STUDIES OF ELECTROPHORETIC DETECTION METHODS FOR SERUM CREATINE KINASE ISOENZYMES

by

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Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science
in the
Chemistry
Program

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August, 1976

ABSTRACT

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This study is concerned with the activity of creatine kinase isoenzymes through electrophoretic detection techniques. It is well documented that creatine kinase may be elevated in a variety of disease states. However, the only concern of the study was with CK elevations due to cardiac involvement, namely acute myocardial infarction.

The initial step was devoted to two methods for the determination of total creatine kinase activity. These two methods included; (1) the kinetic method from the Boehringer Mannheim Corporation (BMC), and the luciferin-luciferase method from Antonik Laboratories. Once the total activity was determined, a standard curve was then developed using control sera from Helena Laboratories.

Completing this phase of the study, it was then necessary to turn all the attention to electrophoresis. Commercially prepared control sera from various manufacturers were obtained and examined for their potential use as creatine kinase isoenzyme markers. The electrophoretic setup used for the analysis was from Helena Laboratories, and the CK

substrate was from BMC. It was discovered that no commercially prepared chemistry control sera could be utilized as a marker for CK isoenzymes, unless a particular control was sold specifically for this purpose.

Another phase of this study involved the reexamination of cord blood sera as potential markers for CK isoenzyme activity. Various cord blood samples were obtained from the maternity wards of area hospitals and examined for their creatine kinase isoenzyme content. It was hoped that the cord blood specimens would contain all three isoenzyme fractions so that the expense of purchasing commercially prepared CK isoenzyme control serum could be alleviated. Ten cord specimens were examined by the fluorometric method of Helena, and only MM and BB fractions were demonstrated.

Migrational studies of the CK isoenzymes were then examined in their relationship to serum protein electrophoresis. It was shown that the MM fraction migrated with the gamma globulin fraction, BB migrated with albumin fraction, and MB migrated with the alpha-2 globulin fraction of total protein. Again the electrophoretic techniques, as outlined by Helena Laboratories were employed.

The final phase of this study involved a comparative examination of various sera from patients diagnosed as having had an acute myocardial infarction by two techniques. These techniques involved the method by Helena, and a bioluminescent method utilizing ATP, luciferin, and luciferase.

The established Helena procedure demonstrated excellent results, with MB fractions of low activity being readily detected by this method. However when luciferin-luciferase system was used, much difficulty was encountered. Most of the problems stemmed from diffusion of the protein molecules during development. In an attempt to correct this diffusion problem, various buffers and solutions were adjusted, optimized conditions were sought, and alterations in technique were employed. However all endeavors proved futile.

Through this study however, much knowledge of the creatine kinase isoenzymes were obtained, as to their activity, mobility, clinical usefulness, and diagnostic importance relating to myocardial infarction.

ACKNOWLEDGEMENTS

At this time I would like to acknowledge the people who have contributed their time and guidance in the completion of this thesis.

First I would like to extend my gratitude to Dr. Leonard B. Spiegel who devoted much of his time and assistance in the thoughts and ideas incorporated in this thesis. By his quest for pertinent information relating to the subject matter, I have gained invaluable knowledge and insight in this aspect of clinical chemistry which will be very beneficial to me in my work at Jameson Memorial Hospital.

I would also wish to extend my gratitude to Dr. Sylvan M. Sax of Western Pennsylvania Hospital for the helpful discussion both Dr. L. Spiegel and myself had with him concerning this project.

I would also like to thank Doctors Marvin Lukin and John D. Van Norman for reading this thesis and for making the necessary corrections required to properly finish this study.

I also would like to extend a great deal of appreciation to Dr. Antonik of Antonik Laboratories in Elk Grove Village, Illinois, for his assistance in the luciferin-luciferase aspect of the thesis and the donation of a total CK kit and a luciferin-luciferase kit for my work.

I would like to thank Mr. Dan Novello of the Youngstown Hospital Association, Northside Unit, whose assistance in obtaining samples necessary for my work proved invaluable.

His guidance and help in the early stages of my work with the fluorometric determinations of CK isoenzymes were extremely beneficial and rewarding.

Finally I would like to thank my lovely wife, Tressa, for her devotion and understanding of my work at Youngstown State University. Without her encouragement throughout my work, my scholastic involvement at YSU would have been troublesome.

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LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
ADP	Adenosine Triphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BB	Brain Fraction of CPK-CPK I
BMC	Boehringer-Mannheim Corp.
CCU	Coronary Care Unit
CK	Creatine Kinase
CP	Creatine Phosphate
DEAE	Diethylaminoethyl
DTE	Dithioerythritol
DTT	Dithiothreitol
ECG	Electrocardiogram
G-6-PD	Glucose-6-Phosphate dehydrogenase
GOT	Glutamic-oxaloacetic transaminase
GPT	Glutamic-pyruvic transaminase
HBD	Alpha-hydroxybutyrate dehydrogenase
HK	Hexokinase
IU/L	International Unit per Liter
LDH	Lactic Acid dehydrogenase
LDH-1, LDH-2	Heart Fractions of LDH Isoenzymes
L-L	Luciferin-Luciferase
MB	Heart Muscle Fraction of CPK-CPK II
MDH	Malic acid dehydrogenase
ME	Mercaptoethanol

MES	2-(N-morpholino) ethanesulfonic acid
MI	Myocardial Infarction
MM	Skeletal Muscle Fraction of CPK-CPK III
MOPS	3-(N-morpholino) propanesulfonic acid
NAD	Nicotinamide-Adenine dinucleotide
NADH	Reduced Nicotinamide-Adenine dinucleotide
NBT	Nitroblue Tetrazolium
PHI	Phosphohexose isomerase
P_i	Inorganic Phosphate
PMS	Phenazine Methosulfate
SMA-12	Technicon's Twelve Channel Sequential Multiple Analyzer
U.V.	Ultraviolet
S.D.	Standard Deviation
TLU	Turner Light Unit

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CHAPTER I

INTRODUCTION

A. Basic Theory of Electrophoresis

Electrophoresis may be defined as the migration of charged particles in a medium under the influence of an external electrical field. Migration of proteins in an electrical field was first perfected by Tiselius, who used a liquid medium. His technique of electrophoresis is known as "moving boundary or free electrophoresis."[1,2] Today "zone electrophoresis" has become popular because it incorporates a stablizing medium which serves as a matrix for the buffer in which the proteins travel and as a structure to which the proteins become attached.[2] Once attached, the proteins may then be stained and examined. Some important support media used today are: chromatography paper, starch gel, Sephadex, agar gel, agarose, polyacrylamide gel, and cellulose acetate.[1,2] However, besides the support media, there exist three other basic components for electrophoretic analysis by zone electrophoresis and these are: sample, buffer solution, and electrical field. A simple zone electrophoretic system is shown in Figure 1.[2]

An important aspect of electrophoretic mobility is the electrical charge of the proteins. Proteins vary in size, shape, and charge. Being amphoteric, proteins, like

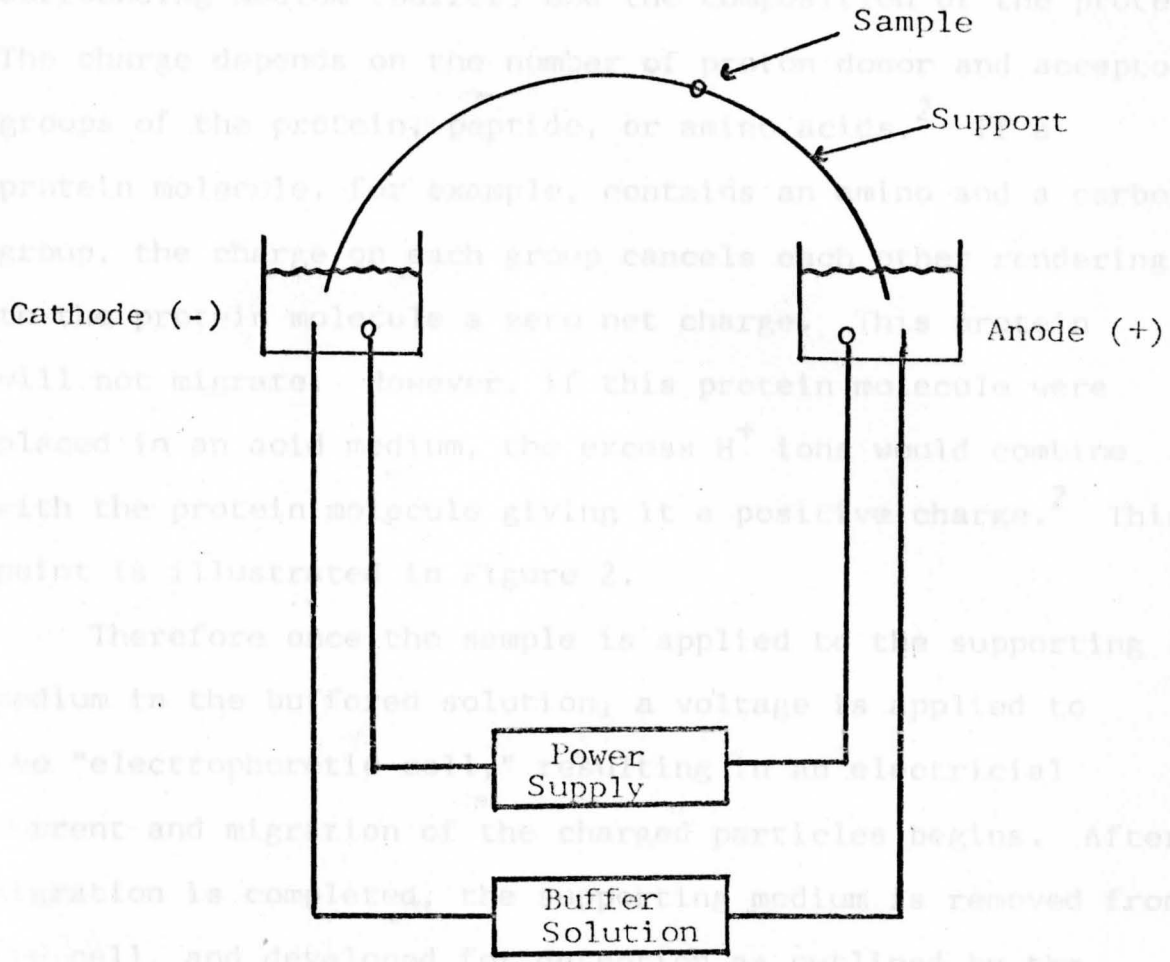


Figure 1. Simple Zone Electrophoresis System.[2]

peptides or amino acids, can have no net charge, a negative charge, or a positive charge, depending upon the pH of the surrounding medium (buffer) and the composition of the protein. The charge depends on the number of proton donor and acceptor groups of the protein, peptide, or amino acids.[2] If a protein molecule, for example, contains an amino and a carboxyl group, the charge on each group cancels each other rendering to the protein molecule a zero net charge. This protein will not migrate. However, if this protein molecule were placed in an acid medium, the excess H^+ ions would combine with the protein molecule giving it a positive charge.[2] This point is illustrated in Figure 2.

Therefore once the sample is applied to the supporting medium in the buffered solution, a voltage is applied to the "electrophoretic cell," resulting in an electricial current and migration of the charged particles begins. After migration is completed, the supporting medium is removed from the cell, and developed for detection as outlined by the particular procedure involved.

B. Creatine Kinase: Its Reaction and Isoenzymes

Creatine kinase (CK; EC 2.7.3.2) the structure of which has not yet been established in its entirety, is an enzyme found in the sera of normal individuals in very small quantities of 0-50 I.U./l. at 25°C and has a molecular weight of 82,600. It is known to catalyze the reversible reaction in which creatine is phosphorylated by adenosine triphosphate (ATP). (See figure 3)

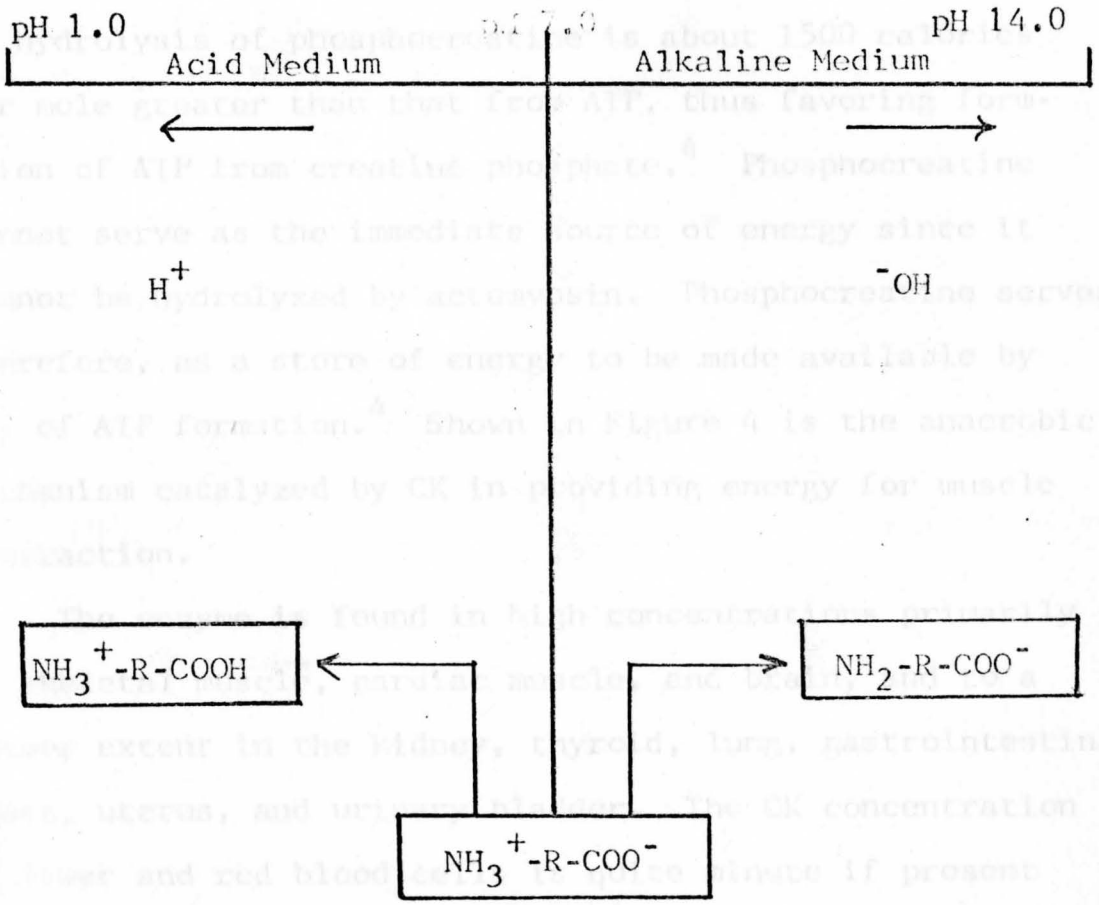


Figure 2. Effect of Buffer Solution on Protein Molecules.[2]

$$\text{ATP} + \text{CREATINE} \xleftrightarrow[Mg^{++}]{CK} \text{ADP} + \text{CP}$$

Figure 3. The CK Reaction[3]

It is then the role of phosphocreatine to provide for the body an anaerobic mechanism for rapid regeneration of ATP. This is accomplished by creatine kinase contained in muscle tissue. At pH 7.0, the free energy made available by hydrolysis of phosphocreatine is about 1500 calories per mole greater than that from ATP, thus favoring formation of ATP from creatine phosphate.[4] Phosphocreatine cannot serve as the immediate source of energy since it cannot be hydrolyzed by actomyosin. Phosphocreatine serves, therefore, as a store of energy to be made available by way of ATP formation.[4] Shown in Figure 4 is the anaerobic mechanism catalyzed by CK in providing energy for muscle contraction.

The enzyme is found in high concentrations primarily in skeletal muscle, cardiac muscle, and brain, and to a lesser extent in the kidney, thyroid, lung, gastrointestinal tract, uterus, and urinary bladder. The CK concentration of liver and red blood cells is quite minute if present at all, so that diseases of these two tissues are unlikely to cause measurably increased serum CK levels.[3,5,6] A listing of multiple causes of total serum CK elevation are shown in Table 1.[6,7]

However, it is now important to evaluate an elevation of serum CK in order to establish a proper diagnosis and

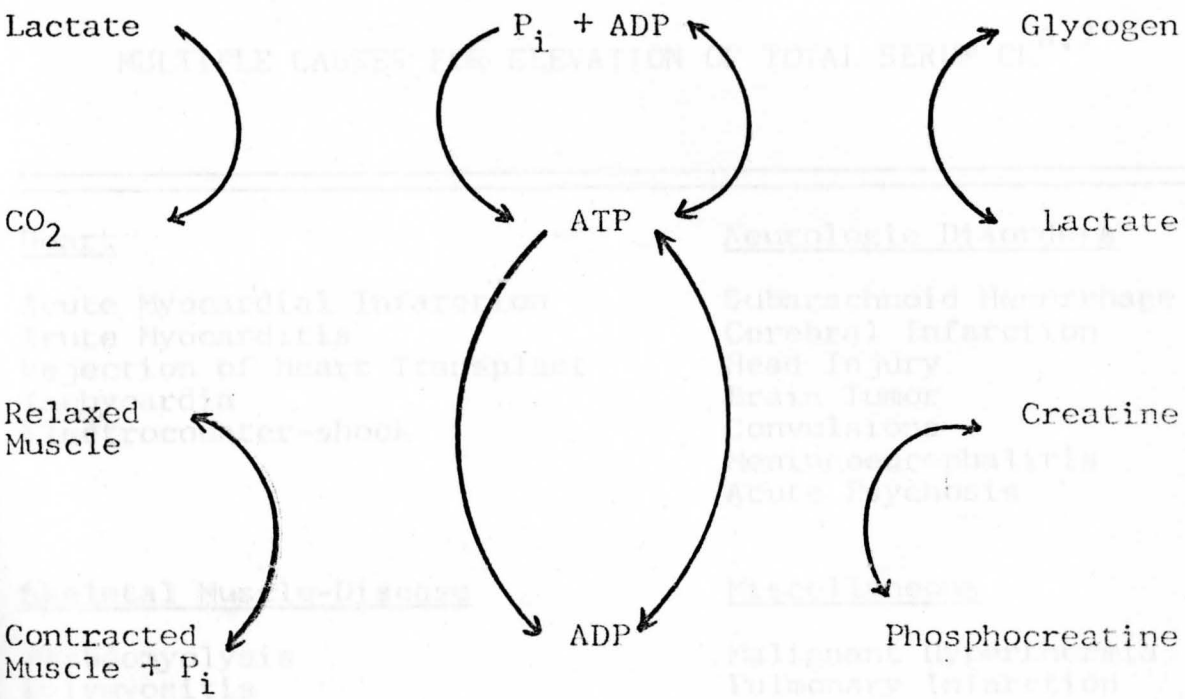


Figure 4. The Anaerobic Mechanism catalyzed by CK in providing energy for muscle contraction.[4]

TABLE I

MULTIPLE CAUSES FOR ELEVATION OF TOTAL SERUM CK[6,7]

Heart	Neurologic Disorders
Acute Myocardial Infarction	Subarachnoid Hemorrhage
Acute Myocarditis	Cerebral Infarction
Rejection of Heart Transplant	Head Injury
Tachycardia	Brain Tumor
Electrocounter-shock	Convulsions
	Meningoencephalitis
	Acute Psychosis
Skeletal Muscle-Disease	**Miscellaneous**
Rhabdomyolysis	Malignant Hyperthermia
Polymyositis	Pulmonary Infarction
Myopathy	Hypokalemia
Muscular Dystrophy	Diabetic Ketoacidosis
Severe Exercise	Electrocountershock
Operative Trauma Skeletal Muscle	Pancreatic Disease
Hypothyroidism	Clofribrate Therapy
Drug Injections-Muscle	

treatment method. When a patient is admitted to the emergency room complaining of chest pains, the attending physician will usually order a total CK analysis. However, suppose the report shows the patient has an elevated CK of 150 I.U./l or 3 times the normal level. Is the report indicative of a true myocardial infarction or is the CK elevated due to prolonged exercise? The attending physician cannot be sure. Although other heart related enzymes can be examined to help in his diagnosis, the physician cannot rely on these tests for 100% accuracy for a true M.I. Elevation of CK will surely occur with acute heart damage, but other clinical conditions will also increase serum levels of this enzyme.

