CHAPTER II

MATERIALS AND METHODS
Three strains of *Sorghum bicolor* (L.) Moench namely, 2219 A, 2219 B and NP3R were used in the present investigations. 2219 B is a fertile (F) strain developed in India. Genetically, it is a Kafir-Shaliu derivative and produces viable pollen. 2219 A is a cytoplasmic male sterile (CMS) strain, developed at Agricultural Research Station, Coimbatore, India, during 1960s. It is derived from 2219 B by backcross technique to a milo type, and therefore, has milo cytoplasm and 2219 B nucleus. Because of this, it is incapable of producing viable pollen.

Presently, 2219 A is used as a functional female in the production of hybrid sorghums, and CHS-6 (Co-ordinated Sorghum Hybrid-6) is one of the successful hybrids evolved by crossing 2219 A with CS 3541.

NP3R is a genic male sterile (GMS) strain from U.S.A.

The seeds (caryopses) of all the three strains were obtained from Sorghum Breeding Research Station, University College of Agricultural Sciences, Dharwad. The seeds were germinated in sterile petri-dishes, and one- to 3-day old seedlings were transferred to pots and grown in the departmental green house.
and the reproductive structures namely anthers, ovarys and caryopses of successive developmental stages, were fixed in different fixatives for a known time duration (see Table-I), specific to the substance or metabolite to be localized. Fixed materials were washed and dehydrated in ethanol series and then twice in n-butanol before cold paraffin infiltration.

2. **Paraffin infiltration and embedding**: Into the medium of pure butanol in vials containing materials, flakes of paraffin were added intermittently for cold infiltration at room temperature. Later, the vials were kept under the table lamp to facilitate higher saturation. Then, the vials were transferred to the thermostat maintained at 50°C, and 5 - 6 changes with molten paraffin were given in order to eliminate butanol. Finally, materials were systematically embedded in pure paraffin employing paper-boat method.

3. **Microtomy**: Paraffin embedded materials were made into small blocks and 8 µm thick sections were cut using 'OPTEX BRAND' microtome (Japan make). Due care was taken to maintain uniformity in the thickness of sections throughout the investigation for qualitative and semi-quantitative assessment of histochemical localization.

4. **Affixing the paraffin ribbons to the slides**: Paraffin ribbons containing sections of plant materials were
## TABLE - I:

Showing the details of fixation and post-fixation procedures

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Biochemical substance or metabolite under investigation</th>
<th>Fixative</th>
<th>Temperature</th>
<th>Duration of fixation</th>
<th>Post-fixation procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Insoluble Polysaccharides</td>
<td>Carnoy's fixative (Acetic-alcohol 1:3)</td>
<td>Room temperature</td>
<td>1 to 6 hours</td>
<td>Washed thrice in 70% alcohol and processed further</td>
</tr>
<tr>
<td>2.</td>
<td>DNA</td>
<td>-do-</td>
<td>-do-</td>
<td>-do-</td>
<td>-do-</td>
</tr>
<tr>
<td>3.</td>
<td>RNA</td>
<td>-do-</td>
<td>-do-</td>
<td>-do-</td>
<td>-do-</td>
</tr>
<tr>
<td>4.</td>
<td>Proteins</td>
<td>-do-</td>
<td>-do-</td>
<td>-do-</td>
<td>-do-</td>
</tr>
<tr>
<td>5.</td>
<td>Histones</td>
<td>NBF, pH 7</td>
<td>-do-</td>
<td>18 hours</td>
<td>Washed in running tap water over night, dehydrated and processed further.</td>
</tr>
<tr>
<td>6.</td>
<td>Ascorbic acid</td>
<td>acidified* alcoholic silver nitrate</td>
<td>0°C to 2°C</td>
<td>7 days in darkness</td>
<td>Washed thrice in 50% alcoholic ammonia at an interval of 15 minutes and processed further.</td>
</tr>
</tbody>
</table>

* Acidified alcoholic silver nitrate acts as both fixative as well as staining reagent.
placed on clean slides, flooded with 0.5 percent gelatin adhesive. Spread using a hot plate, drained out gelatin and dried for two days. \textit{(Preparation of gelatin: 0.5 g of gelatin was dissolved in 100 ml of distilled water in which a pinch of potassium dichromate was added).}

5. \textbf{Deparaffinization and hydration:} Three jars of pure xylene were used to deparaffinise sections, keeping in each for 10 minutes. Then, the slides were air dried at room temperature and passed through down grade ethanol series for gradual hydration of sections. Prior to RNA and DNA staining, slides were coated with 1 percent celloidin, prepared in 1 part ether and 1 part ethanol.

6. \textbf{Histochcmical staining procedures:} Hydrated sections on slides were subjected to specific histochemical staining procedures (described in detail later in this chapter) which are specific to the substance or metabolite localized (see Table-II).

7. \textbf{Post-staining procedure:} Subsequent to staining, the slides were passed through upgraded ethanol and/or butanol series, cleared in xylene and mounted using DPX.

