

CHAPTER 2

MATERIALS AND METHODS

Subjects

Normal subjects : The normal subjects are composed of 238 subjects, 159 males and 79 females. They were blood donors taken from the Thai Red Cross Society and volunteers from the Thai Police Medical Hospital. Their ages ranged from 20 to 48 years for males and 18 to 35 years for females.

The pregnant women : They were 220 pregnant women from the Department of Obstetrics and Gynaecology, Siriraj Hospital . They were taken randomly and their ages ranged from 18 to 42 years.

Patients :

1. Patients with hookworm infection : The studies were performed in 67 patients, 46 males and 21 females. They were admitted into the Hospital for Tropical Disease, Faculty of Tropical Medicine, Bangkok; Hospital of Vachiralongkorn Dam, Tamaung, Kanchanaburi; and the Hospital for the Navy, Sattaheep. Their ages ranged from 7 to 76 years.

2. Patients with malarial infection : There were 167 patients, 122 males and 45 females. They were admitted into the

Hospital for Tropical Disease, Faculty of Tropical Medicine, Bangkok. Their ages ranged from 13 to 55 years.

3. Patients with liver diseases : There were 138 patients, 119 males and 19 females. They were suffered from various liver diseases, i.e. 5 patients with Opisthorchiasis, 15 patients with amoebic liver abscess, 12 patients with hepatomegaly, 8 patients with obstructive jaundice, 12 patients with cirrhosis, 73 patients with infectious hepatitis, 7 patients with carcinoma of the liver and 6 patients with hematoma. Their ages ranged from 12 to 87 years.

Methods

1. Determination of haptoglobin phenotypes

The haptoglobin phenotypes were estimated by the Starch-gel electrophoresis as described by Smithies (1955, 1959).

Principle : A starch gel containing the desired buffer is prepared in a suitable plastic tray. The sample is introduced into a vertical slit in the gel at right angle to the greatest length of the gel. Electrical contact is made to the ends of the gel with filter-paper pads, soaked in a suitable buffer solution, which dip into vessel containing the same solution. Filter-paper bridges in turn connect these vessels to the

electrode chambers. Current is passed for the proper time. The gel is then removed from the tray and sliced along its length in a horizontal plane, and the slices obtained are stained with a protein dye. After washing with dye solvent, the stained separated proteins may be observed, and the type of haptoglobin is defined. The general layout of the apparatus is shown in Fig. 2.

Instruments

1. Electrophoresis Apparatus (Shandon model U 77)
2. Electric stirrer
3. Hot plate (Gallenkamp)
4. Centrifuge (International Portable Refrigerated Centrifuge Model PR-2)
5. pH meter (Beckman)
6. Electrical balance (E. Mettler, Type H 16)

Reagents

1. Starch-hydrolysed supplied by Connaught Laboratories Limited, Willowdale, Toronto, Canada.
2. Buffer (Poulik, 1957)
 - a. Bridge buffer

Boric acid 0.3M 18.54 g / L

NaOH 0.05M 2.0 g / L

pH 8.6

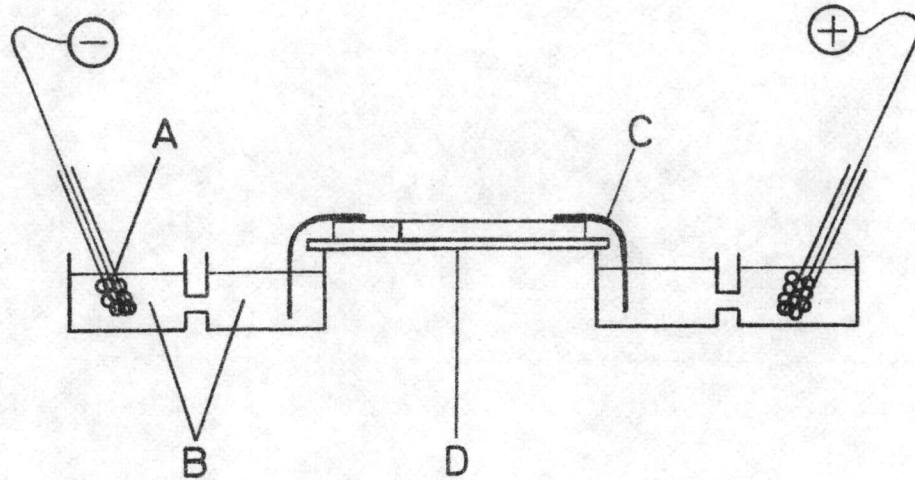


Figure 2. General layout of the electrophoresis apparatus.

