General Techniques

Sahli’s Acid Haematin method (Seiverd, 1966):

Haemoglobin is converted to acid haematin by the action of hydrochloric acid (HCl). It was done as follows:

1. The graduated tube was filled up to 20 mark with N/10 HCl.
2. Blood (0.02 ml.) was added to the tube, mixed thoroughly and was kept for 5 to 10 minutes.
3. Distilled water was added drop by drop, mixing between each addition, until the color matched the standard.
4. The amount of the solution in the graduated tube was observed and result were expressed in gram percentage.

Papanicolaou’s Stain (Papanicolaou, 1942):

Preparation of Vaginal Smear:

Vaginal smear was made by aspiration of the posterior vaginal fornix with a stout walled, slightly curved glass pipette fitted with a rubber bulb. The aspirate was spread rapidly on a clean glass slide. The slides were placed without any delay into a jar of
fixative before drying occurs. After this staining was performed.

Preparation of Stain:

It consists of, (1) Harri’s alum Haematoxylene;
(2) Orange Green-6; (3) Eosin Auzurin 36 or 50.

1. Harri’s Alum Haematoxylin:

Haematoxylin (1.0 gram) was dissolved in 10 ml of Ethyal alcohol (95% C2H5OH); 20 grams of Aluminium Ammonium Sulphate (ammonium alum) in 200 ml. of distilled water; The two solutions were mixed together by heating. To this solution 0.5 gram of mercuric oxide solution was added, that assumed a dark purple color. The solution was cooled by keeping the vessel into the cold water. The solution was thus ready for the use. Addition of 4% glacial acetic acid increased the precision of nuclear staining.

2. Orange Green-6:

Orange green (0.5%) was prepared in 95% alcohol. To this 0.015 gram of Dodeca-Tungstophosphoric acid was added. After filtration it was used.

3. Eosin Auzurin 36:

(a.) Prepared 45 ml. of Light green (0.5%) stain in 95% alcohol.

(b.) Prepared 10 ml. Bismark brown (0.5%) in 95% alcohol.
(c.) Prepared 45 ml. Eosin yellow (0.5%) in 95% alcohol.

All the three solutions were mixed and 0.2 gram of Dodeca-Tungsto Phosphoric acid was added to the solution. One drop of saturated aqueous lithium carbonate was also added to the solution. All the ingredient were mixed thoroughly, filtered and were stored in brown bottles.

Procedure:
1. Smear was removed from fixative and was rinsed in descending grades of alcohol i.e. 80%, 70%, 50%, for 8-10 seconds each.
2. The slides were then stained with Harris’s Haematoxylin for 4 minutes.
3. The slides were washed in tap water for 1-2 minutes.
4. The slides were again differentiated in 0.5% HCl until nuclei were stained.
5. Again slides were washed in tap water for 6-10 minutes.
6. The slides were transferred to 70% alcohol followed by two changes in 90% alcohol for few seconds each.
7. Stained in Orange Green-6 for 2 minutes.
8. Rinsed in three changes of 95% alcohol.
10. Rinsed in three changes of 95% alcohol.
11. Completed dehydration in absolute alcohol and cleared in Xylene.
12. Mounted the slides in DPX and observed under the microscope (100x).

**Protein Estimation:**

Protein was estimated by the modified lowry's method of Less and Paxman (1972). The reagents employed were:

A. Freshly prepared 5% Sodium Duedocyl Sulphate (SDS) in 0.05 N Sodium Hydroxide (NaOH).
B. Copper tartrate solution prepared by mixing 50 ml. each of 2 percent Sodium Tartrate and 1 percent Copper Sulphate (CuSO4.5H2O).
C. Folin Phenol reagent (IN).
D. 2% Sodium Carbonate (Na2Co3).
E. Standard bovine Serum albumin (BSA) (1mg/ml.).

