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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

TOWARDS AN ARTIFICIAL LIVER

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

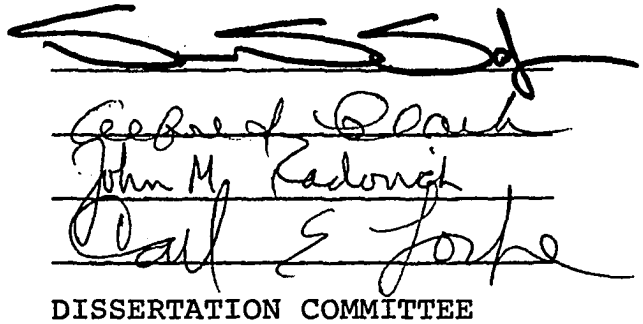
LAWRENCE H. HARE

Norman, Oklahoma

1976

TOWARDS AN ARTIFICIAL LIVER

APPROVED BY



DISSERTATION COMMITTEE

ABSTRACT

Due to recent advances in enzyme immobilization and isolation in conjunction with increasing understanding of soluble, labile cofactor retention and recycling, it now appears feasible to design an extracorporeal hemoperfusion device incorporating an hepatic microsomal oxidase that is capable of duplicating at least one of the biochemical pathways of hepatic detoxification in man. Several designs are proposed, each emphasizing the various advantages of enzyme immobilization, membrane modification for selective isolation of cofactors and reaction media, macromolecular cofactor analogues, and an *in situ* enzymatic cofactor regeneration system.

Enzyme catalyzed oxidation rates on the order of 1.5 μM/min/g catalyst were observed in platelet-free serum, ultrafiltered serum, and organic buffer. An 80% reduction in required cofactor concentration has been achieved through an *in situ* enzymatic regeneration system. Furthermore, the design of a cofactor selective membrane capable of reducing charged $NADP^+$ fluxes by 44% is described. More advanced techniques of cofactor retention and multi-enzyme catalysis are discussed in a recommendations section.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

TABLE OF CONTENTS

CONTINUED

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

TOWARDS AN ARTIFICIAL LIVER

CHAPTER I

INTRODUCTION

Man as Machine

> The human body, as to its natural actions, is truly nothing else but a Complex of Chymico-Mechanical Motions, depending on such Principles as are purely Mathematical. For whoever takes an attentive view of its Fabrick, he'll really meet with Shears in the Jaw-bones and Teeth. . . Hydraulick Tubes in the Veins, Arteries and other Vessels . . . a Pair of Bellows in the Lungs, the Power of the Lever in the Muscles, Pulleys in the Corners of the Eyes, and so on. (1)

The above, an excerpt from the preface of Richard Mead's Treatise, A Mechanical Acount of Poisons, illustrates a fashionable analysis of man strictly in terms of the mechanics of Galileo and Newton. It was published in 1702.

Astonishingly, this is the dictum followed by most biomedical engineers today. The artificial kidney and artificial heart-lung, first realistically proposed in 1948 (5) and 1954 (4), respectively, remain to this day the only clinically accepted artificial organ substitutes. Yet their operation depends strictly upon physical phenomena.

With the advent of biochemistry, essentially a twentieth century creation, efforts have been made to explain the functions of the body in terms of chemical reactions. Engineers have only recently begun to grasp the initiative in this area (23). The total ramifications of applied biochemistry (biochemical engineering) is indeed awe inspiring: Any or all biological phenomena associated with life can in theory be duplicated, singly or completely, in a synthetic system. In an effort to avoid the theological and philosophical disputes inherent in the above statement, it would seemingly be prudent for today's biochemical engineer to bite off a more chewable portion -- but where to begin, and how?

Before we become overly cocksure with the abilities of modern engineering, let us examine two of its more stellar successes and compare them with nature's version.

The heart, judged as a pump, effortlessly adapts to varying loads with extreme reliability. Significantly, the heart-lung machine, which will only take over the functions of the natural mechanism for a few hours, is about a thousand times larger than life.

The kidney may be considered a chemical engineering system, designed and operated with a precision that no engineer would be bold enough to imitate; the artificial kidney is large and clumsy, and offers numerous life-threatening side effects because synthetic materials cannot approach

the performance of the natural organ.