- A Electrode
- B Bridge solution.
- C Filter paper bridge soaked in
bridge solution.
- D Plastic tray contains starch gel.

b. Gel buffer

Tris buffer (THAM) 0.076 M 9.20 g / L

Citric acid 0.005 M 1.05 g / L

pH 8.6

c. Acetate buffer

Acetic acid 1.5 M glacial acetic 42.89 ml to 500 ml

Sodium acetate 1.5 M 102.06 g /500ml

pH 4.6

3. Dye solution

O-dianisidine 0.3 g

Methanol 100.0 g.

(0.15 g /50 ml for each staining)

4. Staining solution

Acetate buffer 50.0 ml

Dye solution 50.0 ml

H₂O₂ 0.4 ml

5. Fixative

Methanol 67.5 ml

Distilled water 67.5 ml

Glacial acetic acid 15.0 ml

All reagents used in the present work were reagent grade of
E. merck,

Procedures

Collection of human sera : To avoid haemolysis, the following procedure has been adopted. About 10 ml of whole blood are withdrawn from a cubital vein with a No. 20 needle. The blood is expelled from the syringe after removing the needle and allowed to run down the side of a centrifuge tube, care being taken to avoid the expulsion of the air bubbles unavoidably present in the syringe. After clotting for about 3 hours at room temperature, the clotted sample are centrifuged for 10 minutes at 2,000 r.p.m., the serum is decanted. The samples so obtained never showed any sign of haemolysis, as judged by their colour.

Sera were used first for the electrophoretic test to determine haptoglobin types and the haptoglobin levels were carried out later by Spectrophotometer. All sera was stored at -20°C until used.

Preparation of the gel :

Gels can be made from starch-hydrolysed at concentrations of 10.5-12.5 g of starch-hydrolysed per 100 ml of gel buffer.

About 16.8 g of starch-hydrolysed is suspended in the 160 ml of gel buffer. The dry starch-hydrolysed is best added to half the volume of gel buffer in a 500 ml conical suction flask

and immediately mixed. The remainder of the gel buffer can then be used to wash traces of powder from the weighing paper into the flask. The mixture is heated over a hot plate with full heat control and constant and vigorous swirling is applied by using an electric stirrer. As the temperature raises, the suspension will turn semi-solid. Further heating is carried out with continued *swirling* until the starch grains are ruptured, semi-solid mass becomes a viscous homogeneous solution. The time of boiling is kept as short as possible (1-2 sec) so that loss of water will not appreciably affect the composition of the gels. The flask is then removed from heat. The swirling is continued for a few moments to ensure a uniform temperature.

In order to remove small air bubbles, degassing is then conducted by applying a negative pressure with a vacuum pump until the contents of the flask bubble vigorously. Standard conditions are essential if the characteristics of the gels are to be reproducible.

The hot starch solution is then poured into a suitable plastic tray, pre-set with a thin celluloid spacer, gel slot maker, which can cut eight rectangular holes, a little smaller than the cross-section of the gel (Fig.3). The gels are ready for use