In the present histochemical studies with fixed
Table - II

Showing the details of methods and control tests employed in the present histochemical studies.

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Macromolecular substance localized</th>
<th>Method adopted</th>
<th>Pre-treatment if any</th>
<th>Reaction reagent</th>
<th>Temperature</th>
<th>Duration of incubation</th>
<th>Colour indication</th>
<th>Control test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total insoluble polysaccharides</td>
<td>PAS method (Petchkiss, 1984)</td>
<td>0.5% periodic acid for 15 minutes</td>
<td>Schiff's</td>
<td>Room Temp.</td>
<td>20 minutes</td>
<td>Magenta red</td>
<td>avoidance of pre-treatment in periodic acid (Cass and Jensen, 1970)</td>
</tr>
<tr>
<td>2</td>
<td>Starch</td>
<td>IKI Test (Johansen, 1940)</td>
<td>-</td>
<td>IKI</td>
<td>-do</td>
<td>10 minutes</td>
<td>Blue or black</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Callose</td>
<td>Aniline-blue visible-light method (Johansen, 1940)</td>
<td>-</td>
<td>0.005% aniline-blue in 50% alcohol.</td>
<td>Room Temp.</td>
<td>24 hrs</td>
<td>Blue</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Lignin</td>
<td>Phenol red test (Johansen, 1940)</td>
<td>-</td>
<td>Saturated aqueous solution in 20% HCl</td>
<td>-</td>
<td>-</td>
<td>Red-violet</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>DNA</td>
<td>Feulgen nuclear reaction (Gomori, 1952)</td>
<td>20 minutes in 5% HCl (Fock, 1969).</td>
<td>Schiff's</td>
<td>Room Temp.</td>
<td>1 hour in dark</td>
<td>Magenta red</td>
<td>DNAase extraction (Pearse, 1960)</td>
</tr>
<tr>
<td>6</td>
<td>RNA</td>
<td>Azure B method (Flav and Himes, 1932)</td>
<td>-</td>
<td>0.025% Azure B in citrate buffer pH 4</td>
<td>50°C</td>
<td>2 hours</td>
<td>RNA-purine</td>
<td>DNAase extraction (Pearse, 1960)</td>
</tr>
<tr>
<td>7</td>
<td>Total proteins</td>
<td>Mercurochrome bromophenol blue method (Maccia et al., 1953)</td>
<td>95% alcohol for 5 minutes</td>
<td>Mercurochrome bromophenol blue in 95 percent alcohol</td>
<td>Room Temp.</td>
<td>15 minutes</td>
<td>Blue</td>
<td>&quot;Trypsin extraction&quot; (Pearse, 1960)</td>
</tr>
<tr>
<td>8</td>
<td>Pituic acid</td>
<td>Ammonical silver nitrate method (Black and Asley, 1964)</td>
<td>10% I2Et</td>
<td>Ammonical silver nitrate</td>
<td>10 min</td>
<td>45 seconds, then in 3% formaldehyde</td>
<td>Aramine rich yellow</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Ascorbic acid</td>
<td>Acidified silver nitrate (Dave et al., 1969)</td>
<td>-</td>
<td>5% silver nitrate</td>
<td>Room Temp.</td>
<td>7 days</td>
<td>Dark brown</td>
<td>Treatment with 10% formaldehyde for 4 hrs prior to incubation</td>
</tr>
</tbody>
</table>
materials, the substances or metabolites localized are:
1. total insoluble polysaccharides, 2. deoxyribonucleic acid (DNA), 3. ribonucleic acid (RNA) 4. total proteins 5. histones both arginine-rich and lysine-rich and 6. ascorbic acid.

8. **Photomicrography**: Photomicrography of the selected sections was made using 'PANATOMIC-X' or 'COPEX PAN' black and white negative films and 'Mirax Laborec' Japan make 35 mm optical camera.

**HISTOCHEMICAL TESTS EMPLOYED ON FIXED MATERIALS**

**Total Insoluble Polysaccharides**

Major total insoluble polysaccharides include starch, cellulose, callose, hemicellulose, mucopolysaccharides and pectins (Lehninger, 1976). Of these, starch, cellulose and callose are known to play significant roles during growth and differentiation of plant tissues. Starch is a storage food material and acts as a reserved energy source for varied metabolic activities of the cells and tissues. Cellulose is a major cell wall material and callose plays significant roles during meiosis in microspore and megaspore mother cells, as well as during pollen mitosis, pollen tube growth and such
other events in cell/tissue differentiation.

