1979

Cardiac isoenzymes.

Michael Royce. Goodwin

University of Windsor

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LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS REÇUE
CARDIAC ISOENZYMES

by

MICHAEL ROYCE GOODWIN

A Major Clinical Chemistry Critique
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario
Canada

1979
CARDIAC ISOENZYMES

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MICHAEL ROYCE GOODWIN

ABSTRACT

This critique reviews the course of development of clinical enzymology as it developed from a science dealing with extracellular enzymes to one dealing with the multitude of enzymes encased within the cellular membranes to those contained within the mitochondrial cristae. A description of some of the basic aspects of cardiac anatomy, physiology and biochemistry is attempted as it relates to an understanding of cardiac isoenzymes.

The section dealing with pathology of the heart covers such topics as description of major types of heart disease, incidence of coronary heart disease, risk factors and related phenomena. The causes of acute myocardial infarction are discussed along with the effect of acute myocardial infarction on cellular respiration. The clinical course of the infarction is described along with traditional means of evaluation.

The next part of the paper deals with isoenzymes from a structural standpoint, but also deals with distribution throughout the human body. A discussion ensues which covers their usefulness in the diagnosis of
acute myocardial infarction. The isoenzymes discussed in detail are creatine kinase, lactate dehydrogenase, aspartate transaminase, and the multimolecular forms of aldolase.

The methods for separation of the isoenzymes occupy a large portion of the paper as they cover in detail those methods in general use in laboratory medicine. Methods include isoenzyme separation by electrophoresis, column chromatography, activation, antibody, chemical and heat inactivation. This is followed by a discussion on choice of methods.

The paper concludes by discussing the diagnostic usefulness of cardiac isoenzymes, in particular their use in cardiac profiling and infarct sizing.
ACKNOWLEDGEMENTS

I would like to thank Drs. T.F. Draisey, R.J. Thibert, and N.F. Taylor for their help and encouragement in the preparation and writing of this critique.

I also wish to extend my personal thanks to Ms. Valerie Franczuk for the expert typing of this manuscript. Her knowledge of thesis layout made my task much easier.

Lastly, I owe my wife Jo an immense debt of gratitude for her forebearance of my continual procrastination and for the extensive time she spent in proof reading and correcting the text.
DEDICATION

TO
MY WIFE
JOCELYN
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ABBREVIATIONS AND DEFINITIONS

ADP  Adenosine Diphosphate
AK   Adenyl Kinase
ALT  Alanine Transaminase
AMI  Acute Myocardial Infarction
AMP  Adenosine Monophosphate
AST  Aspartate Transaminase
ATP  Adenosine Triphosphate
ATPase Adenosine Triphosphatase
CA   Cellulose Acetate
CHD  Coronary Heart Disease
CK   Creatine Kinase
CK-1 Creatine Kinase Isoenzyme 1
CK-2 Creatine Kinase Isoenzyme 2
CK-3 Creatine Kinase Isoenzyme 3
CK-BB Creatine Kinase Isoenzyme 1, Dimer Composition
CK-MB Creatine Kinase Isoenzyme 2, Dimer Composition
CK-MM Creatine Kinase Isoenzyme 3, Dimer Composition
DEAE Diethylaminoethyl
DTT  Dithiothreitol
G6PDH Glucose-6-Phosphate Dehydrogenase
GSH  Glutathione
HBD  Hydroxybutyrate Dehydrogenase
HHHH Tetramer Structure of Lactate Dehydrogenase Isoenzyme 1
I\textsuperscript{125} Radioactive Nuclide with Mass of 125
IU   International Unit
$K_m$  Michaelis-Menten Constant
L  Liter
LDH  Lactate Dehydrogenase
LDH-1  Lactate Dehydrogenase Isoenzyme 1
LDH-2  Lactate Dehydrogenase Isoenzyme 2
LDH-3  Lactate Dehydrogenase Isoenzyme 3
LDH-4  Lactate Dehydrogenase Isoenzyme 4
LDH-5  Lactate Dehydrogenase Isoenzyme 5
Mb  Myoglobin (reduced form)
MbO$_2$  Myoglobin (oxygenated form)
MHHH  Tetramer Structure of Lactate Dehydrogenase Isoenzyme 2
MI  Myocardial Infarction
mL  Milliliter
mM  Millimolar
MHHH  Tetramer Structure of Lactate Dehydrogenase Isoenzyme 3
MMMHH  Tetramer Structure of Lactate Dehydrogenase Isoenzyme 4
MMMMH  Tetramer Structure of Lactate Dehydrogenase Isoenzyme 5
MTT  Methylphenazonium Methosulphate
NAD  Nicotinamide Adenosine Dinucleotide Oxidized Form
NADH  Nicotinamide Adenosine Dinucleotide Reduced Form
NADP  Nicotinamide Adenosine Dinucleotide Phosphate Oxidized Form
NADPH  Nicotinamide Adenosine Dinucleotide Phosphate Reduced Form
pH  Hydrogen Ion Concentration
Pi  Inorganic Phosphate Group
PK  Pyruvate Kinase
PO2  Partial Pressure of Oxygen
r   Correlation Coefficient
RIA  Radioimmunoassay
SAT  Specific Activation Technique
TCA  Tricarboxylic Acid Cycle
μM  Micromolar
V   Volts

Predictive value
The frequency of diseased patients among all patients with positive test results for a particular disease

Sensitivity
The frequency of positive test results in patients with a particular disease

Specificity
The frequency of negative test results in patients without the particular disease
CHAPTER I

INTRODUCTION

Enzymes have been used in clinical chemistry as an aid to diagnosis since the early 1930's (1, 2) when Cherry and Crandall produced a method for lipase in duodenal contents and Norby produced a method for amylase also in duodenal contents. The late 1930's saw methods for amylase in serum and urine (3) and the 1940's saw modification of these existing methods to give them greater sensitivity and specificity (4). This early work formed the basis for our present knowledge of clinical enzymology. The most important aspect of enzymology in this early period was that all determinations were involving enzymes produced by an organ for substrate utilization far removed from the site of production. An example of this is pancreatic amylase produced in the pancreas with site of action in the duodenum; similarly, lipase and trypsin, just to mention a few.

It was not until the 1950's that the significance of truly intracellular enzymes was realized. It was Karmen and his co-workers in 1955 (5) who first suggested a possible correlation between so-called transaminating enzymes and ischemic heart disease, and since this time progress in clinical enzymology has gone hand in hand with the biochemical study of metabolic pathways in an attempt to find organ specific enzymes. This search for organ specificity proved virtually fruitless, since most metabolic processes are
common to all organs of the body. In 1950 Meister (6) observed that beef heart LDH could be separated into two protein components. Two years later, in 1952, Nielands demonstrated enzymatic activity in the two separated proteins (7). In 1959 Wiene and Demeulenaère (8), using agar gel electrophoresis, demonstrated five distinct bands of LDH activity in serum. It was soon realized that a large number of enzymes exist in multiple forms, and the word isozyme, or preferably isoenzyme, was suggested in 1959 by Markert and Møller (9) for these multiple forms. Since that time a clear definition of an isoenzyme has not been forthcoming. A broad definition which best suits our present knowledge might be, "different proteins with similar enzyme activity."

The exact biological significance of isoenzymes has not been elucidated except for a few isolated examples. Most of the early work on the nature of isoenzymes was in connection with lactate dehydrogenase which remains to this day the greatest area of endeavor as to structure and function of isoenzymes. The classic treatises of Latner and Skillen (10) and Wilkinson (11) indicate the scope of our knowledge of isoenzymes.

Even though the exact role of isoenzymes is poorly understood, their use as markers of specific tissue involvement in disease processes has guaranteed their inclusion as a diagnostic tool in clinical enzymology. Iso-enzymes can have the effect of turning relatively non-specific
enzyme tests into organ specific tests. The study of the relationship between a given disease process and a given isoenzyme pattern continues. The most recent work has involved studies of isoenzyme patterns of LDH/CK in acute myocardial infarction (AMI) (12, 13).

The isoenzymes of LDH and CK have been used as part of a cardiac profile, and have been shown to be especially useful for borderline MI and non-MI patients. Cardiac isoenzymes are any isoenzymes which are involved with metabolism of the heart, whether they are associated with membranes, cytosol, or mitochondria of the cardiac muscle cell. Present clinical laboratory practice utilizes mainly LDH and CK isoenzymes, but future research will no doubt encompass other isoenzymes such as the mitochondrial aspartate transaminase isoenzymes. There has also been a recent advance concerning aldolase isoenzymes (14). All of these will need further evaluation both in the laboratory and in the clinical setting.
CHAPTER II

ANATOMY, PHYSIOLOGY AND BIOCHEMISTRY OF THE HEART

The heart beats continuously, resting only during diastole, the duration of which is 0.53 to 0.14 seconds per minute depending on heart rate, from the moment of birth until the time of death. It is an organ with little functional reserve and no regenerative capability; hence, once badly damaged, the chances of survival without medical support are minimal. This observation is supported by the high percentage of fatalities associated with AMI victims before they reach medical help. According to the statistics of insurance companies, heart disease tops the list of causes of death in western society, contributing to an excess of 600,000 deaths per year in North America alone. With this ominous background, it is important to look at the role of the clinical laboratory in the diagnosis of coronary heart disease (CHD). The laboratory is able to assist the physician in his diagnosis by making available to him tests that can indicate firstly, the presence of cardiac injury as soon as possible after it has occurred, and also the extent and size of the myocardial damage. To obtain a proper insight into the overall picture of heart disease it is necessary to review the anatomy, physiology and biochemistry of the heart.
A. ANATOMY AND PHYSIOLOGY

The weight of the average male heart is about 300 grams and the female heart 250 grams, although this varies and is related to the musculature of the person in question. It is located in the thoracic cavity behind the sternum and rib cartilages, between the lungs and immediately above the diaphragm (See Figure 1). The heart is composed of a special type of muscle (known as cardiac muscle) and valves (Figure 2). The heart valves are made of an extremely thin and ultrafine membrane known as the endocardium, which also lines the interior surfaces of the heart. The pericardial cavity is the space in which the heart expands and contracts, and is lined by the same delicate membrane as the inside of the heart. This membrane is called the pericardium.

The external equivalent of the endocardium is the epicardium, the layer of pericardium which is in contact with the heart. The myocardium is in between the endocardium and the epicardium and is the thickest part of the heart wall. It is composed of a special type of muscle called cardiac muscle. Figure 3 shows the relationships between epicardium, myocardium and endocardium.

The heart is divided into four cavities known as atria and ventricles, there being two of each. The left atrium receives oxygenated blood from the lungs. From there the blood passes to the left ventricle, which forces it via the aorta through the arteries to supply the tissues of the body. The right atrium receives the blood after it has
Overall Diagram of Heart Showing its Relation to Other Structures
Overall Diagram of the Heart Showing its Relation to Other Structures
FIGURE 2

Heart Showing Valves and their Relationship to the Atria, Ventricles and Other Structures

FIGURE 2

Heart Showing Valves and their Relationship to the Atria, Ventricles and Other Structures
passed through the capillaries and given up most of its oxygen. The blood now passes to the right ventricle, and then to the lungs for reoxygenation. There are four major valves which are the left and right atrioventricular valves and the aortic and pulmonary valves. The left atrioventricular valve lies between the left atrium and the left ventricle. The right atrioventricular valve lies between the right atrium and the right ventricle. The aortic valve is situated at the orifice of the aorta within the left ventricle, while the pulmonary valve is found at the orifice of the pulmonary trunk in the right ventricle.

It is very important for heart muscle to have a plentiful supply of blood since it is the blood which transports oxygen to the heart for its own use in aerobic tissue metabolism. The heart functions almost exclusively under aerobic conditions, and if forced to function under anaerobic conditions it rapidly loses efficiency. The reason for this is metabolic and will be discussed in detail under the section dealing with metabolism. The blood supply of the heart consists of the coronary arteries, the cardiac veins and collateral circulation which can be further subdivided into cardiac and extracardiac circulation. The exact location of the arteries and veins of the heart can best be visualized by referring to Figure 4 and 5 (15).

If cardiac collateral circulation is obstructed anastomosis can occur. Branches of the coronary arteries seldom anastomose on the epicardial surface of the heart.
FIGURE 3

Scheme of Myocardial Circulation
This data was taken from reference 15 without permission of the author.

FIGURE 4

The Coronary Arteries
This data was taken from reference 15 without permission of the author.
FIGURE 3

Scheme of Myocardial Circulation

FIGURE 4

The Coronary Arteries
Anastomoses do occur in the myocardium, but the vessels involved are small. If the need arises, the anastomoses can enlarge and become functional, blood-carrying vessels. It is worthy of note that intercoronary arterial anastomoses have been found in less than 10 percent of so-called normal hearts, but in excess of 40 percent in anemic hearts, and in 100 percent of hearts with old coronary occlusion (16).

If both coronary arteries are obstructed, there is an extracardiac collateral circulation, but unless it has had extensive preparation time it does not function. The extent of the vascularization can best be observed by reference to Figure 6.

Up to this point in the paper, the discussion has centered on the gross anatomy of the heart. From the biochemist's position, an understanding of the structure of cardiac muscle is important, since this facilitates a clearer understanding of the chemistry and metabolism of cardiac muscle.

Of the three types of muscle that are generally considered, namely skeletal, cardiac and smooth, the myocardium consists of cardiac muscle. The most studied of the muscles is skeletal muscle. There are a lot of similarities in function and structure but there are differences, and it is these differences that will be emphasized.

(1) Structure of Cardiac Muscle

Cardiac muscle is made up of fibrils surrounded by an electrically excitable membrane called the sarcolemma.
FIGURE 5

The Cardiac Veins
This data was taken from reference 15 without permission of the author.

FIGURE 6

Transverse Section of Ventricles Showing Branches of Coronary Arteries Plunging into the Heart Substance
This data was taken from reference 15 without the permission of the author.
FIGURE 5

The Cardiac Veins

FIGURE 6

Transverse Section of Ventricles Showing Branches of Coronary Arteries
Plunging into the Heart Substance
These fibrils, in combination with other fibrils, make up a cardiac muscle fiber. These fibers do not exist as individual muscle cells, but exhibit numerous anastomoses between different parts of the fibers. The myocardium exists as a syncytium (a multinucleated mass of protoplasm produced by merging of individual muscle cells). See Figure 7 for details, which represents cardiac muscle as seen under the light microscope. The fluid within the intramuscle cellular space is known as sarcoplasm and within this fluid is contained the high energy compounds (creatine phosphate and ATP), glycogen and the enzymes of glycolysis.

The sarcomere (Figure 8) is the functional unit of cardiac muscle and is repeated along the length of a fibril at 2.5 μm intervals. When observed under polarized light, the fibrils of cardiac muscle are observed to have alternating bands that are either optically isotropic (I bands) or anisotropic (A bands). A more detailed study of cardiac muscle fibers shows that A bands are thicker than I bands; I bands have a denser zone in their centre known as the Z line; A bands are bisected by a clearer zone called the H band, the centre of which contains another band known as the M line. These features can be seen in Figure 9. The intercalated disc as seen in Figure 7 is the place where two fibers butt. This provides for a strong union between fibers, and also provides for cell to cell cohesion, so that the pull of one contractile unit can be transmitted along its axis to the next. Along the side of the muscle fibers
FIGURE 7

FIGURE 7

Cardiac Muscle as Seen by the Light Microscope

[Diagram showing intercalated disk, nucleus, and fiber with a scale of 10 µm]
FIGURE 8

Cardiac Muscle as Seen Under the Electron Microscope
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FIGURE 8

Cardiac Muscle as Seen Under the Electron Microscope

FIBER

Fibrils
Sarcolemma
Sarcoplasmic reticulum
T system
Terminal cisternae

Mitochondria
Intercalated disk

2 µm

B
Capillary
FIBRIL
SARCOMERE
Arrangement of Filaments as Seen in Extended Cardiac Muscle

FIGURE 9

Arrangement of Filaments as Seen in Extended Cardiac Muscle
next to the discs, the cell membranes of adjoining fibers fuse for considerable distances. These "tight" junctions provide low resistance bridges for the spread of excitation from one fiber to the next.

The muscle fibrils are surrounded by structures made up of a membrane, an extension of the sarcolemma, which appear in electron photomicrographs as vesicles and tubules. These structures form the sarcotubular system, which is made up of a T system and a sarcoplasmic reticulum. The T system in cardiac muscle is located at the Z lines rather than the A-I junction (as in skeletal muscle). Cardiac muscle contains large numbers of elongated mitochondria in close proximity to the myofibrils. The function of the T system is the rapid transmission of the action potential from the sarcolemma to all the fibrils in the muscle. The sarcoplasmic reticulum is involved with calcium movement and muscle metabolism. For overall structural relationships, see Figure 8.

B. BIOCHEMISTRY

(1) Chemistry of Cardiac Muscle

The chemistry of cardiac muscle is the chemistry of the contractile proteins, which are myosin, actin, tropomyosin and troponin.

a) Myosin

Myosin, the most plentiful of the muscle proteins, is a globulin, soluble in weak salt solution but insoluble in water. It is a very large molecule, molecular weight
500,000 daltons, comprising two identical major chains of 200,000 daltons molecular weight each and four light chains of approximately 20,000 daltons molecular weight each. The structural relationships of the two globular regions and the light chains can best be visualized by reference to Figure 10.

Myosin exhibits enzyme activity and is able to function as an adenosine triphosphatase (ATPase). Myosin binds to the polymerized form of actin, another muscle protein, to form a protein complex known as actomyosin, consisting of three myosin molecules and one actin molecule. This interaction is essential to the generation of the force involved in the movements of the thick and thin filaments in muscle contraction (Figure 11).

b) Actin

Actin is a globulin of 60,000 daltons molecular weight and is the major constituent of the thin filaments in cardiac muscle. Actin is found in two forms, G- and F-actin. G-actin is a globular protein, monomeric in structure, and having a molecular weight of 42,000 (Figure 12). Actin G, in the presence of calcium ions and ATP, polymerizes to the fibrous form, F-actin, which under the electron microscope appears as a double stranded helix of actin monomers. During this polymerization ATP is hydrolyzed to ADP and Pi is liberated, since actin, like myosin, has ATPase activity. From this it can be seen that the depolymerization of F-actin to G-actin also requires the addition of ATP.
FIGURE 10

Myosin Molecule (Diagramatic)

LEGEND

G = globular region
L = light chains

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36, p 634.
FIGURE 10

Myosin Molecule (Diagramatic)
Arrangement of Filaments as Seen in Contracted Cardiac Muscle
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from Review of Physiological Chemistry. 16th ed. Lange Medical
FIGURE 11

Arrangement of Filaments as Seen in Contracted Cardiac Muscle

THIN FILAMENT
THICK FILAMENT
FIGURE 12

Schematic Representation of the Thin Filaments, Showing the Spatial Configuration of the Three Major Components
FIGURE 12

Schematic Representation of the Thin Filaments, Showing the Spatial Configuration of the Three Major Components
As mentioned previously, actomyosin is formed when actin and myosin combine. This reaction only takes place under strict conditions of pH, potassium and magnesium ion concentrations. The associated complex is stable in the absence of ATP, so when ATP is added the complex dissociates (Figure 13). It is of interest to note that the ATPase activity of actomyosin is considerably greater than that of either actin or myosin. The dissociation and association of actin and myosin may be steps in the breaking and making of the cross-linkages between the thick and thin filaments of the cardiac muscle fibrils.

c) Tropomyosin and Troponin Complex

These are proteins located in the thin filaments of the muscle, and comprise approximately one third of the muscle mass. Calcium ions are physiological regulators of muscle contraction. These ions exert their effect on the interaction of actin and myosin, and in turn are mediated by tropomyosin and troponin.

Tropomyosin is a double stranded helical rod of 70,000 daltons molecular weight positioned between the double strands of F-actin. Troponin is a complex of three polypeptide chains labelled TpC, TpI and TpT (Figure 12). This complex is located in the thin actin filaments at intervals of about 40 nm, bound to a tropomyosin molecule, and from this position it regulates the activity of about seven actin monomers. A diagrammatic representation of the "assembled thin filament" can be seen in Figure 12.
FIGURE 13

Dissociation of Actomyosin by ATP

LEGEND

The rapid drop of viscosity accompanies dissociation. As ATP is dephosphorylated the actin and myosin reassociate.

This data was taken from reference 101 without the permission of the author.
FIGURE 13

Dissociation of Actomyosin by ATP
Two other proteins, neither of which are contractile proteins, are myoglobin and myoglobin.