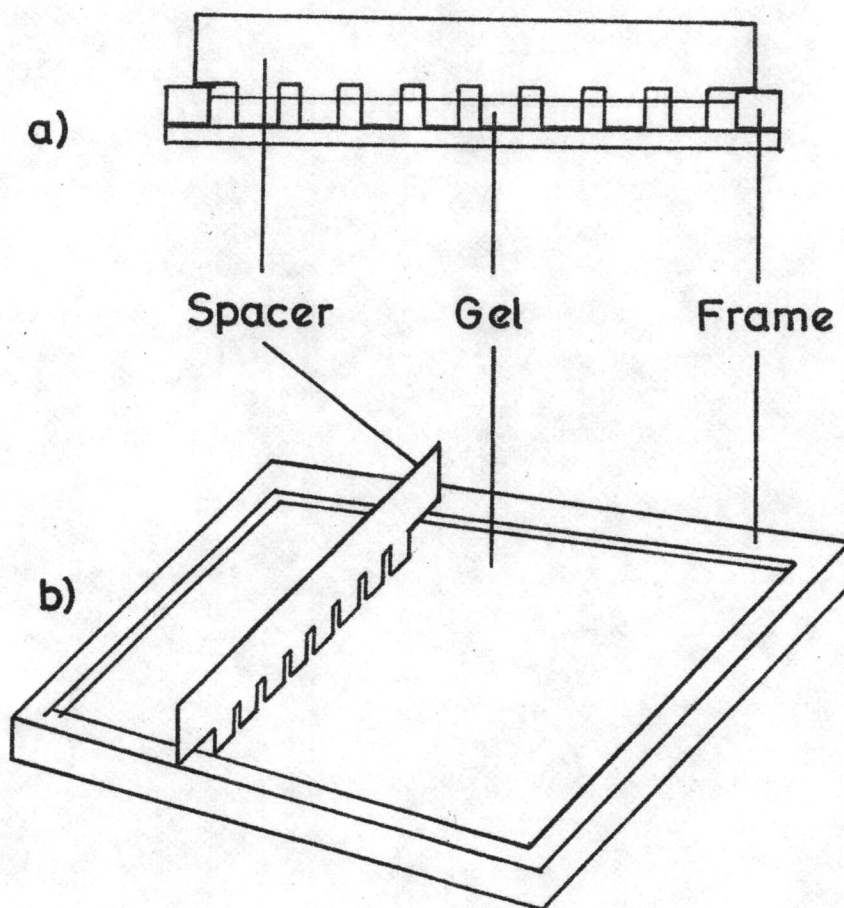


Figure 3. Gel tray and spacer,
a) Cross section.
b) Perspective view.

when they have cooled down to room temperature. The gel slot maker must be taken out very carefully so that the gel is not broken.

The serum for electrophoresis was prepared by mixing one drop of haemoglobin solution (10g. /100ml) to 10 drops of serum (dropped by capillary tubes). The mixture was left for twenty minutes for the formation of the haemoglobin-haptoglobin complex. Usually, the serum mixture was freshly prepared but it may be kept frozen for a week. This mixture was placed on the apparatus (Fig.2).

Electrical connections :

Electrical contacts to the gels are made with filter-paper wads soaked in Bridge buffer solution of approximately the same pH as that of the gels (Fig.2). Care must be taken that the current should run from anode to cathode and the electrode must be alternated in every run.

In the present work, current of 300 voltage was used as the source of power, a serum separation of 8-10 cm was achieved within $2\frac{1}{2}$ -3 hours. It was usually performed at cold room temperature (8°c). The pattern of the electrophoretic separation was affected by the follow factors:-

1. Concentration and composition of buffer solution.
2. Current and effective-voltage.
3. Working distances.
4. Position of starting line.
5. Time to run.
6. Working temperature.
7. Type of electrophoresis tank.
8. Characteristic of filter-paper pads.

By suitable combinations of these variable, it was possible to evolve optimum conditions for achieving the desired electrophoretic separation.

After electrophoresis has proceeded sufficiently long enough, the gel was then put on levelling table and gel slicer (Fig.4), the gel was then split by gel slicer along its length in a horizontal plane before staining . The two slices so obtained were rapidly separated and transferred to a tray for staining with the cut surfaces uppermost.

Staining :

The protein-detecting dye was then poured over the gel and allowed to remain in contact with the gel for 5-10 minutes. Haemoglobin-haptoglobin complex stains dark-brown,

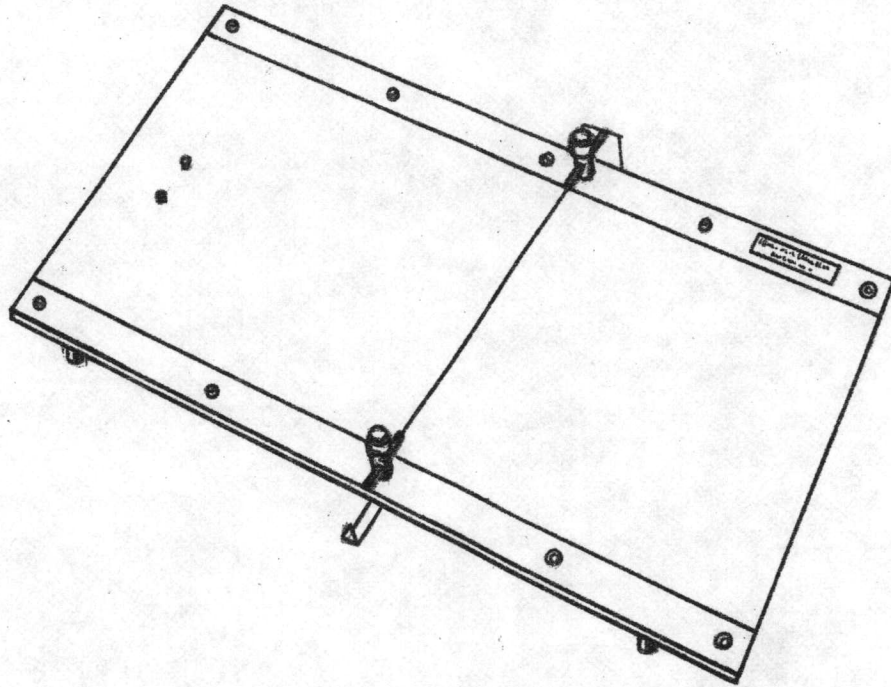


Figure 4. Levelling table and gel slicer

the dye was decanted and the fixative was poured in and left overnight . The haptoglobin phenotypes of each serum was read out next morning.