METHOD FOR LOCALIZATION OF INSOLUBLE
POLYSACCHARIDES (Hotchkiss, 1948 in Jensen, 1962)

Localization of total insoluble polysaccharides was
made by employing Periodic Acid-Schiff's (PAS) method
devised a reaction based on the oxidation of 1,2- glycol
groups of polysaccharides into aldehyde groups with periodic
acid (HIO₄) and its use in histological and histochemical
studies. Aldehyde groups formed in this reaction, react with
leucobasic fuschin (bis-N-aminosulphonic acid) of Schiff's
reagent to produce a visible magenta-red colour-complex.
Based on this reaction, Hotchkiss (1948) devised the method
to localize total carbohydrates of insoluble polysaccharides
in tissue-sections. According to him, PAS-reaction is expected
of any metabolite that fulfils the following four criteria:

1. that contains 1, 2- glycol groups or their
equivalents such as amino-or alkylamino-derivatives or the
oxidation product of CHOH-CO;

2. that does not diffuse away during fixation;
that gives an oxidation product which is not diffusible; and

that which is present in sufficient concentration to give a detectable final colour-complex.

Jensen (1962) has recommended that this procedure is highly suitable for the demonstration of total insoluble polysaccharides because of the following characteristics:

1. The reaction does not cause the breakage of polysaccharide chains.

2. It is specific to polysaccharides, as the chemical basis of staining, due to this reaction, is known.

3. It offers least interference and gives no false localization.

4. It results in a colour-complex which is intense as well as stable.

Since it is not a differential staining method, other tests such as $I_2$KI (Johansen, 1940) and aniline blue methods (Johansen, 1940) were also employed to confirm the presence of starch and callose, respectively.
Procedure of PAS Method: The steps that were followed are as follows:

1. Hydrated sections on slides were treated with 0.5 per cent periodic acid for 15 minutes at room temperature (Preparation of 0.5 per cent periodic acid: 0.5 g of periodic acid was dissolved in 90 ml of distilled water and to this, 10 ml of 0.2 M sodium acetate was added).

2. Sections were washed in running tap water and incubated in Schiff's reagent for 15 minutes at room temperature.

   Preparation of Schiff's reagent: 2 g of basic fuschin was dissolved in 200 ml of 0.15 N HCl. The mixture was agitated for 3 hours at frequent intervals. To this, 1.9 g of sodium metabisulphite was added and agitated frequently and left over night. Later, 1 g of activated charcoal was added, agitated for some time, filtered immediately and stored in refrigerator at 4°C until use. Only colourless preparations were used throughout the studies. Before use, the aliquot of Schiff's reagent was allowed to reach room temperature.

3. Stained sections were washed in distilled water and treated with bleach to remove superfluous stain.

   Preparation of bleach: Bleach was prepared by
dissolving 0.5 g of sodium metabisulphite in 95 ml of distilled water to which 5 ml of 1N HCl was added.

4. Sections were then washed in distilled water, dehydrated, cleared and mounted with DPX.

**Colour Indication:** Polysaccharides stain magenta red or intensely red.

**Control test for PAS reaction:**

The following control test was employed to confirm the presence of PAS-positive substance.

*Avoiding pretreatment with periodic acid* (Cass and Jensen, 1970): Hydrated sections on slides were directly incubated in Schiff's reagent, as in a PAS reaction, but without pretreatment with periodic acid.

**Result:** Known sites such as starch grains and cell walls did not stain magenta red.

**KI confirmatory test for starch** (Johansen, 1940):

In the present study, this test was used to confirm
whether PAS-positive grains were starch grains or not. This reaction was first used by Coventou in 1926 to demonstrate starch. The accumulation of iodine in the centre of the helical starch molecule has been considered as the basis for colour formation (Jensen, 1962).

**Staining procedure:** Hydrated sections on slides were treated with IKI solution that was prepared by dissolving $2 \, \text{g}$ of potassium iodide and $0.2 \, \text{g}$ of iodine in $100 \, \text{ml}$ of distilled water.

**Colour indications:** Older starch grains stained blue-black and newly formed ones, red-violet.

**Aniline-blue method for callose—visible light method** (Johansen, 1940): In the present studies this method was employed to confirm whether additional wall thickening around PMCs is callose or not.

**Procedure:** Fresh and half-cut anthers from young flower buds were kept in $0.005 \, \text{per cent}$ solution of aniline blue, prepared in $50 \, \text{per cent}$ alcohol for $24 \, \text{hours}$, rinsed in water, smeared on a clean slide in a drop of distilled water, covered with a cover slip and observed.
Colour indications: The additional wall thickening of MMCs appeared blue.

Phloroglucinol test for Lignin (Johansen, 1940; Siegel, 1953 in Jensen, 1962): This test was employed to know the pattern of lignification in seedlings, and to test the nature of endotheceial thickenings.

Procedure: Fresh, free-hand sections were placed in a drop of saturated aqueous solution of phloroglucinol in 20 per cent HCl.

Colour indication: Mature xylem elements appeared red-violet.

NUCLEIC ACIDS

Of all the cellular constituents, the nucleic acids namely deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are the most important because the former acts as genetic material encoded with biological information and the latter play their due role in protein synthesis.