In the same vein, there is a good possibility recent advancements in enzyme stabilization, cofactor regeneration, and membrane manufacture may find successful application with a detoxifying liver enzyme to yield an extracorporeal device capable of mimicking a crucial facet of hepatic biochemictry.

The literature is incomplete in many areas, particu= larly those concerning the specialized oxidase enzyme involved. This precludes the immediate synthesis of the various modules presented in the following chapter to construct a viable working model. It is therefore the purpose of this disser- tation to clarify the remaining hurdles and to provide a data base from which such a device could be constructed. Specifically, the research goals are:

1) To show that rapid cofactor regeneration can sig- nificantly lower the total pyridine nucleotide content of the reaction medium without compromising the efficiency of the reaction. The end result of such a goal would be to reduce the flux of the toxic cofactor, and significantly lower capital operating costs.

2) To show that NADP selective membranes are feasible. The objective is once again to reduce toxic cofactor dif- fusion to the patient, without forfeiting high reaction rates.

3) To show that the MFMF oxidase reaction will indeed occur in whole blood, plasma serum, and blood dialysate.

4) To elucidate the efficacy of high molecular weight cofactor analogs in such a system. The primary objective is to reduce toxicity to the patient. Although economics are secondary, the two goals may not be mutually exclusive.

All the above goals have been successfully accomplished in the course of the research for this dissertation.

CHAPTER II

BACKGROUND

The purpose of this chapter is three-fold. Primarily, it provides a condensation and analysis of the diffuse, non-specific, and often contradictory literature concerning current modes of extracorporeal hepatic therapy, in addition to a host of information surrounding a subject most aptly referred to as "enzyme engineering." The continuing theme returns to a discussion of the many possible ways in which these seemingly unrelated topics may coalesce to yield a feasible extracorporeal hemoperfusion device capable of duplicating various critical pathways of hepatic detoxification.

Secondarily, the material here is intended to be food for thought to the reader, regardless of his background. The concept of duplicating biochemical pathways in a synthetic system is as alien to the engineer as it is to the physician.

Lastly, this section provides a justification for what some may consider a rather circuitous and unnecessarily complex means to an end: improved survival in advanced liver disease.

We begin with an elementary discussion of liver function, proceed to an investigation of how the critical functions may be assisted, and then through a logical series of compromises, arrive at a unique proposal for the extracorporeal treatment of

what is presently termed irreversible liver disease.

Liver Function

Excellent engineering predominates again in the liver, where complex processes are carried out at low temperature and without any of the powerful reagents which would be necessary in the laboratory. The liver plays roles in blood storage, bacterial filtration (99% per pass), and in bile production for digestive purposes. In addition, the healthy liver is often considered the chemical processing plant for almost every substance absorbed and distributed by the blood. The blood, which has picked up various substrates through the lungs, skin, alimentary canal, and elsewhere, perfuses the liver via the hepatic portal vein. The hepatic artery, in turn, provides an ample supply of oxygen. Once in the liver, nutrients and other substances are removed from the blood, metabolized, possibly stored for a time, and then released back into the blood. This metabolism is the net effect of a specific sequence of chemical reactions which depend on the substrates (precursors) encountered, and the needs of the body. Because these reactions have heretofore been difficult or impossible to achieve in a manner compatible with a living system, a truly artificial liver exists today only as science fiction.

These transformations are made possible at the proper time and environment by enzymes. These high molecular weight protein molecules posses catalytic activity. They are, by

most definitions, not living, but it is noteworthy that an enzyme, ribonuclease, has been synthesized "from scratch" (6) in the laboratory, and with it researchers have been able to assemble a fundamental precursor to life: RNA.

Due to the fact that many enzymes are non-specific with respect to substrate, yet highly specific to their action on that substrate, a relatively small number of enzymes may catalyze a wide array of reactions. In the liver, for example, amino acids are made into proteins and other nitrogenous compounds; glucose is converted into glycogen and stored, to be converted back into glucose and released when needed. And, drugs and other toxic substances, from which no useful energy or chemical products can be extracted, are detoxified (and in rare cases, activated) (7).

