CHAPTER II

EXPERIMENTAL

This chapter has two sections.

I. Collection of samples

II. Laboratory methods

I. Collection of samples

a) Blood

Most of the blood samples for the study were collected from Trivandrum, a coastal city in Kerala state. Screening was carried out in endemic areas in Trivandrum. The National Filariasis Control Programme Units (NFCP) in Kerala helped in the selection and supply of cases.

By pricking one of the fingers, about 20 cmm of blood was collected at any time between 8.30 p.m. and 10.30 p.m. and thick blood smears were made. The smears were then dried and taken to the laboratory. These smears were stained with Giemsa stain and examined under a microscope for the detection of mf. Mf positive slides were labelled for follow-up studies. Mf positive blood from other districts of Kerala such as Quilon, Allepey, Ernakulam, Trichur, Calicut, Cannanore and Kasargod were collected with the help of the respective NFCP units. Control samples of blood from normal
individuals were also collected.

About 5 ml of heparinised blood was collected from mf positive and control cases for identification of blood groups, separation of serum and separation of mf (To each 10 ml of blood, 0.1 ml of 1% solution of heparin was added). Serum was separated by keeping unheparinised blood for a few hours at room temperature for clotting of blood and separation of serum. The serum was then centrifuged and stored in a refrigerator for further studies.

Along with the collection of blood, a thin blood smear was also taken on a clean slide and this was used for the differential count of white blood corpuscles.

b) Parasite

Human Mf isolation

A number of techniques for isolating mf have been described in literature. The most recent method is the use of membrane filters as described by Bell and Chularerk and Desowitz.

The nucleopore filter technique was the only method used for the collection of mf. This was made possible by the kind gift of a nucleopore-filter set and filters by the Nucleopore Corporation, California and Dr. C.K. Rao of National Institute of Communicable Diseases (NICD), New Delhi.
The heparinised blood was pushed through the filter. The mf collected over the filter was washed using phosphate buffered saline (PBS) and the washings were discarded. The filter disc was then immediately dipped in PBS. The disc was removed, and shaken to get mf in the medium. This was then centrifuged and the supernatant was discarded. The sediment containing the mf was agitated and washed well in PBS. As there were chances of contamination with RBC, the residue was suspended in water. This facilitated lysis of the RBC. The solution was again centrifuged and washed in PBS. The isolated mf was stored in a freezer.

*S. digitata* - Whole worm and mf

Adult *S. digitata* were collected from the local slaughter house. The adult parasites were washed and incubated at 37°C in modified Tyrode solution, the composition being:

- Sodium chloride - 0.8%
- Potassium chloride - 0.02%
- Calcium chloride - 0.02%
- Magnesium chloride - 0.01%
- Sodium hydrogen carbonate - 0.05%
- Glucose - 0.5%

The solution was made up to 500 ml.
The mf released in a period of 3 hours were collected by centrifuging the Tyrode medium. The Tyrode medium was changed every hour. Glucose was added just before every hour of incubation. The mf collected by centrifuging the incubated Tyrode medium, were used for the various studies.

II. Laboratory methods

This section deals with the procedures employed for the analysis of the blood samples.

Preparation of thick blood smear

The finger was cleaned with spirit and a prick was made with a sharp needle. A large drop of blood (about 20 cmm) was evenly spread on a clean glass slide with the help of a needle or the edge of a glass slide to a circle of about $\frac{1}{2}$" diameter. It was then dried in the air.

Preparation of thin blood film

A small drop of blood was placed on a clean slide. Another slide was kept at an angle of 45° on the top of the blood and was drawn forward for spreading the film. The film was then dried in the air.
Preparation of stains

1. Giemsa's stain

This was used for detecting mf in the peripheral blood. The composition being,

- Giemsa's stain powder: 0.6 gm
- Methyl alcohol: 50 ml
- Glycerine: 50 ml

The stain powder was weighed and ground well with glycerine. It was then transferred to a flask and heated in a water bath at 55°C to 60°C for 2 hours or longer, shaking the mixture occasionally. It was then cooled to room temperature and then methyl alcohol was added. The methanolic solution was kept away from light for about two weeks, filtered into small bottles and again stored in the dark. The stock solution was diluted (1 to 10 with distilled water) before use.

2. Leishmann's stain

This was used for staining WBCs differentially. The composition being,

- Leishmann's stain powder: 0.15 gm
- Methyl alcohol: 100 ml

Stain powder was dissolved in methyl alcohol by heating on a water bath in a flask tightly plugged with cotton wool. It was allowed to cool and then filtered.
Before use, the stain was diluted with 2 volumes of distilled water.

**Staining procedure**

a) Mf: After dehaemoglobinization and fixation, the thick blood smears were immersed in diluted Giemsa's stain for 30 min. Excess stain was removed with the help of a small quantity of distilled water.\(^4\),\(^3\)\(^8\)

b) WBC: To the dried thin blood smear 2 drops of Leishmann's stain was added. After 5 minutes it was washed in distilled water.\(^4\) The slide was then dried in air.