Myoglobin is a chromoprotein found in appreciable quantities in cardiac muscle and to a much lesser extent in skeletal muscle. It is a small globular protein having a molecular weight of 16,700 daltons. Myoglobin is able to react with oxygen to form oxymyoglobin (MbO₂), which is in equilibrium with deoxymyoglobin (Mb).

\[
\text{Mb} + \text{O}_2 \rightleftharpoons \text{MbO}_2
\]  

(1)

The position of equilibrium is dependent on the concentration of oxygen in the system. Myoglobin may, therefore, be considered as a storage reserve for oxygen as it is largely in the oxygenated form in which it is present in the myocardial sarcoplasm. If the PO₂ of the muscle cells should fall, then the oxymyoglobin dissociates in an attempt to maintain the cellular PO₂. It is interesting to note that deep diving mammals such as dolphins make use of myoglobin to provide a store of oxygen. Its role in human cardiac muscle during periods of oxygen debt is thought to be similar, but its functional reserve is limited.

Myogen is really not a true protein as such, but is the result of cold water extraction of homogenized muscle and contains mainly the enzymes of glycolysis, that is extra mitochondrial material, and does not include the enzymes of the T.C.A. cycle or respiratory chain.

(2)- Molecular Basis of Contraction

The unit of contraction of muscle, the fibril, is
composed of at least four different proteins which are, as mentioned above, myosin and the actin, tropomyosin-troponin complex. On examination under the electron microscope, it appears that each fibril is build up of two types of longitudinal filaments, the thick myosin filaments and the thin actin, tropomyosin-troponin filaments. Cross section shows the thick fibers surrounded by a hexagonal array of thin fibers. A diagrammatic representation of the thick-thin filaments can be seen in Figures 8 and 11. On contraction, the A band remains the same length but the I band disappears. It has been suggested that on contraction, the individual filaments of myosin and actin complex remain unchanged in length but that a change in length of the muscle (contraction) is brought about by the sliding of the two filament arrays into or out of each other (see Figures 9 and 11). The sliding during contraction is presumably produced by breaking and reforming of the cross-linkages between the myosin and the actin complex, possibly by the formation of actomyosin at the interface of the myosin and the actin complex.

The process by which depolarization of the muscle fiber initiates contraction is called "excitation-contraction coupling." The action potential is transmitted to all the myofibrils in the fiber via the T system. There is evidence to suggest that the action potential in muscle triggers the release of calcium ions from portions of the sarcoplasmic reticulum next to the T system. The interaction of actin and myosin is inhibited by troponin and tropomyosin when calcium
ions are absent. Calcium is bound by the troponin; this has the effect of inhibiting the inhibitory effect on contraction, thereby permitting the actin complex to slide on the myosin. It is plain to see why the troponin and tropomyosin are referred to as "relaxing proteins." During the resting state of the muscle, calcium ions are maintained in the sarcoplasmic reticulum. Since there will be a tendency for the calcium ion to move to an area of lower concentration, for example the sarcoplasm, there will have to be an active transport system for calcium ions. This system is energized by ATP, and has the effect of lowering the concentration of calcium in the sarcoplasm and increasing its concentration in the sarcoplasmic reticulum where it is stored and bound by a calcium binding protein called calsequestrin. When calcium is released there is activation of muscular contraction via the troponin–tropomyosin complex as illustrated below.

\[
\text{Ca}^{2+} \rightarrow \text{troponin} \rightarrow \text{tropomyosin} \rightarrow \text{actin} \rightarrow \text{myosin} \rightarrow \text{actomyosin}
\]

(2)

(3) Metabolism of Cardiac Muscle

Much has been said about ATP and energy in relation to muscular contraction, but up to this point nothing has been said as to the origin or production of the ATP. This section will show the pathways by which the heart produces ATP for its own energy needs.

It has been mentioned previously that mammalian hearts have an abundant blood supply, numerous elongated mitochondria and a high content of myoglobin. Normally less
than one percent of the total energy requirement is met by anaerobic metabolism. On the other hand, this might rise to nearly ten percent under conditions of hypoxia. If forced to function under strict anaerobic conditions, the energy produced is insufficient to maintain sustained ventricular contractions.

In the so-called normal person, the basal calorific needs of the human heart are met as follows:

- Carbohydrates 35%
- Amino acids and Ketones 5%
- Fats 60%

The nutritional state of the person has direct bearing on substrate utilization. For example, after ingestion of large amounts of glucose, more lactate and pyruvate are utilized. On the other hand, during starvation or in an untreated diabetic, carbohydrate usage is reduced and that of lipids is increased. The circulating free fatty acids account for almost 50 percent of the fat utilized.

Now that we have an overall statement of the energy sources, let us consider how these individual substrates are utilized to produce ATP.

It is the hydrolysis of ATP which gives rise to the necessary energy of muscular contraction, to the extent of 7000 calories per mole of ATP. This can be represented as follows:

\[
ATP + H_2O \rightarrow ADP + H_3PO_4 + 29.3 \text{ KJ } \ (3)
\]

From the above it is seen that the catabolism of fats constitutes 60 percent of substrate utilized for energy.
purposes. It is observed that one mole of fatty acid, when oxidized to acetyl-CoA and incorporated into the TCA cycle and mitochondrial respiratory chain, gives rise to 44 moles of ATP per mole of a 6-carbon fatty acid. The pathway for oxidation of fatty acids can be seen in Figure 14. Similarly, for carbohydrates which constitute up to 35 percent of utilizable substrates, one mole of glucose oxidized by aerobic glycolysis, TCA cycle and respiratory chain to CO$_2$ and H$_2$O, gives rise to 38 moles of ATP. Under strict anaerobic conditions, oxidation of glucose stops at the production of lactate. In heart muscle this will continue until the pH falls due to accumulation of lactic acid. It is the associated intra-cellular acidosis which inactivates PFK, which shuts down the lactate production. This method of oxidation is not very effective as it only produces 2 moles of ATP per mole of glucose. It is possible for cardiac muscle to utilize ketones and amino acids but only to an extent of about 5 percent. Their incorporation into the TCA cycle is shown in Figure 15. The overall relationships between the utilizable substrates is best seen by observing Figure 16.

The aforementioned pathways for production of ATP are quite sufficient under normal energy requirement conditions. But if bursts of intense energy are required, then this pathway cannot cope. Thus an alternative source of energy is necessary and is provided by creatine phosphate. This acts by providing a source of high energy phosphate, enabling the prompt resynthesis of ATP. A further source of
FIGURE 14

Pathway For Oxidation of Fatty Acids

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from Review of Physiological Chemistry. 15th ed. Lange
14, p 282.
FIGURE 14

Pathway For Oxidation of Fatty Acids

\[
\begin{align*}
\text{FATTY ACID} & \rightarrow R-CH_2-CH_2-C-OH \\
& \quad \downarrow \text{CoA.SH} \\
& \quad \downarrow \text{ATP} \\
& \quad \downarrow \text{Mg}^{++} \\
& \quad \downarrow \text{AMP + P}_3 \\
& \quad \downarrow \text{THIokinase} \\
& \quad \downarrow \text{ACYL-CoA (ACTIVE FATTY ACID)} \\
& \quad \downarrow \text{ACYL-CoA dehydrogenase} \\
& \quad \downarrow \text{TRANS \ O,\ \alpha\,\beta\,-\,UNSATURATED \ ACYL-CoA} \\
& \quad \downarrow \text{\beta\,-\,HYDROXYACYL-CoA dehydrogenase} \\
& \quad \downarrow \text{\beta\,-\,KETOACYL-CoA} \\
& \quad \downarrow \text{THIOLASE (\beta\,-\,KETOTHIOLESE)} \\
& \quad \downarrow \text{ACYL-CoA + CH}_3-C\sim S-CoA \\
& \quad \downarrow \text{ACYL-CoA} \\
& \quad \downarrow \text{ACETYL-CoA} \\
& \quad \downarrow \text{CITRIC ACID CYCLE} \\
& \quad \downarrow \text{2CO}_2 \\
& \quad \downarrow \text{RESPIRATORY CHAIN} \\
& \quad \downarrow \text{H}_2\text{O} \\
\end{align*}
\]
Incorporation of Amino Acids into the T.C.A. Cycle
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from Review of Physiological Chemistry. 15th ed. Lange
15, p 334.
Incorporation of Amino Acids and Ketones into the TCA Cycle
FIGURE 16

The Relationship Between Utilizable Substrates within Aerobic Respiration

FIGURE 16

The Relationship Between Utilizable Substrates within Aerobic Respiration

- CARBOHYDRATE
  - (GLYCOGEN, GLUCOSE)
  - FRUCTOSE
  - SERINE
  - TRIOSE-P
  - LACTATE
  - MÉTHIONINE + SERINE
  - Cysteine
  - TRYPTOPHAN
  - THREONINE
  - SERINE
  - HYDROXYPROLINE
  - PHENYLALANINE
  - TYROSINE
  - ISOLEUCINE
  - PROPIONYL-CoA
  - PROPIONATE
  - MÉTHIONINE

- FAT
  - TRIACYLGlycerol
  - QA-Glycerol
  - QA-Glycerol
  - FATTY ACIDS
  - THREONINE
  - ISOLEUCINE
  - PHENYLALANINE
  - TYROSINE
  - LEUCINE

- Acetyl-CoA
- Aceto-Acetate
- OXALOACETATE
- ASPARTATE
- CITRATE
- CITRIC ACID CYCLE
- GLUTAMATE
- PROLINE
- ORNITHINE
- HYDROXYPROLINE
ATP in cardiac muscle can be ascribed to the presence of the enzyme myokinase which catalyzes the following reaction:

\[ 2 \text{ADP} \xrightleftharpoons{\text{Myokinase}} \text{AMP} + \text{ATP} \] (4)

It is important to realize that these last two methods of ATP production can only provide ATP for very short periods of time, and once exhausted the heart will be in a state of collapse. Reference to Figure 17 will show formation and breakdown of creatine phosphate and the relationship of these events to ATP in muscular contraction.

The heart is extremely versatile in its substrate utilization and can function under quite a range of metabolic conditions, thus increasing its chances of survival.
FIGURE 17

Formation and Breakdown of Creatine Phosphate and the Relationship of these Events to ATP in Muscular Contraction

Creatine-P \rightarrow \text{Creatine kinase} \rightarrow \text{ATP-Creatine trans-phosphorylase} \rightarrow \text{ADP} \rightarrow \text{energy for muscular contraction} \rightarrow \text{ATP} \rightarrow \text{Pi} \rightarrow \text{energy from aerobic oxidation, lipolysis, gluconeogenesis, and myokinase activity} \rightarrow \text{Creatine-P}

FIGURE 17
CHAPTER III

PATHOLOGY OF THE HEART IN ACUTE MYOCARDIAL INFARCTION

A. MAJOR TYPES OF HEART DISEASE

This paper is mainly concerned with acute myocardial infarction (AMI), but since this is only one of the major types of heart disease, though the most important, it would seem opportune to mention the other kinds. Robins (17) lists the eight classes of cardiac disease which now dominate clinical practice in North America. These are as follows:

(1) Coronary heart disease, which constitutes 80 percent of all cardiac deaths, and includes the form that will be discussed in this paper.

(2) Hypertensive heart disease (9% of cardiac deaths)

(3) Rheumatic heart disease (2 to 3% of cardiac deaths)

(4) Congenital heart disease (approximately 2%)

(5) Bacterial endocarditis (approximately 1 to 2%)

(6) Syphilitic heart disease (approximately 1%)

(7) Pulmonary heart disease (approximately 1%)

(8) Other types, which make up the remaining 4 to 5% of cardiac deaths, including pericardial disease, such as pericarditis, myocardial disease and endocardial disease.

From the above it is obvious that coronary heart disease (CHD) accounts for the highest incidence of all cardiac deaths, and it is this aspect of heart disease that will be discussed.
Definition of Myocardial Infarction

Robins (18) defines myocardial infarction as: "the catastrophic and frequently fatal form of CHD, usually resulting from precipitous reduction or arrest of a significant portion of coronary flow. Almost always, severe atherosclerotic narrowing, often associated with total thrombic occlusion, is present in one or more of the major coronary arterial trunks."

B. INCIDENCE

Acute myocardial infarction is the cause of 20 to 25 percent of all deaths in our Western atherosclerosis-prone society and is the number one killer of males. Running second, but with only half the number of deaths, is cancer. These figures are from 1967. This compares with a 1937 epidemiological study which gave deaths from MI sixth position and a death rate of 4.8 percent (19).

Atherosclerosis, or narrowing of the coronary arteries, is a progressive process which occurs in all individuals, male and female, and reaches a peak between the ages of 80 and 90. Associated with this is the risk of MI, which also peaks out during the eighth decade of life. Males have a definite predisposition to MI which is most striking in the younger age group (45 to 54) where the male/female ratio of deaths is 5:1. MI is uncommon in women of child-bearing age unless there is a history of the disease.

As a result of the Framingham Study (20) correlations
have been drawn between MI and the following factors:

(1) High caloric intake
(2) High intake of saturated animal fats
(3) Raised serum cholesterol levels
(4) Raised serum levels of beta and pre-beta lipoproteins
(5) Hypertension
(6) Obesity
(7) Smoking
(8) Metabolic disorders leading to raised cholesterol levels, such as diabétés mellitus, nephrosis and the genetic lipidemias.
(9) Physical inactivity
(10) Emotional and psychological stress.

Of these, four are classified as "major risk factors." These are:

(1) Raised lipid levels ("cholesterol", beta and pre-beta lipoproteins)
(2) Obesity
(3) Hypertension
(4) Smoking

If three of these factors are present in a given individual then that person is seven times more likely to have a MI than a person in which these risks are absent. With two "major risk factors" present, vulnerability is increased four times and with one factor the risk is double that of an individual who does not have any risk factors.
C. ETIOLOGY AND PATHOGENESIS

(1) Cause

The onset of acute myocardial infarction is usually insidious and is particularly associated with young and middle-aged males (premenopausal females seem to be exempt), who are at risk. The initial cause of MI is arteriosclerotic heart disease which evolves from slow, progressive narrowing of the coronary arteries, usually occurring over the life span of the individual. Associated with this narrowing is atrophy of the myocardium brought about by gradual reduction of blood supply to the myocardium. The commonest form of arteriosclerosis is characterized by deposition of lipid material in the intima of the arteries, in particular the coronary arteries. This form of arteriosclerosis is known as atherosclerosis. A suggested mechanism by which atherosclerosis may be produced is shown in Figure 18. This diagram further suggests a means by which a myocardial infarction may be induced. As indicated above, atherosclerosis is characterized by the deposition of cholesterol esters in the connective tissue of the arterial walls of the coronary arteries. It is of interest to note that people suffering from diseases associated with hyperlipidemia, such as diabetes mellitus, nephrosis and hypothyroidism are found to have premature or more severe forms of atherosclerosis. A possible mechanism showing the inter-relationship between blood lipoproteins (lipid transport proteins) and atherosclerosis is shown in Figure 19.
FIGURE 18

A Possible Mechanism by which Atherosclerosis and Tissue Infarction May be Produced

FIGURE 18

A Possible Mechanism by which Atherosclerosis and Tissue Infarction May be Produced

NORMAL ARTERIAL INTIMA

LIPID DEPOSITS

Fibrosis, calcification

ATHEROSCLEROTIC PLAQUE

Continued proliferation

NARROWING OF ARTERIAL LUMEN

Degenerative changes

Turbulent blood flow

Tendency to clot formation

Stimulus (stress)

THROMBOSIS

Interference with tissue O₂ supply

TISSUE ISCHEMIA

INFARCTION
Postulated Interrelationship Between Plasma Lipoproteins and Atherosclerosis

LEGEND

Chylomicrons are metabolized in a similar manner to VLDL. Only the prominent lipids are shown in LDL and HDL. (VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein; TG = triacylglycerol; PL = phospholipid; C = cholesterol; CE = cholesteryl ester; LCAT = lecithin:cholesterol acyltransferase.) Reprinted without permission of the author from Review of Physiological Chemistry. 15th ed. Lange Medical Publications, Los Altos, California. (1975). Chapter 14, p 321.
FIGURE 19

Postulated Interrelationship Between Plasma Lipoproteins and Atherosclerosis
(2) Effects of Myocardial Infarction on Cellular Respiration

As reviewed previously, the energy metabolism of the myocardium usually follows an aerobic pathway. Once the flow of blood has stopped, the oxygen reserves are used, that is, the oxygen dissolved in the myocardium (0.3 vol.%) and bound to myoglobin (0.5 vol.%). Initially, for a period of about eight seconds or eight contractions (21) the myocardial energy needs are met by the aerobic pathways and the stores of high energy phosphates and lactic acid remain unchanged. As soon as the reserves have been depleted the concentration of creatine phosphate falls and that of lactic acid increases (see Figure 20). From this diagram it can be seen that the energy for contraction, derived from the hydrolysis of ATP, is maintained. After about a minute following tissue ischemia the lack of perfusion of the myocardium results in shortages of the required substrates, fatty acids, glucose and gluconeogenic substrates such as amino acids. There is also concurrent accumulation of end products which causes product inhibition of enzymes. Intracellular levels of ATP, NAD and the cytochromes reduce as the myocardium's oxidative phosphorylation capability diminishes. The conversion from aerobic to anaerobic glycolysis brings about the accumulation of lactic acid within the cardiac cell, thereby causing the intracellular pH to fall, thus bringing about inhibition of enzymatic function. It is likely that the cessation of contractile function is due, not to a lack of ATP, as analysis shows the cell is not depleted, but to the intracellular acidosis which has an
FIGURE 20

Diagram Showing Concentration of Metabolites with Respect to Time Following Ischemia. The P_2O_2 is also indicated

**LEGEND**

This diagram depicts the myocardial contents of creatine phosphate, ATP, ADP, AMP and lactate before and after the onset of ischemia. The dotted line indicates the oxygen tension. This data was taken from reference 21 without the permission of the author.
FIGURE 20

Diagram Showing Concentration of Metabolites with Respect to Time Following Ischemia. The PO$_2$ is also Indicated.
inhibitory effect on the ability of calcium ions to activate contraction. Concurrently, intracellular water and electrolyte homeostasis is altered and the cells lose potassium and magnesium, probably due to failure of the "cation pump" because of depleted levels of ATP. Associated with these changes, and since the integrity of the myocardial cell membrane cannot be maintained, enzymes start to leak out, enzymes such as LDH, AST and CK, initially enzymes of the cytosol. Closely following this, the mitochondria lose their organization, the cristae break down and in their place dense granules of disorganized material appear. Within a period of a few hours of the infarct, the intracellular concentrations of the citric acid cycle intermediates become so low that the metabolic pathways cannot be maintained, and the cardiac muscle cells of the necrotic area die. The increase of acidity in the dead or dying area has the effect of releasing lysosomal enzymes, in particular phosphatases, deoxyribonucleases and other hydrolases, which bring about the degradation process required to remove the debris of dead and dying cells. This process can be slow requiring anywhere from a few days to weeks depending on the size and extent of the infarct.

(3) Clinical Course with Traditional Means of Laboratory Diagnosis

AMI has become a common occurrence in our life style with 600,000 deaths attributed to this cause in 1976. This is a terrible drain on our human resource, especially since AMI
usually strikes an individual in the "prime of life", when the person is reaching his most productive years.

Acute myocardial infarction (AMI) is usually marked by the sudden onset of a crushing, devastating pain originating from under the breast bone in the area of the precordium. This pain centered over the heart often radiates to the jaw, left shoulder and/or arm. Occasionally AMI is dismissed as indigestion. No patient is ever diagnosed as suffering from AMI on enzyme tests alone. The diagnosis is often made as soon as the patient is seen by the physician. The patient is likely to be sweaty, ashen grey, extremely short of breath, anxious and obviously very ill. However, in the majority of AMI's, laboratory data is necessary to confirm or establish the diagnosis. As illustrated in Figure 21, it is generally accepted that in an uncomplicated case of AMI serum myocardial enzymes increase sharply, classically with AST and LDH levels rising to a peak within 12 to 24 hours. The AST soon falls but the LDH remains elevated for 7 to 10 days. Unfortunately, similar increases of enzyme activity are seen in hepatocellular disease and in pulmonary infarction.

Some means of differential diagnosis was required when patients presented themselves with chest pains. The majority of cardiologists, therefore, prefer the estimation of the activity of creatine kinase (CK). Increased activity of this enzyme is encountered earlier (6 to 8 hours) and usually falls to normal within 48 hours, assuming no re-infarction occurs. CK has greater specificity than the other
Sequential Changes in Serum Enzymes after Acute Myocardial Infarction
Sequential Changes in Serum Enzymes after Acute Myocardial Infarction

![Graph showing enzyme activity over time](image-url)
enzymes (AST, LDH, etc.) since its activity is thought to be confined to skeletal and cardiac muscle (21). (It is of interest to note that a mitochondrial form of CK is thought to have been detected in hepatocytes). It was, therefore, reasoned that it was highly unlikely that skeletal muscle disease could mimic the symptoms of a myocardial infarction.