The engineering excellence of living organs, such as the liver, is counterpointed only by a single flaw; they will eventually and irreversibly cease to function. In 1970, thirty thousand people died from cirrhosis alone (8). The future, with its increased industrial contamination and the effects of high population densities, will offer no respite for the liver already overtaxed by alcohol, therapeutic drugs, and food additives (9,15,16,17). Surprisingly, no effective drug therapy exists today for so-called irreversible liver failure (10). Hepatic failures, regardless of the causative disease, are complex entities whose etiologies remain essentially undefined, and treatments have yet

to be established. Currently practiced treatments are conservative, general, non-specific, and of value only in temporary symptomatic relief or general support (12). Indeed, many drugs that one might prescribe cause an increased load on the diseased liver (19). The prognosis of fulminant liver failure is closely linked to the severity of encephalopathy, with survival of those patients in grade-IV coma (unresponsive to verbal commands) being around 10-20% (13, 14). Consequently, liver disease is often characterized by a descending spiral of liver efficiency resulting in hepatic coma and death (11).

One quality of the liver, however, sets it apart from all other organs (with the possible exception of the skin). Given the opportunity to recuperate, the liver is capable of rapid and spontaneous regeneration after disease or trauma (18). Rat liver, as an extreme example, when surgically excised to 25% of its original size, will completely regenerate within three days (20). Even man can survive after 90% hepatic resection (21). Due to the essential functions of the liver, a no-load period in which it may regenerate is currently unattainable after the onset of cirrhosis (22), or potentially lethal intoxications (23). Clearly, clinical medicine would welcome an extracorporeal device that could at least assist in some areas of liver function for a sufficient period of time to allow recovery.

Project Scope

During the protracted development period preceding the invention of the automobile, someone had the amazing foresight to first discover the wheel. In artificial liver research, science is now at the point of determining which part is to be invented first (hopefully with equal foresight).

For the time being, due to biomaterials limitations among others, a permanent hepatic device is unrealistic (24), and probably unnecessary. However, a reasonable objective, a ten-year goal, for example, would be to build a device using all current technology, and anything new discovered during its development, that would mimic at least some functions of the liver -- preferably those most essential to the support of life -- for a period of hours or perhaps days. By then, hopefully, the patient's liver will have recovered sufficiently on its own (or with drug therapy tolerated by the diseased liver when assisted artificially) to effect a complete recovery. What, then, are life-threatening liver deficiencies? Let us review the principal functions of the liver:

1) Protein synthesis and bile production can be considered long term goals of the liver, but not life-threatening in the initial phases of hepatic failure.

2) Glucose release is critical, yet can be

controlled effectively with intravenous injection (25). Additionally, fresh frozen plasma may be administered as a source of coagulation factors although they have dubious effectiveness (25).

3) Blood storage and its resultant effect, blood pressure control and RBC content can also be controlled by the physician.

4) Anti-bacterial functions can be assisted initially through drug therapy alone, but it is significant that massive infection is the almost certain cause of death in chronic liver disease (26).

The remaining liver function, metabolism of endogenous and foreign toxins, remains unassisted with current clinical practices (22). It is no mystery then that physicians and engineers alike have attacked the problem of liver detoxification head-on in the past 10 years. A review of this work follows.

Review of the Literature

Artificial liver research, per se, has found only limited interest. Index Medicus cites one reference for 1976 (27), four for 1975 (28-31) and three for 1974 (32-34). Purported clinical studies are even scarcer. Typically,

three or four case histories are followed in patients with potentially terminal liver disorders. The results of a particular hepatic support therapy are statistically inconsequential not only because of the small sample size, but also due to the fact that the patient usually dies anyway. If the patient has survived, it is not clear whether this was the result of the therapy, or in spite of the therapy. The techniques range from the Frankensteinian to the beastly.

Parabiosis

Eschbach has reported the use of a living partner, either animal or human, as a means of extracorporeal hepatic support (35). Typically, arterial shunts connect the hepatic failure patient to the liver of the healthy parabiotic partner. The patient's blood is returned in a venous shunt from the partner's hepatic portal vein. Other modifications of this technique involve mutual blood transfusions in which intimate mixing of the partner's blood occurs between simple femoral venous shunts (36). Potential parabiotic partners in either case must be closely screened for blood antigen compatibility. Nevertheless, this has not prevented bold attempts to use humans, orangutans, and gorillas as hepatic support partners (37,38,39,40,41). The concept of sedated gorillas as bed partners underscores the desperation of the medical community concerning hepatic failure. Human benefactors, unfortunately, are hard to come by. As might be expected, there is considerable risk to both partners,

and in many hepatic diseases (i.e., serum hepatitis), rapid infestation of the disease is all but guaranteed in the healthy partner (42). Successful parabiosis has not been reported in the literature within the last three years.