Recently, precise procedures for the determination of CK isoenzymes have been developed. It has been well documented that multiple forms of CK exist after electrophoresis.[6,7,8,9,10,38,39] These multiple forms are due to the fact that CK is a dimer (two peptide chains). Both peptide chains originating from skeletal muscle have been designated MM or CK III. CK from the brain has been designated BB or CK I, and the heart muscle of CK containing one peptide chain from skeletal muscle and one peptide chain from brain is designated MB or CK II.[8] All three forms of the CK isoenzymes differ in their electrophoretic mobilities. The brain (BB) fraction moves the farthest and is found near the anode, the skeletal muscle fraction (MM) is the slowest moving and is found near the point of application (cathode),

and the heart muscle fraction (MB) is found between MM and BB. Several other human tissue sources, along with brain, heart, and skeletal muscle, are listed in Table 2 with the percent distribution of each CK isoenzyme.

The MB isoenzyme usually appears in the serum of a patient with an acute myocardial infarction 6 hours after the onset of chest pains, remains elevated for approximately 24-30 hours, and then begins to return to normal.[7,9] Since the MB isoenzyme is a very heat labile enzyme which appears and disappears rapidly; determination of the MB fraction in a suspected case of an acute M.I. should be performed as soon as possible. This identification of the CK (MB) isoenzyme, along with the LDH isoenzyme analysis, provide perhaps the most sensitive and specific procedure available in the clinical laboratory for the diagnosis of an acute myocardial infarction. However, the CK (MB) isoenzyme has the distinct advantage of early M.I. detection (6 hours after chest pains), whereas LDH isoenzyme activity is not readily determined for 48-72 hours after an acute M.I.[9,12] Illustration of this information is seen in Figure 5.

The normal range commonly used for the MB isoenzyme fraction, as outlined by Gambino and Galen,[10] is 0-3% of the total CK activity. In Table 3 are noted other pathological conditions which may show the MB fraction, however it should be noted that in no condition is the normal range of Gambino and Galen[10] exceeded except in the case of an acute M.I.

TABLE 2

PERCENT DISTRIBUTION OF CK ISOENZYMES IN HUMAN TISSUE[9]

TISSUE	BB	MB	MM
Normal Serum	0	0	100
Skeletal Muscle	0	0	100
Heart	0	40	60
Brain	90	0	10
Lung	90	0	10
Bladder	95	0	5
Bowel	100	0	0

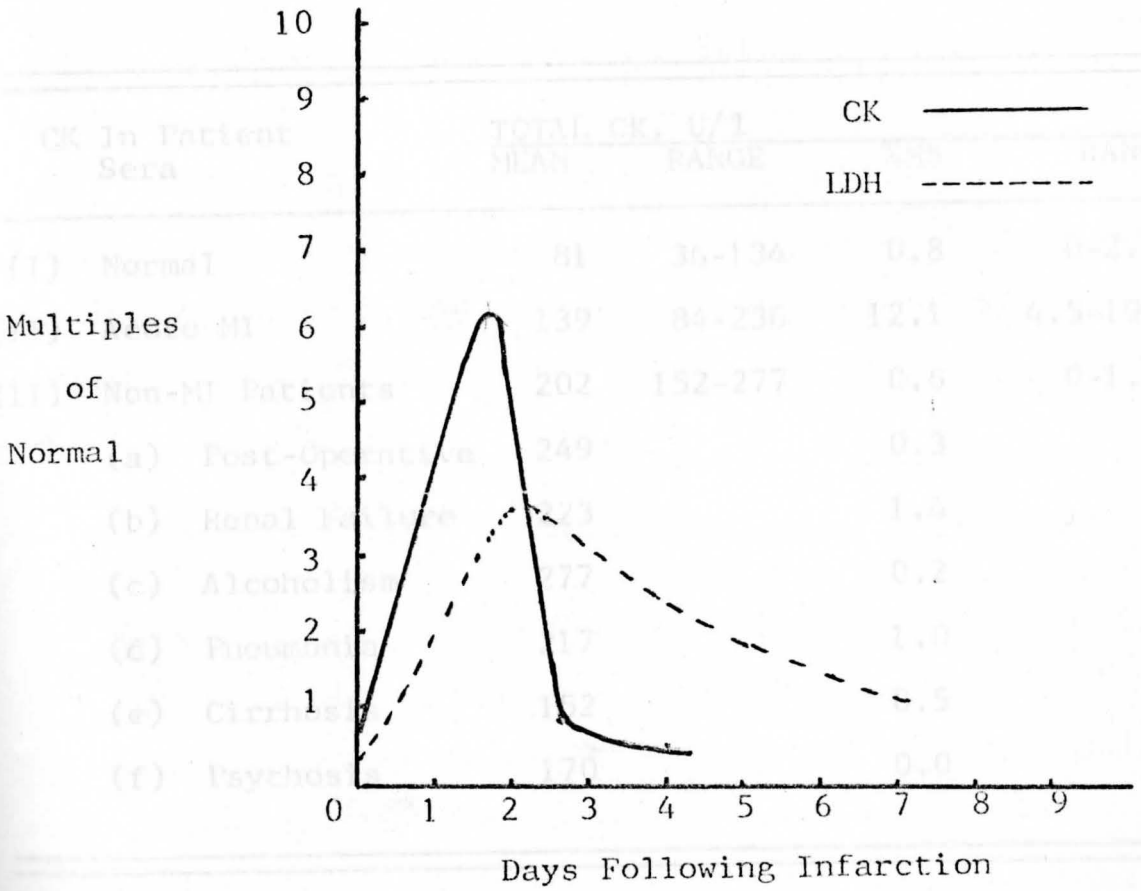


Figure 5. CK and LDH levels in serum following an acute M.I.[9,12]

TABLE 3

CONDITIONS WHERE THE MB ISOENZYME MAY BE DETECTED[9]

CK In Patient Sera	TOTAL CK, U/l MEAN	RANGE	%MB	RANGE
(I) Normal	81	36-134	0.8	0-2.1
(II) Acute MI	139	84-236	12.1	4.5-19.9
(III) Non-MI Patients	202	152-277	0.6	0-1.6
(a) Post-Operative	249		0.3	
(b) Renal Failure	223		1.6	
(c) Alcoholism	277		0.2	
(d) Pneumonia	217		1.0	
(e) Cirrhosis	152		0.5	
(f) Psychosis	170		0.0	

C. The Role of LDH and LDH Isoenzymes in the Diagnosis of Myocardial Infarction

Another important heart related enzyme is commonly called lactic dehydrogenase or usually referred to as LDH, the molecular weight of which is 135,000.[13] The International Union prefers to call it L-Lactate: NAD oxidoreductase, and has given it an easy number, 1.1.1.27. The chemical reaction catalyzed by LDH can be observed by one of two ways: lactate can be oxidized with NAD and the rise of NADH is measured at 340 nanometers, or pyruvate can be reduced with NADH and the fall of NADH is measured at 340 nanometers. (See Figure 6).[13]

$$\text{PYRUVATE} + \text{NADH} \xleftrightarrow{\text{LDH}} \text{LACTATE} + \text{NAD}$$

Figure 6. The LDH Reaction[13]

Elevated serum LDH levels are observed in many conditions such as: megaloblastic anemia, myocardial infarction, metastatic carcinoma, hepatitis, cirrhosis, renal disease, acute leukemia, hemolytic anemia, and infectious mononucleosis, only to name a few.[14] The main thrust of this section will deal with the LDH activity in myocardial infarction. Total serum LDH activity will generally increase 48-72 hours after the onset of chest pains in a suspected case of acute M.I. Once elevated, the activity remains so for approximately 8-10 days before returning to the normal level.[12]

In 1957 it was first recognized that the LDH enzyme could be electrophoretically separated into five protein fractions, and that each of the five isoenzyme fractions represented a tetramer containing varying combinations of two different monomers, H and M, under genetic control. (See Figure 7).

It has been suggested that the two types of LDH isoenzymes (H + M) have different functional roles.[13] The H type LDH is thought to function in cells with a degree of aerobic metabolism, whereas the M type is thought to function in cells with high rate of glycolysis or with low oxygen uptake. Therefore, the H type should be located in muscles ready to perform a sustained activity, whereas the M type would be a constituent of voluntary striated muscle geared for sudden activity. In other words, LDH_5 predominates in anaerobically metabolic tissue since in high concentrations it functions more efficiently than LDH_1; by contrast, LDH_1 is dominant in aerobic tissue where high substrate concentrations do not develop.[13]

Fractionation of the LDH isoenzymes is usually accomplished in one of four methods: electrophoresis, selective heat denaturation, use of substrate analogue, and biochemical inhibition. The most common method is electrophoresis. As mentioned previously, there are five major fractions of LDH isoenzymes separable by electrophoresis. These are designated 1 through 5 in order of descending mobility, such that the fraction closest to the anode is LDH_1 and the fraction closest to the cathode is LDH_5.[2]

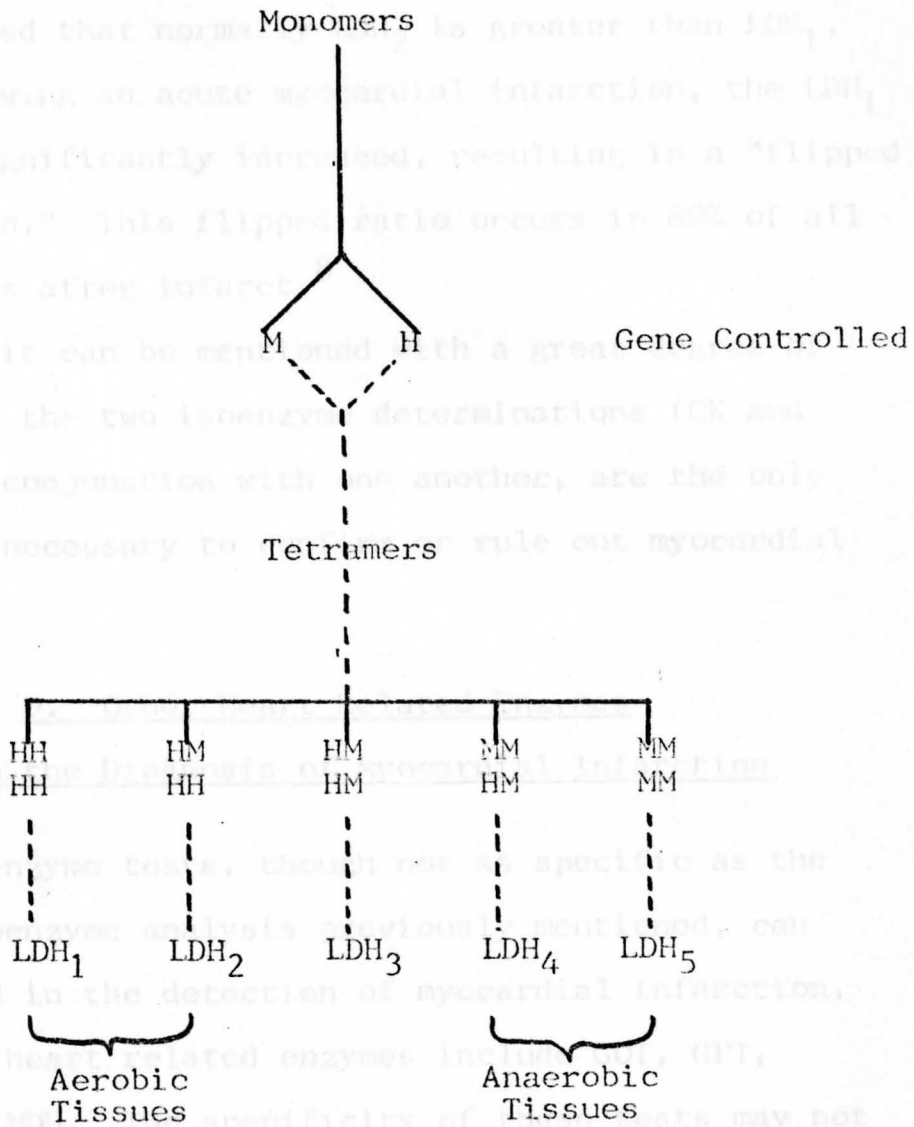


Figure 7. Tetramic Association of LDH Isoenzymes.[13]

Serum LDH isoenzyme values from healthy individuals are relatively constant and are shown in Table 4. From this it is noted that normally LDH_2 is greater than LDH_1. However, following an acute myocardial infarction, the LDH_1 fraction is significantly increased, resulting in a "flipped LDH_1/LDH_2 ratio." This flipped ratio occurs in 80% of all M.I.'s 48 hours after infarct.[8]

Finally, it can be mentioned with a great degree of certainty that the two isoenzyme determinations (CK and LDH), used in conjunction with one another, are the only enzyme assays necessary to confirm or rule out myocardial infarction.

D. Other Heart Related Enzymes Used in the Diagnosis of Myocardial Infarction

Several enzyme tests, though not as specific as the CK and LDH isoenzyme analysis previously mentioned, can be used to aid in the detection of myocardial infarction. Some of these heart related enzymes include GOT, GPT, HBD, PHI, and MDH. The specificity of these tests may not be as great as with the isoenzyme analysis of CK and LDH. However, by the use of these enzymes, the physician will be able to follow the course of the patient's progress.

Electrocardiograms, used by many clinicians in the detection of M.I., may be of little diagnostic value in patients with left bundle-branch block, with old changes which may obscure the interpretations, with intramural

TABLE 4

PERCENTAGE OF LDH ISOENZYMES IN NORMAL SERUM[2]

BAND	MEAN	S.D.	RANGE
LDH_1	22.0	4.6	15.0-30.0
LDH_2	39.0	4.0	33.0-51.5
LDH_3	27.0	4.3	20.0-35.5
LDH_4	7.5	1.0	2.5-13.5
LDH_5	4.5	0.8	0.0- 8.5

infarctions which may not change the ECG pattern, or with diaphragmatic infarctions which frequently are missed on an ECG. Myocardial infarction and pulmonary embolism often exhibit the same clinical symptoms, and often an ECG will not distinguish between the two.[12] Necessary information for a correct diagnosis therefore can be provided by the use of enzyme analysis.

GOT is elevated in 92-98% of the cases of myocardial infarction with elevation beginning shortly after occurrence, usually within 4-8 hours.[12,14] A peak is reached in about 24 hours, and enzyme levels usually return to normal within 4-6 days.[12,14] Observation of the degree of elevation reached is the prime interest to the clinician, as it is roughly proportional to the extent of the infarction.[15] Serial determinations of GOT levels are usually taken for several days following an M.I. due to variation in time required for peak level attainment, and variation in time required for return to normal levels.

The serum value of GOT cannot be used as an accurate indicator in the diagnosis of an acute M.I. Out of many hundreds of cases of myocardial infarction, which have been studied, approximately 5% have shown no elevation.[15] Since GOT is found in all tissues, it is not unexpected that a false positive would occur in certain disease states. Aside from elevations seen in M.I., increased levels have also been seen in pulmonary embolism, congestive heart failure with infarcts of other organs, pancreatitis, skeletal

muscle damage, and myositis. Thus an increase in GOT level is a manifestation of tissue necrosis and is by no means specfific for myocardial infarction.[12,15]

GPT levels usually remain near normal or slightly increased in an acute M.I. Increased levels may be observed when infarction is complicated by prolonged deep shock or congestive cardiac failure resulting in liver damage.[12]

HBD activity in serum is also elevated following a myocardial infarction. It has been reported that elevation of this enzyme is more specific and more prolonged than that of LDH or GOT, returning to normal 16 to 18 days after the onset of the infarction. However, HBD activity is also increased in progressive muscular dystrophy, nephrotic syndrome, malignant melanoma, lymphoma, leukemia, and megaloblastic anemia.[15]

Malate dehydrogenase (MDH), which catalyzes the oxidation of malate, is important in the oxidation process of cell respiration. It is found in cardiac, kidney, liver, and skeletal muscle tissue, as well as in red blood cells. Following an M.I. the level of MDH rises and falls rapidly, much like CK. However, this assay is not widely used in the clinical laboratory.[12]

Finally, the only other enzyme used, which has significance for cardiac activity is the phosphohexose isomerase (PHI). This enzyme catalyzes the reversible conversion of glucose-6-phosphate to fructose-6-phosphate. Elevation of PHI begins within twelve hours and remains

elevated for about a week. Serum PHI activity is also increased by malignancy, and diseases of the heart, liver, and skeletal muscle.[12]

E. Luciferin-Luciferase System

The analysis of CK based on the luciferin-luciferase system involves the phenomenon of bioluminescence. The original work on bioluminescence was done by Raphael Dubois in 1885, on the light emitting organs of a West Indian beetle, Pyroparus. Of the three components needed for luminescence (luciferin, luciferase, and ATP), two were isolated by Dubois, namely luciferin and luciferase. There are many different sources of luciferin-luciferase systems known today, some of which are seen in Table 5.[16]

The firefly luciferase has been crystallized and has a molecular weight of 82,600.[16] The structure of firefly luciferin also had been established and is shown in Figure 8.[16]

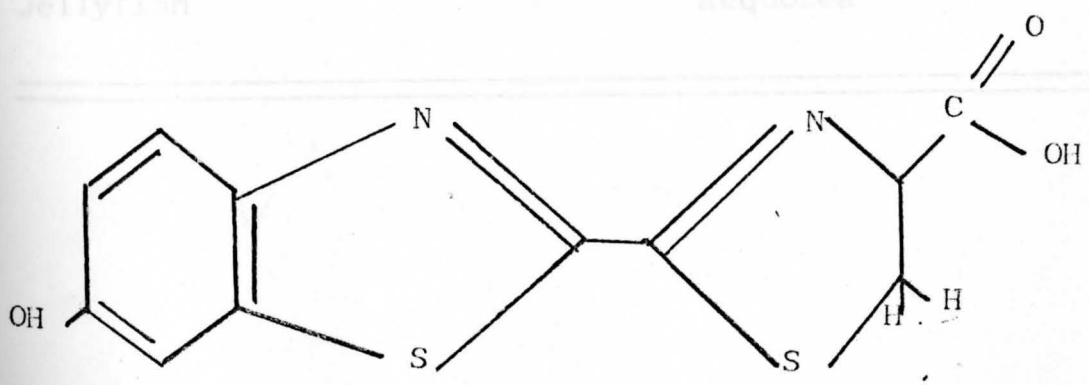


Figure 8. Structure of Firefly Luciferin

TABLE 5

SOURCES OF LUCIFERIN--LUCIFERASE[16]

ORGANISM	SOURCE(S)
Firefly	Photinus Photuris Luciola
Ostracod crustacea	Cypridine Pyrocypris
Bacteria	Achromobacter fischerii Photobacterium fischerii
Protozoa (dinoflagellate)	Gonyaulax polyerda
Sea Pansy	Renilla reniformis
Jellyfish	Aequorea

W. D. Mc Elroy and his colleagues worked out the mechanism of the reaction as shown in figure 9.[16]

This reaction leads to the excited state molecules in figure 10 which can go to an enol form or keto form.[16] The main reaction product, decarboxyketoluciferin, is formed in a single excited state and emits a quantum of light as it goes to the electronic ground state. The enol dianion which is formed emits a yellow-green light of about 562 nm, which is typical to the firefly. The keto forms emit a red light at approximately 614 nm which is not characteristic of any firefly forms.[16]

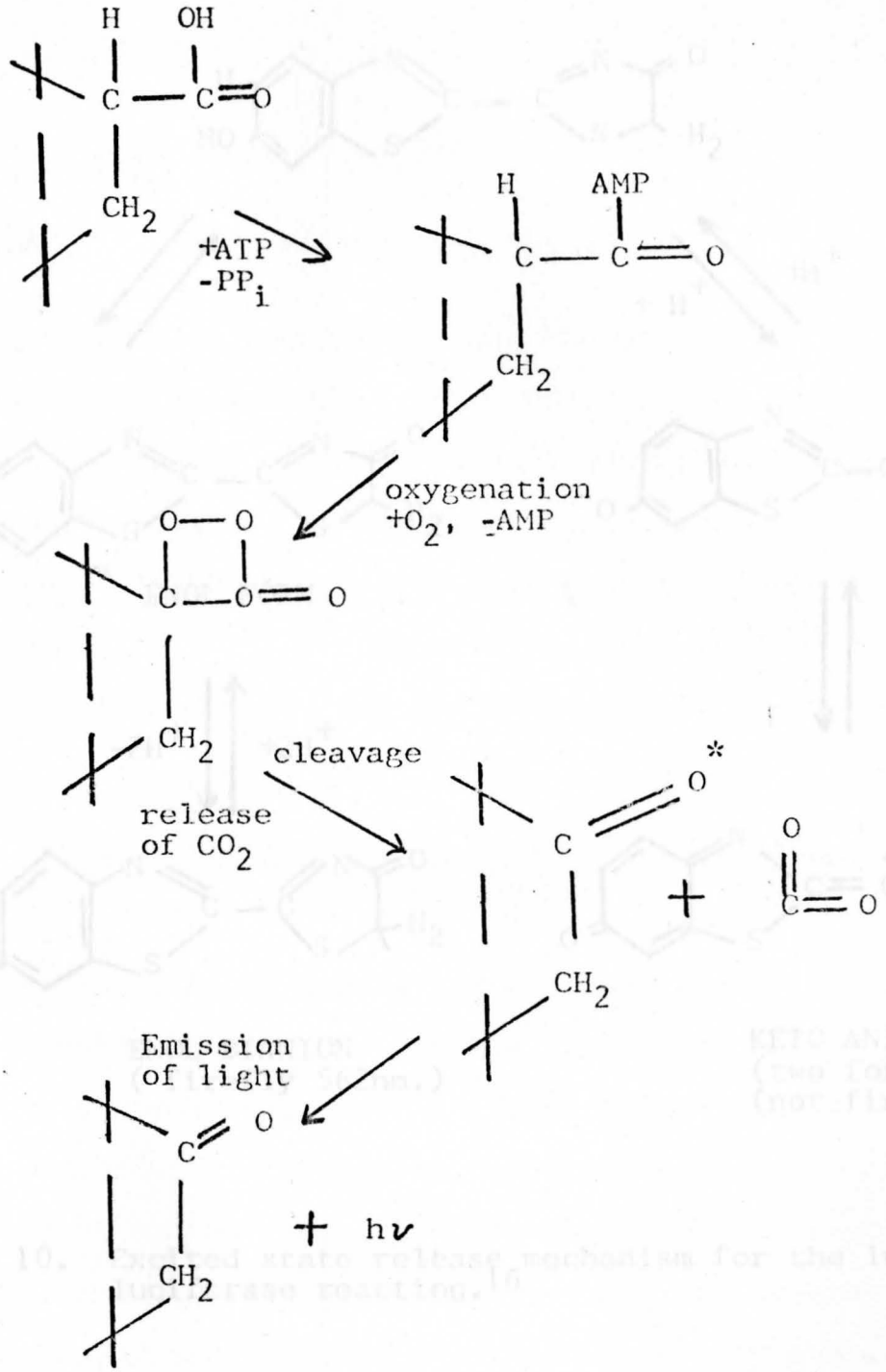


Figure.9. Mechanism of the Luciferin-Luciferase reaction.[16]