2. Determination of serum haptoglobin level.

It was performed by measuring the peroxidase activity of a haptoglobin-methaemoglobin complex with a spectrophotometer (Owen et al., 1960).

Principle : The complex formed by the combination of serum haptoglobins with methaemoglobin has a much greater peroxidatic activity than free methaemoglobin. The peroxidatic activity of complex is measured colorimetrically with a spectrophotometer, and the concentration of haptoglobins is determined by comparing to a calibration curve. The concentration of haptoglobin is expressed as mg of haemoglobin-binding capacity per 100 ml of serum.

Instruments

1. Spectrophotometer (Bausch & Lomb 340)
2. Water bath (Chicago Surgical and Electrical Co.)
3. pH meter (Beckman)
4. Electrical balance (E.Mettler, Type H 16)
5. Super-mixer (Vortex-Genie)

Reagents

1. Guaiacol Reagent

3.75 g of guaiacol (Codex Erba from Carlo Erba) was dissolved in 700 ml of distilled water and 100 ml of 1M glacial acetic acid was added. The pH was adjusted to 4.0 by the addition of 1N NaOH. The volume is finally made up to 1 litre.

2. Hydrogen Peroxide 0.05 M

This is prepared immediately before use by diluting 2.5 ml of 1 M H_2O_2 to 50 ml with distilled water.

3. Methaemoglobin solution

To 8 volumes of thrice-washed packed red cells are added 3 volumes of water and 1 volume of ether. The mixture is shaken and centrifuged. The haemolysate is pipetted off, and its haemoglobin concentration is determined. It is then diluted until the final haemoglobin concentration is 1 g /100 ml.

To 25 ml of haemoglobin solution are added 10 ml of 1 g /litre potassium ferricyanide to convert the haemoglobin to methaemoglobin. After 10 minutes the volume is made up to 500 ml (50 mg of methaemoglobin per 100 ml). The solution can be stored at $4^{\circ}C$ for one week.

Methods

Construction of a Calibration Curve

One ml of methaemoglobin solution is dropped into each of 11 tubes. Pooled normal serum is added to each tube (except the first one) in progressively increasing amounts, from 0.1 ml to 1.0 ml at the interval of 0.1 ml. The volume in each of 11 tubes is made up to 2 ml with 0.9% NaCl solution.

Twelve pairs of test-tubes, each containing 5 ml of guaiacol reagent are placed in a water-bath (25°C) After 10 minutes, 0.1 ml of each of the above serum mixture is added to guaiacol tubes set duplicate and a blank of 0.1 ml of saline is added to the twelfth tube. Immediately thereafter, 1.0 ml of hydrogen peroxide, previously warmed to 25°C , is added to each tube. The contents are rapidly mixed. After 8 minutes, the optical density of the colour developed was measured in a spectrophotometer, which is set at zero with the normal saline solution blank. A wavelength of 470 nm is suitable. As the coloured material fades, especially if exposed to the bright daylight, the reading should be made within 4 minutes after the 8 minute reaction time.

The optical density are plotted against the volume of serum in each tube on the arithmetic graph paper. The gradient of the graph changes markedly when all the methaemoglobin becomes bound. This represents a haptoglobin content with a methaemoglobin-binding capacity of 50 mg/ 100 ml. This point is marked on the abscissa which is then recalibrated accordingly to the Fig.5. It should be noted that the curve does not pass through zero. This is due to the peroxidatic activity of the free methaemoglobin.

The abscissa shows the amount of normal pooled serum in the reaction mixture. At the point of change of gradient the normal pooled serum contains 50 mg haptoglobin per 100 ml. This is marked on the abscissa, and the distance between this point and the origin is recalibrated proportionately to give readings of haptoglobin concentration.