METHODS FOR LOCALIZATION OF NUCLEIC ACIDS

There are many recommended methods for the
cytochemical/histochemical localization of nucleic acids. Of these, the following methods were employed for the in situ localization of DNA and RNA, respectively.


The histochemical staining of nucleic acids in these methods depends upon the properties of two components of the nucleotides namely the pentose sugar and phosphoric acid. It is the deoxyribose sugar present in DNA which is responsible for positive feulgen reaction with Schiff's reagent after mild hydrolysis, whereas phosphoric acid residues are responsible for the basophilic properties of both DNA and RNA with basic dyes.

**Feulgen Nucleic Acid Method for DNA** (Gomori, 1952 in Jensen, 1962): Based on Schiff's reaction for staining aldehydes, this method was first developed in 1924 by Feulgen and Rossenbeck. According to Gomori (1952) this is one of the best histochemical techniques ever devised to demonstrate DNA. In this method, hydrated sections were subjected to mild hydrolysis.
either with 1 N HCl at 60°C or with 5 N HCl at room temperature (Fox, 1969) that removes the purines at the level of purine-deoxyribose glucosidic bonds of DNA. As a result the aldehyde groups of deoxyribose sugar are unmasked and released free to react with Schiff's reagent. This reaction is widely accepted as a specific one for the **in situ** localization of DNA (Sharma and Sharma, 1965). The success of Feulgen reaction, however, depends upon two factors: (1) the fixative used and (2) the duration of hydrolysis. The intensity of the colouration depends on the number of aldehyde groups.

In the present investigation, materials fixed in acetic-alcohol (1:3) were used, and the standardized duration of hydrolysis with 5 N HCl at room temperature is 20 minutes.

**Procedure:**

1. Deparaffinized sections coated with one percent celloidin were hydrolysed at room temperature for 20 minutes in 5 N HCl (Fox, 1969).

2. Slides were rinsed in distilled water and incubated with Schiff's reagent at room temperature for one hour in dark.

3. The slides were rinsed again in distilled water
and treated with bleach and again rinsed in distilled water, dehydrated, cleared and mounted with DPX.

**Control test for DNA: Deoxyribonuclease extraction method**
(Pearse, 1960).

**Procedure:** Deparaffinized and hydrated sections were treated for 24 hours with deoxyribonuclease 1 mg/ml in 0.025 m Veronal buffer at pH 7.5 containing 0.003 M -MgSO₄. The enzyme solution was replaced twice. Slides were washed in water, dehydrated in alcohol and passed to alcohol-ether 1:1 and coated with one percent celloidin. Slides were then hydrated and stained according to Feulgen method as usual.

**Results:** Sites of DNA localization could not be stained.

**RNA and DNA**

**Azure B method** (Flax and Himes, 1952 in Jensen, 1962): In this method, phosphoric acid residues are responsible for staining both RNA and DNA with Azure B, a basic stain. The technique is based on the phenomenon of metachromasia, in which certain cell constituents stain differently from the original colour of the dye. The purple colour of the dye is
termed orthochromatic shade and the second blue-green colour formed in certain areas is called metachromatic shade. With Azure B, (a basic dye of thiazine group), at pH 4, RNA exhibits orthochromatic shade, whereas DNA, metachromatic shade and the proteins are left unstained (Plax and Himes, 1952 in Jensen, 1962). Cell walls do not stain until they contain lignin, in which case they appear green. However, the metachromatic nature of lignin with Azure B is little appreciated by histochemists (Jensen, 1962).

Procedure: 1. Deparaffinized tissue-sections were coated with one per cent celloidin and hydrated.

2. Slides with hydrated sections were incubated in 0.025 per cent Azure B-stain (prepared in citrate buffer of pH 4) at 50°C for 2 hours.

3. Stained slides were rinsed in distilled water, dehydrated in TBA, cleared in xylene and mounted with DPX.

Colour indication: RNA stains purple and DNA, blue-green. Known lignin areas appear green.

Note: Although Azure B stains both RNA and DNA, only the localization of RNA is taken into account in the present
histaechimal assessment, as the results of DNA localization were not clear with this method.

**Control Test for RNA**

**Ribonuclease Extraction Method (Pearse, 1960):**

**Procedure:** 1. Deparaffinized and hydrated sections were incubated for one hour at 37°C in a solution of ribonuclease 0.1 mg/ml in glass distilled water.

2. Washed thoroughly in running water and stained with Azure B method as usual.

**Results:** Sites of RNA localization could not show any staining.

**PROTEINS**

The enormous varieties of proteins do serve as major building materials and also as regulatory molecules that control the diverse activities of a plant organism (Averz, 1976). The organic structure of an organism is built-up through the organized regulation of the formation, degradation and activity of both structural proteins and enzymes, both in space and time. Therefore, histochemical localization of both structural proteins and enzymes (described under fresh
material studies) helps in understanding the chemical constitution and metabolic state of any cell or tissue in an organized structure.

