INTRODUCTION

Coronary artery is a main artery supplying nourishing substances to the heart muscle. This coronary artery arises from the aortic sinus and gives off two main branches; the right and the left. The right coronary artery supplies blood to the entire right ventricle, the posterior half of the interventricular septum, and a part of the left ventricle and gives rise to atrioventricular nodal and posterior descending arteries. The left coronary artery gives rise to the atrioventricular nodal and posterior descending arteries and supplies blood to all of the left ventricle, the entire interventricular septum, and a part of the posterior wall of the right ventricle. Various branches from coronary arteries supply blood to the two atria. In the heart with a balance circulation, which is the most common pattern, each ventricle receives its blood supply from the corresponding named coronary artery, and the posterior branch of the right coronary artery supplies blood practically to all of the interventricular septum while the anterior part receives its blood supply from the left coronary artery.

The most prevalent disease of coronary artery dysfunctions is artherosclerosis which accounts for more than 90% (Lyght et al, 1966). The disease can produce various degrees of narrowing and complete occlusion of the arteries, resulting in ischaemic heart disease.

Myocardial infarction, defined as a damage to a portion of the heart muscle by myocardial ischaemia, is usually the result of coronary artery occlusion. In association with artherosclerosis, coronary thrombosis is a major cause of occlusion (Anderson, 1966; Lyght et al, 1966).

Thrombi are usually found at sites of narrowing in the sclerotic arteries. They are occasionally present in more than one artery and occur in order of descending frequency, in the left anterior descending coronary artery, the right coronary artery and the left circumflex artery. Coronary occlusion is infrequently caused by embolism which mostly involves the left coronary and its branches (Lyght _et al_, 1966). Although emboli usually [illegible] arise from thrombi on artherosclerotic plaques in the root of the aorta, it may also originate from a thrombus in a main coronary artery or from mural thrombi in the left ventricle, left atrium, or left atrial appendage (Lyght _et al_, 1966).

Occlusions frequently occur in the left coronary artery, particularly in its anterior descending branch. The most common site of infarction is the anterior region of the left ventricle near the apex, including two-thirds of the anterior interventricular septum. The next common site, generally related to diseases of the right coronary artery, is the posterior part of the interventricular septum. Infarcts confined to lateral wall of the left circumflex artery are less common. The right ventricle is infrequently involved, and when there is a lesion, it is usually an extension of an infarct of the left ventricle. Infarcts of the atria are frequently unrecognized during life time or even at autopsy. Most of the infarcts occurred in the right atrium are usually associated with infarcts of the left ventricle and often accompanied by mural thrombi. It is believed that the oxygenated blood of the left atrium tends to protect this chamber from infarction (Anderson, 1966; Lyght _et al_, 1966).

Although the myocardial infarction is generally characterized by severe chest pain which is frequently referred to the left arm,

shoulder, back and abdominal region, it is sometimes characterized by a shock, a cardiac dysfunction and a sudden death. A common problem in clinical medicine is the diagnosis of acute chest pain. The historical records, clinical findings, and electrocardiographic evidence are usually sufficient for the diagnosis of those typical cases which are large infarctions. However, smaller infarctions, occur with one of the small coronary arteries, are usually not obviously noticeable. In such patients, severe chest pain may repeatedly occur without changes in the electrocardiogram or little transient lowering, inversion of the T wave may be noticed. Some cases have atypical features or show electrocardiograms which are difficult to interprete. This may be because of previous infarction, left bundle-branch block or previous left ventricular strain. In such instances, clinicians normally need the aid of clinical chemistry to clarify those cases (Griffiths, 1966; Russek and Zohman, 1971).

Clinical enzymology is now generally accepted as a useful diagnostic tool, especially when the clinical findings are complex or confusing or when the usual diagnostic criterias are inconclusive. Many enzymes are common to most tissues and are liberated on damaging or death of the cells in various portions and at different time according to the tissue involved (Rowan, 1970). It would be ideal for diagnostic purpose, if an enzyme is specific for a particular tissue organ or disease processes.

