# THE ELECTROCHEMICAL DETECTION OF LACTATE DEHYDROGENASE

by

Caroline R. DeVincent

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ABSTRACT

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Lactate dehydrogenase is an enzyme of great clinical importance, especially in the diagnosis of myocardial infarction. This study attempted to devise an electrochemical method of detection which would be more efficient and precise than present methods of analysis.

The activity of lactate dehydrogenase was determined by measuring the oxidation of NADH to $NAD^+$ electrochemically. In the absence of enzyme, NADH was oxidized at the working electrode, generating a steady current. When lactate dehydrogenase was introduced, oxidation of some NADH occurred before the electrode was reached, so that at the electrode the concentration of NADH was diminished. This resulted in a drop in current which was proportional to the lactate dehydrogenase activity.

Cyclic voltammograms were run to determine the potential at which to oxidize NADH. This potential was then optimized using the flowing system and electrochemical cell used in the course of this study.

Three flow-through cells were constructed: The compositions of the electrodes were as follows: gold-silver-Ag/AgCl, carbon paste-silver epoxy-Ag/AgCl, carbon paste-stainless steel-Ag/AgCl. Of the three, only the latter showed satisfactory performance.

Although this cell yielded good results at first, the working electrode was soon contaminated by adsorbed species. Attempts were made to remove or reduce the amount of these species, but were unsuccessful. Only by disassembling the cell and applying fresh carbon paste to the electrode surface could the effect of the contamination be avoided.

## ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. Daryl Mincey for his assistance and guidance throughout this project. His suggestions were gratefully welcomed.

I also wish to thank my friends and fellow students who served as sources of inspiration.

Finally, I dedicate this thesis to my parents, without whom I could not have accomplished this work.

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# LIST OF SYMBOLS

| SYMBOL | DEFINITION |
|---|---|
| Arg | arginine |
| cm | centimeter |
| Glu | glutamate |
| His | histidine |
| HPLC | High Performance Liquid Chromatography |
| LD | lactate dehydrogenase |
| L→P, P→L | L-lactate to pyruvate, pyruvate to L-lactate conversions |
| mL | milliliter |
| mmol/L | millimole per liter |
| $NAD^+$ | oxidized form, nicotinamide adenine dinucleotide |
| NADH | reduced form, nicotinamide adenine dinucleotide |
| nm | nanometer |
| TRIS | tris(hydroxymethyl)aminomethane |
| U/L | International Unit per liter |
| v | volts |
| μA | microamp |

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# CHAPTER I

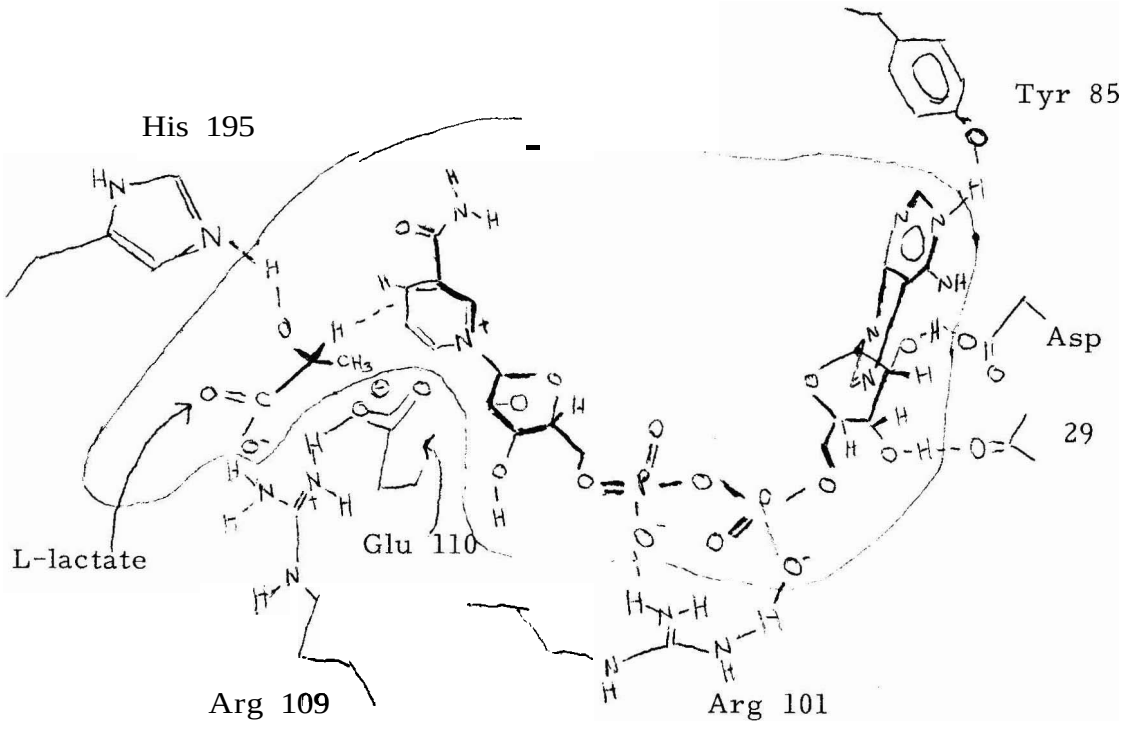
# INTRODUCTION

## The Nature of Lactate Dehydrogenase

Lactate dehydrogenase is an enzyme which is commonly found in the cellular cytoplasm. It is especially predominant in the liver, heart, kidney and skeletal muscle, although it is present in most tissues. In addition, it may also be found in urine and cerebrospinal fluid, but is not present to any great extent in serum.

Lactate dehydrogenase is known to be a tetramer composed of two different kinds of polypeptide chains, H(heart) and M(muscle). The molecule contains no metals or S-S bridges, although cysteine is important for the enzyme's activity. The amino acid sequence has been determined for some of the five major isoenzymes, and is known to consist of 300-350 amino acid residues per chain. Crystallization and X-ray diffraction have been used to determine the three-dimensional structure of lactate dehydrogenase, which has extensive $\alpha$-helical and $\beta$-sheet regions. The coenzyme $NAD^+$ is in open conformation, and binds at several sites.[1]

Figure 1[2] shows the active site of lactate dehydrogenase, at which the binding of $NAD^+$ and L-lactate takes place. As can be seen, the coenzyme is in a distorted 'C' formation, while the L-lactate is positioned so as to be able to transfer a hydride ion to $NAD^+$. Arg 109 of the enzyme neutralizes the carboxylate group of lactate, while His 195 serves as a general base catalyst. Finally, Glu 140 neutralizes the positively charged $NAD^+$.




L-Lactate and NAD+ Binding at the Active Site of LD

Figure 1

The five common isoenzymes of lactate dehydrogenase may be described as follows:

| | | | | | |
|---|---|---|---|---|---|
| LDI | $H_4$ | LD3 | $H_2M_2$ | LD5 | $M_4$ |
| LD2 | $H_3M$ | LD4 | $HM_3$ | | |

LD1 is comprised of four H-chains and migrates fastest to the anode in electeophoresis, while LD5 is the slowest. The M and H chains are different both physically and chemically, and are synthesized by different by different genes. They each, however, have a molecular weight of approximately 35,000 and have four sulfhydryl groups.

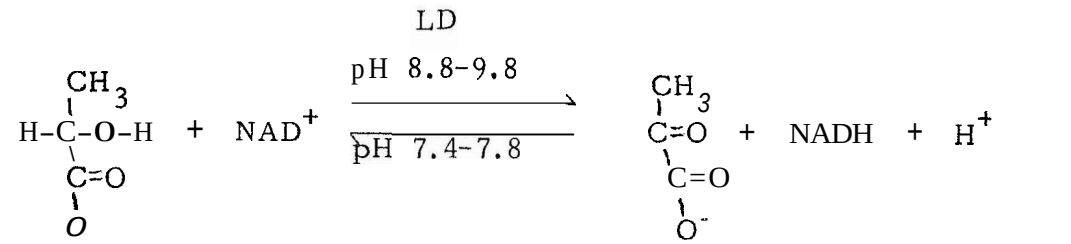
In addition to these two chains and five common isoenzymes, other chains are found, including X, C, E, F, M', H'. The latter four are not present in mammals, while LD-X is found only in the testis and spermatozoa.[3]

## Reactions of Lactate Dehydrogenase

The primary function of lactate dehydrogenase is to catalyze the reaction shown in Figure 2,[4] as discovered by Warburg and von Euler. That is, L-lactate is ixidized to pyruvate while $NAD^+$ is reduced to NADH.

To account for this activity, a flip-flop mechanism has been proposed.[5] That is, as lactate is converted to pyruvate and $NAD^+$ to NADH, the conformation of the enzyme changes. This change is reversible, however, since NADH will dissociate as the enzyme restores itself to its original form. Thus, the conformational changes which occur are not abrupt or well-defined, but are instead of a continuous, gradual nature.

In addition to this primary function, lactate dehydrogenase aids




L-lactate | Oxidized coenzyme I | Pyruvate | Reduced coenzyme I

The LD Reaction

Figure 2

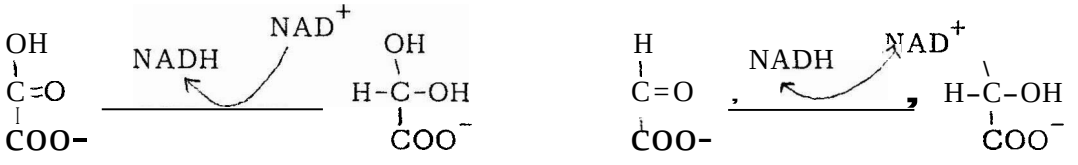
in the reduction of -keto acids to hydroxyacids with NADH. Further, it oxidizes glyoxalate to oxalate and reduces glyoxalate to glycolate, as shown in Figure 3.[6] Finally, lactate dehydrogenase can catalyze the addition of -CN to the nicotinamide ring to produce NAD-CN.

Lactate dehydrogenase is inhibited by pyruvate, with less pyruvate needed at a low pH. This inhibition is caused by the formation of a LD:NAD-pyruvate complex. In addition, lactate dehydrogenase may also be inhibited by a high concentration of lactate, with $H_4$ being the most sensitive.

## Present Methods of Analysis

One of the most common methods of measuring activity is by NADH consumption. In this procedure, as pyruvate converts to lactate, NADH is correspondingly oxidized to $NAD^+$, and the decrease in absorbance at 340 nm is measured. This is generally done at 30°C, pH 7.4. Normal values for this procedure are 95-200 U/L or 7-30 U/L for cerebrospinal fluid. The lactate to pyruvate conversion may also be utilized, at a pH of 9.0-9.5. The L→P and P→L conversions were the first continuous monitoring enzyme assays to be performed.[7]

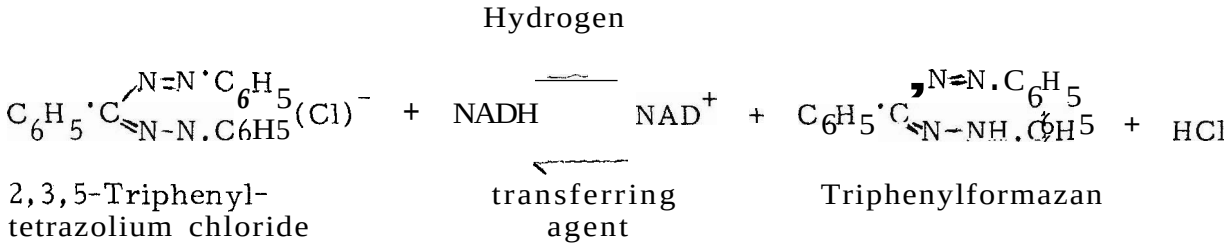
In other methods, colorimetry is used to measure the amount of formazin formed according to the reaction shown in Figure 4.[8] That is, the amount of dye produced is directly proportional to the concentration of NADH, which in turn is directly proportional to the amount of lactate dehydrogenase present. A third method involves the formation of a golden-brown complex of phenylhydrazone which is measured at 440 or 525 nm, as shown in Figure 5.[9]




oxalate glyoxalate glycolate

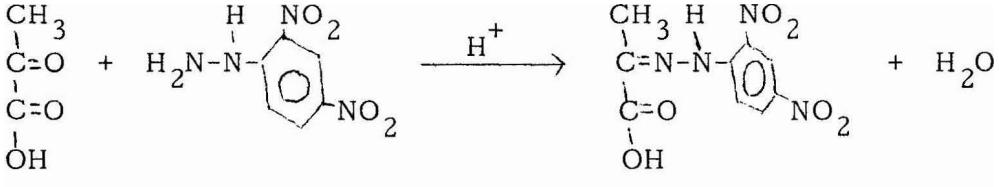
The Glyoxalate Reactions

Figure 3




The Formation of a Formazin Dye

Figure 4



Pyruvic acid | 2,4-Dinitrophenyl-hydrazine | Pyruvate 2,4-dinitrophenyl-hydrazone

OH–

Golden-brown colored form

Formation of a Phenyl hydrazone

Figure 5

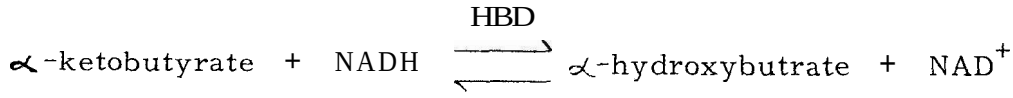
To measure the activities of specific isoenzymes, several methods may be used, the most common being electrophoresis. In this technique, the different amino acid composition of each isoenzyme allows it to be separated by charge. The separated isoenzymes are then incubated with lactate and $NAD^+$ and the bands made visible with UV spectroscopy, fluorometry or colorimetry.