A. Total Proteins

Bromphenol blue method (Mazia et al., 1953) was employed in the present investigation because of the following reasons:

1. Even the minute quantity of proteins can be localized.

2. The dye binds itself to basic proteins even when mercury is absent, and also other proteins when mercury is present.

3. The amount of dye bound is proportional to the quantity of proteins.

Procedure: 1. Deparaffinized slides were brought to 95 per cent alcohol and incubated for 15 minutes at room temperature in mercuric bromphenol blue (Hg-BPB) staining reagent which was prepared by dissolving 10 mg of bromphenol blue crystals in 100 ml of 10 per cent mercuric chloride solution in 95 per cent alcohol.
2. Slides were rinsed in 0.5 percent acetic acid for 5 to 10 minutes, and differentiated in tap-water until the sections turned blue.

3. Then, dehydrated in tertiary butanol series, cleared in xylene and mounted in DFX.

B. HISTONES (BASIC PROTEINS)

Histones are relatively small protein molecules and are highly basic in nature. They are the major constituents of the chromosomes. Histones usually occur in an equal proportion to DNA by weight. Although the specific role of histones in cellular metabolism is yet to be confirmed, they appear to play their significant role in the supercoiling of DNA in eukaryotic chromosomes to serve as: (1) structural elements, or (2) to cover or (3) to repress specific segments of DNA so that the latter are not transcribed (Lehninger, 1976).

There are two standard methods presently available for the localization of histones in tissue-section; These are:


In the present investigation, ammoniacal silver nitrate method was employed because of the following reasons:

1. There is no need for extraction of DNA

2. It is less time consuming

3. By this method, both arginine-rich and lysine-rich histones can be differentially localized.

**Procedure**: 1. Deparaffinized and hydrated sections were pretreated with neutral buffered formalin (NBF) for one hour.

2. Slides were then repeatedly washed in distilled water and incubated at room temperature for 45 seconds in dark with standard ammoniacal silver nitrate reagent which was prepared with utmost care by adding 10 percent aqueous silver nitrate solution drop by drop to 2 ml of liquor ammonia until the first visible and permanent turbidity appeared.

3. Slides were again repeatedly washed in distilled water and developed in 3 percent formalin for two minutes.

4. Finally, the slides were dehydrated, cleared and mounted with DPX.
Colour indications: Arginine-rich histones stain light yellow and lysine-rich histones stain black.

CONTROL TEST FOR HISTONES and NON-HISTONE PROTEINS -

TRYPSIN METHOD (Pearse, 1960):

Procedure: 1. Deparaffinized and hydrated sections were incubated for 1 hour at 37°C in 0.05m phosphate buffer at pH 8.9 containing 1 mg/ml of pure trypsin.

2. washed well in water and dehydrated up to 95 per cent alcohol, and stained by usual mercuric bromphenol blue method for proteins or brought to water, pretreated in NBF and stained with ammoniacal silver nitrate for histones.

Results: Sites of histones and non-histone proteins do not show any staining.

ASCORBIC ACID (AA)

Earlier workers developed methods for the localization of this important growth regulator, in which the tissues were subjected to staining with aqueous silver nitrate, prior to sectioning. The problem of ascorbic acid localization arises not from the final reaction, but from the solubility of ascorbic acid in aqueous solutions. This means that only
freeze-dried tissue sections should be used to avoid solubility of AA. In this way great care has to be taken to prevent the tissue coming in contact with aqueous solution prior to localization (Jensen and Kavaljian, 1956). Keeping these difficulties in view, Dave et al. (1969) devised a method in which both fixation and staining are combined together. The method is known as acidified alcoholic silver nitrate method. According to Dave et al. (1969), at low temperatures, silver nitrate is reduced to silver and there is no shifting of AA because both fixation and staining are simultaneous.

**Acidified-alcoholic silver nitrate method (Dave et al., 1969):**

*Procedure:* 1. Fresh materials were directly placed in black paper-covered vials containing acidified alcoholic silver nitrate solution (prepared by dissolving 5 g of silver nitrate crystals in a mixture of 34 ml of distilled water, 66 ml of absolute alcohol and 5 ml of glacial acetic acid) and kept for incubation for 7 days at 0°C to 2°C.

2. Materials were then washed thrice in 5 per cent liquid ammonia in 50% alcohol at an interval of 15 minutes, each time under the red light in dark room, dehydrated in TBA series, embedded in paraffin and microtomed at 3 μm thickness.

3. The slides were deparaffinized and mounted with DPX.
**Colour indications:** The sites of AA appear dark brown or black in the form of fine silver grains.

**Control test for Ascorbic acid:** Prior to the usual procedure the materials were treated with 10% formalin* for 4 hours.

**Results:** Known sites of AA did not show any localization.

**FRESH MATERIAL STUDIES**

Fresh material studies with respect to localization of enzyme activities were conducted with either unfixed frozen sections or free-hand sections wherever satisfactory frozen sections could not be obtained.