The most commonly and widely used enzymes, as a diagnostic aid in myocardial infarction, are glutamic oxalacetic transaminase (GOT), hydroxy -butyric acid dehydrogenase (HBDH), creatine phosphokinase (CPK) and lactic acid dehydrogenase (LDH) (Nissen et al, 1965; Coodley, 1968;

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Rowan, 1970). The advantages and disadvantages in using different enzymes are discussed below.

Glutamic oxalacetic transaminase is an enzyme that catalyzes the reversible transfer of an amino ($-NH_2$) group from an amino acid to a receptor keto acid, yielding a new amino acid and a new keto acid

$$\text{Aspartate} + \text{Ketoglutarate} \xrightleftharpoons{\text{GOT}} \text{Glutamate} + \text{Oxalacetate}$$

GOT is present in high concentration in heart and liver. The diagnostic value of GOT was first demonstrated by La Due et al, in 1954. Since then, a number of reports supporting its efficiency and usefulness in the diagnosis of myocardial infarction have appeared. Serum values rise within 12 to 24 hours after infarction, generally reach a peak 2 to 10 times of the normal level in 12 to 24 hours, and returns to the normal range within 3 to 6 days (La Due et al, 1956; Stewart and Warburton, 1961; and Rowan, 1970). However, the activity of serum GOT may be increased when either heart or liver tissue is damaged (Batsakis et al, 1960; Rosalki, 1963). Rowan (1970) stated that serum GOT level is a sensitive indicator of any cell damage.

The enzyme CPK functions as part of the high energy-storage system and serves to catalyze the reaction

$$\text{ATP} + \text{Creatine} \xrightleftharpoons{\text{CPK}} \text{ADP} + \text{Creatine phosphate}$$

This enzyme is a muscle specific enzyme. Heart and skeletal muscle are its richest sources. It is also found in lesser amounts in brain tissue, kidney, liver, pancreas, and the red blood cells. The distribution in human tissues has led to the consideration of serum CPK activity as the

most specific and sensitive enzyme test currently available for the confirmation of disease or injury to skeletal muscle and myocardium. It is usually possible to rule out muscular disease on the basis of history from even small infarction (Coodley, 1966; Batsakis, 1966; Griffiths, 1966; Hamolsky, 1967).

Serum level of CPK regularly rises in the course of an acute myocardial infarction. Serial determinations reveal the appearance of abnormal levels as early as 3 to 6 hours reaching its maximum activity usually between 12 to 14 hours after the onset of the infarction. An elevated activity generally persists through the third day (Stewart and Warburton, 1961; Nissen et al, 1965; Vincent and Rapaport, 1965; and Batsakis et al, 1966).

A major disadvantage in using CPK and GOT determinations for the detection of myocardial infarction is that the elevation following infarction lasts only four to five days after the infarct. Care must be taken, therefore, in choosing the time of which blood samples are obtained (Stewart and Warburton, 1961; Rosalki, 1963).

Other disadvantages of the CPK determination such as the inactivation of enzyme activity due to a delay in testing, storing of serum, have also been reported. Therefore, its activity should be determined within a few hours after drawing of blood samples (Nissen et al, 1965; Vincent and Rapaport, 1965). Vincent and Rapaport (1965) also found that the activity of CPK in serum of normal subjects was low and frequently undetectable by spectrophotometric method.

The enzyme HBDH catalyzes the reversible reaction of

$$\text{alpha-Ketobutyrate} + NADH_2 \xleftrightarrow{HBDH} NAD + \text{alpha-Hydroxybutyrate}$$

The high concentration of HBDH in many tissues including kidney, liver, muscle, white and red blood cells, and lung may explain the high sensitivity of the test in the detection of diseases involving most organ systems. The heart muscle is relatively rich in HBDH activity and consequently, heart lesions would be expected to have a greater influence on the activity of this enzyme in serum than those of kidney and liver. The HBDH determination shows a significant advantage over LDH as far as duration of the rise of enzyme level is concerned (Elliott and Wilkinson, 1961; Rosalki, 1963).