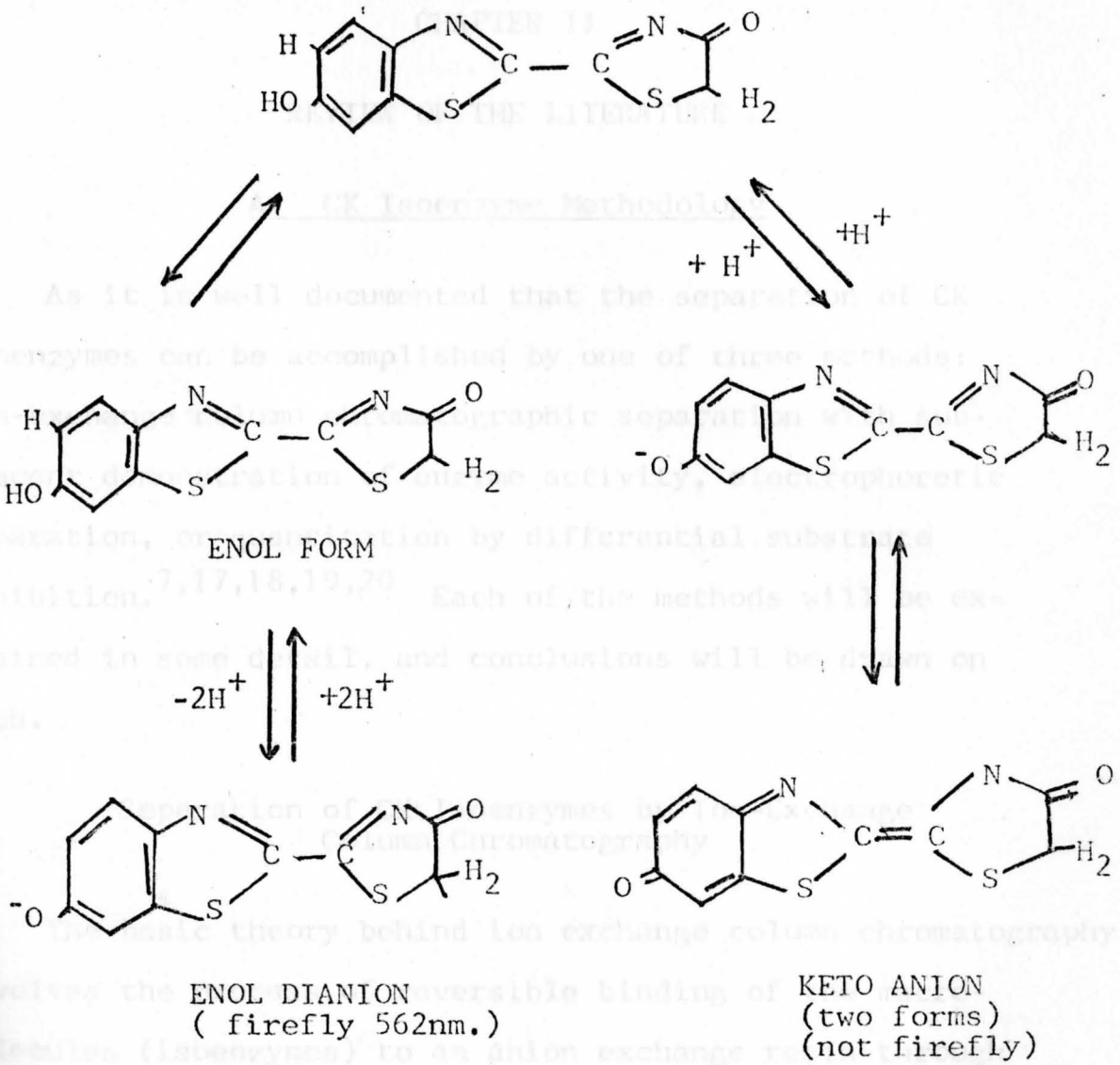


Figure 10. Excited state release mechanism for the luciferin-luciferase reaction.[16]

CHAPTER II

REVIEW OF THE LITERATURE

A. CK Isoenzyme Methodology

As it is well documented that the separation of CK isoenzymes can be accomplished by one of three methods: ion-exchange column chromatographic separation with subsequent demonstration of enzyme activity, electrophoretic separation, or quantitation by differential substrate inhibition.[7,17,18,19,20] Each of the methods will be explained in some detail, and conclusions will be drawn on each.

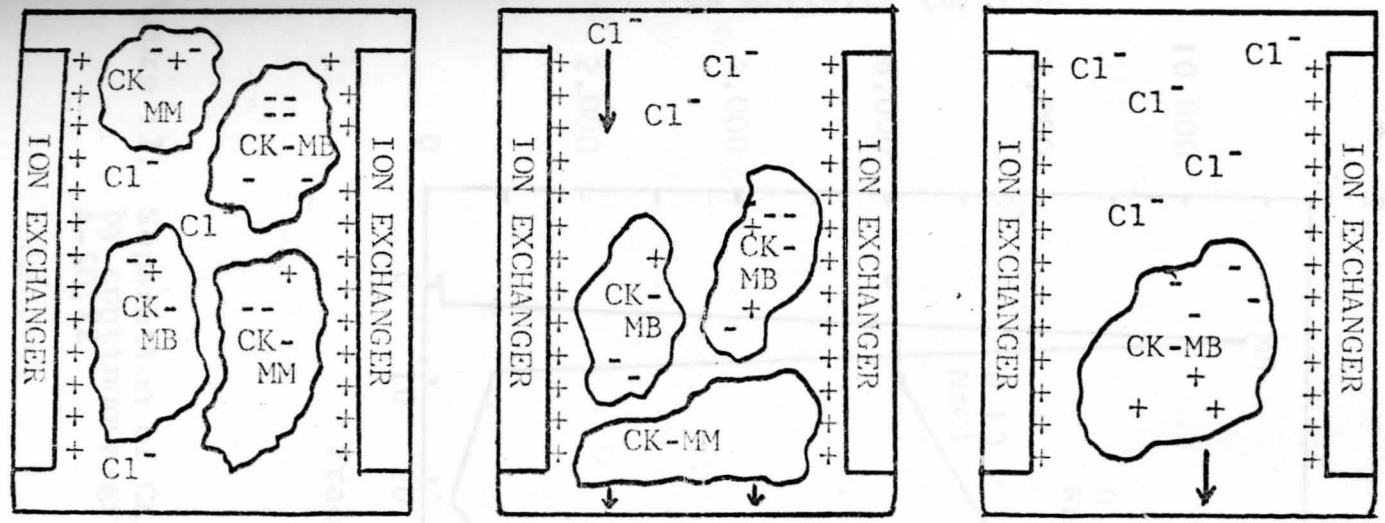
Separation of CK Isoenzymes by Ion-Exchange Column Chromatography

The basic theory behind ion exchange column chromatography involves the process of reversible binding of the macromolecules (isoenzymes) to an anion exchange resin through the formation of electrostatic bonds between charges of opposite sign on the isoenzymes and the anion exchanger. The ion exchanger charge groups are fixed and interact with the opposite charges on the isoenzymes. By changing the conditions, the isoenzymes are released from the bonding and eluted from the column.[20] The first procedure is to elute weakly held isoenzymes by introducing halide ions to take up the fixed charge positions of the ion

exchanger thereby releasing the macromolecules. Additional elution can be obtained by reducing the net negative charge of the macromolecules by lowering the pH. The process is shown diagramatically in figure 11 (a) (b) (c).[20]

Recently Mercer,[21] Nealon and Henderson,[18] and Yasmineh and Hanson[22] have all described ion-exchange column procedures for the separation and quantitation of the isoenzymes of CK. Because the CK activity is determined by a spectrophotometric, kinetic assay, the column assays are thought to be more sensitive than the conventional electrophoretic methods where densitometers or fluorescent scanning is generally the means of quantitation.[23]

This point was illustrated in the work of Yasmineh and Hanson,[22] who used a continuous-gradient elution chromatography on DEAE-Sephadex A-50 indicating that the column technique is more sensitive than electrophoresis on cellulose acetate, especially when the activity of a CK isoenzyme is very low with respect to another, as is the case of the MB isoenzyme after an M.I. In their experiments, one ml of dog heart extract containing 40 U of enzyme activity was applied to a continuous gradient elution on a macro-column (1.2 x 25 cm) of DEAE-Sephadex A-50 as shown in figure 12. As it can be seen, separation of the three isoenzymes, MM (97.2%), MB (2.7%) and BB (0.15%) were excellent.[22] Elution of the isoenzymes were complete at NaCl concentrations of 0.10, 0.20, and 0.30 mol/liter. Yashmineh and Hanson, also ran gradient elution



(a) CK-MM and CK-MB binding to ion exchanger after sample additon. Both MM and MB are in dynamic equilibrium with ion exchanger and the 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 density of chloride ions and reducing the pH (binding capacity of CK-MB) the CK-MB is eluted.

Figure 11. Basic Theory of Ion-Exchange Column Chromatographic Separation of CK Isoenzymes.[20]

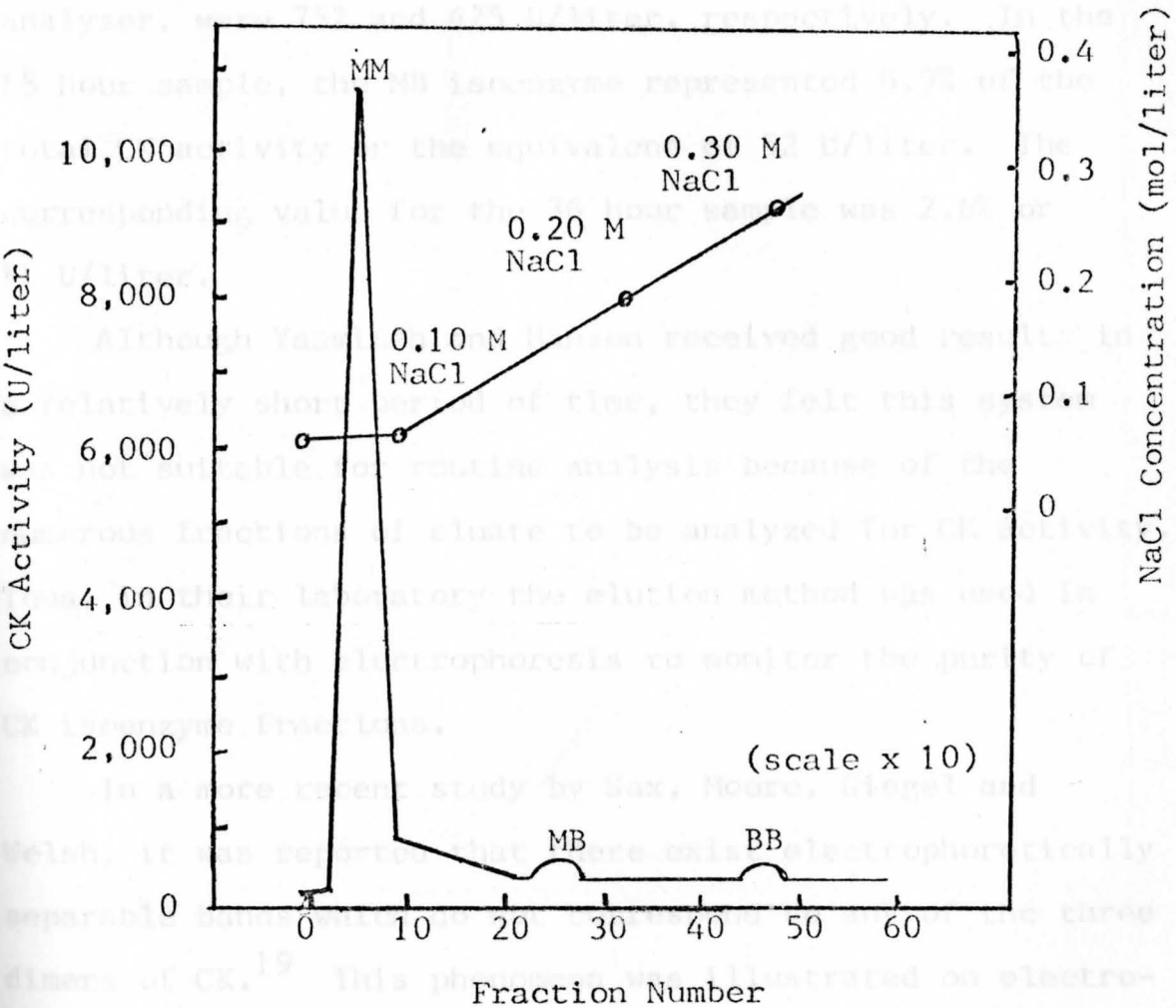


Figure 12. Separation of CK isoenzymes in dog heart extract by continuous-gradient elution of DEAE-Sephadex A-50.[22]

patterns on sera drawn from a patient about 18 hours and 36 hours after the onset of an acute M.I. Figure 13 (a) and (b) illustrate these patterns.[22] Values for the total CK activity, as obtained by a GEMSAEC centrifugal analyzer, were 752 and 425 U/liter, respectively. In the 18 hour sample, the MB isoenzyme represented 6.9% of the total CK activity or the equivalent of 52 U/liter. The corresponding value for the 36 hour sample was 2.6% or 11 U/liter.

Although Yasmineh and Hanson received good results in a relatively short period of time, they felt this system was not suitable for routine analysis because of the numerous fractions of eluate to be analyzed for CK activity.[22] Thus, in their laboratory the elution method was used in conjunction with electrophoresis to monitor the purity of CK isoenzyme fractions.

In a more recent study by Sax, Moore, Giegel and Welsh, it was reported that there exist electrophoretically separable bands which do not correspond to any of the three dimers of CK.[19] This phenomena was illustrated on electrophoretograms of eluates from DEAE-Sephadex columns. Both the low and medium salt eluates demonstrated a single band located between MM and MB. (see figure 14).[19] Sax and et.al., obtained sera from a patient with a known M.I., a high total CK, and an elevated MB and from a patient exhibiting an unexplained MB increase with normal total CK gave identical peak activity eluates from Sephadex G-100.

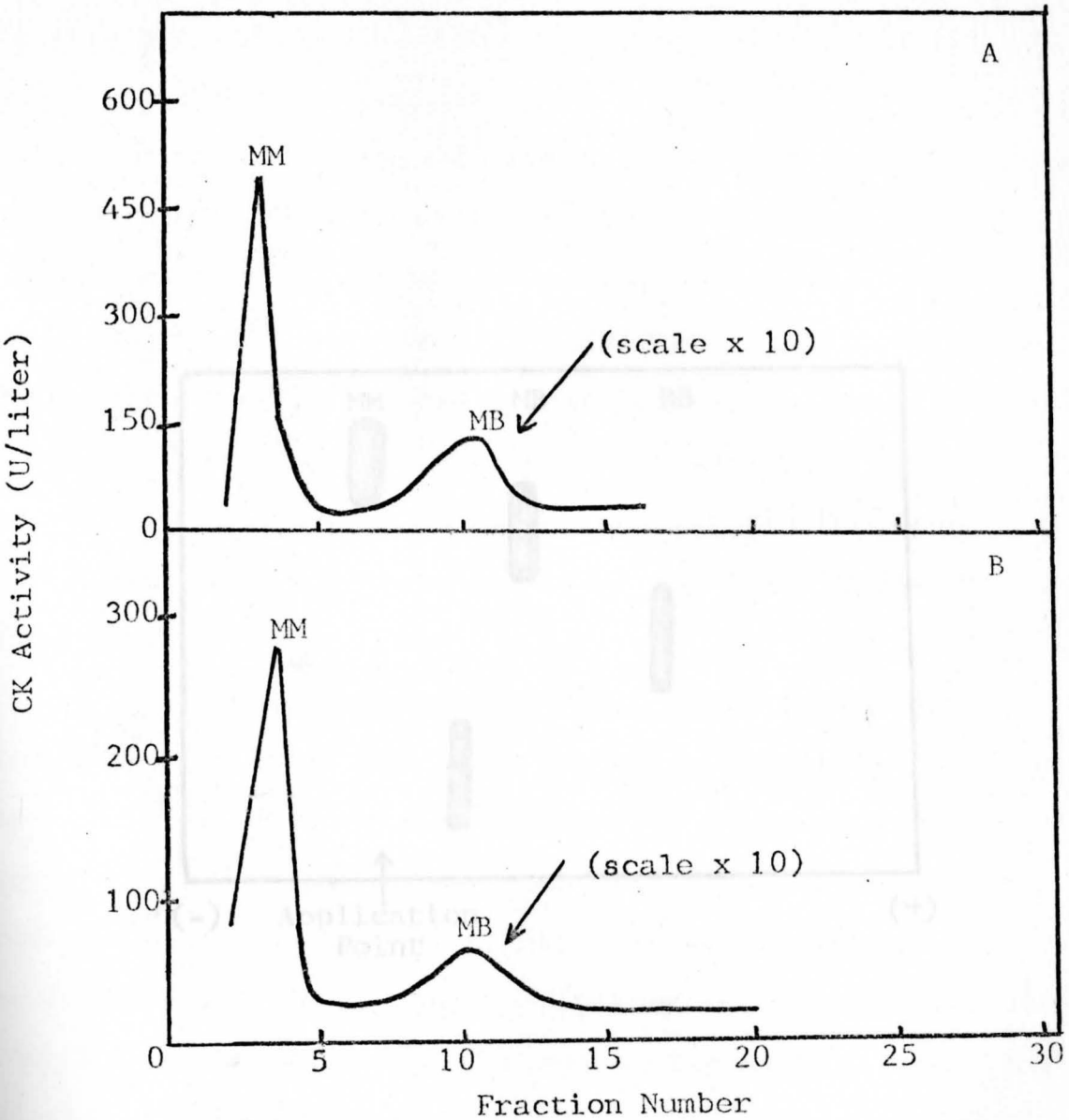


Figure 13. Separation of CK isoenzymes in M.I. Patient by continuous-gradient elution of DEAE-Sephadex A-50.[22]

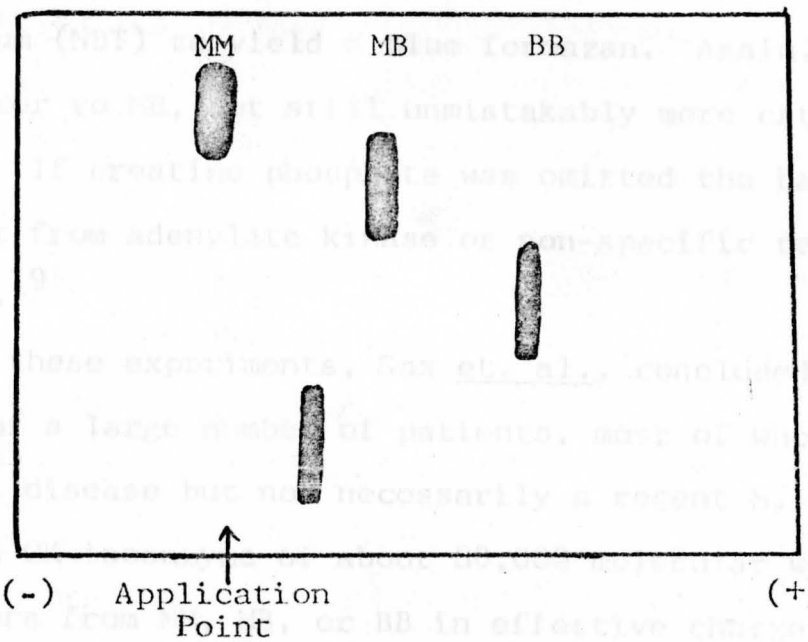


Figure 14. Electrophoretograms of eluates showing abnormal band between MM and MB isoenzymes of CK.[19]

The approximate molecular weights were calculated to be 81,000, indicating that the unusual MB fraction is not a macromolecular complex and that it apparently does not differ in molecular weight from the usual MM and MB isoenzyme.[19] The CK isoenzymes were also separated by electrophoresis using the Dade procedure with cellulose acetate. For development, the resulting NADH reacted with nitroblue tetrazolium (NBT) to yield a blue formazan. Again, a single band, closer to MB, but still unmistakably more cathodal appeared. If creatine phosphate was omitted the bands did not result from adenylate kinase or non-specific tetrazolium reduction.[19]

From these experiments, Sax et. al., concluded that the sera of a large number of patients, most of whom have myocardial disease but not necessarily a recent M.I., contains a CK isoenzyme of about 80,000 molecular weight that differs from MM, MB, or BB in effective charge or conformation or both. On ion-exchange chromatography, when the CK was eluted by both low and medium-salt buffers, the single, unknown, band could be easily mistaken for the MB isoenzyme using this technique. So if using this technique, a falsely positive MB increase should be considered a possibility in patients demonstrating persistent above-normal values for total CK. It is recommended to use a suitable electrophoretic method that clearly demonstrates this unusual isoenzyme to confirm or rule out actual increase in MB.[19]

Separation of CK Isoenzymes by Electrophoresis

CK isoenzymes have been electrophoretically separated on cellulose acetate, polyacrylamide gel, agar gel, or agarose gel. Their activities are either measured spectrofluorometrically after they are eluted from the cellulose acetate strip, or they are measured by a direct reaction of NADH fluorescence of the isoenzymes or by reduction of the dye NBT to produce a blue colored formazan.[6,8,18] Listed below are three common electrophoretic methods used in the laboratory today.