Transplantation

Organ transplantation is, in the strictest sense, (and indeed, as far as the body is concerned) an extracorporeal device. It therefore earns a mention in this review.

The longevity record of 168 days for a whole transplanted liver is currently held by the University of Colorado Medical Center in a 7 year old girl (43). The patient was comatose intermittently during this period. In addition, liver support was assisted by hemodialysis and experimental drug therapy. In the interest of keeping the patient alive, controlled experimentation was not possible, and the issue of hepatic transplantation remains a clouded issue. The consensus of clinicians at this point is that hepatic transplantation should be abandoned until effective immunosuppressive therapy is available. Even then, the demand for donors will far outstrip the supply, as is painfully obvious with kidney transplantation today. (Perkins has proposed an interesting hypothesis that the immunorejection of an organ is directly related to its biochemical complexity, and as such researchers should concentrate their efforts on those components most successfully transplanted (44).

The liver, incidently, is far down on his list for potential tranplantation.) At any rate, the objective of whole liver transplantation is that of permanent hepatic replacement. As stated earlier, this is neither within the scope of this paper nor within the ten-year goal of this project.

Liver grafting is currently enjoying the limelight in the medical literature (5 references in 1976) (45-49). It is included under this heading because it, too, is proposed to be a long range surgical therapy. The graft, ranging in size from one to several cc's of liver tissue may come from a suitable partner or from a non-diseased portion of the patient's own liver. (The liver is often assumed to be homogenous both with regard to structure and to function.) Hepatic regeneration is then activated with drug therapy in the hope that the graft will become active (47). In isolated cases, the surgery alone seems to trigger hepatic regeneration (49). To date, permanent liver grafting between non-identical twins has failed, and this has limited the technique to cases of isolated neoplasms where homografting may be effective (45).

Selective Chemical Adsorbents

Rosenbaum (50,51,52) has reported on the use of a resin adsorption column that somewhat selectively removes foreign compounds in acute, life threatening drug intoxication. Additionally, Willson (42) describes hemoperfusion through charged and uncharged ion exchange resins for the

removal of choleophilic anions from dogs with biliary constriction. Perfusion of the resins "Dowex-1" and "XAD-2" removes a variety of protein bound anions from plasma. Resin hemoperfusion, however, results in extreme hemolysis plus lowered platelet counts within two hours, obviating this technique in patients with liver failure, who already have a bleeding diathesis and often have profound thrombocytopenia (24). Weston has attempted resin perfusion with plasma obtained from an Aminco continuous cell-separator in dogs, but losses of blood platelets was not demonstrably lower than in whole blood perfusion (24,25,82).

The adsorbent properties of activated carbon have been known for many years. Typically, it will remove from blood water soluble compounds such as aminoacids and creatinine (55). Unfortunately, most blood toxins are lipid soluble. In addition, hemolysis remains troublesome, coupled with the catastrophic effects of small carbon particle emboli. Two methods have greatly increased the biocompatibility of activated carbon; both involve containment within polymer fibers. Chang has successfully microencapsulated both charcoal and ion exchange resins in collodion and nylon membranes (53). This will be covered in more detail later. Bruck has achieved considerable success in coating individual charcoal particles with a variety of polymers, including hydrogels (54). In both instances, a penalty must be paid due to the exclusivity and rate-limiting characteristics of such polymer films.

Dialysis

Direct hemodialysis is probably still the most clinically acceptable method of extracorporeal hepatic support (56,57). Peritoneal dialysis has found less acceptance, but the principles involved and net effect are much the same. Potential toxins are removed from the blood due to a concentration gradient across a synthetic membrane or the peritoneum. Lack of selectivity has been offset by the expertise gleaned from artificial kidney research. Recent evidence, however, indicates that neither peritoneal dialysisn or hemodialysis seem to be effective at improving the level of consciousness in level IV coma (25,58,59). The pathogenesis of the resulting encephalopathy is not clear, although one possibility is that the healthy liver synthesizes unknown factors necessary for brain function. This hypothesis is supported by data collected from crossed-hemodialysis experiments in which CCl_4 induced acute hepatic coma in pigs was improved by dialysis against the hemodialysate of a healthy pig, whereas hemodialysis against a synthetic medium was ineffective (60). Zieve, et al., (61,62), support the more likely conclusion that an accumulation of some unknown toxin is a more likely causation of the encephalopathy. Their work suggests the synergism between ammonia, short chain fatty acids, and mercaptans. Others point to changes in the metabolism of plasma-amino acids which may lead to the accumulation of false neurotransmitters

in the brain (59). The ineffectiveness of dialysis indicates that low molecular weight water soluble toxins such as ammonia are not totally responsible. Other toxins may either be lipid soluble and bound to plasma proteins, high molecular weight compounds, or selectively charged. Hemodialysis with different membrane characteristics is always a continuing subject of research.