**Microscopy**

Microscopic examination was carried out for the detection of mf and differential count of WBC.

a) Mf: Dark ground illumination microscopy was found more useful for detecting mf in fresh blood, especially when the whole slide had to be screened. Microscopic detection, identification and examination of blood parasites were done using the stained blood smear technique.

b) WBC: The differential count carried out refers to the relative number of the five different types of WBC (neutrophil, basophil, eosinophil, lymphocyte and monocyte). The percentage of individual types was determined by counting 100 white cells.
c) Blood grouping: Blood grouping was performed according to the method of Dacie and Lewis, using antisera purchased from Associated Labs, Private Ltd., India.

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE was carried out in a home made Hoeffer slab gel apparatus using the methods of Laemmli, Fairbanks et al. and Studier. The size of the gel was 15 cm x 12 cm x 0.075 cm. Electrophoretic mobility depends on both molecular charge and size, so that the resulting protein pattern is characteristic of the specimen. All reagents and gels were made using the Laemmli system. The same technique was used for the separation of isoenzymes with slight modifications.

Composition of separating gel solution for isoenzymes

- Acrylamide:Bis (30:0.8) - 4.95 ml
- 1.5 M Tris HCl (pH 8.8) - 3.75 ml
- Ammonium persulphate (1%) - 0.75 ml
- Distilled water - 5.55 ml
- TEMED - 5.25 μl

Total - 15.00 ml
Composition of stacking gel solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide:Bis (30:0.8)</td>
<td>1 ml</td>
</tr>
<tr>
<td>0.5 M Tris HCl (pH 6.8)</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Ammonium persulphate (1%)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>2.75 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5.5 ml</strong></td>
</tr>
</tbody>
</table>

The electrophoretic tank and glass plates were washed and rinsed with distilled water. The Teflon tubing was kept in position between the two plates separated by Teflon spacers and fixed with clips on to the electrophoretic tank.

The separating gel was poured into the space between the two plates avoiding any air bubble and leaving sufficient space for the stacking gel. Distilled water was layered on the top of the separating gel before gel formation to get an even surface.

After pouring separating gel for polymerisation, it was overlayed with water and kept overnight. Next day, the surface was washed thoroughly with distilled water and the stacking gel was poured. Perspex spacers (comb) were used for cutting the wells. The spacers were removed after one hour as also the Teflon tube.

The wells were washed with the electrode buffer by means of a syringe and a needle and the electrophoretic tank
was filled with the buffer in both the compartments.

The composition of electrode buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>3 gm</td>
</tr>
<tr>
<td>Glycine</td>
<td>14.4 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Samples were diluted with a diluting buffer in the ratio of 1:1.

The composition of sample diluting buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.5 M Tris HCl (pH 6.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Crystal violet (0.5 %)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

The electrophoretic tank fitted with the glass plates in position was kept inside the fridge and current applied for half an hour at 100 V for 30 minutes as pre-electrophoresis to stabilize the apparatus. Switched off the current and applied the samples in the wells by means of a microsyringe or micropipette. Switched on the current for half an hour at 50-75 V and then at 100-150 V for 3 to 4 hours.

On completion of electrophoresis, the current was switched off and gels were removed and stained in their respective substrate systems. After staining, the gels were kept in 7% acetic acid and stored in the refrigerator.
Staining mixture for different enzymes

1. Lactate dehydrogenase

Sodium lactate (1 to 10 dilution) - 5 ml
NAD - 15-20 mg
Phenazine methosulphate - 0.2 ml (2 mg/ml)
Nitro blue tetrazolium - 10-15 mg
Tris buffer pH 7.5 (0.5 M) - 10 ml
Water - 20 ml

2. Malate dehydrogenase

1 M Malic acid (1.5 gm in 10 ml neutralize with 1N NaOH to pH 7.5) - 5 ml
NAD - 15-20 mg
Phenazine methosulphate - 0.2 ml (2mg/ml)
Nitro blue tetrazolium - 10-15 mg
Tris buffer pH 7.5 (0.5 M) - 10 ml
Water - 20 ml

Alcohol dehydrogenase

Isopropyl alcohol - 1.5 ml
NAD - 15-20 mg
NBT - 10-15 mg
Phenazine methosulphate - 0.2 ml (2 mg/ml)
Phosphate buffer pH 7.0 - 10 ml
Water - 20 ml
4. Alkaline phosphatase

Fast blue salt RR - 12.5 mg
Sodium 1-naphthyl phosphate - 12.5 mg
Tris buffer pH 7.5 - 1 ml diluted to 25 ml with water

5. Esterase

Sodium 1-naphthyl phosphate - 12.5 mg
Fast violet B salt - 12.5 mg
Tris buffer pH 7.5 - 1 ml
Water - 24 ml