The first electrophoretic method employs the use of a cellulose acetate support medium. The strip is pre-soaked in a buffer for 10-20 minutes. Serum is applied to the strip and electrophoresed for 8-9 minutes at 350 volts. The strip is then incubated at 37°C with the CK isoenzyme substrate which utilized the following reactions.[8]

(1) Creatine Phosphate + ADP $\xleftrightarrow{CK}$ Creatine + ATP

(2) ATP + Glucose $\xleftrightarrow{HK}$ Glucose-6-Phosphate + ADP

(3) Glucose-6-Phosphate + NAD $\xleftrightarrow{G\text{-}6\text{-}PD}$ NADH + Phosphogluconate

The amount of fluorescence of NADH produced by each CK isoenzyme is measured on a fluorescent densitometer.

Another alternate detection method employed, using the same electrophoretic system as outlined above, utilizes an agar tray containing a nitroblue tetrazolium (NBT) dye and phenazine methosulfate (PMS), in which the cellulose acetate is layered and allowed to develope. The NBT dye

is reduced producing a blue formazan at the site of isoenzyme activity on the strip. The reaction step for this alternate procedure is shown below:

$$NADH + NBT \xleftarrow{PMS} \xrightarrow{} Formazan\ (blue)$$

The membrane strip may then be scanned in a densitometer using an interference filter between 575-600 mm.[24]

Another electrophoretic method employs the use of an agarose gel. In this procedure, serum is applied to thin agarose gel plates, electrophoresed, and is then layered with 1.0 ml of solution containing the reactants as outlined by the previous cellulose acetate method. After development, the gel plate may then be scanned with a Turner fluorometer mounted with a TLC automatic scanning door, or observed visually with a U.V. light source.[7,17] Again the amount of NADH detected is directly proportional to the amount of CK activity present.

The final electrophoretic procedure to be demonstrated incorporates the use of a polyacrylamide gel. The polyacrylamide disk gel is electrophoresed in a Canalco Model 12 apparatus at room temperature in a Tris-glycine buffer at pH 9.2 for 50 minutes under a constant voltage of 10 volts per tube.[5] After electrophoresis, the gels are immersed for 45 minutes at 37°C in a substrate solution similiar in reactants to that discussed in the above two procedures. Directly after substrate incubation, the CK bands are developed by submerging the gels in a solution

of NBT and PMS. The total activity of samples are then determined by the Conn and Anido fluorometric technique.[5,19] Patterns obtained by electrophoresis utilizing this technique are shown in figure 15.[5]

However simple and straightforward the methods described above may seem to be, the scanning technique employed with each had drawn some strong criticism. Several reasons why quantitative scanning may tend to be inaccurate were pointed out by R. Roberts et. al.,[25] and G.A. Moss.[23] These include:[25] (1) Isoenzymes may be distributed asymmetrically in the electrophoretic supporting medium so that selection of the position used for scanning may influence results, (2) activity of individual isoenzymes may be underestimated because of limited diffusion of substrate into the supporting medium or loss of NADPH or dye from the medium, and (3) apparent activity of specific isoenzymes may deviate from linearity with respect to time or actual activity because of disparate activities of one or more isoenzymes in a mixture. It is for these reasons that Roberts et. al., has developed and evaluated a procedure for quantitative analysis of CK isoenzymes designed to eliminate these difficulties. The systematic procedure employed by Roberts et. al., is outlined below:[25]

1. CPK isoenzymes were separated by cellulose acetate electrophoresis.
2. After electrophoresis, sections of the cellulose acetate strips containing each isoenzyme were cut out, immersed in 0.5 to 3 ml of the CPK assay medium in a disposable cuvette, and incubated at 25°C with constant shaking.

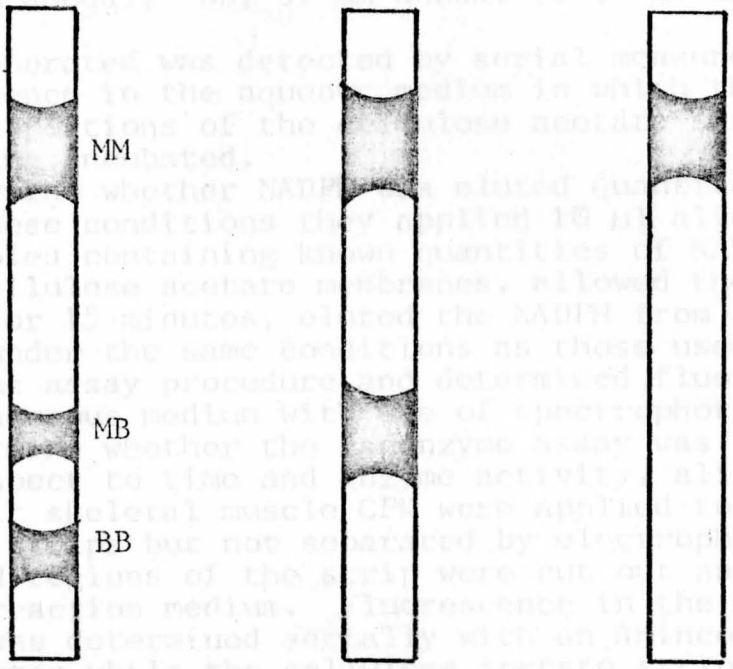


Figure 15. Patterns of Polyacrylamide gel electrophoresis.[5]

Incubation time and volume were chosen to provide linear reaction rates and sufficient fluorescence from NADPH generated by the isoenzymes on each selected portion of the strip. Analysis of 100 samples, each sections containing the three isoenzymes indicated that the enzymes were contained with the following regions: MM, 4mm cathodal to 12 mm anodal; MB, 12 mm anodal to 37 mm anodal; BB, 37 mm anodal to 47 mm anodal.

3. NADPH generated was detected by serial measurement of fluorescence in the aqueous medium in which the selected portions of the cellulose acetate strips were being incubated.
To determine whether NADPH was eluted quantitatively under these conditions they applied 10 µl aliquots of samples containing known quantities of NADPH to moist cellulose acetate membranes, allowed the samples to dry for 15 minutes, eluted the NADPH from the strips under the same conditions as those used in the isoenzyme assay procedure and determined fluorescence in the aqueous medium with use of spectrophotometry.
To determine whether the isoenzyme assay was linear with respect to time and enzyme activity, aliquots of rabbit skeletal muscle CPK were applied to cellulose acetate strips but not separated by electrophoresis. Selected regions of the strip were cut out and incubated in CPK reaction medium. Fluorescence in the aqueous medium was determined serially with an Aminco-Bowman fluorometer while the cellulose acetate strip was removed transiently from the solution.
To assess recovery of isoenzyme activity from cellulose acetate strips, fluorescence detected with aliquots added to the medium itself was compared with fluorescence detected when corresponding aliquots were assayed in cellulose acetate strips immersed in incubation medium.
To determine whether electrophoresis altered recovery of CPK activity, aliquots of rabbit skeletal muscle CPK were subjected to electrophoresis before fluorometric assay of CPK activity in appropriate regions of the cellulose acetate membrane. Percent recovery was calculated by comparison with activity observed when corresponding aliquots were assayed in solution. Recovery of MM and MB isoenzymes in serum samples was determined in the original sample and in serial dilutions.
Reliability of the assay was examined with human myocardial supernatant fractions containing MM and MB-CPK. Activities of each were determined 10 times from aliquots of the initial sample and 10 times each from aliquots of the sample diluted to contain 0.2 and 0.1 IU/ml. Accuracy of the method was assessed with solutions formulated with selected quantities of BB and MB isoenzymes.

The method described includes several advantages over the conventional scanning procedures. Since isoenzymes are assayed separately, reaction conditions can be adjusted to achieve linearity with respect to time and activity of each isoenzyme. All NADPH generated contributes to fluorescence since it is eluted into the aqueous reaction medium. Availability of substrate to each isoenzyme in the supporting medium is faciliated by constant shaking and immersion of the supporting medium into the reaction solution. The problem of non-uniform distribution of the sample is eliminated since the entire region of the supporting medium encompassing each isoenzyme is utilized. Reactions are performed kinetically. The method detects individual isoenzymes with an activity level of less than 0.002 IU/ml, and hence is capable of quantifying isoenzymes even when total serum CPK activity is not increased. Results appear to be reproducible.[25]

Detection of CK Isoenzymes by Kinetic Methods

The method conventionally used for the detection of CK activity in sera is that of Oliver,[26] and modified by Rosalki.[27] However, this assay cannot be used at very low concentrations of substrates due to a lack of sensitivity.[28] Eppenberger et.al., demonstrated that the MM and BB isoenzymes from both rabbits and chickens exhibited different kinetic properties.[29] In the reaction CP + ADP $\longleftrightarrow$ creatine + ATP, Km values were found to be lower for both ADP and Creatine phosphate (CP) with the BB isoenzyme. When creatine

phosphate was present in excess the BB isoenzyme was inhibited more than the MM isoenzyme. When reaction rates were compared at high versus low substrate concentrations, different ratios of activity for the three isoenzymes were observed.[28,29]

Based on these results Witteveen, Sobel and DeLuca[28] developed a kinetic method to assay CK at very low substrate concentrations by measuring the amount of ATP production with a firefly light-emitting reaction. Thus the reaction CP + ADP $\longleftrightarrow$ creatine + ATP is followed by coupling the ATP formed with the firefly luciferase reaction:[28]

$$\text{ATP} + \text{luciferin} + O_2 \xleftrightarrow{E} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + h\nu,$$

where E is luciferase.

Under selected conditions the light emission at any moment is directly proportional to the amount of ATP present at that instant. When CK, ADP, and CP are present in the reaction medium, the rate of increase of light emission is proportional to the rate of ATP formation. By using the Rosalki method for CK activity at high substrate concentrations and the luciferin-luciferase reaction at low substrate concentrations, it is possible to determine the amount of the three isoenzymes in a sample.

Witteveen et.al.,[28] measured the total CK activity spectrophotometrically based on the method of Rosalki. The luciferase-coupled assay was measured by light emission in an Aminco-Chemglo photometer. The emission of light

as a function of time was recorded on a Beckman recorder. To initiate the reaction 0.02 ml of ADP and 0.02 ml of CK sample were added and the baseline light emission was recorded. If myokinase was present in the sample, light emission was observed before the addition of CP. This background light emission was subtracted from the final light emission observed upon the addition of CP. The light output was measured for 15 seconds and was linear over this period. A typical CK-luciferase assay is shown in Figure 16.[28] Activity was expressed in arbitrary units. The presence of myokinase in the serum seriously interfered with the luciferin-luciferase system at high substrate concentrations. The Rosalki system, which includes 0.01 M AMP, inhibited the myokinase. They found that this concentration of AMP also inhibited luciferase and could not be included in the luciferase assay. However, at low concentrations of ADP used routinely with the luciferase assay, the myokinase did not interfere. It was for this reason that the luciferase assay was used on serum samples at low substrate concentrations and the Rosalki assay at high substrate concentrations.[28]

When samples of MM and BB that had the same activity in the Rosalki assay were assayed at low substrate concentrations with the luciferase system, BB isoenzyme exhibited a four-fold higher activity than MM. MB exhibited intermediate activity under these conditions.

In conclusion, Witteveen et.al.,[28] found that the human BB isoenzyme showed a higher activity than MM at lower

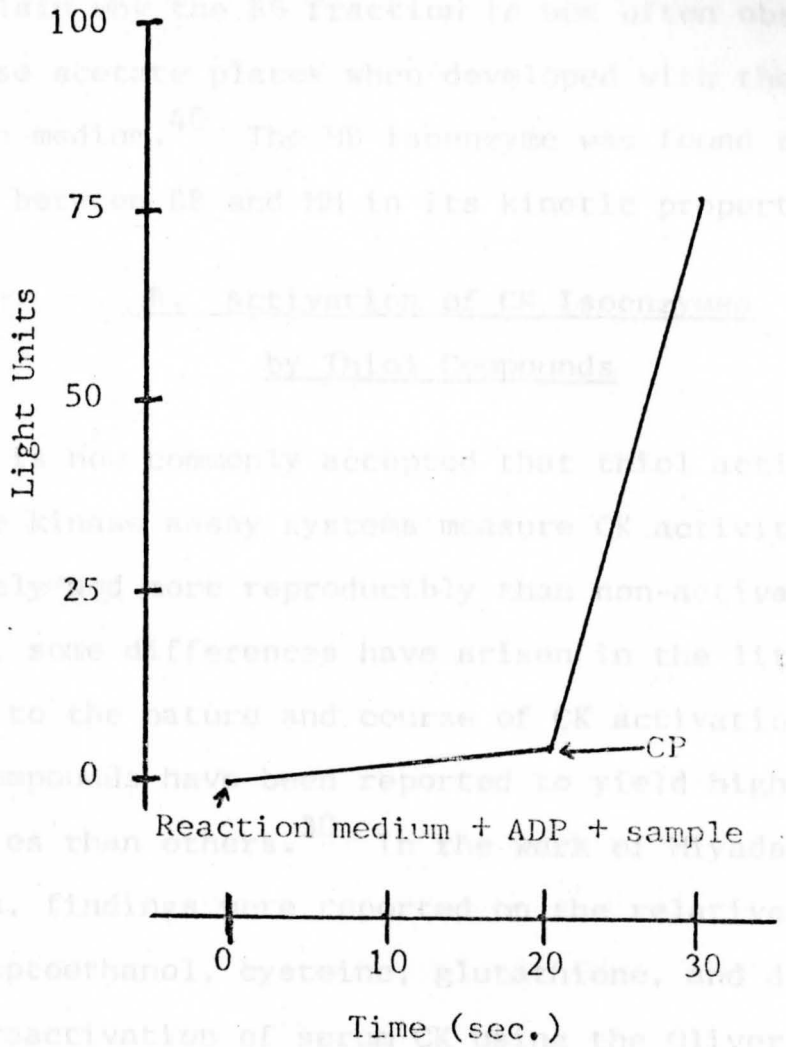


Figure 16. Representative tracing of a bioluminescence assay. Slope of line depends upon CK activity in sample.[28]

substrate concentrations, while increasing the substrate concentration caused inhibition of the BB fraction. This may explain why the BB fraction is not often observed on cellulose acetate plates when developed with the Rosalki reaction medium.[40] The MB isoenzyme was found to be intermediate between BB and MM in its kinetic properties.

B. Activation of CK Isoenzymes by Thiol Compounds

It is now commonly accepted that thiol activated creatine kinase assay systems measure CK activity more accurately and more reproducibly than non-activated systems. However, some differences have arisen in the literature in regards to the nature and course of CK activation. Some thiol compounds have been reported to yield higher enzyme activities than others.[30] In the work of Miyada, Dinovo and Nakamura, findings were reported on the relative effectiveness of mercaptoethanol, cysteine, glutathione, and dithiothreitol in the reactivation of serum CK using the Oliver-Rosalki method.[30]

Miyada, et. al., pooled human elevated CK sera and bubbled oxygen through the serum for 30-45 minutes to inactivate CK. The concentrations of the thiol compounds used in their study were 0.5, 1.0, 5.0, and 10.0 mM. The BMC reagent system was used for their study because the activator could be added separately and thus would allow for selective use of different activators. Then 0.1 ml

of the pooled serum containing the inactivated CK was added to 2.50 ml of substrate at zero time. The reactants were mixed and aspirated into temperature controlled cuvettes. Absorbance measurements were obtained at 340 nm on the Gilford 300-N Spectrophotometer with Data Lister 4008. All of their reactions were carried out at 37°C including the pre-incubation of reagents.[30]

As a result of their work, it was shown that all four thiol compounds did indeed reactivate serum CK, and that CK activity increased with increasing activator concentration until maximum activity is attained.[30] However, Dinovo and Nakamura found that all four thiol compounds maximally reactivate CK at different concentrations. They demonstrated that although reaction linearity was observed for all activators and at all concentrations, the reaction rates with lower activator concentrations were only a fraction of the maximum rate. This attainment of linearity without attaining maximum enzymatic activity was observed with all activator systems and they feel that it reflects either exhaustion of thiol activator or the attainment of an active-inactive enzyme equilibrium.

They also tabulated their results to show that the lag time using the Oliver-Rosalki method for CK measurement is dependent upon the specific activator as well as its concentration.[30] (see table 6) Here they defined lag phase as the time interval (lag time) for an enzyme reaction to attain linearity, which includes the time necessary to activate the enzyme as well as the time to attain steady

TABLE 6

CK ACTIVITY AND LAG TIME VERSUS THIOL CONCENTRATION[30]

Thiol Tested		Thiol Concentration (mmol/liter) 0.5	1.0	5.0	10.0
DDT	Activity (U/liter)	590	717	721	768
	Lag time (min)	11	8	3	1
Glutathione	Activity	337	616	713	759
	Lag Time (min)	14	12	5	3
Cysteine	Activity	438	675	781	818
	Lag Time (min)	7	6	3	1-2
Mercaptoethanol	Activity	548	793	985	784
	Lag Time (min)	13	9	4	2-3

levels of intermediates when using a coupled enzyme system such as the Oliver-Rosalki method.[30] Therefore from table 6, it can be seen that lag time decreases with increasing concentrations of thiol activators, but that it is never eliminated.

In another study by Morin[31] using a macroporous strong anion exchanger for separating MM and MB isoenzymes of creatine kinase, it was also demonstrated that certain thiol activators markedly enhance the activity of BB and MB. In table 7, Morin points out a comparison of sulfhydryl activation on the three isoenzymes of CK as contrasted to glutathione.[31] In his study, Morin, also demonstrated that MM is relatively stable up to 47°C. MB and BB, however, become very unstable at 44°C, losing virtually all activity in 10 minutes. Unfortunately, MB hybridizes rapidly at this temperature to form the more stable MM and also shows a short-lived increase in activity because of the formation of BB.[31] The mean loss of activity in 30 sera for MB was 24% in 2 hours at ambient temperature, and 84% to 100% loss in 24 hours refrigerated. The prompt addition of 10 mM of DTT or ME per liter allowed 96% recovery of activity after 24 hours refrigeration.[31]

Finally, Morin, points out that enhancement of activity by certain thiol compounds merits further study, both to determine whether the increased activity merely represents activation of inactive enzyme, a shift in active-inactive enzyme equilibrium, or an enhancement of the activity of

TABLE 7

COMPARISON OF SULFHYDRYL ACTIVATION ON ISOENZYMES[31]

Isoenzymes	Cysteine	DTE	DTT	ME
MM	1.00	1.06	1.05	1.08
MB	1.04	1.40	1.43	1.41
BB	1.03	1.91	1.89	1.92

already active enzyme, and to further assess its potential as a tool in quantitating isoenzymes.[31]

C. Elevated CK Levels Can be Misleading

Although elevated serum creatine phosphokinase activity is well known to be a sensitive detector of myocardial necrosis, caution must be used and consideration given to the many conditions that may affect it. The clinician must becognizant of these pathological conditions, and be careful not to be misled in his diagnosis.