Microcells

Chang has investigated a series of biomedical applications for his "artificial cells" since their initial preparation in his laboratory in 1956 (71). Chang has repeatedly suggested their use in an artificial kidney and an artificial liver (53,63-67). Their preparation is typically the result of condensation polymerization occurring at the interface of two dispersed immiscible fluids or through the physical phenomenon of coacervation. He has successfully encapsulated any number of exchange resins and activated charcoal in addition to their potential in straight dialysis (68,69). At first, enzyme microencapsulation proved unfeasible due to the harsh preparatory conditions. In recent years, however, many enzymes have been successfully immobilized by this technique while retaining activity, furthering the potential utility of the invention for enzyme replacement therapy, artificial blood cells, and tumor therapy. In a recent paper (70), Chang has reported the microencapsulation of an oxido-reductase enzyme system which cycles

automatically, regenerating the enzymes and the coenzymes involved while consuming necessary substrates which pass through the membrane. This method offers great potential in duplicating complex enzyme systems, much like those that occur in the liver.

The artificial cells may be administered by direct i.v. injection, peritoneal injection, oral consumption, or perfused in an extracorporeal device. The latter case has been explored most thoroughly in the literature. Techniques for modification and optimization of the membrane properties are abundant. There nevertheless exist upper bounds for molar fluxes and molecular weight cutoffs, but the high surface area available (2,500 cm^2/ml) (67) often offsets their limitations.

Extracorporeal Biological Devices

The methods discussed up to this point cannot be expected to replace or substitute for the liver's complex enzymatic functions, many of which still remain unknown. Because of the lack of understanding of the total scope of hepatic functions, utilization of biological tissue has been attempted by various investigators. The biological systems employed include healthy donor blood (72-75,78), as a transmitter of liver functions, and liver tissue itself, in various forms of preparation (10,76-81). These techniques have largely met with failure due to preparation and procurement complexities, storage and preservation, and

immunology. Various methods of liver cell culturing and preservation exist. Essentially, they fall into two categories: (1) blood perfusion of large surface areas of healthy liver; and (2) blood perfusion over cultured colonies of hepatomas (85-87). (Normal liver tissue will not replicate successfully in vitro) (83,178)

In the first case, rapid degradation of catalytic properties due to the presence of proteolytic enzymes in the tissue is observed regardless of the method of preparation. This is a naturally occurring process in all non-living tissue, which necessitates the excision, preparation, and use of any hepatic tissue within 15-30 minutes. The elevated temperatures (37-38 C) at which all of these analyses have been conducted undoubtedly accelerate the rate of isozymic decay. This may be unnecessary. Sofer has shown that the optimum temperature for at least one hepatic oxidase enzyme is well below physiological temperature (84). Perhaps increased half lives could be observed in these hepatic support devices at lower operating temperatures without sacrificing catalytic activity. This has not been shown in the literature.

The second case sidesteps the decay problem by maintaining a steady-state in vitro cell culture which is then directly (or indirectly, across a membrane) perfused with the patient's blood. Yet the concept of continuously exposing one's blood to a thriving, malignant cell culture may

offer a cure worse than the disease. The question of incomplete or abnormal liver function in the hepatoma has also been raised (85).

Critical Analysis of the Current Literature in Review

1) No universally acceptable technique exists for extrahepatic support.

2) One method makes an attempt to duplicate actual hepatic biochemical pathways, with little positive effect.

3) One reference strives to remove protein-bound toxins (pesticides, drugs, and potentially numerous unknown endotoxins).

4) All other techniques stress removal of free, low molecular weight toxins, which is not the primary function of a normal liver. One primary mode of detoxification in the liver is to convert lipophilic, protein-bound compounds to hydrophilic products which can then be excreted by the kidneys. Removal of critical endotoxins remains unexplored.

