ELECTROCHEMICAL DETECTION OF LDH ISOENZYMES

by

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ABSTRACT

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This study deals with the design, construction and application of an electrophoresis apparatus, which implemented a twin electrode potentiostat as a detector of LDH isoenzymes, separated by agar gel electrophoresis.

A design of the twin electrode potentiostat was drawn. From this design a dual sided circuit board was constructed by the method of "circuit board photo-fabrication." Various electronic components were then soldered onto side 1 of the circuit board. Designs of the front and rear panels of the twin electrode potentiostat were drawn. From these designs, the twin electrode potentiostat was completely assembled.

An electrophoresis cell including an electrode system was designed and constructed. The electrode system here consists of two gold mini-grid working electrodes and silver mini-grid auxiliary electrode.

A cyclic voltammetry study was carried out in a cell with the twin electrode potentiostat, using two platinum electrodes for the working electrodes, a platinum

electrode for the auxiliary electrode and a S.C.E. for the reference electrode. Similar studies were carried out in the electrophoresis cell. These studies illustrated an oxidation-reduction mechanism with the twin electrode potentiostat.

Finally, a study using the twin electrode potentiostat as a detector of LDH isoenzyme separation, in serum, by agar gel electrophoresis, was carried out. This study illustrated the separation of the five LDH isoenzymes as plotted on an x-y recorder.

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I with to thank Dr. Thomas Ridgeway of the University of Cincinnati for the donation of the much needed dual sided circuit board of the twin electrode potentiostat.

I wish to thank the Media Center of Youngstown State University for the clear plastic and negative transparencies needed to construct the dual sided circuit board.

I wish to thank the Physics Department of Youngstown State University for permitting the use of their metal lathe, drill press and band saw.

For assistance in obtaining serum samples necessary to finish this study, I wish to thank Vicki Mincey, Medical Technologist of St. Elizabeth Medical Center.

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LIST OF ABBREVIATIONS

ABBREVIAVION	DEFINITION
AST	Aspartate Amino Transferase
C	Capacitor
CPK	Creatine Phosphokinase
CM-Cellulose	Carboxymethylcellulose
CR	Diode
DEAE-Cellulose	Diethylaminoethylcellulose
2,4-DNPH	2,4-dinitrophenylhydrazine
DIP	Dual Inline Pin
E	Potential
g	gram
INT	2-p-iodophenyl-3-p-nitrophenyl tetrazolium
H	Ohms
K	Kiloohm
i	Current
LD	Lactate Dehydrogenase
LDH	Lactate Dehydrogenase
LDH_1	First Lactate Dehydrogenase Isoenzyme
LDH_2	Second Lactate Dehydrogenase Isoenzyme
LDH_3	Third Lactate Dehydrogenase Isoenzyme
LDH_4	Fourth Lactate Dehydrogenase Isoenzyme
LDH_5	Fifth Lactate Dehydrogenase Isoenzyme
M	Megaohms
M	Molarity
µF	Microfarad
µL	Microliter

mg	Milligram
mV	Millivolt
NAD^+	Nicotinamide Adenine Dinucleotide-Oxidixed
NADH	Nicotinamide Adenine Dinucleotide-Reduced
P	Pyruvate
pI	Isoelectric Point
PMS	Phenazine Methosulfate
R	Resistor
S.C.E.	Saturated Calomel Electrode
V	Volt
W	Watt
Z	Chip Holder
ZD	Zener Diode

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CHAPTER I

INTRODUCTION

Basic Theory of Electrophoresis

Electrophoresis is perhaps the most powerful tool a researcher or clinician has to monitor life processes. It is the primary method used to analyze proteins and since proteins are literally the very building blocks of life itself, their detection and analysis is essential to any study of living processes. The most dramatic use of electrophoresis is as a diagnostic tool. As such, it offers the clinician a vast array of knowledge that is just beginning to be understood.[1]

Electrophoresis is simply the movement of charged molecules in an electric field. Three ingredients are necessary: (1) an electric field, (2) a charged particle, and (3) a medium in which the movement may occur. A Russian physicist, Reuss, first described the electrokinetic movement of colloids in 1809. L. Michailis, named the migration of colloidions in an electric field "electrophoresis" and the terminology has been used since.[1,2]

A. Tiselius is generally considered the modern day father of electrophoresis. His "moving boundary" electrophoresis of proteins in a U-tube greatly increased electrophoresis potential. Moving boundary electrophoresis

is commonly called "free electrophoresis" since it occurs strictly in a liquid without the benefit of a solid matrix.[1]

P. Konig, in 1939, reported using electrophoresis on paper strips saturated with an electrolyte to isolate a yellow pigment from snake venom and thus the beginning of a form of electrophoresis which utilized a solid matrix to hold the electrolyte. Tiselius suggested the name "zone electrophoresis" to encompass all forms of electrophoresis on a solid support medium and this form of electrophoresis has become highly used.[1]

Any solid material which can absorb or hold an electrolyte is a potential medium for zone electrophoresis Paper was first used, and is still popular today. Others, including agar gel, starch, acrylamide gel and cellulose acetate have proven successful since. The major advantage of zone electrophoresis lies in the stabilization of the migration, consequently the equipment and technique are much simpler.[1] A representative electrophoresis system is shown in figure 1.

Agar Gel Electrophoresis (AGE)

Agar gel electrophoresis has been successfully applied to the analysis of serum proteins, hemoglobin, lactate dehydrogenase (LDH) isoenzymes, lipoproteins, and other substances. In fact, this gel medium parallels cellulose acetate in versatility and convenience, and generally competes with other media for applicability

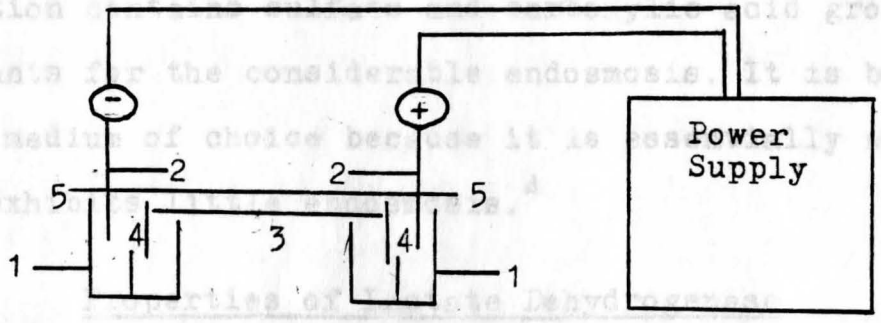


Figure 1. A Schematic Of A Typical Electrophoresis Apparatus.[3]

Two buffer boxes (1) with baffle plates contain the buffer used in the process. In each buffer box is an electrode (2), either platinum or carbon, the sign of which is fixed by the mode of connection to the power supply. The electrophoresis support (3) on which separation takes place is in contact with buffer by means of wicks (4). The whole apparatus may be covered (5). Direct current power supply may be either constant current (adjustable) or constant voltage (adjustable), both, or constant wattage.

to routine clinical laboratory demands.[4]

Impure or even purified agar is composed of at least two fractions; agaropectin and agarose. The former fraction contains sulfate and carboxylic acid groups and accounts for the considerable endosmosis. It is becoming the agar medium of choice because it is essentially neutral and exhibits little endosmosis.[4]

Properties of Lactate Dehydrogenase

Lactate Dehydrogenase (EC 1.1.1.27; L-lactate: NAD oxidoreductase) is a hydrogen transfer enzyme which catalyzes the oxidation of L-lactate to pyruvate with the mediation of NAD^+ as hydrogen acceptor. The reaction is reversible and the reaction equilibrium strongly favors the reverse reaction, namely the reduction of pyruvate to lactate (P→L).[5] (See Figure 2.)

The optimum pH for the lactate to pyruvate (L→P) reaction is 8.8 to 9.8; for the (P→L) reaction it is 7.4 to 7.8. The optimal pH's vary slightly with the source of enzyme and depend on the temperature, as well as on substrate and buffer concentrations.[6]

Definition of Isoenzymes

Enzymes exist in multiple molecular forms that have similar catalytic properties, but differ from one another in other respects. Isoenzyme is the name used to describe the multiple moleculal forms of a particular enzyme. It would not be possible to distinguish these

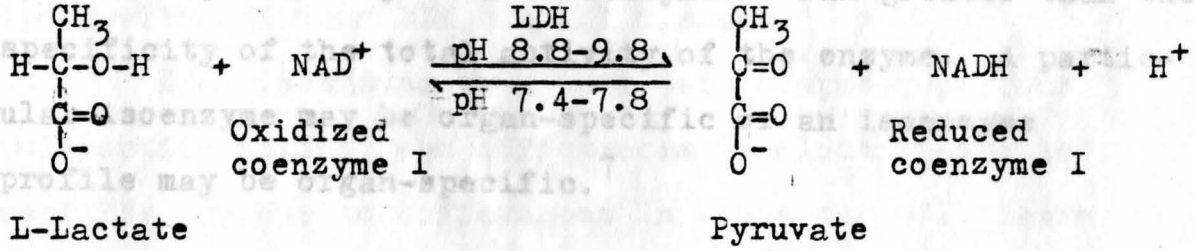


Figure 2. The LDH Reaction[5]

multiple molecular forms or isoenzymes, by measuring total enzyme activity since by definition they all catalyze the same chemical reaction. In order to identify and quantitate isoenzymes, we must take advantage of the difference in physical properties between isoenzymes. This is most conveniently accomplished using electrophoretic techniques. Once separated, each isoenzyme may be quantitated individually by measuring its reaction with the specific substrate. The value in separately measuring isoenzyme activity is that the specificity of an isoenzyme is far greater than the specificity of the total activity of the enzyme. A particular isoenzyme may be organ-specific or an isoenzyme profile may be organ-specific.[7]

Lactate Dehydrogenase Isoenzymes

Lactate Dehydrogenase (LDH) is an enzyme found in virtually all human tissues. The liver and skeletal muscle have the greatest concentration, followed by the heart. LDH is also present in red blood cells, kidney, lung, pancreas, and brain. Normal human serum contains small amounts of LDH due to normal tissue breakdown, but increases significantly following tissue damage.[8]

The fact that LDH, like most other enzymes, is found in numerous organ systems, limits its usefulness in confirming any specific disease. An elevated total LDH activity may indicate that something, somewhere is wrong but does not indicate which organ is damaged. It is possible in many cases to determine which organ is causing

the increase by examining the isoenzyme profile of LDH.[8]

Following electrophoresis, five isoenzymes of LDH can be demonstrated in human serum. Each isoenzyme is designated by a number which is related to its electrophoretic mobility. The fastest moving fraction (most anodic) is designated LDH_1 and is found primarily in heart muscle. The slowest moving (most cathodic) is LDH_5 and is found primarily in liver and skeletal muscle. The others, LDH_2 LDH_3 and LDH_4 are found to varying degrees along with LDH_1 and LDH_5 in all tissues.[9]

Each isoenzyme is a tetramer (composed of four polypeptide chains) and differences in electrophoretic mobility are due to differences in these chains. There are only two different polypeptide chains and a combination of these two chains make up the structure of the isoenzymes.[10]

The H polypeptide chain is so named because it is isolated from heart muscle and the M chain is isolated from skeletal muscle. LDH from heart muscle (LDH_1) is composed of four identical H chains while skeletal muscle and liver LDH (LDH_5) is composed of identical M chains.[8] The polypeptide composition of the five LDH isoenzymes is shown in Table 1. Table 2 shows the appropriate distribution of LDH isoenzymes in homogenates of various tissues and normal serum.

TABLE 1

COMPOSITION OF LDH ISOENZYMES[7,10]

ISOENZYME	COMPOSITION OF TETRAMER	
LDH_1	H H H H	H_4
LDH_2	H H H M	H_3M
LDH_3	H H M M	H_2M_2
LDH_4	H M M M	HM_3
LDH_5	M M M M	M_4

TABLE 2

DISTRIBUTION OF LDH ISOENZYMES IN HUMAN TISSUES (PERCENT)[7,11]

TISSUE	LDH_1 H_4	LDH_2 H_3M	LDH_3 H_2M_2	LDH_4 HM_3	LDH_5 M_4
Serum	25	35	20	10	10
Heart	40	35	20	5	0
Kidney	35	30	25	10	0
Brain	25	35	30	10	0
Lung	5	10	35	35	15
Thyroid	5	10	30	30	25
Bladder	5	10	40	35	10
Uterus	5	20	50	20	5
Bowel	5	30	45	10	10
Spleen	5	15	30	30	20
Liver	0	5	10	15	70
Skeletal Muscle	0	0	10	30	60
Erythrocytes	39	36	11	4	2

Although all LDH isoenzymes catalyze the same reversible reaction, (See Figure 2.), there is a difference amoung the isoenzymes as to substrate preference or which way the reaction will proceed. Tissues engaged primarily in aerobic metabolism contain more H than M chains and prefer the lactate → pyruvate reaction while tissues more involved in anaerobic metabolism possess more M chains and favor the pyruvate → lactate reaction.[12]

Diseases of the liver and skeletal muscle can be detected by demonstrating elevated LDH_5 activity. The relationship of LDH_2, LDH_3, and LDH_4, to various pathologies has not been well documented. The primary use of LDH is in the detection of myocardial infarction.[12]

LDH_2 is found in highest concentration in normal human serum. The normal ratio of LDH_1/LDH_2 is therefore less than 1. Following myocardial infarction, there is a substantial elevation in LDH_1 so that the LDH_1/LDH_2 ratio following MI is generally greater than 1. The use of this LDH_1/LDH_2 ratio to confirm a myocardial infarct will be discussed below.[12]

The LDH level begins to rise approximately 12-24 hours following myocardial infarction, frequently reaching levels two to three times (or greater) the upper limit of normal. Peak activity is usually reached on day 3-4 and activity may remain elevated for as long as two weeks after infarction. In 80% of the patients following an infarct, the "flipped" LDH_1/LDH_2 ratio will be found 48

hours after infarction.[12] Table 3 lists the LDH isoenzyme reference values.

Clinical Significance of LDH Isoenzymes

Elevated Total LDH:

The measurement of LDH is clinically useful in the recognition of myocardial infarction, rising two to ten times its normal value within 48 to 72 hours of the apparent onset of infarction. It remains elevated for five to ten days, a distinctly longer period than that usually found for AST or CK.[14]

Serum LDH activity is increased in cirrhosis, hepatitis and metastatic involvement of the liver. It is also elevated in cases of pulmonary embolism, progressive muscular dystrophy, megaloblastic anemia, infectious mononucleosis, and certain renal conditions such as infarction, transplantation and homograft rejection.[14]

Elevated levels of urine LDH occur in cancer of the kidneys or bladder and in glomerulonephritis, malignant hypertension, lupus nephritis, acute tubular necrosis, renal transplantation and homograft rejection, and sometimes in pyelonephritis.[14]

Elevations of Specific Isoenzymes:

The LDH isoenzymes released into the blood when tissue necrosis occurs alter the pattern to reflect that of the damaged tissue. Thus in myocardial infarction the proportion of activity of isoenzymes one and two increases

TABLE 3

LDH ISOENZYMES REFERENCE VALUES[13]

COMPOSITION	NAME	NORMAL VALUES	ORIGIN
H H H H	LDH_1	15-26%	Heart muscle, red blood cells, or kidney
M H H H	LDH_2	28-37%	Heart
M M H H	LDH_3	17-25%	Lung and other tissues
M M M H	LDH_4	9-18%	Many tissues
M M M M	LDH_5	7-17%	Skeletal muscle and liver

in serum while in liver damage there is an increase in the activity of isoenzyme five. A knowledge of the clinical history is required for proper interpretation of the pattern. Erythrocytes and kidney have an isoenzyme pattern similar to that of heart muscle, and so the serum LDH pattern will be essentially the same in myocardial infarction, pernicious anemia and renal infarction. LDH_3 elevation may be seen in extensive pulmonary pneumonia or advanced cancer.[13] Table 4 lists the LDH isograms in various diseases.

