulgen Absorbance (10²)

Time

4 6 8 10 12 14 16

0 0.2 0.4 0.6

PASPALIDIIUM (shoot) 0.45

LIMNOPYTON (shoot)
Chemistry of the Feulgen hydrolysis:
(Kasten, 1967; Pearse, 1972; Jordanov, 1976; Gabe, 1976):
Hot acid causes rapid release of purine bases (adenine and guanine) than pyrimidine bases (cytosine and thymine). As a result, aldehyde groups are produced from deoxyribose sugar. Secondly, the aldehyde groups are progressively removed from the tissue into the hydrolysing solution. Thus Feulgen reaction is always a compromise between these two actions. At a short period of hydrolysis, purine glycoside links get broken and are available for Schiff's binding. With the increase of the hydrolysis time pyrimidine bases are also released. But at this period, purine bases lost its compensating point and its aldehyde groups get released into the hydrolysing solution, as a result there is a pause in the stain intensity (Fig. 1). Before reaching the stain intensity to ground level, aldehyde groups from pyrimidine bases are available to Schiff's reagent, giving most intensive reaction. This is the virtual optimum period of hydrolysis. The loss of stain intensity with the increase in hydrolysis time can be attributed to the lability of sugar aldehyde attachments and instability of furanose forms (Pearse, 1972). About the experimental conditions of hydrolysis there are different opinions. Some authors (Jordanov, 1976; Dutta, 1977, 1978) are of the opinion that the conditions of
hydrolysis (in HCl at 60°C) mentioned by Feulgen and Rossenbeck (1924) are difficult to maintain. A little departure from its optimum time stamps sharp differences in the staining. However, hydrolysis at room temperature with 5 N HCl for long time has led to correct DNA estimations. On the other hand, Lyndon (1978) in his personal communication writes that any slight deviation from room temperature makes fluctuations in Feulgen cytophotometry. But the original method (1 N HCl at 60°C) by slight modification gave precise results. To arrest Feulgen hydrolysis at the optimum time, slides with sections were dipped in cold 1 N HCl for one minute then washed with distilled water (D.W.) 2 minutes. The staining schedule for DNA localization is as follows. Carnoy's fixed material was used.

1. Sections were deparaffinized in xylene and hydrated to water by passing through downgrade series of ethanol.
2. Slides were rinsed with D.W. at 60°C and transferred to 1 N HCl at 60°C. 3. After attaining optimum reaction of hydrolysis (12th minute in case of Limnphyton and 10th minute in case of Paspalidium) slides were briefly rinsed with chilled HCl and then washed with D.W. 2 minutes.
4. Slides were kept in Schiff's reagent at room temperature (25 ± 5°C) in dark for 30 minutes. 5. Rinsed
with D.W. 6. Bleached with freshly prepared sulfurous acid by giving two changes of 10 minutes each. 7. Washed under tap water for 5 minutes to remove bleaching solution from the tissue, and finally rinsed with D.W.
8. Dehydrated through ethanol series, cleared in xylene and mounted in DPX (BDH India) using coverglass No. '0'.
Blue star. 9. Measured the dye absorbance at 565 nm.

Control slides were prepared either by omitting hydrolysis step from the above schedule or by extracting DNA from the hydrated sections with cold 0.5 M perchloric acid for 24 hr at 4°C prior to hydrolysis (Jensen, 1962).

Schiff's reagent

Schiff's reagent was prepared by adopting the method of Leuchtenberger (1958). One gram of basic fuchsin (Harleco, U.S.A. C.I. 42510) was dissolved in 200 ml of boiling glass distilled water for 5 minutes. The solution was cooled to 50°C, and before being filtered through Whatman No. 1 filter paper 20 ml of 1 M HCl was added and the solution was further cooled to room temperature (25 ± 5°C). At this temperature 2 gms. of potassium metabisulphite was added, stored in a tightly stoppered bottle overnight. The solution appeared straw yellow colour. To this 0.5 gms. of activated charcoal was added, shaken rapidly for 1 minute, filtered through coarse
filter paper and stored in a tightly stoppered amber
coloured bottle at 5°C. The reagent thus prepared was a
colourless liquid (pH 2.5).

Sulfurous acid:

Added 10 ml 1 N HCl to 10 ml 10 percent potassium
metabisulphite (BDH- India) and diluted by adding 180 ml
D.W.