Other methods of determining isoenzyme concentrations include column chromatography, heat stability (LD1 is least affected by heat), urea inhibition and HBD. The latter involves the fact that lactate dehydrogenase will catalyze the reaction shown in Figure 6,[10] and can thus be referred to as hydroxybutyrate dehydrogenase, or HBD. Since the isoenzymes of LD exhibit differing activities using $\alpha$-ketobutyrate as a substrate, the decrease in NADH absorbance at 340 nm can be used to quantitate this activity, and thus allow the concentration of isoenzyme to be assessed.

## Clinical Significance

Although lactate dehydrogenase is present in most tissue, its concentration in serum is insignificant in a healthy individual. However, when tissue is damaged, the injured cells are known to 'leak' enzyme into the serum, thereby increasing the concentration. Thus, an increase in serum lactate dehydrogenase is of great importance in the diagnosis of many disease states.

Lactate dehydrogenase levels are elevated 3–6 times the normal value in myocardial infarction. That is, as cardiac cells are destroyed due to insufficient blood supply, their enzymes will diffuse into the plasma. Detection of this increase is often a more definitive diagnosis than an electrocardiogram.




The HBD Reaction

Figure 6

As can be seen in Table 1 and Figure 7,[11] within 48-72 hours, lactate dehydrogenase will rise to its maximum level and remain elevated for as long as twelve days. Confirmation of this increase, along with that of the other enzymes listed, can then be used to verify the suspected myocardial infarction.

In addition to myocardial infarction, elevated serum levels may be seen in liver disease, renal disease, pernicious and megaloblastic anemia and muscular dystrophy, among others. Table 2[12] gives a more complete listing of diseases in which lactate dehydrogenase is known to rise, and indicates which isoenzymes are specifically involved. Thus, because each tissue has a specific lactate dehydrogenase pattern, the injured tissue may be identified by this pattern, which is a consequence of both the synthesis and degradation of a specific isoenzyme.

There are two ways in which LD levels may be utilized. First, total change in LD level can confirm injury to a specific organ or tissue. For instance, if an organ is known to be dampged, the severity may be assesed by noting the change in LD serum level. However, if the specific tissue damaged is not known, it may be more beneficial to study the isoenzyme pattern rather thab the change in total LD. Thus, for instance, a rise in LD1 and LD2 would indicate heart disease, while a rise in **LD5** would indicate disease of the lever.[13]

## Declaration of the Problem

It is apparent that the detection of LD is an integral part of many diagnostic procedures. Thus, it would seem essential to utilize the fastest, most reliable method available for this detection. Presently, measurement of NADH absorbance at 340 nm and electrophoresis for isoenzyme

TABLE 1

Enzymes in the Diagnosis of Myocardial Infarction

| Name | Usual Rise above Upper Limit of Normal | Time for Maximum Rise | Time for Return to Normal |
|---|---|---|---|
| Creatine kinase | 5-8 x | 24 hrs. | 2-3 days |
| Isoenzyme #2 | 5-15 | 24 | 2-3 |
| Aspartate aminotransferase | 3-5 | 24-48 | 4-6 |
| Lactate dehydrogenase | 2-4 | 48-72 | 7-12 |
| Isoenzyme #1 | 2-4 | 48-72 | 6-12 |
| Isoenzyme #2 | 1.1-3 | 48-72 | 6-10 |

Rise in Serum Enzymes Following Myocardial Infarction

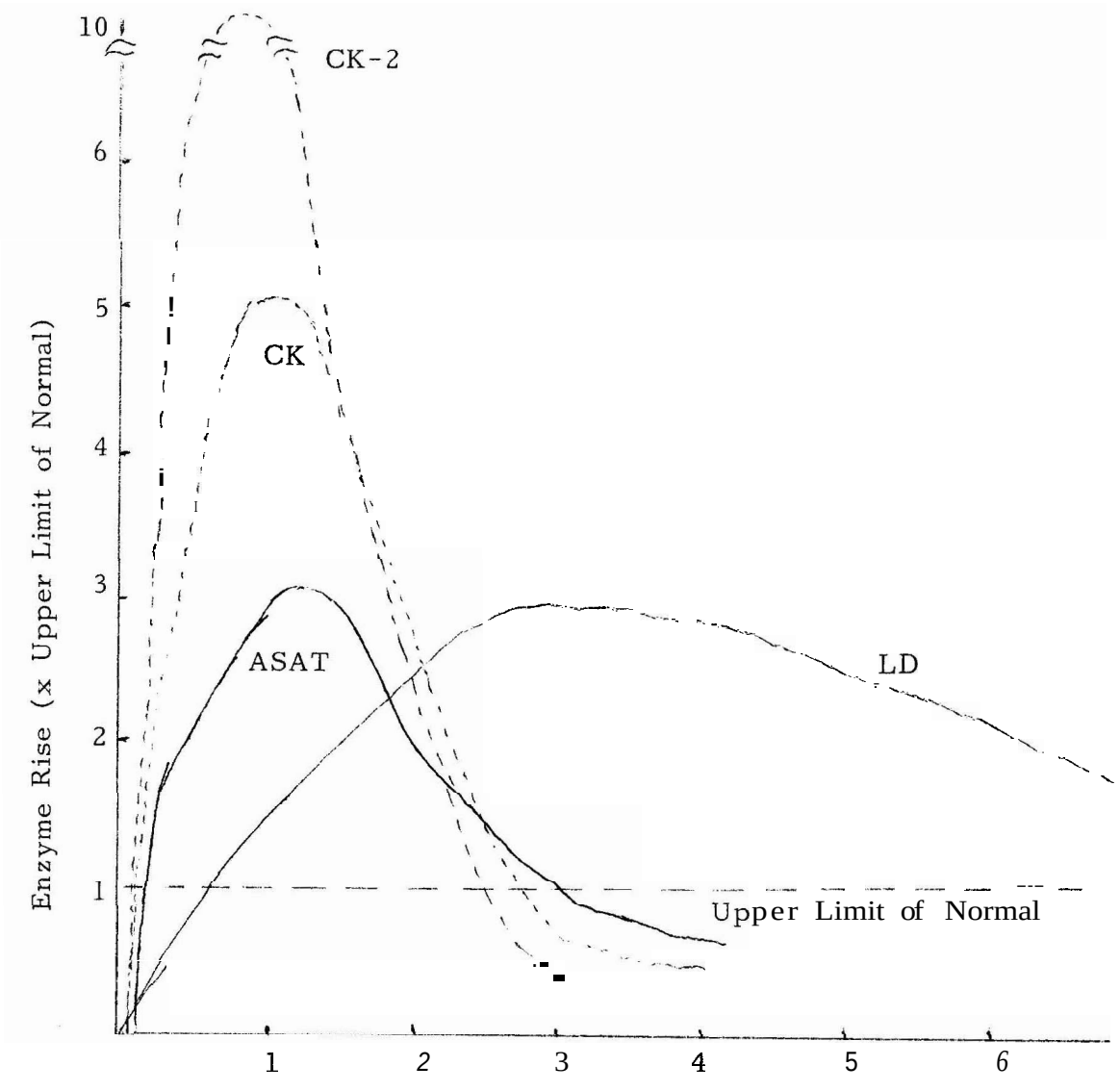



Figure 7

TABLE 2

LD ISOGRAMS IN VARIOUS DISEASES

| | LD1 | LD2 | LD3 | LD4 | LD5 |
|---|---|---|---|---|---|
| Cardiovascular | | | | | |
| Myocardial Infarct | + | + | | | |
| Myocardial Infarction with hepatic congestion | + | + | | | + |
| Pulmonary Infarct | | + | | | |
| Rheumatic Carditis | + | + | | | |
| Inactive Rheumatic Fever | Normal | | | | |
| Myocarditis | + | + | | | |
| Congestive Heart Failure (decompensated) | + | + | + | + | + |
| Shock | + | + | + | + | + |
| Angina Pectoris | Normal | | | | |
| Percarditis | Normal | | | | |
| Heart Block with Strokes-Adams syncope | Normal | | | | |
| Hepatobiliary | | | | | |
| Hepatitis | | | | | + |
| Cirrhosis, Active | | | | | + |
| Cirrhosis, Inactive | Normal | | | | |
| Hepatic Concestion | | | | | + |
| Acute Extrahepatic Obstruction | Normal | | | | |
| Hematologic | | | | | |
| Pernicious Anemia | + | + | + | | |
| Hemolytic Anemia | + | + | + | | |
| Sickle Cell Anemia | + | + | + | | |
| Megablastic Anemia | + | + | + | | |
| Lymphoma & Leukemia (with hemolysis) | + | + | + | | |
| Anemias without Hemolysis | Normal | | | | |
| Miscellaneous | | | | | |
| Infectious Mononucleosis | | | + | + | + |
| Adenocarcinoma of Colon | + | + | + | + | + |
| Renal Tubular and Cortical Necrosis | + | + | + | + | + |
| Skeletal Muscle Necrosis | | | | | + |
| Dermatomyositis | | | | | + |
| Various Malignant Neoplasms | + | + | + | + | + |

separation appear to be the most popular techniques. However, with recent developments in the field of electrochemistry, an electrochemical mode of detection should not only be feasible, but would also allow small concentrations to be measured more precisely. Thus, by using an electrochemical cell and a potentiostatic detector, LD should be detectable in smaller quantities and in a fast, reliable manner.

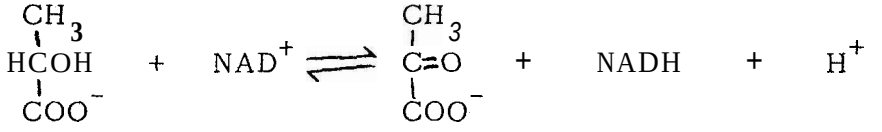
Thus, it is the aim of this work to explore various cell designs and electrode materials in order to devise an optimal cell. Further, an attempt is made to determine the correct applied potential and concentrations of materials used. Finally, response time and peak definition are optimized.

# CHAPTER II

# HISTORICAL REVIEW

## Discovery and Isolation

Although it was known that the final product of glycolysis in the muscle is lactic acid, it was not until the 1930's that Warburg and von Euler discovered the actual reaction shown below:



lactate pyruvate

In 1940, Straub reported the first purified sample of enzyme, and the tetrameric nature of the molecule was established by Markert and Moller in 1964 and a complete three-dimensional structure in 1970.

Since LD is present in the cytoplasm, homogenization or osmotic shock will free the enzyme. Isolation may then be accomplished using several techniques. Straub extracted the enzyme, adsorbed it on a calcium phosphate gel, and fractionated it using acetone and ammonium sulfate. In the early 1960's, Jecsai achieved crystallization using only ammonium sulfate fractionization. Ion-exchange chromatography, however, is the predominant method of isolation today. In the early 1970's, $NAD^+$ was linked to a column packing. Isolation of the enzyme was then accomplished by displacing it with a specific nucleotide. Still better results were obtained using a column which carried a bound substrate analog such as oxamate. This procedure yields single large crystals. Finally, stable preparations may be obtained by binding the enzyme to an insoluble matrix.[14]

## Methods of Analysis

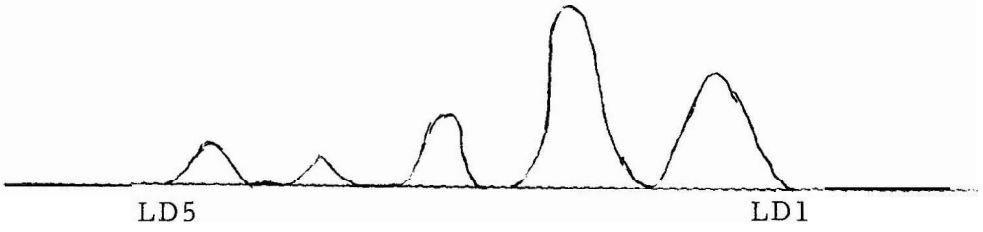
Wroblewski and LaDue devised the continuous monitoring assay described in Chapter I.[15] In this method, which was subsequently revised by Henry, the P→L reaction is used, and the decrease in absorbance of NADH at 340 nm is measured. The activity unit was defined as the amount of enzyme necessary ti change the absorbance by 0.001/min., using a cuvet with a light path of 1.0 cm and a total volume of 3.0 mL.