Introduction to Electrochemistry

Electrochemistry is a well developed specialty area of chemistry with a complete set of theories and quantitative relationships. In many respects, it is one of the oldest specialties of classical physical chemistry and traces its origins to the midnineteenth century.[15]

During the past two decades the dynamics and mechanisms of electron transfer processes have been studied by numerous groups throughout the scientific world. This has been made possible by applying transition state theory to electrochemical kinetic processes. As a result, both the kinetics of the electron transfer process (from solid electrode to the solution species), as well as of pre- and postchemical homogeneous processes, can be characterized quantitatively. This has brought about a much better understanding of heterogeneous electron-transfer

TABLE 4

LDH ISOGRAMS IN VARIOUS DISEASES[9]

	LDH 1	LDH 2	LDH 3	LDH 4	LDH 5
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				
Pericarditis	Normal				
Heart Block with Strokes-Adams syncope	Normal				
Hepatobiliary					
Hepatitis					+
Cirrhosis, Active					+
Cirrhosis, Inactive	Normal				
Hepatic Congestion					+
Acute Extrahepatic Obstruction	Normal				
Hematologic					
Pernicious Anemia	+	+	+		
Hemolytic Anemia	+	+	+		
Sickle Cell Anemia	+	+	+		
Megalablastic 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	+	+	+	+	+

mechanisms.[15]

The so-called Renaissance of electrochemistry has come about through a combination of modern electronic instrumentation and the development of a more pragmatic theory. Many challenging applications of modern electrochemistry have been undertaken during the past 20 years as a result of this Renaissance. Within the area of physical chemistry there have been numerous thermodynamic studies of unstable reaction intermediates. In addition, there have been extensive studies of the kinetics of electron-transfer processes both in aqueous and in non-aqueous media. The electrochemical characterization of adsorption phenomena has been of immense benefit to the understanding of catalytic processes.[16]

Some of the most exciting applications of electrochemistry have occurred in the areas of analytical chemistry and biochemistry. The control of an oxidation or reduction process through electrochemistry is often more precise than is possible with chemical reactions.[16]

Statement of Problem

Electrophoresis, the separation of charged particles in an electric field, has successfully been employed for years to fractionate biologically significant materials such as serum proteins, hemoglobin and enzymes. The major difficulty in any electrophoretic technique has been the detection and quantitation of the separated

fractions. For some, such as serum proteins and hemoglobin, a dye, usually Ponseau S, can easily be administered to the electrophoresis plate to stain the resolved fractions. The intense red spots, which correspond to the separated components, can then be quantitized using a densitometer.[17]

On the other hand, an enzyme such as LDH has been considerably more difficult to detect and quantitate. These isoenzymes resolve dramatically in an electrophoretic field. In the case of LDH, the differing mobilities are caused by charge/mass substitutions, giving each isoenzyme a different isoelectric point (pI). The isoelectric point of a protein is the main determinant of its migration rate in an electrical field.[8]

LDH activity may be detected employing colorimetry or fluorescence. Colorimetric methods available are of two types. In the first group, pyruvate is reacted with 2,4-dinitrophenylhydrazine (2,4-DNPH) to form the corresponding phenylhydrazone, which has a golden brown color at alkaline pH.[18]

The second group of colorimetric procedures is based on the reduction of such dyes as 2,6-dichlorophenolindophenol or 2-p-iodophenyl-3-p-nitrophenyl tetrazolium chloride (INT) by the NADH formed in the forward reaction. Phenazine methosulfate (PMS) serves as an intermediate electron carrier between the NADH and the dyes.[18]

Inasmuch as NADH shows strong fluorescence, both

the L⟶P and P⟶L reactions can be monitored fluorometrically, providing a considerably increased sensitivity. These methods are two point assays, and they are standardized against solutions of NADH or against enzyme solutions of known activity. The NADH formed in the L⟶P reaction can be determined by using diaphorase to reduce resazurin to resorufin, which can also be measured fluorometrically.[18]

It is proposed that a simpler, more precise and accurate method of detecting the isoenzyme electrophoretic bands of LDH be electrochemically employed using a twin electrode potentiostat. Unlike a conventional potentiostat, a bipotentiostat allows the application of two potentials to two independent electrodes.[19]

CHAPTER II

LITERATURE REVIEW

Methods

LDH isoenzymes can be separated and detected. There is much discussion in the literature concerning techniques for separation and detection of these isoenzymes. Chromatography and Electrophoresis are the two techniques most often used for LDH separation and detection. The results of various workers in this area are discussed below.

Electrophoretic Techniques

Electrophoretic Separation:

Several electrophoretic systems have been used in previous attempts to separate LDH isoenzymes. Electrophoretic separation of LDH has been studied using paper, starch block, cellulose acetate, agar gel, starch gel, or polyacrylamide gel as the electrophoretic medium.

Lactate dehydrogenase was already of considerable diagnostic importance when it became one of the first enzymes known to exist in multi-molecular forms. It was natural therefore that much of the impetus during the early stages of isoenzyme investigation should come from the clinical laboratory and, as paper electrophoresis was at that time the principal means for the routine

examination of serum proteins, several of the early isoenzyme separations were carried out by this technique.[20] These have included human and animal tissue lactate dehydrogenases.[21,22,23] The isoenzymes of human serum and tissue lactate dehydrogenase have also been separated by continuous paper electrophoresis.[24,25,26] The amount of material which can be applied to a paper strip is strictly limited, but when it is required to separate larger quantities the technique of continuous paper electrophoresis may be employed.[27,28,29,30]

Starch block electrophoresis was first introduced by Kunkel and Slater,[31] and its first application in the isoenzyme field was that of Vesell and Bearn,[32] who separated the isoenzymes of human serum lactate dehydrogenase. Starch block electrophoresis has proved to be a very useful procedure for enabling individual lactate dehydrogenase isoenzymes to be separated in quantities sufficiently large to permit the study of their properties.[33,34,35] It has also been employed for the separation of lactate dehydrogenase isoenzymes as an aid to the differential diagnosis of malignant and benign effusions.[36]

The most useful electrophoretic medium for the clinical laboratory is cellulose acetate. Cellulose acetate membranes have several advantages over paper as media for electrophoresis, since absorption of proteins is very much reduced and albumin 'tailing' is practically

eliminated.[37,38,39,40] Cellulose acetate has been extensively used for the clinical investigations of lactate dehydrogenase isoenzymes.[23,41,42,43,44,45] Cellulose acetate is also available in gel form in blocks and strips ('Cellogel' manufactured by Chemetron of Milan, Italy). It has proved satisfactory for the separation of the isoenzymes of lactate dehydrogenase.[46,47] Cellogel appears to have certain advantages over other media in that it is not necessary to prepare the gel immediately before use, and that relatively small amounts of the expensive staining reagents are required.[46,47]

Although Arrhenius studied ionic transport in agar gels as long as 1901, it was not until nearly fifty years later that protein fractionation by agar gel electrophoresis was reported,[48] and the first application of this technique to isoenzyme separation was that of Wieme.[49] Agar gel has several advantages over paper as a support medium for zone electrophoresis; since it does not absorb proteins, it can be prepared in a homogeneous state, it is transparent and the separated protein fractions can be determined by direct photometric measurement. It has high resolving power, and agar gel electrophoresis may readily be adopted for use on an ultramicro scale. It has the particular advantage that during isoenzyme separation passive diffusion is less than with paper or cellulose acetate. An excellent account of the technique and applications of agar gel electrophoresis has been

compiled by Wieme.[50] Wieme applied this procedure to the fractionation of mouse and rat tissue lactate and sorbitol dehydrogenases and to the lactate dehydrogenases of human pathological sera.[49,51,52,53] Other investigators have also used this technique for the study of isoenzymes of lactate dehydrogenase from a variety of tissues and species.[54,55,56,57]

Since the introduction of polyacrylamide gel as a medium for electrophoresis,[58] it has already found a considerable number of applications in the study of molecular heterogeneity of enzymes. Polyacrylamide has advantages over other gels in that it is optically clear, and suitably stained bands can be determined by scanning procedures. It can be used over a wide pH range. Complications due to electroosmotic flow do not normally occur with this medium. Polyacrylamide gel has high resolving power and, like starch gel, behaves as a molecular sieve, enabling proteins to be separated according to their molecular sizes as well as to their charges.[59] Unidimensional acrylamide gel electrophoresis has been applied to the separation of lactate dehydrogenase.[60,61,62] The lactate dehydrogenase isoenzymes have also been separated by a two dimensional technique.[59]

Electrophoretic Detection and Determination:

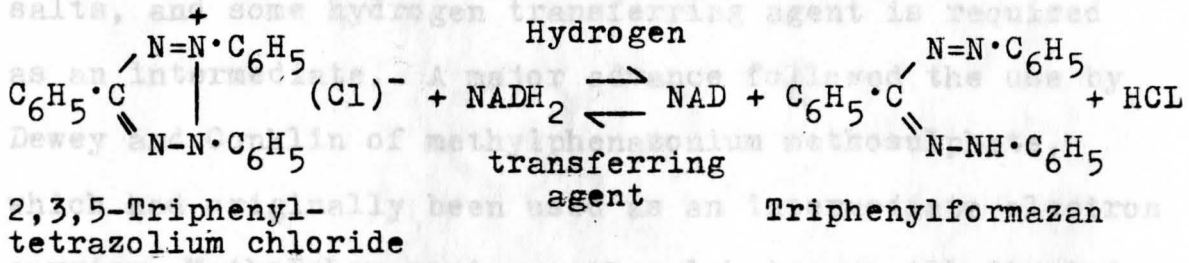
Several methods have been devised for the detection of isoenzymes separated by electrophoresis without recourse to their elution. Among these is the technique

of 'enzymoelectrophoresis' which is used for the detection of LDH isoenzymes in agar gel.[49]

Enzymoelectrophoresis has a number of advantages over the histochemical procedures described below, since spreading of the isoenzyme bands is kept to a minimum and there is no risk of enzyme action being affected by extraneous chemical substances required in staining procedures. Enzymoelectrophoresis provides quantitative information which is likely to be more reliable than that obtained by scanning a stained gel. On the other hand, this technique requires a specially modified spectrophotometer which may not be readily available in many laboratories.[53]

Tetrazolium salts, particularly triphenyltetrazolium chloride,have been used during the past two decades for the colorimetric detection of lactate dehydrogenase.[63] (See Figure 3)

Markert and Moller overlaid a starch gel lactate dehydrogenase electropherogram with an agar gel containing phosphate buffer, lactate, NAD, hydrazine, diaphorase, methylene blue and a tetrazolium salt. (See Figure 4) The reactions involved are quite complex. The pyruvate formed as a result of the enzyme action combines with the hydrazine, and so is removed from the scene, while the $NADH_2$ formed at the same time is oxidized back to NAD by the diaphorase, the hydrogen being transferred by the methylene blue to the tetrazolium salt which as



2,3,5-Triphenyltetrazolium chloride

Triphenylformazan

Figure 3. Reaction For The Colorimetric Detection Using A Tetrazolium Salt.[63]

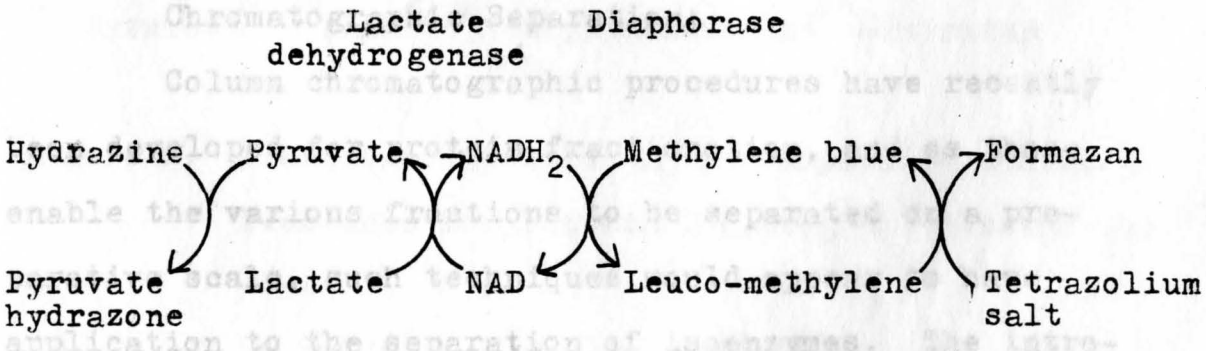


Figure 4. Reactions Involved In The Detection Of Lactate Dehydrogenase Isoenzymes By The Technique Of Markert And Moller.[64]

a result is reduced to the formazan.[64]

$NADH_2$ does not react directly with tetrazolium salts, and some hydrogen transferring agent is required as an intermediate. A major advance followed the use by Dewey and Conklin of methylphenazonium methosulphate, which had originally been used as an intermediate electron carrier. Methylphenazonium methosulphate, as illustrated in figure 5, catalyses the reaction between $NADH_2$ and the tetrazolium salt, thereby rendering unnecessary the use of diaphorase and methylene blue. Moreover, when methylphenazonium methosulphate is used gels stain satisfactorily when treated with an aqueous solution of the reagents. Reactions involved in the simplified staining procedure for LDH isoenzymes can be seen in Figure 6.

Chromatographic Techniques

Chromatographic Separation:

Column chromatographic procedures have recently been developed for protein fractionation, and as these enable the various fractions to be separated on a preparative scale, such techniques would appear to have application to the separation of isoenzymes. The introduction of the ion exchange celluloses, carboxymethylcellulose (CM-Cellulose) and diethylaminoethylcellulose (DEAE-Cellulose) for protein fractionation has been followed by the use of DEAE-Cellulose for the separation of the LDH isoenzymes.[66]

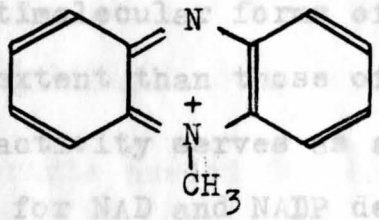


Figure 5. Methylphenazonium methosulphate.[65]

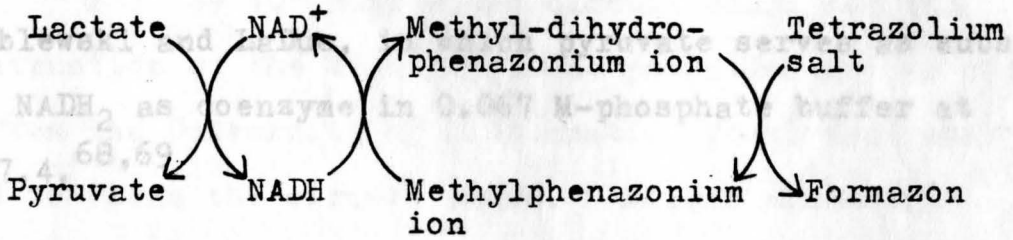


Figure 6. Reactions Involved In The Simplified Staining Procedure For Lactate Dehydrogenase Isoenzymes.[65]

DEAE-Sephadex has been used for the chromatographic separation of LDH isoenzymes.[67]

Chromatographic Detection and Determination:

Since the multimolecular forms of LDH have been studied to a greater extent than those of other enzymes, determination of its activity serves as a good example of the procedure used for NAD and NADP dependent dehydrogenases. This enzyme catalyses the reversible oxidation of lactate to pyruvate in the presence of NAD. Its activity may be determined spectrohpotometrically with either lactate or pyruvate as substrate, provided that the appropriate oxidized or reduced form of the coenzyme is incorporated into the reaction mixture. The most convenient procedure is that of Kubowitz and Ott modified by Wroblewski and LaDue, in which pyruvate serves as substrate and $NADH_2$ as coenzyme in 0.067 M-phosphate buffer at pH 7.4.[68,69]

CHAPTER III

MATERIALS AND APPARATUS

Materials

Necessary materials needed for constructing the working transparencies of the dual sided circuit board such as pre kut tape shapes (donut pads), pre kut stik ons (chip mounting pads) and precision slit tape were received from Bishop Graphics Inc., of Westlake Village, California. The Working Transparency clear plastic and negative transparancies were obtained from the Media Center of Youngstown State University.

A 7" by 10" dual sided circuit board for the construction of the twin electrode potentiostat was obtained from the University of Cincinnati. Steel wool was used to clean the circuit board. Various electronic components such as resistors, capacitors, chip holders, operational amplifiers, transistors, diodes, zener diodes, DIP switch connectors, on-off switches, 2-position switches, 3-position switches, 5-position switch, 10-position switches, potentiostats, colored insulated wire, BNC connectors and terminal connectors were obtained from Newark Electronics of Chicago, Illinois. For the housing of the twin electrode potentiostat, a metal cabinet was also obtained from Newark. After designing the cabinet, it was painted with Stambaugh's white enamel spray paint.

Dry transfers (Kabel Bond), manufactured by Mecanorma of Holland, were used to label the various components on the front and rear panels of the metal instrument cabinet. The cabinet was then sprayed with Stambaugh's clear enamel spray paint in order to protect the lettering. Plexiglas-rod, supplied by Baker Plastics Inc., was used to construct the electrophoresis cell. A septum, supplied by Fisher Scientific Co., was incorporated in the electrophoresis cell.

In constructing the electrode system of the electrophoresis cell, copper foil (J.T. Baker Chemical Co., Lot # 7438517), teflon spacers (Thermwell Products Co.), gold mini-grid (Buckbee-Mears Company), and silver mini-grid (Buckbee-Mears Company) were used.

All reagents used such as Agarose (J.T. Baker Chemical Co., Lot # 320103), NAD (Nutritional Biochemicals Corporation, Lot # 1687), Pyruvic Acid (Cal.Biochem, Lot # 5510), Potassium Chloride (Mallinchrodt Inc., Lot # 6858), NADH (Cal. Biochem, Lot # 800732), HCl, Ethanol, Ferric Chloride, Kodak Photo Resist, Type-3 (Kodak, Lot # 8102166), Kepro Developer (Kepro Circuit Systems Inc.) and KPR black dye (Kodak) were reagent grade.

The buffer used for the agar gel electrophoresis system and agar gel preparation (Tris-EDTA-Boric Acid pH. 8.2-8.6) was received from Helena Laboratories of Beaumont, Texas. The buffer was diluted with the specified amount of distilled deionized water as outlined in Chapter

IV, stored in the refrigerator when not in use, and prepared once every month.