RNA:

(Tepper and Gifford, 1962):  

Histochemical detection of RNA was achieved by using
pyronin G alone. However, the specificity of pyronine
to localize RNA is in question (Kurnick, 1955), because
in animal tissue pyronine binds with DNA when it is not
competitively inhibited by methyl green. But Tepper
and Gifford (1962) demonstrated the specificity of pyronin
reaction to RNA in plant tissue by enzyme digestions and
extractions of RNA from the tissue. Furthermore, many
workers (Heslop-Harrison, 1970; Corson and Gifford, 1969;
Molder and Owens, 1972; Patel et al. 1978) showed the
use of pyronin reaction to detect RNA in plant tissues.

Histochemical analysis of RNA has its own limitations in
quantitative terms, because some types of RNA (m-RNA and
s-RNA) are lost during fixation and dehydration. The RNA
available to the dye is r-RNA. Since r-RNA constitutes 80-90% of the total cellular RNA (Busch and Smetana, 1970) the staining reaction is accessible to quantitative terms. The staining schedule is as follows:

1. Carnoy's fixed material was used. 2. Sections were deparaffinized and hydrated through downgrade series of ethanol:DW. 3. Kept in pyronin-G dye for 7 minutes at room temperature (25 ± 5°C). 4. After giving a brief rinse with D.W. slides were blotted with blotting paper and dehydrated through n-butanol (BDH-India). 6. Cleared in xylene and mounted in DPX. 7. Measured the dye absorbance at 500-570 nm. Control slides were prepared by extracting RNA from the hydrated sections with 1 N perchloric acid at 4°C for 12 hrs (Jensen, 1962). In another control hydrated sections were incubated in RNase (pH 6.3) for 60 minutes at 50°C (Tepper and Gifford, 1962; Lyndon, 1978). In both the controls there was a complete loss of pyroninophilia in the sections.

Pyronin reagent was prepared by dissolving 2 gms. of pyronin-G (Fluka-Swiss. B.No. 565 238) in a 100 ml of double distilled water and equal volume of acetate buffer (pH 3.5) was added to it. The dye impurities were extracted by repeated washing with chloroform until the lower layer in the separating funnel becomes colourless.
RNAse was prepared by dissolving 25 mg. of RNAse crystals (Nutritional biochemical Corporation, Cleveland, OHIO 1-23OR control no-3183) in 50 ml double distilled water and adjusted to pH 6.8 with 1 n NaOH (Lyndon, 1978).

TOTAL NUCLEIC ACIDS
(Gabe, 1976; Lyndon, 1978):

Gallocyanin-chrome alum specifically stains total nucleic acids (Gabe, 1976; Lyndon, 1978; Brown and Scholtz, 1979). But the chemical significance is not known. However, the structures containing basophilia take on a bluish black colour. There is indeed a stoichiometric relationship between the stain intensity and the amount of nucleic acids (Brown and Scholtz, 1979). Dye binding in nuclei after treatment with ribonuclease is attributable to DNA (Kiefer, 1970; Lyndon, 1978). Thus individual amounts of RNA and DNA can be obtained. The staining schedule is as follows.

1. Carnoy's fixed material was used. 2. Sections were deparaffinized, hydrated by passing through ethanol: D.W.series. 3. Incubated the sections in gallocyanin chrome-alum (pH 1.6) overnight at 40°C. 4. Rinsed with acid water (pH 1.6) to remove excess stain. 5. Dehydrated,
cleared in xylene and mounted in DPX.

Cytoplasm and nucleus showed bluish black colour and absorbance at 575 nm was measured to obtain total nucleic acid content. To obtain the value for DNA alone, the sections were treated with RNase before staining (step 3). The optimum time of incubation in RNase was judged after trying with different periods and 60 minutes at 50°C was found optimum. Further period of incubation (70 minutes) showed a diminution in nuclear staining (Table 1). The details of RNase preparation are the same as described earlier in connection with pyronin technique.

Gallocyanin-chrome-alum reagent (Pearse, 1972),

1. Dissolved 600 mg of gallocyanin (Gurr Ltd. England, B.N. 17279) in 200 ml D.W. by shaking for 1 minute, filtered and the filtrate was discarded. 2. The extracted residue from the filter paper was dissolved in a freshly prepared 200 ml 5% chrome-alum (Chromic potassium sulphate. BDH, India) in a boiling water bath for 30 minutes. The pH of the dye was adjusted to 1.6 with 1 N HCl and final volume made to 200 ml with D.W. The following table depicts the time course extraction of RNA from the tissue with RNase at 50°C and 60 min. of incubation in RNase was found optimum for complete removal of RNA.
Table 1

<table>
<thead>
<tr>
<th>Time of incubation in minutes</th>
<th>Stain intensity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Nucleus</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>+++</td>
<td>+++</td>
<td></td>
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<tr>
<td>60</td>
<td>+++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

+ Positive reaction  
- negative reaction

TOTAL PROTEINS  
(Mazia et al., 1953)

Mercuric bromphenol blue method is used for the identification of insoluble proteins, as all the soluble ones are lost during fixation. It is stoichiometric and highly specific (Berlyn and Miksche, 1976; Gabe, 1976). The staining schedule is as follows.