6. Glucose 6-phosphate dehydrogenase

Glucose 6-phosphate - 0.00056 M
Magnesium chloride - 0.005 M
NADP - 0.00013 M
Nitro blue tetrazolium - 0.00012 M
Phenazine methosulphate - 0.0013 M
Tris buffer pH 8.0 - 0.05 M

7. Isocitrate dehydrogenase

Isocitric acid trisodium salt - 0.0077 M
NADP - 0.00026 M
Manganese chloride - 0.001 M
Phenazine methosulphate - 0.00046 M
Nitro blue tetrazolium - 0.00021 M
Tris buffer pH 8.0 - 0.2 M
8. Glutamate dehydrogenase

Glutamic acid - 0.25 M  
NAD - 0.0015 M  
Phenazine methosulphate - 0.000163 M  
Nitro blue tetrazolium - 0.00043 M  
Sodium phosphate buffer (dibasic) pH 9.0 - 0.125 M

9. Glutamic-Oxaloacetic Transaminase

0.04 M DL-aspartic acid  
0.005 M α-ketoglutaric acid  
50 mg pyridoxal phosphate

in 100 ml of 0.034 M phosphate buffer pH 7.0. Just before use, 126 mg of Fast violet B salt is added.

Effect of heat on isoenzymes

The effect of temperature on isoenzyme pattern was studied by heating the serum samples at different temperatures.

The relative mobility of the isoenzymes of all samples with reference to a known indicator dye (crystal violet) was calculated as follows:

Relative Mobility, RM = \frac{\text{Distance moved by the band}}{\text{Distance moved by the marker dye}}
A typical experiment

(a) For this study was selected one sample each from mf positive, control and patient (mf negative) group. The mf positive had a high mf count and the patient had distinct clinical manifestation of chronic filariasis.

(b) 0.5 ml of serum was taken from each case, mixed with 0.5 ml of serum diluting buffer and kept in the refrigerator.

(c) An enzyme gel was made ready.

(d) The temperature of the water bath was set at 50°C. After completing the experiment, if there was no change in the isoenzyme pattern, the temperature was raised to 60°C. On the other hand, if the isoenzyme pattern was completely denatured the temperature was lowered to 40°C.

(e) A plastic packing strip was taken, holes were made on it and the serum samples kept in small tubes were put on those holes and heated in the water bath.

(f) Eighteen small tubes were taken. Pipetted out 0.1 ml of the diluted serum samples into each of the tubes as follows (The tubes should have the numbers marked on them). Tubes 1-6 mf positive; tubes 7-12 control and tubes 13-18 mf negative. All these tubes were kept in the refrigerator.
(g) After noting the time, tube numbers 1, 7 and 13 were inserted into the plastic base and kept in the water bath (to immerse the serum portion of the tube).

(h) After 15 minutes tubes 2, 8 and 14 were kept in the water bath.

(i) Ten minutes after step (h), tubes 3, 9 and 15 were kept in the water bath.

(j) Five minutes after step (i), tubes 4, 10 and 16 were kept in the water bath.

(k) Five minutes after step (j), tubes 5, 11 and 17 were kept in the water bath.

(l) After 5 minutes or after the elapse of 40 minutes the heating pad was taken out of the water bath and kept in ice cold water.

(m) After cooling, the tubes were taken from the water bath and kept along with tubes 6, 12 and 18.

(n) Immediately after this, electrophoresis was carried out with these samples.
Effect of pH on isoenzyme patterns

The effect of pH on the isoenzyme patterns of LDH, MDH, ADH and alkaline phosphatase were studied from mf positive, control and patient (mf negative) sera at different pH (pH 7, 8 and 9). The serum diluting buffer was prepared by using Tris buffer pH 6.8, 8 and 9. The diluted serum was incubated at 37°C for 1 hour. The remaining procedure is the same as that described above.
Protein estimation with the Folin-Ciocalteu Reagent

The method described below is that of Lowry et al. The final colour is the result of Biuret reaction of protein with copper ion in alkali and reduction of phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.

Reagents

1. Reagent A: 2% Na₂CO₃ in 0.1 N NaOH
2. Reagent B: 0.5% CuSO₄·5H₂O in 1% sodium potassium tartrate
3. Reagent C: Alkaline copper solution
   Mixed 50 ml of Reagent A with 1 ml of Reagent B.
   Discarded after 1 day.
4. 0.1 N NaOH
5. Folin-Ciocalteu reagent (Folin and Ciocalteu) Refluxed gently for 10 hours a mixture consisting of 100 gms of sodium tungstate (Na₂WO₄·2H₂O), 700 ml water, 50 ml of 85% Phosphoric acid and 100 ml of concentrated hydrochloric acid in a 1.5 litre flask. Boiled the mixture for 15 minutes without condenser to remove excess bromine. It was then cooled, diluted to 1 litre and filtered. The reagent should have no greenish tint.
6. Diluted Folin's reagent
   1 ml of Folin Ciocalteu reagent was diluted to 3
7. Albumin standard

10 mg of bovine serum albumin was dissolved in 25 ml of 0.1 N NaOH.