All water used in this study, either for reconstitution of buffer or preparation of solutions, was distilled deionized.

All serum samples used in this study were obtained from Saint Elizabeth Medical Center.

Apparatus

A Kepro, BTX-200, Ultra-Violet Exposure Frame was used to expose the Kodak Photosensitive resist on the copper board to near ultraviolet radiation. The Kepro, BTE-202, Double Side Spray Etch was used to etch both sides of the copper board.

In making the agar gel, A Thermolyne, 1000, heat-stir apparatus was used. The entire electrophoresis cell, including electrode system, and twin electrode potentiostat were originally designed and constructed by my thesis advisor, Dr. Daryl W. Mincey, and myself at Youngstown State University. A Bio-Rad, 1420B, Constant Power Supply was used. Heath's, IR-5207, X-Y Recorder was used to monitor potential and current. Sabtronics, 2015A, Digital Multimeters were used to monitor the potential and current of each working electrode.

Throughout this study, a metal lathe and drill press, located in B-13, Ward Beecher Science Hall, Youngstown State University was extensively used.

CHAPTER IV

EXPERIMENTAL

Circuit Board Construction

A design for the twin electrode potentiostat is incorporated in Appendix A. From this design, a double sided circuit board was constructed through the process of circuit board photofabrication.

The steps involved in circuit board photofabrication were:[70]

1. A master 14" by 20" master artwork draft of each side of the twin electrode potentiostat, incorporating components only, was drawn from the design in Appendix A.
2. A 14" by 20" working transparency was constructed from the master artwork. This working transparency consists only of electronic components, not circuitry. Pre kut tape shapes (donut pads) and pre kut stik ons (IC chip pads) were transferred onto the working transparency with the aid of a light box.
3. From the 14" by 20" working transparency, two identical 7" by 10" negative transparencies were obtained from the Media Center of Youngstown State University.
4. From the master artwork showing the components and circuitry of each side of the circuit board, the circuitry was taped on the negative transparencies using precision slit tape. Both negative transparencies were taped, representing each one's specific side fo the circuit board.
5. The negative transparencies were temporarily scotch taped onto the copper board. A hole was drilled through each corner of the copper board, piercing both negative transparency and copper board per hole.

These holes were used for alignment purposes only.

6. Both sides of the copper board were cleaned free of oxide films and fingerprints by first scouring with steel wool and then wiping clean with trichloroethylene.

7. To complete a chemical surface treatment satisfactorily, the surfaces of the copper board must be dry. A time/temperature cycle of 120°C for 10 minutes was used.

8. Kodak Photo Resist, Type 3, was applied evenly with the aid of a Kim-Wipe across the first side of the copper board surface to obtain a thin consistent coating.

9. The photoresist was prebaked on the first side of the copper board at 120°C for 10 minutes. The purpose of the prebake was to remove any residual photoresist solvent that had not been eliminated by air drying.

10. Steps 7 and 8 were repeated for the second side of the copper board.

11. The copper board was allowed to cool to room temperature. After 15 minutes elapsed, the negative transparencies were scotch taped onto the board, being careful that component perforations and drilled holes align properly.

12. Kodak photoresists are sensitive to a particular range of the light spectrum. The peak spectral sensitivities of these resists are in the near ultraviolet, between 280 and 550 millimicrons. The Kodak photosensitive resist was exposed to near ultraviolet light in a Kepro, BTX-200, Ultra-Violet Exposing Frame. Each side of the copper board was exposed for 2 minutes.

13. The photosensitive resist coatings were developed. The copper board was submerged in Kepro developing solution for 2 minutes.

14. The entire copper board was rinsed with a slow running stream of water.

15. The copper board was placed in the oven at 120°C for 10 minutes to facilitate drying.

16. KPR black dye was sprayed with light and even strokes onto both sides of the copper board.

17. The copper board was washed with ethanol to rid the board of excess dye.

18. The circuitry of both sides of the copper board were examined for pinholing and/or image defects.

19. Both sides of the copper board were etched in a Kepro, BTE-202, Double Side Spray Etch. The etchant is a solution of ferric chloride in HCl.

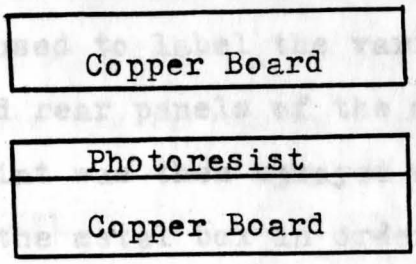
Figure 7 illustrates the fabrication of printed circuit material.[70] A circuit board illustration of each side of the twin electrode potentiostat is incorporated in Chapter V.

Circuit Board Assembly

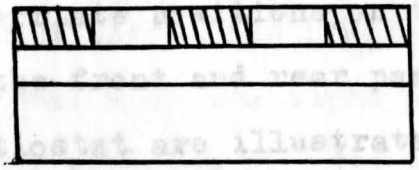
Once the double sided circuit board was etched, the next step was to drill the component pin holes. Then various components were placed into the appropriate pin holes and soldered on both sides of the circuit board.

Dual Electrode Potentiostat Front and Rear Instrument Panel Design and Construction

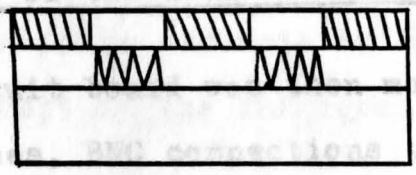
The next step was to lay out an appropriate design for the front and rear panels of the twin electrode potentiostat. An adequate design was drawn. The various component openings such as switches, potentiostats, BNC connectors and terminal connectors were sketched on the front and rear panels of the metal box as specified, drilled, reamed, then filed to a snug fit. The front and rear panels of the twin electrode potentiostat were



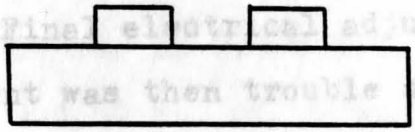
Copper board coated with photoresist.



Transparency in place on the surface.



The photoresist receiving exposure.



The exposed, developed photoresist layer.

Figure 7. The Fabrication Of Printed Circuit Material.[70]

Kodak photosensitive resist is a liquid formulation of resins. When applied to a Copper Surface and allowed to dry, they become sensitive to relatively short exposures to ultraviolet radiation. Ehen exposed to ultraviolet radiation, through a photographically reduced transparency, the resist "cross-links" or polymerizes in the areas that are struck by ultraviolet radiation. The image formed by the ultraviolet radiation and defined by the clear areas of the transparency is insoluble in the developing bath. The opaque areas of the transparency act as a mask and allow those portions that have received no exposure to be dissolved in the developer. The soluble portions wash away and leave a tough, chemically resistant image in minute relief on the surface.

then painted with white enamel spray paint. Now dry transfers were used to label the various components on the front and rear panels of the metal box. Clear enamel spray paint was then sprayed over the front and rear panels of the metal box in order to protect the delicate dry transfers. The components were installed into their appropriate positions on the metal box. A design of both the front and rear panels of the twin electrode potentiostat are illustrated in Chapter V.

Final Assembly of the Twin Electrode Potentiostat

The circuit board was then mounted onto the metal cabinet. Switches, BNC connections and terminal connectors were soldered into their appropriate positions on the circuit board. Final electrical adjustments were made and the instrument was then trouble shot.

Electrophoresis Cell Design and Construction

A design for the electrophoresis cell was drawn. The cell was milled from a solid plexiglas-rod using a metal lathe. An electrode system, incorporated within the cell, was then constructed using gold mini-grid for the two working electrodes and silver mini-grid for the auxiliary electrode. Strips of copper foil were incorporated between each electrode for proper electrical contact from the cell. Teflon spacers were used between all metal-cell and metal-metal junctions. All teflon spacers

and copper foil strips had a hole bored through the center to allow proper migration. A design of the electrophoresis cell and electrode system can be seen in Chapter V.

Preparation of Solutions

The first solution prepared was the buffer used for electrophoresis and in the preparation of the agar gel. The buffer was a Helena Supre Heme, Tris-EDTA-Boric Acid Buffer, pH 8.2-8.6. Two packets of buffer were reconstituted in 250 ml. of distilled deionized water to yield an ionic strength of 0.10. Once prepared, this buffer was kept in the refrigerator, and prepared monthly.

The following solutions were prepared for use in this study: 500 ml. of 0.5 M KCl, 50 ml. of 10 mmolar NAD in 0.5 M KCl and 50 ml. of 10 mmolar NADH in 0.5 M KCl.

Preparation of the Agar Gel

The Steps Involved in the Preparation of the Agar Gel were:[71]

1. 1.00 g. of agarose was weighed.
2. 50.0 ml of Tris-EDTA-Boric Acid Buffer and 3.0 ml. of 0.5 M KCl was diluted with distilled deionized water to 100.0 ml. in a volumetric flask.
3. The solution was transferred to a 500 ml. Erlenmeyer flask.
4. The volumetric flask was placed upside down with its long neck in the mouth of the Erlenmeyer to reduce

evaporation during boiling.

5. The flask was placed on a hot water bath implementing a Thermolyne-1000, heat/stir apparatus.

6. After 5 minutes, 1.00 g. of agarose was added.

7. After exactly 20 minutes, the erlenmeyer was taken out of the hot water bath and cooled under running tap water until the solution felt comfortably warm.

8. 25 mg. of NAD, and 0.40 g. of pyruvate was added to 25 ml. of gel.

9. The electrophoresis cell was poured with the contents of the erlenmeyer.

The agar gel was ready for electrophoresis 24 hours after preparation.

Background of the Twin Electrode Potentiostat

A cyclic voltammetry study of NADH was carried out employing the twin electrode potentiostat. Two platinum electrodes were used for the working elecrtodes, platinum electrode for the auxiliary electrode and S.C.E. for the reference electrode.

A study of NAD^+ was carried out in the electrophoresis cell. Two gold mini-grid electrodes were used for the working electrodes, silver mini-grid electrode for the auxiliary electrode and S.C.E. for the reference electrode is shown in Figure 9.

A study of 0.5 M KCl (background) was carried out in the electrophoresis cell consisting of the gold-silver mini-grid electrode system as can be seen in Figure 8.

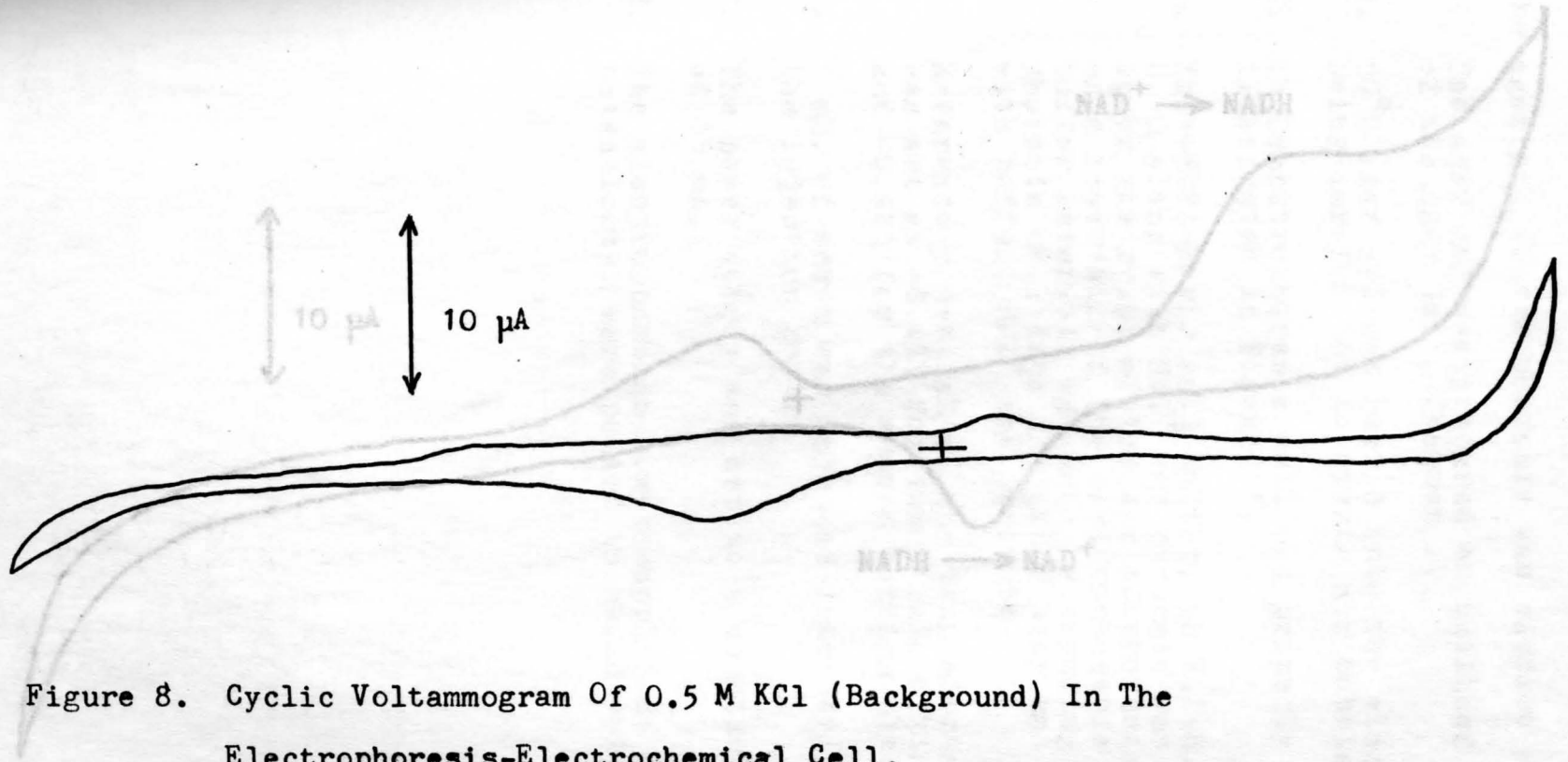


Figure 8. Cyclic Voltammogram Of 0.5 M KCl (Background) In The Electrophoresis-Electrochemical Cell.

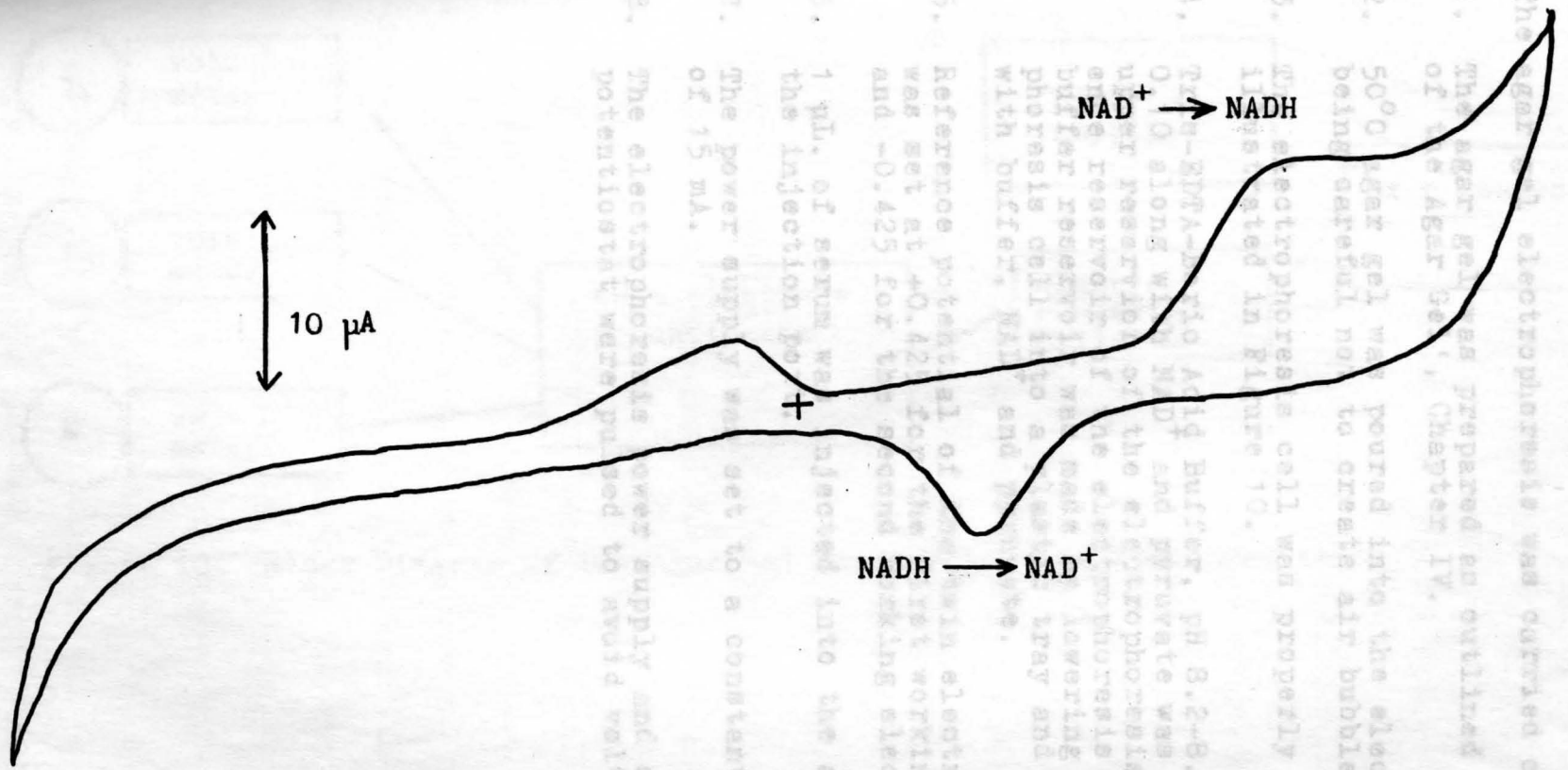


Figure 9. Cyclic Voltammogram Of 10 mmol. NAD^+ In 0.5 M KCl Supporting Electrolyte In The Electrophoresis-Electrochemical Cell

Agar Gel Electrophoresis

The agar gel electrophoresis was carried out as follows:

1. The agar gel was prepared as outlined in 'Preparation of the Agar Gel', Chapter IV.

2. 50°C agar gel was poured into the electrophoresis cell, being careful not to create air bubbles.