**FROZEN SECTIONS**

Although, standard paraffin processing procedures are capable of producing excellent sections, suitable for microscopic histochemical studies, majority of them do have drawbacks of affecting the histochemical localization of enzyme activity because routine paraffin processing at about

* Treatment with formalin devitaminizes the tissue.
58°C brings about denaturation of most of the enzymes within the tissues, and thus cause complete or incomplete loss of demonstrable enzymes. For this reason, it is imperative to obtain sections of plant materials without processing through routine histological paraffin procedures.

**OBTAINING FROZEN SECTIONS**

Frozen material sections could be obtained by employing any one of the following prescribed techniques.


2. Cryostat method.


Of these, the third method was employed because of the following advantages:

1. Convenience because the need for using carbon dioxide is eliminated.

2. Accurate temperature control.

3. Optimum cutting temperature could be maintained indefinitely.

4. Even serial sections could be cut at 5 μm thickness.
PROCEDURE OF OBTAINING FROZEN SECTIONS
ON A MICROTOME WITH THERMO-MODULES ATTACHMENT

The thermo-module method involves the following steps:

1. Attachment of the thermo-modules to the microtome.

2. Preparation of 15 per cent antifreeze gelatin embedding medium.

3. Embedding anthers in antifreeze gelatin medium.

4. Fixing the gelatin block to the main thermo-module.

5. Cutting the frozen sections.

6. Picking up the frozen sections.

1. Attachment of the thermo-modules to the microtome: The main thermo-module was fixed to the block-holder of the standard Erma microtome and the remaining four accessory modules were fixed on either side of the blade in pairs.

2. Preparation of 15 per cent gelatin antifreeze embedding medium (Knox, 1970): 15 g of gelatin was dissolved in 100 ml of 0.8 per cent Dimethyl Sulfoxide (DMSO) in distilled water and solidified by keeping it inside a refrigerator at 10°C for 1 or 2 hours before use.
3. Embedding anthers in 15 per cent gelatin antifreeze embedding medium: The solidified gelatin was taken out of the refrigerator and allowed to become semi-solid at room temperature for 15-20 minutes. In this semi-solid medium, anthers ranging from 2 mm to 5 mm length were vertically embedded in a row. Again the container was kept inside the refrigerator for solidification for about 30 minutes.

4. Cutting the frozen sections: The container was removed from the refrigerator and cubic blocks of material, embedded, in gelatin, were cut. A single cubic block containing anthers was fixed to the main thermo-module. The block and the blade were cooled to about -30°C for 10-15 minutes and then 15 µm thick sections were cut.

5. Picking up the frozen sections: The frozen sections were either taken directly on the slide by holding the latter nearer to the blade edge or by picking the sections with a camel brush and placing them on a slide flooded with distilled water. The excess distilled water was drained off carefully before subjecting the sections to histo-enzymological tests.

STAINING THE FRESH-SECTIONS FOR ENZYME ACTIVITY

Although various procedures are available for the
demonstration of the activity of many enzymes in tissue sections, in the present investigation, only staining procedures for localization of enzyme activities of glucose-6-phosphate dehydrogenase (G6PDH), succinate dehydrogenase (SDH), cytochrome oxidase, peroxidase and non-specific esterase were adopted, and the sources of these are listed in table III.

**GLUCOSE-6-PHOSPHATE DEHYDROGENASE**

(D. glucose-6-phosphate: NADPH oxidoreductase, 1.1.1.49, G6PDH).

It is a widespread soluble plant and animal enzyme, which catalyzes the following biochemical reaction.

\[
\text{Glucose-6-phosphate} + H_2O + NADP^+ \rightarrow \text{glucose-6-lactone-6-phosphate} + \\
\text{NADPH} + H^+
\]

It is one of the important enzymes of the hexose monophosphate shunt (pentose shunt) and plays significant role in the regulation of sugar metabolism. It depends on this enzyme whether glucose shall undergo glycolysis or be utilized via the pentose shunt. The pentose shunt has the following physiological significances:
Table - III:

Showing the methods employed for the localization of enzyme activities.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Enzyme Activity Localized</th>
<th>Method Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>G-6-Phosphate dehydrogenase</td>
<td>Pearse (1972)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bancroft (1975)</td>
</tr>
<tr>
<td>2.</td>
<td>Succinate dehydrogenase</td>
<td>Pearse (1972)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bancroft (1975)</td>
</tr>
<tr>
<td>3.</td>
<td>Cytochrome oxidase</td>
<td>Burstone (1959)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bancroft (1975)</td>
</tr>
<tr>
<td>4.</td>
<td>Peroxidase</td>
<td>Isaac and Winch (1947)</td>
</tr>
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<td></td>
<td></td>
<td>Jensen (1962)</td>
</tr>
<tr>
<td>5.</td>
<td>Non-specific esterases</td>
<td>Gomori (1950)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bancroft (1975)</td>
</tr>
</tbody>
</table>
1. Pentoses formed can be utilized for the synthesis of ribonucleic acids (Lojda et al., 1979).