Briefly, the procedure is as follows: A TRIS buffer (57.5 mmol/L, pH 7.4 at 30°C) is utilized, although phosphate buffers have also been employed. This buffer is then used to prepare NADH and pyruvate solutions. After the solutions have been prepared, buffer, NADH and serum are pipetted into a cuvet and incubated for approximately 15 min. Next, pyruvate is added and absorbance readings taken in 0.5 minute intervals for 3-6 minutes. It should be noted that all procedures are carried out at 30°C, and at the buffer pH of 7.4.

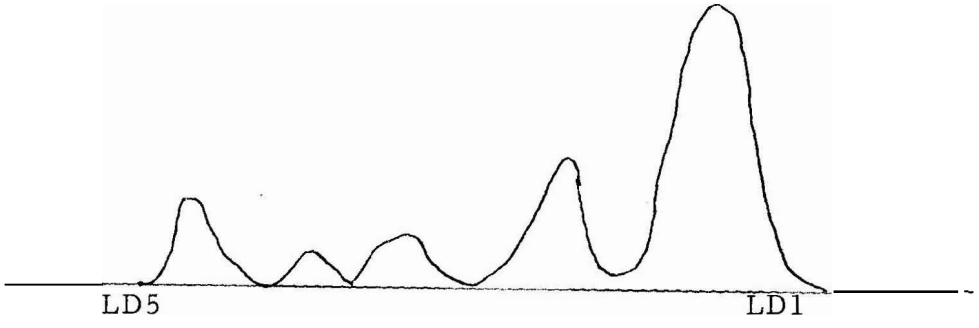
The opposite reaction, L→P, pH 9.0-9.5, has been suggested by Amador as an alternate method. Greater stability of reagents is achieved using this procedure, since lactate and $NAD^+$ are more stable than pyruvate and NADH. Further, NADH solutions tend to develop inhibitors which prevent accurate measurement of activity. However, both procedures are used since actual determinations of accuracy and precision have not been completed.[16]

To measure LD isoenzymes, electrophoresis is commonly used. Support media may include polyacetate, cellulose acetate or agarose, and visualization may be accomplished using UV spectroscopy, fluorometry or colorimetry. One typical procedure would be the following:[17] Serum

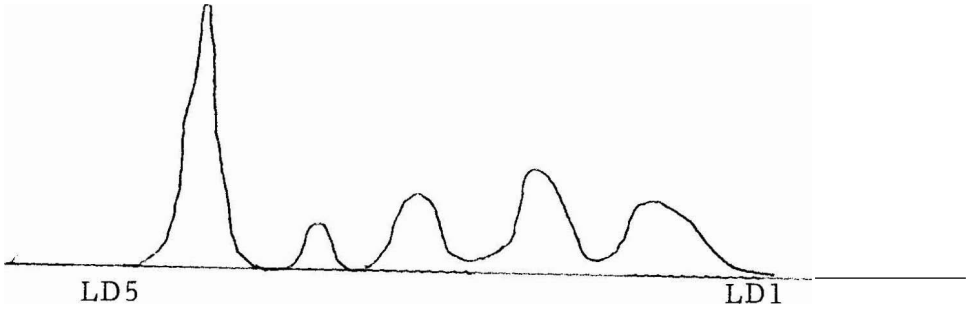
isoenzymes are separated on a cellulose acetate medium. This membrane is then placed on an agar gel which contains lactate, $NAD^+$, buffer, nitro-blue tetrazolium and phenazine methosulfate. As NADH forms, electrons are transferred to phenazine methosulfate, which then acts to reduce the nitroblue tetrazolium to a formazin dye. The intensity of the dye may be measured using densitometry, and is directly proportional to the amount of isoenzyme present. Typical densitometric tracings are shown in Figure 8.[18]




Normal




Myocardial Infarction




Alcoholism

Typical Densitometric Tracings

Figure 8

# CHAPTER III

# METHODOLOGY

## Cyclic Voltammetry

Cyclic voltammetry is a straight-forward, relatively simple electrochemical method which provides a good deal of information about redox systems. As the potential changes at a fixed rate during a scan, characteristics of the system are elucidated, and can be used to determine the direction in which further study should proceed. That is, information is provided not only on the oxidized and reduced species, but also on any intermediate species and the degree of reversibility of the system. Thus, cyclic voltammetry can be the first in a series of experiments employed in the characterization of a redox system.

In this method, the current flowing at an electrode is measured as a function of applied potential. That is, as potential varies with time, current flow will also vary due to the reactions undergone by the redox system. Essentially, a potentiostat controls the working electrode potential by regulating the voltage applied across the auxiliary and working electrodes. In this fashion, a plot of potential $E(t)$, vs. current $i(t)$, is obtained.

The current under consideration is faradaic in nature. That is, it can be classified as chemical, or as producing changes in the oxidation state of a species in the system. The rate of reaction is measured directly from the current flow, and is dependent upon two variables. These are, mass transport, or the rate at which a species reaches the

electrode, and charge transfer, or the rate at which the transfer of electrons occurs,

Mass transport may occur in any of the following ways: diffusion, migration and/or convection. However, the latter is eliminated in cyclic voltammetry by using a hydrostatic solution, while the penultimate is repressed by adding a supporting electrolyte. Thus, diffusion remains as the primary form of mass transport, and the current obeys the following equation:[19]

$$i(t) = nFAD \left. \frac{\partial C(x,t)}{\partial (x)} \right|_{x=0}$$

n-number of electrons
A-area of electrode
F-96,500 C/faraday
D-diffusion coefficient
C-concentration

In the actual practice of cyclic voltammetry, the steps listed below are followed. First, the working electrode is given an initial potential at which no reaction occurs. Then, the potential is changed over time such that either reduction or oxidation takes place. Following the completion of the reaction or reactions, the scan is reversed and the reactions of products and/or intermediates produced as a consequence of the forward scan may be observed. Thus, the potential sweep is 'cycled' such that a complete picture of the redox system is obtained.

Under experimental conditions, little current is initially produced. As reduction begins to occur, however, current increases to some maximum, after which it again decreases. That a peak should result is explained by the fact that as sample is reduced, a depletion layer of the sample develops around the electrode and fresh sample must diffuse through this layer before reduction can occur. That is, as the surface concentration of sample decreases (due to the depletion layer), current is controlled by diffusion.

At a set potential, scan direction is reversed and oxidation takes place. Thus, an anodic peak is formed just as the cathodic peak was initially formed. The entire procedure may be cycled repeatedly, although only reversible reactions will produce reproducible voltammograms.

## Continuous Flow

Continuous flow provides an improvement over simple batch sample analysis. This is especially important in clinical applications, where samples from a large number of patients must be examined in a fast, cost-efficient manner. Continuous flow answers these needs, according to Mottola,[20] for the following reasons: reagents can be better utilized; precision and accuracy can be achieved even with large numbers of sample; and automation minimizes human error. Thus, this method is in widespread use in clinical laboratories.

The major problem in continuous flow is sample integrity. As solution flows through a system, it will not maintain discrete 'plugs' of sample, but will diffuse this sample throughout. This is due to the higher viscosity at the walls of the tubing, which results in uneven flow and longitudinal mixing.

To correct this problem, several approaches have been tried. The oldest and most common method is air segmentation, in which air 'plugs' are introduced between samples such that dispersion is restricted.[21, 22, 23] Immediately prior to the cell, the solution is debubbled to prevent erratic electrode response.

A variation of this technique is demonstrated by Patton, et. al.,[24] who used bubble-gating to avoid the loss of solution which inevitably accompanies debubblers. In this procedure, the segmented stream is

permitted to enter the cell, but is detected only when the chamber is filled with liquid. Briefly, this involves a square-wave-type pattern in which a colorimetric detector varies between approximately 0 %T for air and some finite value for the sample. The fluctuations in signal are used to synchronize the filling of the cell with data acquisition and storage. Thus, although both methods described above use segmented flowm the problem of air in the detector is resolved differently.

A third method used to avoid sample diffusion is nonsegmented continuous flow.[25,26] Here, the typical sample stream of the Auto Analyzer (sample-air-wash solution) is connected by a valve to a continuously flowing 'reaction' stream. By regulating the valve, injections of sample can be made and the nonsegmented stream can then be analyzed. To maintain integrity of sample, high flow rates, small bore tubing and low sample volumes are needed.

## Electrode Composition

Gold has often been chosen for use as a solid electrode. In addition to being very pure and easily fabricated, it is quite resistant to oxidation.[27] Further, it has a relatively large overpotential for hydrogen evolution but does not adsorb hydrogen to a significant extent.

At a positive potential of 1.4-1.5 v, gold forms an oxide film due to chemisorbed oxygen. This oxide is reduced at approximately 1.2 v. In addition to the formation of oxide, gold is known to oxidize readily when halides or cyanide is present. Thus, at positive potentials, gold complexes form, changing the electrode surface, and rendering it unsuitable for use. Although gold was considered for this study as a possible electrode material, it was rejected due to this and other reasons later explained.

Another electrode which was considered was the carbon paste electrode.[28] As with other carbon electrodes, it is easy to use, easy to reproduce, has little electrical resistance and a useful potential range, which is quite large. Thus, carbon paste electrodes have shown themselves to be both useful and versatile.

The electrode is constructed as follows: into an inert material (Plexiglas, Teflon) a depression is cut. An electrical contact is inserted, and the depression filled with carvon paste. The paste itself is comprised of graphite powder and a mulling liquid such as mineral oil or bromoform. The choice of the mulling liquid must be made on the basis of the solvents to be passed over the electrode; the appropriate selection should be virtually insoluble in these solvents. Care must be exercised when packing the electrode so that a uniform surface is obtained and no unfilled holes remain. To renew the surface, a procedure which should be carried out before each experiment, the top layer of paste should be discarded and the depression filled with fresh carbon paste.

A third electrode used in this study was reticulated vitreous carbon. This is similar to the carbon paste previously described, but is more versatile in both resistance to solvent attack and range of potential.

The electrodes described above are the working electrodes. However, two other electrodes are necessary to complete the circuit. These are the reference and auxiliary electrodes. In this study, the former was chosen to be a Ag/AgCl reference electrode. The latter was originally a silver epoxy plug situated immidiately adjacent to the working electrode, but was changed to a stainless steel length of small-bore tubing placed some distance from the working electrode.

## Electrochemical Cell

The design of the electrochemical cell is of crucial importance in the execution of any electrochemical experiment. The actual construction of the cell, as well as the choice and placement of electrodes all have an effect upon its performance. Thus, factors such as potential, current flow and type of solution must be taken into account so that the correct cell design will be chosen.

For the purpose of this study, a three-electrode, controlled potential system was designed. Under low-current conditions, the three electrodes can be fitted to one compartment, and the cell resistance is the resistance of the solution between the working and auxiliary electrodes. Thus, it is dependent upon the distance between and the area of these electrodes, as well as the solution conductivity. To keep current and potential density uniform, the auxiliary and working electrodes should be placed in a symmetrical arrangement.[29]

In thin-layer, controlled--potential electrochemical detection, a potential difference is applied between the working and reference electrodes. This potential serves to oxidize or reduce a species by allowing the electrode to function as an electron sink or source. For example, as a solution comes in contact with the electrode surface, oxidation or reduction occurs in a thin layer of molecules adjacent to this surface. The resultant current is measured, and is directly proportional to the concentration of species undergoing reaction. It should be noted that although only a very small amount of sample is converted, attempts to increase efficiency by using larger electrodes result in increased electrolyte conversion and decreased signal-to-noise ratio.

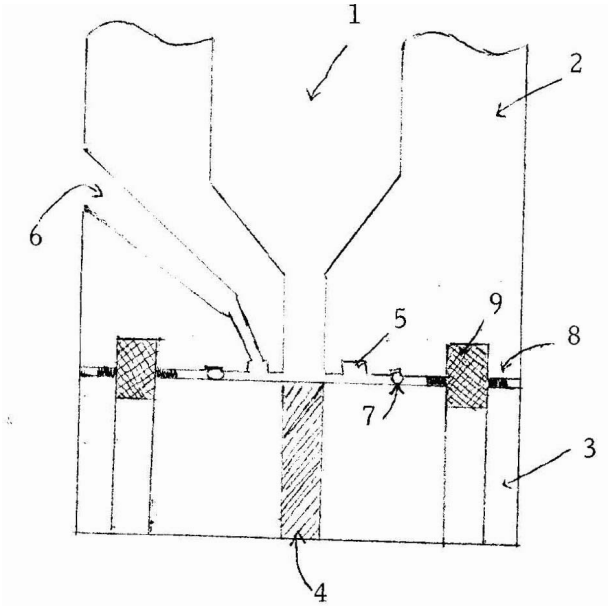
Although the choice of electrode material is crucial to an electrochemical experiment, the cell itself must be constructed of an inert material. In general, the material should be easy to fabricate, durable, stable and inert to solvents and reagents. Glass, Teflon, Kel-F, Nylon and acrylates such as Lucite or Plexiglas have all been used in cell manufacture. In this study, Plexiglas was chosen as the material from which the cell was constructed.