The procedure was carried out as follows:

1. To 50 microlitre of the sample or standard containing (10-100 microgram protein), 0.5 ml. of solution A was added and kept at room temperature for 3 hours, vortexing several time to dissolve the samples.
2. 2.5 ml. Copper Carbonate solution (prepared by mixing 50 ml. of 2% Na2Co3 and 1 ml. of Copper Tartrate solution.) was added to each sample, vortexed and incubated for 15-20 minutes.
3. 250 microlitre Folin reagent was added and mixed immediately.
4. Readings were taken at 750 nm. after 45 minutes.

Leucocyte Migration Inhibition Test (Jain et al. 1980):
Erythrocytes were removed by incubating spleen cell suspension with dextran solution (6%) for 30 minutes at 37°C.

Capillary tube migration:
Blue tipped plain capillary tubes of 1.10 mm internal diameter and 75.0 mm length were used for packing the cells. The Leucocyte suspension was filled in the capillary tubes and one end sealed with plasticine. The capillary tubes were then centrifuged at 500 rpm. for 2 minutes. They were cut at the cell fluid interface and the cell containing portion fixed in the culture chambers with the help of tetracyclin ointment. MEM (with antigen or without antigen) was introduced into the chambers by means of a syringe. Each chamber was then sealed with a cover slip by applying vacuum grease. The chambers were placed in a petridish and incubated at 37°C for 18 hours.

Expression of Results:
The migrations were observed after projection
and the whole image of the migration field was traced on a butter paper. These migration areas from the papers were cut and weighed in an electric balance. The percent migration inhibition was calculated by the formula:

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\text{Percent Migration Inhibition} = \frac{\text{Mean of migration in presence of test antigen}}{\text{Mean of migration in absence of antigen}} \times 100
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Counter Current Immunoelectrophoresis (CIEP):

CIEP was carried out in 0.75 percent agarose gels on microscopic (75 x 80 nm) slides. The slides charged with antigen and antiserum were electrophoresed at 5mA/slide for 2 hours. After completion of electrophoretic run, the slides were dried at 37 degree centigrade overnight and stained with amido black 10B solution (1% in 10% acetic acid) for 15 minutes. Destaining was done with 7.5% acetic acid solution until the background became clear.

Enzyme Linked ImmunoSorbent Assay (ELISA):

Antibodies to malarial antigen of \( P. berghei \) were evaluated by indirect ELISA method of Voller et. al. (1976). For micro-ELISA, the antigen (0.75 microgram/ml.) diluted in Carbonate-Bicarbonate buffer (pH-9.6) were added to 96 welled microtitre plates (Cooke Microtitre M29 AR). After overnight incubation at 4°C, the plates were washed three times in Phosphate buffered saline.
pH-7.2 (PBS) containing 0.05% Tween-20 (PBS-Tween). Serial two fold dilutions of serum were made in the antigen coated plates using PBS-Tween supplemented with 0.5 % bovine serum albumin (Sigma, USA). The starting serum dilution was 1/20 and the final volume of diluted serum in each well was 0.1 ml. or 100 microlitre. After incubation at 37°C for 2 hours in a humidified atmosphere the plates were washed three times with PBS-Tween, and a 0.1 ml. of 1 in 3000 diluted horse radish peroxidase conjugated antimouse immunoglobulin (Sigma, USA) was added to each well. After 1 hour incubation at 37°C in a humidified atmosphere, the plates were again washed three times with PBS-Tween and 0.1 ml. of substrate solution was added. The substrate solution containing 0.05% (W/V) O-Phenylenediamine (Sigma) in 0.1 M citrate buffer and 0.01% hydrogen peroxide was prepared immediately before use. After 30 minutes incubation in the dark at room temperature, the reaction was terminated by adding 25 microliter of 4N Sulphuric Acid (H2So4). The colour intensity indicative of the antibody concentration in the serum was determined by ELISA reader (Dynatech) at 492 nm and Log2 values were calculated.