2. By the direct oxidation of glucose, NADPH is produced which is utilized in the biosynthesis of many important metabolites such as fatty acids, lipids etc. (Lojda et al., 1979).

3. The pentose phosphate pathway is also capable of generating erythrose, a tetrose that is used in the biosynthesis of complex phenolic compounds (cited in Noggle and Fritz, 1977).

METHOD FOR LOCALIZATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY (PEARSE, 1972 in BANCROFT, 1975):

Reagents required:

1. Nitro-blue tetrazolium (NBT)
2. Tris buffer, pH 7.4
3. Glucose-6-phosphate (Disodium salt)
4. Nicotinamide adenine dinucleotide phosphate (NADP)
5. 0.05 M Magnesium chloride.

Preparation of stock solutions:

1. Stock Glucose-6-phosphate substrate solution: 304 mg of G-6-phosphate was dissolved in 0.8 ml
of distilled water to which 0.06 ml of 1 N HCl was added and the pH was adjusted to 7.1. The solution was kept frozen until use.

2. Stock NBT solution: 10 mg of NBT (4 mg/ml) was dissolved in 2.5 ml of distilled water to which 2.5 ml of 0.2 M tris buffer pH 7.4 was added. To this, 1.0 ml of 0.05 M Magnesium chloride was added followed by addition of 3 ml distilled water. The pH was adjusted to 7.0 using stock buffer and kept frozen until use.

Preparation of incubating medium: This was prepared fresh by mixing 0.9 ml of stock NBT solution and 0.1 ml of Glucose-6-phosphate stock substrate solution. To this 2 mg of NADP was added and dissolved by shaking.

Incubating procedure:

1. Freshly cut frozen sections or free-hand sections were covered with one or two drops of incubating medium and incubated for 30 minutes at room temperature, covered with a coverslip and photographed immediately.

Results: Sites of G6PDH activity exhibited purple formazan deposits.

Control test: Control tests were conducted either with heat
inactivated sections incubated in the normal incubating medium, or fresh sections incubated in incubating medium without glucose-6-phosphate.

**Results:** In both the cases, usual sites of G6PDH activity could not exhibit purple formazan deposits.

**Succinate Dehydrogenase**

(Succinate acceptor oxido-reductase, 1,3,99,1, SDH)

Succinate dehydrogenase is another important dehydrogenase. Unlike G6PDH it is structurally bound and is associated with mitochondria and catalyzes the following biochemical reaction of the Kreb's cycle.

\[
\text{Succinate} + \text{acceptor} \rightarrow \text{fumarate} + \text{acceptor}
\]

This is one of the most vulnerable enzyme and belongs to the so called succinate oxidase system, also called respiratory chain, and does not need NADPH unlike G6PDH. It is flavoprotein enzyme; the flavin group is bound to the protein by main valencies. Each flavin group has four iron atoms. The enzyme possess SH groups on which the enzyme activity depends. Histochemical localization of SDH activity gives valuable information about the Kreb's cycle i.e., Tricarboxylic acid cycle, also known as citric acid cycle.
METHOD FOR LOCALIZATION OF SUCCINATE DEHYDROGENASE ACTIVITY (PEARSE, 1972 in Bancroft, 1975):

Reagents required:

1. Nitro-blue tetrazolium (NBT)
2. Tris-buffer, pH 7.4
3. Sodium succinate
4. 0.05 M magnesium chloride.

Preparation of stock solutions:

1. Stock succinate substrate solution: This was prepared by dissolving 6.75 g of sodium succinate in 8 ml of distilled water to which 0.05 ml of 1 N hydrochloric acid was added. The pH was adjusted to 7.1 with 1 N hydrochloric acid. The solution was kept frozen until use.

2. Stock NBT solution: (As described for G6PDH)

Preparation of incubating medium: To 0.9 ml of stock NBT solution, 0.1 ml of stock succinate substrate solution was added.

Incubating procedure: Freshly cut frozen sections or hand-cut
sections were covered with 1 or 2 drops of incubating medium and incubated for 30 minutes at room temperature. Then covered with a cover slip and photographed immediately.

**Results:** Sites of SDH activity exhibited purple formazan deposits as in case of G6PDH.

**Control test:** Control tests were conducted either with heat inactivated sections incubated in normal incubating medium, or in incubating medium without sodium succinate i.e., substrate.

**Results:** In both the cases usual sites of SDH activity could not exhibit purple formazan deposits.

**CYTOCHROME OXIDASE**

(Cytochrome c oxidase; ferrocytochrome c; oxygen oxidoreductase; 1.9.3.1)

This enzyme has been demonstrated for many years and is considered to be the characteristic of the mitochondrial membrane to which it is relatively firmly bound. It is highly active in cells containing large number of mitochondria. Thus, high activity of this enzyme can be taken as a parameter to assess the degree of oxidative metabolism (Lojda et al., 1979). Cytochrome oxidase is responsible for termination of respiratory chain. This enzyme actually
catalyzes the transfer of electrons from substrate to molecular oxygen, yielding water and hydrogen peroxide as the end products.