1. FAA fixed material was used. 2. Sections were deparaffinized in xylene and hydrated through ethanol: D.W.
series. 3. Hydrated sections were stained in mercuric bromphenol blue for 15 minutes at room temperature. 4. Kept the sections in cold tap water for 2 minutes. 5. Removed excess stain in 0.5% glacial acetic acid. 6. Washed under tap water which leads to the development of blue colour. 7. Dehydrated through ethanol series cleared in xylene and mounted in DPX. 8. The dye absorbance was measured at 575 nm.

Mercuric bromphenol blue reagent

Bluish positive reaction is appeared in the cytoplasm and nucleus, indicate the sites of distribution of proteins. Dissolved 50 mg bromphenol blue (BDH Poole England) and one gram of mercuric chloride (Analar S.D. India) in 100 ml 2% acetic acid. Control preparations were made by deaminating (50 ml 10% TCA + 50 ml 10% sodium nitrite for 20 minutes at room temperature) the hydrated sections (Jensen, 1962).

SULPHHYDRYL GROUP PROTEINS
(Barrnett and Seligman, 1952)

Protein bound sulphydryl groups were localized with Robert's (1960) modification of Barrnett and Seligman's (1952) DDD (2,2, dihydroxy-6,6' dinaphthyl disulphide) reaction followed by coupling with fast blue- B(4-
benzylamino 2,5 dimethoxy aniline). The specificity and quantitative relationship of this reaction is well established (Roberts, 1960; Fosket and Miksche, 1966; Sippel, 1978; Nohammer, 1977, 1978). DDD simultaneously reacts with the protein bound SH and SS groups. However, the reaction with SH groups is many times quicker than the SS groups. Nohammer (1977) observed in Ehrlich ascites tumor cells the binding of DDD with SH groups was complete within 7 hr, while the reaction with SS groups took 14 days. Because of the high reactivity of thiols (SH) in contrast to the relative inertness with disulphides (SS) a call for careful distinction between these two counterparts. But the method we adopted accounts for both SH and the rereduced SS groups. Before staining, sections were treated with thioglycollic acid (pH 8.5) which reduces the SS groups into SH. Further more, during fixation and dehydration some of the native SH groups will be oxidized to SS. These oxidized groups again are reduced to SH groups by thioglycollic acid treatment. SH groups have the property of auto oxidation when they are exposed to environmental oxygen. To prevent this, material immediately after collection was fixed in 10 % TCA in 80 % ethanol overnight. Thus, by maintaining these
conditions both SH and SS were made accessible for quantitative terms. The staining schedule is given below.

1. TCA fixed material was used. 2. Sections were dewaxed in xylene and hydrated by passing through ethanol: D.W. series. 3. Incubated the slides in 0.5 M thioglycollic acid (pH 8.5) (E. Merck Germany) at 50°C for 1 hr. 4. Sections were washed with D.W. (3 times). 5. Placed the sections in D.D.D* (2,2 dihydroxy-6,6' dinaphthyl disulphide) reagent for one hr. at 56°C. 6. Sections were brought to room temperature, rinsed with D.W. and finally given a brief rinse with acid water (pH 4.0) to convert the byproducts of reaction into free naphthals. 7. Dehydrated through ethanol series brought to absolute ethanol. 8. Kept in solvent ether (Alembic Chemicals, India) to remove free naphthals. 9. Again sections were hydrated through ethanol: D.W. series. 10. Slides were placed in freshly prepared 1% w/v fast blue B (E. Merck, Germany) in phosphate buffer (pH 7.4) at room temperature for 5 minutes. 10. Washed the sections in running tap water for 5 minutes and finally with D.W. 11. Dehydrated through upgrade ethanol: D.W. series, cleared in xylene and mounted in D.P.X. 12. Measured the dye absorbance using a filter having spectral range 480-520 nm. (Nohammer, 1978).
Control: After thioglycollic acid treatment slides were transferred to 0.1 M aqueous solution of N-acetylmaleimide (Fluka A.G., Swiss) adjusted to pH 7.4 with NaOH, for 4 hrs at 37°C (Bock, 1978).