Procedure

1 ml of protein solution was mixed with 5 ml of reagent C and kept for 10 minutes at room temperature. 0.5 ml of diluted Folin phenol reagent was added, mixed well and optical density read at 670 nm exactly after 30 minutes. The protein in the solution was calculated along with the bovine serum albumin standard made under identical conditions by using the formula,

\[
\frac{\text{Optical density of test}}{\text{Optical density of standard}} \times \text{Concentration of standard}
\]

Protein Estimation with the Alkaline Copper Reagent

Reagents

1. Alkaline copper reagent

Prepared by mixing 50 ml of 2% sodium carbonate in 0.1 N NaOH and 1 ml of 0.5% CuSO4 in 1% sodium potassium tartrate.

2. Folin's reagent

1 ml of Folin's reagent was mixed with 2 ml of water.
3. 0.1 N NaOH

4. Albumin standard

400 mg of bovine serum albumin was dissolved in 100 ml of 0.9% saline.

Procedure

0.05 ml of serum precipitated in 2 ml of 10% TCA and centrifuged. Precipitate was dissolved in 10 ml of 0.1 N NaOH. To all tubes added 5 ml of copper reagent and mixed immediately. The tubes were kept for 30 minutes and optical density (OD) estimated at 670 μm against a blank.

Standard: 0.2 and 0.4 ml aliquot taken. Made up to 1 ml with 0.1 N NaOH.

Estimation of Serum Protein

In a test tube 0.5 ml of serum and 9.5 ml of 22.7% Na₂SO₄ solution were taken. Mixed and transferred 2 ml into another tube, marked total protein (TP). Closed tightly with cotton plug. Shook the rest and waited for 10 minutes. Then filtered through a Whatman No.1 filter paper. If the filtrate was not clear, it was filtered repeatedly through the same filter paper and collected the final filtrate in another test tube. Transferred 2 ml of sodium sulphate into a third tube marked blank. Added 5 ml of working Biuret reagent to all the three tubes and mixed well. Waited for
10 minutes and read at 555 μν. Obtained the result from the graph.

Subtracted the values of albumin from total protein to get values of globulin.

Normal protein = 7-9 gm/100 ml of serum
Normal albumin = 2.8-4.8 gm/100 ml of serum

Lactate dehydrogenase (LDH): Colorimetric method of King

The colorimetric method is based on the formation of pyruvate dinitrophenylhydrazone. King used lactate and measured the increase in colour as pyruvate is formed.

1. Glycine buffer, 0.1 M
   Dissolved 7.505 gms of glycine and 5.85 gms of NaCl in distilled water and made to a litre.

2. Buffered substrate
   Added 4 gms of lithium lactate, 125 ml of the glycine buffer and 75 ml of 0.1 N NaOH.

3. Solution of NAD
   Dissolved 10 mg in 2 ml of distilled water.

4. 2:4-dinitrophenylhydrazine reagent
   Dissolved 200 mg of the reagent in hot normal hydrochloric acid and made to a litre with this acid.

5. Sodium hydroxide, 0.4 N

6. Standard sodium pyruvate solution
   Dissolved 11 mg sodium pyruvate in 100 ml of
buffered substrate solution. This contained 1 micromole of pyruvate per ml.

7. Solution of NADH₂

1 micromole per ml of buffered substrate (disodium salt, mol.wt. 710).

Technique

Pipetted 1 ml of buffered substrate and 20 μl serum (0.1 ml of serum diluted 1 to 5 with water) into each of two tubes. Added 0.2 ml of distilled water to one (the blank) and placed both tubes in a water bath at 37°C. Allowed to reach the temperature of the bath, then to the other tube (the test) added 0.2 ml NAD solution and shook to mix. Exactly 15 minutes after adding the NAD, added 1 ml of the dinitrophenylhydrazine reagent to each, shook to mix, and left in the water bath for further 15 minutes. Then they were removed from the bath and to each was added 10 ml 0.4 N NaOH and read at 440 mμ within one to five minutes of adding the hydroxide. For the standard curve the following tubes were set up.

<table>
<thead>
<tr>
<th>I.U. per litre</th>
<th>0</th>
<th>167</th>
<th>333</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>ml of NADH₂ in substrate</td>
<td>0</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
</tr>
<tr>
<td>ml of pyruvate solution</td>
<td>0</td>
<td>0.05</td>
<td>0.1</td>
<td>0.15</td>
</tr>
<tr>
<td>ml of buffered substrate</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>ml of NAD solution</td>
<td>0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>ml of water</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Spectrophotometric method (Wroblewski and La Due, 1955)

LDH reduces pyruvate to lactate in the presence of NADH₂. The change in optical density at 340 nm as the coenzyme is oxidized, provides a measure of the enzyme activity.