3. The electrophoresis cell was properly connected as illustrated in Figure 10.

4. Tris-EDTA-Boric Acid Buffer, pH 8.2-8.6, ionic strength 0.10 along with NAD^+ and pyruvate was added to the upper reservior of the electrophoresis cell and reference reservoir of the electrophoresis cell. A lower buffer reservoir was made by lowering the electrophoresis cell into a plastic tray and filling it with buffer, NAD^+ and pyruvate.

5. Reference potential of the twin electrode potentiostat was set at +0.425 for the first working electrode and -0.425 for the second working electrode.

6. 1 µL. of serum was injected into the agar gel through the injection port.

7. The power supply was set to a constant current mode of 15 mA.

8. The electrophoresis power supply and dual electrode potentiostat were pulsed to avoid voltage crossover.

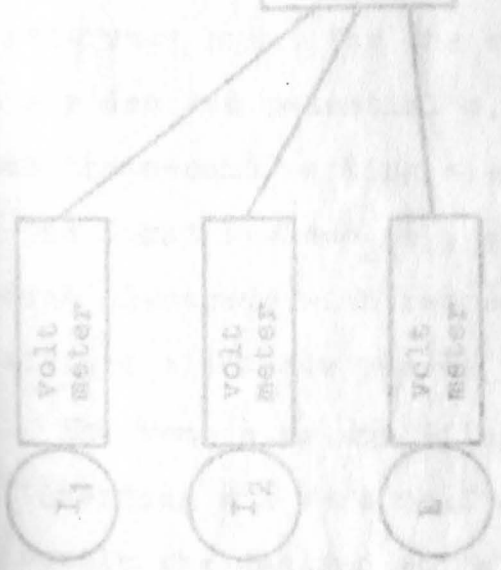


Figure 10. Block Diagram Of The Agar Gel Electrophoresis Cell

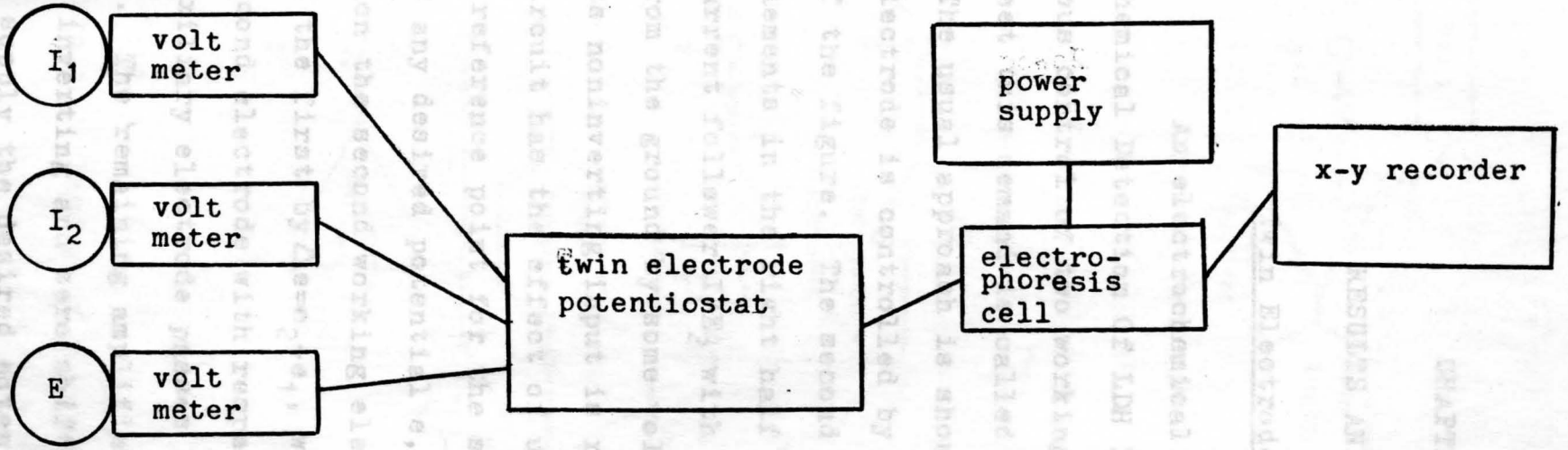


Figure 10. Block Diagram Of The Agar Gel Electrophoresis Instrumentation.

CHAPTER V

RESULTS AND DISCUSSION

Twin Electrode Potentiostat

An electrochemical experiment such as "The Electrochemical Detection Of LDH Isoenzymes" requires simultaneous control of two working interfaces. A device that will meet this demand is called a twin electrode potentiostat. (The usual approach is shown in Figure 11). The first electrode is controlled by the elements in the left half of the figure. The second electrode is controlled by the elements in the right half of the figure. One finds a current follower I/E_2 with a summing point held away from the ground by some voltage difference Δe, because its noninverting input is referenced to ground by Δe. This circuit has the effect of using the first electrode as a reference point for the second. We can set the first at any desired potential e, with respect to the reference, then the second working electrode is offset with respect to the first by $\Delta e = e_2 - e_1$, where e_2 is the potential of the second electrode with respect to the reference. The auxiliary electrode passes the sum of currents i_1 and i_2. The remaining amplifiers (Ext. In. and Δe) serve as inverting and zero shifting stages. They allow one to supply the desired potential e_2 at the imput without

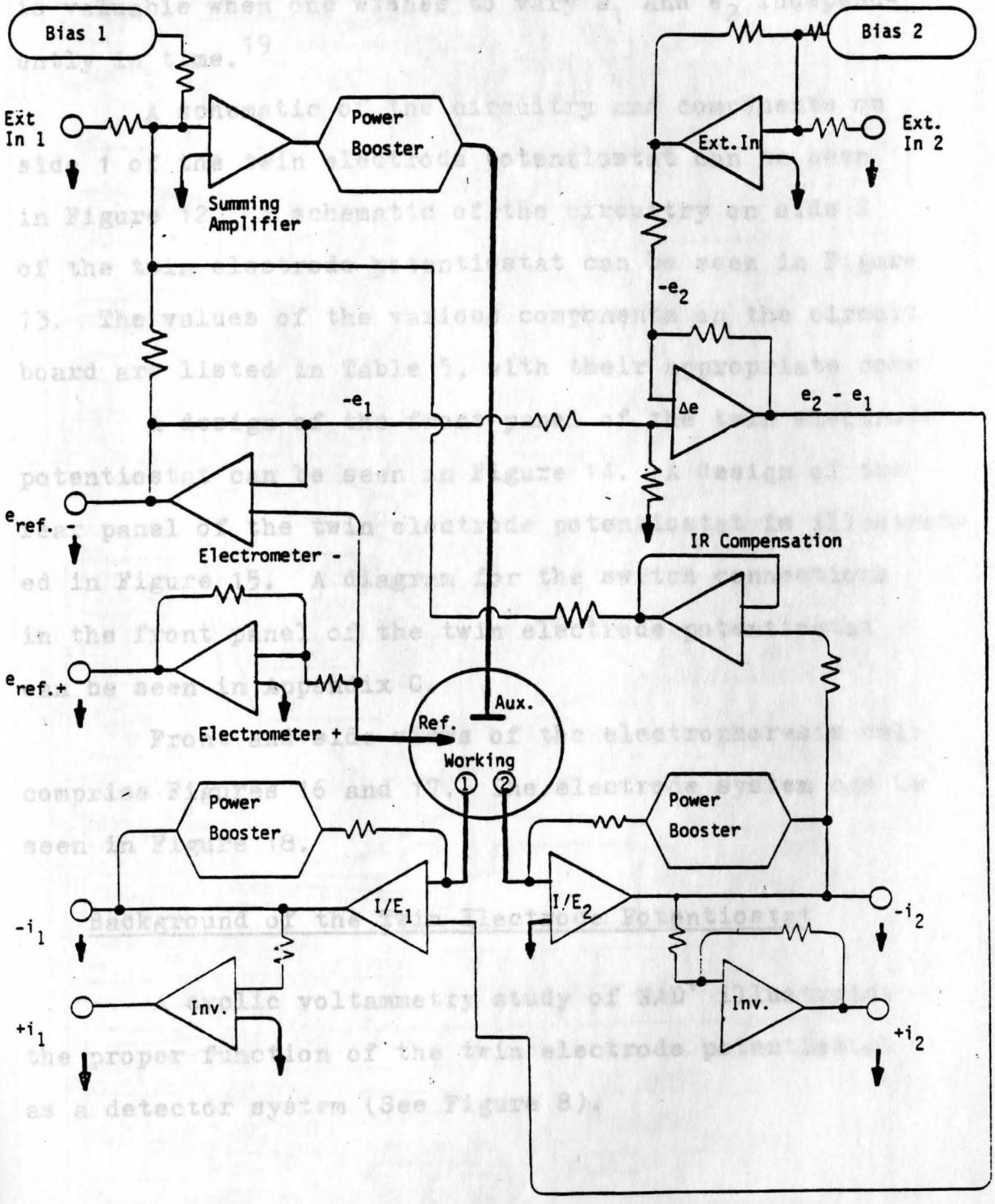


Figure 11. Design Of A Typical Twin Electrode Potentiostat.

without concern for the value of e_1. Such a convenience is valuable when one wishes to vary e_1 and e_2 independently in time.[19]

A schematic of the circuitry and components on side 1 of the twin electrode potentiostat can be seen in Figure 12. A schematic of the circuitry on side 2 of the twin electrode potentiostat can be seen in Figure 13. The values of the various components on the circuit board are listed in Table 5, with their appropriate code.

A design of the front panel of the twin electrode potentiostat can be seen in Figure 14. A design of the rear panel of the twin electrode potentiostat is illustrated in Figure 15. A diagram for the switch connections in the front panel of the twin electrode potentiostat can be seen in Appendix C.

Front and side views of the electrophoresis cell comprise Figures 16 and 17. The electrode system can be seen in Figure 18.

Background of the Twin Electrode Potentiostat

A cyclic voltammetry study of NAD^+ illustrates the proper function of the twin electrode potentiostat as a detector system (See Figure 8).

Figure 12. Side 1 Of The Circuit Board Comprising The Twin Electrode Potentiostat.

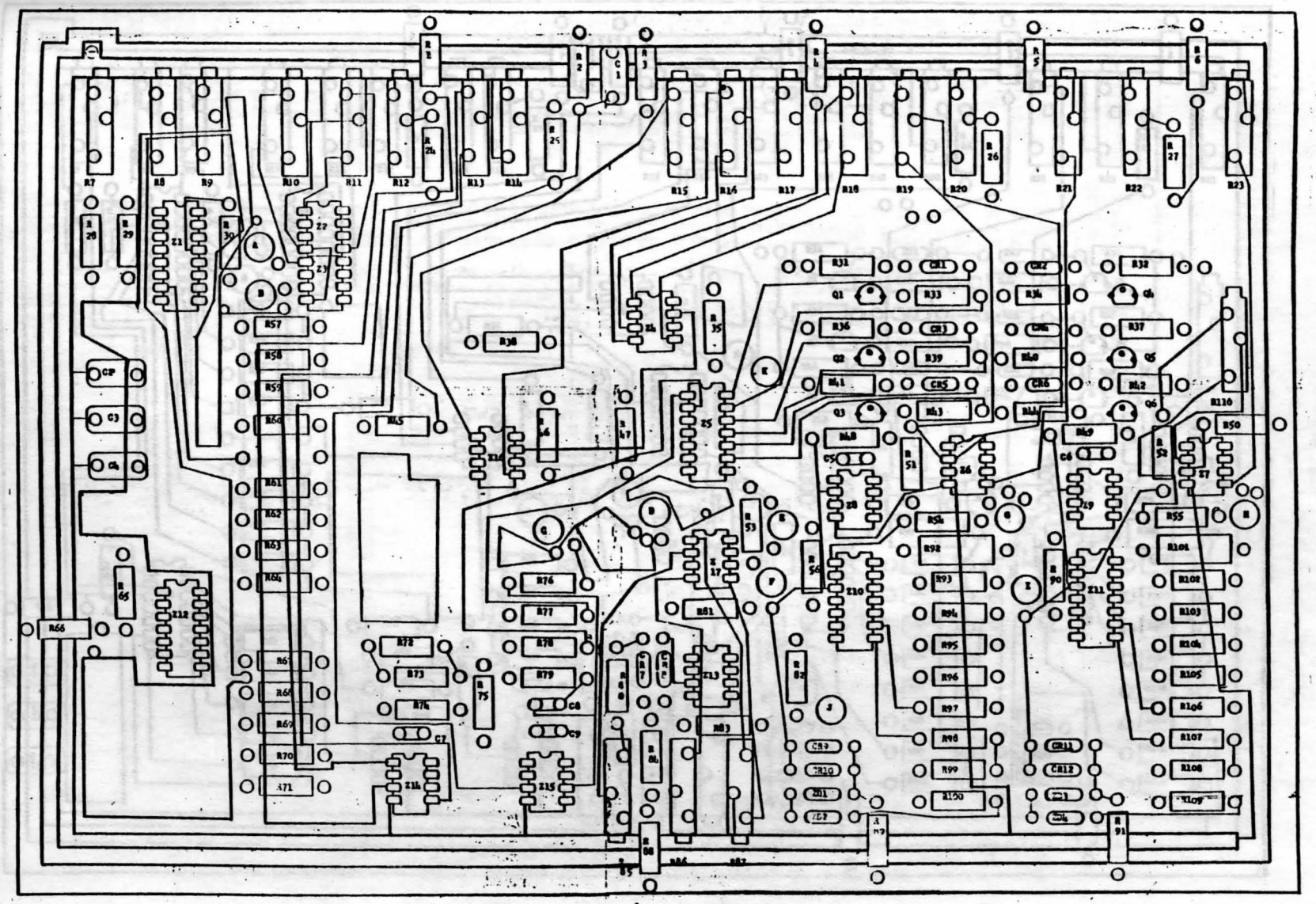


Figure 12. Side 1 Of The Circuit Board Comprising The Twin Electrode Potentiostat.

