CHAPTER I

GENERAL METHODOLOGY
A. INSTRUMENTATION:

I. THE OPTICAL MICROSCOPES:

Three different types of optical microscopic systems have been used for the investigations presented in the dissertation. These are: (i) Bright-field microscopy, (ii) Fluorescence microscopy and (iii) Polarizing microscopy. The salient features of the instruments used are as follows:

1. The Bright-field Microscope:

The instrument which was mainly used for this purpose is a Reichert 'Biozet' trinocular microscope equipped with 10X & 40X plane-field achromatic objectives and compensating type of paired oculars for the observation path. The 100X oil-immersion objective is a semi-apochromat ('fluorite') with a built-in iris diaphragm giving a maximum numerical aperture of 1.30. A Carl Zeiss C 12.5X compensating projective was mostly used for the photographic recordings. The aplanatic substage condenser of N.A. 1.4 is centrable to give Koehler's illumination with a 6 Volt 5 Amp compact filament tungsten lamp provided with a variable transformer.
2. **The Fluorescence Microscopes** :

(a) **The Basic Instruments** :

For fluorescence observations and photomicrography, two different microscopes have been used in this work, these are:

(i) A Carl Zeiss Jena 'Fluoval' trinocular microscope with arrangements for work in both the transmitted light and incident light (i.e., epi-illumination) paths — The apochromatic objectives of the microscope as well as the achromatic-aplanatic condenser can be interchanged for observations under the phase optics or in conjunction with a cardioid dark-ground condenser. The exciter and barrier filters (as specified below) are incorporated in a box-type filter magazine and a rotatable turret-type disc respectively.

(ii) An Olympus 'EC-Tr' trinocular microscope — This was converted into quite an efficient transmitted light fluorescence microscope in conjunction with a Carl Zeiss (Oberkochen) fluorescence illuminator with several exciter filters. The barrier filters can be incorporated in the tube below the microscope head either singly or in fixed
combinations. The microscope has an 1.2 N.A. bright-field Abbe condenser and a set of achromatic objectives of the usual magnifying powers of 10X, 40X & 100X. The condenser and the oil-immersion 100X objective of this microscope were interchanged with a Carl Zeiss 1.2/1.4 N.A. immersion type cardioid dark-field condenser and an 100X oil immersion objective of the same make with built-in iris diaphragm (having a maximum N.A. of 1.25) whenever required. The trinocular head (as in the Fluoval microscope) is capable of attaching a photomicrographic camera permanently without disturbing the binocular observation set-up.

(b) The Illuminators:

The illuminator of the Fluoval microscope consists of a 200 Watt high pressure mercury vapour arc lamp, HBO 202, which can be conveniently centered with the microscopic axis. As stated earlier, the illuminating beam from this UV-rich source can be channelized either through the substage condenser for trans-illumination or through the objective under use (acting in lieu of the regular condenser) for epi-illumination by means of a slidable deflecting element.
and a partially reflecting mirror set at 45° angle. This built-in illuminator has also arrangements for simultaneous mixing of white light from a tungsten filament lamp via the substage path through a bright-field or a phase-contrast condenser alternatively.

The illuminator which was used with the Olympus microscope is a Carl Zeiss "Model II Fluorescence Illuminator". This is rigidly mounted on a large circular base plate over which the microscope can be mounted and centered. The light source in this illuminator is an Osram HBO 200 W/4 high pressure mercury vapour arc lamp.

Both the UV-light sources, viz., HBO 202 of Carl Zeiss Jena and HBO 200 W/4 of Carl Zeiss (Oberkochen) have more or less the same luminance of about 33,000 Stilbs and a luminous flux of 9,500 lumens approximately. The spectral energy distributions of these super high pressure mercury vapour lamps are characterized with prominent maxima at 365.02/365.48 nm and 366.29/366.33 nm; 404.70/404.80 nm; 434.80/435.30 nm; 546.10 nm and 577.00/579.10 nm.

(c) **The Exciter Filters** :

The Carl Zeiss (Oberkochen) fluorescence
illuminator has provisions for the following exciter filters:

(1) **KG 1/2 mm**: Heat-absorbing filter — a sharp-cutting filter passing wave bands between 300 nm to 800 nm approximately.

(2) **BG 38/2.5 mm**: Red-absorbing blue filter — with a sharp cut at about 300 nm and a gradual slope beyond 600 nm.

The filters (1) and (2) were kept permanently in the light path in order to protect the other filters from high temperature and to cut off the unnecessary and disturbing 'leakage' of red wavelengths, particularly with the exciter filters BG 3 and UG 5.

(3) **BG 12/3 mm & 4 mm**: Blue-violet transmitting exciter filters — these have peak transmission at about 400 nm (extending between 330 to 500 nm, so that the mercury spectral bands at 365, 404 and 435 nm approx. are fully covered).