It is reported that electrocardiographic changes are even more definitive than serum enzyme activities in detecting AMI. The hallmark of the AMI is elevation of the ST segments in the leads overlying the area of infarction. It is of interest that changes in ionic composition of the extra cellular fluid of the heart can be detected by electrocardiography. Sobel and Shell (22) compiled data from 13 literature studies of CHD and found elevations of CK, AST and LDH in 27 percent of patients with prolonged ischemic chest pain but without electrocardiographic changes. Another limitation of electrocardiographic studies is difficulty in following and detecting a re-infarction.

Other diagnostic methods are available such as coronary arteriography, vector cardiography, determination of the lactic acid concentration in the venous effluent of the coronary sinus, and others. It is apparent that there is no lack of methods for diagnosing AMI; what is lacking are effective methods of prevention and cure.
CHAPTER IV

ISOENZYMES IN DIAGNOSTIC ENZYMEOLOGY

Isoenzymes, by definition, are multiple molecular forms of an enzyme with closely related substrate specificities. They share some chemical and physical properties but differ in electrophoretic mobility, Michaelis Constant, and they have variable substrate specificity, immunological characteristics and thermal stability. It is these differences which have led to the development of methods able to detect the presence of these multi-molecular forms.

Recently, the International Union of Biochemistry produced a list of recommendations enabling a more standardized method of classification of those enzymes which exist in multi-molecular form (23). The advent of methods able to separate the multiple forms of enzymes has provided diagnostic enzymology with a very powerful tool capable of providing the physician with a "biochemical biopsy", thus helping him to pinpoint the diseased organ more rapidly than was possible in the past. This technique is far less traumatic on the patient than the "old biopsy" methods as all that is required is a few milliliters of the patient's blood.

A. ISOENZYMES

(1) Structure

As in other areas of scientific endeavour, several
theories relating to structure have been proposed. Those isoenzymes referred to in this paper as cardiac isoenzymes (lactate dehydrogenase and creatine kinase) are thought to exist as combinations of different types of subunits. On the other hand, the various alkaline phosphatases contain differing amounts of sialic acid and it is thought that this accounts for the difference in electric charge and hence mobility in an electric field exhibited by the alkaline phosphatase isoenzymes. Conformational differences and association with different carrier proteins have been suggested as accounting for the structure of other isoenzymes (24).

The most popular theory concerned with explaining the structure of isoenzymes is the subunit theory. This theory suggests that isoenzymes result from a combination of subunit of more than one type. The majority of this work has been derived from the study of the isoenzymes of lactate dehydrogenase.

It was observed by Vesell and Bearn, 1957 (25), Wieland and Pfleiderer, 1957 (26), and by Sayre and Hill, 1957 (27) that lactate dehydrogenase of animal and human origin existed in five major forms separable by electrophoresis. These forms were given the numbers 1 to 5 inclusive. LDH-1 is the fastest moving fraction and travels in the inter-albumin-alpha-1-globulin position; the LDH-2 travels in the same zone as the alpha-2-globulin; the LDH-3 travels in the beta-globulin region; while the LDH-4 and -5
travel in the gamma-globulin region. See Figure 22 for details. It is known that the molecular weight for each LDH fraction is in the order of 135,000 daltons. Another striking feature of the LDH fractions is the uniformity of spacing of the electrophoretogram. This regular difference in net electrical charge suggests that the amino-acid composition of LDH-1 differs from that of LDH-2 by the same increment as that between other pairs of neighbouring isoenzymes, a concept which is supported by Wieland and Pfleiderer. "Finger printing" of each isoenzyme reveals the striking regular differences in the amino-acid composition of the four isoenzymes, LDH-1, LDH-2, LDH-3, and LDH-5 (see Figure 23). The gradual increase in the content of lysine and arginine, and the regular fall in aspartic and glutamic acid moieties from LDH-1 to LDH-5 explain the regular changes in electrophoretic mobilities. It was observed by Appella and Markert in 1961 (28) that treatment of the five electrophoretically separated isoenzymes with 12M-urea or 5M guanidine resulted in each isoenzyme splitting into four subunits. It was then realized that each LDH isoenzyme was a tetramer composed of four monomers. Finger printing indicated that the monomers of LDH-1 were all the same, as were all the monomers of LDH-5, except that the monomers of LDH-1 differed from the monomers of LDH-5. Cohn et al. 1962 (29) realized that the synthesis of monomers of LDH was influenced by the type of metabolism prevailing at the cellular level. In the anaerobic state as occurs in
Relative Mobilities of the Lactate Dehydrogenase Fractions in Relation to Serum Proteins

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*The Definitive Cardiac Profile*, Helena Laboratories, Beaumont, Texas, Page 2.
FIGURE 22

Relative Mobilities of the Lactate Dehydrogenase Fractions in Relation to Serum Proteins
FIGURE 23

Differences in the Number of Molecules of Certain Amino Acids per Molecule of Enzyme in Four Human Lactate Dehydrogenase Isoenzymes
This data was taken from reference 11 without the permission of the author.
FIGURE 23

Differences in the Number of Molecules of Certain Amino Acids per Molecule of Enzyme in Four Human Lactate Dehydrogenase Isoenzymes.
muscle, he labelled each LDH-5 subunit monomer (M). In the aerobic state as occurs in the heart, he labelled each LDH-1 subunit monomer (H). Each monomer was observed to have a molecular weight of 34,000; thus, four monomers give rise to the LDH tetrameric structure and the tetramer molecular weight of 4 x 34,000 to equal 136,000 (compared to 135,000). See Table I for a summary of the characteristics of LDH. Markert, in 1963 (30) electrophoresed a mixture of equal amounts of purified ox-heart isoenzymes LDH-1 and LDH-5. Subsequently the presence was observed of all five isoenzymes in approximately the calculated proportions of 1:4:6:4:1, which would be expected if random reassociation had occurred. Electrophoresis of LDH-1 and LDH-5 separately showed the presence of the individual isoenzyme only (see Figure 24).

Similar reasoning lead Dawson, Eppenberger and Kaplan in 1965 (31) to conclude that creatine kinase had a dimeric structure. They suggested that the muscle enzyme may be composed of two identical subunits (M-M), the brain enzyme of two identical, but different from muscle, subunits (B-B), and the hybrid molecule of two subunits, one of either kind (M-B). The hybrid enzyme is intermediate in kinetic properties and in electrophoretic mobility between the M-M and the B-B dimers. Hence, the nature of the multiple forms of creatine kinase resembles that of lactate dehydrogenase, in that hybrid enzymes occur in vivo.
TABLE 1
Characteristics of Lactate Dehydrogenase

<table>
<thead>
<tr>
<th>Class</th>
<th>Subunits</th>
<th>Band</th>
<th>M.W. ($10^3$)</th>
<th>Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>H</td>
<td></td>
<td>34</td>
<td>Aerobic</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td></td>
<td>34</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Tetramer</td>
<td>HHHH</td>
<td>LDH-1</td>
<td>135</td>
<td>Aerobic</td>
</tr>
<tr>
<td></td>
<td>HHHM</td>
<td>LDH-2</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HHMM</td>
<td>LDH-3</td>
<td>135</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>HMMM</td>
<td>LDH-4</td>
<td>135</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMMM</td>
<td>LDH-5</td>
<td>135</td>
<td>Anaerobic</td>
</tr>
</tbody>
</table>

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Cawley, L. P. from Electrophoresis and Immunelectrophoresis.
FIGURE 24

Starch Gel Electrophoresis of Purified Ox Heart Lactate Dehydrogenase

LEGEND

Note LDH-1 and LDH-5 showing single bands only. The centre run is that of a mixture of LDH-1 and LDH-5 which have been subject to dissociation and reassociation.

This data was taken from reference 11 without the permission of the author.
Starch Gel Electrophoresis of Purified Ox Heart Lactate Dehydrogenase

FIGURE 24

LDH-5
MIXTURE (1+5)
LDH-1

1
2
3
4

ORIGIN

5

(+) (-)
More recent work on LDH and CK indicated that the structure of these two isoenzymes is not as simple as was first thought, since under certain conditions up to 5 bands have been observed for LDH-5 instead of the usual one. Similarly for other fractions, LDH-4 (4 bands), LDH-3 (3 bands), LDH-2 (2 bands), and LDH-1 (1 band) were found. This phenomenon was observed on starch gel electrophoresis, a method of electrophoresis which has a molecular-sieving effect. The samples had also been reacted with beta-mercaptoethanol and formaldehyde (see Figure 25). Wieland and Pfleiderer (32) were the first to observe the extra bands exhibiting LDH activity, while Boyd (33) attempted an explanation of this phenomenon, suggesting that M subunits consisted of 2 polypeptide chains, one enzymatically active and one enzymatically inactive. Likewise, M subunits consisted of another two polypeptide chains, one showing enzyme activity, the other no activity. Combinations of these could theoretically lead to a large number of electrophoretically different molecular subunits.

An even more recent study by Wevers et al. (1977) (34) on the dimeric structure of creatine kinase describes a similar phenomenon. It is of special interest at this time since these extra bands were obtained simply by extending the time of electrophoresis and by changing the buffer twice. The bands of activity described in Wevers' paper numbered 5, 3 MM (MM1, MM2, MM3, MB1 and MB2) all were obtained from patients with elevated CK. Another extremely
FIGURE 25

Diagram of Sub-bands of Lactate Dehydrogenase Isoenzymes

LEGEND

This diagram represents a starch gel electrophoretogram of lactate dehydrogenase after sample was reacted with 2-mercaptoethanol (A) and with formaldehyde (B).

FIGURE 25

Diagram of Sub-bands of Lactate Dehydrogenase Isoenzymes
interesting facet of this particular paper was the fact that samples drawn from a patient at 1, 2, 24, 40 and 56 hours after a transmural infarction showed different band patterns within the MM sub-bands. It was also observed that patients with transmural infarction exhibited two MB bands on extended electrophoresis. The relevance of these findings to diagnostic enzymology, although extremely interesting, remains to be seen. See Figure 26 for details.

(2) Enzyme Distribution and Tissue Specificity

The search for organ specific enzymes has led to the accumulation of a large volume of literature on the distribution of various enzymes in different tissues of the body, the most recent work relating to isoenzyme composition of the different tissues. Figures 27, 28 and 29 show the relative enzyme activities in human organs for the enzymes lactate dehydrogenase, creatine kinase and aspartate aminotransferase, respectively. Note that all determinations of the relative amounts of enzymes in different organs have been based largely on enzyme "activity" and not enzyme concentration. These results will, therefore, be very much method-dependant. Not only are they method-dependant, but also highly dependant on the treatment they receive during analysis, as the isoenzymes show varying degrees of stability; for example, MM is more stable than MB, which is more stable than BB (35). The latest literature information relating to isoenzyme distribution is shown in Figures 30 and 31. This information has been derived from
FIGURE 26

Diagram of Creatine Kinase Electrophoretogram Showing Effect of Prolonged Electrophoresis

LEGEND

A. Traditional electrophoretogram of a serum sample after an infarction.
B. The same sample after 90 min electrophoresis. The CK-MB band has disappeared from the gel.

This data is taken from reference 34 without the permission of the authors.
Diagram of Creatine Kinase Electrophoretogram Showing Effect of Prolonged Electrophoresis
FIGURE 27

Lactate Dehydrogenase Activity in Human Organs
Lactate Dehydrogenase Activity in Human Organs

<table>
<thead>
<tr>
<th>Organ</th>
<th>Activity (U per Gram of Organ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skeletal muscle</td>
<td>147</td>
</tr>
<tr>
<td>Liver</td>
<td>145</td>
</tr>
<tr>
<td>Heart</td>
<td>124</td>
</tr>
<tr>
<td>Kidney</td>
<td>109</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>83</td>
</tr>
<tr>
<td>Pancreas</td>
<td>50</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>33</td>
</tr>
<tr>
<td>Lung</td>
<td>27</td>
</tr>
</tbody>
</table>
FIGURE 28

Creatine Kinase Activity in Human Organs

FIGURE 28

Creatine Kinase Activity in Human Organs

[Bar graph showing creatine kinase activity in different organs: Skeletal muscle, Brain, Heart, Smooth muscle, Kidney, Liver, with values 670, 350, and others]
FIGURE 29

Aspartate Transaminase Activity in Human Organs

Aspartate Transaminase Activity in Human Organs

FIGURE 29

Aspartate Transaminase Activity in U per Gram of Organ
FIGURE 30

Electrophoretic Patterns of Lactate Dehydrogenase Isoenzymes in Various Normal Human Tissues

This data was taken from reference 36 without the permission of the author.
FIGURE 30

Electrophoretic Patterns of Lactate Dehydrogenase Isoenzymes in Various Normal Human Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Isoenzyme Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1</td>
</tr>
<tr>
<td>Lung</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>Heart</td>
<td>1 2</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>Kidney Cortex</td>
<td>1 2 3</td>
</tr>
<tr>
<td>Kidney Medula</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>Normal Serum</td>
<td>1 2 3 4 5</td>
</tr>
</tbody>
</table>
FIGURE 31

Distribution of Creatine Kinase Isoenzymes in Extracts of Tissue Obtained from Patients at the Time of Surgery

This data was taken from reference 37 without permission of the authors.
FIGURE 31

Distribution of Creatine Kinase Isoenzymes in Extracts of Tissue Obtained from Patients at the Time of Surgery

Creatine Kinase Activity in U per Gram of Tissue

- Total CPK
- MM CPK
- BB CPK
- MB CPK

Skeletal Muscle
Heart
Brain
G.I. Tract
Lung
Kidney
Liver
Spleen
RBC
research performed by the following workers: Papadopoulos et al. (1967) (36); Sobel et al. (1976) (37); Tsung (1975) (38) and Wilhelm et al. (1976) (39). The patterns shown by LDH, in my opinion and experience, are highly reproducible and give specific information under controlled conditions, while those of CK are used in an attempt to confirm or rule out specific myocardial damage, or less frequently brain damage. It can be seen from the information on the presence of the MB subunit, that workers disagree on its distribution and also on its relative activity. I do not think there is any doubt that it is more prevalent than was at first thought, and as more definitive methods are developed this will be confirmed.

Enzyme activity detected in the serum following acute myocardial infarction reflects the loss of enzymes from damaged myocardial cells. The first event following the damage is the escape of enzymes from the cells of the myocardium into the interstitial space. There are varying rates of leakage from damaged cells which are not necessarily related to the molecular size of the enzymes in question. There is even evidence to suggest that there may be intracellular differential binding of enzymes. Once in the interstitium, enzymes are subject to inactivation, and a large proportion of the liberated enzymes never reaches the general circulation. Spilled enzymes are transported via the lymphatic and coronary circulations of the heart to the general circulation. The rate of transport from the inter-
stitial space may vary from enzyme to enzyme and these rates are not known for specific enzymes. Once enzymes have reached the general circulation there are differences in clearance rates of the individual enzymes. Several mechanisms for the removal of enzymes from the circulation have been postulated and include urinary excretion (40, 41), biliary excretion (41), uptake by the reticuloendothelial system (42), and progressive increase in the Km of circulating enzymes (43). A recent theory suggests that circulating enzymes are inactivated in the serum and the inactivated products are hydrolyzed in the lumen of the gut with the amino acids produced as a result of the hydrolysis being reabsorbed into the body's amino acid pool (44-46). The most recent paper of Painter, Sobel and Roberts indicates that clearance of CK is due to removal of protein rather than a denaturation or inhibition (47). It is academic as to which mechanism is correct since the end result is a certain level of circulating enzyme which is available for detection and possibly quantitation.

The cells of the myocardium are best suited to function under conditions of high oxygen tension, while those of skeletal muscle operate maximally under conditions of extremely low oxygen tension. This basic difference between the two types of tissue has lead to the evolution in the respective tissue of different types of enzymes best suited to function under the prevailing metabolic conditions. Such an example is lactate dehydrogenase which, as mentioned
previously, is composed of four monomers giving a tetrameric structure consisting of H and M monomers. The H tetramers are more prevalent in tissues of high oxygen tension while the M tetramers are more prevalent in tissues of low oxygen tension, such as skeletal muscle. Thus, it can be seen that the detection and identification of isoenzymes can assist in the identification of a specific organ. This can be clearly seen in Figure 32.

B. ISOENZYMES USED IN THE LABORATORY DIAGNOSIS OF ACUTE MYOCARDIAL INFARCTION

This section is intended to act as an overall review of those isoenzymes which are currently being used in diagnostic enzymology as an aid to the laboratory diagnosis of acute myocardial infarction (AMI).

(1) Creatine Kinase Isoenzymes

It was in 1964 that Deul and Van Breeman (48) found that the CK derived from extracts of human brain and human skeletal muscle differed in their electrophoretic mobilities. The CK from the brain was given the letter B while that from muscle, M. The brain CK moved toward the anode while the muscle CK remained near the point of application. A year later Dawson and his co-workers (49) presented data which indicated that CK exists as a dimer and that skeletal muscle is composed of two identical subunits MM, and that brain is also formed from two but different subunits, BB. Dawson also studied heart muscle and found that it contained
FIGURE 32

Altered Serum Lactate Dehydrogenase Isoenzyme Patterns Indicating Specific Tissue Pathology

This data was taken from reference 36 without the permission of the author.
FIGURE 32

Altered Serum Lactate Dehydrogenase Isoenzyme Patterns Indicating Specific Tissue Pathology

<table>
<thead>
<tr>
<th>Clinical Diagnosis of Serum Donor</th>
<th>Serum Isoenzyme Patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis</td>
<td></td>
</tr>
<tr>
<td>Pulmonary Embolism</td>
<td></td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td></td>
</tr>
<tr>
<td>Acute Exercise</td>
<td></td>
</tr>
<tr>
<td>Nephritis</td>
<td></td>
</tr>
<tr>
<td>Nephrotic Syndrome</td>
<td></td>
</tr>
<tr>
<td>Heart and Liver Injury</td>
<td></td>
</tr>
</tbody>
</table>
MM but also a hybrid consisting of one M subunit and one B subunit, MB. This hybrid was observed to have kinetic and electrophoretic mobilities halfway between the MM and BB isoenzymes. He was unsure as to whether the hybrid occurred naturally or was formed in vitro. Dawson observed striking differences in the activities between muscle and brain CK using different amounts of either creatine phosphate or creatine, further suggesting differences in substrate affinities of the isoenzymes.

Numerous methods have been developed to detect and to quantitate the isoenzymes of CK and these will be discussed in some detail later in this paper.

Lounis (50) noted that the MM and MB isoenzymes have different "optimum" pH's as well as different K_m's. No difference in substrate optima were observed. These differences give an indication of the difficulties involved in designing the so-called "optimized" methods for analysis of sera which may contain a mixture of all three isoenzymes.

The composition of the creatine kinase isoenzymes can be seen in Table 2.

From the clinical point of view, the most important diagnostic parameter in the interpretation of CK isoenzyme levels is the presence or absence of the CK-2 isoenzyme, which is the heart hybrid isoenzyme. BB, the brain fraction, is almost never seen in the serum, even after a cerebrovascular accident, due to the fact that enzymes only cross the blood-brain barrier under extreme conditions. This
statement by Galen (51) has recently been questioned by Itano (52) and Vladutiu and Schachner (53) and revolved around the method of detection, with one based on concentration (RIA), the other on enzyme activity. These authors pointed out that since enzyme activity had been used to define the absence of BB, this might in fact be in error since the BB activity could be diminished due to specific inhibitors, or even albumin, at 37 degrees centigrade. However, this inhibition is not important with RIA techniques. They also point out that if RIA is used, BB could possibly be detected in all individuals. It is of interest to note that elevations of CK following meningitis and encephalitis have been shown to be of skeletal origin. In the right clinical setting, the presence of CK-MB in the serum indicates damage to the myocardium. The CK-MB was detected in the 48-hour period following the acute myocardial infarction in all patients, except those in which the AMI was so severe that perfusion of the myocardium is very limited and no removal of cellular debris can occur (54). A total CK of 0 to 40 percent as CK-MB has been reported in AMI. Timing is a critical factor in the detection of CK-MB isoenzymes. The MB isoenzyme is most frequently detected between 4 and 8 hours post infarct. Both CK-MM and CK-MB increase with rising CK activity, but the CK-MM proportionately more than the CK-MB. As the acute phase resolves, the CK-MB decreases rapidly and is usually undetectable after 72 hours. The presence of CK-MB is not 100 percent specific for myocardial damage since it is found
in certain muscular dystrophies, polymyositis, and in patients with significant myoglobinuria. However, these conditions should present no diagnostic problem to the physician. The extremely high CK values observed in malignant hyperthermia give isoenzyme patterns in which all three fractions are seen. Similarly, cord blood serum also contains the three isoenzymes. Patients with severe burns show only CK-MM. The presence of CK-MB is observed following carbon monoxide poisoning, as the carbon monoxide is a cardiotoxic substance.