5) Toxicity, hemolysis, immunological complications, and other side effects of varying degree in each case compromise any net detoxification of which the device may be capable.

A Proposal

Attempts to duplicate isolated examples of synthetic liver function in vitro with purified liver enzymes have been infrequent (25,88,89,90). Likewise, reproduction of

detoxifying liver metabolism has been thwarted by the unavailability of the necessary hepatic enzymes.

If one or more of these enzymes could be incorporated in an extracorporeal shunt reactor, it would provide a test bed on which to run successive tests evaluating the reactor's effect on various substrates. It would at the least be a major step toward temporary hepatic support, and could conceivably see clinical use in detoxification of specific substrates transformed by the particular enzyme.

The reactions of concern here are catalyzed by a class of "mixed function oxidases"; enzyme complexes that require oxygen and a hydrogen-carrying coenzyme, are nonspecific, and which oxidize a substrate as shown in Figure 1. They are not only critical as an end in themselves, but also as intermediate steps in overall liver function; for example, after the crucial oxidation step, conjugation may occur. With few exceptions, either the oxidized or the oxidized/conjugated compound is devoid of substantial biological activity.

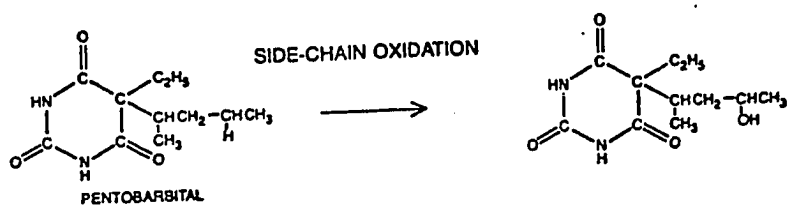
Exceptions to this rule have surfaced in recent years, no doubt due to the cataclysmic changes occurring in the environment. Man's biochemistry has been attacked by a great variety of new drugs, agricultural chemicals and pollutants against which the body has no natural defensive mechanism. Up to a point, the liver can deal with toxic substances using its available enzymes in its customary

Figure 1. (7) Typical Oxidation Reactions Encountered in the Liver. Transformations such as these are the primary pathways of detoxification, yet their duplication in the laboratory, or expecially in an artificial organ, is difficult without the specific enzymes required in each case.