Stuart et al (1965) stated that the diagnostic efficiency of serum HBDH determination is greater than that of serum GOT determination, especially in the period of 3 to 10 days after the acute episode. They also found that HBDH was elevated in some patients with myocardial ischaemia.

Coodley (1968) found that serum HBDH was increased in acute liver congestion, liver damage, renal necrosis, and various types of anemia.

LDH, L-Lactate:NAD oxidoreductase, E.C.1.1.1.27, an enzyme of the glycolytic cycle, is almost universally distributed in the body. It catalyzes the reversible transformation of pyruvate to lactate and requires nicotinamide adenine dinucleotide (NAD) as an oxidizable-reducible coenzyme for the reaction according to the following equation

$$\text{Pyruvate} + \text{NADH}_2 \xleftrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}$$

(Richterich et al, 1963; Wootton, 1964).

LDH is specifically reacts with L-lactate. The D-isomer is inactive and unable to interfere with the oxidation of L-lactate even at a substrate concentration corresponding to half saturation of the enzyme (Neilands, 1952). Takenaka and Schwert (1956) found that the enzyme forms a complex with one form of the coenzyme and the resulting binary complex then forms a ternary complex with the substrate. The binary complex obtained from the binding of four active sulfhydryl groups on one LDH molecule with the coenzyme. Kaplan et al (1956) found that the nicotinamide group ($-CO-NH_2$) is not essential for NAD activity.

The reaction catalyzed by the crystalline LDH of beef heart can be represented by the following sequences

$$\text{E} + \text{NAD} \longleftrightarrow \text{E.NAD}$$

$$\text{E.NAD} + \text{Lactate} \longleftrightarrow \text{E.NAD.Lactate}$$

$$\text{E.NAD.Lactate} \longleftrightarrow \text{E.NAD}_2\text{.Pyruvate} \longleftrightarrow \text{E.NADH}_2 \quad + \text{Pyruvate}$$

$$\text{E.NADH}_2 \longleftrightarrow \text{E} + \text{NADH}_2$$

The kinetic behavior of this system is accounted for by a model in which the proton generated during the oxidation of lactate is accepted by an uncharged imidazole groups on the enzyme surface. The proton which must be supplied for the reduction of pyruvate is supplied by a charged imidazolium group. Further, an unionized sulfhydryl is essential for the binding of lactate to the enzyme-NAD complex and for the binding of

$NADH_2$ to the enzyme. The rate-limiting step in the forward direction of the over-all reaction is the rate of dissociation of $LDH.NADH_2$ complex

$$E.NADH_2 \longleftrightarrow E + NADH_2$$

(Winer and Schwert, 1958).

The molecular weight of the native enzyme LDH varies from 135,000-145,000. It can be splitted into subunits of molecular weight 36,000 ± 1,600 according to the species of sources (Neilands, 1952; Appella and Markert, 1961; Jaenicke, 1964; Appella and Zito, 1968).

The heterogeneity of the enzyme was first introduced by Neilands (1952). He stated that the enzyme is composed of two types of subunits which are referred to as "A" (anode) and "C" (cathode). The proposal became more significant when the concept of "isozyme" (isoenzyme) was introduced by Markert and Moller (1959) who used the term to refer to multiple forms of enzymes. Cahn et al (1962) suggested "M" and "H" for referring two types of the subunits since M subunit predominates in skeletal muscle and H subunit predominates in heart muscle. These types of subunits are beleived to combine in various tetrameric association, yielding five molecular species with different electrophoretic characteristic (see Table 1). All of them possess LDH activity (Appella and Markert, 1961; Cahn et al, 1962). Cahn et al (1962) also proposed a term "hybrid" to call the three intermediate forms of LDH since these hybrids differ qualitatively and quantitatively from the "parenteral" types.