Creatine phosphokinase MB isoenzyme can be quantitated by several methods, all of which will be discussed later, in chapter 5, section A. An appraisal will also be made on the value of quantitation.

(2) Lactate Dehydrogenase Isoenzymes

In the late fifties, Vesell and Bearn (25), and Sayre and Hill (27) reported on the electrophoretic heterogeneity of human serum lactate dehydrogenase. Wieland and Pfleiderer (26) about the same time had described five LDH fractions in human tissues. Over the ensuing years, other investigators (36) (55) demonstrated characteristic organ profiles for these LDH fractions which had been termed isoenzymes by Wroblewski and his co-workers (56). Each isoenzyme is a tetramer composed of H or M or both subunits. The composition of the five LDH isoenzymes is seen in Table 3.
TABLE 2
Composition of the Creatine Kinase Isoenzymes

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK₁</td>
<td>BB</td>
</tr>
<tr>
<td>CK₂</td>
<td>MB</td>
</tr>
<tr>
<td>CK₃</td>
<td>MM</td>
</tr>
</tbody>
</table>

TABLE 3
Composition of the Lactate Dehydrogenase Isoenzymes

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH-1</td>
<td>HHHHH</td>
</tr>
<tr>
<td>LDH-2</td>
<td>HHHMM</td>
</tr>
<tr>
<td>LDH-3</td>
<td>HHMM</td>
</tr>
<tr>
<td>LDH-4</td>
<td>HHMM</td>
</tr>
<tr>
<td>LDH-5</td>
<td>MMMMM</td>
</tr>
</tbody>
</table>
The homotetramer composed of the H monomers is found in brain, heart, and erythrocytes, while the homotetramer consisting of the M monomers is observed in skeletal muscle and the liver. As discussed previously, each tetramer differs by one unit of surface charge so that electrophoretically the isoenzymes are found evenly spaced across the globulin region of human serum from alpha-1 to the far gamma-globulins. Besides differing in electrophoretic mobility, LDH isoenzymes vary in heat stability, those with high proportions of H monomers being more stable than those with a greater preponderance of the M monomers. There are also differences as to optimum substrate concentration and also reaction rates with coenzyme analogs. Mention has been made previously about the prevalence of particular subunits in tissues of differing metabolic potential, with H subunits in tissue which is associated with aerobic function and M subunits in tissue which is mainly concerned with anaerobic metabolism. Everse and Kaplan (1973) (57) point out in their paper that there is evidence to suggest that the reaction lactate $\rightleftharpoons$ pyruvate proceeds in opposite directions in heart and in skeletal muscle. The anaerobic skeletal muscle isoenzyme (MMMM) might be called pyruvate reductase since very little oxidation of lactate to pyruvate takes place in this tissue. On the other hand, the isoenzyme present in the myocardium (HHHH) is truly lactate dehydrogenase since it catalyzes the oxidation of lactate to pyruvate.
The most important diagnostic parameter in interpreting LDH isoenzyme patterns is the relationship between the isoenzyme fractions. There are basically two types of patterns which have been used extensively, although intermediate patterns are being interpreted in the light of new findings. The pattern which relates to myocardial damage is an increase of LDH-1 in relation to LDH-2, especially when the ratio LDH-1/LDH-2 is one or greater. When this occurs there are few diagnostic possibilities which could explain the elevation. This is the so-called "flipped LDH" that is seen following AMI, acute renal infarction (renal cortical necrosis) and in hemolysis such as that associated with prosthetic heart valves and megaloblastic or sickle cell anemias. AMI leads to the "flipped" profile usually within 12 to 24 hours post infarction and is present in 80 percent of patients with MI within 48 hours of the acute episode. It is of interest to note that the "flipped" profile can occur even though the total LDH has not exceeded the "upper limit of normal". It is, therefore, a sensitive indication of acute myocardial damage.

One of the advantages of using LDH isoenzymes is that its use is not just limited to the diagnosis of myocardial damage, since a predominance of the LDH-5 isoenzyme can indicate acute liver necrosis, acute muscle injury, acute dermatitis, and prostatic adenocarcinoma. It has been suggested that the concomitant rise of LDH-1 and LDH-5 following AMI is a result of congestion of the liver.
Others have suggested it is a result of disuse atrophy of the skeletal muscles. A summary of these patterns can be seen in Figures 33 and 34.

Several intermediate patterns have been observed and are shown in Figures 33 and 34. These include patterns observed in pulmonary infarction, leukemia, Hodgkin's disease, and in certain cases of malignancy. The overall clinical significance of all these patterns can be seen in Table 4. One drawback in the use of LDH isoenzymes as an aid to clinical diagnosis is the not uncommon finding of a so-called "isomorphic pattern". This is observed when the total LDH is elevated but the relative percentages remain within the normal range (see Figures 33 and 34).

One of the problems associated with current LDH isoenzyme methods is that they are relatively time consuming, and require specialized equipment and some degree of expertise on the part of the technologist. Attempts were, therefore, made to find another test which retained the specificity for the LDH isoenzyme but could be performed more rapidly. It had been observed by Rosalki and Wilkinson (58) that in addition to pyruvate, a number of other oxoacids can act as substrates for LDH and undergo reduction in the presence of NADH. The first to be introduced to clinical use was 2-oxobutyrate which is reduced to 2-hydroxybutyrate by LDH. This activity was given the nomenclature "alpha-hydroxybutyrate dehydrogenase" by its authors who first reported its significance. Some of the early literature
FIGURE 33

Some Significant Lactate Dehydrogenase Isoenzyme Patterns
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FIGURE 33

Some Significant Lactate Dehydrogenase Isoenzyme Patterns

MYOCARDIAL INFARCTION

HEMOLYSIS megaloblastic and sickle cell anemias

ACUTE LIVER NECROSIS
ACUTE MUSCLE INJURY
ACUTE DERMATITIS
PROSTATIC ADENOCARCINOMA
FIGURE 34

Some Significant Lactate Dehydrogenase Isoenzyme Patterns (continued)
Reprinted without permission of the authors, Turner, G. K. and Associates
FIGURE 34

Some Significant Lactate Dehydrogenase Isoenzyme Patterns (continued).

PULMONARY INFARCTION

LEUKEMIA
HODGIN'S DISEASE
(in certain cases)

MALIGNANCIES
(in certain cases)
TABLE 4

Clinical Significance of Lactate Dehydrogenase Isoenzymes

<table>
<thead>
<tr>
<th>LDH ISOENZYME FRACTION</th>
<th>NORMAL RANGE (% of total)</th>
<th>INTERPRETATION</th>
<th>DECREASED</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 HHHH</td>
<td>17-27%</td>
<td>Myocardial infarction</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hemolysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leukemia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pernicious anemia</td>
<td></td>
</tr>
<tr>
<td>2 HHHM</td>
<td>28-38%</td>
<td>Adenocarcinoma of the colon</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Testicular carcinoma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Renal cortical necrosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ Pulmonary infarction</td>
<td></td>
</tr>
<tr>
<td>3 HHMM</td>
<td>19-27%</td>
<td>+ Pulmonary infarction</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>with LDH-1 and LDH-2 in renal cortical necrosis</td>
<td></td>
</tr>
<tr>
<td>4 MMMH</td>
<td>5-16%</td>
<td>With LDH-5 in hepatic necrosis, renal tubular and cortical necrosis and certain malignant tumours</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>With LDH-3 and LDH-4 in infectious mononucleosis, hepatic congestion, hepatitis, prostatic adenocarcinoma, skeletal muscle necrosis, dermatomyositis, testicular carcinoma, multiple myeloma, renal cell carcinoma</td>
<td>Muscular dystrophy, certain cases of neurogenic muscular atrophy</td>
</tr>
</tbody>
</table>

documented the value of this "enzyme" in diagnosing myocardial infarction as it was supposed to represent LDH-1 activity; however, raised values were also observed in patients suffering from hepatocellular disorders (59) and in 70 percent of patients undergoing orthopedic surgery, reflecting surgical damage to skeletal muscle (60). This confirms the fact that HBD is not the same as LDH-1, since H subunits are found in four of the five LDH isoenzymes, which probably accounts for its lack of specificity. Even with the test's obvious shortcomings, Goldberg (61) uses HBD along with CK, AST, and ALT as an admission screen to rule out or to follow the course of AMI. He further recommends this regimen as being optimal for diagnosis, for prognosis and for the early detection of complications such as cardiac failure (62).

On the other hand, Galen (60) in his paper states "the HBD determination does not improve the diagnostic information significantly enough to warrant its routine determination."

It would appear at this time that no test is as yet available that will replace the determination of LDH isoenzymes.

(3) Aspartate Transaminase Isoenzymes

Two isoenzymes of AST have been demonstrated by electrophoresis of heart and liver cell extracts. The isoenzyme with the greatest mobility on electrophoresis is known as AST-1 and is present in the cytosol. The slower form of AST termed II is found in the mitochondria.
Following either cardiac or liver cell damage, AST-I appears in the serum. If the damage is extensive, involving the mitochondria, then AST-II is observed. It would appear that AST isoenzyme methods have a somewhat limited use in differentiating between liver and cardiac problems. AST activity is nonspecific and relatively nonsensitive. Its determination offers no unique information. As a test of cardiac damage it is redundant, unnecessary, and should no longer be used (63).

(4) Others

Although the main direction of isoenzyme analysis in relation to myocardial infarction has been with lactate dehydrogenase and creatine kinase, other approaches have been tried. It is reasonable to assume that if a set of special metabolic circumstances exist such as metabolism within the myocardium, then some unique arrangement of other metabolic enzymes might exist. It was this idea that led Pfleiderer and his co-workers (14) to examine the distribution of the aldolase isoenzymes in human tissues. They developed an immunotitration method by which they could quantitatively estimate the different isoenzymes of the aldolase found in human tissue. They determined the distribution patterns of aldolase A, B, and C in extracts of human organs and tissues as well as in normal and pathological sera. The results of their work can be reviewed by reference to Tables 5 and 6.

For the work of Pfleiderer to be useful in the
TABLE 5

Quantitative Relationships of Aldolase Isoenzymes A, B and C in Miscellaneous Organs and Tissues

<table>
<thead>
<tr>
<th>DISEASE</th>
<th>ALDOLASE (%) total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
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<td>Spermatozoa</td>
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This data was taken from reference 14 without permission of the author.
<table>
<thead>
<tr>
<th>DISEASE</th>
<th>ALDOLASE (% total activity)</th>
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<tr>
<td></td>
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This data was taken from reference 14 without permission of the author.
diagnosis of myocardial infarction it must allow for a specific pattern to show when a particular disease is present, that is the pattern must be different from that of other organ disorders. Table 6 shows marked differences in the different pathological sera investigated. Progressive muscular dystrophy shows a predominance of aldolase A, and myocardial infarction sera show higher proportions of aldolase B and C. Patients with hepatitis seem to have a predominance of the aldolase B isoenzyme. It would appear from this paper that persons from the healthy population have a similar distribution of aldolase isoenzymes to persons having suffered a myocardial infarction, although the healthy population seem to have a higher proportion of the C type aldolase. It has been suggested that progressive muscular dystrophy is a result of muscle failing to mature; if this is the case then the aldolase distribution of newborn children and the progressive muscular dystrophy sera might be expected to be similar. Table 6 shows them to be similar. Time will tell if aldolase isoenzyme activities will have any significant impact on the diagnosis and treatment of myocardial infarction.
CHAPTER V

METHODS FOR ISOENZYME SEPARATION

Any method for the determination of isoenzyme fraction activities can be divided into three parts - separation, detection and quantitation.

Wilkinson (11) in his book cites in excess of 300 references just on separation of isoenzymes, and over 110 references on the detection of the multi-molecular forms of isoenzymes. Methods of separation comprise basically two groups, both of which rely on the physical differences between the particular enzyme subunits. One group relies on the separation by electrophoresis on such mediums as paper, cellulose acetate, agar gel, agarose, polyacrylamide gel, column electrophoresis and isoelectric focusing. Chromatographic techniques include separation on ion-exchange celluloses, paper (DEAE-paper), hydroxylapatite columns, gel filtration and DEAE-Sephadex columns. Other methods used in isoenzyme separation are not true separation techniques as the "purified" form of the isoenzyme is not obtained, and rely on differential activation or inactivation by heat or chemicals of dilutions of human serum. The homogeneous enzyme subunits such as LDH-1 (HHHH) and CK-3 (MM) show a high degree of immunochemical specificity, and antibodies show little cross-reaction with the specific antigens, although they react nonspecifically with the heterogeneous
enzyme subunits such as LDH-3 (HHMM) and CK-2 (MB). It is of interest to note that immunochemical methods measure "concentration" of the subunits which may be different from the "activity" of the enzyme due to inhibitors, even albumin, at 37 degrees centigrade (64). This is important since enzymes can be measured in terms of their catalytic activity or in terms of their concentration (65).

The majority of work concerning the detection of separated isoenzymes has hinged on the histochemical staining of the electrophoretic support medium in situ. This has been performed by the use of tetrazolium-staining techniques or by the use of fluorescent producing or reducing methods. Another method which measures the rate of formation of a given product for a stated isoenzyme fraction has been used (66), but requires a specially modified spectrophotometer. It, however, has a number of advantages over the usual histochemical staining methods. Isoenzyme band spreading is kept to a minimum and there is little risk of enzyme action being affected by other substances being used in the staining reaction. Moreover, "enzymoelectrophoresis" (67) provides quantitative information which is more reliable than the scanning of a stained gel (compare kinetic versus end-point enzyme methods). Column methods require the actual measurement of the activity of the given isoenzyme, such as LDH or CK activity present in eluted fraction.
Quantitation usually requires the use of a scanning densitometer for electrophoretic strips, either visible or fluorescent, depending on the method. Column methods usually rely on measurement of activity of the different eluted fractions. Activation or inactivation methods rely on a "before and after" activity as a measure of relative enzyme activity. Finally, RIA methods measure the concentration of the actual immunochemical entity, assuming this is meaningful; for example, the difference between LDH-1/LDH-5 (HHHH)/(MMMM), using the detection of the H or M subunits, or CK-3/CK-2 (MM)/(MB) using the detection of the B subunit as a measure of the presence of MB fraction. This may or may not be valid (68).

A. CREATINE KINASE METHODS

Methods that will be discussed in this section are current ones, or ones that are likely to be accepted as routine methods in clinical diagnostic enzymology.

1) Electrophoresis

Deul and Van Breeman in 1964 (48) were the first researchers to electrophorese CK. The support medium they used was agar gel, while in 1967 Dawson, Epenberger and Kaplan (49) used starch gel to separate the subunits of CK. Today starch gel tends to be used only as a research tool, due to the inherent difficulty in preparing adequate gels. Rosalki (69) in 1965 was one of the first researchers to use cellulose acetate electrophoresis as a technique for the
separation of CK isoenzymes. This technique is one of the main methods to be described in this paper. The other electrophoretic technique which has become well accepted is that of agarose-gel electrophoresis which was first utilized by Sjovall and Voight (70) in 1964. Many other researchers also used this definitive technique. These include: Jacobs, Heldt and Klingenburg, 1964 (71); Kar and Pearson; 1965 (72); Brownlow and Gammock, 1967 (73). The first person to bring the agarose-film technique into the realm of the clinical chemist was Elevitch, et al. in 1964 (74).

a) Agarose

This method uses a one-percent solution of agarose in barbital buffer at pH of 8.6 and an ionic strength of 0.025 M. The gel film is supported on Mylar plastic backing and has pre-formed serum inoculation wells. The volume of serum necessary for CK isoenzyme determinations is 2 μl. These gels are available commercially (75). The inoculated film is electrophoresed for a period of 20 minutes. At the end of this time period a fluorogenic substrate solution is spread over the entire film and the film incubated for a period of 20 minutes at 37 degrees centigrade. This incubation is carried out in a humidity chamber to prevent the gel from drying out. The gel is dried at 75 degrees centigrade for a length of time not exceeding 15 minutes. The dried gel when observed under near-ultraviolet light (365 nm) exhibits blue-white fluorescent bands at the site of the CK
isoenzymes. The sites of enzymatic activity can be visualized in several ways. Two of the more popular methods can be seen in the following series of reactions. These series of reactions are used to localize the zones of enzymatic activity.

$$\text{Creatine phosphate} + \text{ADP} \xleftrightarrow{\text{CK}} \text{Creatine} + \text{ATP} \quad \text{(5)}$$

$$\text{MgCl}_2, \text{L-cysteine}$$

$$\text{ATP} + \text{Glucose} \xleftrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP} \quad \text{(6)}$$

$$\text{Glucose-6-phosphate dehydrogenase}$$

$$\text{Glucose-6-phosphate} + \text{NAD}^+ \xleftrightarrow{\text{}} \text{NADH} + 6-\text{Phospho-gluconate} \quad \text{(7)}$$

The CK isoenzyme sites appear as blue-white fluorescent bands (NADH fluorescence) under near ultraviolet light (365 nm). The MgCl₂ is required in the reaction as the Mg²⁺ is a cofactor, and the L-cysteine is an activator of the CK reaction. The reaction is carried out in a MES buffer (morpholino-ethane-sulphonic acid) at a pH of 6.2. AMP is included in the reaction mixture as this inhibits myokinase which is frequently present in human serum and catalyzes the following reaction:

$$2 \text{ ADP} \xleftrightarrow{\text{Myokinase}} \text{AMP} + \text{ATP} \quad \text{(8)}$$

The presence of adenosine monophosphate results in product inhibition of the myokinase. There is also a 5 to 10 percent inhibition of CK by the presence of 5 mM of AMP.
per liter (76). This reaction sequence is based on the methods of Rosalki, (77) and Hess et al. (78).

Another coupled enzyme reaction based on the so-called "forward reaction" is shown below. It is also a fluorogenic method, and was derived by Tanzer and Gilvarg in 1959 (79).

$$\begin{align*}
\text{ATP} + \text{Creatine} & \xrightleftharpoons{\text{CK}} \text{Phosphocreatine} + \text{ADP} \quad (9) \\
\text{ADP} + \text{PEP} & \xrightarrow{\text{Pyruvate Kinase}} \text{Pyruvate} + \text{ATP} \quad (10) \\
\text{Pyruvate} + \text{NADH} & \xrightleftharpoons{\text{LDH}} \text{Lactate} + \text{NAD}^+ \quad (11)
\end{align*}$$

This method would show a reduction of fluorescence at the site of enzyme activity. This is not very convenient, as the fluorescent scanning would be from high to low, and the chances of obtaining a uniform background fluorescence would be rather remote due to the lack of uniformity in spreading the substrate material, and also unequal absorption of substrate material into the gel.

Another earlier method (90) utilized the above reaction sequences (5) and (6) but instead of visualizing in the near UV, the visible range was used as the bands were purple-blue in colour. The reaction sequences were identical to reactions (5) and (6) above, with the following additional reactions which can be seen in the three following reactions.

$$\begin{align*}
\text{Glucose-6-phosphate} + \text{NADP}^+ & \xrightarrow{\text{6-PDH}} \text{6-Phosphogluconate} + \text{NADPH} \quad (12) \\
\text{NADPH} + \text{phenazine methosulphate} & \xrightleftharpoons{\text{PMS}} \text{NADP}^+ \text{reduced PMS} \quad (13)
\end{align*}$$
Reduced PMS $\rightarrow$ Nitroblue tetrazolium (NBT) $\rightarrow$ "FORMAZAN" $\rightarrow$ PMS (14)

The "FORMAZAN" is the purple-blue colour that is formed in situ. This method is perhaps more convenient in some respects but it still lacks the inherent sensitivity of the fluorescent method.

b) Cellulose Acetate

This method of isoenzyme separation is identical to agarose gel electrophoresis in that it has no molecular sieve properties and relies only on the charge differences of the individual isoenzymes to produce the observed migration rates.

The cellulose acetate method of electrophoresis entails the soaking of the CA strip in the requisite buffer, usually barbital, pH 8.6, 50 mM/L. The soaked strips are then blotted dry with filter paper, inoculated with the patient's serum, and suspended in the electrophoresis cell. The voltage is applied, and separation allowed to proceed until adequate separation has been achieved, usually anywhere from 8 minutes to an hour, depending on the system. Obviously for a given buffer the higher the applied voltage the greater the separation. Care should be exercised due to the extreme lability of the CK-MB due to heat build up at higher voltages. Cooling of the electrophoresis cell can help to reduce the loss of the enzymatic activity from heat build up.