**Method**

Mixed 2.7 ml of phosphate buffer, 0.1 ml of serum and 0.1 ml of NADH₂ solution and allowed the mixture to remain at room temperature for 30 minutes to destroy endogenous substrate. Added 0.1 ml of pyruvate substrate, mixed well and measured the OD of the solution at 340 nm in a spectrophotometer. Took readings at 30 or 60 second intervals for 3 to 6 minutes, according to the activity of the specimen. During the period of observation the fall in OD should be linear. Calculated the change in OD per minute.

Equimolar amounts of substrate and coenzyme are involved in the reaction. It is known that 1µ mole of NADH₂ in 3 ml of reaction mixture has an optical density of 2.1. Thus the rate (µmole per minute) of coenzyme oxidation is,

\[
\frac{\text{OD change per minute}}{2.1}
\]

This is the reaction rate caused by the enzyme present in 0.1 ml of serum. The activity of the enzyme contained in 1 litre of serum is

\[
\frac{\text{OD change per minute} \times 1000}{2.1 \times 0.1}
\]
Hence serum LDH = OD change per minute x \(10^3\) x 4.8

The determination should be carried out as near 25°C as possible. The various fractions of LDH have differing temperature coefficients so that it is difficult to correct activity to a standard temperature.

Solutions

Buffer (pH 7.4)

Dissolved 7.55 gm of anhydrous disodium hydrogen phosphate and 1.81 gm of anhydrous potassium dihydrogen phosphate in water and made it to 1 litre. Checked the pH with a meter or by using indicator papers. Stored at 4°C.

Reduced nicotinamide adenine dinucleotide (NADH_2)

Dissolved 2.5 mg of NADH_2 in 1 ml of phosphate buffer. Prepared freshly for each batch of tests.

Sodium pyruvate substrate (23mM)

Dissolved 125 mg in phosphate buffer and made to 50 ml. Divided into 2 ml portions and stored at -15°C.

Heat stability

The isoenzyme present in greatest proportion in heart muscle, LDH-1, has been shown to be more heat resistant than that in liver. Wroblewski and Gregory (1961) treated the serum as follows. To 2 ml of unhaemolysed serum added 0.2 ml NADH (2.5mg/ml). After 20 minutes, placed 0.5 ml of this solution in each of three small test
tubes. Left one unheated (I), one at 57°C (II) and the other at 65°C (III), for 30 minutes. Removed, cooled rapidly and determined the activity by the technique of Wroblewski and La Due. I gives total LDH activity, I-II mainly LDH-5 and formed 10 to 30 % in normal persons but 33 to 85 % in acute liver disease, whereas III was mainly LDH-I constituting 20 to 40 % in normal sera but 45 to 65 % in myocardial infarction and II-III were mainly intermediate isoenzymes LDH-2 and LDH-4.

The effect of heat on the activity of LDH was determined by a slight modification of the original method. In the original method, the conditions used for the heat effect were preheating the sera for 30 minutes at 65°C. From our experience we found out that a preheating time of 40 minutes at a slightly lower temperature of 60°C was a preferred condition. However the results were comparable.

Determination of Serum Alkaline Phosphatase

Method of King and Armstrong

Reagents

1. Disodium phenyl phosphate, 0.01 M

Dissolved 0.545 gms of disodium phenyl phosphate in water and made up to 250 ml. Heated quickly to boil. Cooled, added a little chloroform and kept in the refrigerator.
2. Sodium carbonate-sodium bicarbonate buffer 0.1 M
   Dissolved 4.09 gms of anhydrous phenyl phosphate in water and made up to 250 ml.

3. Buffered substrate for use
   Prepared by mixing equal volumes of solutions 1 and 2. This had a pH of 10.

4. Phenol reagent of Folin and Ciocalteu
   To 1 volume of phenol reagent added 2 volumes of water.

5. 20% sodium carbonate solution
   Dissolved 20 gms of anhydrous Na₂CO₃ in water and made up to 100 ml.

6. Standard phenol stock solution
   10 mg of phenol per 10 ml of 0.1 N HCl.

7. Diluted phenol standard for use
   Diluted the stock standard 1 in 10 to obtain a standard solution containing 10 mg of phenol per 100 ml of solution.

8. Standard phenol solution and reagent
   0.5 mg phenol was dissolved in 100 ml of water. Took 2.5 ml of the diluted standard, added 15 ml of the diluted phenol reagent and made up to 50 ml with water. This was prepared fresh daily.