Figures 9[30] and 10[31] show typical cell designs. In the first, fast electrode reactions may be studied. All electrodes are positioned near each other, such that there is a uniform distribution of current over the working electrode, and the reference electrode is low-resistance. Figure 10 shows a thin layer cell which may be used in conjunction with liquid chromatography. Here, a Plexiglas cell contains a working electrode in a low dead volume region, while the reference (Ag/AgCl) and auxiliary (stainless steel) are placed in a separate compartment and not in the thin layer region. A cell of similar design was chosen to be used in this project.

Thus, many factors go into the design of an electrochemical cell. The correct choice of electrode material and cell design depends upon the course of study undertaken, and must be adapted to the goals of that study. This, then, is the crucial step in any electrochemical experiment,

## Summary of Methods

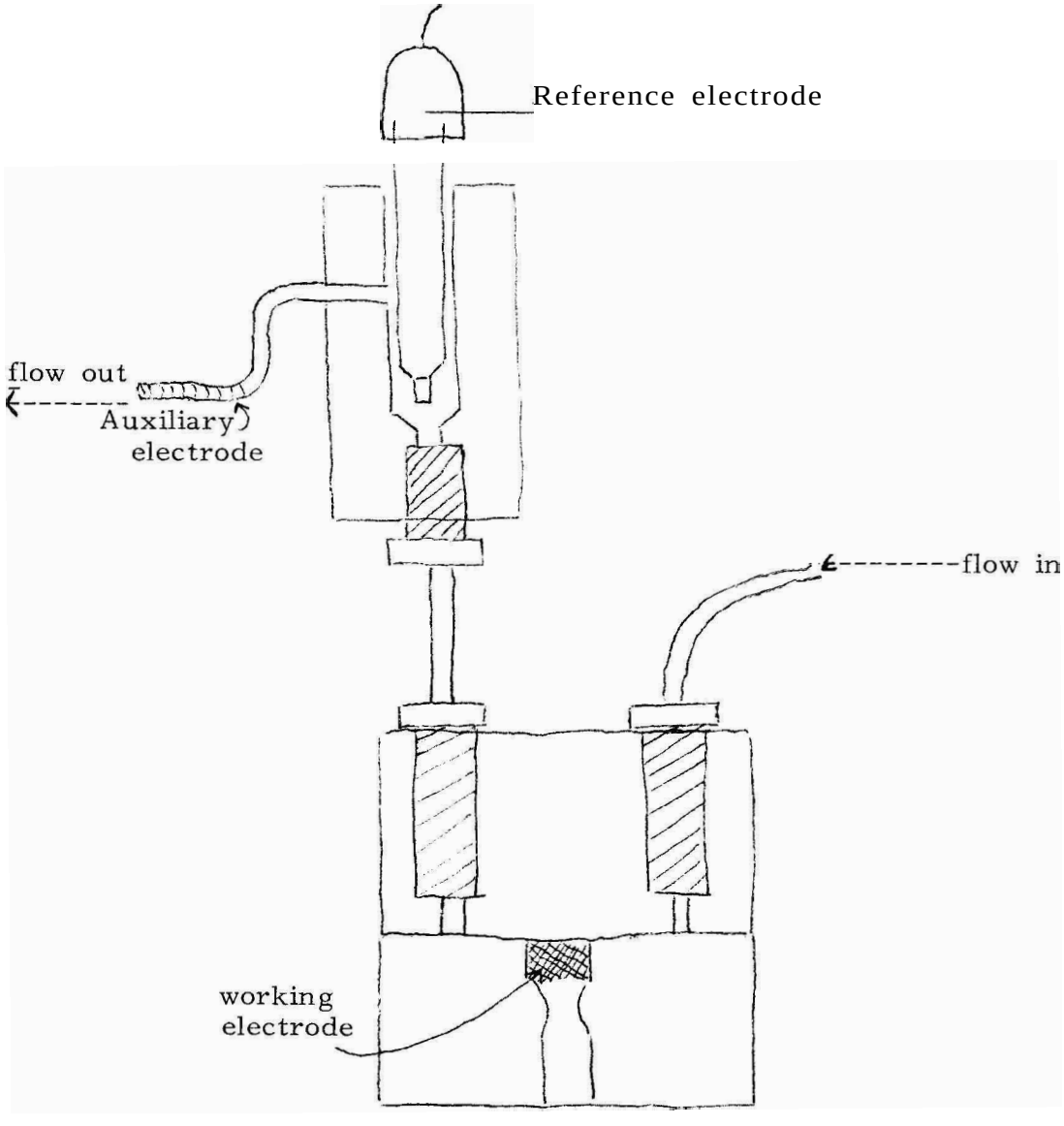
In the course of this work, several electrochemical methods were used. Cyclic voltammetry was used to determine the correct potential at which to oxidize NADH to $NAD^+$. Several cell designs were tried in




1) counter electrode chamber
2) Kel-F top
3) Teflon bottom
4) working electrode
5) reference electrode groove
6) reference electrode
7) Viton O-ring
8) stainless steel spacer
9) stainless steel locating pin

Cell for Fast Electrode Reactions

Figure 9




A Thin-layer Cell

Figure 10

order to obtain maximum efficiency. Further, gold, carbon paste and reticulated vitreous carbon were all tried as working electrodes. Finally, experiments were performed using a continuous flow system.

# CHAPTER IV

# MATERIALS AND APPARATUS

## Materials

Table 3 provides a list of the reagents used in this study. All were used without further purification.

Refrigerated control samples of human serum provided the lactate dehydrogenase, which was stable for 24 hours following dissolution. β-NADH (disodium salt, trihydrate) was stored in a dessicator at $0^{o}C$, and was stable in solution for 72 hours if refrigerated. Pyruvate solutions were also refrigerated and were stable for 3-4 weeks.

## Apparatus

### Cyclic Voltammetry

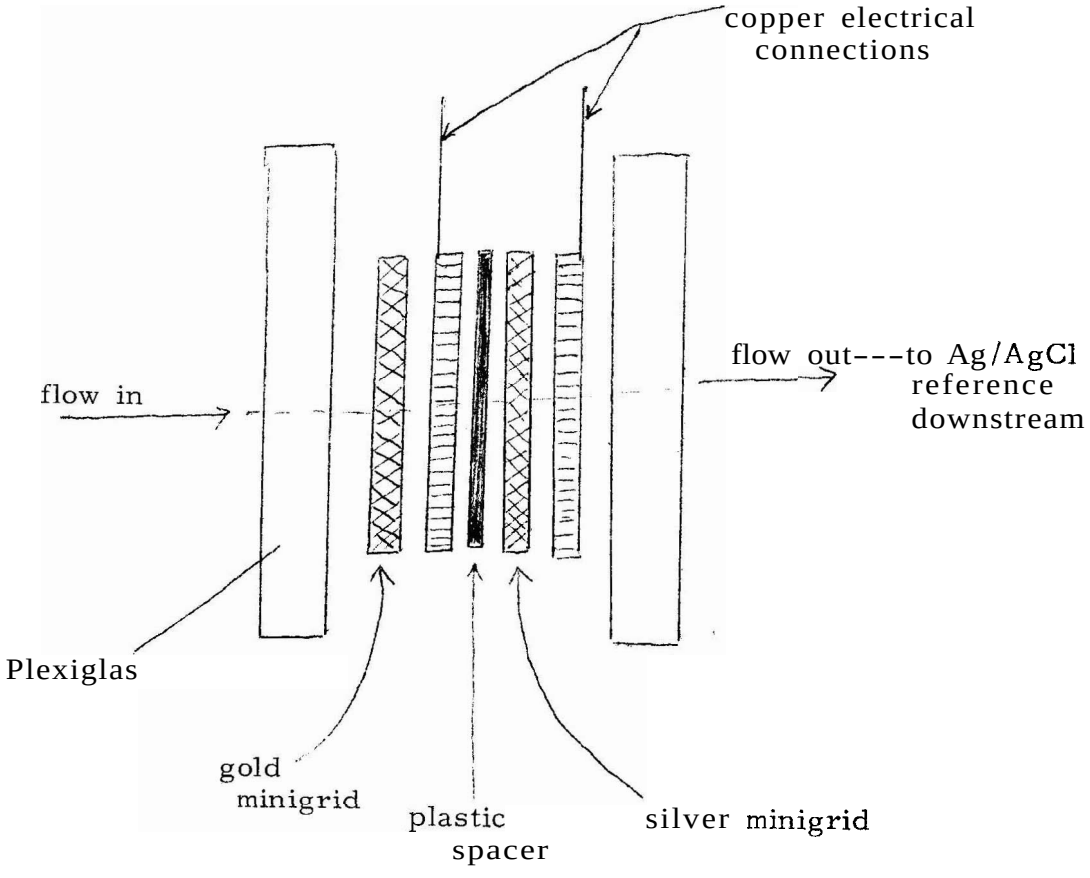
All cyclic voltammograms were obtained using a BAS cyclic voltammograph, model CV-1B and a Houston Instrument Model 200 X-Y Recorder. The cell consisted of Pt working and auxiliary electrodes and a Ag/AgCl reference.

### Cells

Three electrochemical cells were constructed during the course of this study. The first is shown in Figure 11, and consisted of a gold minigrid working electrode, a silver minigrid auxiliary and a Ag/AgCl reference. This design was discarded for two main reasons: the oxidation

TABLE 3

| Material | Grade | Manufacturer |
|---|---|---|
| NADH disodium salt, 3 1/2 $H_2O$ | ---------- | Calbiochem-Behring |
| $NAD^+$ | ---------- | Sigma Chemical Co. |
| Pyruvate sodium salt | Reagent | Eastman |
| Lactate | Reagent | Allied Chemical |
| TRIS | Reagent | Eastman |
| KCl | Certified ACS | Fisher Scientific Co. |
| LD | Normal Enzyme Verifier | DuPont Co. |
| | Elevated Enzyme Verifier | DuPont Co |
| | Precinorm U | Boehringer Mannheim Diagnostics, Inc. |




Gold-Silver-Ag/AgCl Cell

Figure 11

of gold in the presence of halides and the inability of the thin foil to withstand the pressure of the flowing sample,

Figure 12 shows the second of the three cells devised. Here, carbon paste was used as the working electrode, a Fisher Ag/AgCl electrode was the reference, and stainless steel tubing served as the auxiliary. As can be seen, the solution flowed over the working, reference and auxiliary in that order, and the auxiliary was a substantial distance from the working electrode.

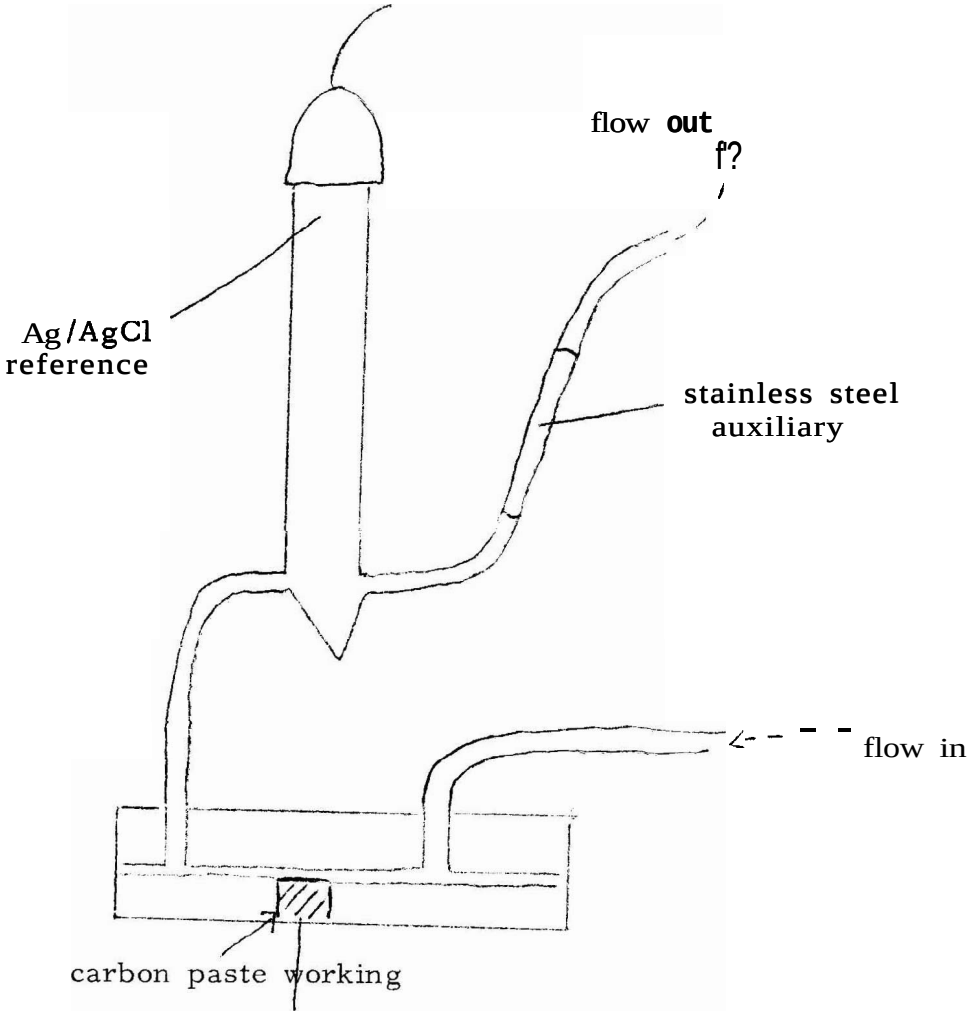
Finally, Figure 13 shows another carbon paste working electrode. Here, however, the auxiliary was a silver epoxy placed no more than 1 cm from the working electrode. The reference was the same Ag/AgCl electrode as before.