It is well known that creatine phosphokinase is found in various organs of the body with the highest concentrations in skeletal muscle, myocardium, and brain, and to a lesser extent in the kidney , bladder, bowel, lung, uterus, and gastrointestional tract.[3,9]

Nevin, et. al., showed in their study of fifty consecutive patients admitted to the coronary care unit (CCU) that of the 15 patients with transmural infarction, the mean maximum CK level was 605 IU/liter using an ultraviolet spectrophotometric method (modified Oliver-Rosalki method) in which the normal range of CK was reported to be 0 to 50 IU/liter. (see table 8) Maximum CK level was generally achieved within the first 24 hours. All patients had maximum elevations at least twice the upper limits of normal.[3]

Of 12 patients with prolonged chest pain due to coronary disease, the mean maximum CK level was 146 IU/liter. The

TABLE 8

MAXIMUM CK IN 50 CONSECUTIVE CCU ADMISSIONS[3]

	Final Diagnosis		
	Transmural MI	Subendocardial Infarction	No Cardiac Ischemia
# of patients	15	12	23
Mean CK (IU/L)	605	146	69
Range (IU/L)	100-1,500	14-580	16-215
% Twice Normal	100	25	22

pathological findings in these patients at the time of CCU discharge was subendocardial infarction or coronary insufficiency.[3]

The third group consisted of 23 patients who had symptoms due to disorders other than acute coronary disease such as congestive heart failure, syncope, pericarditis, arrhythmias, or cholecystitis. In this group the mean maximum CK level was 69 IU/liter.[3]

Of the five patients in the non-coronary category with CK elevation greater than two times normal, two had tachycardia, two had received recent intramuscular injections, and one had experienced chest pain after strenuous snow shoveling. Tachycardia, injections, and exercise all are potential causes of elevated CK level that must be excluded before making a diagnosis of myocardial necrosis.[3]

Elevations in CK levels may also be observed in muscle disorders such as, in all forms of muscular dystrophy, as well as polymyositis and other myopathies. Several clinical forms of myopathy occur in alcoholic patients whose CK level may be elevated in about 75% of the cases and may achieve levels as much as 8 to 29 times normal. The pathogenesis of alcoholic myopathy is unknown, but alcohol apparently has a direct toxic effect.[3]

Muscle injury due to trauma or surgery, may cause markedly increased CK levels. After an operation the maximum CK level occurs from 24 to 48 hours, and normal results may not be observed for four or five days.

Intramuscular injections are an important cause of serum CK elevation.[3] Some injectable drugs that have been known to elevate CK are listed in table 9.

A rare, but frequently fatal, cause of high CK level is the syndrome of malignant hyperthermia, in which seemingly normal people develop a temperature as high as 112°F during general anesthesia, apparently because the anesthetic precipitates an acute metabolic reaction in skeletal muscle. It is believed that this tendency in some families is genetically transmitted as an autosomal dominant trait.[3]

Another important area of CK elevation is found in neurologic and psychiatric disorders. It is well documented that the brain, after heart and skeletal muscle, is the richest source of CK.[3,7] Among these neurologic disorders that are capable of CK elevations as much as 100 times normal are subarachnoid hemorrhage, stroke, head injury, brain tumor, convulsions, meningitis, and encephalitis.[3,7] In patients suffering from acute psychosis, elevations in CK level were 2 to 35 times normal, but rarely were abnormal values seen in neurotics or long-term psychotics.

Other conditions which may cause elevated CK levels, but to a lesser extent than those previously mentioned, are hypokalemia, pulmonary disease, hypothyroidism, prolonged coma, eclampsia, hemorrhagic pancreatitis, gangrene of the gall bladder, pancreatic carcinoma, diabetic ketoacidosis, electric countershock, urologic procedures, radiotherapy, sleep deprivation, and dissecting aneurysm.[3,7]

TABLE 9

INTRAMUSCULAR INJECTIONS WHICH ARE KNOWN TO CAUSE ELEVATED CK LEVELS[3]

Carbenicillin disodium
Ampicillin trihydrate
Chlorpromazine hydrochloride
Lithium carbonate
Furosemide
Morphine sulfate
Meperidine hydrochloride
Chlordiazepoxide hydrochloride
Trimethobenzamide hydrochloride
Dexamethasone

So as it can be seen that, because of the lack of specificity of CK determinations, the clinician must be aware of the many conditions which affect the results. Referral to other more specific tests such as CK Isoenzymes may be the rule of thumb.

CHAPTER III

MATERIALS AND APPARATUS

A. Materials

All serum samples used in this study were obtained from either the Jameson Memorial Hospital or the Youngstown Hospital Association, Northside branch. The samples were procured from each hospital after their daily run was completed. Only those samples which exhibited abnormally high values of CK were studied for a possible diagnosis of M.I. These samples were usually aliquoted in 0.5 to 1.0 ml quantities and if not analyzed the same day, they were kept frozen for no longer than one week.

Also obtained from the Jameson Memorial Hospital and St. Elizabeth's Hospital were various cord blood specimens from the hospital's maternity ward. These samples were especially beneficial in this study, for it was believed that the cord sera contained all three fractions of the CK isoenzymes, namely MM, MB, and BB. With this possibility in mind, it was thought that the cord sera could be pooled and used as CK isoenzyme markers. All cord blood was obtained from the maternity ward usually within one hour after delivery. The specimen was then centrifuged at 2800 rpm for 10 minutes, aliquoted in 1.0 ml volumes and frozen

until electrophoretic analysis was performed. Again, no sample was kept frozen for more than one week before the analysis was performed.

Other samples used in this study included various commerically prepared control reference sera as those listed below:

Phadebas Reference Human Serum
SMA Reference Serum
Precipath E
Validate
Validate A
Enza-Trol
Helena Control Sera
Sigma CPK Isotrol
Kemtrol (Normal)
Kemtrol (Abnormal)

These control sera were reconstituted by following the labeled instructions printed on each vial. Once reconstituted, the analysis on each was carried out the same day.

Antonik Laboratories, of Elk Grove Village, Illinois sent two kits in order to assist in this study. One kit contained 48 tubes for total CK analysis. Each tube was reconstituted for use by the addition of 3.5 ml of deionized water. The other kit obtained from Antonik Laboratories contained 24 tubes of luciferin-luciferase to be used in the developer system of the bioluminescence technique. These Antonik tubes were stored in the cold room ($5^{o}C$) when not in use.

Also received from the Boehringer Mannheim Corporation (BMC) were two kits for total CK analysis from their U.V.-10 system. These vials, when reconstituted with 2.0 ml

of 20% sucrose, were used in the developer system of fluorescence using the Helena technique. These vials were reconstituted, as needed, each day and discarded. The kit was stored in the cold room when not in use. Stability of the BMC kit was approximately one year.

All water used in this study, either for reconstitution of the control sera, reconstitution of buffers, or the preparation of solutions, was distilled-deionized.

The buffer used for electrophoresis system was received from Helena Laboratories of Beaumont, Texas. Their buffer was diluted with the specific amount of deionized water as outlined in Chapter IV, stored in the cold room when not in use, and prepared once every month.

All other reagents used such as the sucrose, dithiothreitol, triethanolamine, ADP, creatine phosphate, glacial acetic acid, and magnesium acetate were reagent grade.

Finally, the heat inactivated serum used for the dilution of the control reference specimens was obtained from the Jameson Memorial Hospital. This heat inactivated serum was prepared fresh daily by pooling the sera from the hospital's morning run, and heating it to 56^{o}C for 30 minutes in a water bath.

B. Apparatus

The apparatus used for the determination of the total CK value by kinetics was the Perkin Elmer Double Beam Spectrophotometer Model 124D at the Jameson Memorial Hospital

in New Castle, Pennsylvania.

For the fluorescence technique of CK isoenzymes the system by Helena Laboratories of Beaumont, Texas was used. This system contained all the necessary items to carry out the electrophoresis. To scan the fluorescent strip, the Quick Scan Flur-Vis densitometer by Helena was also used.

In the bioluminescence technique for the total CK values a Turner Model #111 Fluorometer was used. To scan the cellulose acetate strip for luminescence of the luciferin-luciferase system a piece of cardboard paper with black tape wrapped around it was inserted to block the primary U.V. light source. The moist cellulose acetate plate was then taped on a Turner Camag TLC Scanner, Model 2 and scanned for luminescence. For a quantitive measurement of the luminescence strip, a Linear Instruments Integrator Recorder Model #252 from Linear Instruments Corporation, Irvine, California was used.

CHAPTER IV

EXPERIMENTAL

A. Preparation of Solutions

During this study a number of solutions were prepared. All water used in the preparation of these solutions and/or buffers was distilled-deionized, unless otherwise indicated.

The first solution prepared was the HR buffer used for electrophoresis. Originally Helena Laboratories said to dissolve one packet of HR buffer in 2000 ml of deionized water. Electrophoretic determinations were run using both buffers, and no appreciable differences in migration or resolution were noted. Once prepared, this buffer was kept in the cold room, and prepared monthly. Helena Laboratories suggest changing the chamber buffer daily, but the same buffer may be used up to 10 times in a single day.[8]

A twenty percent solution of sucrose (w/v) was prepared for use in the reconstitution of the BMC-CKP substrate vial and the luciferin-luciferase tube. The sucrose solution was not essential in the reactions of either system, but was used to decrease the amount of diffusion in both systems. The solution was kept in the cold room, and kept approximately one month or until mold growth was observed.

Another buffer employed in this study was a 0.100 M triethanolamine (M.W. 149.14 gm/mole) solution. This was prepared by adding 1.33 ml of the triethanolamine to 80 ml of 20% sucrose, adjusting to pH 7.0 with 5% glacial acetic acid, and adding additional 20% sucrose to bring to the mark using a 100 ml volumetric flask. This solution was also kept in the cold room and prepared once a month.

The initial developer for the luciferin-luciferase system was prepared in 10 ml aliquots and kept in the cold room for 2-3 weeks. The constituents of the developer and their concentrations are listed below:

0.035 M Creatine Phosphate

0.030 M Magnesium Acetate

0.001 M ADP

0.005 M DTT

However Szasz, et. al.,[32] re-examined all kinetic factors relevant to an optimal and standardized enzyme assay at 30° and 25°C.

They found that the optimum pH range for creatine kinase activity falls between pH 6.5 and 7.0 with pH optimum at pH 6.7.[32] In a comparison among five different buffers (table 10), all at 100 mmol/liter concentration and pH 6.7, showed almost identical creatine kinase activity in imidazole and triethanolamine, whereas in MOPS, MES, and PIPES buffers slightly to significantly lower activity was measured.

TABLE 10

EFFECT OF TYPE OF BUFFER ON CREATINE KINASE ACTIVITY[32]

Imidazole Acetate	Triethanolamine Acetate	Sodium MOPS	Sodium MES	Sodium PIPES
166 u/l	165 u/l	163 u/l	159 u/l	150 u/l

Further studies showed the creatine kinase activity increases up to 30 mmol of creatine phosphate per liter, and at greater concentrations inhibition was observed. ADP concentrations of 2 mmol per liter gave maximum creatine kinase activity, but no inhibition of activity was seen up to 4 mmol per liter of ADP.[32] Another essential item for creatine kinase activity is magnesium ions. Without added magnesium salts only 10% of the maximum activity was observed (Figure 17). The highest activity was obtained with concentrations of 10 to 20 mmol/liter.[32] The final substance pertinent to their study was the reactivation of creatine kinase in serum by the addition of sulfhydryl reagents. Szasz, Gruber, Bernt et.al., pointed out that such a sulfhydryl compound must quickly reactivate the enzyme without interfering with the measurement, as well as possessing qualities of practicability, solubility, and stability. Of the 27 thiol compounds they tested, N-acetyl cysteine was most suitable at a concentration of 20 mmol per liter.[32]

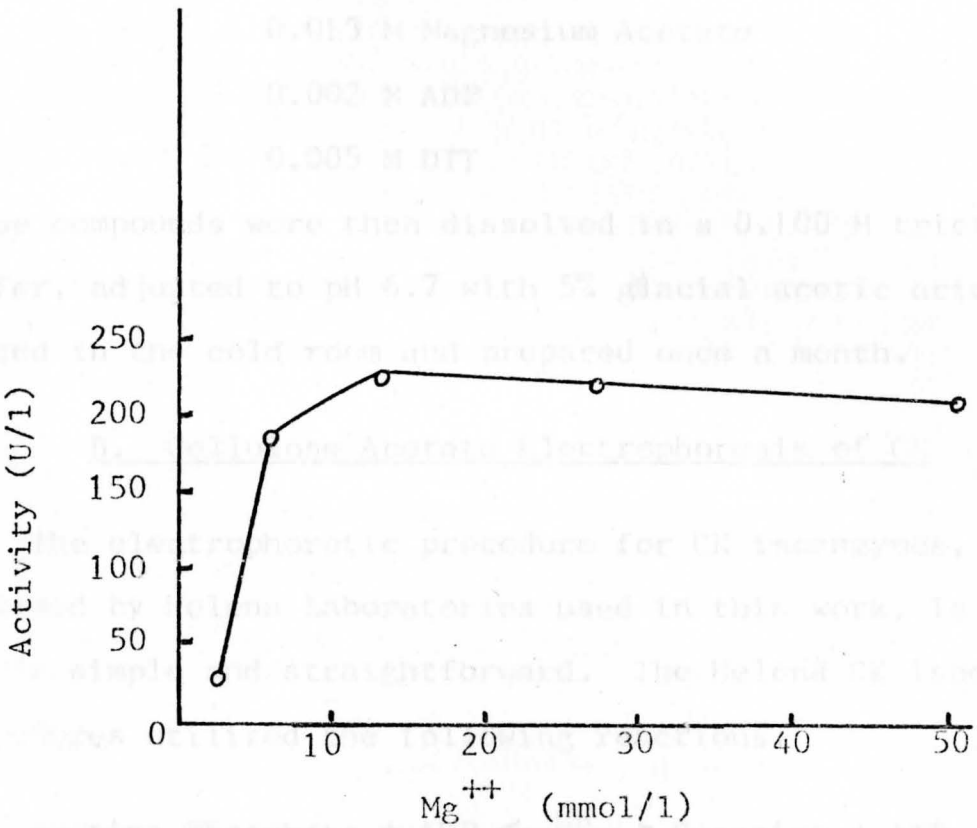


Figure 17. Effect of Magnesium ions on Creatine Kinase activity.[17]

Taking the suggestions as outlined by Szasz, et. al., a new developer was prepared for the luciferin-luciferase system. The constituents of the developer and the new concentrations are listed below:

0.025 M Creatine Phosphate

0.015 M Magnesium Acetate

0.002 M ADP

0.005 M DTT

These compounds were then dissolved in a 0.100 M triethanolamine buffer, adjusted to pH 6.7 with 5% glacial acetic acide, stored in the cold room and prepared once a month.

B. Cellulose Acetate Electrophoresis of CK

The electrophoretic procedure for CK isoenzymes, as outlined by Helena Laboratories used in this work, is rather simple and straightforward. The Helena CK isoenzyme procedures utilized the following reactions:

1. Creatine Phosphate + ADP $\xleftarrow{CK}$ Creatine + ATP
2. ATP + Glucose $\xleftarrow{HK}$ Glucose-6-Phosphate + ADP
3. Glucose-6-Phosphate + NAD $\xleftarrow{G\text{-}6\text{-}PD}$ NADH + Phosphogluconate

The amount of fluorescence of NADH, which is directly proportional to CK activity, is measured on a Helena Quick Scan Flur-Vis densitometer. The total time needed for the procedure is approximately 45 minutes.[8]

In the results section which follows, it can be assumed that all serum samples and controls were electrophoresed

exactly as shown below:

1. Pour 50 ml of buffer (HR diluted to 1800 ml) in each of the outer compartments of the electrophoresis chamber. Wet two disposable paper wicks in the buffer, then drape one over each support bridge making sure it makes buffer contact. Chamber is now ready for electrophoresis, but should be kept covered while not in use.

2. Wet the required number of Titan III-XW Zip Zone Plates (each plate holds 4 samples) by lowering a rack of strips into buffer (HR diluted to 1800 ml). Soak strips approximately 20 minutes prior to use. Prior to wetting a strip, it may be coded by marking on the glossy, hard (Mylar) side with a Sharpie Marker or grease pencil. It is suggested that the mark be placed in one corner so that it can later be used to help distinguish the multiple samples. At this time, for each strip to be used for electrophoresis, soak an additional Titan III-XW strip for the development phase.

3. Using the Microdispenser, fill every other well in the Sample Well Plate with 5 µl of sample. For economy and convenience, do not change the bore with each new sample, merely wash it out with water. Cover the Sample Well Plate with a glass slide if it is not used within 5 minutes. Note: A Helena CK Isoenzyme Control should be run on every plate. Another enzyme, Myokinase, which catalyses the reaction

$$2\ ADP \xleftrightarrow{Myokinase} AMP + ATP$$

is often present in human serum. Since myokinase utilizes ATP, as does the CK reaction, it will also produce fluorescence when present. One myokinase isoenzyme migrates with albumin just ahead of CK II (MB). Even though the myokinase band is completely separated from CK II, an inexperienced observer might confuse myokinase activity for a myocardial infarction. The Helena CK isoenzyme control shows you exactly where CPK migrates and prevents confusion with myokinase.[8]

4. Load the applicator by depressing the tips into the sample wells 3 or 4 times. The first loading should be wiped off with a paper towel. This primes the applicator and the second loading is much more uniform. Cleaning the Zip Zone Applicator prior to sample loading with Zip Zone Prep (dilute 5 drops of concentrate with 100 ml of water) insures a uniform sample loading and therefore uniform application. Once the applicator is loaded, it must be used within 15 seconds. Move quickly through the next two steps.

5. Remove the wetted XW plate from the buffer with finger tips and blot firmly once. Quickly place the strip in the Aligning Base cellulose acetate side up. One end of the strip should align to the top of the Aligning Base (Helena Laboratories printed on the bottom) to yield an application 1 inch off the cathodic end. The Sharpie pen mark should be on the left side.

6. Depress the applicator tips once more into the sample wells to insure maximum pick up. Now promptly transfer the applicator to the Aligning Base. Press the button quickly and hold the button down 5 seconds. Repeat 3 more times for a total of 4 applications.

7. Quickly place the strip, cellulose acetate side down, in the chamber, turn timer to 8 minutes and adjust voltage to 350 V. Power must be applied within 2 minutes after a strip has been placed in the chamber. Power is automatically cut off at the end of the time period.

C. Development Technique

The developing substrate to be used in the utlraviolet (U.V.) technique was not from Helena Laboratories, but from BMC Cat. #15790. The constituents of this developer are listed in table 11. The development phase of the cellulose acetate plates was carried out exactly as shown below:

The Steps for Ultraviolet Development Were:

1. The developing substrate should be prepared 10 minutes prior to use or immediately before the start of electrophoresis. Add 2 ml of 20% sucrose to a vial of BMC CK substrate and mix thoroughly. Note: Sucrose is not essential in the reactions and only acts to decrease diffusion.

2. Two minutes before the electrophoresis run is complete, firmly blot a wetted Titan III-XW plate and place cellulose acetate side up, on a blotter pad. Let plate air dry for 30-60 seconds. Pipette 1 ml of substrate onto surface of plate, and spread over the surface by tilting the blotter. Let substrate soak into pores of the membrane for 1 minute.

3. Immediately after electrophoresis, remove the electrophoresed strip from the chamber. Lightly blot and layer it, cellulose acetate side down, over the substrate strip. The best technique is to touch one edge of the strip to the solution first, then gradually lower the remaining portion of the strip onto the development plate in such a manner as to avoid excessive strip movement while the strip is in contact with the substrate solution.

4. Blot off the excess substrate solution. Press the plates and blotters between glass slides.

5. Place into an incubator at 37°C for 30 minutes. Note: Temperatures up to 45°C can be used and will increase activity. However, an increased amount of isoenzyme diffusion and background fluorescence may be encountered at higher temperature.

6. After the development is complete, blot and quickly dry the strip with a hair dryer until the complete strip turns white.

D. Detection and Interpretation of Results

In this study a U.V. light source was used to visualize CK activity. However, since a small amount of CK II (MB) activity is often found in normal serum, this method is not very reliable in detecting mild myocardial infarcts. Quantitation, therefore, was carried out in the Helena Quick Scan Flur-Vis densitometer to accurately measure the scanning patterns. By this method the CK isoenzyme activity was reported in terms of % of total or IU/l.

