Chapter 2

MATERIALS AND METHODS

1. Chemicals

Nicotinamide adenine dinucleotide, nitro blue tetrazolium, phenazine methosulfate, and DL-lactic acid were biochemical grade from BDH Laboratory Chemical Division, England.

Difco Special Agar-Noble was from Difco Laboratories, U.S.A.

Other chemicals were reagent grade from BDH Laboratory Chemical Division, England; E. merck Ag. Darmstadt, West Germany; and J.T. Baker Chemical Co., U.S.A.

2. Instruments

Chromoscan Recording and Integrating Densitometer, Joyce-Loebl Company Limited, England.

Electrophoretic apparatus, Shandon Southern SAE 2525, Shandon Southern Instruments Limited, England.

Fisher Automatic Titrimeter, Fisher Scientific Company, U.S.A.

Gilson Omnibath, Universal Electric Company, Great Britain.

International Clinical Centrifuge, International Equipment Company, U.S.A.

Spectrophotometer

1. Hitachi Perkin-Elmer, UV-VIS Spectrophotometer, U.S.A.

2. Spectronic 20, Bausch & Lomb, Bausch & Lomb Incorporated, U.S.A.

Thermostat Waterbath, Elconap Laboratory Apparatus, Electric Heat Control Apparatus Company, U.S.A.

3. Subjects

3.1 Normal subjects

Plasma of normal subjects were obtained from The Blood Bank of The Thai Red Cross Unit, Chulalongkorn Hospital.

3.2 Patients

The patients were chosen from those who were conclusively diagnosed myocardial infarction by cardiologists. They were admitted into The Intensive Care Unit of Department of Medicine, Chulalongkorn Hospital, between January and April, 1973.

4. Collection and Storage of Plasma Samples

Venous blood samples of both the patients and normal subjects were taken after a fast period of 12 hours. Disodium ethylene diamine tetraacetate, 1mg /ml blood, was used as an anticoagulant (Henry, 1968). Whole blood was centrifuged twice at 2,000 and 3,000 rpm. in an International Clinical Centrifuge to eliminate blood cells; hemolyzed plasmas were discarded. The plasma samples for electrophoresis and lipids determinations were kept at 4°C and those for the determination of LDH activity were kept at -5°C (Erickson and Morales, 1961).

5. Preparation of Reagents

5.1 Buffer solutions

5.1.1 0.05 M Barbital buffer, pH 8.6 (Cawley, 1969)

Diethylbarbituric acid	1.84	gm
Sodium diethylbarbiturate	10.30	gm
Distilled water	1.0	L

5.1.2 Agar solution, 0.8% w/v;

Difco Special Agar-Noble was mixed in the barbital buffer (5.1.1) and warmed in a boiling water-bath until the agar dissolved. A large quantity of a stock agar solution was prepared, divided into suitable portions and stored in a refrigerator.

5.1.3 Glycine buffer, pH 8.7 (Ressler and Joseph, 1962)

Glycine	3.75	gm
Lithium chloride	1.32	gm
Distilled water, to make	1.0	L

The pH was adjusted to 8.7 with 1 N NaOH

5.1.4 Sodium pyrophosphate buffer, pH 8.8;

Sodium pyrophosphate. 10 H_2O	24.78	gm
Distilled water, to make	1.0	L

The pH was adjusted to 8.8 with 1 N NaOH

5.1.5. Urea-sodium pyrophosphate buffer, pH 8.8;

Sodium pyrophosphate. 10 H_2O	24.78	gm
Urea	133.50	gm
Distilled water, to make	1.0	L

The pH was adjusted to 8.8 with 1 N NaOH

5.2 Fixing solutions

5.2.1 For protein and lipoprotein electrophoretogram (Cawley, 1969)

Glacial acetic acid	100.0	ml
Methyl alcohol, to make	1.0	L

5.2.2 For LDH isoenzymes electrophoretogram;

Glacial acetic acid	1	part
Distilled water	5	parts
Methyl alcohol	5	parts

5.3 Staining solutions

5.3.1 For protein

Ponceau S	0.20	gm
3% aq. Trichloroacetic acid, to make	100.0	ml

5.3.2 For lipoprotein (Dyerberg and Hjorne, 1970)

Sudan Black B	0.40	gm
Ethyl alcohol	120.0	ml
Zinc acetate	4.0	gm
Distilled water	80.0	ml

The solution was filtered before use.

5.3.3 For LDH isoenzymes

Sodium lactate, 1 M	0.5	ml
Nicotinamide adenine dinucleotide, 10 mg /ml	0.8	ml
Nitro blue tetrazolium, 10 mg /ml	0.4	ml
Phenazine methosulfate, 0.2 mg /ml	0.8	ml
Glycine buffer, pH 8.7	20.5	ml

The solution was freshly prepared because the solution

of phenazine methosulfate is sensitive to light (Warburton and Waddecar, 1966).

5.4 Destaining solutions

5.4.1 For protein and LDH isoenzymes (Cawley and Eberhardt, 1962)

Glacial acetic acid	5.0	ml
Distilled water, to make	100.0	ml

5.4.2. For lipoprotein (Cawley, 1969)

Ethyl alcohol	55.0	ml
Distilled water, to make	100.0	ml

5.5 Nicotinamide adenine dinucleotide (NAD) solution

NAD	263.5	mg
Distilled water, to make	100.0	ml

The pH was adjusted to 7.0 with 1 N NaOH

5.6 Lactate solution

DL-Lactic acid (88%)	23.8	ml
Distilled water, to make	100.0	ml

The pH was adjusted to 8.8 with 1 N NaOH

5.7 Preparation of the gel layer

Microscopic glass slides(76 x 26 x 1mm.) were used as supporting medium. They were cleaned in 2% potassium dichromate in concentrated sulfuric acid, rinsed with tap water and then with distilled water. The cleaned slides were dried in an oven and stored in a tight container until use.