Once separation is complete, detection is brought
about by a histological *in situ* staining technique. Another similar piece of CA strip is impregnated with the reaction mixture and the two strips are laid one on top of the other. The sandwiched strips are then incubated in a humidity chamber at 37 degrees for between 20 and 30 minutes. The strips are removed and dried for approximately 15 minutes in a warm air oven. Alternatively, they are allowed to air dry in a fume hood. It is important to realize that the same colour reactions that have been used on agarose can be used on CA except that colourimetric methods using tetrazolium salts as chromogens are not really sensitive enough to detect low but significant levels of CK-2 that may be present in borderline increases of total creatine kinase. The fluorogenic method of Elevitch et al. (1972) (81), therefore, becomes the method of choice. See reactions (5) (6) (7) for a description of the multi-stepped reactions leading to detection of the isoenzyme activity.

c) Polyacrylamide

Anido et al. (1967) (82) used 5 percent polyacrylamide gel columns with Tris-glycine buffer at a pH of 9.2. He electrophoresed activated serum for a period of 50 minutes, using an applied voltage of 120 volts. Following electrophoresis, the gels were submerged for 45 minutes at 37 degrees centigrade in a mixture containing 50 mM of Tris, 28 mM of magnesium acetate, 18 mM of glucose, 1.3 mM of ADP, 1.3 mM of NADP, 10 mM of AMP, 10 mM of creatine phosphate, 4 IU/ml of hexokinase and 2 IU/ml of glucose-6-
phosphate dehydrogenase. All concentrations relate to a liter. It is interesting to note that these quantities are far from optimum (Sasz 1976) (76). The technique used by Anido and his co-workers is essentially that of Elevitch (1972) (81). The patterns derived by Anido et al. were similar in all respects to those of workers using either agarose or CA even though polyacrylamide gel is known to have molecular sieve properties.

The most popular of all the electrophoresis methods for creatine kinase isoenzymes would appear to be the agarose method, closely followed by the cellulose acetate method, both detecting the areas of enzyme activity by fluorometric means. There are commercial methods available which use both the agarose (75) and the CA (83). Polyacrylamide does not seem to have the same following that has been enjoyed by the other two methods. However, several papers have appeared in the literature covering the use of this technique in relation to CK isoenzymes (84-86). They attest to the usefulness of the method. The lack of popularity is probably due to the fact that the method is more involved than the other two methods and does not offer any apparent advantage.

(2) Columns

One of the main drawbacks of the electrophoretic techniques is that they require a large financial investment, especially if a scanning densitometer is involved. They also require a reasonably high level of analytical expertise.
All of these factors make the electrophoretic techniques expensive for those laboratories wishing to perform this test. The time involved is another factor to consider, as the fastest electrophoretic method takes a minimum of 65 to 70 minutes per run. The agarose method requires a run comprising 7 tests and one control for it to be economical. This is not always possible, so if less tests are run then the cost of the test rises. One way around this problem is to have a method that can be run either individually or in large numbers, and the method that best fits into this description is a column method, since one or two can be run at the same time or 20 can be run simultaneously. Assay times are much less than electrophoretic methods and only require comparatively basic laboratory equipment including a spectrophotometer with an effective band pass of 8 nm or less and the capability of producing monochromatic light at 340 nm. This is the most sophisticated equipment that is required to perform CK-isoenzymes by column. The literature contains numerous references to creatine kinase isoenzymes by column chromatography (87-91). The column methods can be considered under three basic headings. These are isolation of the isoenzymes from their serum matrix, separation of the individual isoenzymes from one another, and finally the quantification of the separated isozyme fractions.

a) Isolation

The general principle of the isolation is the reversible binding of the isoenzymes to an anionic ion-exchange
resin through the formation of electrostatic bonds between charges of opposite sign on the isoenzyme and the ion-exchange resin. The charges on the ion exchanger are fixed and interact with the dynamic equilibrium of the ionic charges on the isoenzymes. The pH of the system is such that the proteins are negatively charged. Under the conditions of the system the isoenzyme with the highest proportion of the B monomers has the greatest negative charge and is, therefore, bound by a larger number of exchanger positions, so BB > MB > MM. See Figure 35 for details (92).

b) Separation

The elution of the weakly held isoenzyme (MM) is achieved by the addition of an increased concentration of chloride ions at the same pH. The greater density of chloride ions competes for the binding sites occupied by the MM isoenzyme, resulting in the displacement of the MM isoenzyme from the column. The more tightly bound CK-MB and CK-BB (if present) are retained on the column. See Figure 35 for details.

The elution of the B containing isoenzymes is brought about by decreasing the pH of the system and by increasing the concentration of the chloride ions. This has the effect of reducing the binding capacity of the CK-MB/BB and it is eluted from the column. See Figure 35 for details. The eluted CK-MB can now be quantitated. It should be noted that some techniques elute both MB and BB in the same fraction, so that LDH-1, 2 can be assayed from the
FIGURE 35

Diagrammatic Representation of Ion-Exchange Process

LEGEND
(a) CK-MM and CK-MB binding to ion exchanger after sample addition. Both isoenzymes are in dynamic equilibrium with the ion exchanger and chloride ions. The more highly charged CK-MB is bound by more exchanger positions.

(b) Addition of more chloride ions at same pH successfully competes for the CK-MM binding sites and washes it from the column. The more closely held CK-MB is retained.

(c) By addition of higher concentration of chloride ions and reducing the pH (binding capacity of CK-MB) the CK-MB is eluted.

This data was taken from reference 93 without the permission of the author.
FIGURE 35

Diagrammatic Representation of Ion-Exchange Process
same procedure. If BB is a concern, the conditions of the method can be adjusted so that BB is eluted separately (93).

c) Quantitation

The actual method by which the enzyme activity of the various CK isoenzyme fractions is assigned has produced many papers by a multitude of authors (77-79, 94-99) in the last few years. This section of the paper will attempt to review the development of the CK methods up to and including those in current use.

Methods for the estimation of CK activity have been based on the following reaction:

\[
\text{Creatine + ATP} \xrightarrow{\text{CK}} \text{Creatine phosphate + ADP} \\
\text{pH 9.0}
\] (15)

This is known as the forward reaction and was used by Tanzer and Gilvarg (79) in their estimation of CK activity. Two other enzymes, pyruvate kinase (auxiliary enzyme) and LDH (indicator enzyme) are used in this coupled enzyme method. The reaction sequences are as follows:

\[
\text{ADP + Phosphoenolpyruvate} \xrightarrow{\text{PK}} \text{ATP + Pyruvate} \\
\text{Pyruvate + NADPH + H}^+ \xrightarrow{\text{LDH}} \text{Lactate + NAD}^+ 
\] (16) (17)

The activity of the CK is determined indirectly by following the decrease in absorbance at 340 nm. This method was anything but optimal since the normal (reference) range for this method was stated as being 0 to 1 \(\mu\text{M/min/liter}\) at 37 degrees centigrade (compared to 0 to 160 \(\mu\text{M/min/liter}\) at 37 degrees for the Scandinavian method (100)). One of
the shortcomings of the so-called forward reaction is that
the equilibrium of the reaction is very much to the left,
since the standard free energy of hydrolysis of the creatine
phosphate is larger by 3 kilocalcs than that of ATP (101).
The reverse reaction should then be the reaction of choice.
The majority of methods have in fact used the reverse reaction.
An example of an estimation using the reverse reaction is as
follows:

\[
\text{Creatine phosphate + ADP} \xrightarrow{\text{CK, pH 6.7}} \text{Creatine + ATP} \quad (18)
\]

\[
\text{ATP + Glucose} \xrightarrow{\text{HK}} \text{Glucose-6-phosphate + ADP} \quad (19)
\]

\[
\text{Glucose-6-phosphate + NADP}^{+} \xrightarrow{\text{G6PDH}} 6-\text{Phosphoglucuronate + NADPH + H}^{+} \quad (20)
\]

The auxiliary reaction is mediated by hexokinase
(HK) and the final reaction indicated by glucose-6-phosphate
dehydrogenase. In this series of reactions the activity of
CK is proportional to the increase of absorbance as NADP is
reduced to NADPH at 340 nm. The absorption spectra of the
coenzyme can be seen in Figure 36. This type of method was
originally proposed by Oliver in 1955 (79). From reaction
18 for the reverse equation, it can be seen that there are
two products of the reaction. This means that there must
be another way of monitoring it, in fact creatine can be
assayed colorimetrically by reaction with diacetyl and alpha-
naphthol. This reaction was utilized by Hughes in 1962 (94).
Sax and Moore (95) in 1965 reacted creatine with ninhydrin
Absorption Spectra of the Reduced and Oxidized Forms of Nicotinamide Adenosine Dinucleotide
Absorption Spectra of the Reduced and Oxidized Forms of Nicotinamide Adenosine-Diphosphate

FIGURE 36

[Graph showing absorption spectra for NAD+ and NADH]
to give a fluorescent product, the intensity of which was proportional to the activity of creatine kinase. It would seem that the colorimetric methods are not as acceptable in terms of accuracy and precision as the kinetic 340 nm methods. Pryce et al. (102), performed a survey of commercially available creatine kinase kits and determined that of the 19 kits studied, 7 were found to be unacceptable. Of those kits found to be unacceptable, 6 were colorimetric end point methods.

On detailed examination of the literature it is evident that the method of choice for CK activity measurement is that one based on the original method of Oliver (79). All acceptable methods in current use are modifications of this initial method. Over the years since the introduction of this spectrophotometric method, attempts have been made to "optimize" the conditions of the assay. The most extensive work on CK methodology has been carried out by Gabor Szasz and his colleagues (35, 76, 103 - 105). This work by Szasz led to the publication of the recommended method by the Scandinavian Society for Clinical Chemistry and Physiology (100).

The fourth paper (105) is of particular interest as it is the one that deals with the substrate affinities of the different isoenzymes of creatine kinase. Szasz observed that it is possible to measure all three isoenzymes in serum under identical reaction conditions with a maximum of 3 percent inhibition of the MB isoenzyme under optimum
conditions of 30 mM of creatine phosphate and 2 mM of ADP. He confirmed the findings of others that the substrate affinities of isoenzymes increases with the increase in proportion of the B monomer. A paper by Morin (107) suggested that the magnitude of the difference was not as great as observed by Szasz. On the other hand, a paper by Wong and Smith (108) indicated the differences in substrate affinities of the three isoenzymes was quite marked. Szasz suggests that the differences might be due to different conditions of assay or might be due to differences in method of preparation of the isoenzymes used in the comparison. He also suggested that the inherent imprecision in calculation of the $K_m$ value might well be another contributing factor to the observed differences.

As mentioned previously, myokinase or adenylate kinase is an enzyme present in serum and has a wide distribution among the different organs, with decreasing activities in this sequence: skeletal muscle, brain, liver, heart, kidney, small intestine, spleen, and lung (103). Since the reaction catalyzed is

$$2 \text{ ADP} \xrightarrow{\text{AK}} \text{ ATP} + \text{ AMP}$$

(21)

it is apparent that the presence of AK in the serum will generate additional amounts of ATP and give rise to an apparent increase of CK activity. In sera of healthy individuals the AK ranges between 0 to 50 U/liter at 25 degrees centigrade. It is greatly increased in hemolyzed
sera. It is, therefore, essential to suppress the activity of the AK when measuring CK activity. The usual method of inhibiting the activity of the AK is by including AMP in the reaction mixture. Unfortunately, AMP also inhibits CK activity so it was necessary to compromise between "increased" activity due to AK activity or "decreased" activity due to CK inhibition. Szasz in his paper (103) suggested the use of another substance in addition to AMP. The chemical he investigated was diadenosine pentaphosphate, a recently described potent inhibitor of red cell and muscle AK, but with much less effect on other types of AK. He suggested the combined use of AMP and diadenosine pentaphosphate, and found the mixture of AK inhibitors gave 97 percent inhibition of muscle and red cell AK, and 95 percent inhibition of liver AK. The overall inhibition of CK is about 5 percent. Other authors have proposed the use of fluoride, namely Rosano et al. (108) and Morin (110), but the only trouble with using fluoride is that refrigerated solutions of reagent precipitate magnesium fluoride after several hours. Meiatlini (111) and his colleagues proposed the use of a combination of AMP and fluoride and claim they have overcome both the inhibition of CK by AMP and also the insolubility problem of fluoride. The optimum concentration of AMP found by these researchers was 2 mM/L (compared to 5 mM in Szasz's paper), and the fluoride concentration of 6 mM (compared to 25 mM in Morin's paper). However, these conditions really do not give any better performance than does Szasz's method; in
fact, they may be somewhat worse since Szasz found that
25 mM/L of fluoride inhibited CK by 6 percent, compared to
his formulation which gave a maximum CK inhibition of
5 percent.

There are two other aspects of total CK methodology that have received a great deal of attention recently in the literature. These are the effect of thiols on isoenzyme activity (35, 112 - 114) and the effect of chelators on CK activity (106, 115). Morin in his paper (112) considered the causes of CK isoenzyme inactivation which he observed to be due to such things as body temperature, which directly affects the rate of decay of the CK isoenzymes. This form of inactivation he found to be irreversible. Urate and catecholamines caused inactivation of the CK isoenzymes (presumably by oxidation of the sulfhydryl groups), the homogeneous dimer MM being less susceptible to inactivation than the MB or BB dimers. The urate inhibition was found to be reversible by use of suitable thiol compounds while the reversibility of the catecholamines was unpredictable. An interesting paradox is that both urate and catecholamines also have an insulating or protective effect on the CK isoenzymes. The age of the sera was observed to have a marked effect on reactivation of the CK isoenzymes. Several thiols have been suggested for the reactivation of creatine kinase. These include cysteine, glutathione, dithiothreitol or dithioerythritol, N-acetyl cysteine, and mercaptoethanol.
Morin states that in effectiveness mercaptoethanol, dithiothreitol and dithioerythritol are superior to the others listed above. Cysteine deteriorates rapidly in solution, thereby reducing the effective stability of the reagent. Glutathione, on the other hand, renders the assay susceptible to glutathione reductase interference. Mercaptoethanol has a higher efficiency of activation than does N-acetylcysteine, states Morin in his paper. Drawbacks to the use of the "Threitols" as reactivators of CK activity include microprecipitation of albumin during analysis, thereby causing false elevations of CK activity. Hence, Morin's final conclusion is that mercaptoethanol is the superior reactivating reagent for use in the assessment of CK activity.

Szasz in his study (35) found N-acetylcysteine to be superior to mercaptoethanol since at levels of thiol concentration that Szasz deemed necessary to provide adequate reactivation, mercaptoethanol became turbid after 3 hours at 30 degrees and 37 degrees centigrade, and after 24 hours at 25 degrees centigrade. N-Acetylcysteine in the same concentration caused turbidity only after 48 hours at 37 degrees centigrade and at lower temperatures did not disturb the assay for several days. From Szasz's study it is apparent that he considers the loss of activity of 5 percent to be negligible, as it lies within the range of method imprecision. Accordingly, permissible storage without benefit of thiol protection should be limited to 15 minutes for the BB isoenzyme at 37 degrees centigrade and 28 days for
the native MM at -20 degrees centigrade. Table 7 gives an overview of the tolerable storage of CK isoenzymes before activity measurement in the presence of 0 and 50 mM N-acetylcysteine. It is apparent from Table 7 that admixture of thiols to all sera submitted for CK assay is unnecessary. In fact, the MM isoenzyme native to serum requires no thiol protection, a fact also confirmed by Morin (112). At temperatures above 30 degrees centigrade thiols cannot delay the inactivation of any isoenzyme and may, in fact, cause turbidity in some sera. To cool the specimens as soon as possible after venipuncture is obligatory. Szasz found that thiols greatly increase the stability of the BB and MB isoenzymes at 30 degrees, 25 degrees and 4 degrees centigrade. The concentration of N-acetylcysteine required to attain this stability is in the order of 5 mM of serum.

Nealon and Henderson in their paper (113) arrived at similar conclusions to both Morin and Szasz, but added that "a storage of all CK isoenzymes at any temperature must be done with sulfhydryl protecting agent present." To minimize loss of activity during thawing it should be performed at 37 degrees centigrade for a period of time not to exceed 2.5 minutes. This was confirmed by Szasz.

From a clinical standpoint, Nierenberg (114) found that the absence of CK-MB in one patient with a clinically massive MI and a total CK of 2000 IU/L raised doubts about the sensitivity of this assay as to the way in which it was
TABLE 7

Tolerable Storage of Creatine Kinase Isoenzymes before Activity Measurement

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Purified prep. in serum matrix N-Acetylcysteine, mM/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native MM</td>
</tr>
<tr>
<td>37°C (min)</td>
<td>120</td>
</tr>
<tr>
<td>30°C (hours)</td>
<td>12</td>
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<tr>
<td>25°C (hours)</td>
<td>48</td>
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<tr>
<td>4°C (days)</td>
<td>14</td>
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<tr>
<td>-20°C (days)</td>
<td>28</td>
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</table>

This data was taken from reference 35 without the permission of the author.
being performed. Their study indicated that a reasonable compromise of different recommendations was to bring serum samples to a final concentration of mercaptoethanol of 5 mM. It was estimated that thiol groups remain active (reduced) for at least 72 hours when the mercaptoethanol is diluted with Tris-hydroxymethylmethylamine buffer with a pH of 7.0 and when stored at 4 degrees centigrade.

Rollo and Davis studied the effects of sulfhydryl compounds on the stability of CK in unfrozen serum and found that mercaptoethanol and N-acetyl cysteine led to an accelerated loss of the native CK activity in the sera studied. This appears to be in contrast to others (35, 112 - 114) who found the opposite to be the case. The difference, I feel, might be due to the length of time before the treated sera were frozen. Rollo further observed that the addition of cation chelators to the serum markedly improved the stability of the endogenous CK activity. Szasz (106) suggests that the apparent improved stability of the endogenous CK activity is due to the enhanced stability of the thiol reagent by the chelator in removing cations that potentiate breakdown of the thiol reagent. He (Szasz) concluded this after observing that the thiol groups of N-acetyl cysteine could be recovered almost completely after storage for a week at 25 degrees centigrade, and even three weeks at 4 degrees centigrade.

From the foregoing it is apparent that there is some difference in opinion as to the best way to handle
specimens for CK isoenzyme assay. It is, therefore, required that any lab that contemplates doing CK isoenzymes check out a method suitable to their environment so that the stability of the CK isoenzymes may be maximized.

Of all the published methods to date, Szasz's work seems to give the most accurate (102) and precise results, and this would appear to be the method of choice for the determination of total creatine kinase and also the individual isoenzymes independent of the method of separation, as long as they are in a suitable medium in which enzymatic activity can be measured.

(3) Activation

The main drawback of the column and electrophoretic methods is the time involved in the performance of the separation and the assay of the CK isoenzymes. An alternate approach pioneered by Rao et al. (116) and researched by Colman and McCroskey (117) is that of "differential activation" which takes advantage of the fact that CK isoenzymes are not activated equally by dithiothreitol and glutathione. The technique assumes that glutathione activates only the MM dimer and that dithiothreitol activates both MM and MB isoenzymes. Rao concluded from this observation that the total CK activity (derived from activation by both GSH and DTT) minus the activation due to the GSH alone was equal to the isoenzyme CK-MB. If this method works and gives results which are clinically useful, it would represent a giant step
in the methodology which has arisen around CK isoenzymes, as the procedure can be completed within 10 to 15 minutes of receiving the patient's serum sample. The big question with this method is - are we really measuring the dimer CK-MB, or is it that GSH has such a low redox potential that it is inefficient in activating CK-MB? Before we answer this question, let us consider some of the properties of the activating agents GSH and DTT (See Figures 37 and 38).

Glutathione is a tripeptide made up of glutamic acid, cysteine and glycine, and has the primary structure indicated in Figure 37, which derives its reducing properties from the sulphhydryl group present on the cysteine residue. From its structure it can be seen that GSH is a monothiol. Its activating effect is concentration dependant, being greatest in the range 8.7 to 15 mM/L for CK-MM and in the 8.7 to 9.7 mM/L range for CK-MB. It should be noted at this point that the activating effect of GSH on CK-MB only occurs in isolation, and when CK-MB is mixed with CK-MM GSH it has no activating effect on the CK-MB dimer.

In contrast to GSH, DTT has a uniform effect on CK-MB and CK-MM even in admixture. Its ability to activate CK isoenzymes appears to be independant of concentration since no difference in activating capacity is observed over the concentration range 12.5, 25 and 50 mM/L. It is interesting to take a look at the proposed mechanisms of reduction postulated by Haugaard (118) and Cleland (119). It is thought that the different redox potentials of the two thiol
FIGURE 37

Structure of Glutathione

CH₂-C-N-CH₂-C-N-CH₂-COOH

FIGURE 38

Structure of Dithiothreitol

H-C-SH
H-C-OH
H-C-OH
H-C-SH
compounds play an important role in their differing effects on the isoenzymes. DTT is structurally different from GSH in that it is a substituted tetritol and has two sulphydryl groups. Structure can be seen in Figure 38. The relative redox potentials at pH 7.0 are GSH -0.25 V, and -0.33 V for DTT. Glutathione activates CK by reducing the disulphide bonds of the creatine kinase in two stages:

\[ \text{EN} \cdots \text{S} \cdots \text{EN} + \text{GSH} \rightleftharpoons \text{EN} \cdots \text{S} \cdots \text{EN} + \text{S} \quad \text{(inactive enzyme)} \]

\[ \text{EN} \cdots \text{S} \cdots \text{G} + \text{GSH} \rightleftharpoons \text{G} \cdots \text{S} \cdots \text{G} + \text{EN} \cdots \text{S} \quad \text{(active enzyme)} \]

Due to the fact that the redox reaction is reversible and that equilibrium constants are close to unity, a considerable amount of glutathione is required to completely activate CK. Unfortunately, GSH concentrations greater than 10 mM for CK-MB and 15 mM for CK-MM decrease the activating capacity of this reducing agent. At high GSH concentrations the rate of reaction decreases due to the accumulation of oxidized GSH which favors the reverse reaction of (19) and (20).

