Chapter 4

DISCUSSION

There were a number of reports described the various supporting media and the conditions used for the separation of LDH isoenzymes. Vessel (1958) introduced starch gel as a supporting medium in the electrophoretic separation of the LDH isoenzymes. This method was found to be complicated and time consuming. It was considered not suitable for clinical investigations. A number of investigators had tried to overcome the disadvantages, proposing several materials and methods for clinical investigations (Allen, 1961; Barnett, 1962; Van Der Helm, 1962; Richterich et al, 1963; Mull and Starkweather, 1965; Lance, 1966; Emery, 1967). The work described in this thesis suggested the use of Difco Special Agar-Noble as a supporting medium.

Cawley (1969) showed that the mobility of plasma proteins varied directly with the concentration of buffer salts and voltage applied. These findings were supported by the results obtained from these studies (results section 3.1 page 27).

When the electrophoresis was performed between 25°- 37°C, at any concentration of barbital buffer or agar used in the experiments, the plasma proteins moved considerably far but the bands obtained were diffused. At 37°C or above, the agar medium tended to melt especially when a long time and a high current were used.

A good separation of plasma proteins was obtained in one and a half hour when the electrophoresis was carried out at 25°C with 0.8-

1.5 % w/v agar, 0.05 M barbital buffer, pH 8.6, and a current of 7 mA. per slide. In order to avoid a deterioration of LDH isoenzymes, the time used in the electrophoresis was reduced to 1 hour. The plasma protein bands obtained under these conditions were well separated.

Ressler and Joseph (1962) found that some of LDH isoenzymes are relatively unstable at high temperature. It is important, therefore, to keep a constant temperature during the electrophoretic procedure. The requirement could be obtained using a circulating Omnibath.

The point of application of plasma samples was also important for the separation of LDH isoenzymes. A distance of 4 cm from the anode was found to be satisfactory to obtain separated bands of all five LDH isoenzymes. If the distance was longer than that mentioned above, the LDH-5 would moved to the area behind the gamma-globulin near the edge of slide causing a difficulty in the detection of both LDH-4 and LDH-5. Moreover, the agar at the end of slide tended to slide off during staining and destaining and it was inconvenient to manipulate.

The electrophoretograms obtained under these conditions showed a clear separation of LDH isoenzymes. Therefore, there was no need to add albumin to the buffer or medium system as suggested by Wieme (1966). It was found that the agar slides could be preserved in a closed humid chamber in a refrigerator for 2 days without damaging effect.

When various formulae of the staining solutions for LDH isoenzymes were compared, it was found that the best result was obtained with the formula of Fritz et al (1970) slightly modified. All of the LDH isoenzyme bands could be stained within 30 minutes with the formula given by Yakulis et al (1962) but the time needed in the destaining

process was too long and the background obtained was not clear. This may due to the high concentrations of nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS) in the solution compared to those present in the formula of Fritz et al (1970). When the modified Fritz et al (1970)'s formula was used, the staining process took 1 hour and the destaining process required not more than 30 minutes. They were shorter than those needed in the method of Yakulis et al (1962). Another advantage was that the background of the electrophoretograms was clear (see Figure 2, page 31).

There were many advantages in using the method of Dyerberg and Hjorne (1970) to stain the lipoproteins. The background obtained was clear and only 30 minutes was sufficient both for the staining and destaining processes. The method of Cawley (1969) required longer time and the dye also precipitated out during the process. The time needed in the method of Cawley (1969) was as long as 2 hours in both the staining and the destaining process and it also gave unclear background.

In the fixing process of LDH isoenzymes, the electrophoretograms were fixed after they had been stained and destained. The fixing solution consisted of methanol, water, and glacial acetic acid in a ratio of 5:5:1. Although this mixture contained a higher proportion of water, the presence of glacial acetic acid destained the gel yielding clear electrophoretogram of LDH isoenzymes.

The electrophoretograms for the fixation of lipoproteins and proteins were fixed before the staining process. It was found that the drier the gel the easier it was to manipulate in both the staining and destaining processes.

The electrophoretic procedure used in these studies could be easily performed and the time needed was much less than the electrophoretic systems used by Strandjord et al (1962), Yakulis et al (1962), Emery (1967), and Fritz et al (1970). It is reasonable to suggest the procedure for routine clinical laboratory. Further, the Difco Special Agar-Noble was cheaper than cellulose acetate. Dried electrophoretograms can be kept in the dark for as long as 1 year without changes in colour. The barbital buffer solution can also be used for routine work of serum or plasma protein electrophoresis. The relative positions of LDH isoenzymes to that of plasma proteins was similar to that reported by Van Der Helm (1961) (see Figure 5).

The electrophoretograms obtained from plasma of normal subjects shown in Figure 6 were similar to those reported by several investigators (Vessel, 1958; Markert and Moller, 1959; Appella and Markert, 1961; Blanchaer, 1961; Wroblewski and Gregory, 1961; and Van Der Helm, 1962). Ten microlitres of plasma samples was used throughout this investigation and this was based on the findings of Van Der Helm (1962). He used 2 to 10 microlitres of serum for electrophoresis and established a linear relationship between the total area under peaks obtained from a densitometer and the total activity of the isoenzymes obtained from spectrophotometric method. He also showed that the activity of each isoenzyme could be obtained by calculating the area under peak of the isoenzyme as a percentage of the total activity obtained spectrophotometrically.