A portion of the stock solution from 5.1.2 (page 20) was resolution by heating in a boiling water bath and then left at room temperature until the temperature was approximately 40°C. The agar solution (3 ml) was spread on a cleaned glass slide which was laid on a levelling table in order to obtain a uniform layer. The gel was left for setting at room temperature for about 15 minutes and then kept in a humid chamber for at least 1 hour before use. The gel can be kept for 2 days without damaging effects.

A slot was prepared using a razor blade. The gel was cut transversely to a length of 1 cm at the distance 4 cm from the anode site.

5.8 The electrophoretic running procedure.

A Shandon Southern SAE 2525 coupling with Gilson Omnibath was used. The temperature of circulating water was set at 24°± 1°C. Barbital buffer solution (160 ml)was poured into both electrode vessels. Six slides of agar were placed on metallic plate in the electrophoretic chamber into 2 rows. Whatman No. 3 filter papers were used as paper wicks. They were placed between pairs of slides and at the end of slides with longer parts immersed in solution and shorter parts resting on gel layer.

The gels were prerun for 15 minutes with a current of 7 mA. per gel strip. A rectangular piece of rigid filter paper (Whatman No. 3) 1 cm long was inserted into the slit to dry the groove. Ten microlitres of plasma was applied into the slot of the gel using a micropipette. Electrophoresis was carried out within 8 hours after the

blood samples were collected at 24° ± 1°C for 60 minutes with a current of 7 mA. per gel strip.

5.9 Staining, destaining, fixing, and drying procedures

5.9.1 LDH isoenzymes;

Two of the six agar slides obtained from one electrophoresis run were used. The electrophoretograms were immediately stained for 1 hour in the dark at 37° ± 1°C in the solution 5.3.3 (page 21). Gentle agitation was applied at intervals of 15 minutes to prevent deposition of the dye which sometimes occurred when deeply stained bands were developed (Warburton and Waddecar, 1966). The reaction was stopped in the dark by immersing the gels three times in the destaining solution 5.4.1 (page 22) for 10 minutes and fixed in the fixing solution 5.2.2. (page 21)for 10 minutes. The gels were dried at 37°C under filter paper. The isoenzymes were semiquantitated by scanning on Chromoscan Recording and Integrating Densitometer.

5.9.2. Lipoprotein and protein;

Four agar electrophoretograms from each run were immediately fixed as soon as the electrophoresis was completed in the fixing solution 5.2.1 (page 21) for 20 minutes. These gels were dried at 37°C under filter paper.

Two dried electrophoretograms were stained for lipoprotein in the staining solution 5.3.2 (page 21) for 20 minutes and destained in the solution 5.4.2 (page 22) until the background was clear. The protein bands were used as markers for LDH isoenzymes (Van Der Helm, 1962).

After destaining, the electrophoretograms were dried at 37°C under filter paper.

5.10 Determinations of lactic acid dehydrogenase isoenzymes activity

5.10.1 Total lactic acid dehydrogenase isoenzymes (TLDH)

TLDH activity was determined spectrophotometrically using lactate as a substrate.

The method used was based on the method of Amador et al (1963) with certain modifications. The reaction mixture consisted of

Sodium pyrophosphate buffer, pH 8.8	2.70 ml
Nicotinamide adenine dinucleotide solution	0.10 ml
Plasma	0.10 ml

(Dilution was necessary for plasma with very high activity)

The reaction mixture was incubated at 25°C for 10 minutes. Then 0.1 ml of 1 M sodium lactate solution was added and mixed by gentle inversion. The mixture was transferred immediately into a Perkin Elmer cuvette of 1 cm light path. The increase in absorbance at 340 nm was recorded every 30 seconds for 5 minutes. All reactions were carried out at room temperature and determinations were performed in duplicates.

One unit of LDH activity was defined as an increase in absorbance of 0.001 per minute per milliliter of plasma at 25°C.

5.10.2 Heat-stable lactic acid dehydrogenase isoenzymes (HLDH)

The method used was based on those of Amador et al (1963), and Bell (1963). The reaction mixture consisted of 2.6 ml sodium pyrophosphate buffer, pH 8.8, and 0.2 ml of plasma. This mixture was

incubated at 60°C for 60 minutes and the reaction stopped by placing the tubes in an ice bath. HLDH activity was determined as described in the determination of TLDH activity.

5.10.3 Urea-stable lactic acid dehydrogenase isoenzymes (ULDH)

The method used was based on the method of Emerson and Wilkinson (1965). ULDH activity was determined as described in the procedure for the determination of TLDH activity. Urea-sodium pyrophosphate buffer pH 8.8 was used instead of sodium pyrophosphate buffer pH 8.8.

5.11 Determinations of plasma lipids

5.11.1 Plasma cholesterol

The determination of cholesterol was based on the colorimetric methods described by Parekh & Jung (1970) and Jung & Perekh (1971). Ferric acetate-uranium acetate and sulfuric acid-ferrous sulfate reagents were used respectively as a protein precipitant and a developing colour substance.

5.11.2 Plasma triglycerides

The colorimetric method of Fletcher (1968) was used for the determination of triglycerides. Triglycerides were saponified and then oxidized to glycerol and formaldehyde. Formaldehyde then formed a yellow substance with acetylacetone.