It is, therefore, obvious that the redox capacity of glutathione is somewhat limited in its ability to completely activate CK isoenzymes. On the other hand, dithiothreitol leads to complete and irreversible reduction of the disulphide bonds in the CK isoenzyme molecules. This arises because of the formation of a stable ring compound, oxidized dithiothreitol, which is not so easily reduced due to the inherent stability of the ring structure. The mechanism of this
reaction might be represented by the equation shown below:

$$\text{EN}----S----S----\text{EN} + \text{HS}----\text{CH}_2----(\text{CHOH})_2----\text{CH}_2----\text{SH} \rightarrow$$

$$\text{EN}----\text{SH} + \text{EN}----S----S----\text{CH}_2----(\text{CHOH})_2----\text{CH}_2----\text{SH}$$

(active enzyme)

(24)

Rao in his paper (116) indicated that it was not clear why GSH did not activate CK-MB in admixture and suggested that it might be that the reducing capacity of the GSH is too weak to reduce both CK-MB and CK-MM completely. This author found excellent correlation between CK-MB activated admixture with CK-MM by DTT and the same CK-MB activated by GSH in isolation ($r = 0.998$). From this observation he reasoned that the mechanism of action of the DTT could not be explained solely on the difference of reducing capacities.

Earlier work of Haugaard (118) and Cleland (119) suggested that the DTT specificity for CK-MB may be related to one of its enolic hydroxyl groups, which forms an ester type of activated addition compound with the carboxylate groups of the predominantly acidic MB molecule. They further indicate that the "ester" type linkage is essential for the activation of CK-MB in serum. Since glutathione is a
predominantly acidic tripeptide with no enolic hydroxy
group, it cannot form an active ester type of linkage with
CK-MB and hence its reducing efficiency is much less than
that of DTT. Somer and Konttinen (120) emphasized the
importance of DTT for CK-MB activation and found that the
amount of MB activity measured in serum following electrophoresis was dependant on the sulphhydril compound used in the
incubation medium. Electrophoretically, an MB band was not
observed in the presence of GSH or in the absence of other
sulphhydril group compounds, but with the addition of dithio-
theitol a clear distinct band was observed. Rao stated
that he found favorable comparisons of his "Selective Activ-
ation Technique" (SAT) with those by electrophoresis/spectro-
photometry \( (r = 0.998) \) and fluorometry \( (r = 0.996) \). All his
comparisons were with sera which had total CK values in
excess of three times the upper limit of normal. He did not
appear to show any comparisons in the normal range or in that
grey area between normal and borderline abnormal; therefore,
his paper failed to give any indication of the discriminating
power of this test.

Very little support for this method has appeared
in the clinical chemistry literature. An initial enthusiastic
response was shown in a paper by Wlodarski, Howard and
Trainer (121) where they stated that the SAT is more
sensitive but less specific than the electrophoresis method
and may, therefore, be used as a screening test with
confirmation of positive results by electrophoresis.
However, in October of 1977 (122) Trainer withdrew his support for this method and indicated that his experience with this test since publication of his paper suggested that the sensitivity of this test for MI was 68 percent, an extremely low sensitivity. He further stated that he found the test relatively insensitive and nonspecific for MI. Similar findings have been observed by other authors (123, 124).

As indicated before, the measurement of CK-MB by this method entails dual measurements and the difference between these readings is an indication of the CK-MB present. This fact in itself can lead to analytical problems. For example, let us assume that we have a CK of 500 and a coefficient of variation of 5 percent for the CK analysis, which means that the standard deviation is 25 IU/L or 50 IU/L for 95 percent confidence limits. If we now compare this with the normal range of the Selective Activation Method (most recent information from Calbiochem indicates that the cutoff is now 20 IU/L), we find that this value of 20 IU/L is well within the experimental error of the method. Very strong criticism of this method has been voiced by Morin in a letter to Clinical Chemistry (125) in which he states, "I do not question that there is a difference in activity between treated and untreated sera or even that the difference is often greater if MB is present. All of the evidence indicates that the difference represents inactive creatine kinase and that the relative
proportion in recovery of MM, MB and BB in this inactive fraction depends on too many uncontrolled variables to be a reliable quantitative index of MB. This consideration and the high incidences of false positives and negatives already noted by myself and others (126, 127) all support my conclusion that the method does not have adequate resolution to offer a reliable diagnostic index."

In answer to the question posed earlier in this paper, I feel that the letter written to the editor of Clinical Chemistry by Morin (125) answers quite succinctly the question as to the validity of the selective activation technique.

(4) Antibody

Methods to date have utilized separation techniques followed by enzymatic activity measurement of the separated isoenzyme, or have depended on differential activation of the CK-MB and CK-MM isoenzymes. Another approach makes use of the immunochemical properties of the individual monomers and causes anti-sera to be generated in sheep or goats which can inhibit or precipitate its respective antigen (CK-M or CK-B). This approach allows expression of CK isoenzymes in terms of activity or concentration. The activity methods can arise from precipitation, and hence inactivation of the selected monomer, of one or other of the dimer monomers, CK-M or CK-B, or by immunoinhibition of the same. Once this precipitation or immunoinhibition has occurred, the residual enzymatic activity
can be determined. The other approach which expresses CK isoenzymes in concentration terms is the standard competitive protein binding radioimmunoassay. Separation of the free CK-M or CK-B from the bound, tagged CK-M or CK-B is achieved by either ammonium sulphate precipitation or by a double antibody procedure. These, then, are the so-called antibody methods which will now be discussed in detail.

(a) Precipitation/Inhibition Techniques

The two different immunochemical methods that may be used jointly with kinetic assays of CK isoenzyme in serum are as follows:

i) The measurement of serum CK activity before and after immunoprecipitation with specific anti-CK-M monomer and anti-CK-B monomer precipitating antibodies permits quantitation of the CK isoenzymes MB (128, 129). The main problem with this approach is the length of time involved in performing the assay. The specimen requires initial dilution to a specified range of total CK activity. Also necessary in this method is an incubation at 4 degrees centigrade for 15 to 18 hours to ensure completeness of precipitation, and a centrifugation step before the actual measurement of residual CK-MM and CK-BB activities in the supernatant fluid. This method, therefore, is not really suitable for use in a routine laboratory and will not be discussed further.

ii) A much simpler and more rapid method uses the measurement of creatine kinase in the absence and presence
of a specific anti-CK-M monomer inhibiting antibody (130, 131). The difference in enzymatic activity is due to the presence of B monomers which is assumed to come from cardiac muscle. Measurable CK-BB activity in serum is extremely rare and, therefore, determination of serum CK-B is a valid measure of CK-MB activity (131). The basic principle of this method can be seen in Figure 39. The measurement of the enzymatic activity performed by Szasz's method (76) was discussed in detail earlier in this paper. When carried out at 37 degrees centigrade, Gerhardt et al. (130) found that the rate of immunoinhibition of human CK-M-subunits corresponded to a half-life of 38 seconds when samples were incubated as per conditions of the test. Under these selected conditions, immunoinhibition of serum CK-M subunit activity up to 1800 U/L was 99 percent complete within 5 minutes using undiluted samples. Sample myokinase was found to constitute a potent source of falsely increased serum CK-B activity, even though two AK inhibitors are used in the reaction mixture. However, a frequency analysis of sample AK activities demonstrated that AK activities of more than 8 IU/L will occur in about 10 percent of the cases. The authors consequently deemed it necessary to measure the individual sample blank AK rates. The routine procedure that they developed thus included three separate measurements; determination of total CK and of CK-B in the absence and presence of antibody, respectively. The authors corrected the CK-B activity in two ways. Since immuno-
FIGURE 39

Schematic Representation of an Immunoinhibition Method for Creatine Kinase Isoenzymes

This data was taken from reference 130 without permission of the author.
Schematic Representation of an Immunoinhibition Method for Creatine Kinase Isoenzymes

FIGURE 39
inhibition was only 99 percent, a value of 1 percent of the total CK activity was subtracted from the measured CK-B activity. Similarly, the individual sample AK activity was measured and subtracted from the apparent CK-B activity. I personally feel that all of the subtractions decreased the precision of the method. These authors concluded that they had a viable method for CK-MB isoenzymes. They also authored a paper on the clinical usefulness of their technique and concluded that they had a "highly selective test for AMI." However, I am inclined to agree with Morin that the test lacks resolution (132) due to the additive imprecision of the blanks and approximations for the 99 percent immunoinhibition.

b) Radioimmunoassay

Of all the methods mentioned to date, RIA probably gives the most accurate values since it is capable of measuring all forms of the isoenzymes, activated and non-activated, and does not rely on activity measurements with all its inherent problems. The main drawback with this method is the length of time involved in performing the assay. However, the RIA methods are of great importance and will, therefore, be discussed in some detail.

The most popular of all the RIA methods for CK isoenzymes seems to be the double antibody technique. This method involves incubating $^{125}$I labelled CK-BB mixed with patient serum containing the CK isoenzymes, with dilute
anti-CK-BB serum derived from sheep or goats. The CK-antiserum forms a soluble antibody-antigen complex with all material containing the B monomer as CK-MB and CK-BB independent of whether or not it is natural or radioactive. The amount of tagged-CK-BB occurring at equilibrium in the soluble antibody-antigen complex will depend on the initial concentration of natural CK-B derived from the patient's serum. This initial incubation usually takes 17 to 24 hours, and is carried out at 4 degrees centigrade. At the end of this period of time a second antibody is used to precipitate the antibody-antigen complex. This is an antispecies gamma-globulin derived in another animal than the one used to produce the first antibody. A non-immune serum is usually used to increase the bulk of the precipitation. The incubation required for the precipitation of the antibody-antigen complex usually takes one hour at 4 degrees centigrade. After this time the precipitates are centrifuged out and their radioactivity determined. The radioactivity of each tube is then expressed as a percentage of the blank tube (BO) and this percent bound is then plotted as ordinate versus log of the concentration as abscissa. See Figure 40 for percent bound versus log concentration in ng/tube CK-B. A slight variation on this technique making the method more sensitive is referred to as sequential saturation analysis, the difference being that the addition of the tracer (labelled CK-BB) is left until the end of the first incubation step. Several
researchers have used this approach to determine the concentration of the CK isoenzymes (133 - 138).

The reason that this method can be utilized for the detection of CK-MB is based on the following assumptions:

i) High cross-reactivity for the B monomer in the MB dimer when using anti-CK-BB antiserum, is seen.

ii) The level of BB dimer in the serum from patients suspected of having a myocardial infarction is low or negligible.

iii) The CK-BB dimers in serum are negligible in "normal" people.

Recently, however, papers are appearing which indicate that the CK-BB is more prevalent than was at first thought, and has been observed in such cases as renal failure (139), surgery of the central nervous system (140), and malignant hyperthermia (141). Such tests, therefore, have to be used with extreme caution and under the appropriate clinical conditions to reduce their nonspecificity.

One final point is worth noting. Neumeiere (138), and his collaborators indicate that the avidity of anti-CK-BB is complete for the CK-MB dimer, while Zweig and his co-workers (142) found that the cross-reactivity of anti-CK-BB was only 3 to 17 percent when one might have expected
FIGURE 40

Standard Curves for CK-B Assay

LEGEND

(a) 8-80 ng/ml.
(b) 40-800 ng/ml.

This data was taken from reference 138 without the permission of the author.
FIGURE 40

Standard Curves for CK-B Assay

A

\[
\frac{B}{B_0} \quad \text{vs.} \quad \text{CK-B (ng/ml)}
\]

B

\[
\frac{B}{B_0} \quad \text{vs.} \quad \text{CK-B (ng/ml)}
\]
50 percent. They suggested that the reason for this might be that M and B in combination do not elicit the same response as B and B do in combination.

In conclusion, it is Morin's opinion that the performance expected of the immunological methods is best realized by the RIA methods rather than by those utilizing immunoinhibition (132).

These, then, are the methods currently in use in the field of clinical chemistry for the study of the isoenzymes of creatine kinase. The actual method of choice will be discussed in the next chapter following the discussion of methods currently available for the study of the isoenzymes of lactate dehydrogenase.

B. LACTATE DEHYDROGENASE METHODS

Lactate dehydrogenase was one of the first enzymes that was found to exist in multimolecular forms (6). Today it is one of the most frequently ordered enzyme tests that is utilized by the physician. Even so, its full capability as a diagnostic agent is not being maximized since the number of requests for LDH isoenzymes is minimal. There are probably many reasons for this, not the least of which is the need to familiarize the clinician with the interpretation of the results.

The methods for LDH isoenzymes are numerous although not as commercially exploited as CK isoenzymes. This, however, may be just a matter of time as the popularity
of CK/LDH profiling comes of age. Methods include electrophoresis, column chromatography, use of inhibitors, and heat inactivation. The last two mentioned techniques depend on a before and after activity measurement. All in all there are quite a few methods to choose from. As in the discussion on CK isoenzymes, only those relative to clinical chemistry will be discussed. The first two methods for the determination of LDH isoenzymes can be conveniently divided into separation (both by physical means) and detection. With electrophoresis, one extra step is required, that being quantitation by scanning densitometry or a qualitative assessment by visual inspection. The chromatographic method has its identification and quantitation in the same analytical step, that is the measurement of LDH activity in the column effluents.

1) Electrophoresis

Separation of LDH isoenzymes by electrophoresis has been reported using agar (143), starch (32), cellulose acetate (144), acrylamide gel (82) or agarose gels (74) as the electrophoretic support medium. The most popular of these support media are agarose and cellulose acetate, with acrylamide gel gaining in popularity. In my experience the easiest medium to work with is the agarose gel, since the material can be obtained prepoured in a plastic well with a mylar back. All that is required is for the film to be peeled from the hard plastic backing plate and the preformed gel to be inoculated. The thinness of the
gel allows for improved precision of isoenzyme quantitation. Uniformity of thickness allows for even penetration of the substrate into the gel. Agarose has a high chemical purity and gives excellent protein resolution. Agarose gel, in contrast to cellulose acetate, stabilizes the isoenzymes within the gel matrix and it is, therefore, unnecessary to use a precipitating histochemical stain to locate the reaction sites. In agarose gel films, the LDH isoenzymes separate into zones of equal area and hence the error resulting from differing reaction rates in zones of varying widths is greatly reduced.

The actual separation technique requires one μl of serum to be inoculated into the preformed well on the gel. The gel is then placed inside the electrophoresis cell for the length of time required to give adequate separation of the LDH isoenzyme bands. At the end of this time the gel is removed and prepared for spreading of the substrate. The buffer used in this application is barbital, pH 8.6, ionic strength 0.05 M. This is the basic separation step and is common to all electrophoretic methods. This is also the method of Elevitch (74).

Once separation of the LDH isoenzymes has taken place the next step is their location. This can be achieved by one of two basic methods:

a) by use of a locating agent which absorbs in the visible range of light.

b) by use of a locating agent which absorbs in the ultraviolet end of the light spectrum.
a) The substrate-colour reagent which is allowed to react with the separated isoenzymes is composed of the following chemicals:

i) lactate

ii) NAD⁺

iii) methylphenazonium methosulphate (MTT)

iv) nitroblue tetrazolium

v) Tris buffer 0.1 M pH 9.2

This sequence of reactants can be best visualized by the following:

\[ \text{Lactate} \xrightarrow{\text{NAD}^+} \text{methyl-dihydro-phenazonium} \xrightarrow{\text{tetrazolium salt}} \text{tetrazolium} \]

\[ \text{Pyruvate} \xrightarrow{\text{NADH}} \text{methylphenazonium} \xrightarrow{\text{formazanion}} \text{formazan} \]  

(25)

This type of reaction sequence can be used on most electrophoretic support media. It was used by Latner and Skillen (145) and modified by Wilkinson (146) by using the tetrazolium salt produced by Tsou in 1956 (147). The advantage of this tetrazolium salt over the others is that it remains more compactly at the site of its production than does MTT and also gives sharper definition of the isoenzyme bands (146). These bands can be scanned in a scanning densitometer.

b) The main drawback of the tetrazolium salt technique is the lack of sensitivity. A much superior sensitivity is obtained by the use of Elevitch, Kelly and Feichtmeir's method (74) which utilizes a fluorogenic reagent film. The reaction sequence can best be followed by observing this equation:
Lactate + NAD$^+$ $\xrightarrow{\text{LDH}}$ Pyruvate + NADH + H$^+$  \hspace{1cm} (26)

Following the electrophoresis the LDH isoenzymes are incubated at 37 degrees centigrade with a liquid reagent film containing lactate and the non-fluorescent coenzyme, NAD. The amount of fluorescent reduced coenzyme (NADH) that is formed is proportional to the amount of lactate converted to pyruvate. The fluorescence of the NADH can be measured in a scanning fluorometer.

It is worthy of note and applicable to both the aforementioned techniques that the following is considered:

i) Variation in conditions might lead to serious inaccuracies, for example, prolonged incubation might lead to over estimation of the minor bands after substrate exhaustion of major bands.

ii) Diffusion of dye occurs from zones of high intensity to zones of lesser intensity, thereby over-estimating the lesser zones.

iii) Errors might also be introduced by use of a fixed substrate concentration, since isoenzymes may differ considerably in their substrate affinities (148).

But ii) above does not apply to method b) as applied to agarose, since the bands are fixed in the matrix of the gel and cannot diffuse out. Both of the aforementioned staining techniques can be applied to cellulose acetate but there are less interferents on the agarose gel for the fluorescent technique. Both sets of staining reactions are carried out in a humidified chamber at 37 degrees centigrade.
and in the dark, as they are light sensitive.

The five peaks which represent the individual isoenzymes of LDH can be quantitated by either calculating the area under each peak and expressing each as a percentage of the total area, or by measuring the peak height and using this as a representative of peak activity. McKenzie and Henderson ably justified this approach (148). Hence, final laboratory diagnosis depends on both visual and quantitative examination and comparison with reference values. It is of interest to note that similar LDH isoenzyme values are obtained when lactate or pyruvate is used as the substrate. This, of course, suggests that the methods have the same accuracy (149). It is the opinion of Henderson (149) that the agarose thin-gel technique appears to give a substantially unbiased estimate of both LDH-1 and LDH-5. This can only be a provisional conclusion since there are no reference methods for LDH isoenzymes or even LDH activity. The estimation of LDH isoenzymes by electrophoresis is a definitive technique as the actual separation can be readily visualized and it is this person's opinion that it is the best method for use as a reference procedure.

(2) Columns

While electrophoresis has been taken as the most definite technique, it has several drawbacks. One of these is the expense of the equipment required for the analysis. Another is that runs usually require a minimum number to
keep down the cost of each determination. The time involved is an important factor, so if more rapid methods are available they may become the methods of choice under a given set of conditions. Ion-exchange chromatography is also a physical method of separation and allows for separation of the isoenzymes based on their relative charges. The current methods available vary in their diagnostic efficiency as some methods are only able to isolate the "cardiac end" while others isolate the "liver end". This has the advantage that one can select the optimum reaction conditions since the liver (LDH-5) has a different substrate concentration that is optimal. For pyruvate LDH-1 it is 1.2 and for LDH-5 it is 3.5 mM/L (149).