## Detector and Recorder

The detector used in this study was a EG&G Princeton Research Polarographic Analyzer, Model 364. To insure the correct potential was applied to the working electrode, a Keithly 169 DMM multimeter was used. All data were redorded on a Fisher Recordall, Series 5000.

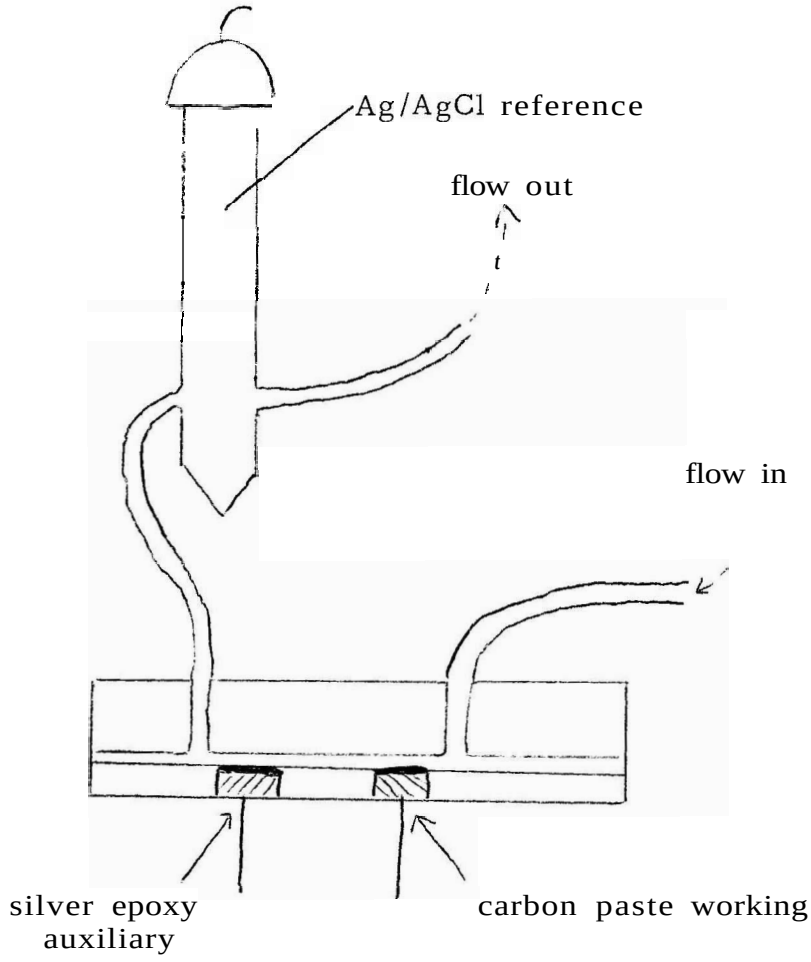
## Continuous Flow

Continuous flow of sample was made possible by the use of a Technicon AutoAnalyzer Sampler and Proportioning Pump. The Sampler provided the means of introducing small samples of LD intermittently, and the Pump allowed LD, pyruvate and NADH to flow in an even, constant fashion through the cell.




Carbon paste–Stainless steel–Ag/AgCl Cell

Figure 12




Carbon paste-Silver epoxy-Ag/AgCl Cell

Figure 13

# CHAPTER V

# PROCEDURE AND RESULTS

## Preparation of Solutions

The following solutions were needed for this study: NADH, pyruvate and LD samples. A TRIS buffer solution was used to dissolve the NADH and the pyruvate, while the serum sample containing LD was dissolved using distilled water.

The TRIS buffer was made by dissolving 60.6 g tris(hydroxymethyl) aminomethane in water, and adjusting the pH to 7.4 using concentrated hydrochloric acid. A working buffer was then prepared by diluting 115 mL of the original buffer to 1000 mL with water and again adjusting the pH to 7.4. This buffer had a concentration of 57.5 mmol/L and was made 1 M in KC1 to provide a supporting electrolyte.

A 5.58 mmol/L NADH solution was made by dissolving 0.87 g Q-NADH in 200 mL of working buffer. A 14 mmol/L pyruvate solution was made using 0.775 g sodium pyruvate in 500 mL of working buffer. KC1 could be added to either of these solutions if the supporting electrolyte concentration was not great enough.[32]

## Cyclic Voltammetry

Cyclic voltammetry was the first experiment performed, as it was necessary to determine the correct potential to apply across the working electrode. A solution of working buffer was prepared as described above, and nitrogen bubbled through to rid it of oxygen. The electrodes

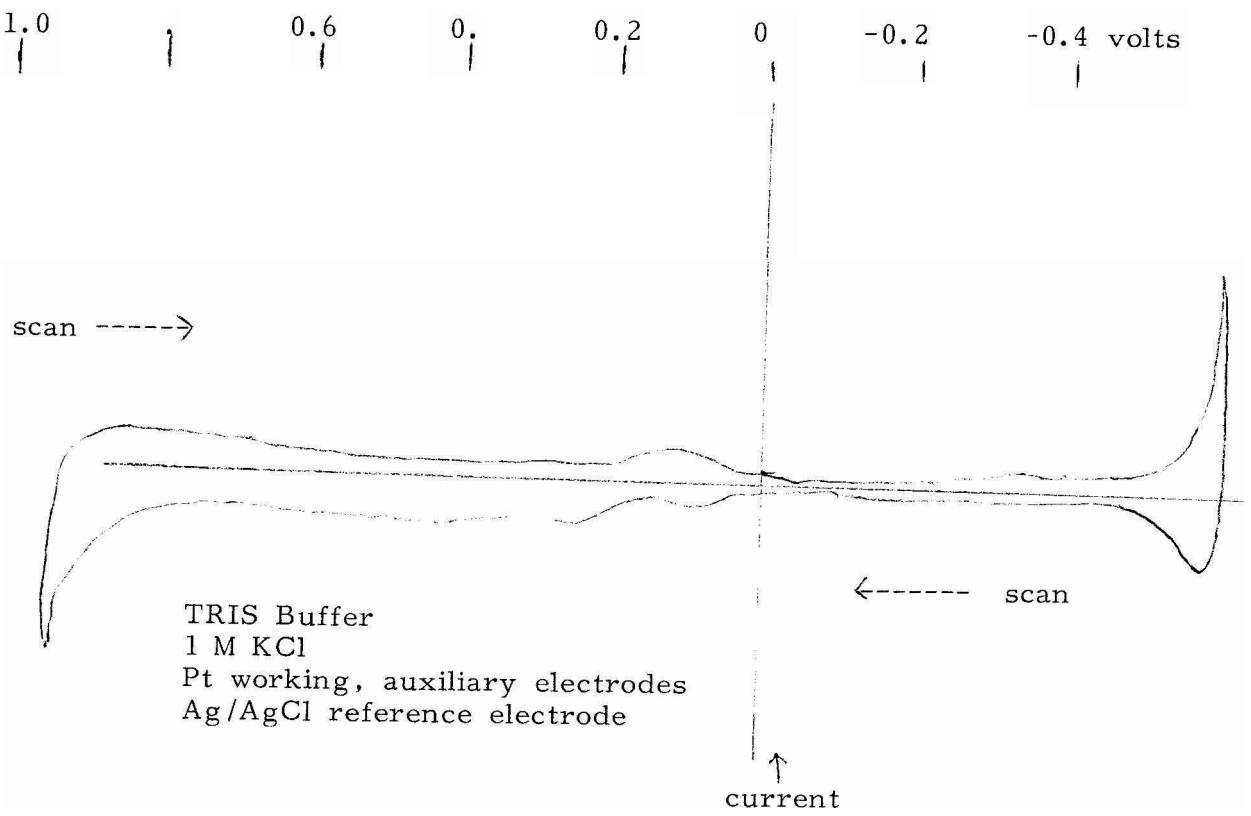
(Pt working and auxiliary, Ag/AgCl reference) were then immersed in the solution, and a voltammogram obtained. The results in Figure 14 show that no significant background reactions were detected using this buffer. Next, a solution of deoxygenated NADH was used. As seen in Figure 15, at approximately +0.6 v, NADH is oxidized to $NAD^+$, while $NAD^+$ is reduced at +0.1 v. Thus, a potential near 0.6 v was determined to be the correct potential at which to conduct further studies.

## Set-Up of Apparatus

All experiments were performed using a Technicon AutoAnalyzer to proportion solutions and provide a continuous flow. The set-up of the system is shown in Figure 16. As can be seen, four separate streams were combined to make the sample stream. Pyruvate and NADH were always present, so that a steady baseline was obtained. LD samples, however, were interspersed with pure water to insure complete recovery between reactions. Air served to separate the sample stream into 'plugs', thereby insuring sample integrity.

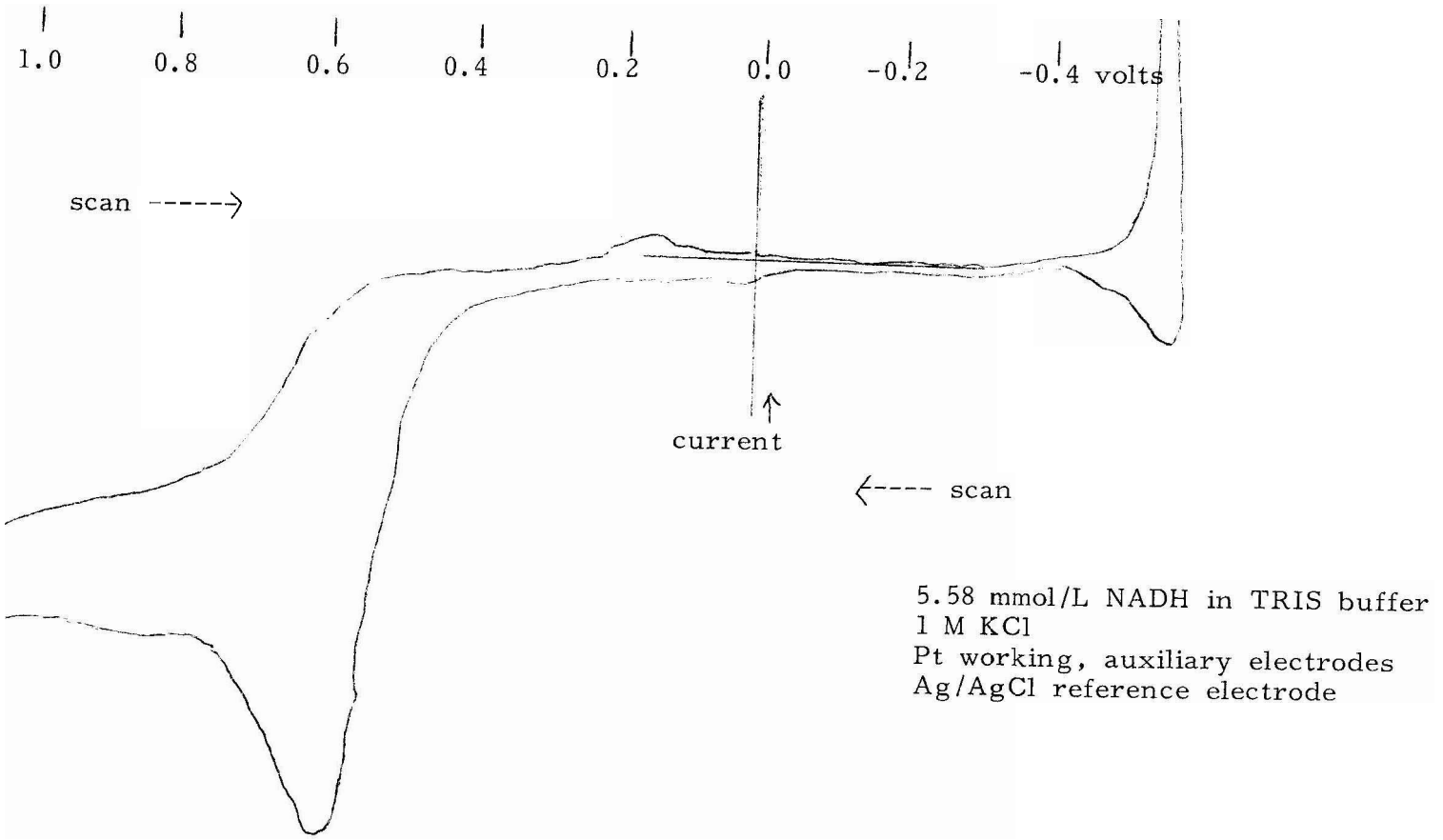
NADH entered the stream at a point close to the electrochemical cell. This further guaranteed that the reactant would not be diffused, but would be constantly present at the electrode in the same concentration.