Technique
   Pipetted 6 ml of the buffered substrate into a test
tube, and placed in a water bath at 37°C for a few minutes. Added 0.3 ml of serum, preferably without removing from the bath. Mixed, corked and allowed to remain in the bath exactly for 15 minutes. Then it was removed from the bath and added 2.7 ml of the diluted phenol reagent immediately. At the same time a tube was set up for control, containing 6 ml of substrate and 0.3 ml of serum, to which was added 2.7 ml of diluted phenol reagent. Mixed well and centrifuged. Took 4 ml of supernatant fluid from each and added 1 ml of 20% sodium carbonate. Put up a standard prepared by adding 1 ml of sodium carbonate solution to 4 ml of the standard phenol and reagent. Placed the three tubes in the 37°C water bath for 15 minutes and read in the colorimeter. As blank, 1.2 ml of diluted phenol reagent and 1 ml of 20% sodium carbonate were added to 2.8 ml of water. A red filter was used with transmission at 680 μm.

Calculation

\[
\frac{\text{Reading of unknown}}{\text{Reading of standard}} \times 15
\]

\[
KA = \text{Units of phosphatase per 100 ml of serum}
\]

\[
\text{I.U. per litre} = KA \text{ units} \times 7.1
\]

Normal range 22-92 I.U. per litre
Transaminases in serum (E.J. King)\textsuperscript{5,3}

Reitman and Frankel, 1957\textsuperscript{5,4}

Solutions

1. 0.1 M Phosphate buffer (pH 7.4)

6.985 gms of potassium monohydrogen phosphate (K$_2$HPO$_4$) and 1.345 gms of potassium dihydrogen phosphate (KH$_2$PO$_4$), were dissolved in distilled water and made to 500 ml. The pH was checked.

2. Stock α-keto-glutaric acid (20 mM)

0.0584 gms of α-keto glutaric acid was dissolved in minimum water, the pH was adjusted to 7.4 (with 1N NaOH) and the volume made up to 25 ml.

3. Aspartic acid (222 mM)

2.955 gms of DL-aspartic acid was dissolved in approximately 20 ml of 1N NaOH, the pH adjusted to 7.4 and the volume made up to 100 ml with phosphate buffer pH 7.4.

4. Alanine (222 mM)

1.977 gms of DL-alanine was dissolved in 20 ml of distilled water, the pH adjusted to 7.4 with 1N NaOH (about 0.5ml) and made up to 100ml with phosphate buffer of pH 7.4.

5. GOT substrate (2mM α-keto glutarate in aspartic acid)

One volume of stock α-keto glutarate was diluted with 9 volumes of buffered aspartic acid solution.

6. GPT substrate (2mM α-keto glutarate in alanine)

One volume of stock α-keto glutarate was diluted
with 9 volume of buffered alanine solution.

7. Sodium pyruvate (2mM)

11 mg of sodium pyruvate was dissolved in 50 ml of phosphate buffer and kept frozen.

8. 2,4-dinitrophenylhydrazine (1 mM)

19.8 mg of 2,4-dinitrophenylhydrazine was dissolved in 10 ml of concentrated hydrochloric acid and made to 100 ml with distilled water. The solution was kept in a dark bottle at room temperature.

9. 0.4 N NaOH

Dissolved 8 gms of NaOH in water and made up to 500 ml.

METHOD

Test

1 ml of GOT or GPT substrate was placed in a test tube in the 37°C water bath for 3 minutes, when 0.2 ml of serum was added. The mixture was gently shaken and kept in the water bath for 60 minutes for GOT estimation or 30 minutes for GPT estimation. The tubes were removed and added 1 ml of 2,4-dinitrophenylhydrazine solution and allowed to stand at room temperature for 20 minutes. 10 ml of 0.4 N NaOH was added and the contents of the tubes were mixed by inversion. The OD was read after 10 minutes at 505 μm or with an Ilford green (624) light filter. The colour was
stable for at least an hour.

**Control**

In another tube was taken 1 ml substrate and 0.2 ml serum; immediately, 1 ml of 2,4-dinitrophenylhydrazine was added. The conditions of the colour reaction were the same as for test.

**Standard**

0.4 ml of Sodium pyruvate solution was added to 0.6 ml of substrate and 0.2 ml of water. This was equivalent to 40% conversion. Then 1 ml of 2,4-dinitrophenylhydrazine was added and the procedure followed as for the test.

**Blank**

1 ml of substrate was added to 0.2 ml of water and the procedure followed as for the standard and test.

**Calculation**

The percentage of the substrate which had been converted in the test was calculated.

\[
\text{Percent conversion} = \frac{\text{Reading of test minus control}}{\text{Reading of standard minus blank}} \times 40
\]

The transaminase activity (units per ml) was then obtained from the percent conversion using the standard curve.
Standard curve

<table>
<thead>
<tr>
<th>Percent conversion</th>
<th>GOT (Units per ml)</th>
<th>GPT (Units per ml)</th>
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<tbody>
<tr>
<td>10</td>
<td>16</td>
<td>19</td>
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<td>20</td>
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<td>30</td>
<td>58</td>
<td>56</td>
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<tr>
<td>40</td>
<td>81</td>
<td>80</td>
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All sera higher than 80 units must be suitably diluted and the estimation repeated.