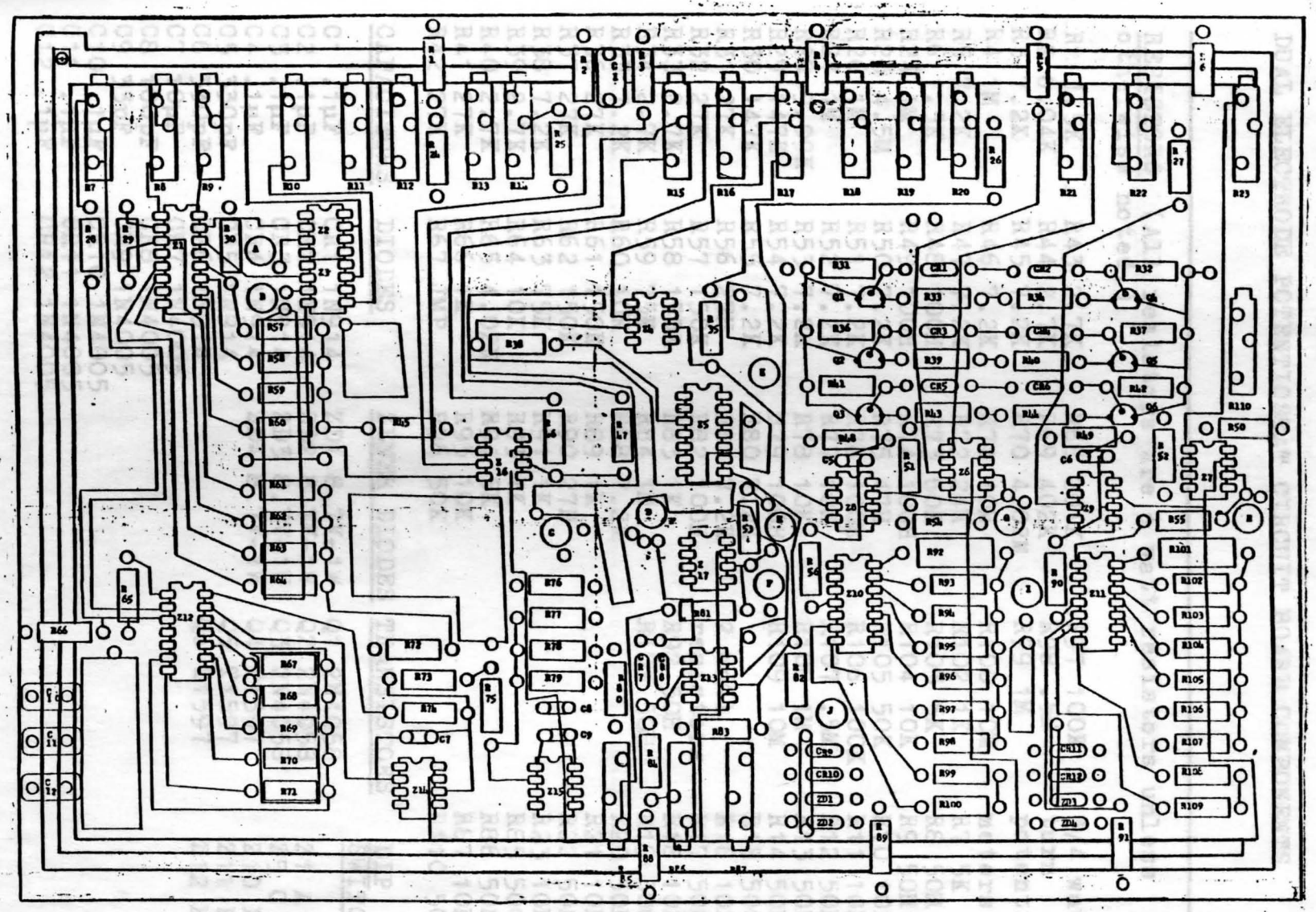


Figure 13. Side 2 Of The Circuit Board Comprising The Twin Electrode Potentiostat.

TABLE 5

DUAL ELECTRODE POTENTIOSTAT CIRCUIT BOARD COMPONENTS

RESISTORS (All resistors are ½ watt resistors unless otherwise noted.)

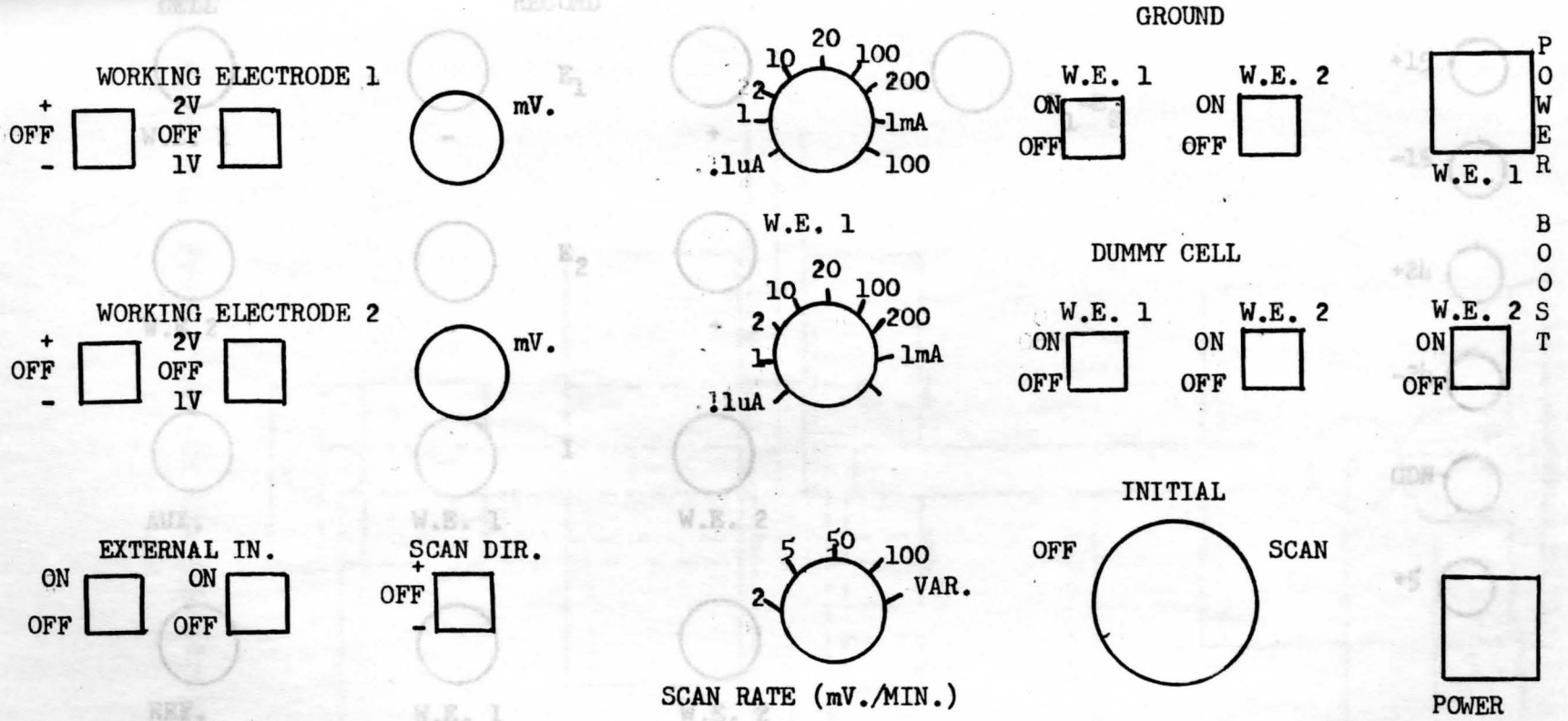
R1 1.5K	R43 2.7K	R68 201K	R97 100K	3/4 watt 15
R2 6.04K	R44 2.7K	R69 402K	R98 .5M	turn
R3 7.2K	R45 7.2K	R70 4.02M	R99 1M	potentio-
R4 1M	R46 7.2K	R71 10M	R100 10M	meters
R5 7.2K	R47 7.2K	R72 24M	R102 1K	R7 5K
R6 1.5K	R48 100H	R73 60K	R103 5K	R8 50H
R24 1M	R49 100H	R74 100H	R104 10K	R9 50H
R25 4.5M	R50 7.2K	R75 47H	R105 50K	R10 10K
R26 1M	R51 7.2K	R76 100H	R106 100K	R11 10K
R27 1M	R52 7.2K	R77 10K	R107 .5M	R12 50K
R28 9.09K	R53 7.2K	R78 10K	R108 1M	R13 50K
R29 147K	R54 7.2K	R79 100H	R109 10M	R14 50K
R30 147K	R55 7.2K	R80 7.2K		R15 500H
R31 27K	R56 27H	R81 7.2K	2 watt	R16 10K
R32 27K	R57 150K	R82 100H	resistors	R17 500H
R33 2.7K	R58 150K	R83 1K	R92 50H	R18 10K
R34 2.7K	R59 75K	R84 1M	R101 50H	R19 500H
R35 7.2K	R60 10K	R88 7.2K		R20 50K
R36 27K	R61 150K	R89 1K		R21 10K
R37 27K	R62 150K	R90 27H		R22 50K
R38 7.2K	R63 75K	R91 1K		R23 10K
R39 2.7K	R64 10K	R93 1K		R85 500H
R40 2.7K	R65 4.02K	R94 5K		R86 50K
R41 27K	R66 1K	R95 10K		R87 10K
R42 27K	R67 JMP	R96 50K		R110 500H

CAPACITORS	DIODES	ZENER DIODES	TRANSISTORS	DIP SWITCHES
C1 .1µF	CR1 1N914	ZD1 8.7V-1W	Q1 2N4058	
C2 .1µF	CR2 1N914	ZD2 8.7V-1W	Q2 2N4058	Z1 A
C3 .1µF	CR3 1N914	ZD3 8.7V-1W	Q3 2N4058	Z5 C
C4 .1µF	CR4 1N914	ZD4 8.7V-1W	Q4 T1597	Z10 D
C5 330pF	CR5 1N914		Q5 T1597	Z11 E
C6 330pF	CR6 1N914		Q6 T1597	Z12 B
C7 10µF	CR7 1N4005			
C8 100pF	CR8 1N4005			
C9 33pF	CR9 1N4005			
C10 .1µF	CR10 1N4005			
C11 .1µF	CR11 1N4005			
C12 .1µF	CR12 1N4005			

TABLE 5

DUAL ELECTRODE POTENTIOSTAT CIRCUIT BOARD COMPONENTS

OPERATIONAL AMPLIFIERS (OA)	BNC CONNECTIONS
Z2 741	A E_1 ext.
Z3 741	B E_2 ext.
Z4 3140	C AUX.$_{out.}$
Z6 3140	D REF.$_{in.}$
Z7 3140	E $+E_{out.}$
Z8 3140	F I_1 in.
Z9 3140	G I_1 out.
Z13 3140	H I_2 out.
Z14 3140	I I_2 in.
Z15 301	J $-E_{out.}$
Z16 3140	K ΔE
Z17 3140	



GEMINI
TWIN ELECTRODE POTENTIOSTAT

Figure 14. Front Panel Of The Twin Electrode Potentiostat.

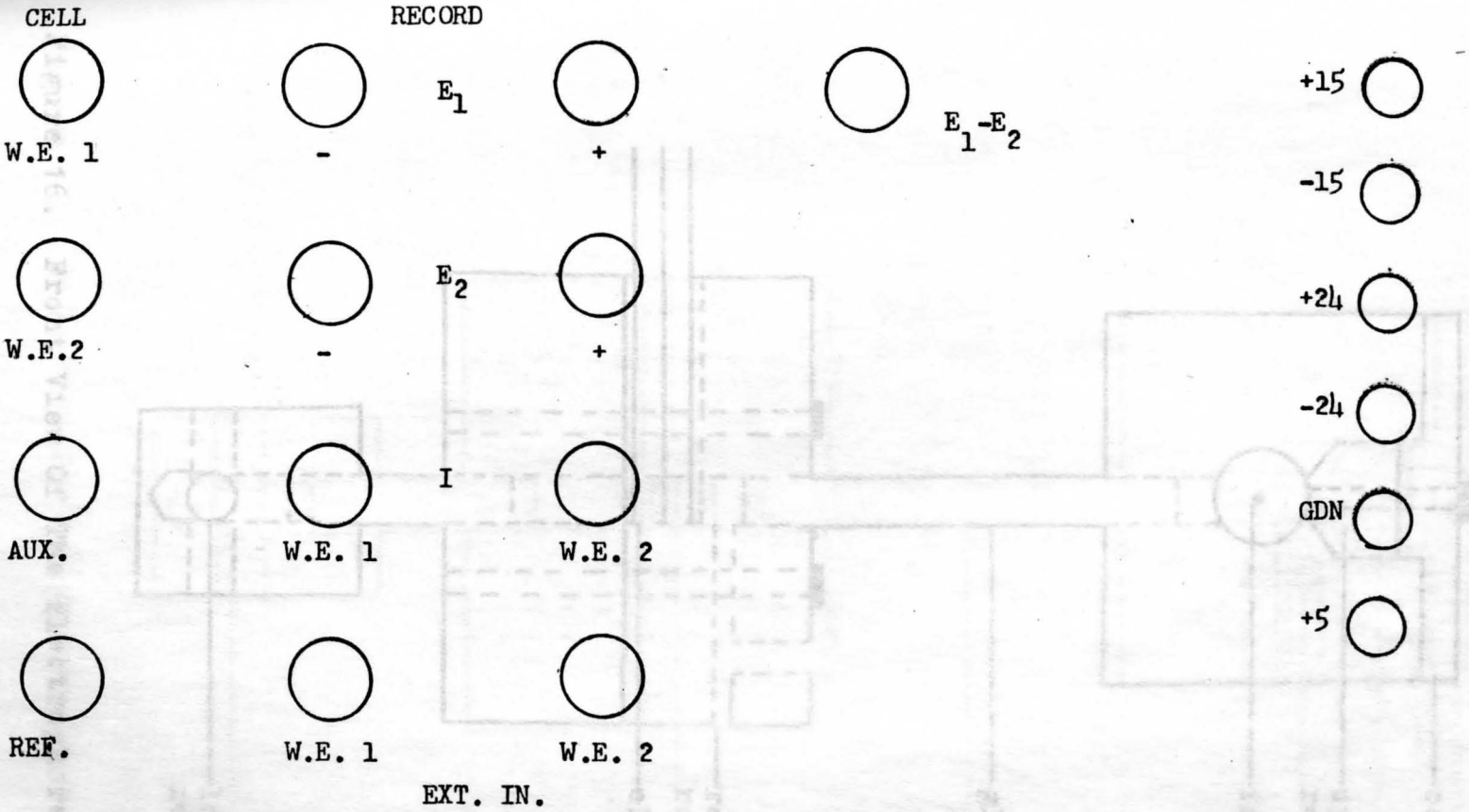


Figure 15. Rear Panel Of The Twin Electrode Potentiostat.

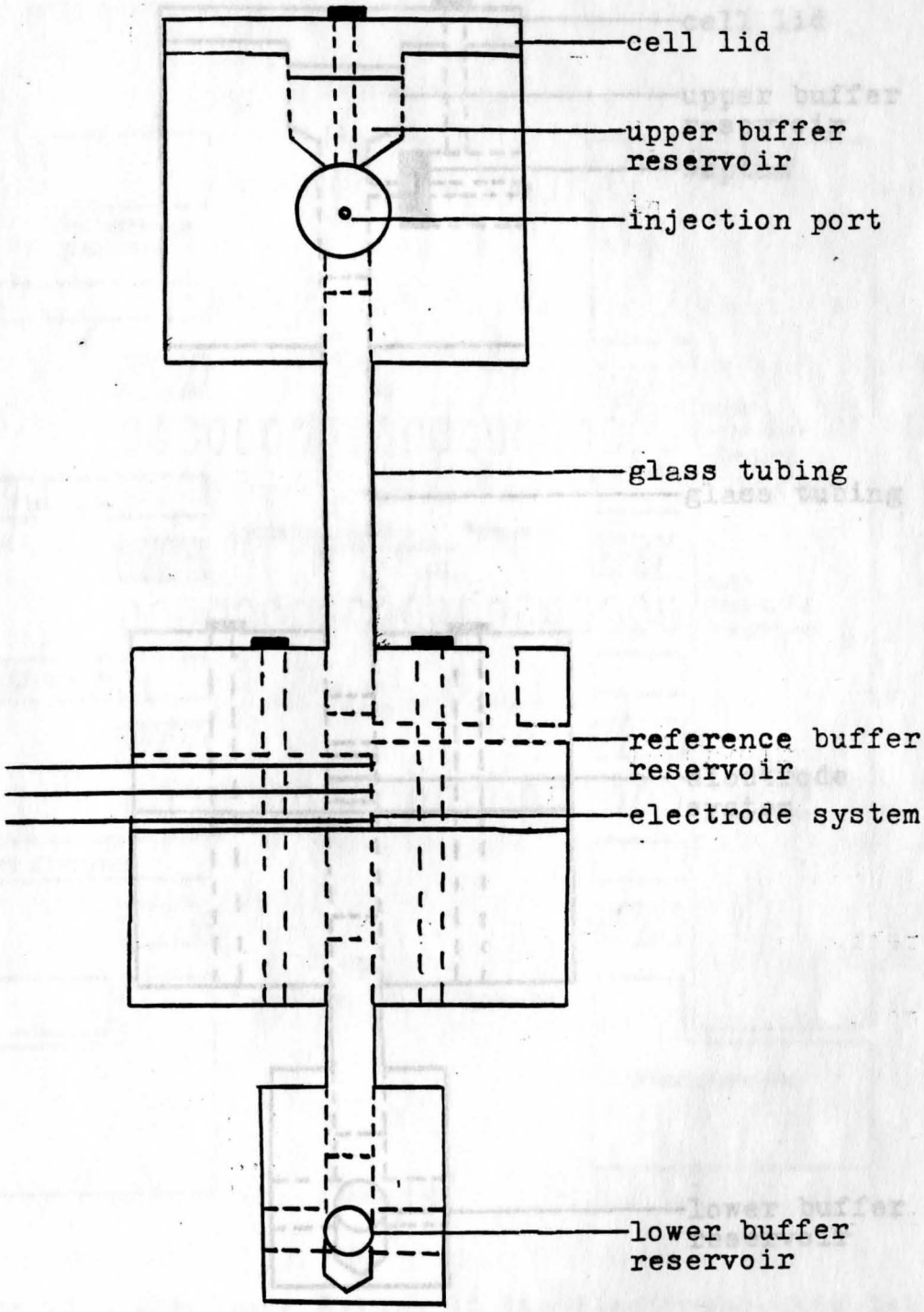


Figure 16. Front View Of The Electrophoresis Cell.