Immediately prior to the cell, the stream was debubbled, insuring that no air would cross the electrode and give erratic, false responses. While a representative sample went to the cell, the remainder passed to a waste receptacle. After the sample had reacted, it too was directed to the same receptacle.



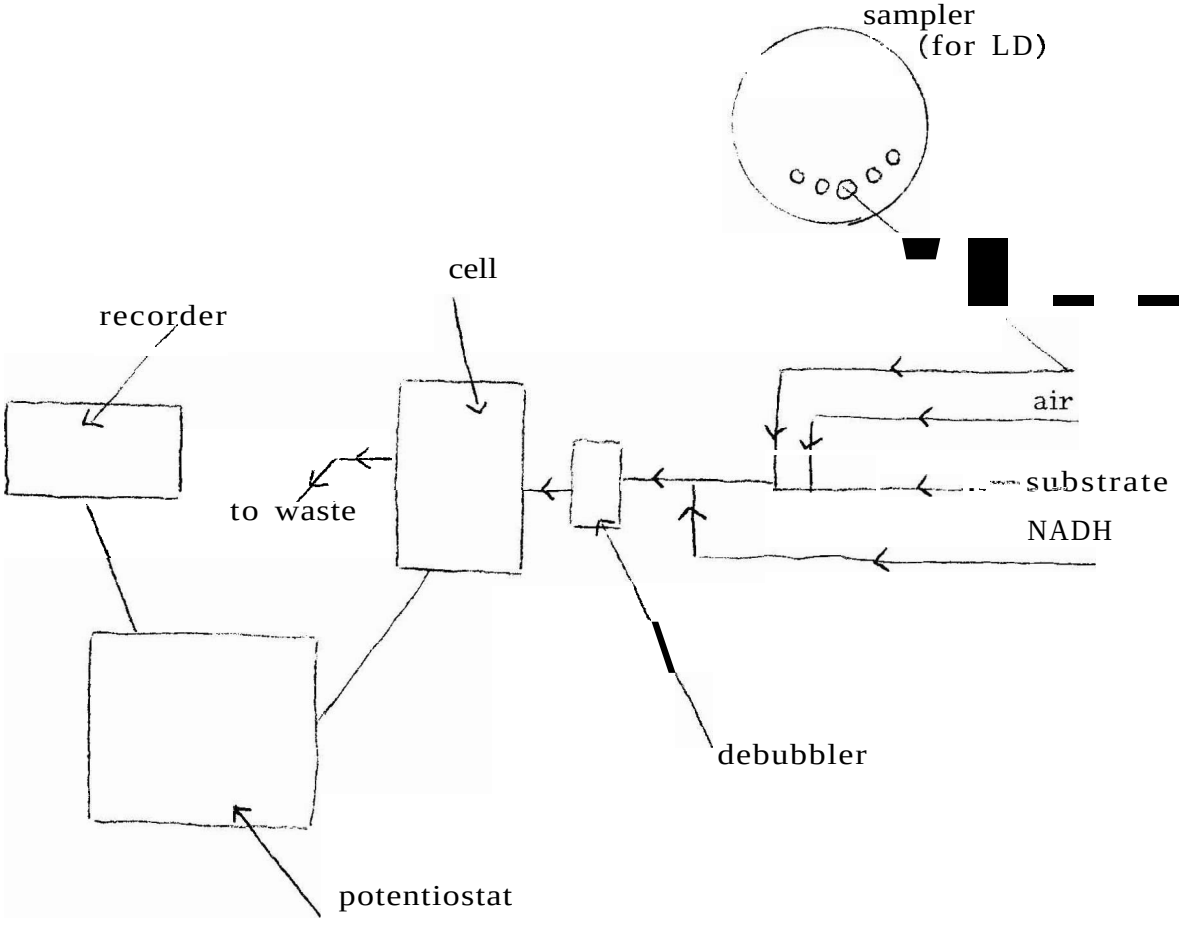

Cyclic Voltammogram of Buffer

Figure 14




Cyclic Voltammogram of NADH in TRIS Buffer

Figure 15




Set-up with AutoAnalyzer

Figure 16

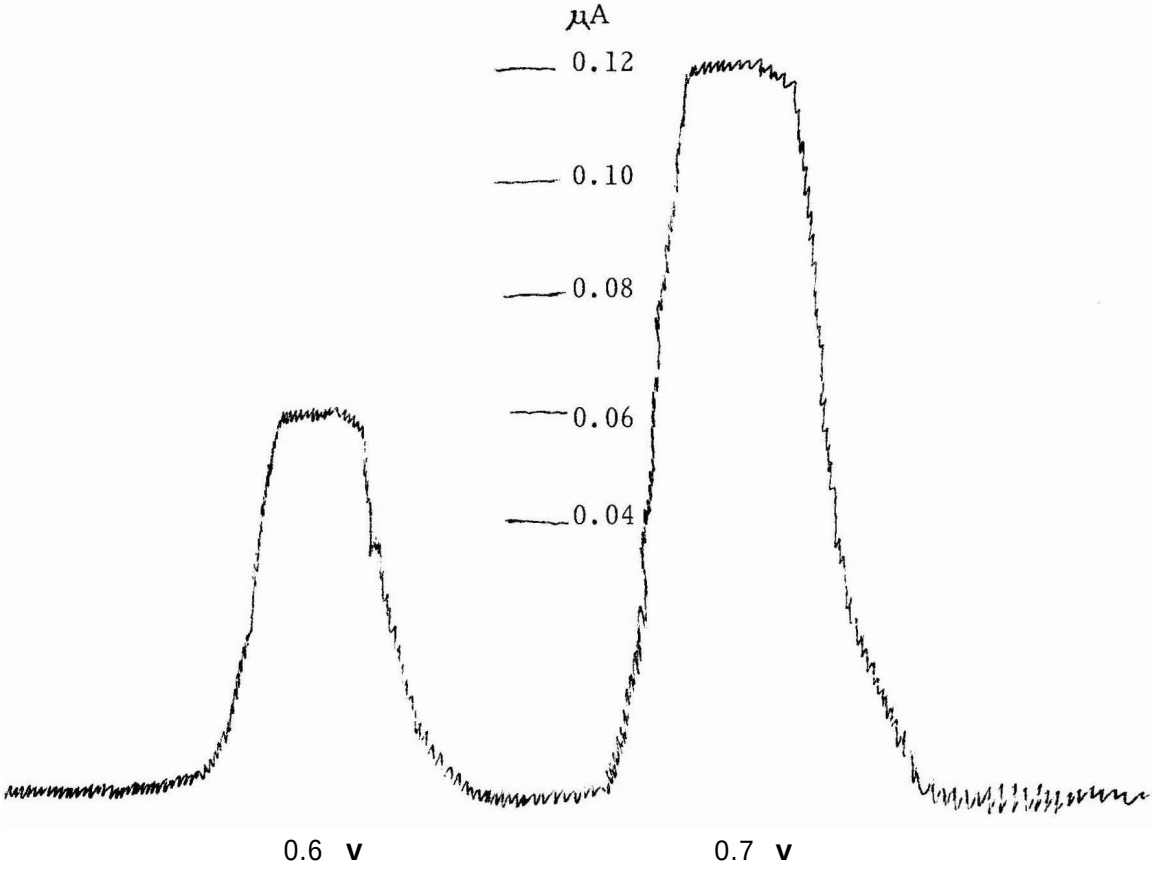
## Optimization of Potential

Although cyclic voltammetry had shown 0.6 v to be the potential at which NADH was oxidized to $NAD^+$, this had to be modified to compensate for the different electrodes used in the actual experiments. Thus, sample was first run through the cell at a potential of 0.6 v, generating a set of data. The potential was then changed to 0.7 v, and the resulting peaks compared with the former. In this fashion, potential values between 0.6-0.8 v were considered, and the optimum was found to be 0.7 v. This value was then used to generate all data obtained during this study. A comparison of responses at 0.6 and 0.7 v is shown in Figure 17.

## Operation and Principle of Cells

Although three different electrochemical cells were used, all operated on the same principle. Thus, a general description of this principle shall follow, and can be applied to any cell described below.

In general, NADH and pyruvate were run continually through the cell. At the working electrode, NADH was oxidized to $NAD^+$, thereby generating a constant current. This steady current was used as the baseline. Intermittently, however, LD samples were introduced. When this happened, the enzyme converted pyruvate to lactate and NADH to $NAD^+$ prior to the stream reaching the working electrode. Thus, less reaction occurred at the electrode, and current dropped in accordance with the concentration of the enzyme. When all sample plugs containing LD had passed through the cell, current rose again to the level of the baseline and remained there until the next sample of LD was introduced.




5.58 mmol/L NADH

Optimization of Potential

Figure 17

Thus, peaks were formed whenever enzyme was present in the solution stream. The intensity of these peaks depended on the concentration of the enzyme, permitting direct measurement of LD activity from the area under a peak.

It was found that LD samples could not be introduced in succession, as this did not provide adequate recovery time. That is, the response time of the detector was not such that the wash between samples (using the AutoAnalyzer Sampler) allowed complete recovery. Thus, samples had to be separated by cups containing pure water so that peaks could be obtained.

## Gold-Silver Electrodes

This cell consisted of a gold foil working, silver foil auxiliary and Ag/AgCl reference electrode. Although it was actually constructed, it was not able to be used. This was primarily due to the fact that the thin foil electrodes were unable to withstand the flow of sample solution. Thus, this first design had to be laid aside and a new cell constructed.

## Carbon Paste-Silver Epoxy Electrodes

In an effort to construct a cell capable of heightened sensitivity, a carbon paste working electrode was combined with an auxiliary composed of silver epoxy. Since the two were situated in close proxomity, the circuit could quickly be completed and response would be rapid. However, it was soon determined that this cell was unable to provide the needed sensitivity, since the current output could not be reduced below 100 $\mu$A without the recorder pen going off-scale. It was therefore determined that the auxiliary electrode needed to be placed at a further distance from the

working electrode, and in such a position that the reference electrode was between the others, rather than following the auxiliary.

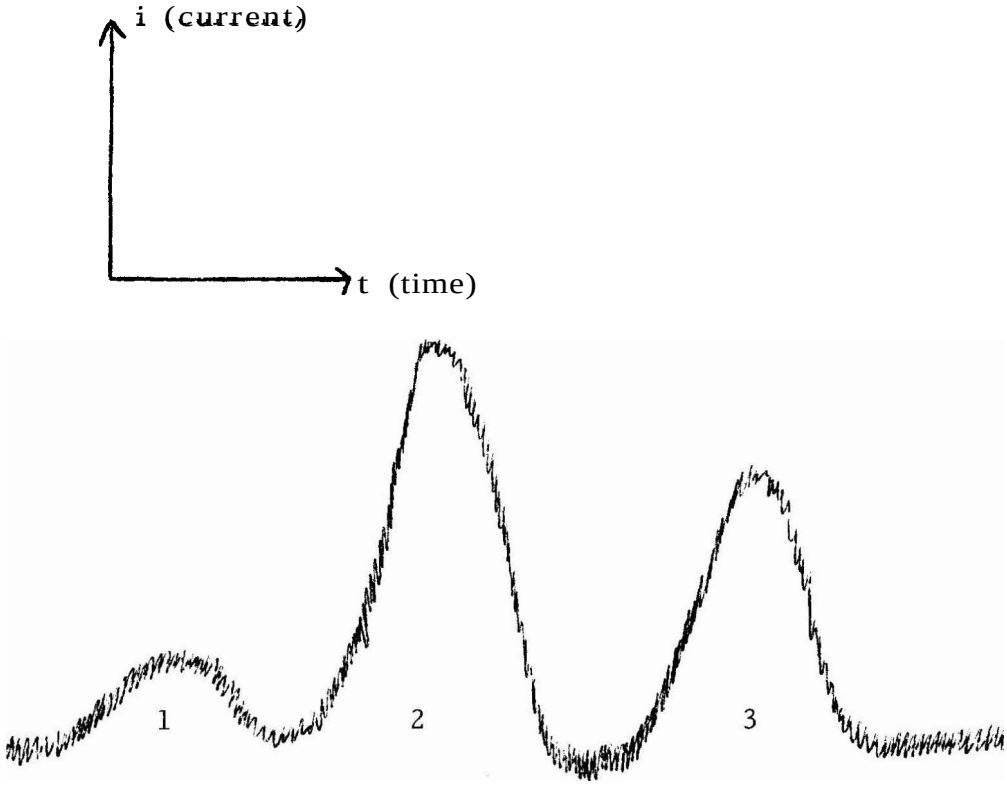
## Carbon Paste-Stainless Steel Electrodes

This cell design is shown in Figure 12. It proved to be quite versatile and attained current output of 2 μA. Using this cell at a potential of 0.7 v, Figure 18 was obtained. Here, a constant flow of NADH and pyruvate yielded a steady baseline. Samples of LD were introduced via the AutoAnalyzer Sampler, and resulted in the formation of peaks due to the chemical (as opposed to the electrochemical) oxidation of NADH. It should be noted that inadequate recorder recovery time prevented the introduction of successive serum samples. Rather, several water washes had to be placed between each sample to allow the baseline time to be reestablished.