Malic Dehydrogenase (MDH)\(^{55}\)

Assay Method (Mehler et al. 1948)\(^{56}\)

Reagents

- 0.25 M glycylglycine buffer, pH 7.4
- 0.0015 M NADH$_2$
- 0.0076 M oxaloacetate, pH 7.4. This solution was prepared freshly.

Procedure

The reaction mixture contained 0.3 ml of buffer, 0.1 ml of NADH$_2$ (0.15 micromole), 0.1 ml of oxaloacetate (0.76 micromole), enzyme and water to a final volume of 3.0 ml. The reaction was carried out at room temperature and started by the addition of either oxaloacetate or enzyme. Readings of OD were made against a blank containing all
components except NADH at intervals of 15 seconds for 1 or 2 minutes. The decrease in optical density ($\Delta \log_{10} I_0/I$ at 340 nm) between 30 and 45 seconds after the start of the reaction was used to calculate the enzyme activity. The amount of enzyme used in a test was adjusted so that the rate of decrease of optical density, for the period between 30 and 45 seconds, did not exceed 0.025.

**Definition of Unit and Specific Activity**

One unit of enzyme is defined as that amount which causes a decrease in OD of 0.01 per minute under the above conditions. Specific activity is expressed as units per milligram of protein.

**Purification and characterization of LDH from *Setaria digitata***

**Materials and Methods**

Adult *S. digitata* were collected from the local slaughter house and worms were washed in Tyrode solution and homogenized with ice-cold KCl (150 mM; 1:10 w/v) using a tissue homogenizer. The homogenate was centrifuged at 10000g (15 mins) for obtaining soluble fraction.

The soluble fraction was brought to 35% saturation with ammonium sulphate, allowed to stand for 2 hours and the precipitate was removed by centrifugation at 10000 g for 15
mins. The supernatant was taken to 70% saturation of ammonium sulphate and kept for 2 hours and centrifuged at 10,000 g for 15 mins. The supernatant was taken to 100% saturation of ammonium sulphate and kept for 2 hours and centrifuged. The precipitate at 35%, 70% and 100% saturation were dissolved in 10 mM tris HCl and dialysed at 4°C for 2-3 days. The LDH assayed in the whole homogenate precipitates and supernatants at 35%, 70% and 100% saturation of ammonium sulphate according to the method of Kornberg. The assay mixture contained tris-HCl buffer (pH 7.4) 200 mM: KCl, 100 mM; Pyruvate, 5 mM; NADH, 0.24 mM and the enzyme protein. The decrease in absorbance of NADH was measured at 340 nm using silica cuvette of 1 cm light path in Schimadzu UV-240 spectrophotometer. The unit for the dehydrogenase was calculated using the extinction coefficient of 6.22 x 10⁶/cm²/mol for reduced NAD. Specific activity is defined as the amount required to catalyze the transformation of one n mole of substrate or formation of product per min. per mg protein. Protein was estimated according to Lowry et al.

Since LDH is also present after 70% saturation of ammonium sulphate, the concentration of ammonium sulphate was changed.

The soluble fraction was brought to 40% saturation with ammonium sulphate, allowed to stand for 2 hours and the
precipitate was removed by centrifugation at 10,000 g for 20 mins. The precipitate was dissolved in 10 mM Tris-HCl. The supernatant was taken to 80% saturation of ammonium sulphate and kept overnight and centrifuged at 10,000 g for 25 mins. The precipitate was dissolved in 10 mM tris-HCl buffer, pH 7.4, and dialyzed overnight at 4°C. The dialyzed enzyme was passed through a Sephadex G-200 column (100 x 1 cm) and elution was carried out with 10 mM tris-HCl buffer (pH 7.4). The fractions rich in LDH activity were pooled, concentrated by precipitation with ammonium sulphate and dialyzed. PAGE was carried out in the home made Hoeffer slab gel apparatus using the methods of Laemmli, Fairbanks et al. and Studier.

**Antigenic studies with *S. digitata***

**Materials and Methods**

**Antigen Preparation**

The bovine filarial worm *S. digitata* were obtained from the slaughter houses. The worms were washed in saline or Tyrode solution thoroughly and incubated in Tyrode solution at 37°C for 3-4 hours, changing the Tyrode every hour. The worm incubated Tyrode solution was centrifuged at 3000-4000 rpm for 15-20 minutes and the mf which were found in the residue were separated. The filtrate was saturated with ammonium sulphate and kept for some time. Then the solution
was centrifuged at 3000-4000 rpm for 15-20 minutes. The residue obtained was dialysed in water using the dialysing tube. The dialysed residue was taken and it was the excretory-secretory material. The sheath of the worm was separated by simple dissection (sheath preparation).

The whole worm, sheath, mf and excretory-secretory materials were homogenized with saline (1 gm/10 ml), containing 0.5% phenol. The solution was centrifuged below 1000 rpm for 2-3 minutes. The filtrate was used as the antigen.