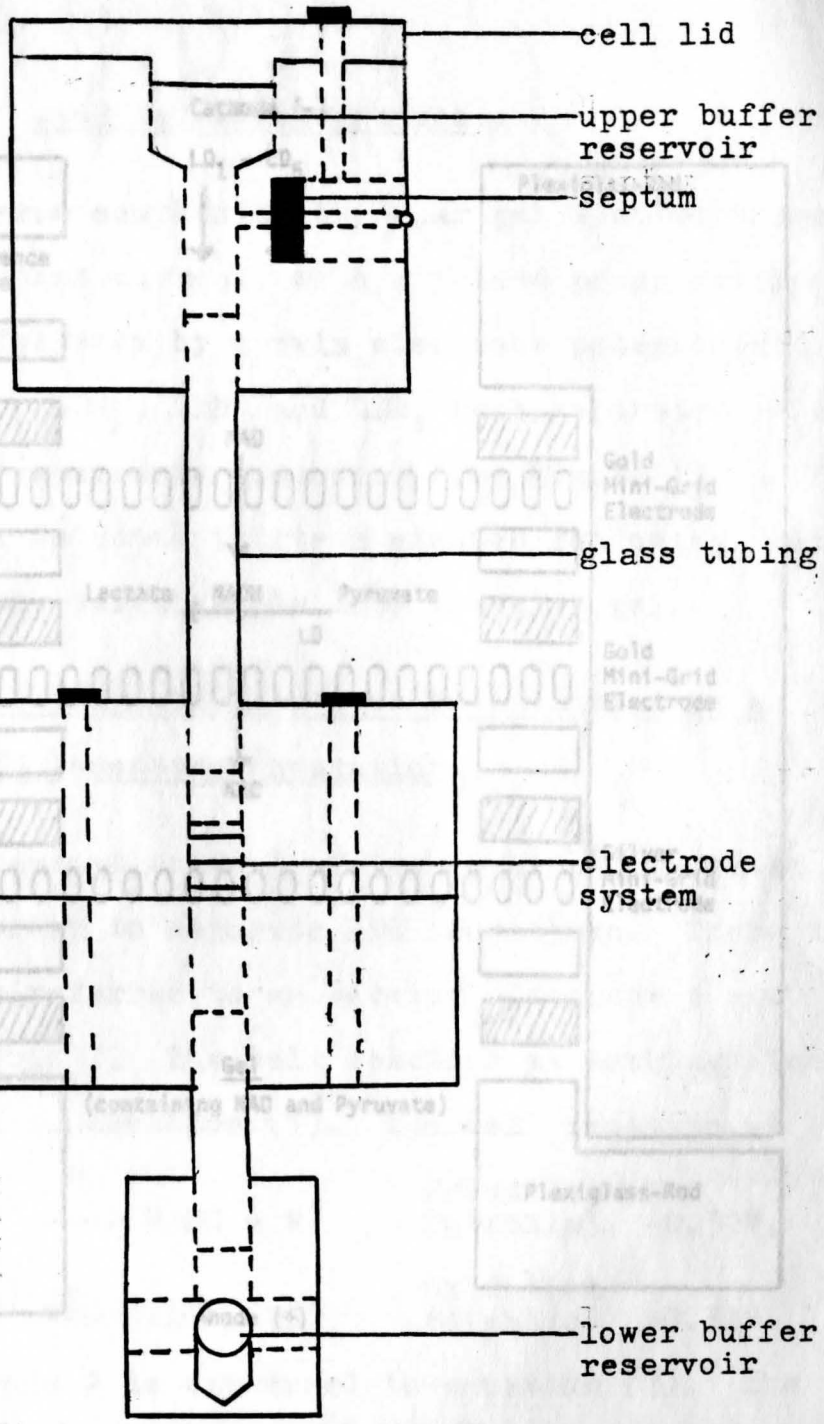


Figure 17. Side View Of The Electrophoresis Cell.

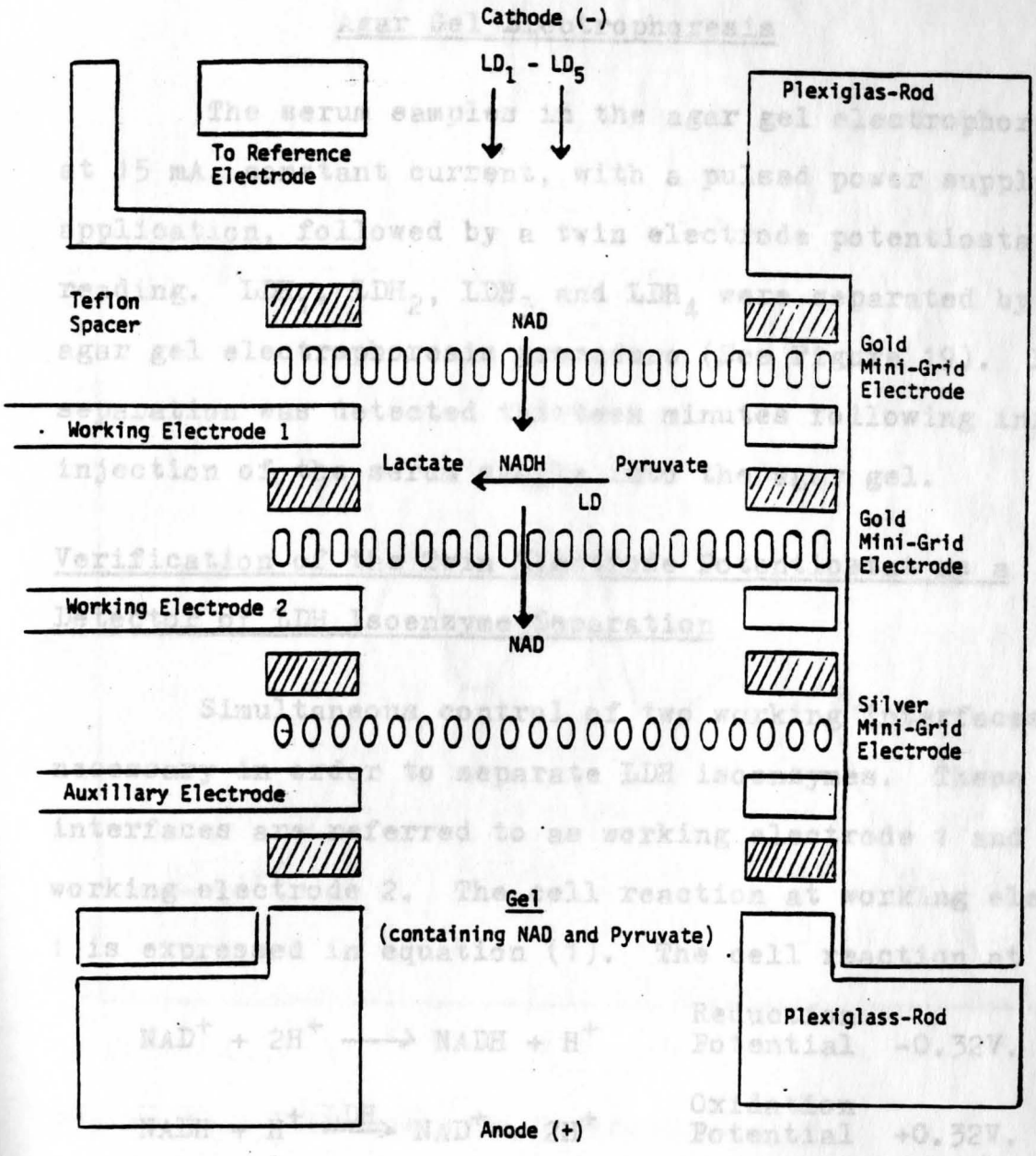


Figure 18. Electrode System Of The Electrophoresis Cell Showing The Cell Reaction.

(See Figure 9).

Agar Gel Electrophoresis

The serum samples in the agar gel electrophoresed at 15 mA. constant current, with a pulsed power supply application, followed by a twin electrode potentiostat reading. LDH_1, LDH_2, LDH_3 and LDH_4 were separated by this agar gel electrophoresis procedure (See Figure 19). LDH_1 separation was detected thirteen minutes following initial injection of the serum sample into the agar gel.

Verification of the Twin Electrode Potentiostat as a Detector of LDH Isoenzyme Separation

Simultaneous control of two working interfaces is necessary in order to separate LDH isoenzymes. These two interfaces are referred to as working electrode 1 and working electrode 2. The cell reaction at working electrode 1 is expressed in equation (1). The cell reaction at

$$NAD^+ + 2H^+ \longrightarrow NADH + H^+ \qquad \text{Reduction Potential } -0.32V. \qquad (1)$$

$$NADH + H^+ \xrightarrow{LDH} NAD^+ + 2H^+ \qquad \text{Oxidation Potential } +0.32V. \qquad (2)$$

working electrode 2 is expressed in equation (2). The second electrode is monitored for LDH isoenzyme separation. Without the simultaneous control of two working electrodes, LDH isoenzymes couldn't be separated. As a result, a one electrode potentiostat couldn't be used to separate LDH isoenzymes.

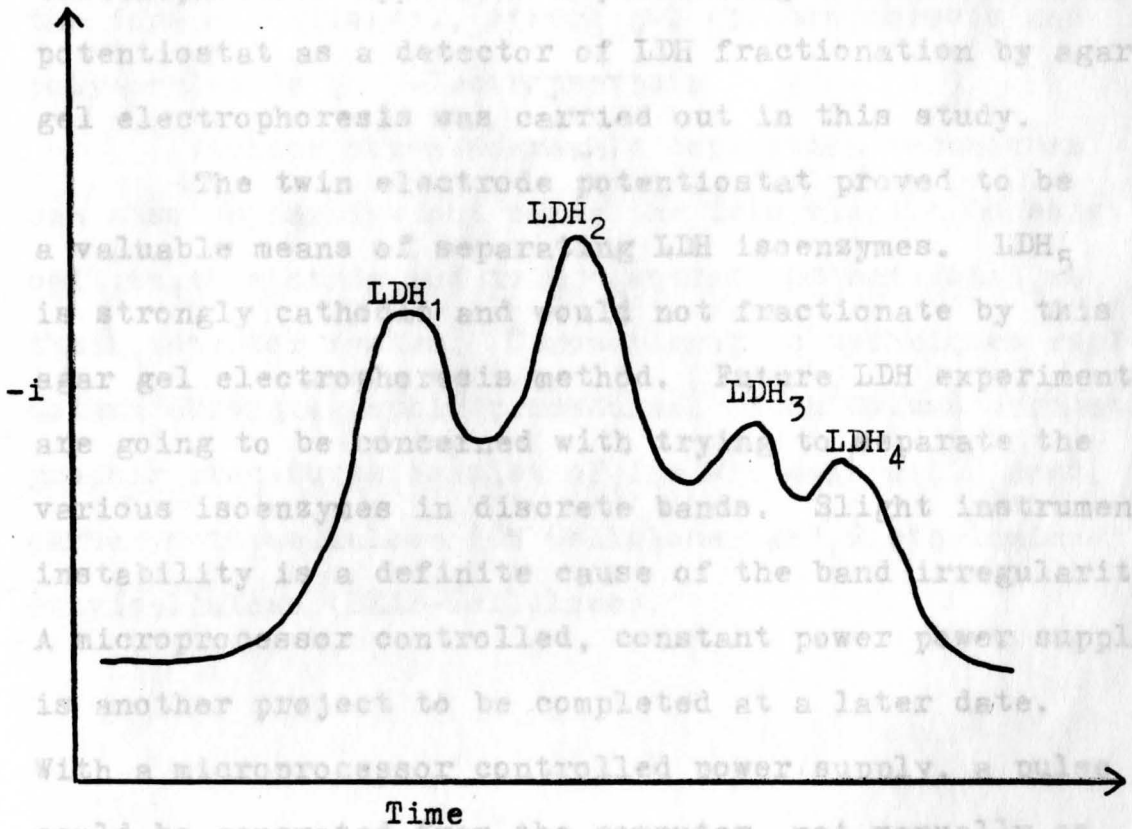


Figure 19. LDH Isoenzyme Separation.

CHAPTER VI

CONCLUSIONS

The design, construction and application of a electrophoresis apparatus implementing a twin electrode potentiostat as a detector of LDH fractionation by agar gel electrophoresis was carried out in this study.

The twin electrode potentiostat proved to be a valuable means of separating LDH isoenzymes. LDH_5 is strongly cathodic and would not fractionate by this agar gel electrophoresis method. Future LDH experiments are going to be concerned with trying to separate the various isoenzymes in discrete bands. Slight instrument instability is a definite cause of the band irregularities. A microprocessor controlled, constant power power supply is another project to be completed at a later date. With a microprocessor controlled power supply, a pulse could be generated from the computer, not manually as was done in this study.

In future work, several separation methods of lactate dehydrogenase as well as other enzymes which occur in multiple isoenzyme forms, such as malate dehydrogenase, amylase, isocitrate dehydrogenase and creatine kinase could be employed to this experimental set up, using the twin electrode potentiostat as its detector system.

A great number of electrophoretic separation techniques, in addition to agar gel, can be carried out using the electrophoresis cell in this study and twin electrode potentiostat as their detector system. Such electrophoretic separation techniques are starch block electrophoresis, cellulose acetate electrophoresis (in the form of cellogel), starch gel electrophoresis and polyacrylamide gel electrophoresis.

Various chromatographic separation techniques can also be carried out using the twin electrophoresis cell in this study and twin electrode potentiostat as their detector system. Chromatographic techniques employ column chromatographic procedures. Such column chromatographic procedures consist of ion exchange cellulosed, carboxymethycellulose (CM Cellulose) and diethylaminoethylcellulose (DEAE-Cellulose).