(4) **BG 3/4 mm**: Ultraviolet/Blue-violet transmitting exciter filter — the transmission of this filter extends from about 270 to 400 nm, with a wide high peak between 320 and 400 nm. It is an efficient filter for
excitation around 365 nm which is passed almost without any attenuation. It is also richer in UV excitation than the BG 12 filters.

(5) **UG 5/3 mm**: Ultraviolet transmitting exciter filter (broad-band) — this filter has a pass-band from about 220 to 420 nm, with a wide high peak between 300 to 370 nm, the mercury lines at 365 being transmitted freely.

(6) **UG 1/3 mm**: Ultraviolet transmitting exciter filter (narrow-band) this filter has pass-band from about 300 to 400 nm; and as a result, the 365 nm group of mercury UV lines are transmitted almost exclusively.

The names of the filters KG 1, BG 38, BG 12 etc. designate the coloured glass types according to Schott's Catalogue, while 2 mm, 2.5 mm, 3 & 4 mm etc. designate the thickness of the glass for a particular filter.

The glass types and the spectral transmissions of the Carl Zeiss Jena exciter filters for the 'Fluoval' microscope are more or less similar. The filters B 223 and the B 224 are BG 12 glasses of thickness 2 and 4 mm; while U 204 and U 205 are UG 1 glasses of thickness 2 and 4 mm. Thus, they serve the same functions as those of the types (3) and (6) respectively as given above.
(d) **The Barrier Filters**

The characteristic features of these filters are a sharp cut towards the shorter wavelengths but an unimpeded (actually about 90 per cent) passage of light thereafter, towards the longer wavelengths. The barrier filters supplied by Carl Zeiss (Oberkochen) and used with the Olympus microscope are designated by the numbers 41, 44, 47, 50 and 53. The wavelength region in which the transmission of a particular filter is about 10 per cent has been chosen as the index to name the filter. Thus, for example, the barrier filter No. 50 transmit 10 per cent of the incident radiation at about 500 nm, while No. 41 does so at about 410 nm and no exciting or fluorescent light shorter than this wavelength is allowed to pass through thereafter.

The barrier filters 50 and 53 were found to have intrinsic fluorescence due to UV radiation causing disturbing flares. However, when the filters 44 and 47, which cut out UV, were put in front of them, the flares were eliminated.

The barrier filter combination of No. 47 and No. 50, when used together in the microscope tube, was found to be best suited for most of the observations and photomicrographic work carried out in these studies,
particularly with the fluorochrome Acridine Orange.

The revolving barrier filter turret of the Fluoval microscope has the filters numbered as (i) G 242, (ii) G 243 (iii) G 245, (iv) G 247 and (v) G 249. Of these, the last two, which are themselves combinations as (OG 1/1 mm + GG 9/1 mm) and (OG 4/1 mm + GG 9/1 mm) were found to be best suited for the studies reported here.

(e) **Exciter & Barrier Filter Combinations** :

The best combination of filters for individual cases were worked out on the basis of trial and error, keeping in view that the whole range of fluorescence emission had to be covered in order to make a correct interpretation. For example, if the barrier filters No. 53 or No. G 249 (orange) were used with the exciter filters No. BG 12/4 mm or No. B 224 (which are the same in their spectral transmission characteristics) in order to secure an absolutely dark background, rendering the desirable high contrast, the actual green colour of a specific component would appear orange or at the best yellow. Hence, the selection of a barrier filter of a lighter colour, i.e., transmitting more towards the shorter wavelength, e.g., No. 50 or No. 47 (or No. G 247 instead of No. G 249) was found necessary for true rendering of
the green colour. The lightening of the background was then corrected either by increasing the thickness of the BG 12 glass to 6 mm or 8 mm instead of 4 mm, or by using the 'darker' UV transmitting filter UG 1/3 mm singly with the blue-absorbing BG 38/2.5 mm, or in addition to the BG 12/4 mm. If the background did not turn sufficiently dark in spite of the use of such filter combinations, dark-ground illumination had to be taken recourse to, even though that meant a comparatively low fluorescence yield calling for a longer time of photomicrographic exposure.

3. The Polarizing Microscope:

For the purpose of study of birefringence phenomena of the starch granules in pollen grains, no elaborate polarizing microscopic equipment with strain-free optics was needed. Converting the Reichert 'Biozet' bright-field microscope into a simple polarizing set-up by the addition of two polarization filters and a quartz compensator was found to adequately serve the purpose.

The filter polarizer and analyzer used for these studies are manufactured by Carl Zeiss (Oberkochen). The first filter (polarizer) was placed on the exit pupil of the built-in illuminator of the microscope, while the second filter (analyzer) was placed over the projective
in the vertical tube of the trinocular head meant for photomicrography. Binocular observation was thus excluded, but monocular observation through the focusing telescope of the attachment camera was quite satisfactory for necessary adjustments and photomicrography. In order to obtain the 'crossed-filter' position, the analyzer was not disturbed but the more easily accessible polarizer was rotated as and when necessary.