**Antibody preparation**

The whole worm, sheath, mf and excretory-secretory antigen preparation were injected into white albino rabbits.

I rabbit - Whole worm  
II rabbit - Sheath  
III rabbit - mf  
IV rabbit - excretory-secretory material

Seven subcutaneous injections of the above preparations were given to each rabbit. Four injections with Freund's complete adjuvant and 3 with Freund's incomplete adjuvant. Eight days after the last injection, blood was taken from the ear of the rabbit. Serum was separated and this serum was used as the antiserum against the antigens.

Antigen-antibody reaction was carried out in agarose gel on immunodiffusion plates according to the
method of Ouchterlony 1.5% agarose was prepared in normal saline. The solution was warmed until dissolved and poured into the plates and it was left at 40°C for the gel to set. Then using the gel punch G7, 7 holes were made one at the centre and 6 holes around it.

The antigen i.e. whole worm 1/20 dilution, sheath 1/20 dilution, mf neat excretory-secretory neat were taken in the central hole separately. The antisera with different dilutions 0, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024 and 1/2048 were taken.

After 48 hours the antisera were applied once more in the surrounding wells.

Gel-diffusion Technique

Double Diffusion in gel (Ouchterlony technique)

(Ouchterlony, 1962)57,58

Materials
1. 0.1 M NaCl + 0.05 M phosphate + 0.01 M NaN₃ (pH 7.1-7.3)
2. Difco Agar
3. Microscope slides 1"x3" or 2"x3" or 35x10mm plastic (Falcon) disposable petridishes
4. Pattern cutter mould
5. Pasteur pipettes
6. Serum and antiserum to be tested

BSA, anti BSA, IgG, Anti IgG and Normal goat serum
Method

Weighed out agar to make 1% w/v solution in PBS. Heated it in a water bath to boiling. Pipetted the clear agar solution on a glass slide or petridish. Allowed it to set on a levelling board and kept in the refrigerator till use. Punched out pattern using the 'laboratory mould' or any other suitable cutter. Removed agar from wells with suction. Filled the wells with desired reagents and labelled them correctly using adhesive tape or 'Magic Marker' and allowed to diffuse in a moist chamber at room temperature (Diffusion time: usually 12 to 24 hours at room temperature but should be observed up to 7 days of which 24 hours must be at 40°C). Read and recorded the results of the appearance of precipitin lines using a viewing box.

Indirect Immunofluorescence Method

The presence of antibodies was tested by the indirect immunofluorescence method (IFT). The worms were processed in two different ways.

1. Whole worm

2. The whole worms were cut at the hind tip and pressed down to release all the egg and mf in it.

These worms were then fixed in cold acetone (stored at -20°C) for 10 minutes. The fixed worms were incubated with the antisera against various fractions for 30
minutes at room temperature. This was followed by thorough washing in PBS. The worms were mounted on a glass slide in 95% glycerine and observed under Leitz Orthoplen (ultraviolet microscope). The readings were recorded as:

- no fluorescence
+ weak fluorescence
++ moderate fluorescence
+++ strong fluorescence

The egg and mf were washed in PBS and smears were made on glass slides. These smears were processed in the same manner as for the whole worm and observed and graded under the UV microscope.

**Implantation of whole worm into white rats**

White albino rats of the *sprague dawley* strain were implanted with *S. digitata* whole worms (2 or 3) in their peritoneal cavity by making a paramedian incision and the wound was closed in layers.

The worms were incubated in PBS medium for half an hour, washed in PBS medium containing antibiotic and suspended in the same medium before implantation.

The animals were closely observed. Subsequently, on the 1\(^{st}\) day, 2\(^{nd}\) day, 3\(^{rd}\) day, 4\(^{th}\) day and so on one rat was cut and maximum blood collected for separating the serum to do A/G ratio and the remaining serum was freezed. The
abdomen was opened up and the worms inside the peritoneal cavity were taken and incubated in PBS medium for half an hour, centrifuged and mf counted. The peritoneal cavity was washed with PBS medium and mf counted. Glycogen and protein were estimated in the liver and kidney of the rat and also the worm tissues.

**Implantation of mf into white rats**

White albino rats of the *sprague dawley* strain were implanted with *S. digitata* mf by injecting the mf into the peritoneal cavity. The mf were suspended in an antibiotic containing PBS medium before injection.

DEC was administered (2mg/100gm rat) to two groups of rats (mf positive and control) for 12 days. Differential count (DC) was taken during the 12 days of DEC treatment for control rats, DEC administered normal rats, mf positive rats and DEC administered mf positive rats on 2nd, 3rd, 4th, 6th, 9th and 13th day of DEC treatment.

The activities of LDH, MDH, alkaline phosphatase, GOT and GPT were determined in mf positive and control rat sera.

The A/G ratio was determined in the serum from normal rats, mf positive rats, DEC fed normal rats and DEC fed mf positive rats.