Interpretation of the results were obtained in one of two ways, visually or by fluorescent scanning. When the U.V. lamp was used to visualize the CK isoenzyme activity the following scan pattern in figure 18 by Helena was used as a representative sample.

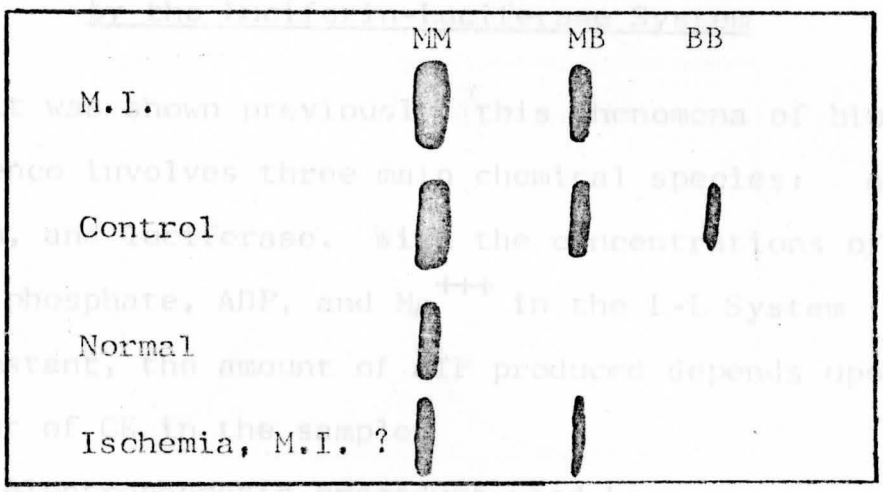


Figure 18. Representative CK isoenzymes pattern by Helena Laboratories.

When the results were obtained by fluorescent scanning, the normal range, set up by Gambino and Galen, were followed (MB fraction, 0-3%).[9] Three typical patterns obtained on the Quick Scan Flur-Vis densitometer are shown in figure 19.

Once each sample was scanned, the area under each peak was integrated and the results were reported out as a % of the total or in IU/Liter.

E. Electrophoretic Procedure as Outlined by the Luciferin-Luciferase System

As it was shown previously, this phenomena of bioluminescence involves three main chemical species: ATP, luciferin, and luciferase. With the concentrations of creatine phosphate, ADP, and Mg^{+++} in the L-L System being constant, the amount of ATP produced depends upon the amount of CK in the sample.

The electrophoretic procedure used here was exactly the same as previously outlined in the section dealing with the Helena System. Briefly, serum was applied four times to a buffered cellulose acetate Titan III-XW plate, electrophoresed for 8 minutes at 350 volts.

The Steps in the Luciferin-Luciferase Development Method were:[34]

1. As electrophoresis continues, 0.3 ml to 0.7 ml of distilled water is added to reconstitute the luciferin-luciferase.
2. The Titan III-XW plate is then blotted, after which the entire amount of reconstituted luciferin-luciferase is added to the plate which is to be developed.

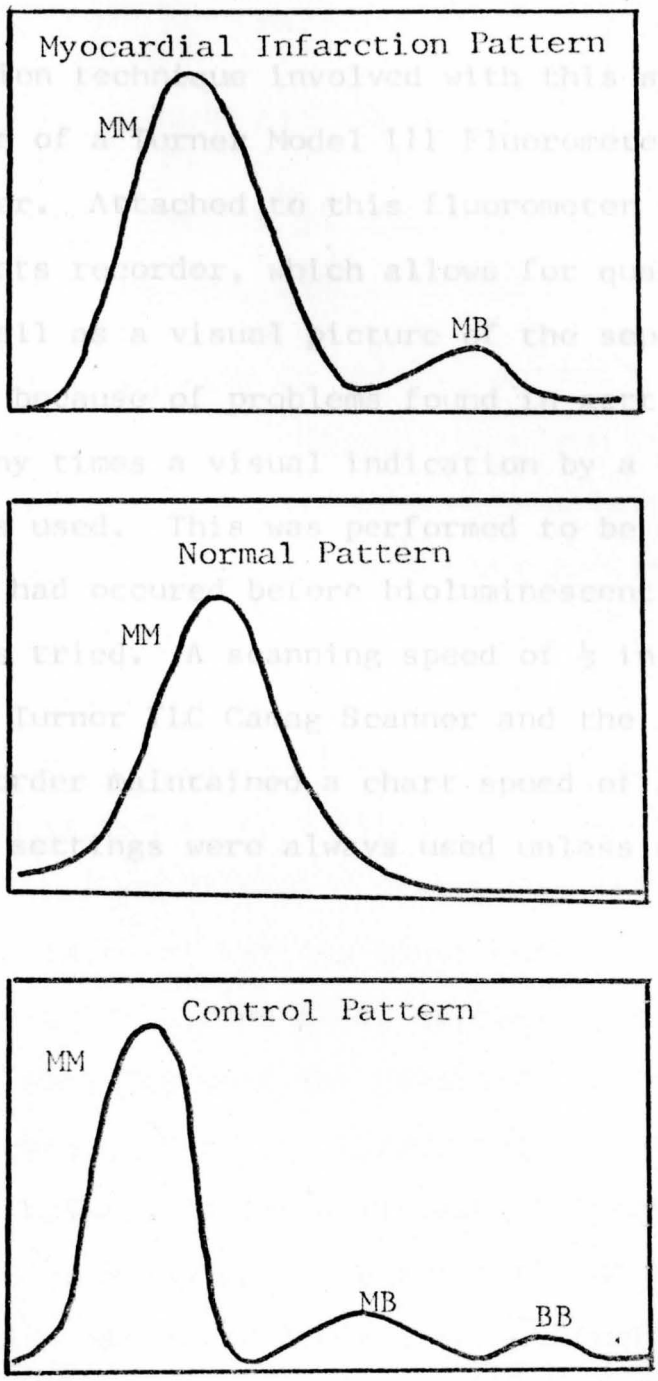


Figure 19. Quick Scan Flur-Vis densitometer patterns.

3. The Titan III plate is then placed on the Turner Camag Scanner and analyzed over periods up to 1½ hours.

The detection technique involved with this system employed the use of a Turner Model 111 Fluorometer with Camag TLC scanner. Attached to this fluorometer was a Linear Instruments recorder, which allows for quantitation of results as well as a visual picture of the separations obtained. Also because of problems found in getting good separations, many times a visual indication by a U.V. light source was used. This was performed to be sure that separation had occured before bioluminescent detection of the peaks was tried. A scanning speed of ½ in./min. was used on the Turner TLC Camag Scanner and the Linear Instruments Recorder maintained a chart speed of 2 in./min. These above two settings were always used unless otherwise noted.[34]

CHAPTER V

RESULTS AND DISCUSSION

A. Determination of the Total CK Value of the Helena Control

The initial step taken in this study was to determine the total CK activity of the Helena Isoenzyme Control Serum. The control serum was purchased from Helena Laboratories in Beaumont, Texas. According to the package insert, the control, as analyzed by the Clinicard System had a total CK activity of 4240 I.U. with a normal range of 4-205 I.U. This value was to be otained if the control vial was reconstituted with 0.5 ml of distilled or deionized water.

However, since no Clinicard System was available for comparing CK activity of various other control sera with the Helena control sera, two other methods were used. The two procedures employed were the UV-system CK from Boehringer Mannheim Corporation (Cat. No. 15790) and CK system from Antonik Laboratories, Elk Grove Village, Illinois.

The method of assaying CK activity by the BMC procedure utilized creatine phosphate and ADP as substrates instead of creatine and ATP. The method was initially devised by Oliver,[26] later modified by Rosalki,[27] and further modified for optimal substrate conditions by Bergmeyer.[41] It is on this modification that the BMC assay is based.[35]

This methodology has a faster reaction rate in the so called "reverse reaction" which makes the test more sensitive, thereby allowing the test to be performed with smaller sample volumes.

In this system, the formation of NADH is measured kinetically, where the rate of formation is directly proportional to the CK enzyme activity.

The components of the BMC substrate system are listed in table 11, and those of the BMC buffer system are listed below:

100 mM Triethanolamine buffer, pH 7.0
20.8 mM D-glucose
10.4 mM Magnesium acetate

To prepare the working solution, 2.5 ml of buffer were added to one vial of CK substrate and the vial was mixed by gentle inversion. This solution is stable for approximately 5 hours at ambient temperatures, and 24 hours at $4^{o}C$.[35] Then 0.1 ml of the Helena Control serum was added to the reconstituted vial and incubated for five minutes at $30^{o}C$. After incubation, the contents of the vial were poured into a 1.0 cm cuvet. The wavelength used was 340 nm and the temperature of the sample compartment of the double beam spectrophotometer was $30^{o}C$. The cuvet was then placed in the spectrophotometer and the change in absorbance per minute was recorded. Recordings of at least three absorbance changes were taken for the determination

TABLE 11

COMPONENTS OF THE BMC SUBSTRATE SYSTEM[35]

CONCENTRATION	COMPONENT
2.6 µmoles	ADP
25.9 µmoles	AMP
1.6 µmoles	NADP
91.0 µmoles	Creatine Phosphate
>3 U	Hexokinase
>3 U	G-6-PDH
>23 µmoles	Glutathione, reduced

of the mean Δ A/min used for the calculations. The instrument did not have an integrator pen connected with it, so all readings were taken visually and recorded manually.

However, when readings were taken on the Helena Control serum, the activity was too great to accurately measure by the manual method and only a range of 900 to 1200 I.U. was obtained. Another 0.1 ml sample of Helena control was taken and diluted with 0.9 ml of deionized water to give a 1:10 dilution. This dilution was then thoroughly mixed on a vortex mixer. Another vial of CK substrate was diluted with buffer and 0.1 ml of the 1:10 dilution of Helena control was added. The vial was incubated and analyzed as mentioned previously. This time six absorbance readings were taken at one minute intervals resulting in a mean value of 151.6 or 152 I.U. (See Table 12). The value of the Helena control undiluted, therefore, would be approximately 1520 I.U.

Another dilution of the Helena control was utilized to see if consistency in results could be obtained. This time a 1:20 dilution was prepared by adding 50 µl of control to 0.95 ml deionized water. The sample was added to a BMC CK vial reconstituted in the manner previously mentioned. All reaction times and temperatures were also followed as outlined above. Again six absorbance readings were taken at one minute intervals resulting in a mean value of 78.8 I.U. or 79 I.U. (See Table 13).

TABLE 12

BMC DETERMINATION OF HELENA CK CONTROL ON A 1:10 DILUTION

TIME (MINUTES)	CONCENTRATION (I.U.)
1	148
2	152
3	152
4	154
5	152
6	152

TABLE 13

BMC DETERMINATION OF HELENA CK CONTROL
ON A 1:20 DILUTION

TIME (MINUTES)	CONCENTRATION (I.U.)
1	80
2	79
3	78
4	79
5	78
6	79

So by mutliplying the mean value obtained by the dilution factor, a resulting activity of 1580 I.U. for the Helena Control was observed. The value from the 1:20 dilution and the 1:10 dilution were then coupled together, and a value of 1550 I.U. was used for the total activity of the undiluted Helena Control.

The other method used for determining the total CK activity of the Helena Control was from Antonik Laboratories. This method utilizes the firefly chemicals luciferin and luciferase. Antonik Laboratories list several advantages of their system which are listed below:[33,34]

1. Highly specific-The firefly chemicals produce light only with ATP. No selective hydrolysis as in the colorimetric methods.
2. Sensitivity-The firefly method is the most sensitive for ATP that is known.
3. Kinetic method-The kinetic method or the time rate method is preferred since different concentrations of the enzyme produce different curves. This method presents actual production (activity) not accumulation, of the end product.
4. Direct approach-Coupled reactions are desireable only when NONE of the reactants can be measured directly. The Antonik Procedure measures one of the reactants directly.
5. Dependable accuracy-Easy to use-Due to the simplicity of operation, the test is less open to error than found in more complex reactions.
   a. Reconstitute the tube with water.
   b. Add sample. Incubate.
   c. Place in photometer and read the light emission.
6. Economical-Technologists spend less work hours by far than previously existing procedures.
7. Ideal for stat-Because of the simplicity, the day and night crews use the same procedure.

The kit contained individual tubes of the freeze dried substrate which contained creatine phosphate, ADP, luciferin, luciferase, dithiothreitol and magnesium MOPS buffer.[33] For analysis, 3.5 ml of deionized water was added to the lyophilized substrate tubes, which included a tube for each unknown sample and one tube for a blank. Only one blank tube was needed for each batch. The tubes were then inverted 5 or 6 times until a solution was obtained. After the tubes reached room temperature, 0.1 ml of sample was added to tube number one, a stop watch was started, the tube was again inverted, and the other samples were added at 30 second intervals. To the blank was added 0.1 ml of 0.85% saline. After exactly 15 minutes incubation at room temperature, each sample was read in a timed sequence in a fluorometer which had been zeroed with the blank. A Turner Fluorometer Model 111 was used and a piece of cardboard (3" x 3") wrapped with black tape blocked off the U.V. light source. All dilutions of controls or serum samples more than 5X the normal range were diluted either with heat inactivated serum (56^{o}C for 30 minutes) or 0.85% saline. Table 14 illustrates typical results obtained when a 1:10 dilution of the Helena control was assayed by the Antonik CK kit. Results are expressed in Turner Light Units (TLU).

From Table 14 it can be observed that as the volume of the Helena CK control increases with increasing concentration, the CK activity is far too great to be measured

TABLE 14

ANALYSIS OF HELENA CK CONTROL (1:10) DILUTION
USING THE ANTONIK KIT

TUBE	SUBSTRATE VOLUME (ml)	ml OF CONTROL (1:10 DILUTION)	SCALE READINGS IN TLU				
			5min	10min	15min	20min	25min
1	3.5	0.1	10	26	57	94	100
2	3.4	0.2	23	96.5	100		
3	3.3	0.3	84	100			
4	3.2	0.4	100				

with any degree of accuracy. Thus the 3.5 ml substrate to 0.1 ml sample ratio was used throughout the study. Using this method and the 1:10 dilution of the Helena CK control a value of 120 I.U. was obtained from a standard curve. The undiluted control value, multiplied by the dilution factor, gave 1200 I.U.

B. Establishment of a Standard Curve

Once the total values of the Helena CK control were determined using the BMC method , a standard reference or response curve was needed.

The first item though, was to observe what type of activity would be seen with heat inactivated serum as the diluent instead of deionized water. For this a 1:20 dilution was prepared by adding 50 µl. of Helena CK control serum to 0.95 ml of heat inactivated serum. Varying volumes of the 1:20 dilution were used with varying volumes of substrate. Table 15 illustrates the Turner Light Units obtained at various timed intervals.

Again from this table it is shown that the 3.5 ml substrate to 0.1 ml control ratio is preferred. Also slightly increased TLU readings were observed using the heat inactivated sera as opposed to deionized water as the diluent. This may indicate that the deionized water may have a slight inhibitory effect on the CK analysis of Antonik.

TABLE 15

ANALYSIS OF HELENA CK CONTROL (1:20 DILUTION)
USING HEAT-INACTIVED SERUM

TUBE	SUBSTRATE VOLUME (ml)	ml OF CONTROL (1:20 DILUTION)	SCALE READINGS IN TLU				
			5min	10min	15min	20min	25min
1	3.5	0.1	12	24	37	57	86
2	3.4	0.2	7	27	68	100	
3	3.3	0.3	13	56	100		

The analysis that need be investigated before a standard curve could be constructed, was to check out at what time interval does linearity begin. This was accomplished by using a 1:30 dilution of the Helena control and varying the volumes of both the control and substrate. Table 16 illustrates the values obtained from such a procedure and figure 20 illustrates the curves obtained.

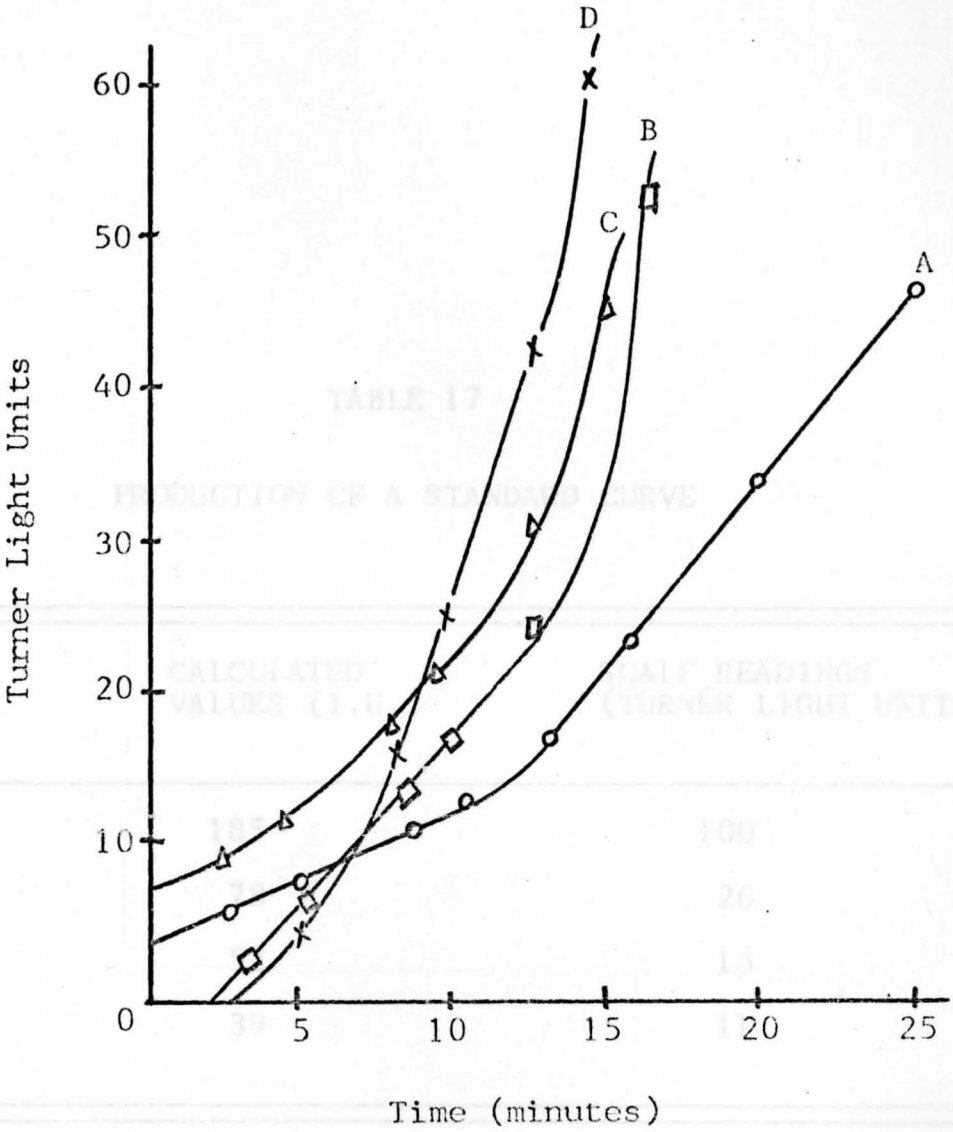
From this data, two conclusions can be drawn: (1) once again 3.5 ml substrate volume and 0.1 sample volume ratio are most consistent in regards to reaction rate, and (2) linearity using the Antonik kit begins approximately 12 minutes after the addition of the sample to the substrate and continues to 25 minutes.

Once all conditions had been satisfied, a standard curve was constructed using dilutions of 1:10, 1:20, 1:30, and 1:40. All dilutions were made with heat inactivated serum. The substrate to sample ratio was 3.5 ml to 0.1 ml, and the incubation time was exactly 15 minutes. All readings were taken at 30 second intervals. Results of standard curve are shown in table 17 and illustration of the curve is shown in figure 21.

TABLE 16

ANALYSIS OF HELENA CK CONTROL
USING A 1:30 DILUTION

TUBE	SUBSTRATE VOLUME (ml)	ml OF CONTROL (1:30) DILUTION	SCALE READINGS IN TLU				
			5min	10min	15min	20min	25min
1	3.5	0.1	6	12	22	34	50
2	3.4	0.2	8	25	56	100	
3	3.3	0.3	4	21	60	100	
4	3.2	0.4	5	30	91	100	



A= 0.1 ml of the 1:30 dilution
B= 0.2 ml of the 1:30 dilution
C= 0.3 ml of the 1:30 dilution
D= 0.4 ml of the 1:30 dilution

Figure 20. Time of reaction vs. TLU of activity.