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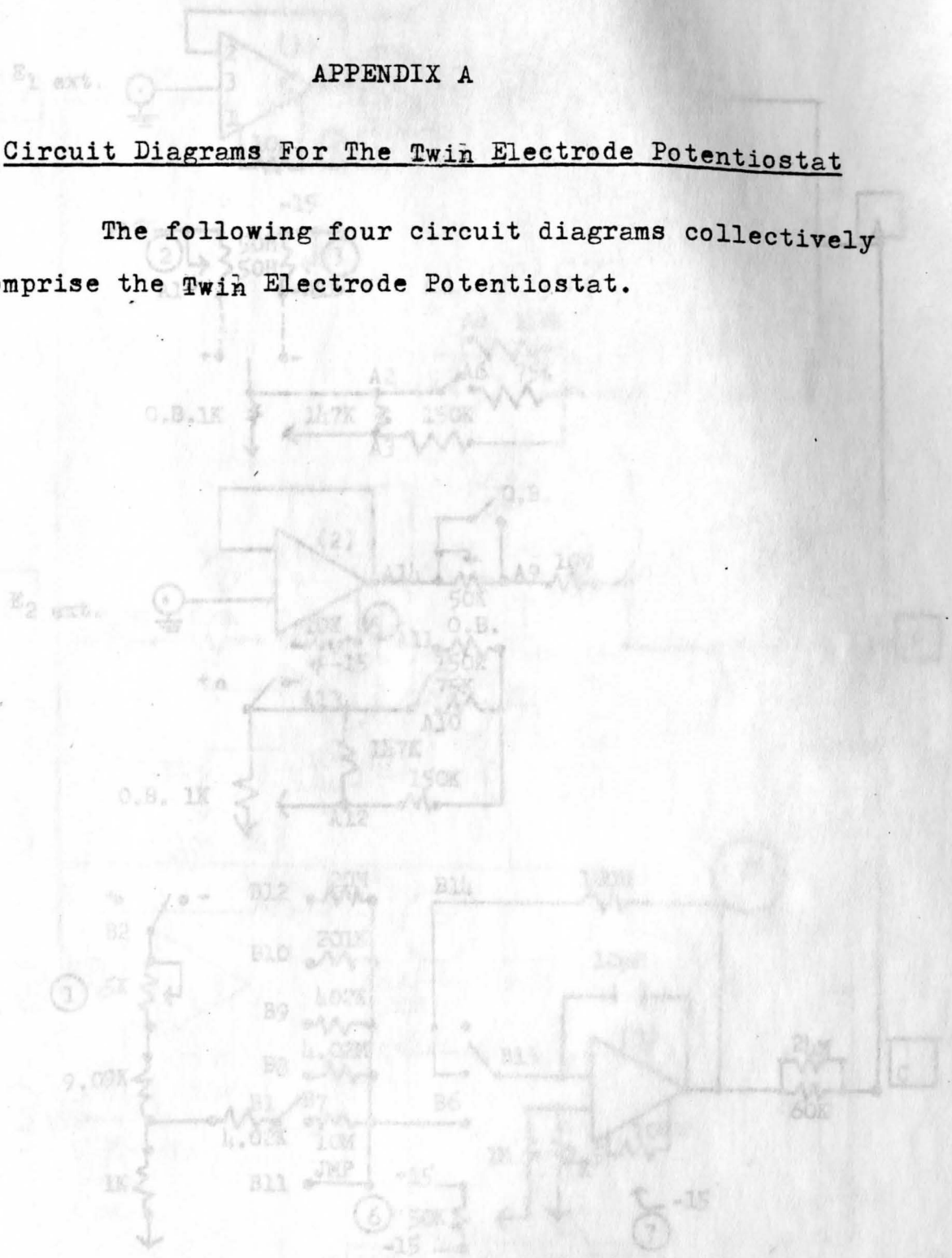
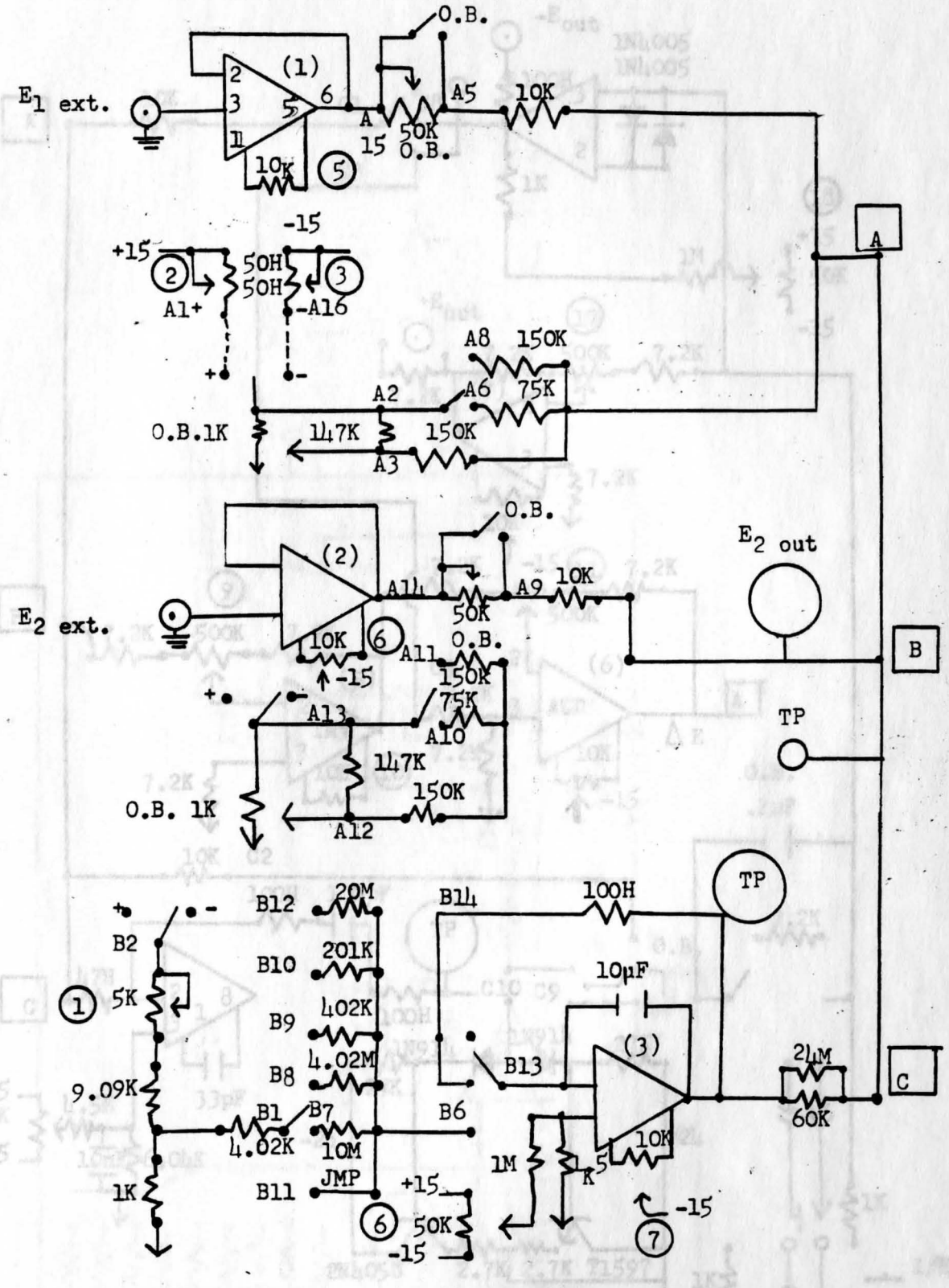
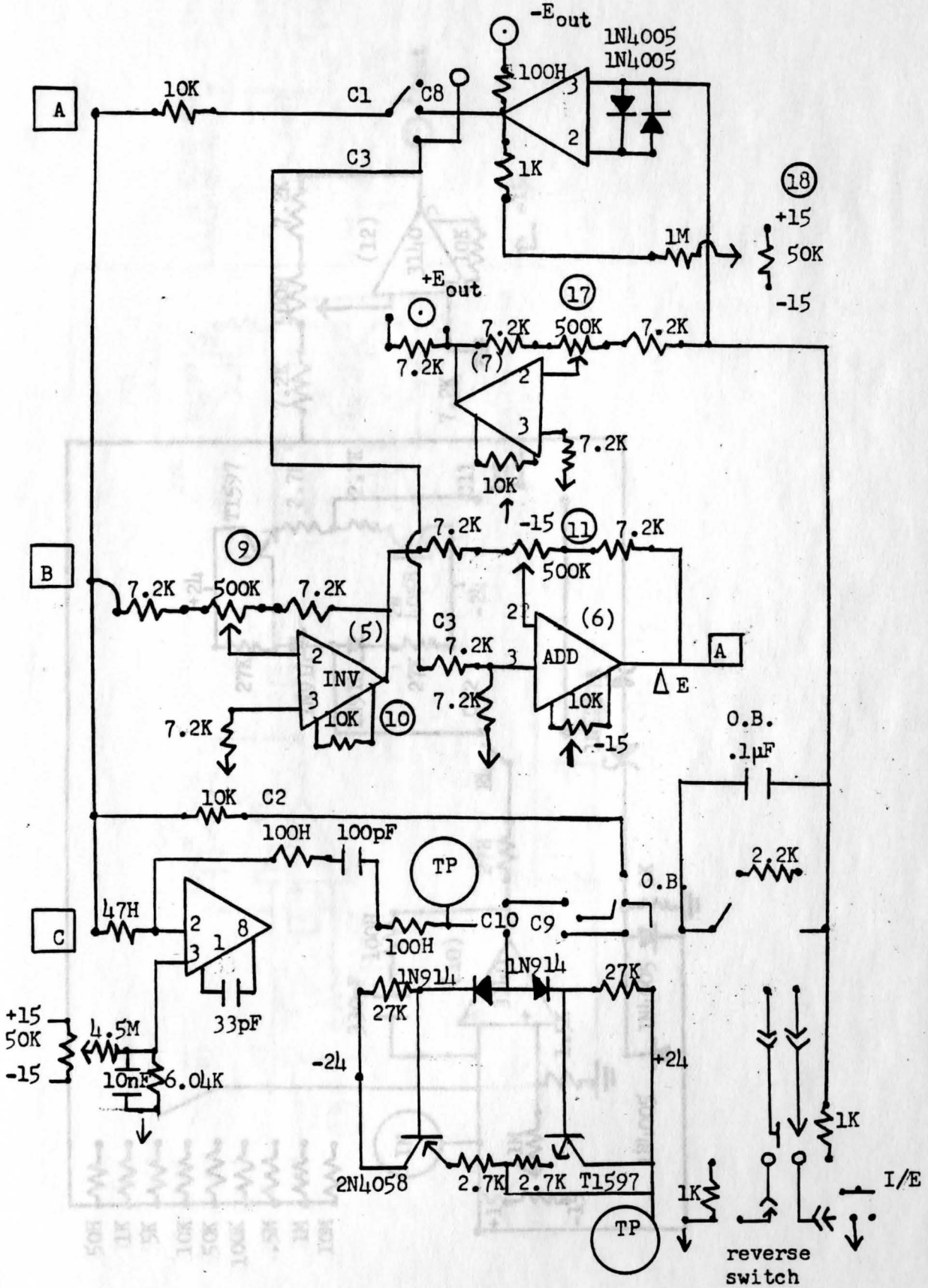
APPENDIX A

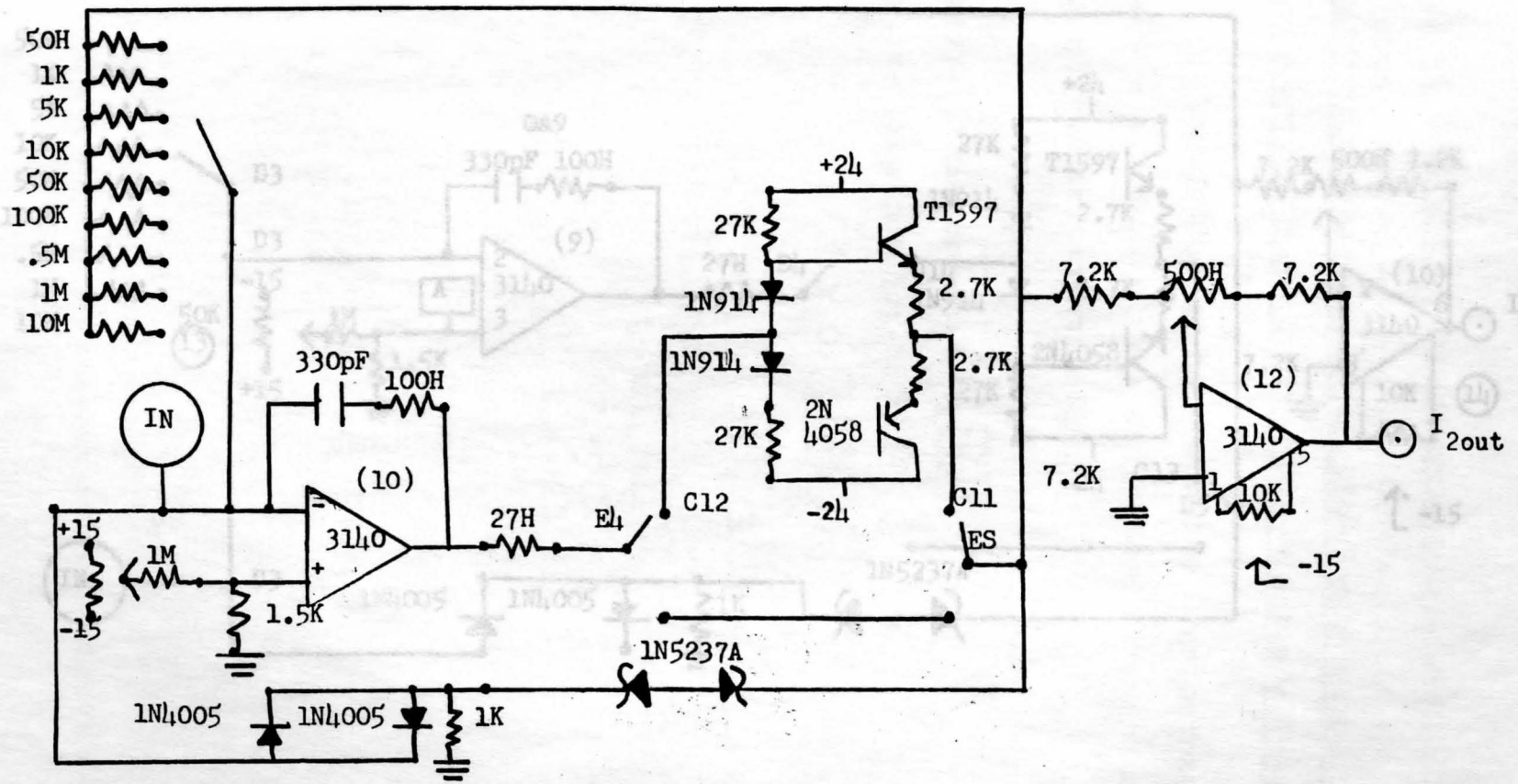
Circuit Diagrams For The Twin Electrode Potentiostat

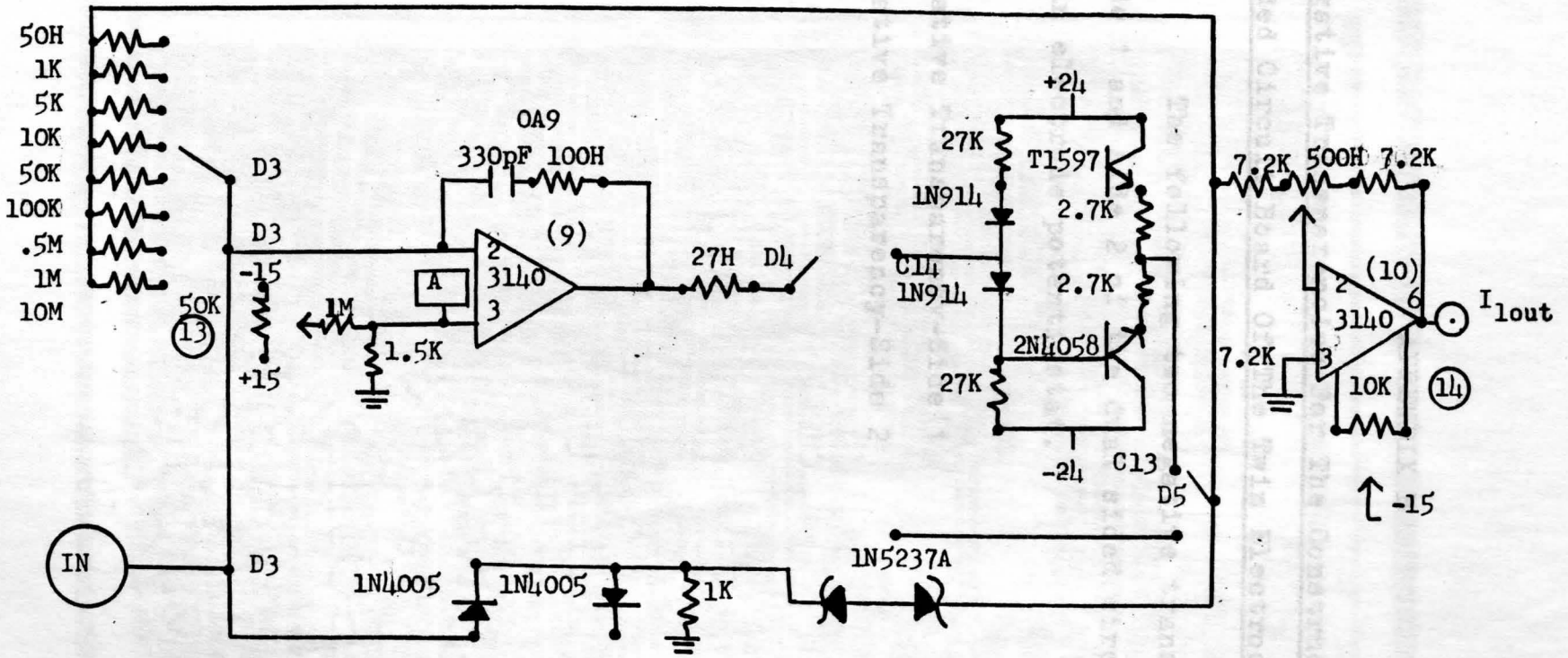
The following four circuit diagrams collectively comprise the Twin Electrode Potentiostat.