Researchers in the field of ion-exchange chromatography of isoenzymes include Mercer (150), Lederer et al. (90) and Yasmineh (89), and they all have their own variation of the ion-exchange chromatographic technique, varying in size of columns, buffers used, pH, and number of wash and elution steps. Table 8 summarizes two of the available methods. One of the interesting features of the column methods is that they have the potential for performing simultaneous determinations of CK-MB and LDH-1, 2 and LDH-5. If this, in fact, can be obtained with the required degree of sensitivity and specificity for the isoenzyme in question, then this is definitely a major advance. The unfortunate part of speed up and miniaturization of chromatographic techniques is that this drive for efficiency tends to
### TABLE 8

Summary of Two Column Methods for Lactate Dehydrogenase Isoenzymes

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>LEDERER</th>
<th>MERCER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resin</td>
<td>Sephadex A-50</td>
<td>Sephadex A-50</td>
</tr>
<tr>
<td>Buffer</td>
<td>pH 8.0, 50mM/L Tris.</td>
<td>pH 8.0, 50mM/L Tris.</td>
</tr>
<tr>
<td>Amount of serum</td>
<td>1.0 ml</td>
<td>0.25 ml</td>
</tr>
<tr>
<td>Eluate 1</td>
<td>0.1 M NaCl pH 8.0 (LDH-5,-3,-4)</td>
<td>0.1 M NaCl pH 8.0 (LDH-5,-4,-3)</td>
</tr>
<tr>
<td>recovers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluate 2</td>
<td>0.1 M NaCl pH 8.0 (discarded)</td>
<td>0.15 M NaCl pH 8.0 (LDH-2)</td>
</tr>
<tr>
<td>recovers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluate 3</td>
<td>0.2 M NaCl pH 8.0 (discarded)</td>
<td>0.2 M NaCl pH 8.0 (LDH-1)</td>
</tr>
<tr>
<td>recovers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluate 4</td>
<td>0.5 M NaCl pH 7.0 (LDH-1&amp;2)</td>
<td></td>
</tr>
<tr>
<td>recovers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column Material</td>
<td>Polypropylene</td>
<td>Glass</td>
</tr>
</tbody>
</table>
reduce the overall resolution of the method leading to a loss of sensitivity and specificity. However, the search continues and the present day field of clinical chemistry is being flooded with modifications of the above mentioned authors' original methods. Hopefully, continued research will lead to a column which has a minimum number of the aforementioned problems.

The column component is only the first stage in the assay of the LDH isoenzyme. The second stage depends on the kinetic determination of the LDH activity in the different eluates. This assay method should be formulated according to the Scandinavian recommended method for LDH assays (151).

As mentioned previously, lactate dehydrogenase catalyzes the following reaction:

\[
\text{CH}_3\text{C}O\text{COOH} + \text{NADH} + H^+ \rightarrow \text{CH}_3\text{CHOH}\text{COOH} + \text{NAD}^+ \\
\text{pyruvate} \quad \text{L-lactate}
\]  

(27)

From this reaction sequence it can be seen that pyruvate or lactate can be used as the substrate. The forward reaction is based on pyruvate as substrate and from a mechanistic point of view this reaction is to be preferred as the equilibrium of the reaction lies well on the lactate side of the reaction sequence. The disadvantages of the forward reaction are that lactate is more stable than pyruvate, NAD is more stable than NADH, and it is also cheaper. Based on this reaction, two types of methods have been used to determine the activity of lactate dehydrogenase. One method
is kinetic and measures the rate of increase or decrease of absorbance of the coenzyme. Wroblewski and La Due 1955 (152), were responsible for the forward reaction, pyruvate to lactate, while Wacker in 1956 (153) developed a method based on the reaction lactate to pyruvate. The other type of method is colorimetric, based on the reverse reaction and uses the formation of pyruvate dinitrophenylhydrazone as a measure of LDH activity. This method is an endpoint type of method and was developed by King in 1959 (154). On the other hand, the loss of pyruvate dinitrophenylhydrazone was used by Cabaud and Wroblewski in 1958 (155), this also being a colorimetric endpoint method.

The method used to determine the activity of column effluents is usually kinetic and based on either the forward or reverse reaction, mainly depending on preference, although a case could be made for pyruvate if the main interest was the optimization of a method for LDH-5 isoenzyme. Lederer (156) and Mercer (150) used the method of Wacker (153) for the determination of the LDH activity in their column effluents. A recent paper by Buhl and Jackson in 1978 (157) stated that either method is equally reliable; however, for interinstrument and interlaboratory comparison, results from the lactate to pyruvate reaction seem more reliable. At this point in time there does not seem to be a multitude of papers that have appeared in the literature as in the case of CK methods. It would, therefore, seem opportune to use methods for LDH which conform to the recommendations of
the Scandinavian Society for Clinical Chemistry (151).

Another technique, while not strictly a column method, entails the selective adsorption of LDH-1 and LDH-2 onto DEAE-cellulose. This test, devised by Hess and Walter in the early sixties (158), gives a simple method by which the cardiac isoenzymes can be separated from the other non-cardiac isoenzymes. This method uses dialyzed serum which is treated with a suspension of DEAE-cellulose in phosphate buffer at pH 6.0 for a period of 10 minutes. The ion-exchange cellulose is then removed by centrifugation. The activity of the supernatant is then compared with that of the untreated serum. Hess and Walter observed that in diseases such as AMI and hemolytic anemia, less than 15 percent of the total activity remained in the supernatant. This indicated that approximately 85 percent of the activity must have belonged to the LDH-1 and LDH-2, since the aforementioned diseases are associated with release of the isoenzymes of LDH, that is LDH-1 and LDH-2. In comparison, 70 percent of the activity remained when patients were known to be suffering from viral hepatitis, this being associated with LDH-5 increases.

This last mentioned test has not as yet found popularity in North America; although it was sold by Boehringer-Mannheim Corporation (159) about ten years ago, as a kit. These are the methods presently available for the determination of LDH isoenzymes using ion-exchange principles.
(3) Chemical

The chemical methods of LDH isoenzyme determination include the isoenzyme's behaviour towards inhibitors such as oxalate and urea, and also there will be brief mention of alternate substrates, although these have been found to have minimal diagnostic significance in terms of myocardial damage. Selective precipitation by the addition of acetone or chloroform will also be discussed under this heading.

a) Inhibitors

In 1965 Emerson and Wilkinson (160) studied the effect of 0.02 M oxalate and 0.2 M urea on the serum activity of LDH. They observed the following:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Effect of Oxalate</th>
<th>Effect of Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Patients</td>
<td>61±3.5% inhibition</td>
<td>52±6% inhibition</td>
</tr>
<tr>
<td>Liver Disease</td>
<td>39±15% inhibition</td>
<td>78±9.5% inhibition</td>
</tr>
<tr>
<td>Myocardial</td>
<td>72±4% inhibition</td>
<td>31±12% inhibition</td>
</tr>
</tbody>
</table>

It can be seen that the fastest moving isoenzymes, LDH-1 and LDH-2, are much more affected by oxalate than the slower isoenzymes LDH-4 and LDH-5, while the reverse is the case with urea. It was suggested by these authors that, while urea might be of greater diagnostic value, the two inhibitors are complementary. Several reports by Konttinen and Lindy in 1967 (161-163) indicate that the sensitivity of the urea inhibition test can be improved by increasing the concentration of the pyruvate substrate. Under these
conditions it is reported that the inhibitory effect of urea on LDH-1 and LDH-2 is almost entirely eliminated, whereas LDH-4 and LDH-5 remain urea sensitive. This modification is reported to make the test more discriminatory than existing tests in the diagnosis of diseases that cause increases in the heart or liver isoenzymes.

b) Alternate Substrates

Pyruvate is not the only keto acid that is acted upon by lactate dehydrogenase; in fact, the next homologue of pyruvate has been used. Its action which was studied by Elliott and Wilkinson (1961) (164) is represented as follows:

\[
\text{CH}_3\text{CH}_2\text{CO.COOH} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{CH}_3\text{CH}_2\text{CHOH.COOH} + \text{NAD}^+ \quad (28)
\]

The above reaction can be reacted either way depending on conditions, just the same as when using pyruvate or lactate. In this particular example, the reverse reaction using \(\alpha\)-hydroxybutyrate is used and Elliott and Wilkinson used the ratio of LDH/HBD activities to assist in the diagnosis of disease processes. They found that the normal person had a ratio of 1.18-1.60, below 1.18 in most cases of myocardial infarction, and above 1.60 in liver disease. Elliott and Wilkinson claim that this indicates that the LDH-1 isoenzyme found to a greater degree in heart muscle and red blood cells is more active against \(\alpha\)-hydroxybutyrate than is the LDH-5 isoenzyme found to a greater extent in liver. However, a number of cases have been observed in which HBD has been
increased in non-cardiac conditions such as hepatocellular disorders. This fact tends to reduce its usefulness as a reliable indicator of myocardial damage (60).

c) Selective Precipitation

It was reported in 1963 by Latner and Turner (165) that precipitation of the serum proteins with acetone allowed a proportion of the fast moving isoenzymes of LDH to remain in solution in an active form. At the time it was thought that this might form the basis for a diagnostic test. However, up to this time no one seems to have taken this suggestion seriously.

(4) Heat Inactivation

Just as with other enzymes, lactate dehydrogenase has isoenzymes which are differentially affected by heat. It has been observed that the various LDH isoenzymes differ in their ability to resist heat inactivation. Wroblewski and Gregory (166) in 1961 took advantage of this difference in properties to decide whether the LDH was of liver or heart in origin. The test entails heating the serum in a water bath at 60 degrees centigrade for 60 minutes and then comparing the LDH activity before and after heating. These two researchers found that after heating, if the patient had the isoenzymes present in their normal proportions, then there was 30-60 percent of the activity remaining. In cases of AMI, more than 60 percent of the activity remains after heating. If the patient is suffering from some other form of disease such as liver disease, the percentage remaining
after heating is less than 30 percent, even if the total activity is increased. These observations indicate that the fast moving isoenzymes, LDH-1 and LDH-2, are more resistant to heat than the liver isoenzyme.

The aforementioned constitutes a review of methods for LDH isoenzymes that have been used to assist the physician in arriving at the correct diagnosis. It should have been noticed that no consideration has been given to methods involving immunological techniques. This is because an antibody to H antigen will react with LDH-1, LDH-2, LDH-3 and LDH-4 and an antibody to M antigen will react with LDH-5, LDH-4, LDH-3 and LDH-2. This cross reaction virtually eliminates immunological techniques as discriminating methods for the differentiation of cardiac and liver diseases.

C. DISCUSSION ON CHOICE OF METHODS

Methods for the detection and quantitation of isoenzymes are controversial, especially when applied to the isoenzymes of CK. This situation is not hard to understand since we still do not have standard methods by which the overall activity of the enzyme can be determined. Until we can say with certainty that we have so many weight units of the enzyme just as we can with the primary standard of analytical chemistry, the situation is not going to improve. In the meantime we are left with highly refined techniques and instrumentation which may or may not be
measuring what we say it is measuring. The clarification of this enigma is then the job of the clinical enzymologist. In an attempt to assist in this difficult task, the selection of a method which is suitable for use in the clinical laboratory will be attempted. The selection of a suitable method for CK will be dealt with first, followed by the selection of a method for LDH.

(1) Creatine Kinase
   
   As already discussed, methods for creatine kinase isoenzymes can be summarized as follows:
   
   a) Electrophoresis
   b) Column
   c) Selective Activation
   d) Immunological
      i) Non radioimmunoassays
      ii) Radioimmunoassays
   e) Differential substrate techniques
   
   There are currently on the market commercial products for all of the aforementioned techniques with the exception of the differential substrate method. This procedure by Witteven et al. (167) lacks absolute specificity and isoenzyme discrimination especially at high enzyme activities, mainly due to the fact that enzyme methods are so influenced by the environment that highly reproducible methods are not readily available. This applies also to other methods such as selective activation and immunological
methods which rely on a "before and after" technique for determining the activity of the MB isoenzyme. It is important to remember that the MB isoenzyme constitutes only 40 percent of the total CK activity, even in heart muscle. Also the release of MB and MM from the myocardium is considerably diluted by the overall blood volume as soon as it finds its way into the general circulation. These two methods measure the activity before and after the minor component has been either activated (SAT) or inactivated. This usually means the difference between two large numbers. Let us consider the following results from a SAT method. Total CK is 500 IU/L. CK before DTT activation equals 475 IU/L, therefore the difference due to CK-MB is 25 IU/L. Reference range is 0 to 15. Going on this criteria, this result is positive and the patient may have had an infarct. However, let us look a little closer. The state of the art as far as methodology is concerned gives us a coefficient of variation of about 5 percent plus or minus one percent. Therefore, this 500 IU/L value could be 450 to 550 IU/L (95 percent confidence limits). It can be seen that the 25 IU/L difference is well within experimental error and, therefore, is hardly a reliable indicator of the presence of CK MB.

Another criticism of the SAT method is that we are not sure what is happening in this method, even though a theory was developed by Rao and his coworkers (116). Morin was very critical of this technique (125) (132) as well as the immunoinhibition methods. This now leaves us with
three possible methods of choice-RIA, column and electrophoresis. As discussed previously, RIA methods are of two types, having been developed for both CK-MM and CK-BB. The CK-BB has been used to determine CK-MB concentration with the assumption that CK-BB does not exist in serum at levels that are going to interfere. This, unfortunately, is not the case. Further problems arise with these methods since they are time consuming (72 hours), expensive, and labour intensive. Galen, (168) has stated that "they may be useful in the research setting to develop models and regimens for infarct size reduction since they do offer a high degree of sensitivity, accuracy and precision in the absence of CK-BB. RIA is not likely, however, to become a routine method for analyzing CK isoenzymes." This now leaves us with the choice of two types of methods for the determination of CK isoenzymes, both of which have a host of followers who extol the virtues of their chosen technique.

The clinical chemist is, therefore, left with having to decide which method or methods best suit his laboratory environment and yet gives the physician the necessary sensitivity and specificity that he needs to assist him in the diagnosis of acute myocardial infarction. It is doubtful if any method presently available fulfills all of a clinical chemist's requirements. Electrophoresis on agarose would appear to be the best electrophoretic procedure since diffusion of substrate into the agarose is quite adequate, as opposed to cellulose acetate where diffusion
may be limited, thereby giving rise to underestimation of a specific isoenzyme. Another possible drawback to electrophoretic techniques using fluorescent intensity scanning is that the range of linear activity per band is optimal when 6 nmol of NADH or less is present. However, Roberts et al. (169) described a technique which entailed elution of the separated bands of activity followed by kinetic assay in which the range of linear activity was up to 60 nM per band. This technique is not really suitable for routine use.

The problem with electrophoresis is not one of specificity, as the method exhibits more than adequate specificity, but with sensitivity. It is in this area that the sensitivity of column methods has its advantage. However, carryover of one fraction to another has always been a problem with chromatographic methods, especially once we try to miniaturize them and make them available for so-called routine use. Therefore, it is imperative, before any column method is used routinely, that the carryover characteristics of MM on MB and MB on BB are fully understood, and if there is significant carryover of any of these isoenzymes that the method be discarded. Concentration of each eluate followed by electrophoresis is required to elucidate carryover problems.

One answer might be to screen patients using column techniques. Those who turn up positive could be further evaluated by means of agarose-gel electrophoresis. This would help to overcome the problems of sensitivity and
specificity. However, if one method is to be used, most investigators consider electrophoresis on agarose gel with fluorometric detection to be the most definitive method of separating CK isoenzymes, and they recognize that it is probably only a semi-quantitative method of reporting isoenzyme activity. This semi-quantitative measure is adequate in most cases as was shown recently in a review on cardiac profiling (170) when it was reported that visual examination of separated patterns of CK isoenzymes showed the same diagnostic precision as that of densitometric scanning. The only time when "plus and minus" results are not good enough is when infarct sizing is required and in this field we are still in our infancy. A short discussion on infarct sizing will follow in Chapter VI.

(2) Lactate Dehydrogenase

The choice of a method for the determination of lactate dehydrogenase is comparatively easy as there is only one definitive method available and that is electrophoresis. The electrophoretic method of choice is that of agarose gel followed by fluorogenic detection. Cellulose acetate has been used with and without fluorogenic detection, but does not seem to have the same degree of sensitivity and reproducibility as does agarose. This method does suffer from some of the problems exhibited by the CK:agarose method, such as different substrate affinities and possible asymmetrical distribution along the axis of scan. Even with these shortcomings, a highly definitive pattern
showing a "flipped LDH" pattern or an increase in fraction of LDH-5 are easily seen. Quantitation by scanning seems justified since reference ranges derived by Elevitch (171) along with a series of definitive patterns also produced by Elevitch (Figure 33) are readily available and can be used in the detection of other disease processes besides heart related conditions.

All other methods as yet have been lacking in their power of discrimination. Even the chemical method using an alternate substrate (alpha-hydroxybutyrate) does not improve the diagnostic information significantly to warrant its routine use in clinical enzymology. It was observed that patients suffering from hepatic congestion following myocardial infarction also had an increase in HBD, hence its reduced discriminating power. In fact, when evaluated in the setting of the coronary care unit, HBQ/LDH ratio had a predictive value of only .75 percent.

The method not mentioned in this discussion is the chromatographic method using Sephadex A-50. To date there seems to be no great move in this direction, although recently one of the larger enzyme-kit manufacturers (172) is marketing a column method for LDH isoenzymes. This method allows for isolation of the five individual isoenzymes of LDH. Procedures of this nature do not lend themselves to routine use due to the multitude of steps required to perform the assay and the time required to complete the assay.
It remains to be seen if column methods for LDH isoenzymes make any inroads into the field of routine clinical chemistry.
CHAPTER VI

DIAGNOSTIC USEFULNESS OF CARDIAC ISOENZYMES

In this final main chapter, now that we have established what cardiac isoenzymes are and the fact that it is possible to separate them and quantitate them in terms of activity as well as concentration, it is time to see how they can be used in the ongoing battle against disease.

Until comparatively recently a diagnosis of acute myocardial infarction was based on the following medical criteria:

- Pathological Q-Wave in ECG
- Classical History
- SGOT
- CPK
- LDH
- HBD

This would give a typical enzyme picture as shown in Figure 41. However, a recent paper by Galen, Reiffel and Gambino (12) compared the relative efficiency of serum enzyme and isoenzyme measurements in the diagnosis of acute myocardial infarction. In this study, discharge patients were classified by cardiologists as either having or not having suffered an acute myocardial infarction on the basis of clinical history and electrocardiographic criteria, without knowledge of the enzyme studies. On the basis of
Serum Enzymes Following Myocardial Infarction

Figure 41

Serum Enzymes Following Myocardial Infarction

[Graph showing activity x upper limit of normal over days after onset of AMI for CK, AST, HBD, and LDH.]
this study they concluded that the combined use of CK and LDH isoenzymes provided the greatest discrimination between the two clinical groups (MI versus non-MI). They further concluded that the routine use of SGOT and HBD activities could be abandoned in the setting of a CCU if CK and LDH isoenzymes were available. Their paper, therefore, formed the basis for the so-called admission cardiac profile.

Another aspect of cardiac isoenzymes is their potential usefulness in the determination of infarct size. Sobel, Roberts and Larson (37) worked with the creatine kinase MB isoenzyme using it as a measure of the amount of enzyme release, which when substituted in a model equation, is reported to give a direct indication of histological infarct size. In contrast, Roe in his review article (54), "Validity of estimating infarct size from serial measurement of enzyme activity in the serum", doubted if this type of model works without some means of correcting for the degree of perfusion of the infarcted area. The aforementioned are the main areas of interest as far as cardiac isoenzymes are concerned and these will now be dealt with in some detail.