DDD reagent: Dissolved 100 mg of DDD (Koch Light Labs, England) in 60 ml absolute ethanol. Took 15 ml from this stock solution and added to 35 ml veronal buffer (pH 8.5).

HISTONES
(Alfert and Geschwind, 1953; Black and Ansley, 1964)

To achieve histone localization two staining techniques, Alfert and Geschwind's (1953) method of alkaline fast green (FG) and ammoniacal silver reaction (AS) of Black and Ansley (1964, 1966) have been employed. Alkaline fast green dye binding is purely based on isoelectric point of basic proteins. At pH 8.0, histones have a charge and binding with the dye because of their high basic nature, but the net charge of other proteins is nil at this pH value (8.0) and thus unavailable to dye binding. Before reacting with fast green it is obligatory to extract nucleic acids to unmask the binding groups of histones. Ammoniacal silver reaction needs no extraction of nucleic acids. But, before staining tissue should be treated with neutral buffered formalene (pH 7.0). According
to Black and Ansley (1966) alkaline fast green combines with bonds of histone molecules which are normally associated with DNA, while AS reaction involves histone groups not so bound. The chemistry of AS reaction is ambiguous, but the qualitative changes in histones can be detected. AS reaction varies from yellowish to black colour, yellowish staining is due to guanido groups of lysine (lysine rich) and blackish staining point the presence of guanido groups of arginine (Black and Ansley, 1964, 1966; Marciniak and Olszewska, 1979). The recent report by Oda et al. (1979) on the mechanism of AS reaction with histones contradicts the involvement of guanidinium groups of arginine. According to these authors AS staining is based on the amino groups with formaldehyde. The following gives the staining schedule of the individual techniques.

Alkaline fast green reaction (Alfert and Geschwind 1953)
1. Carnoy's fixed material was used. 2. Sections were deparaffinized in xylene and hydrated through ethanol:
D.W. series. 3. Kept the slides in 5% (w/v) TCA (E. Merck, Germany) for 15 minutes at 90°C. 4. Washed under running tap water for 5 minutes to remove TCA from the tissue and finally rinsed with D.W. 5. Kept the slides in 0.1% (w/v)
fast green at room temperature (Harleco, U.S.A. C.I. 42053) for 30 minutes. The pH of the dye 8.0 - 8.1 was adjusted with dilute NaOH. 6. Rinsed with D.W. dehydrated through ethanol; D.W. series, cleared in xylene and mounted in DPX. Measured the absorbance of the green chromophore using a filter having spectral range 625-630 nm.

Control: Deaminated (conditions were the same as described in bromphenol blue technique) sections showed negative staining. Ammoniacal silver reaction (Black and Ansley, 1964, 1966).

1. NBF fixed material was used. 2. Sections were dewaxed in xylene and hydrated by passing through ethanol; D.W. series. 3. Kept the slides in natural buffered formalene (pH 7.0) for 1 hr. at room temperature (25 ± 5°C). 4. Washed the sections thoroughly 3 times with D.W. 5. Kept the slides in freshly prepared ammoniacal silver reagent for 10 seconds at room temperature. 6. Again washed with D.W. to remove excess reagent. 7. Kept the slides in 3% formalene (v/v) for 3 minutes. 8. Washed with D.W. dehydrated through ethanol, cleared in xylene and mounted in DPX. 9. Measured the dye absorbance at 480-520 nm.

Ammoniacal silver reagent: This is prepared by adding 10% (w/v) silver nitrate (BDH, India) to concentrated
ammonium hydroxide (S&M, India) until the first permanent turbidity appears. The ratio usually appeared 10:1 (v/v) respectively as Black and Ansley (1964) noted.

Control: Deaminated and acetylated slides showed negative response.

Studies on the Localization of Nonhistone Chromosomal Proteins (Acidic Proteins)

To the best of knowledge there is only one specific technique by Smetana and Dusch (1956) in the literature to localize acidic nuclear proteins or non histone chromosomal proteins (NHCP). These authors reported the use of toluidine blue "O" (Allied Chem. Nat. Aniline Div.) at pH 9.0 to detect acidic nuclear proteins in cancerous tissue of Walker 256 carcinosarcoma. We tried our material (NEF fixed) using toluidine "O" (E. Merck, Germany, 0.1, 52040) at pH 9.0 but there was unspecific binding with the cell walls, cytoplasm and nucleus (Fig. 2, A).