TABLE 17

PRODUCTION OF A STANDARD CURVE

DILUTION	CALCULATED VALUES (I.U.)	SCALE READINGS (TURNER LIGHT UNITS)
1:10	185	100
1:20	78	26
1:30	52	15
1:40	39	11

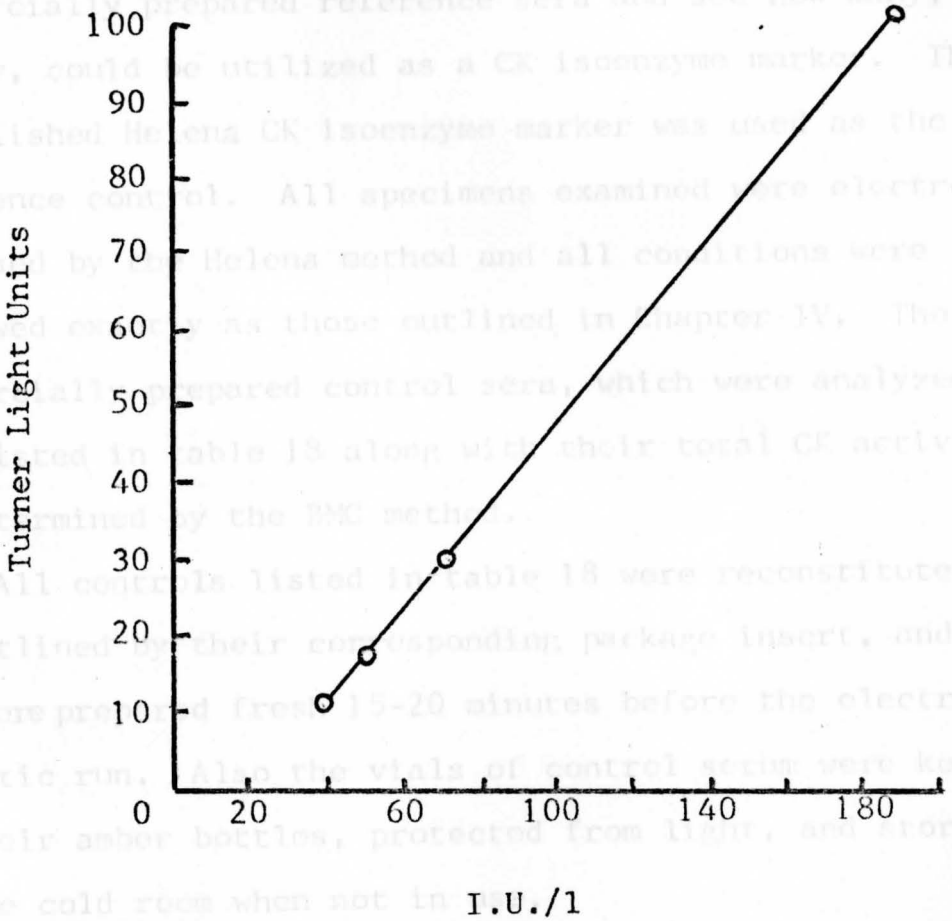


Figure 21. Standard curve using Antonik system.

C. The Role of Commercially Prepared Control Sera as CK isoenzyme Markers

It was next decided to experiment with various commercially prepared reference sera and see how many, if any, could be utilized as a CK isoenzyme marker. The established Helena CK isoenzyme marker was used as the reference control. All specimens examined were electrophoresed by the Helena method and all conditions were followed exactly as those outlined in Chapter IV. The commercially prepared control sera, which were analyzed are listed in table 18 along with their total CK activity as determined by the BMC method.

All controls listed in table 18 were reconstituted as outlined by their corresponding package insert, and all were prepared fresh 15-20 minutes before the electrophoretic run. Also the vials of control serum were kept in their amber bottles, protected from light, and stored in the cold room when not in use.

Each control sample was electrophoresed three times on different cellulose acetate strips, and each was developed with the CK substrate from BMC (Cat. No. 15790). The samples were observed under the U.V. light source for isoenzyme band migration, and compared to the migration of the Helena control.

The Helena control exhibited 3 bright and distinct isoenzyme bands, a heavy MM band, a weak BB band, and an intermediate MB band. All of the control specimens, however,

TABLE 18

COMMERCIALLY PREPARED CONTROL SERA AND TOTAL CK VALUE [a]

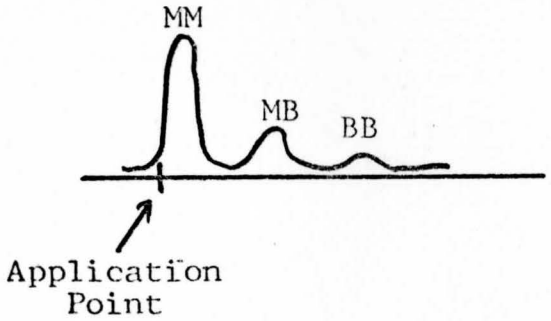
CONTROL	BMC (I.U./l)
Precipath E	86
Validate	15
Validate A	94
Kemtrol (Normal)	20
Kemtrol (Abnormal)	141
Enza-trol	54
Sigma CPK Isotrol	970
Phadebas Reference Serum	52

a-Control sera were received either unassayed or assayed by methods other than BMC.

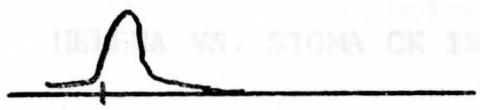
exhibited only a heavy to moderate MM band at or near the point of application. No other commerically prepared control serum electrophoresed by the Helena system was shown to contain a MB or BB band. In figure 22 are representative tracings of some of the control specimens electrophoresed with the Helena control, showing only a MM band at the point of application.

The only control specimen which gave results comparable to those obtained by the Helena control was a control purchased from the Sigma Company called, "Sigma CPK Isotrol." It was then decided to run a comparison study on both CK isoenzyme controls. Three cellulose acetate strips were used and one sample from each control (Helena and Sigma) was applied to each strip. After electrophoresis both controls were examined under the U.V. light source and both exhibited three clear isoenzyme bands. Quantitation was accomplished by scanning with the Helena Quick Scan Flur-Vis densitometer and computation was performed manually by integrating the area under each peak. Results of this computation are shown in table 19 and representative scans are shown in figure 23.

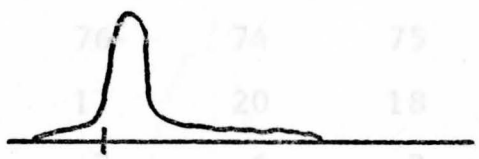
After examining the results of table 19 and figure 23, several conclusions can be made; (1) it was clearly demonstrated that both methods gave very consistent results as to the % value of each of their constituents, (2) both controls contain an adequate amount of MB and BB, (3) either control could be utilized as a CK marker with



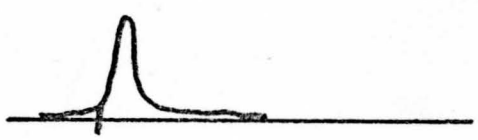
Helena Control



Precipath E



Validate-A



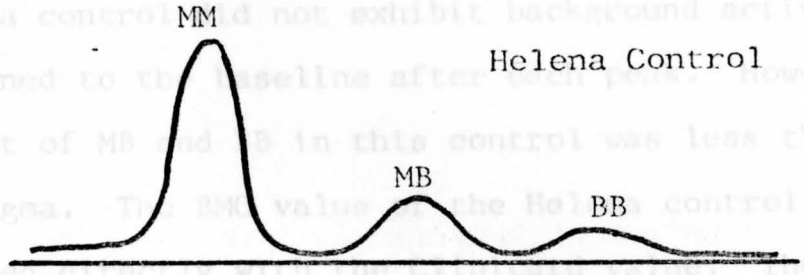
Kemtrol (abnormal)

Figure 22. Electrophoresis of commercially prepared control sera.

TABLE 19

HELENA VS. SIGMA CK ISOENZYME CONTROL

	HELENA			SIGMA		
	#1	#2	#3	#1	#2	#3
% MM	76	74	75	50	50	51
% MB	17	20	18	36	36	35
% BB	7	6	7	14	14	14



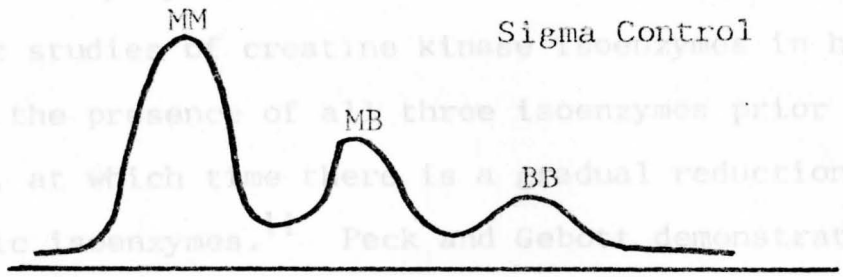


Figure 23. Electropherogram of Helena vs. Sigma CK Isoenzyme Control.

great satisfaction. The only differences that existed between the two controls were the higher % MB and BB exhibited by the Sigma control, and the slight amount of background seen in the tracings of the Sigma control. The Helena control did not exhibit background activity and returned to the baseline after each peak. However, the amount of MB and BB in this control was less than in the Sigma. The BMC value of the Helena control cannot be compared directly with the Clinicard value. The unassayed Sigma control was 970 I.U./l when assayed by the BMC method.

D. Cord Sera as CK Isoenzyme Markers

In a study by Peck and Gebott, it was stated that ontogenic studies of creatine kinase isoenzymes in humans revealed the presence of all three isoenzymes prior to maturity, at which time there is a gradual reduction of the anodic isoenzymes.[11] Peck and Gebott demonstrated that the adult tissue isoenzyme pattern, which is predominantly MM, was found in children of four years of age, and that normal children have all three isoenzymes at ten years of age.[11] Recognition of the transformation of CK isoenzymes in human tissues led Peck and Gebott to investigate both the total enzyme and isoenzyme activity in cord sera. Table 20 reflects both the total activities and relative isoenzyme percentages observed after evaluation of twenty cord sera. These results led them to believe that

TABLE 20

ISOENZYME DISTRIBUTION IN NORMAL HUMAN CORD SERA[11]

ISOENZYME	MEAN (%)	RANGE (%)
MM (CPK_3)	82	54 - 93
MB (CPK_2)	4.8	2.3 - 20
BB (CPK_1)	13	1.2 - 43
	MEAN (I.U./L.)	RANGE (I.U./L.)
TOTAL ACTIVITY	145	68 - 261

cord sera could be employed as control specimens for the analysis of CK isoenzymes.[11]

In various other studies by the Corning Medical Research Center, it was pointed out that cord sera exhibited only a MM and slight BB band on electrophoresis.[9] Also in agreement with this concept was Dr. Sylvan M. Sax, of West Penn Hospital in Pittsburgh, Pennsylvania. Dr. Sax, in his work also demonstrated only two electrophoretic bands with cord sera, namely, MM and BB. (private interview)

It was then decided to investigate the possibility of utilizing cord sera as a CK isoenzyme marker. Cord sera used in this study was obtained from three sources: Jameson Memorial Hospital, Youngstown Hospital Association North Side Unit, and St. Elizabeth Hospital. Most of the cord sera samples were obtained from each hospital's maternity ward as soon as possible after delivery. The cord sera was immediately centrifuged at 3000 rpm for 10 minutes, aliquoted in 1.0 ml quantities, and frozen. Analysis of the cord sera was performed as soon as possible, and no analysis was performed after one week in the frozen state. The method of analysis used was the Helena CK procedure and all specifics were followed as outlined in Chapter IV. Total CK activity was also performed on each cord specimen using the BMC methodology at 30°C. A total of ten specimens were examined. The results of which are shown in table 21, and a representative cellulose acetate plate, as viewed under a U.V. light source, is shown

TABLE 21

HELENA ISOENZYME DISTRIBUTION OF CORD SERA

SPECIMEN #	ISOENZYME FRACTIONS (%)		
	MM	MB	BB
1	100	0	0
2	97	0	3
3	98	0	2
4	100	0	0
5	100	0	0
6	97	0	3
7	99	0	1
8	100	0	0
9	100	0	0
10	100	0	0

in figure 24. All cord sera was electrophoresed with a Helena Isoenzyme Control to assure that migration had indeed occurred.

The total CK activity of all ten cord sera was found to be in the range of 44-60 I.U.

Also analyzed in this study were two cord blood specimens collected with the anticoagulant, ethylenediamine tetraacetic acid (EDTA). These specimens, however, only demonstrated a weak MM band at the point of application and the total CK activity corresponded to that obtained with the cord sera previously mentioned.

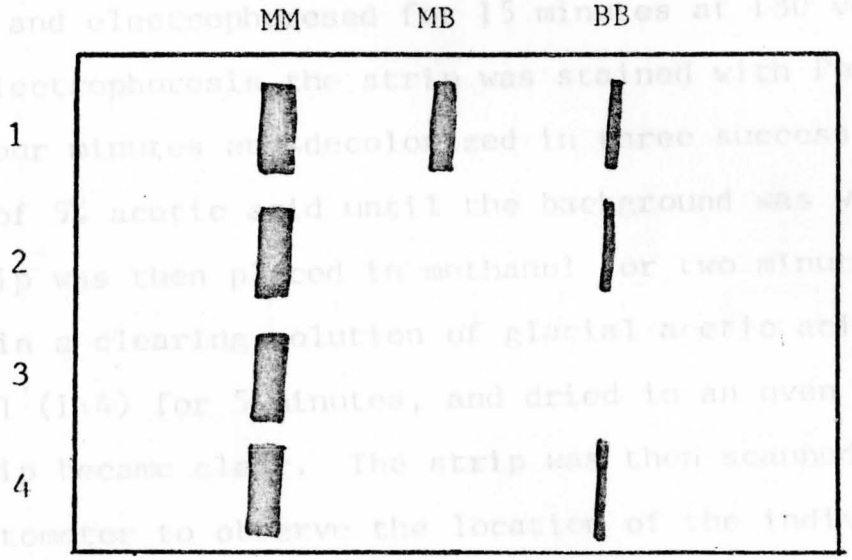
It was concluded then that cord sera contains only an MM fraction and possibly a BB fraction, but no MB activity was observed in any of the cord sera analyzed. This observation coincided with the work of Dr. Sax and the Corning Medical Research Center.

E. Serum Protein Migration In Correlation With CK Isoenzyme Migration

The next analysis undertaken was to investigate the migrational tendency of the CK isoenzyme with respect to serum protein migration.

To accomplish this, one strip was electrophoresed for CK isoenzyme activity using the Helena control serum, and one strip was electrophoresed for serum protein analysis using a Protein Electrophoresis Standard by the Gelman Company.

The Helena CK isoenzyme analysis was again performed to the exact procedure as outlined in Chapter IV. The



Position #1 Helena Isoenzyme Control
Position #2 Cord Sera, patient #3
Position #3 Cord Sera, patient #5
Position #4 Cord Sera, patient #7

Figure 24. Representative cellulose acetate plate of cord sera.

Helena control sera was used to assure that all three CK isoenzyme fractions would be present.

The serum protein analysis employed, was also a Helena procedure. The HR buffer used was diluted to 750 ml with deionized water. Only one application of the Gelman standard was applied to the cellulose acetate strip. The strip was then placed in the electrophoretic chamber and electrophoresed for 15 minutes at 180 volts. After electrophoresis the strip was stained with Ponceau S for four minutes and decolorized in three successive washes of 5% acetic acid until the background was white. The strip was then placed in methanol for two minutes, soaked in a clearing solution of glacial acetic acid: methanol (1:4) for 5 minutes, and dried in an oven until the strip became clear. The strip was then scanned in a densitometer to observe the location of the individual protein fractions.

The two strips were then aligned so that both points of serum application corresponded. From this it was learned that the MM isoenzyme fraction migrated in the gamma globulin region, the MB isoenzyme fraction migrated in the alpha-2 region, and the BB isoenzyme fraction migrated in the albumin or pre-albumin region.[42] (See Figure 25)

F. Alterations of the Original Luciferin-Luciferase Procedure

A significant portion of this study was devoted to the task of testing the Luciferin-Luciferase procedure,

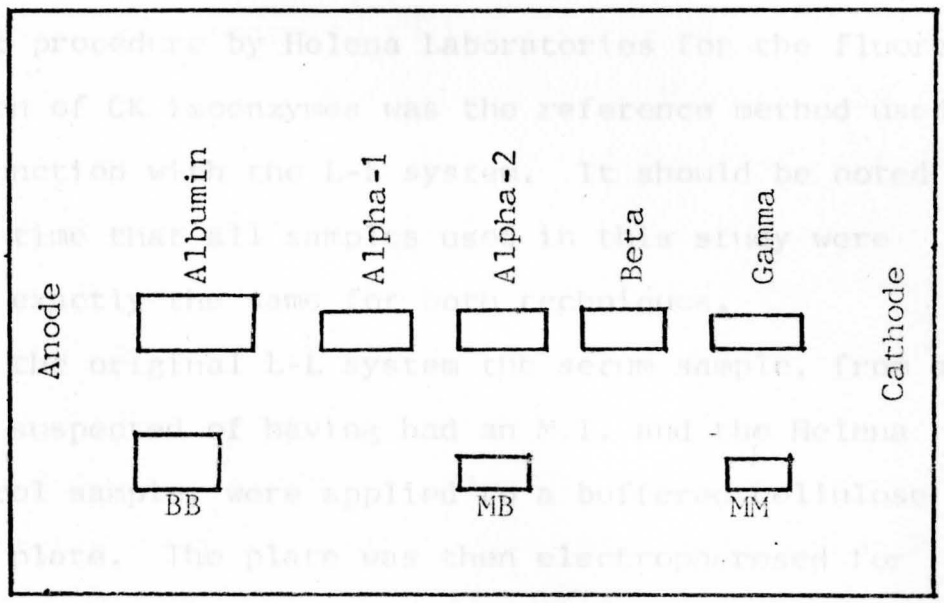


Figure 25. Correlation of electrophoretic migration of CK isoenzymes and serum proteins.

worked on by Randy D. Walter in his Master's thesis at Youngstown State University.[34] The procedure was designed to quantitate the electrophoresed creatine kinase isoenzymes by the reaction of ATP and luciferin in the presence of luciferase. The bioluminescence produced by the reaction was then detected by a fluorometer and scanned on a recorder for visual inspection. The established electrophoretic procedure by Helena Laboratories for the fluorescent detection of CK isoenzymes was the reference method used in conjunction with the L-L system. It should be noted at this time that all samples used in this study were treated exactly the same for both techniques.

In the original L-L system the serum sample, from a patient suspected of having had an M.I. and the Helena CK control sample, were applied to a buffered cellulose acetate plate. The plate was then electrophoresed for 9 minutes at 330 volts. After electrophoresis the strip was blotted and layered with 1.0 ml of a developing solution containing creatine phosphate, magnesium ion, ADP, and DTT. This strip was then sandwiched with another buffered cellulose acetate strip, pressed between two glass slides and incubated at 37°C for 30 minutes. Approximately 5 minutes before development was completed, a tube containing luciferin-luciferase, donated from Antonik Laboratories, was reconstituted with 1.0 ml of deionized water and inverted several times to insure proper reconstitution. After development, the strip was removed from the oven and

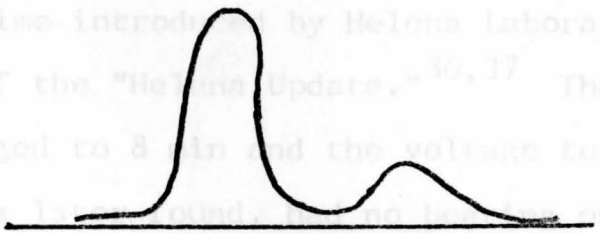
0.5 ml of the reconstituted L-L buffer was applied to the entire strip. The strip was then immediately placed on the Turner TLC Camag Scanner, which in turn was placed on a Turner Fluorometer Model 111 and the wet plate was scanned for luminescence. The scan lever on the scanner was set at position number 1 (1.2 cm/min at 60 Hz). To record the luminescence a recorder from Linear Instrument Corporation was connected to the fluorometer. The chart speed setting on the recorder was maintained at 2 in./min. These two positions on the scanner and recorder were always employed unless otherwise noted.

Also analyzed along with the L-L system was a plate containing the same samples but analysis was performed with the Helena methodology as outlined in Chapter IV. The results of this determination are shown in figure 26.

Observing the two scans, good resolution and detection was shown with the Helena procedure, however, extremely poor resolution and detection was encountered with the L-L system due to an excessive amount of diffusion.