A. CARDIAC PROFILING

Creatine kinase and lactate dehydrogenase have been used concomitantly in the study of acute myocardial infarction for a good many years but until recently they suffered from lack of specificity, since total CK and LDH
are increased in a large number of unrelated diseases (See Table 9 for a brief list of some of these disorders). With the inception of methods to determine the multimolecular forms of these enzymes, great advances were made in the capability of the clinical laboratory to aid in the diagnosis of AMI. The simultaneous use of CK and LDH combines the sensitivity of CK with the specificity of LDH. It is apparent that a myocardial incident has occurred when the CK-MB is present and a "flipped LDH" pattern is observed within the first 48 hours after the onset of the symptoms. Optimally, three samples are taken from the patient - on admission, at 24 hours and at 48 hours. It is necessary for total CK and LDH to be performed initially, and if increased, both CK and LDH isoenzymes are then performed. The "flipped LDH" pattern has never been observed to precede the appearance of the CK-MB. It is apparent that these criteria for an infarct do not have to occur in the same patterns, only within the 48-hours window. All MI's cause substantial increases in both LDH-1 and LDH-2, even though the "flip" as such may not be seen. An increase in LDH-1 should be taken as an indication of MI in the proper clinical setting. It is evident that increases in LDH-1 may not be observed in all three samples, presumably due to the fact that the peak activity of LDH does not occur until the third or fourth day after the acute episode. On the other hand, following cellular breakdown, the enzyme CK rapidly enters the blood stream (1 to 48 hours). The liberated
<table>
<thead>
<tr>
<th></th>
<th>LDH 1</th>
<th>LDH 2</th>
<th>LDH 3</th>
<th>LDH 4</th>
<th>LDH 5</th>
</tr>
</thead>
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<tr>
<td><strong>Cardiovascular</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Myocardial infarct</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hepatic congestion</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary infarct</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Rheumatic carditis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Inactive rheumatic fever</td>
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<td>Normal</td>
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</tr>
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<td>Myocarditis</td>
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<td></td>
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<td>Congestive heart failure</td>
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<tr>
<td>(decompensated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shock</td>
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<td>Normal</td>
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<td></td>
<td></td>
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<td>Heart block with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stokes-Adams syncope</td>
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<td>Hepatitis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cirrhosis, active</td>
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<td></td>
<td></td>
<td>+</td>
<td></td>
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<tr>
<td>Cirrhosis, inactive</td>
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<td></td>
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<td>+</td>
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<td>Hepatic congestion</td>
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<td>Acute extrahepatic</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>obstruction</td>
<td></td>
<td></td>
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<tr>
<td><strong>Hematologic</strong></td>
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<td></td>
</tr>
<tr>
<td>Pernicious anemia</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hemolytic anemia</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Megal blastic anemia</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoma and leukemia</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>(with hemolysis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Anemias without hemolysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infectious mononucleosis</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Adenocarcinoma of colon</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Renal tubular and cortical necrosis</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Skeletal muscle necrosis</td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Dermatomyositis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Various malignant</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>neoplasms</td>
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TABLE 9 (Continued)

Lactate Dehydrogenase and Creatine Kinase in Various Diseases

<table>
<thead>
<tr>
<th>Condition</th>
<th>MM CPK₁</th>
<th>MB CPK₂</th>
<th>BB CPK₁</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active myocardial damage including:</strong></td>
<td>++-----</td>
<td>trace--</td>
<td>+++-----</td>
</tr>
<tr>
<td>subendocardial infarct</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>infarct extension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cardiac surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myocardial ischemia</td>
<td>+------</td>
<td>trace</td>
<td></td>
</tr>
<tr>
<td>Active skeletal muscle damage including:</td>
<td>++-----</td>
<td>0--</td>
<td></td>
</tr>
<tr>
<td>Duchenne's muscular dystrophy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extensive rhabdomyolysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymyositis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early dermatomyositis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myoglobinuria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe ischemia of extremities due to vascular disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rocky Mountain Spotted Fever</td>
<td>+--</td>
<td>0--</td>
<td></td>
</tr>
<tr>
<td>Reye's syndrome</td>
<td>+--</td>
<td>0--</td>
<td></td>
</tr>
<tr>
<td>Normal Serum</td>
<td>0--</td>
<td>0--</td>
<td></td>
</tr>
<tr>
<td>Brain injury</td>
<td>0--</td>
<td>0--</td>
<td>trace--</td>
</tr>
<tr>
<td>Biliary atresia</td>
<td>+------</td>
<td>0--</td>
<td></td>
</tr>
<tr>
<td>Malignant tumors (usually with metastases)</td>
<td>++</td>
<td>0--</td>
<td>0--</td>
</tr>
<tr>
<td>Severe shock</td>
<td>+------</td>
<td>0--</td>
<td>0--</td>
</tr>
<tr>
<td>Chronic renal failure</td>
<td>+--</td>
<td>0--</td>
<td>0--</td>
</tr>
</tbody>
</table>

enzyme is highly labile and is no longer detectable in the blood after 48 hours unless there has been a re-infarction. Due to the relatively short half-life of CK, its presence in the blood stream indicates active tissue damage.

In Galen's paper (173) he states that in 80 percent of cases, the diagnosis of MI or non-MI can be made on the basis of isoenzymes alone. In the remaining 20 percent of cases, only CK-MB is evident, the LDH pattern not being observed. These patients may have had MIs, intermediate syndromes, coronary insufficiency or crescendo angina, and will demonstrate electrocardiographic evidence of myocardial ischemia. Hence the presence of CK-MB indicates severe myocardial ischemia, which may or may not be associated with infarction. It is readily apparent that the combined criteria facilitate optimal patient management.

After 48 hours in a patient with chest pains, if the CK-MB fails to appear, one can rule out MI with 100 percent accuracy and vacate a coronary care bed with complete confidence. A "flipped LDH" pattern in the absence of CK-MB indicates hemolysis or renal infarction. Table 10 indicates the diagnostic capability of the combined isoenzyme analyses. It is used to rule out myocardial infarction during the acute 48 hours period following the episode. It should be noted that once diagnostic criteria have been met, there is no need for further testing to document the diagnosis. If after 24 hours the criteria are met, then the diagnosis is positive and testing can be discontinued. Further testing
TABLE 10

Combined Isoenzyme Analysis: Rule out Myocardial Infarction*

<table>
<thead>
<tr>
<th>CK-MB absent</th>
<th>CK-MB present</th>
<th>CK-MB present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal LDH</td>
<td>Normal LDH</td>
<td>Flipped LDH</td>
</tr>
</tbody>
</table>

100% predictive value that there is no MI.
Both MI and non-MI cases**
100% predictive value that there is MI

*During acute 48 hour period following episode.
**Non-MI cases reflect clinical and electrocardiographic evidence of ischemia.

This data was taken from reference 173 without the permission of the author.
might be performed if extension or re-infarction is suspected. A typical case is described in Figure 42. A series of profiles is shown in Figure 43.

In conclusion, it is evident that massive MI's do not need isoenzyme tests for confirmation, and in these cases this profile will be used to detect extension or re-infarction. The usefulness of this profile lies in the detection of MI patients who are borderline cases. If the patient has no detectable CK-MB and a normal LDH 1:2 ratio through the three-day sample period following a clearly defined episode, then the physician can assure the patient that he did not suffer an AMI from the episode in question. The advantage of this type of profile would appear to be two-fold. Firstly, if the test is negative following apparent MI-like symptoms, the patient can be reassured that he has not had a "heart attack" with all its social and economic ramifications. The advantage to the hospital is that once the patient has been classified as non-MI-he can be transferred to another, less expensive area where the patient can be examined for other disease processes which mimic AMI. The unfortunate part about this type of testing is that it does not guarantee the patient that he will not develop an AMI in the near or distant future. It is of passing interest to note that a research team at the John Hopkins Medical School in Baltimore (174) has developed a test to determine those people who have a high risk of sudden death from cardiovascular disease. The test involves
FIGURE 42

Isoenzyme Series Myocardial Infarction with Case History

LEGEND

The following data goes with the respective photographs.

<table>
<thead>
<tr>
<th>Date</th>
<th>Time</th>
<th>CK Total IU</th>
<th>LDH Total IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>11-11-73</td>
<td>0110</td>
<td>130</td>
<td>181</td>
</tr>
<tr>
<td>11-11-73</td>
<td>0615</td>
<td>813</td>
<td>323</td>
</tr>
<tr>
<td>11-11-73</td>
<td>1215</td>
<td>1707</td>
<td>607</td>
</tr>
<tr>
<td>11-11-73</td>
<td>2115</td>
<td>2114</td>
<td>808</td>
</tr>
<tr>
<td>12-11-73</td>
<td>0625</td>
<td>2135</td>
<td>891</td>
</tr>
<tr>
<td>12-11-73</td>
<td>1830</td>
<td>1350</td>
<td>818</td>
</tr>
<tr>
<td>14-11-73</td>
<td>2300</td>
<td>447</td>
<td>1452</td>
</tr>
<tr>
<td>16-11-73</td>
<td>2330</td>
<td>244</td>
<td>891</td>
</tr>
</tbody>
</table>

A 67 year old male with no previous history of myocardial infarction but prior 6 month history of angina with exercise was admitted to the hospital 6 hours after the onset of substernal non-radiating chest pain. He was diaphoretic but without nausea and vomiting. E.C.G was consistent with acute anterior - inferior myocardial infarction. He was a known hypertensive patient with history of resected adeno-carcinoma of the colon 10 years prior to this admission.

Admission physical examination revealed a pulse of 120, blood pressure of 180-120/130 and afebrile. Neck vein distension, pedal edema, pulmonary rales and liver edge 2 cm below the right costal margin were noted. The liver size decreased on the second hospital day. (note LDH-5 returning to normal) Increased chest pain was noted on the second day and improved. (note LDH-3 pulmonary in sample 6.)

Reprinted without permission of Corning from Cardiac Isoenzymes a Case History. Corning Medical, Medfield, Massachusetts. (1975).
FIGURE 42

Isoenzyme Series Myocardial Infarction with Case History.

CK-ISOENZYME FRACTIONS

LDH ISOENZYME FRACTIONS

PATIENT SAMPLE #  1  2  3  4  5  6  7  8

CK-MB

CK-MM

LDH-1
LDH-2
LDH-3
LDH-4
LDH-5
FIGURE 43

Examples of Cardiac Profiling

FIGURE 43

Examples of Cardiac Profiling

Normal CPK₂ & Normal LDH (No MI)

Increased CPK₂ & Flipped LDH (MI)

Increased CPK₂ & Normal LDH
(possible MI, definite Ischemia)
electrical stimulation of the right ventricle a millisecond or so before it would normally beat. In healthy no-CHD patients, the stimulation causes one premature beat, while in CHD-prone people, two premature beats were observed. This test is in the realm of preventive medicine. Along with its counterpart, HDL-cholesterol (175), it should help to improve the long-term health of the population. The admission cardiac profile on the other hand will allow the physician to reach a more definitive diagnosis of MI, more rapidly.

B. INFARCT SIZING

The idea of infarct sizing, which has given rise to considerable controversy, was first suggested by Witteveen et al. in 1970 (176). The hypothesis was that the observed enzyme value was a reflection of the rate of enzymic release into the blood from the infarcted myocardium and the rate of enzymic clearance from the blood. That is to say that each enzyme estimation is the dynamic equilibrium of simultaneous gain and loss of the enzyme in question. It can be seen that it is important to know the rate at which the enzyme is disappearing from the blood so that an estimate of the total amount added to the blood can be obtained. To obtain this information, partly purified creatine kinase from dog myocardium was injected into dogs and the rate of clearance was determined by taking serial determinations of CK over a period of time. This enabled decay to be represented
as percent activity disappearing per minute and was designated "the decay constant"; $K_d$. The "decay constant" allowed for correction of enzyme that was concomitantly disappearing and reappearing as it was released from the myocardium. The cumulative activity of CK released by the heart or the appearance function can be calculated by the integration formula:

$$\int_0^T f(t)\,dt$$

(29)

where $f(t)$ is rate of change of CK activity due to enzyme being released from the heart. This gives an estimate of activity per ml. of blood. To determine the total amount of enzyme liberated it is required to know the volume of the space in which the enzyme is distributed. Shell et al. (177) suggested that 11.4 percent of the body weight in grams would be equivalent to the distribution volume in milliliters. Nachman et al. (178) estimated it as being equal to the plasma volume. Therefore, $\int_0^T f(t)\,dt \cdot K_w \cdot BW$ represents the total amount of enzyme in the distribution space ($K_w \cdot BW$), where $K_w$ is the designation for the percentage of body weight (BW) in which the enzymes are distributed.

It is apparent that only about 15 percent of the enzyme released from the myocardium actually reaches the circulation, namely the distribution space. Shell et al. (177) measured CK in normal and infarcted myocardium to see how much of the enzyme was missing from the infarcted area. The ratio of the amount of enzyme in the distribution space compared to that missing from the myocardium was an index
\( \left( K_r \right) \) of the proportion reaching the blood. Hence:

\[
\text{Estimated myocardial} = \int_0^T f(t) dt \cdot K_{MW} \cdot BW \quad \text{CK depleted} \quad K_r
\]

Finally, one needed to know how much enzyme represents one gram of infarct \( (CK_D) \). This was determined to be 540 U/g for patients by Sobel et al. (179). Thus, from the total amount of enzyme depleted from the myocardium, one could estimate the size of the infarct in grams, or as termed by Shell et al., gram-equivalents \( (g\text{-eq}) \) (177).

\[
\text{Infarct size, g\text{-eq}} = \int_0^T f(t) dt \cdot K_{MW} \cdot BW \quad \text{CK depleted} \quad K_r \cdot CK_D
\]

With this model, one could estimate the extent of the infarction from serial estimation of creatine kinase activity following myocardial infarction. The early work of Shell and his co-workers (177) showed an excellent correlation between such estimates and infarct size determined by measuring depletion of CK from infarcted myocardium. This author did not test the association with histologic infarct size. However, because of this good correlation the method was applied both experimentally and clinically. Current review of the literature illustrates the large numbers of scientific papers that have been produced on the merits of infarct sizing based on the serial measurement of CK and CK-MB (180 - 182). These papers showed good correlation with the clinical assessment of the status of patients who were in coronary care units. Other studies have utilized this type of infarct sizing to evaluate
the utility of different methods of clinical intervention. An example of pharmacological intervention can be demonstrated by a paper presented at the Scientific Sessions of the American Heart Association. This intervention used propranolol as a means of reducing creatine kinase release after AMI (183). These workers used total CK, rate of enzyme release and peak measured activity as an index of infarct size and rate of myocardial necrosis. From the information that they had gathered they concluded that propranolol in the dose used and with an uncomplicated transmural infarction reduced the infarct size and decreased the rate of myocardial necrosis.

To assess the affect of a pharmacological or mechanical intervention, one must have some way of predicting, rapidly, the final size of the infarct had there been no intervention. To this end, Shell et al. (184) designed a curve fitting technique. Knowing the early components of the post infarct curve allowed one to project the remainder of the curve and thereby produce an estimate of infarct size. This type of curve fitting assumes a family of post infarct curves with one basic shape and also that the slope of the upslope gives an indication of the ultimate shape, that is the steeper the upslope the larger the infarct, the shallower the slope the smaller the infarct. The infarct size predicted (ISP) is compared to the infarct size observed (ISO) (calculated from serial measurements after occlusion). Figure 44 illustrates three comparisons of ISO versus ISP.
Current Interpretation of the Influence of Clinical Intervention on the Size of Myocardial Infarction Based on Comparison of Observed and Predicted Enzyme Curves

This data was taken from reference 54 without the permission of the author.
FIGURE 44

Current Interpretation of the Influence of Clinical Intervention on the Size of Myocardial Infarction Based on Comparison of Observed and Predicted Enzyme Curves
and how they are used to evaluate the effects of intervention. If ISP > ISO, then tissue was salvaged. On the other hand, if ISO > ISP then the infarct has extended. This concept forms the basis for evaluating the usefulness of interventions during the course of myocardial infarction.

Conversely, Roe (54) in his paper seems to refute all findings on infarct sizing by declaring that "it is virtually impossible to distinguish a small from a moderately large histological infarct. Small anatomic infarcts were frequently associated with large amounts of enzyme in the distribution space than was so for larger infarcts." He further stated that "the most logical explanation for this discrepancy would be that blood flow to the ischemic region plays a role in enzyme egress from myocardium. Apparently, flow may influence not only the amount of enzyme but also the rate with which enzymes appear in peripheral blood." He concluded his paper by saying "evaluation of therapeutic interventions on the course of myocardial infarction with predicted and calculated estimates of size should be approached cautiously until the relationship of the mathematical model to the biological events are better understood."

The conclusions reached by panalists attending a recent profiling (122) was that infarct sizing will not be exact for some time to come due to imprecisions of present methodology and also due to a lack of understanding of the physiology of the myocardium during infarction. Those
present felt that early CK-MB values (1 to 5 hours after episode) are needed—for accurate infarct sizing. It was observed that most MI patients do not get to the hospital within the required time frame. Any intervention therapy that might be used must be started soon after the onset of the infarction. Since most patients wait 4 to 12 hours after myocardial infarction before entering hospital, the isoenzyme determinations would not be much help to the clinician in administering initial therapy. So one of the problems is that cardiologists cannot rely on patients getting to the hospital in time to measure CK-MB at maximum levels. Finally, it was observed that large MB peaks represent massive cardiac injury but small peaks do not necessarily represent minor cardiac damage.

In spite of obvious problems related to infarct sizing, the research continues as evidenced by papers presented at the American Heart Association's Scientific Sessions held in November, 1977 in Miami Beach, Florida (183). It would, therefore, appear that, due to the polarization of ideas on infarct sizing, it will be a long time before this technique will be accepted as a routine tool in the treatment of patients suffering from acute myocardial infarction.
CHAPTER VII
SUMMARY

Clinical enzymology is a branch of medical laboratory science that has experienced a phenomenal growth rate over the past ten to fifteen years. Routine enzymology now accounts for approximately 50 percent of the normal workload of the average 400 bed hospital. Thus clinical enzymology, with its humble beginnings, has surpassed all other fields of endeavor in that branch of laboratory medicine known as clinical chemistry. The early years of clinical enzymology brought us methods for the detection of intracellular enzymes in body fluids, particularly blood serum. It is in this matrix that the enzymes resulting from catabolism, aging and trauma are to be found. The ease in which a suitable sample of blood serum can be obtained has done much to facilitate the acceptance of clinical enzymology as a branch of laboratory medicine. The drawbacks to the use of the total activities of enzymes in blood serum lies in the question of specificity for a particular disease process. The majority of tissues in the body have a metabolism which is similar, if not identical, to that of other tissues in the body. This fact has led to great confusion for the clinical chemist and physician alike since it is impossible to tell from total enzyme activity measurements the exact origin of the enzyme in question. Therefore, methods had to be devised
which would supply this highly relevant information. The way in which this conundrum was solved was by the identification of the fact that some enzymes, if not all enzymes, exist in multimolecular forms known as isoenzymes, these being "different proteins with similar enzymatic activity". This discovery opened up a new branch of clinical enzymology which enabled the physician to perform a "biochemical biopsy" on his patient without the associated problems of a surgical biopsy. The notion that body organs can have specific isoenzyme patterns altered the course of clinical enzymology. So, instead of trying to find an organ-specific enzyme, the emphasis changed and the isoenzyme patterns themselves were found to be useful as a means in the detection of an acute myocardial infarction and possibly in the assessment of the extent of the resulting damage.

As a general background to cardiac isoenzymes let us consider the fact that approximately one million people die each year in North America from diseases of the cardiovascular system, accounting for 55 percent of all deaths. Between the years 1950 and 1970 the greatest increase in deaths from heart attacks was in young men 25 to 44 years of age. In men 45 to 64 years of age there was only a minimal increase in such deaths. It, therefore, becomes essential that the clinical laboratory have suitable methods by which the physician can rapidly diagnose the presence and the extent of an acute myocardial infarction so that suitable treatment can be initiated. It is important to exclude the
possibility of AMI, primarily because of its social impact on the patient and his family, but also because of the cost of maintaining a patient needlessly in a coronary care unit, in these days of budgetary restraint.

Methods available for the separation of isoenzymes include such diverse techniques as electrophoresis, ion-exchange chromatography, antibody techniques and chemical methods. In the author's opinion the most definitive method of separation is that of electrophoresis, specifically by agarose. Both creatine kinase and lactate dehydrogenase isoenzymes can be separated by this method. The agarose gel method of protein electrophoresis has been chosen as a "proposed selected method" (185). The most sensitive method of detection of the separated isoenzymes, whether they are creatine kinase or lactate dehydrogenase isoenzymes, is one using fluorogenic detection. This type of detection is most suited to agarose, although lactate dehydrogenase methods on cellulose acetate have used a fluorogenic detection technique. However, in the case of creatine kinase, even though electrophoresis is the most specific, it is not necessarily the most sensitive. Into this category fall the column methods. These certainly are more sensitive but with this increase in sensitivity comes a decrease in specificity. A compromise approach might be to use the more rapid column method as a screening tool and when positive findings are observed, to confirm the positive report by the more specific electrophoretic method. This approach is economically
unfeasible in most cases. The answer in most instances is to conduct clinical trials with the method of choice and to evaluate the performance of the test under the conditions of the existing environment. Another shortcoming of some column methods for creatine kinase isoenzymes is that some do not allow for the separation of CK-MB and CK-BB.

The use of creatine kinase isoenzymes and lactate dehydrogenase isoenzymes in tandem is popularly known as a cardiac isoenzyme profile. The presence of CK-MB and a "flipped LDH" can result under the appropriate clinical setting in 100 percent predictive value of myocardial infarction. Conversely the absence of CK-MB gives 100 percent predictive value that there is no myocardial infarction. This only applies during the acute 24 hours phase following the episode.

Much interest has been shown over the past decade in the use of creatine kinase, specifically the CK-MB dimer, as a means to assess the size of an infarct prior to medical intervention. However, it is the considered opinion of many eminent cardiologists that infarct sizing will not be exact for a long time. They arrive at this conclusion due to the fact that large MB peaks represent massive cardiac injury; however, small peaks do not necessarily represent minor cardiac injury. An additional problem is that cardiologists cannot rely on patients getting to the hospital in time to measure MB at its maximum level. A further impediment to the cause of infarct sizing is that the
maximum level of CK-MB from a slowly evolving infarct may be very low, thus giving misleading values.

It is apparent from the extensive literature that the intelligent use of creatine kinase and lactate dehydrogenase isoenzymes will result in more rapid and definitive diagnosis of myocardial infarction and related conditions.
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