Test

Serum should be free from haemolysis. To two 0.25 ml volumes of concentrated or diluted (see below) serum is added an equal volume of methaemoglobin solution (test) and of distilled water (blank), respectively, 0.1 ml of

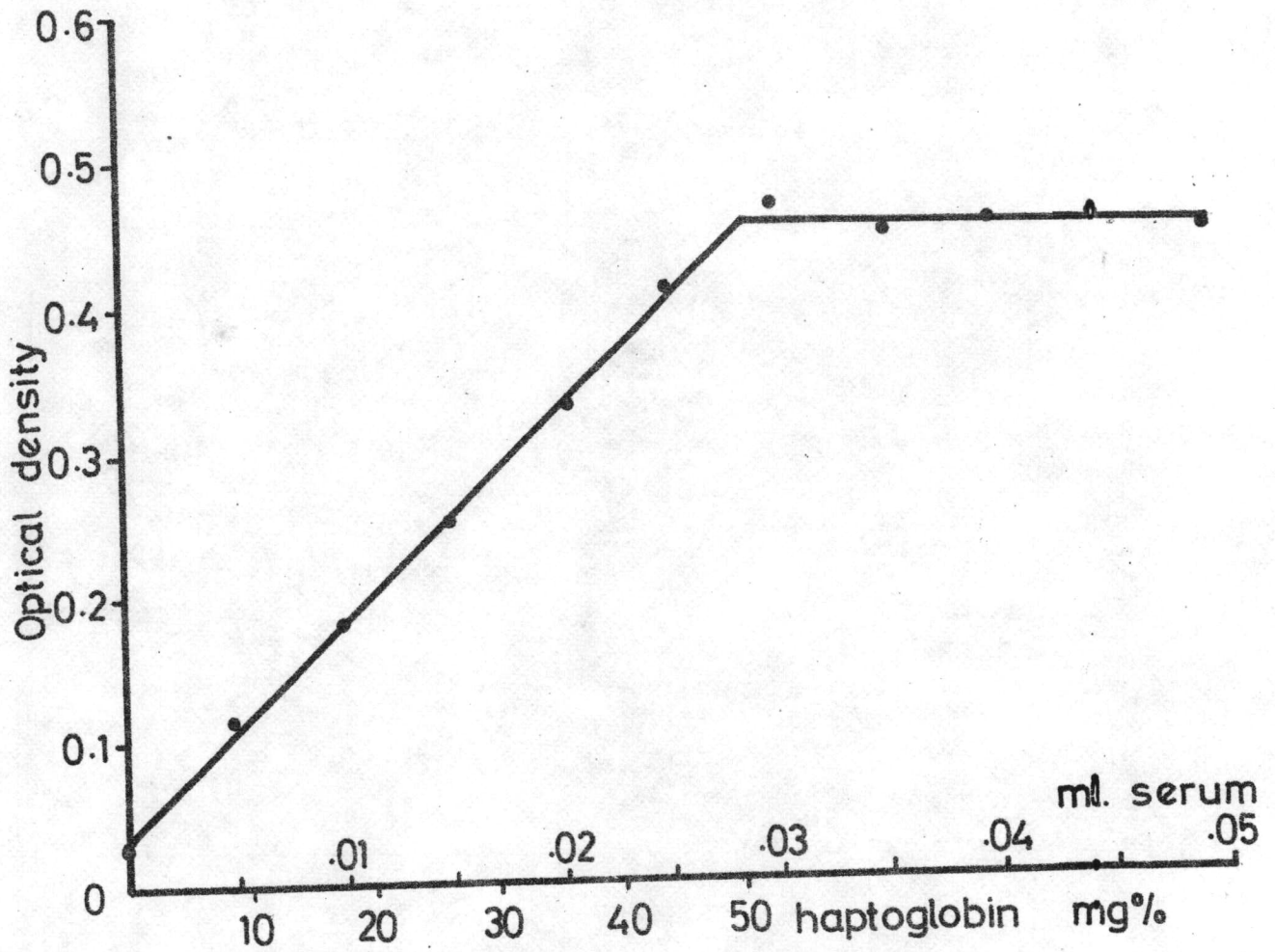


Figure 5. Calibration graph for estimation of haptoglobin concentration.

each mixture is added to 5 ml of previously warmed guaiacol reagent and the reaction developed with 1.0 ml of hydrogen peroxide as described above. After 8 minutes, the colour developed in each tube is measured, the instrument being set at zero with water. The blank reading is subtracted from the test reading and the concentration of haptoglobin is read off from the calibration curve. If the reading is too near or exceed the maximum, the test should be repeated with suitable diluted serum and the value read from the calibration curve multiplied accordingly.