Surprisingly, esterified sections also showed positive reaction with the dye. Hence, these results are not reproducible. We attempted to localize non histone chromosomal proteins using fast green FCF at pH 5.0. This method essentially follows the method of Alpert and Geschwind's (1953) technique to localize histones, except a change in pH of the dye.
The staining schedule is given below:

1. Carnoy's fixed material was used. 2. Slides were deparaffinized and hydrated through xylene ethanol series and brought to distilled water. 3. Slides were kept in trichloroacetic acid (5\% TCA w/v) at 87-90°C for 15 minutes to extract nucleic acids. 5. Washed under running tap water for 3 minutes to remove TCA from the tissues, and finally gave a brief rinse with distilled water. 5. Before keeping in the dye, slides were kept in pH adjusted distilled water (pH 5.0) to avoid any change in the pH of the dye. 6. This staining reaction was allowed at room temperature (28 ± 5°C) by keeping the slides in 0.1\% fast green (pH 5.0) for 30 minutes. 7. Slides were dehydrated through ethanol:D.W., cleared in xylene and mounted in DPX.

Effect of K-ion concentration on fast green dye binding in the cell:

The localization pattern of proteins with the fast green dye represented a function of the pH values of the dye. At pH 2.6 there was intensive staining in the cell, no differential staining from the cytoplasm to the cell nucleus was observed. Similar staining pattern obtained at pH 3.6 too, but the intensity of dye binding was relatively less. Afterwards, there was a gradual loss in
cytoplasmic staining, but the nucleus maintained its strong affinity to the dye. There was no cytoplasmic staining at pH 5.0, but nuclear staining was very much prominent. From pH 5.0 to 8.0, there was a diminution in nuclear staining but cytoplasmic staining reappeared till the pH value 7.0. There was absolutely no cytoplasmic staining at pH 8.0 and dye binding confined to nucleus only. At pH 9.0 there was positive reaction neither in cytoplasm nor in nucleus (Table 2). Thus these findings show that fast green staining is a function of the pH which represents remarkable features for the identification of different species of proteins having different isoelectric points. The following table showing the effect of H ion concentration on fast green dye binding to a cell. + indicates the positive reaction, - indicates negative reaction.
Table - 2

Pastgreen dye binding at different H ion concentration at room temperature (28 ± 5°C)

<table>
<thead>
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<th>pH</th>
<th>Localization pattern</th>
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<tr>
<td></td>
<td>Cytoplasm</td>
<td>Nucleus</td>
<td></td>
</tr>
<tr>
<td>2.6</td>
<td>+++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>4.0</td>
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<td>-</td>
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<tr>
<td>9.0</td>
<td>-</td>
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</table>

In further studies, we stained the slides at two pH values one towards acidic side (pH 5.0) and one towards basic side (pH 8.0). The proteins staining at pH 8.0 are the basic proteins (Alfert and Geschwind, 1953) and those reacting at pH 5.0 are the proteins having acidic nature. This was further confirmed by treating the slides with the following reagents after TCA treatment (after the II step in the staining schedule) and before keeping
in the dye at pH 5.0 and 8.0 (refer table - 3).

Acetylation: This serves as control for histones, because nonacetylated proteins get stained with fast green at pH 8 (Sauter, 1969; Jordanov, 1976; Jensen, 1962). After acetylation there was absolutely no positive reaction at pH 8.0 (Fig. 2,D) but the reaction at pH 5.0 was undisturbed.

Treatment with dilute acid: Basic proteins are labile to dilute acid (0.25 N HCl) (Black and Ansley, 1964; et al. Bonner, 1968; Sasaki, 1978; Holtzan, 1965). Similar localization pattern was observed as found after acetylation.

Dilute alkali treatment: Acidic proteins are alkali labile (Sasaki, 1978; Elgin et al. 1971). Dilute alkali (0.003 N NaOH) treatment showed a little loss in the staining intensity at pH 5.0 but there was no complete loss (Fig. 2,E). But, this treatment showed no influence on staining at pH 8.0.

Deamination: The amino groups of proteins are being extracted from the tissue and this renders reacting groups unavailable to the dye (Jensen, 1962). Deaminated slides showed staining neither at pH 8.0 nor at 5.0).
Fig. 2. Studies to localize nonhistone chromosomal proteins (NHCP).