Cahn et al (1962) found that different tissues from the same animal have a characteristic distribution of the five electrophoretic

TABLE 1

Characteristic of Lactic Acid Dehydrogenase

Class	Subnits	Electrophoretic Band	Subunit Monomer and Electrical Charges	M.W.x10^3	Principle Organ Localization	Metabolism
Monomer	H			35		Aerobic
	M			35		Anaerobic
Tetramer	HHHH	LDH-1	definite net negative charge	134	Heart, High aerobic metabolism	Aerobic
	HHHM	LDH-2	moderate negative charge	134		Intermediate
	HHMM	LDH-3	slight negative charge	134		Intermediate
	HMMM	LDH-4	very slight negative charge	134		Intermediate
	MMMM	LDH-5	no electrical charge	134	Skeletal,high anaerobic metabolism	Anaerobic

Revised from Markert and Moller, 1961; Cahn et al, 1962; and Reeves et al, 1969.

bands but the distribution are species specific. He indicated that these M and H types of LDH are under the control of different genes. This statement was supported by the work of Ressler and Tuttle (1966).

The skeletal muscle enzyme operates in the presence of relatively high level of pyruvate, an anaerobic breakdown product of carbohydrates for utilizable energy during vigorous exercise when the oxygen becomes limiting. Under these conditions, the reduction of pyruvate to lactate with NADH + H^+ as a reducing agent occurs. This results in a large production of lactic acid (Cahn et al, 1962; Fritz, 1967). The LDH isoenzyme from skeletal muscle (LDH-5) is an allosteric protein and a regulatory enzyme. It is activated by citrate, cis-aconitate, isocitrate, alpha-ketoglutarate, succinate, fumarate, malate, aspartate, and glutamate, and inhibited by oxaloacetate. This isoenzyme is also extremely sensitive to changes in pH within the range of 6.2 to 7.8 and completely destroyed when heated for 3 minutes at 60°C (Fritz, 1965; 1967).

The heart enzyme depends more on aerobic metabolism than the skeletal muscle enzyme. There is no sudden requirement of energy of the heart. Pyruvate metabolism in the heart is directed toward oxidation rather than toward reduction to lactate (Cahn et al, 1962). The LDH isoenzyme from rabbit heart muscle (LDH-1) is apparently neither allosteric nor regulatory enzyme. It is unaffected by changes of pH within the range 6.2 to 7.8. Its catalytic activity is stable when heated at 60°C for 3 minutes (Fritz, 1965; 1967).

The differences between LDH-1 and LDH-5 about substrate inhibition will decrease as much as the reaction undergo near physiological pH and temperature. At pH 8.3, both LDH-1 and LDH-5 react with lactate as

as well as pyruvate, and at 40°C, they both similarly react with pyruvate (Vessel, 1966).

Dissociation of LDH from chicken heart and beef heart into subunits has been achieved with sodium dodecyl sulfate (Sabato and Kaplan, 1966). The dissociation of the enzyme into subunits is accompanied by loss of enzymatic activity without marked changes in the structure of the subunits. Reduced nicotinamide adenine dinucleotide and its acetyl pyridine analogue protect the enzyme from dissociation. The coenzyme also protect the catalytic activity. Four molecules of coenzyme appear to bind to one molecule of enzyme and the prevention of dissociation by the reduced coenzymes may be due either to a locking of subunits or to a conformational changes. The LDH are apparently elongated molecules (Sabato and Kaplan, 1964; Pesce et al, 1964).

The attachment of subunits may be not due to hydrogen bonding or hydrophobic bonding as stated by Cahn et al (1962) because of the stability in 8 M urea. It may be due to salt linkages that can be splitted by guanidine hydrochloride. However, the interaction of the four subunits is essential for stability as well as to give the molecule the tertiary structure. It may also be necessary for the catalytic function and all four subunits seem to be necessary for enzymatic activity (Cahn et al, 1962; Jaenicke, 1964).