The compensator, manufactured by the same firm as that of the filter polarizers, as used for these studies is a red 1st order quartz retardation plate. This filter-type plate is mounted in a metallic disc with a handle and could be very conveniently placed in the substage filter-carrier and rotated as required.

**II. THE SCANNING ELECTRON MICROSCOPE**

A Hitachi Model S-530 electron microscope (SEM) was used in course of these studies. The instrument is maintained by the 'Central Instrumentation Facility' of the University of Burdwan.

The relevant specifications of this SEM are:

Resolution — 60 Å; Acceleration voltage — 1 to 25 KV; Magnification — 20 to 150,000 X.

Gold coating of the specimens were done by sputtering with an IB-2 Ion Coater, manufactured by E.I.K.O. Engineering, Japan.
The electron micrographs were taken on OROW NP-22 (ISO 125/22°) black & white film by a 120 format Mamiya 300 camera attached to the SEM.

**B. PHOTOMICROGRAPHY**

The following photomicrographic equipments and materials were used in course of these studies:

1. **THE CAMERAS**:

   Three different cameras were used for taking the bright-field and fluorescence photomicrographs in colour or black & white. These are:

   (a) An Olympus PM-6 35 mm format attachment camera — This camera could be used interchangeably with either the Biozet bright-field or the Olympus fluorescence microscopes. It has a beam-splitting prism making available 80 per cent light for photography while the rest 20 per cent goes to a side tube for the purpose of observation and focusing. The image on the film plane is reduced in this equipment by a factor of X 0.33.

   (b) A Carl Zeiss (Oberkochen) attachment camera — This has a large fixed beam-splitting prism, the Basic Body I, which also, like the Olympus camera, permits 80 per cent light from the microscope to the film plane. It has
interchangeable backs for either the standard 35 mm film cassette or the 120 size roll films. The magnification achieved on the 35 mm film (24 x 36 mm) is reduced by a factor of X 0.4, while that on the 120 roll film (16 exposures — 40 x 56 mm) is 0.5 times in this camera.

The larger field of the focusing telescope and the availability of long time ('T') exposure facility rendered this camera advantageous over the other two.

(c) A 35 mm 'mf' camera attachment — This is fitted to the basic body 'mf' of the Carl Zeiss Jena Fluoval microscope. This camera cannot normally be interchanged and was invariably used with this fluorescence microscope. It is provided with several specially designed projection eyepieces, of which K 3.2:1 and K 4:1 were mostly used.

2. **PHOTOGRAPHIC FILMS & PAPERS**:

(a) **Black & White Negative Films**:

The 35 mm and 120 size roll films which were used in these studies were manufactured by OROW and were of two speed ratings, viz., NP-22 (ISO 125/22°) and NP-27 (ISO 400/27°). Although the faster films mean shorter exposures, which is particularly useful for fluorescence photomicrography that usually requires much longer
exposure times than in bright-field work, they generally produce larger grains and thus restrict enlargement to a maximum of about three times. For this reason, generally NP-22 films were used for bright-field work and NP-27 films were preferred for fluorescence work.

(b) **Colour Negative Films** :

Only the 35 mm colour films were used. These were manufactured either by Kodak, viz., Kodak Gold 100 (ISO 100/21°) and Kodacolor VR 200 (ISO 200/24°) or by Fuji, viz., Fujicolor HR 100 (ISO 100/21°) and Fujicolor Super HR 200 (ISO 200/24°). Of these, Kodak Gold 100 was found to give the best colour rendition, but the others were also quite satisfactory. No graininess in the prints, even of the negatives from the faster films, after enlargements of about 3.5 times were normally encountered.

(c) **Bromide Paper** :

Agfa 'Brovira' papers of the Normal and Hard grades, single weight and glossy types, were used for enlargement of the black & white photomicrographs. They were found to be quite satisfactory when properly processed.

(d) **Colour Printing Paper** :

Colour enlargements of fluorescence and other
photomicrographs were made on Fuji and Sakura papers. The former brand was found to give better colour rendering.

3. **PROCESSING**:

Processing of the colour negative films and the enlargements from them were done by colour processing laboratories.

The processing of black & white materials as well as enlargements, however, were done personally in the dark room of this department. The Kodak film developer Formula D-19 was used for high contrast, whereas D-76 was found more suitable where finer grains and higher resolutions were called for. For developing the bromide papers either Kodak Formula D-72 or Agfa Formula 108 were used.
GENERAL REFERENCES


Carl Zeiss, Brochure No. 41-300-e. (1968) 'Microscope Illuminators, Lamps and Lamp Sockets'; Carl Zeiss, Oberkochen, West Germany.