(A) Toluidine blue stained meiocytes of Limnophyton anther sac for acidic nuclear proteins according to the method of Smetana and Busch (1966). Note the cytoplasmic staining in the cells (x480).

(B) Fast green (pH 5.0) stained nonhistone chromosomal proteins (acidic proteins). The dye binding is confined to nucleus only. There is no sign of cytoplasmic staining (x480).

(C) Fast green (pH 8.0) stained histones (according to the method of Alfert and Geschwind, 1953) at tetrad stage during microsporogenesis in Limnophyton (x480). The staining intensity is relatively poor when compared to that of the FG (pH 5.0) staining of nonhistone chromosomal proteins.

(D) Fast green (pH 8.0) staining after acetylation. Note the negative reaction (x314).

(E) After methylation fast green (pH 5.0) showed no dye binding (x380).

(F) Fast green (pH 5.0) staining after treating with 0.003 N NaOH (x314). There is no absolute negative reaction.

TP - tapetum, TT - tetrad.
Methylation: Carboxyl groups will form ester bonds by methylation. Thus -COOH groups are blocked (Smetana and Buch, 1966; Pearse, 1972). There was a complete loss of staining at pH 5.0 and at pH 8.0 there was a little loss (Fig. 2,F). These staining patterns after the above said treatments were depicted in table 3.

From the above pretreatments, staining at pH 5.0 are the proteins other than histones evidenced by acetylation and by dilute acid treatment. They are mostly acidic in nature, because methylation showed negative reaction at this particular H-ion concentration. Furthermore, it has no influence on the staining reaction at pH 8.0. It is known that proteins responsive to fastgreen at pH 8.0 are histones (Alfert and Geschwind, 1953). Acetylated and dilute acid treated slides did not show staining at pH 8.0. Thus these two treatments served as a strong control for basic proteins. The reason for not giving complete negative reaction after dilute alkali treatment is accountable since all types of acidic proteins are not alkali labile, some are resistant to dilute alkali, their residual nature. Complete recovery of NHC proteins even through differential centrifugation technique is also helpless. These factors can be attributed to the persistance of positive reaction even after alkali treatment. Thus,
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conditions: (Followed after TCA extraction of NAS)</th>
<th>Use of the treatment</th>
<th>Staining pattern at pH 5.0</th>
<th>Staining pattern at pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deamination</td>
<td>50 ml 10% TCA + 50 ml 10% sodium nitrite for 12-15 mnts. at R.T.*</td>
<td>To remove NO₂ groups</td>
<td>No staining</td>
<td>No staining</td>
</tr>
<tr>
<td>Methylation</td>
<td>90 ml methanol + 1 ml 1 N HCl for 15-20 hrs. at R.T.*</td>
<td>To block COOH groups</td>
<td>No staining</td>
<td>+</td>
</tr>
<tr>
<td>Acetylation</td>
<td>50 ml 50% ethanol + 50 ml 50% acetic anhydride for 20-24 hrs at R.T.*</td>
<td>To mask arginine and lysine groups</td>
<td>+++</td>
<td>No staining</td>
</tr>
<tr>
<td>Dilute acid HCl</td>
<td>0.25 N HCl for 1 hr. at R.T.*</td>
<td>To extract acid soluble material (basic proteins)</td>
<td>+++</td>
<td>No staining</td>
</tr>
<tr>
<td>Dilute alkali NaOH</td>
<td>0.003 N NaOH for 1-2 mnts. at 0-4°C</td>
<td>To extract alkali soluble material (acidic proteins)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Untreated</td>
<td>Kept slides directly from TCA to dye after washing with DW</td>
<td></td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

* Room temperature 28 ± 5°C
fast green at pH 5.0 specifically binds with NHC proteins (Fig. 2,B).

INSOLUBLE SUGARS
(Hotchkiss, 1948)