E1 ext.
(1)
2
3
6
11
5
10K
5
A
15
50K
O.B.
O.B.
A5
10K
A
+15
-15
2
50H
50H
3
A1+
-A16
A8
150K
A2
A6
75K
O.B.1K
147K
150K
A3
E2 ext.
(2)
O.B.
E2 out
A14
A9
10K
50K
10K
6
-15
A11
O.B.
150K
B
A13
75K
A10
TP
147K
O.B. 1K
150K
A12
TP
B12
20M
B14
100H
B2
B10
201K
10μF
1
5K
B9
402K
(3)
B8
4.02M
B13
24M
C
9.09K
B1
B7
B6
60K
4.02K
10M
10K
1K
B11
JMP
1M
R
5
+15
6
50K
-15
-15
7


-E out
1N4005
1N4005
100H
A
10K
C1
C8
C3
1K
18
+15
50K
-15
1M
+E out
17
7.2K
500K
7.2K
7.2K
(7)
2
3
7.2K
10K
7.2K
-15
11
7.2K
9
B
500K
7.2K
500K
7.2K
(5)
2
INV
3
10K
10
C3
7.2K
2
(6)
3
ADD
A
ΔE
7.2K
10K
-15
0.B.
.1μF
10K
C2
100H
100pF
TP
2.2K
0.B
C
47H
2
3
1
8
C10
C9
100H
1N914
1N914
27K
27K
33pF
8
+15
50K
-15
4.5M
10nF
6.04K
-24
+24
1K
2N4058
2.7K
2.7K
T1597
TP
1K
I/E
reverse
switch

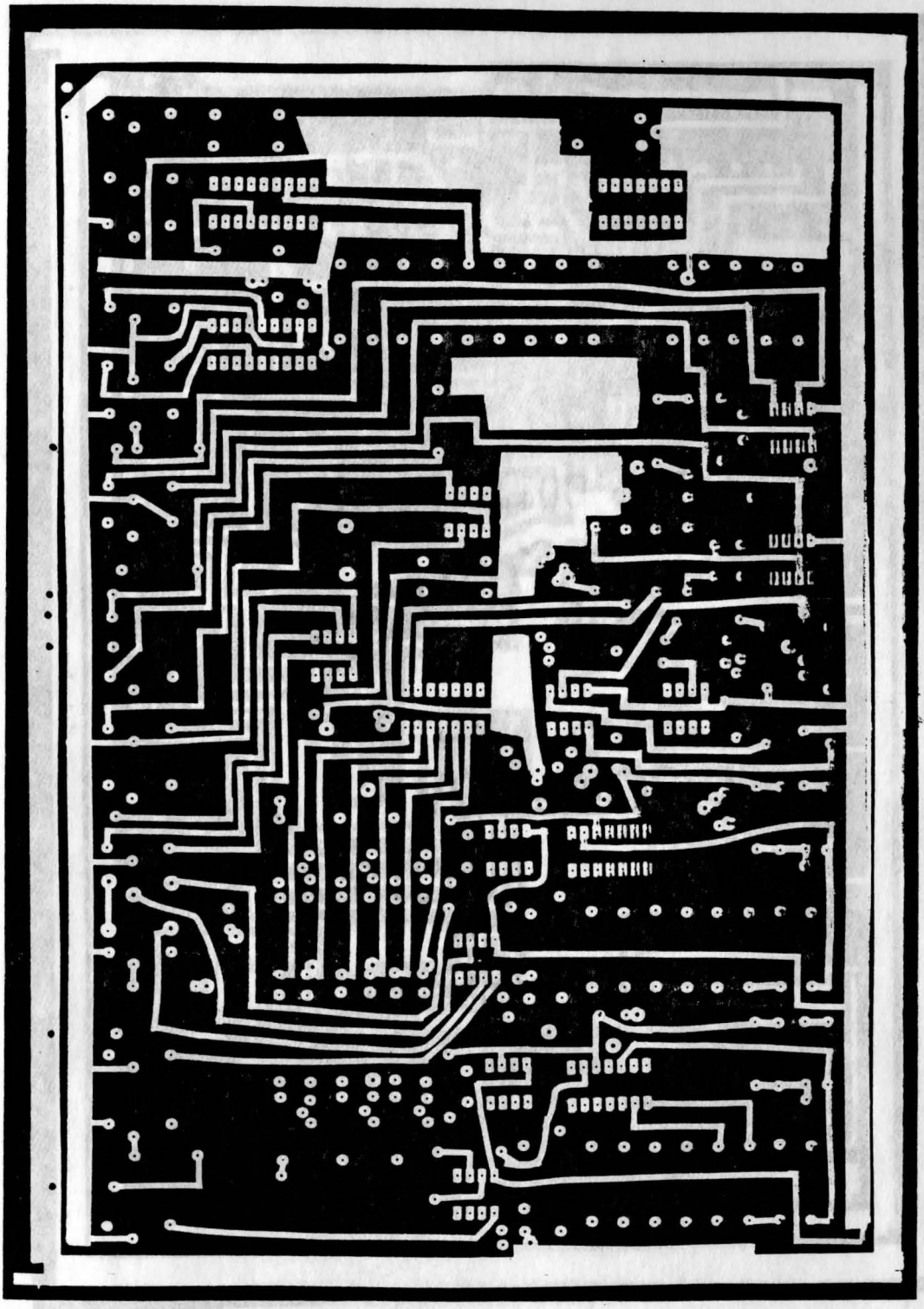

50H
1K
5K
10K
50K
100K
.5M
1M
10M
IN
330pF
100H
(10)
3140
+15
-15
1M
1.5K
27H
E4
C12
+24
T1597
27K
1N914
1N914
2.7K
2.7K
2N
4058
27K
-24
C11
ES
1N5237A
1N4005
1N4005
1K
7.2K
500H
7.2K
(12)
3140
7.2K
10K
I_{2out}
-15

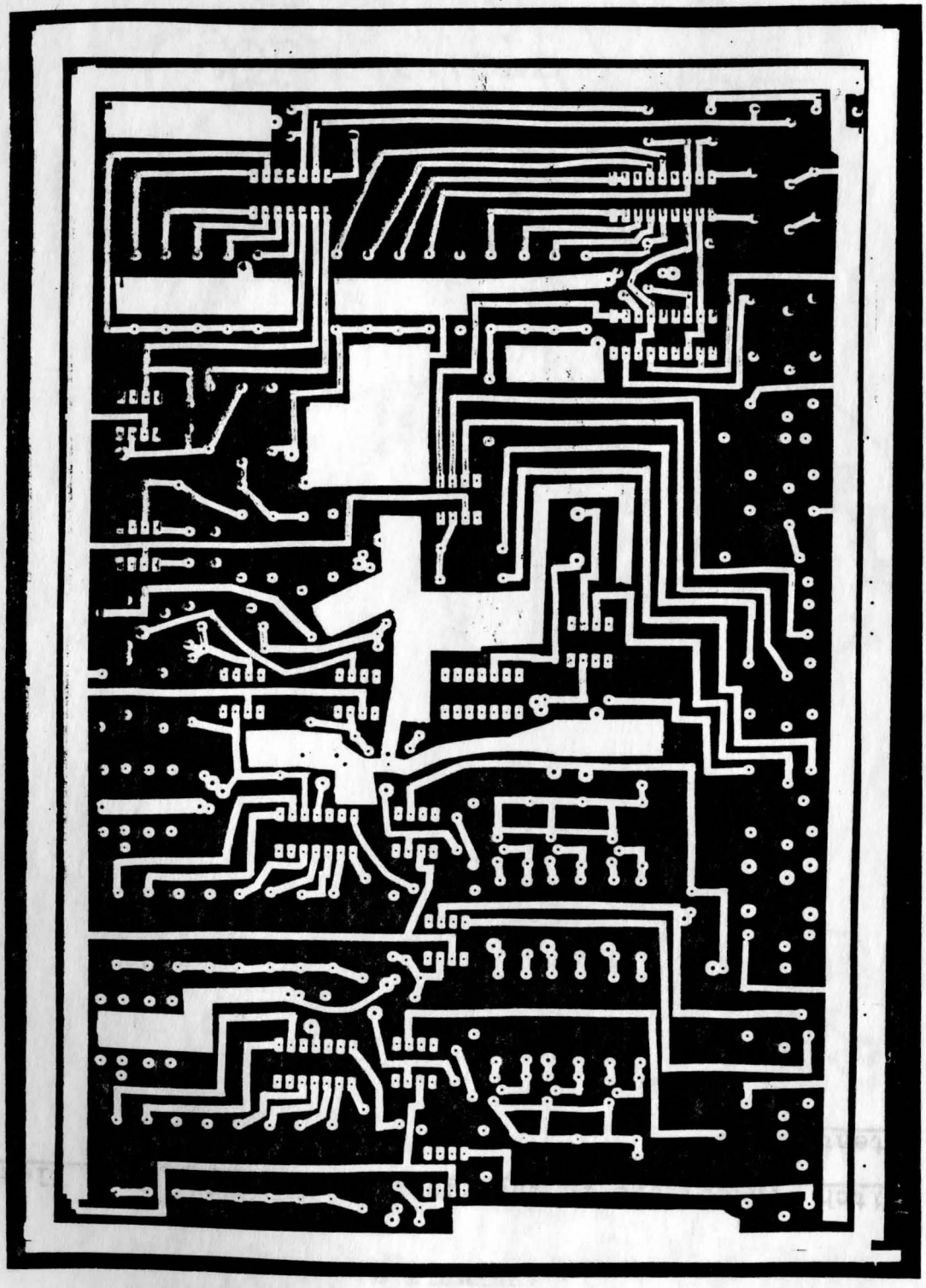

50H
1K
5K
10K
50K
100K
.5M
1M
10M
D3
D3
-15
50K
13
+15
1M
1.5K
A
OA9
330pF 100H
2
3140
3
(9)
27H
D4
+24
27K
T1597
1N914
2.7K
C14
1N914
2.7K
2N4058
27K
-24
C13
D5
7.2K 500H 7.2K
(10)
2
3140
6
3
7.2K
10K
14
Ilout
-15
IN
D3
1N4005
1N4005
1K
1N5237A

APPENDIX B

Negative Transparencies For The Construction Of The Dual Sided Circuit Board Of The Twin Electrode Potentiostat

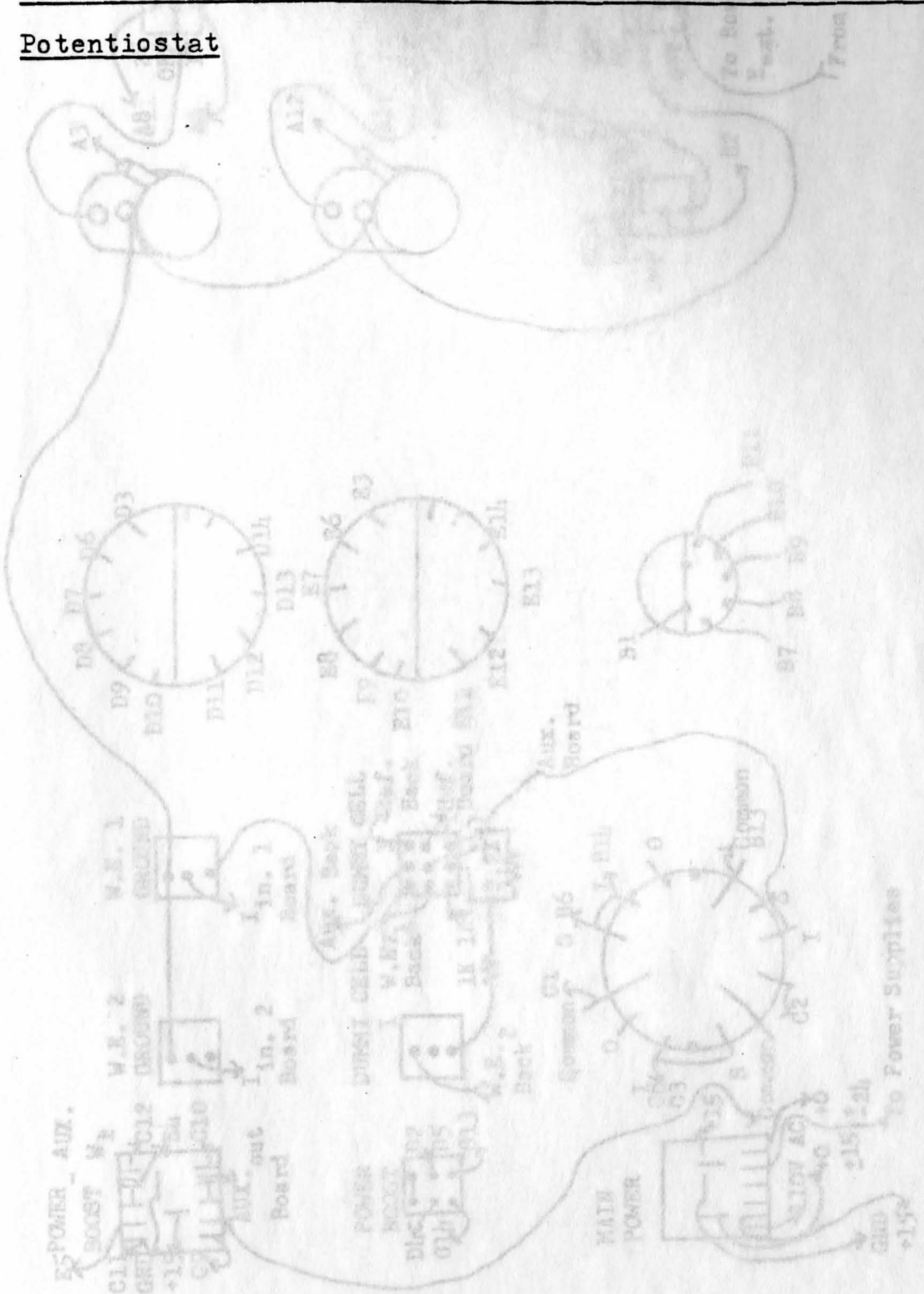
The following two negative transparencies comprise side 1 and side 2 of the dual sided circuit board of the twin electrode potentiostat.

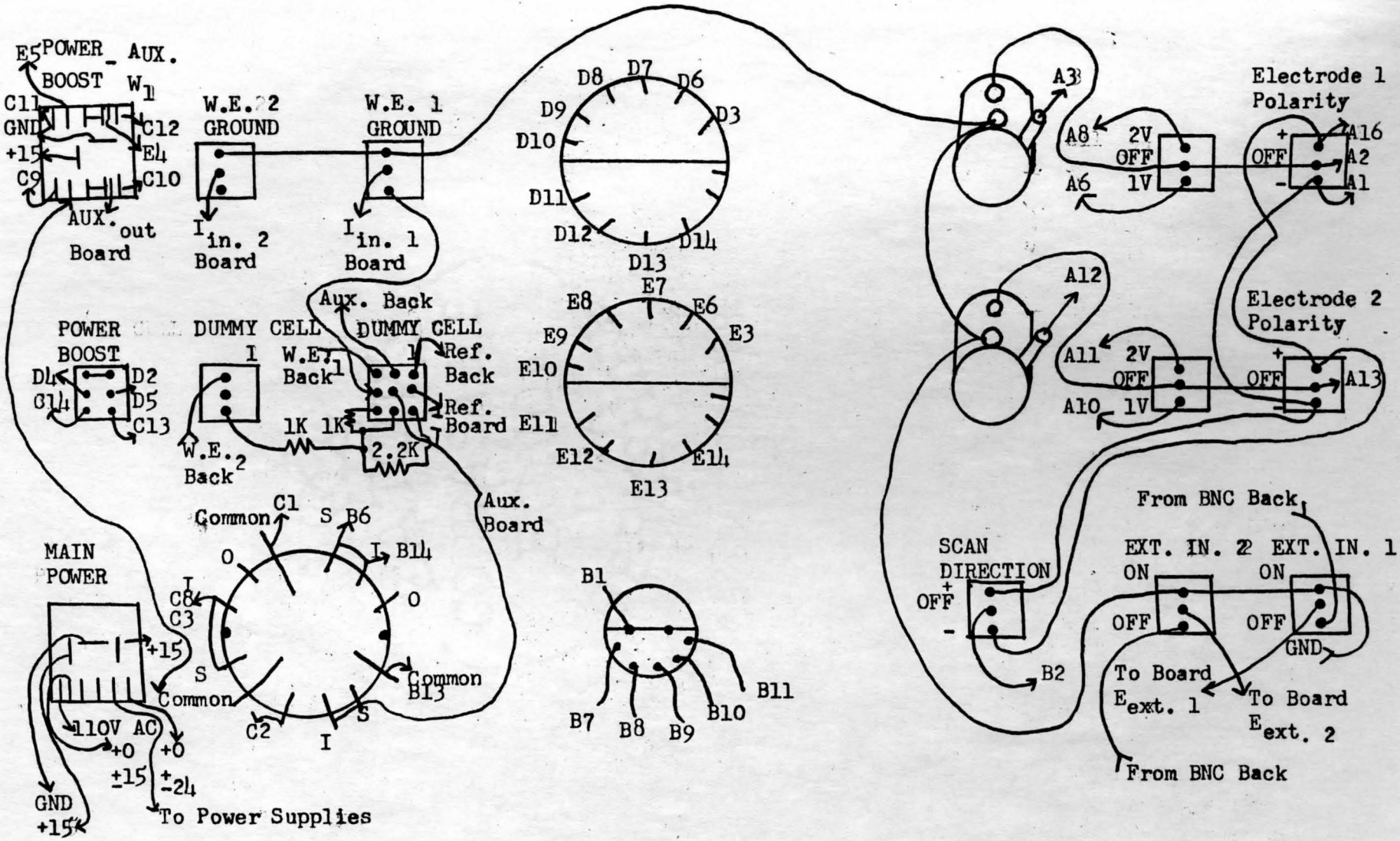




APPENDIX C

Switch Connections In The Front Panel Of The Twin Electrode Potentiostat




E5
POWER BOOST
AUX. W1
C11
GND
+15
C9
C12
E4
C10
AUX. out Board
W.E. 2 GROUND
I in. 2 Board
W.E. 1 GROUND
I in. 1 Board
D8 D7 D6
D9
D10
D11
D12
D3
D14
D13
Aux. Back
E8 E7 E6
E9
E10
E11
E12
E3
E14
E13
POWER BOOST
D4
C14
D2
D5
C13
DUMMY CELL I
W.E. 1 Back
DUMMY CELL I
Ref. Back
Ref. Board
1K 1K
2.2K
W.E. 2 Back
Aux. Board
Common
C1
S B6
I B14
O
I
C8
C3
S
Common
+15
110V AC
+0
+0
±15
±24
GND
+15
To Power Supplies
MAIN POWER
Common B13
C2
I
S
B1
B7 B8 B9 B10 B11
A3
A8
2V
OFF
1V
A6
Electrode 1 Polarity
+
OFF
-
A16
A2
A1
A12
A11
2V
OFF
1V
A10
Electrode 2 Polarity
+
OFF
-
A13
From BNC Back
SCAN DIRECTION
+
OFF
-
B2
EXT. IN. 2
ON
OFF
EXT. IN. 1
ON
OFF
GND
To Board E ext. 1
To Board E ext. 2
From BNC Back