Figure 18 was the result of several experiments, all of which produced similar results. The LD concentrations indicated by the peaks were: peak 1, from a normal serum sample- approximately 130 U/L; peak 2, from a pathological sample- 180 U/L; peak 3, from a pathological sample- 158 UIL.

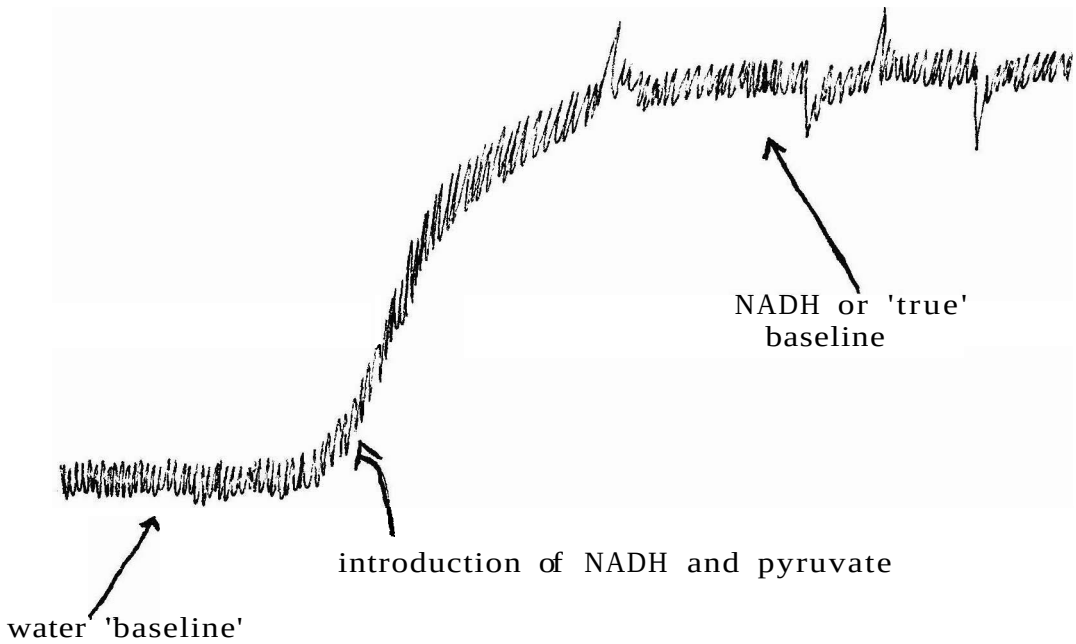
Each ,day, water was run through the cell for approximately fifteen minutes. This provided time for the detector to warm up, and also served as a wash for the electrode surfaces. After this time, pyruvate and NADH were introduced, and allowed to constantly flow. Again, at least a few minutes were necessary to permit this new system to stabilize. Using the recorder, the water 'baseline' and the true baseline could be followed, as seen in Figure 19. When the true baseline had leveled, the system was ready for the introduction of enzyme.

Sensitivity was an important consideration during the course of this study. While it had been previously determined that +0.7 v was the




Current Response to LD Samples in TRIS Buffer
using the Electrochemical Cell

Figure 18




Comparison of Baselines due to Water Wash and to Constant NADH Flow

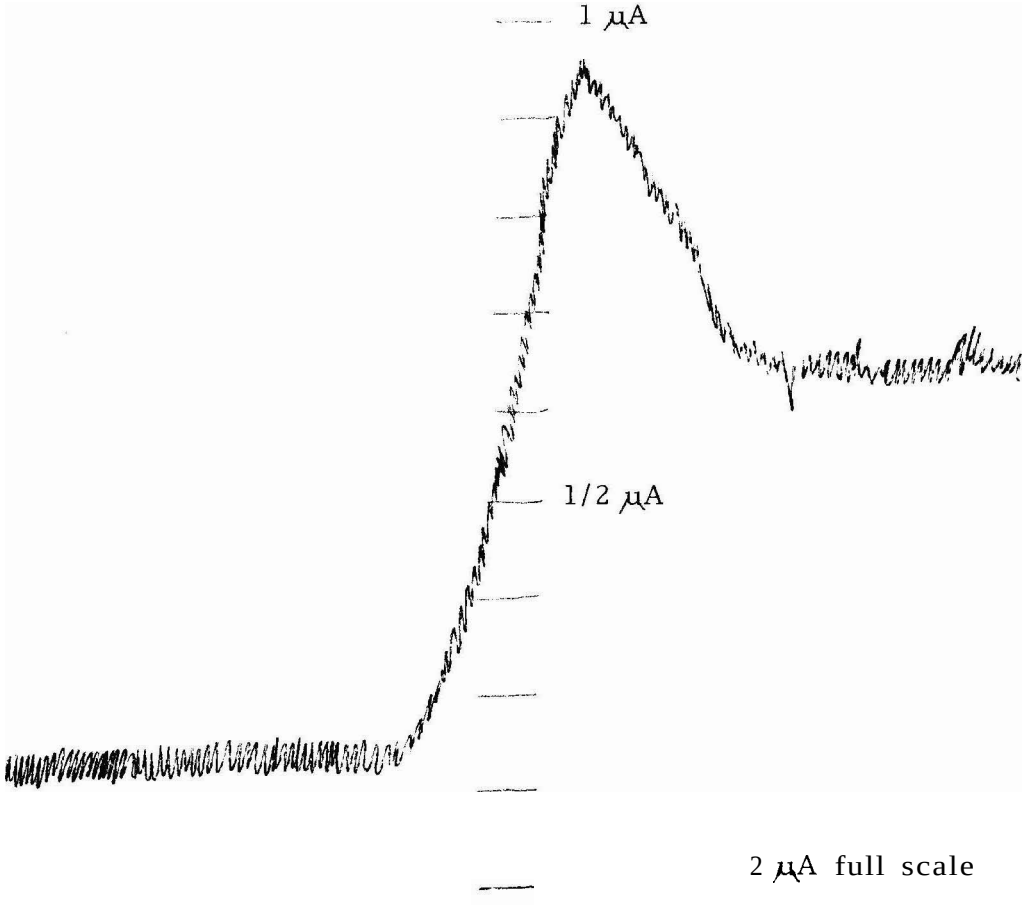
Figure 19

optimal potential at which to oxidize NADH, increased peak height could also be achieved by minimizing the current and voltage output as seen by the recorder. Thus, it was found that a sensitivity of 1.0 v full scale and 2 μA full scale could be obtained. Attempts to further increase the sensitivity were fruitless, as the pen went off-scale.

Although the cell performed well at first, after a period of time it was found that the baseline was not recovered following removal of a sample of LD. That is, although the baseline may have been stable before the peak, after a sample of LD a new, elevated baseline appeared. This new baseline was also stable, but did not allow an accurate measurement of peak area.

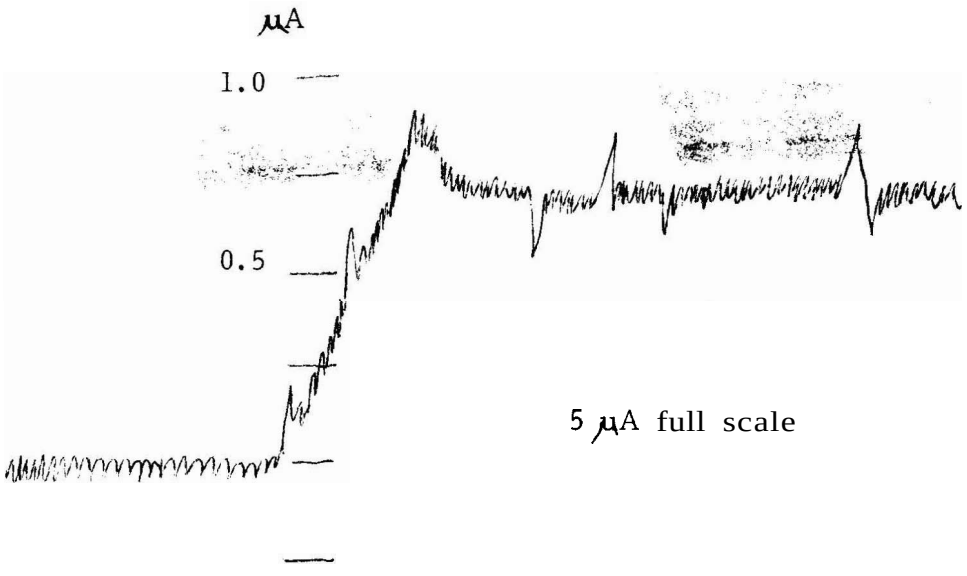
Figures 20 and 21 show this new baseline formation. In the former Figure, there is an attempt to recover the original baseline, while the latter merely remains at the height to which it originally rose. This difference was attributed to the decreased sensitivity of the latter Figure (5 μA vs. 2 μA full scale).

Since it appeared that adsorption onto the surface of the working electrode was the major cause of this baseline shift, attempts were made to isolate the source of contamination. To determine if NADH was the contaminant, as well as determine if greater sensitivity could be obtained, the reverse, L→P, reaction was tried. Here, the same principle applied, except $NAD^+$ and lactate were used rather than NADH and pyruvate. From the cyclic voltammograms, it was found the $NAD^+$ to NADH reduction occurred at +0.15 v. Thus, this potential was used rather than the +0.7 v used previously. Another change was made in the pH, since the new reaction proceeded at a pH of 9 rather than 7.4. Other than these changes, the remainder of the set-up was unaltered.




Incomplete Baseline Recovery

Figure 20




Incomplete Baseline **Recovery**

Figure 21

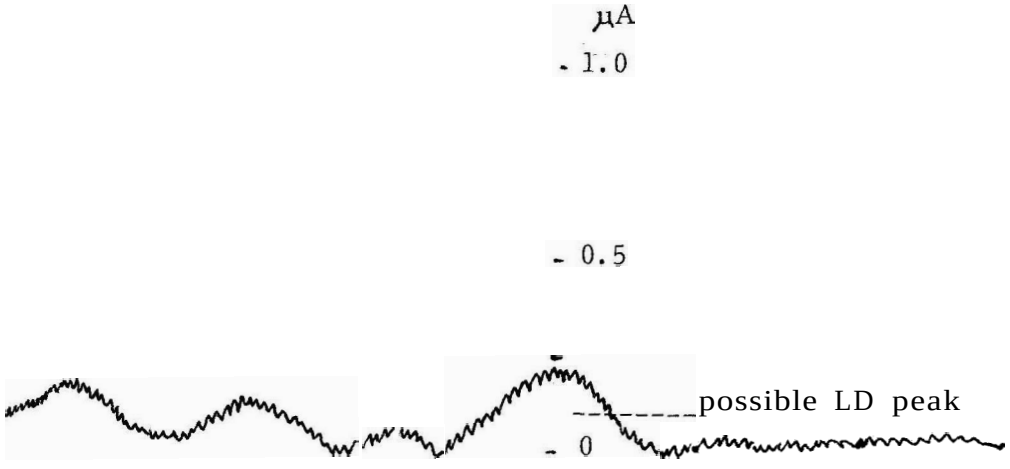
The results of this experiment were not encouraging. Although it was known that $NAD^+$ would adsorb to the electrode surface,[33] it was hoped that results could nevertheless be obtained. However, this was not the case, as can be seen in Figure 22, which shows only a slight drop in current. Subsequent samples failed to produce even this small drop. Thus, it was concluded that $NAD^+$ was probably the major source of electrode poisoning as seen in the previous experiment.

Since the working electrode was now contaminated, the cell was taken apart and a new surface given to the electrode. This was done by removing one-third to one-half of the carbon paste and replacing it with fresh material. The cell was then reassembled and allowed to stabilize using water. Afterwards, NADH and pyruvate were run through and the baseline established as before.

Although a fresh surface had been prepared, it was found that contamination quickly occurred. In an effort to counteract this effect, several methods were tried. One method involved diluting the 5.58 mmol/L NADH with buffer to produce a solution of approximately 2.8 mmol/L. It was anticipated that the amount of $NAD^+$ now present would not contaminate the electrode as readily. Yet, as seen in Figure 23, the electrode still became contaminated even at this reduced concentration.