Localization of insoluble sugars was carried out by periodic acid–Schiff’s reaction of Hotchkiss (1948) as detailed by Jensen (1962). For light microscopic study PAS reaction is effective (Chao, 1977) and it is accessible for cytophotometry after removing starch from the tissue (Jona and Pfa, 1977). It gives intensive colour. However, its specificity has been questioned (Diboll, 1967). Periodic acid oxidation is a critical step where 1:2 glycol linkage releases the free aldehyde groups. If the oxidation prolongs long time other complex molecules like polyphenols and proteins also mimic the reaction giving false PAS positive staining (Bara and Anderson, 1965). PAS reaction yielded yellow colour where proteins reacted and red colour with polyphenols (Considine and Knox, 1972; Lingle et al. 1977). However, success on the method depends on a large extent on the colouristic purity of the stained chromophore (magenta colour). The method to localize polysaccharides is as follows.
1. Carnoy’s fixed material was used. 2. Sections were dewaxed in xylene and hydrated by passing through ethanol;
D. w. aeries. 3. Hydrated slides were kept in 0.5 % (w/v) periodic acid (Fiedel, Germany) for 10 minutes at room temperature after trying for different periods for its colouristic purity. 4. Washed thoroughly under running tap water for 5 minutes. 5. Kept in Schiff's reagent for 10 minutes at room temperature. 6. Excess stain was removed by keeping in 2 % sodium metabisulphite (BDH, India) for 2 minutes. 7. Washed again under running tap water for 5 minutes. 8. Dehydrated in ethanol, cleared in xylene and mounted in DPX.

The characteristic magenta colour indicates the sites of polysaccharides; starch is localized in a granular form. As Jona and Koa (19??) felt, the wide dispersal of starch granules strongly interfered with cytophotometric readings hence we have noted the qualitative changes in the localization pattern for polysaccharides. Schiff’s reagent was the same as prepared for Feulgen nuclear staining. In case of control preparations, oxidation by periodic acid was omitted and strictly followed the remaining steps in the above staining schedule. Another control was prepared according to the method of Feder and O'Brien (1968) using cinnamone. A saturated solution of cinnamone (5,5-dimethyl cyclohexamane 1,3-dione; No. 281 79, BDH Poole, England) was prepared by adding 0.5 g of
dime done to 100 ml of D.W. Stirring with a magnetic stirrer for 8 h. filtered through whatman No. 1 filter paper. After periodic acid oxidation, the slides were transferred to dime done reagent for 15 hr. at room temperature and kept in D.W. for 30 minutes giving changes for each 10 minutes. Now sections were kept in Schiff's reagent. No PAS positive reaction was observed.

NUCLEOLUS
(Trump et al., 1961)

1. Carnoy's fixed material was used. Sections were deparaffinized in xylene and hydrated through ethanol: D.W. series. 2. Placed the slides in 0.1 % w/v toluidine blue 'O' (E. Merck, Germany) prepared in 2.5 % (w/v) sodium carbonate (BDH, India) solution (pH 11.3). 3. Differentiated in D.W. till nucleolus is clearly visible. 4. Dehydrated through a series of alcohol, cleared in xylene and mounted in DPX. Toluidine blue selectively bound with the nucleolus, appeared blue in colour (Fig. 30).

CYTOPHOTOMETRY

The cytophotometer used in this piece of work was designed in our laboratory. This is a Pollister microspectrophotometer type (1952) and follows Lambert-Beer's laws of light absorption. The main components in the
cytophotometer are Köhler illumination (15 W 6 V bulb fitted with a Leitz Wetzlar microscope), photometric device (micro ammeter) and a light dependent resistor (L.D.R.) (Phillips, Holland). Microscope and L.D.R. are kept inside a dark chamber. Köhler bulb receives current from the main supply through a series of stabilizers to resist against the fluctuations in the current. Over the Köhler illumination, narrow band (wave length) filters were kept to get the light of required wavelength. Thus filtered light passes through the stained preparation (slide on the platform of the microscope). A part of the light being absorbed by the chromophore and the transmitted light passes through the objective of the microscope and reflected by the mirror on to a LDR kept parallel to it.

Working of LDR: On applying current to one end of the LDR, it does not allow the current to pass through it when it is in complete darkness. But, when a beam of light fall on LDR, it looses resistance according to the intensity of the light to which it is exposed. Thus there exists a positive correlation between the intensity of light and the decrease in the resistance of LDR (Phillips-Daža Hand Book, 1970). These differences between the flow of
Fig. 3. Different assemblies of cytophotometers.

(A) In this assembly microscope and LDR were kept in a dark chamber. In the dark chamber microscope is separated from the LDR by a wooden box to avoid the interference of the light source of microscope with that of LDR. Light source is Kohler illumination (KI) which is connected from the main power supply through stabilizers (S). The transmitted light through the stained preparations pass through the eye piece of the microscope reflects by a mirror on to the LDR. The LDR is connected with a microammeter (uA) to record current. (see the circuit diagram depicted in figure 4).