Presently, a number of procedures are available for the demonstration of cytochrome oxidase of which, the metal chelation method of Burstone (1959, in Bancroft, 1975) is important because it has an advantage of producing intense colour reaction and is considered as more specific than the original Nadi-reaction. The enzyme oxidizes the Nadi-reagent, a mixture of α-naphthol and N-phenyl-p-phenylenediamine. The reaction product is then chelated with cobalt ions.

**METAL CHELATION METHOD FOR LOCALIZATION OF CYTOCHROME OXIDASE ACTIVITY**

(Burstone, 1959 in Bancroft, 1975)

Reagents required:

1. α-naphthol
2. N-phenyl-p-phenylenediamine
3. Absolute alcohol
4. Distilled water
5. 0.2 M tris buffer of pH 7.4
6. Cobalt acetate.
Preparation of incubating medium:

Solution 1: 10 mg of α-naphthol and 10 mg of N-phenyl-
p-phenylenediamine were dissolved in 0.5 ml of absolute alcohol and then 35 ml of distilled water was added. To this, 15 ml of 0.2 M Tris buffer of pH 7.4 was also added.

Solution 2: 500 mg of cobalt acetate was dissolved in 5 ml of formaldehyde and 45 ml of distilled water.

Incubating procedure:

1. Frozen or free-hand cut sections were incubated in solution 1 for 15 minutes at room temperature and the incubating solution was carefully drained off.

2. Then sections were treated with 1 per cent cobalt acetate (solution 2) for one hour, washed carefully with distilled water and mounted in dilute glycerine and photographed immediately.

Result: Sites of cytochrome oxidase activity exhibited blue-black colouration.

Control test: Heat inactivated sections did not exhibit blue-black colour with above mentioned procedure.
PEROXIDASE

(Donor: hydrogen peroxide oxidoreductase, 1.11.1.7)

The enzyme catalyzes the following reaction:

\[
\text{Donor} + H_2O_2 \rightarrow \text{oxidized donor} + H_2O.
\]

or

\[
AH_2 + H_2O_2 \rightarrow A + 2H_2O
\]

Peroxidases are haemoproteins that catalyze the oxidation of various substances by means of hydrogen peroxide. And they are thought to function in the oxidation of IAA (Siegel and Galston, 1955; Stonier et al., 1979), hydrogen peroxide decomposition (see Zaffren and Hall, 1973), ethylene biosynthesis (Mapson and Wardale, 1972), hydroxylation of proline in the wall (Ridge and Osborne, 1970), lignification (Hepler et al., 1972; Harkin and Obst, 1973; Fielding and Hall, 1978) and ion translocation (DeJong, 1966).

The most popular method to demonstrate peroxidase activity is benzidine method, in which benzidine is oxidized to a blue-brown colour. Although benzidine is now recognized as a carcinogenic substance, in the present investigation benzidine itself was used with utmost care because, 1,3-diamino benzidine could not be obtained.
BENZIDINE METHOD FOR THE LOCALIZATION OF
PEROXIDASE ACTIVITY:

Reagents required:

1. Benzidine
2. Hydrogen peroxide
3. 0.1 M phosphate buffer of pH 7.0
4. Ammonium chloride, and
5. Distilled water.

Preparation of incubating medium:

5 ml of saturated benzidine, 5 ml of 1% hydrogen peroxide and 1 ml of 5% ammonium chloride were mixed and used immediately.

Incubation procedure: Fresh sections were incubated in the incubation medium for 5 minutes and photographed immediately.

Result: Sites of peroxidase activity exhibited blue or brown colour.

Control test: Air dried and heat-inactivated sections could not show any enzyme activity at the known sites.
NON-SPECIFIC ESTERASES

Esterases are capable of hydrolyzing esters. If the substrate is a simple ester such as αC-naphthyl acetate, the hydrolyzing enzyme is called a non-specific esterase.

NAPHTHYL ACETATE SUBSTRATE METHOD (Gomori, 1950 in Bancroft, 1975)

Reagents required:

1. αC-naphthyl acetate
2. Acetone
3. 0.2 M phosphate buffer of pH 7.4
4. Diazonium salt East blue B

Preparation of incubating medium:

5 mg of αC-naphthyl acetate was dissolved in 0.1 ml of acetone. To this 10 ml of 0.2 M phosphate buffer was added and thoroughly mixed. Further, to this, 30 mg of Fast Blue B was added. The solution was filtered and used immediately.

Incubation method: Sections were incubated for 30 seconds in the incubating medium, washed carefully in the tap water
for 5 minutes and mounted in glycerine jelly.

**Result:** Sites of enzyme activity exhibited reddish-brown colour.

**Control:** Heat inactivated sections could not exhibit any colouration at the known sites.
REFERENCES