Determinations of the activities of plasma or serum LDH is valuable in establishing the diagnosis of acute myocardial infarction since experimental and clinical myocardial infarction are associated with a characteristic rise in serum LDH activity (Wroblewski and Ladue, 1955).

Serum LDH activity level was found to be elevated in situations where tissue necrosis occurs in AA-sarcoma from rats, neoplasm, granulocytic leukemia, carcinoma of pancreas and gall bladder, myocardial infarction, infective hepatitis, obstructive jaundice, necrotizing pancreatitis, and skeletal muscle trauma and/or surgical trauma resulting in tissue necrosis. It has been demonstrated that serum LDH activity alteration associates with tissue necrosis, the rapidity with which it occurs, the rate of loss of enzyme from the tissues, the accessibility of the lost enzyme to circulation, and the normal range of serum LDH (Wroblewski and Ladue, 1955; Borroughs and Hill, 1956; Hsieh and Blumenthal, 1956; Wroblewski, 1958).

Although it is generally an accepted fact that the determination of activity of LDH in the serum has been a useful guide in clinical study of pathologic conditions, it is also a well documented fact that the activity of serum LDH may be elevated in such diverse conditions as mentioned above. This lack of specificity between disease state and the level of enzyme in serum has seriously limited its usefulness, and has led to searchs for a more refined method of measuring LDH.

It was apparent from literature studies that LDH was initially determined as the total LDH activity both in sera and tissues. This was not only found to be nonspecific but also have tended to become no longer useful as knowledge of the isoenzymes of LDH grows. These studies indicated that LDH in serum is composed of several forms of LDH. Each one may originate from different tissue. The LDH isoenzymes pattern varies with the tissue studied (Cahn et al, 1962), with the development state (Neilands, 1952; Markert and Moller, 1959) and with specific disease

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(Wroblewski and Ladue, 1955; Hsieh and Blumenthal, 1956; Wieme and Maercke 1961; Reeves et al, 1969). The confirmation of organ-specific diagnosis has been improved considerably by the difference in electrophoretic mobility due to the total charge differences of the basic and acidic amino acids (Pesce et al, 1964).

Various media have been used for the separation of the LDH isoenzymes. Vessel (1958), Allen (1961), Ressler and Tuttle (1966), and Emery (1967) reported the separation of LDH on starch gel electrophoresis. Van Der Helm (1961), Cawley and Eberhardt (1962), Ressler and Joseph (1962), Yakulis et al (1962), and Mull and Starkweather (1965) used agar as a medium in electrophoresis. Barnett (1962), Lance (1966), and Warburton and Waddecar (1966) reported a method for separation and quantitation of LDH isoenzymes on cellulose acetate. Richterich et al (1963) separated LDH isoenzymes by adsorption-elution column chromatography on Sephadex-DEAE. The use of disc electrophoresis of LDH isoenzymes was reported by Davis (1964), and Dietz and Lubrano (1967).

Normal human serum shows 3 to 5 bands in electrophoresis. In certain diseases, as many as 5 bands can be identified (Vessel, 1958; Markert and Moller, 1959; Appella and Markert, 1961; Blanchaes, 1961; Wroblewski and Gregory, 1961). Serum of patients who were attacked with myocardial infarction shows a marked increase in LDH-1 whereas the increase of LDH-4 and LDH-5 are more apparent in serum of patients with cirrhosis of the liver (Van Der Helm, 1961).

In electrophoretic method, nitro blue tetrazolium (NBT) has been used to form an insoluble purple formazan with the isoenzymes by the following reaction

L-Lactate + NAD ⟷ Pyruvate + $NADH_2$

$NADH_2$ + Phenazine methosulfate ⟷ NAD + Phenazine methosulfate (reduced)

Phenazine methosulfate (reduced) + NBT ⟷ Formazan + Phenazine methosulfate

(Van Der Helm, 1961 ; Cawley, 1969).