(B) In this assembly the transmitted light directly falls on LDR housed in a wooden box over a microscope. See the outline diagram of this assembly in figure 4.

uA : microammeter           C ; condenser
F  : filter                  HLDR : housed LDR
K  : key (switch)            KI : kohler illumination
LDR : light dependent resistor
PA : photographic attachment
S  : stabilizer              ST : stage of the microscope
TPS : transistor power supply
TR : transformer
the current are recorded by a microammeter connected to the other end of the LDK. If more current is flowing (implies more loss of the resistance of LDR) microammeter records more reading and less current records less reading (because of a gain in the resistance of LDR).

Stain intensity is related with ammeter readings because of the interdependence of all these parameters as shown below:

Stain intensity \( \propto \) light absorbance

\[
\text{Stain intensity} = \frac{1}{\text{Transmitted light}}
\]

Transmitted light \( \propto \) current passed through LDR

Current released by LDR \( \propto \) ammeter reading

On adjusting the control preparation (control slide) on to the LDR more light will fall on the LDR because of more transmittance of filtered light through the slide. Thus higher reading will be recorded on the ammeter. Reverse is the case with stained preparation.

Working of the instrument: Instrument kept on working by putting on the main switch. Allowed to stabilize the current for 15 minutes. 2. Put the slide on the microscope and adjusted the required cell/nucleus on the LDR. The LDR was removed to take dimensions of the cell/nucleus
(camera lucida drawings). 3. Again put the LDR in its original position on the mirror image of the required region. Noted the ammeter reading. 4. Now, blank portion of the slide was projected on the LDR, again ammeter reading was noted.

The logarithmic difference between the readings of blank and stained region will give the extinction value (Pollister et al., 1969).

\[ \text{Extinction value} = \log \text{of blank reading} - \log \text{of stained reading}. \]

Content or total dye binding is the product of extinction value and corresponding area/volume. \( \text{Content} = \text{extinction value} \times \text{area or volume}. \)

The number of readings measured for a parameter range from 15 to 20 (both stained and blank readings) in a section. Thus 2-3 slides were exposed for one parameter. Nuclei or cells near edges of the sections were omitted since they are more prone to damage in preparation of the tissue. Those cells/nuclei which have come completely in the selected section thickness were only subjected for measurements. In nuclear measurements to avoid light scatter from cytoplasm LDR was covered with a diaphragm having
apertures of different diameters. Thus the exposing surface of LDR always kept smaller than the dimensions of the image falling over it.

In a particular set of experiment exposed surface of the LDR was kept constant. The instrument was checked for its performance and sensitivity in a manner suggested by Pollister et al. (1969) and Ruthman (1970).

The results of two such experiments are as follows:

1. Lambert's law was checked by measuring the extinction values (dye absorbance) of the stained material at different section thickness. A linear relationship was found.

2. Beer's law was checked by measuring the extinction values of fast green dye of different concentrations. A linear relationship was obtained. In a series of publications by Shah and his school (1975, 1978, 1980) discussed the results obtained by this cytophotometer. Evans (1976) in his personal communication supported our cytophotometric estimations with his own data. The magnification of the projected image on to the LDR was noted by calibrating with the help of a micrometer slide (10 microns). Thus area of the surface of the cells and nuclear volume were
Fig. 4. The diagramatic representation of the two different assemblies of the cytophotometers given in the figure 3 A and B.

UA : microameter
B : battery
C : condenser
D : diaphragm
F : filter
K : key (Switch)
LDR : light dependent resistor
O : objective eye piece
P : power supply
PM : plain mirror
S : stage of the microscope
SV : side viewer
T : transformer
Fig. 4
calculated. For nuclear volume we employed different formulae depending on the shape of the image: $\frac{4}{3} \pi r^3$ for spheroidal nuclei (where $r$ is the radius) and $\frac{4}{3} \pi a^2 b$ (where $a$ and $b$ are the minor and major radii) for elliptical nuclei. The assembly of cytophotometer is given in figure 3, A. In this LDR was kept in a dark chamber and the image of the tissue was projected on to a LDR with the help of a mirror. There are some chances of experimental error because of stray light while working with the instrument. Hence, an improvement over this assembly has been made, where the LDR is kept in a completely closed wooden box (Fig. 3, B). The circuit diagrams of both the types are depicted in the figure 4A, B.

Photographs of stained preparations were made using photomicroscope-I with planapochromatic lenses. Carl-Zeiss, Germany. Ilford Pan F 35 mm film was used to record the images. A strict control of the time of exposure for both negatives and positives was maintained to permit the visual qualitative comparison of the stain intensity.