The reliability of the methods used in the determination of LDH activities is of critically important in the diagnosis of myocardial

infarction. The methods should be simple, reproducible and accurate. These requirements could be fulfill using the method described by Amador et al (1963, 1965) with some modifications. They found that in the assay using lactate as a substrate, the reaction rate was linear over a wide range of activities, and the activity was directly proportional to the amount of serum used. The method could be use to tell the abnormality of the enzymic activity in serum. Various fractions of LDH isoenzyme, however, posses different temperature coefficients (Amador et al, 1963) and it is difficult to correct their activities to values at a standard temperature. Amador et al (1963, 1965) and Wootton (1964) suggested to carry out the determination at 25°C.

Of interest was that plasma samples which were kept at -5°C for 30 days were as good as the fresh samples and this was agreeable to the reports of Lazaroni et al (1958).

Although an incubation of plasma at 60°C for 60 minutes was considered to be the best condition for the separation of heart from liver LDH isoenzymes (Bell, 1963), it appeared from the work in this thesis that the values obtained under the above conditions were lower than those reported by Strandjord and Clayson (1961), Bell (1963), and Auvinen and Konttinen (1971). When the incubation of plasma was performed in 2 M urea as reported by Emerson and Wilkinson (1965), it was also found that the values of urea-stable activity were lower than those reported by Emerson and Wilkinson (1965), Lindy and Konttinen (1967), and Auvinen and Konttinen (1971). The low values of KLDH and ULDH activities may resulted from the use of plasma instead of serum. Fibrinogen may be an interfering substance in the determination as previously

pointed out by Wroblewski and Gregory (1961) who protested the idea of using plasma in the determination of HLDH activity.

Bell (1963) stated that, at low level of activity, there may be an error of as high as 15% in expressing the HLDH fraction as a percentage of TLDH.

Because of the relatively high difference in the ULDH activity in plasma of normal subjects and that in plasma of patient with myocardial infarction compared to the difference in the HLDH activity, the determination of the former isoenzyme was considered to be more useful. In addition, the determination of ULDH activity was quicker than that of HLDH. However, the use of either TLDH, ULDH, or HLDH alone may be not clinically sufficient. A combination of the determinations would be more useful although the cost should also be considered.

It must be realized that enzymic diagnosis had both technical and clinical limitations. Technical limitation was due to hemolysis of red blood cells which are rich in LDH isoenzymes (Wroblewski and Ladue, 1955; Mellick, 1961; Erickson and Morales, 1961; and Amador et al, 1965). Improper storage of serum was also a problem (Wroblewski and Ladue, 1961; Estrin and Agress, 1963). Clinical error may resulted from either the uncertainty in the duration that myocardial infarction had happened or an elevation of the level of LDH isoenzymes which caused by other diseases.

An anticoagulant such as oxalate or heparin also affected the determination of LDH isoenzymes activity (Wroblewski and Ladue, 1955; Hsieh and Blumenthal, 1956; Novoa et al, 1959). Serum should be used to

avoid this problem. However, plasma was used throughout the experiments to avoid too much blood taken from the patients. The use of disodium edetate did not effect the determination and this was agreed with the work of Henry (1968).

Enzyme activity is generally expressed in terms of arbitrary units but the specific activity of enzymes varied among laboratories (Erickson and Morales, 1961; Richterich et al, 1963; Amador et al, 1963; and Fritz et al, 1970). Therefore, the data from different laboratories cannot be directly compared. The activity of the isoenzymes expressed as a change in optical density in a unit time requires no mathematical calculation. This is clinically useful because the relative activity of the isoenzymes in plasma of normal subjects and patients could be compared.

Studies of historical records showed that patients were often suffered from chest pain and the pain sometimes radiated to the left arm. Hypertension or hyperlipidemia also occurred in some cases. All these records should be taken into consideration together with the results from enzymic determinations in the diagnosis of the disease.

Although Enger and Ritland (1970) and Thomson and Wootton (1970) admitted that cholesterol is a factor in the development of coronary artery disease, the plasma cholesterol of the patients with myocardial infarction found in this study was not significantly different from those of the normal subjects. This may be because of the wide range found in plasma of both patients and normal subjects. It is not possible therefore, to use this determination as a diagnostic aid in the diagnosis of myocardial infarction.

The methods of Parekh and Jung (1970), Jung and Parekh (1971) and Fletcher (1968) for the determination of cholesterol and triglycerides required only small amount of plasma. These two methods were reliable and simple enough to be used in routine laboratories for determination of plasma lipids of the patients with myocardial infarction.

The use of Sudan Black B in zinc acetate for the staining of lipoproteins as reported by Dyerberg and Hjorne (1970), and Magnani and Howard (1971) was found to be satisfactory.