Plasma LDH activity can be determined spectrophotometrically with either lactate or pyruvate as substrate, using the difference of maximum light absorption at 340 nm of the nicotinamide adenine dinucleotide. $NADH_2$ shows a maximum light absorption at 340 nm, whereas the absorption of NAD at this wavelength is negligible. The determination is easily performed by measuring either the rate at which $NADH_2$ is oxidized (decrease in optical density at 340 nm) or NAD reduced (increase in optical density at 340 nm). The reduction of pyruvate to lactate is favorable in the pH range of 7 to 8, whereas the reverse reaction favored the pH from 8.8 to 10 (Erickson and Morales, 1961; Amador et al, 1963; Amador et al, 1965).

In order to avoid the tedious work in electrophoresis, several modified methods for the determination of LDH isoenzymes have been reported. Strandjord and Clayson (1961) stated that heat-stable LDH (HLDH), the activity remained after an incubation in a water-bath at 60°C for 60 minutes, appears to arise mainly from heart muscle. Hence, determination of the serum HLDH activity permits the diagnosis of myocardial infarction especially in the presence of acute hepatocellular injury (Strandjord and Clayson, 1961; Wroblewski and Gregory, 1961; Bell, 1963; Peters and Davis, 1969; and Auvinen and Konttinen, 1971).

Emerson and Wilkinson (1965) have introduced the use of oxalate or urea as an aid in the enzymatic diagnosis of heart and liver diseases. In sufficient concentrations of urea, a protein denaturant, LDH isoenzymes with different subunits reacts differently to the denaturants. The activity of LDH-4 and LDH-5 has been shown to be inhibited by a certain concentration of urea which show little effect on LDH-1 and LDH-2. 2 M urea is the most suitable concentration for the differentiation of cardiac isoenzymes (LDH-1 and LDH-2) from the isoenzymes of liver and skeletal muscle (LDH-4 and LDH-5) (Emerson and Wilkinson, 1965; Lindy and Konttinen, 1967). The serum LDH of patients with liver disease was inhibited by 2 M urea to a greater degree and that of the patients with myocardial infarction to a lesser extent than that of normal persons. This substantiated the use of urea as inhibitors in the investigations.

Since myocardial infarction is closely related to artherosclerosis and thrombosis, the work in this thesis also concerned electrophoretic separation of lipoprotein simultaneously with LDH. Sudan Black B in zinc acetate was used to stain lipoprotein bands (Dyerberg and Hjorne, 1970). Plasma cholesterol and triglycerides were also examined. The determination of plasma cholesterol was based on the methods of Parekh and Jung (1970) and Jung and Parekh (1971). Ferric acetate-uranium acetate and sulfuric acid-ferrous sulfate reagents were used respectively as a protein precipitant and a developing colour substances. Plasma triglycerides was carried out by the method of Fletcher (1968), using acetylacetone to form colour substance dihydrolutidine derivatives with formaldehyde.

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To the best of the author's knowledge, attempts to use the determinations of changes in the activity of LDH isoenzymes and plasma lipids concentrations as a diagnostic aid of myocardial infarction have never been reported in Thailand. The work in this thesis suggests the use of a few practical methods as clinical means for diagnosis myocardial infarction which is potentially a fatal disease in Thai people, bearing in mind that tediousness and cost of the methods should also be taken into consideration.

Experimental Protocol

The investigation can be divided into four parts

Part 1: Comparative studies of various concentrations of Difco Special Agar-Noble and the preservation of agar gel, width of slit, application zone, amount of plasma used, temperature and time consumed in electrophoresis

Part 2: Comparative studies of various dye-staining methods for LDH isoenzymes and lipoproteins

Part 3: Comparative studies of various destaining method, fixing solution and drying temperature

Part 4: Investigation of changes of LDH isoenzymes level in terms of TLDH, ULDH, HLDH, cholesterol, triglycerides and lipoproteins in plasma of patients with myocardial infarction taken at different days after the onset of the attack