The first step taken to try and correct this diffusion involved adding 1.0 ml of 20% sucrose to reconstitute the luciferin-luciferase substrate. It was thought that by the addition of the 20% sucrose, which is used to curtail diffusion in the Helena procedure, the diffusion problem might be solved. Another electrophoretic analysis, using both methods were examined. The unknown serum sample used in this batch was from a 52 year old male diagnosed as

A. Helena Fluorescent System



B. L-L System

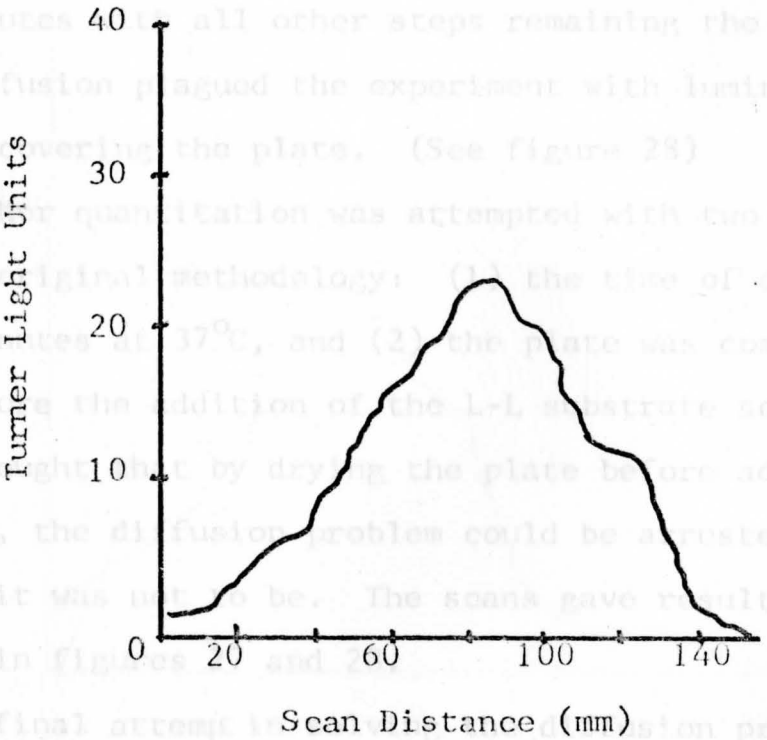


Figure 26. Original scan of L-L system vs. Helena Fluorescent System on a patient diagnosed as having an acute M.I. A is a fluorescence scan by Helena; B is a bioluminescence scan of the L-L system.

having an acute myocardial infarction. The results of the experiment are shown in figure 27.

A slight modification of the electrophoretic procedure was at this time introduced by Helena Laboratories in the publication of the "Helena Update."[36,37] The electrophoresing time was changed to 8 min and the voltage to 350. This change, it was later found, had no bearing on any results with either method. It was felt, though, that perhaps the developing time ATP build up was too long causing excessive diffusion at this step. The time for development was cut for the luciferin-luciferase method from 30 minutes to 15 minutes with all other steps remaining the same. Again diffusion plagued the experiment with luminescent activity covering the plate. (See figure 28)

Another quantitation was attempted with two changes from the original methodology: (1) the time of development was 15 minutes at 37°C, and (2) the plate was completely dried before the addition of the L-L substrate solution. It was thought that by drying the plate before adding the substrate, the diffusion problem could be arrested. Again, however, it was not to be. The scans gave results similiar to those in figures 27 and 28.

The final attempt in solving the diffusion problem was tried after incorporating the ideas of Szasz, Gruber, and Bernt.[32] The pH of the developing solution was lowered from 7.0 to 6.7, and the other constituents of creatine phosphate, ADP, magnesium ion and DTT were adjusted as

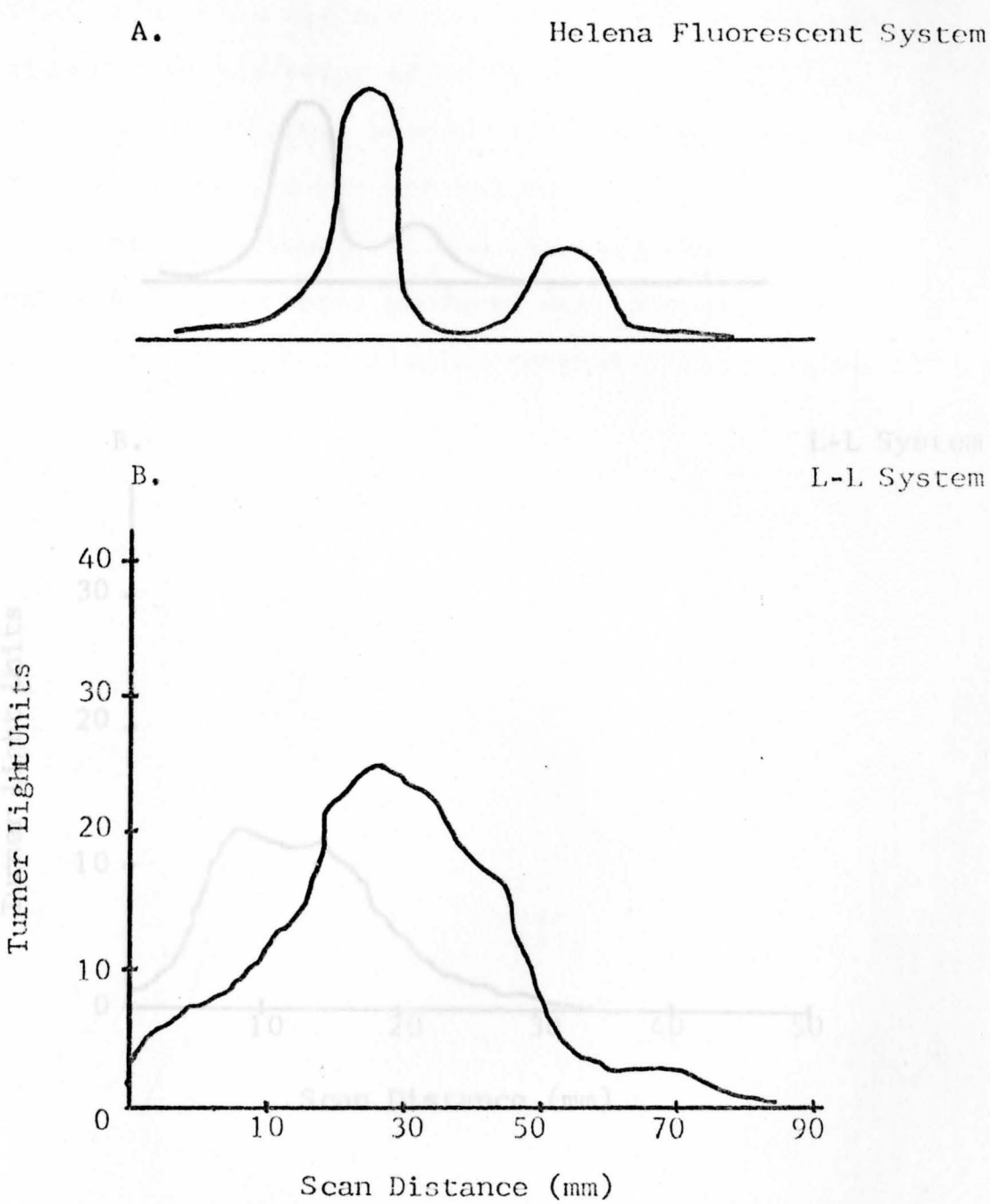


Figure 27. Scans of CK analysis on a 52 year old male with an acute M.I. Total CK activity was 189 I.U. A is a fluorescence scan, B is a bioluminescence scan.

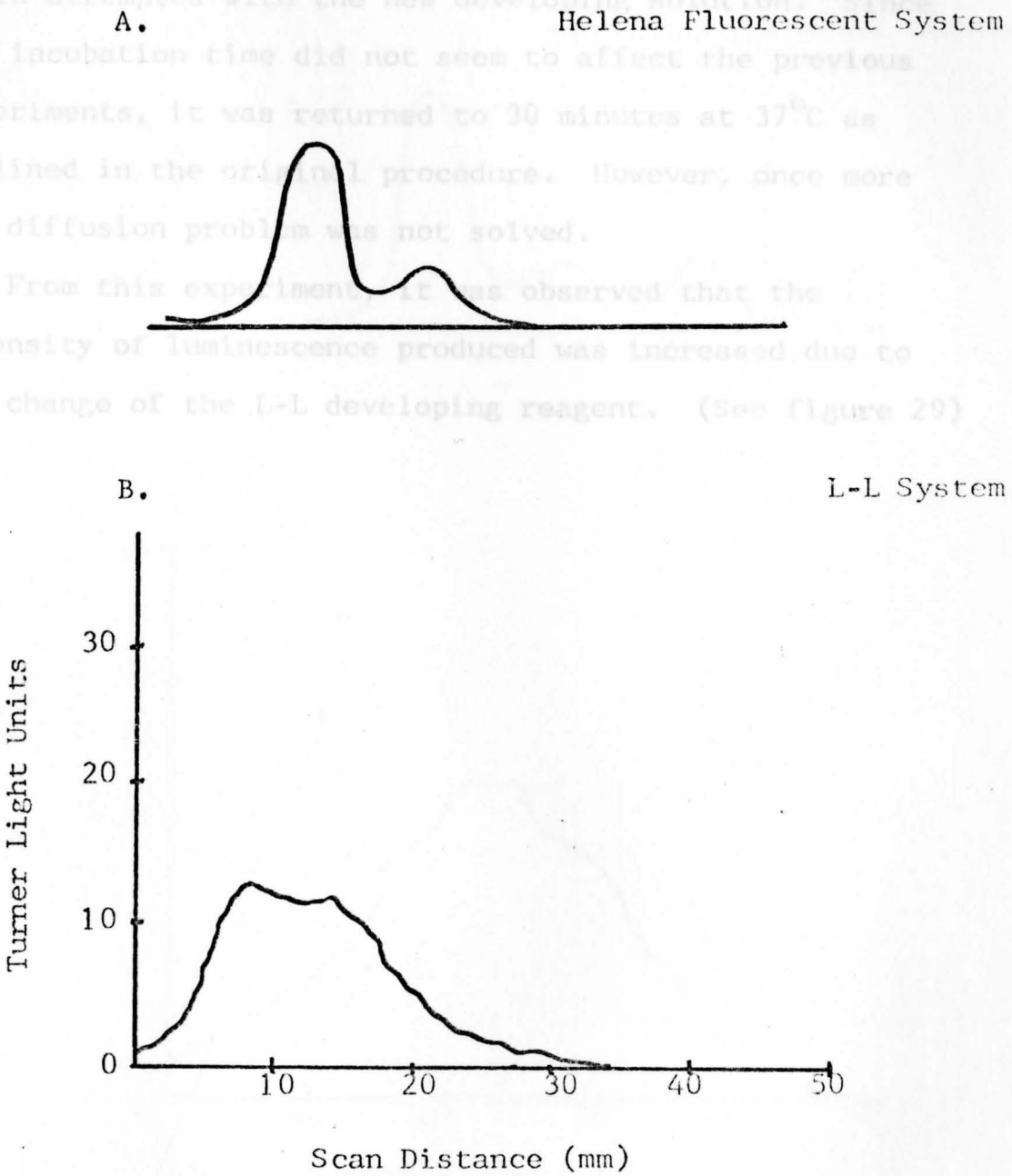
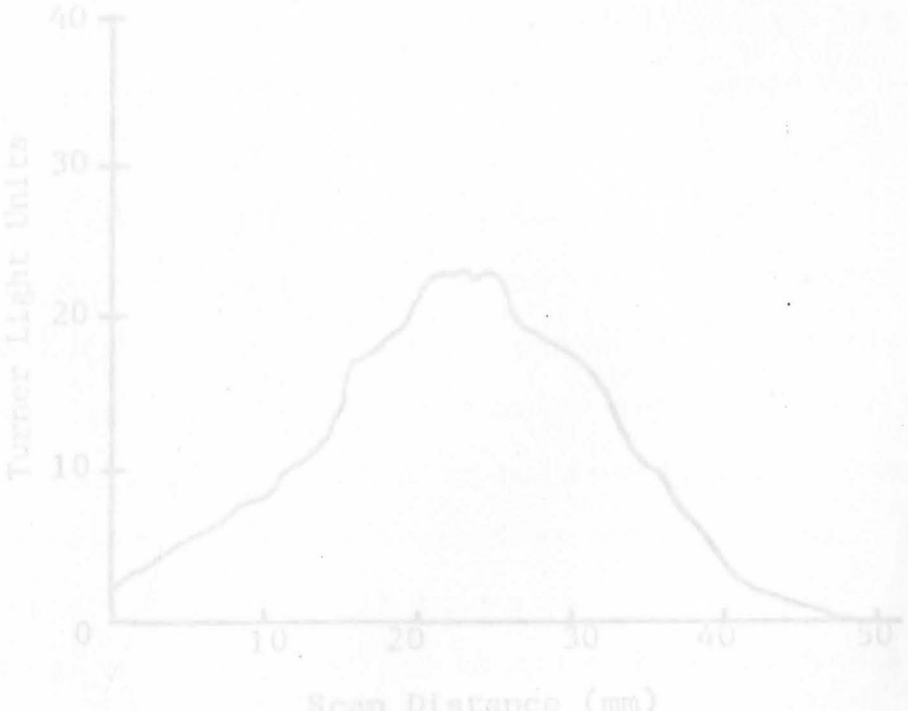


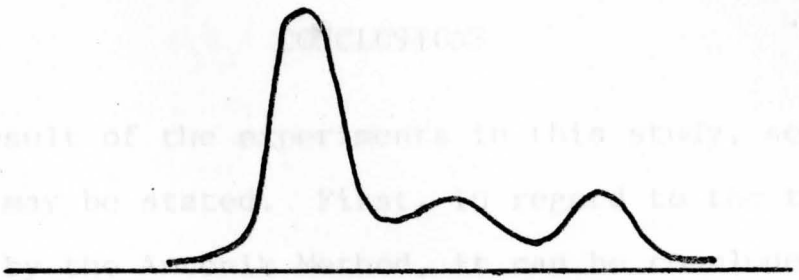
Figure 28. Scans of CK analysis with 15 minutes incubation of the L-L developer. A is Helena Fluorescent scan; B is a L-L scan.

outlined in Chapter IV. An electrophoretic analysis was again attempted with the new developing solution. Since the incubation time did not seem to affect the previous experiments, it was returned to 30 minutes at 37°C as outlined in the original procedure. However, once more the diffusion problem was not solved.

From this experiment, it was observed that the intensity of luminescence produced was increased due to the change of the L-L developing reagent. (See figure 29)



A. Helena Fluorescent System



B. L-L System

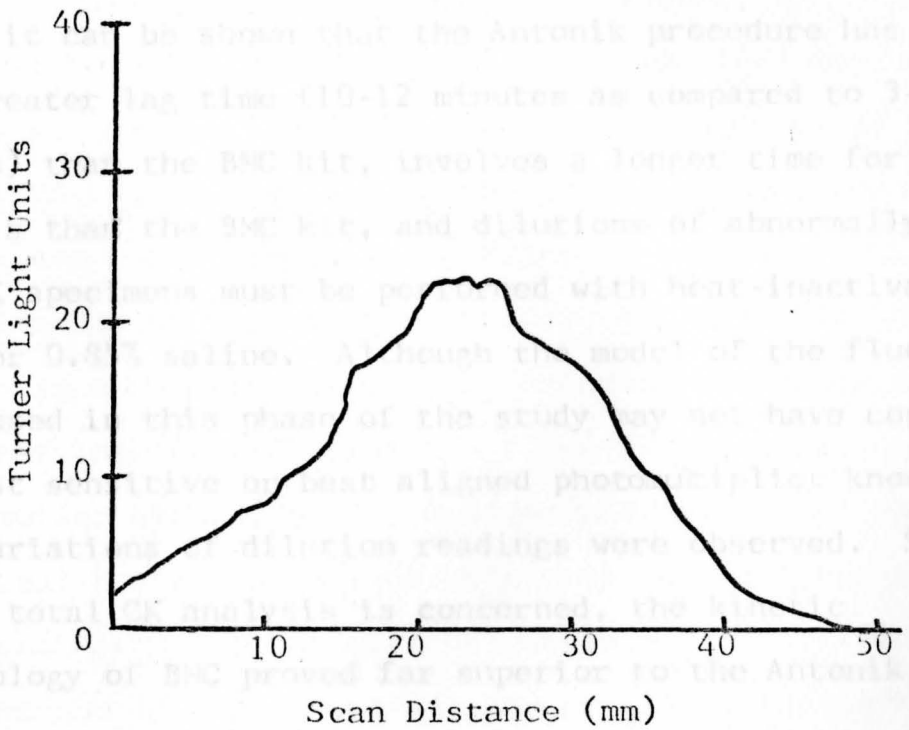


Figure 29. Scans of CK analysis with new L-L developer. A is Helena Control scan; B is L-L scan.

CHAPTER VI

CONCLUSIONS

As a result of the experiments in this study, several conclusions may be stated. First, in regard to the total CK analysis by the Antonik Method, it can be concluded that the test does possess the qualities of having a direct approach, easy to use, economical, and ideal for stat use. However when compared to the BMC kinetic methodology, it can be shown that the Antonik procedure has a much greater lag time (10-12 minutes as compared to 3-5 minutes) than the BMC kit, involves a longer time for analysis than the BMC kit, and dilutions of abnormally high CK specimens must be performed with heat-inactivated serum or 0.85% saline. Although the model of the fluorometer used in this phase of the study may not have contained the most sensitive or best aligned photomultiplier known, many variations of dilution readings were observed. So far as total CK analysis is concerned, the kinetic methodology of BMC proved far superior to the Antonik procedure.

The second area in which some conclusions can be drawn involves the preparation of a standard curve. First of all, it should be mentioned that the standard curve was to be drawn using the Antonik procedure, for no curve was

needed for the BMC procedure since the measurement of activity was expressed in the change of absorbance per minute. It was at this time that experiments were made to see if the time, dilutions and conditions as expressed by the Antonik Method were indeed optimum for this particular study. It was concluded that the mixing of 3.5 ml reconstituted luciferin-luciferase to 0.1 ml of unknown or control provided the most linear results once the lag time was achieved. Also the incubation time of 15 minutes proved adequate, because specimens of low concentration had not achieved linearity before 15 minutes, and after 15 minutes abnormally high serum concentrations would be off scale. It was for this reason that dilutions of 1:10, 1:20, 1:30, and 1:40 of the Helena control were used in the exact procedural outline of Antonik for the establishment of the standard curve. Any dilution made for use in the production of the standard curve was prepared from heat inactivated serum (56^{o}C for 30 minutes).

Another area of study involved the testing of various commercial control sera routinely used in the laboratory. This examination of the various controls was initiated to determine if they possessed any or all CK isoenzyme fractions to be used as electrophoretic markers. The conclusions drawn from this phase of the study clearly demonstrated that no routine commercial control possessed the ability to be used as a CK marker. This was probably due to the fact that (1) many controls, although they are

of primate tissue origin, contain mainly skeletal muscle, (2) many controls are composed of rabbit muscle, or (3) that the labile fractions of MB and BB are destroyed in the lyophilization process. It was for one or all of these reasons that only an MM isoenzyme fraction was observed upon electrophoresis.

Experiments involving electrophoresis were also utilized in the analysis of cord blood. It was suggested in the literature[11] that cord sera contains all three isoenzyme fractions, to a varying degree, and can be utilized as a CK marker. Analysis of ten cord sera samples electrophoresed by the Helena procedure showed only a MM band and in a few instances a BB band.

Electrophoretic studies were also performed on cord plasma with EDTA as the anticoagulant, but only a MM band was observed. From this it was concluded that cord sera could not be employed as an adequate marker for CK isoenzyme analysis.

Another experiment was performed to study the relationship of CK isoenzyme migration to that of total protein migration by the use of the Helena electrophoretic technique. From this analysis it was demonstrated that the MM fraction of CK isoenzyme migrated in the gamma globulin region, the MB fraction migrated in the alpha-2 region, and the BB fraction migrated in the albumin or pre-albumin portion.

The final area in which conclusions may be drawn deals with the comparative study of the Helena methodology

with the luciferin-luciferase technique. Much difficulty was observed in this analysis. The Helena procedure performed beautifully throughout the entire course of this study. Trouble, however, was encountered when the luciferin-luciferase detection method was attempted. The main core of the problem stemmed from diffusion of the protein molecules. Several techniques were attempted to try to solve the diffusion problem, but all attempts proved unsuccessful. These attempts were: (1) reconstitution of the luciferin-luciferase substrate with a 20% sucrose solution, (2) changing the pH and constituents of the developing solution to what is believed to be optimum conditions, (3) drying the cellulose acetate plate incorporated with ATP before the addition of the luciferin-luciferase substrate, and (4) changing the incubation time of the developing system from 30 minutes to 15 minutes.

It is for this reason that the conclusion can be made that the luciferin-luciferase detection system is not suitable for clinical use at this time due to the massive diffusion problem. Perhaps studies can be initiated with agar gel electrophoresis using the L-L system, and diffusion may possibility be curtailed.

It is therefore recommended that if clinical determination of CK Isoenzyme analysis is needed, an established procedure, as the one from Helena Laboratories, should be employed.

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