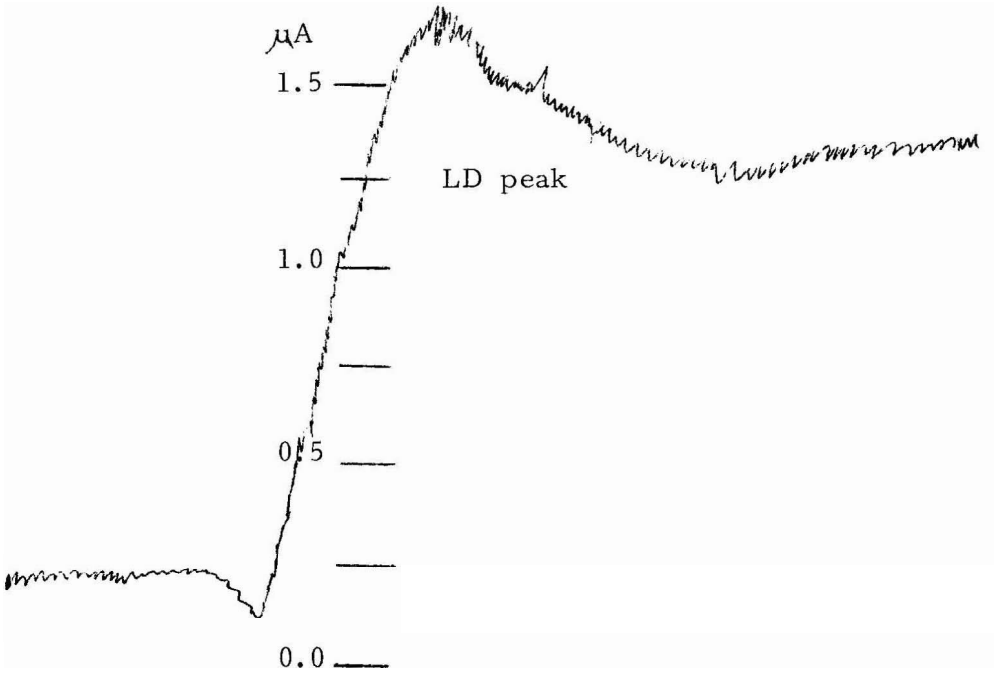
Next, a copious amount of wash solution (water) was passed through the cell between LD samples. In addition, sensitivity was decreased to 5 μA. It was hoped that by washing the electrode thoroughly, the baseline would gradually be reestablished. Figure 24 shows this was a false assumption, however, since the elevated baseline never fell, but was used as the true baseline by the next sample.

A similar attempt to stabilize the electrode was made using pyruvate as the wash solution. This ensured that no foreign substances



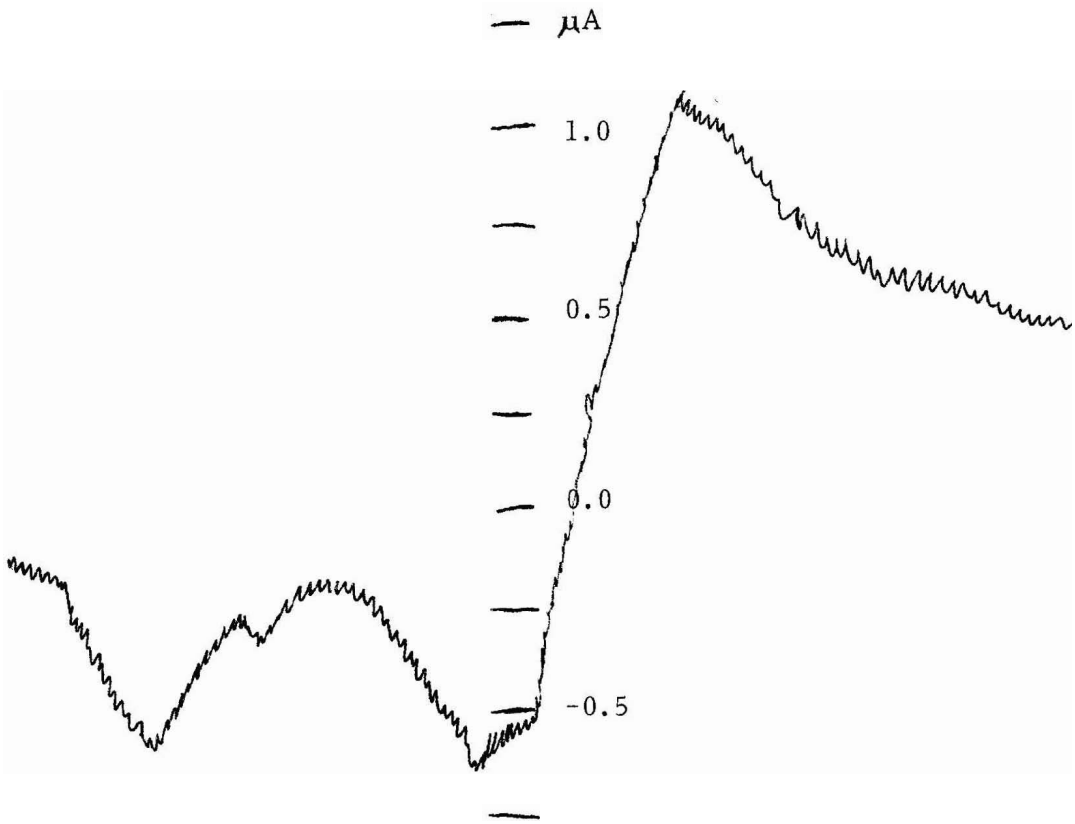

Reverse, L→P, Reaction

Figure 22



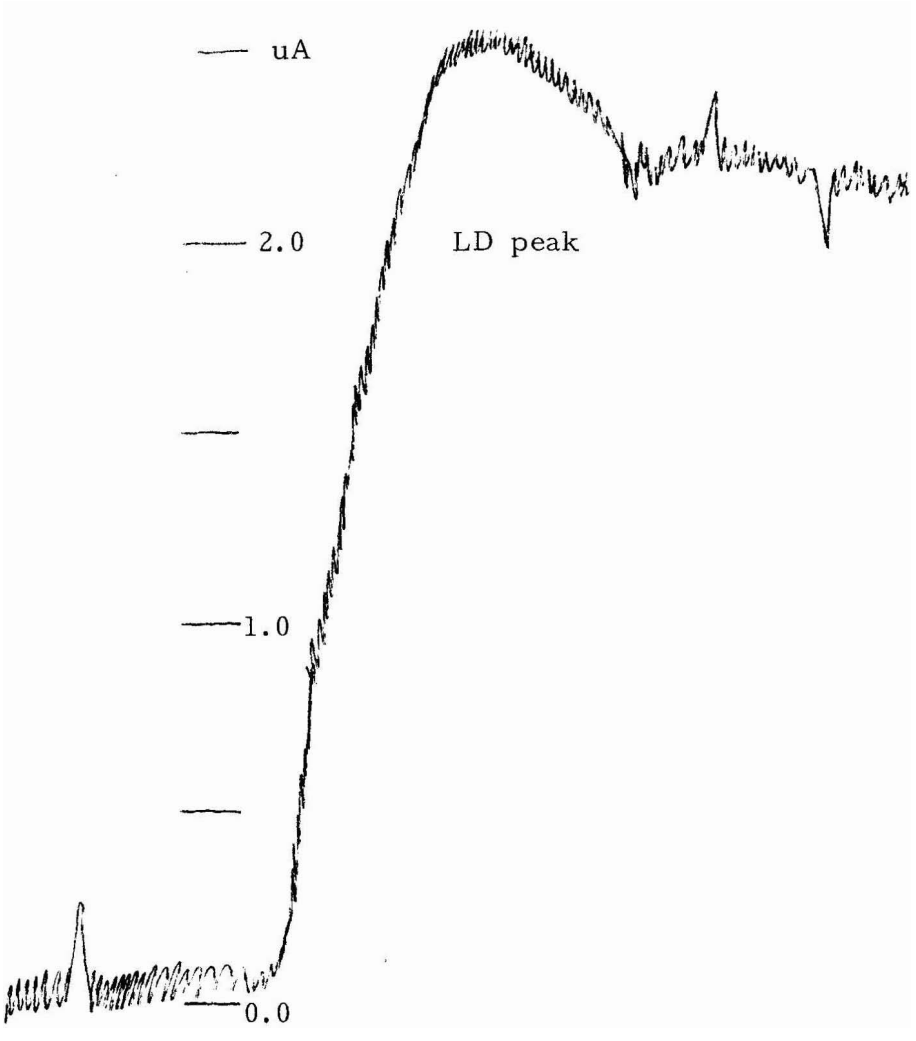

Contamination of Electrode Surface at Reduced Concentrations

Figure 23



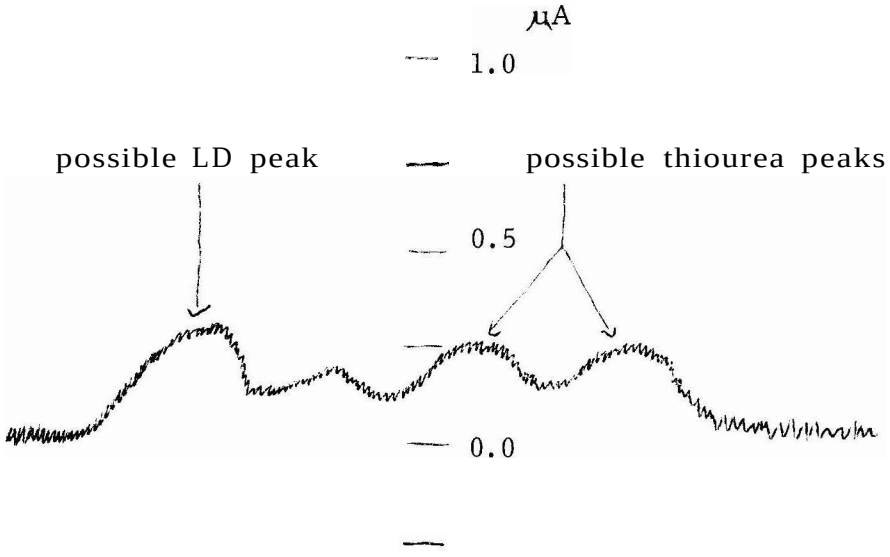

Failure to Recover Baseline Using Water as a Wash

Figure 24




Failure to **Recover** Baseline Using Pyruvate as a Wash

Figure 25




Use of Thiourea to Strip Electrode

Figure 26

# CHAPTER VI

# DISCUSSION OF RESULTS AND CONCLUSIONS

## Cyclic Voltammetry

Cyclic voltammetry provided the means of determining the potential at which NADH was oxidized to $NAD^+$. This value was found to be +0.6 v. The voltammograms were obtained using platinum working and auxiliary electrodes, and a Ag/AgCl reference. This experiment served as the foundation on which the remainder of this study was built.

## Optimization of Potential

Using 0.6 v as a starting point, the potential was optimized by recording the response of $NADH \rightarrow NAD^+$ at varying potential settings. In this manner, 0.7 v was found to be the optimal potential. That it was different from that found by cyclic voltammetry can be explained by the difference in the types of working and auxiliary electrodes used in each case.

## Gold Minigrid Cell

This cell had to be rejected since the thin foils which were used as the electrodes could not withstand the pressure of a flowing system,

## Carbon Paste-Silver Epoxy Cell

This cell was of sound design, but could not provide the necessary sensitivity. This was due to the proximity of the auxiliary and wor-

king electrodes, as well as to the position of the reference in the solution stream. That is, the reference should ideally have been positioned between the working and auxiliary electrodes.

## Carbon Paste-Stainlesssteel Cell

This cell proved to be sensitive and versatile, The current output could be reduced to 2 μA and the flow could be maintained in a steady, even fashion. Response to LD samples was fairly rapid and measurable current differences could be attained. After a time, however, adsorption of serum proteins and/or $NAD^+$ onto the working electrode surface was evidenced by the inability to recover the baseline after a sample of LD had been detected. To counteract this, several methods were tried, including reducing the concentration of NADH, washing the electrode with wate and pyruvate and using thiourea to strip the adsorbed species from the electrode. None of these methods were successful, however, and disassembly of the cell and regeneration of the surface proved to be the only feasible method of attaining the desired results.

## Conclusions

This study investigated the use of an electrochemical cell to detect the enzyme LD. This was accomplished indirectly by monitoring the conversion of NADH to $NAD^+$ at a working electrode. As NADH was oxidized at a potential of +0. 7 v, a steady output of current resulted. This formed the baseline for the subsequent experiments. When LD was introduced to the sample stream, NADH was converted to $NAD^+$ prior to reaching the working electrode. Thus, less reaction occurred at the electrode and the current dropped. By monitoring the drop in current, the activity of LD could be determined.

Although the method developed was fairly straight-forward, problems arose due to adsorption of serum proteins on the electrode surface. To remove the proteins, several methods were suggested. One of these was HPLC. Another was affinity chromatography, in which LD would be adsorbed onto the column and then eluted with $NAD^+$, nicotinamide or adenine. Finally, dialysis would be used to separate the LD from the $NAD^+$, so that a relatively pure, protein-free sample could be obtained. Since these techniques were beyond the scope of this study, attempts to separate the proteins prior to electrochemical detection are left for future study.

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