ADMINISTERED DRUGS

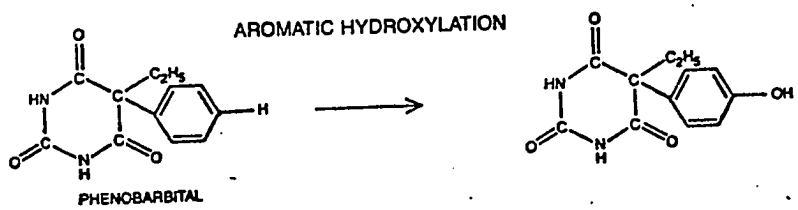
TRANSFORMED PRODUCTS

SIDE-CHAIN OXIDATION



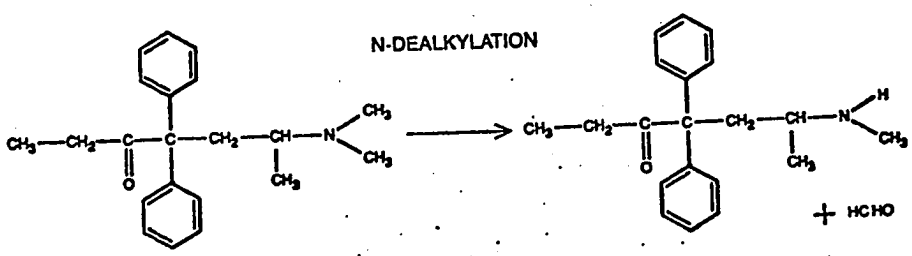
PENTOBARBITAL

AROMATIC HYDROXYLATION



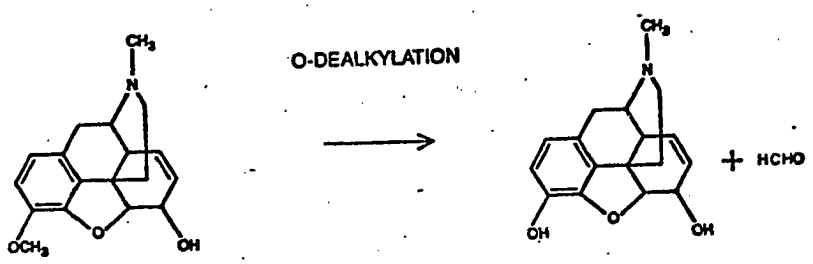
PHENOBARBITAL

N-DEALKYLATION



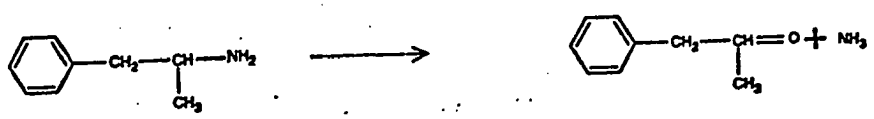
METHADONE

O-DEALKYLATION



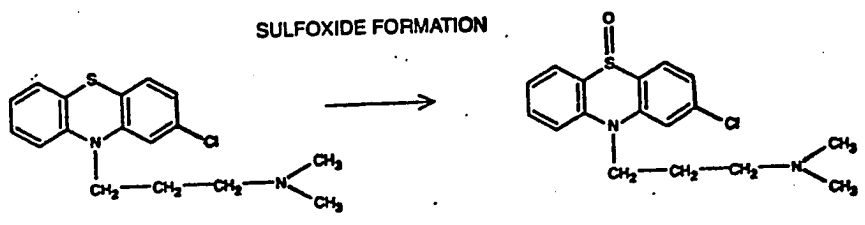
CODEINE

DEAMINATION



AMPHETAMINE

SULFOXIDE FORMATION



CHLORPROMAZINE

way to promote chemical changes which may make the unwanted materials less dangerous or more soluble and therefore more easily disposed of in the urine. For example, the endotoxin ammonia (formed in the breakdown of proteins and highly toxic) is converted to urea, which is relatively harmless, and easily excreted. But when the chemical defenses of the liver are challenged by a man-made chemical, the outcome may or may not prove successful. Barbiturates are converted to less toxic chemicals, but tetraethyl lead and the insecticide parathion, which are non-toxic in themselves, are converted to highly poisonous compounds by enzyme action in the liver. This activation may be circumvented in those particular cases where these known intoxications exist by the prudent selection of hepatic enzymes in the shunt reactor.

A rare opportunity was offered this laboratory when Professor D. M. Ziegler of the University of Texas' Clayton Foundation agreed to supply large quantities of a mixed function microsomal flavoprotein (MFMF) he exclusively isolated from hogs (91,92,93). Until 1975, it was the only example of a purified hepatic detoxifying enzyme. These drug metabolizing enzymes are present predominantly on the smooth surface filaments of hepatocyte endoplasmic reticulum. The metabolic role of the oxidase is yet to be elucidated, but based upon its extremely high concentrations in liver tissue (up to 20% of total E.R. protein), it is believed to be

of significance in liver detoxification.

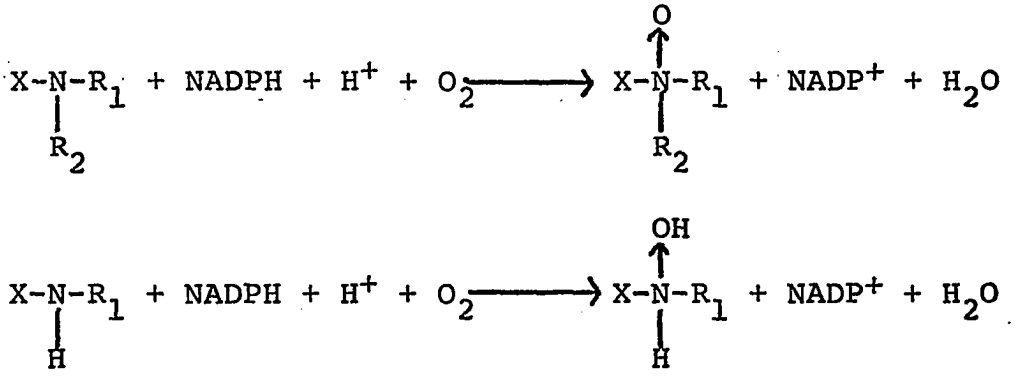
As a result, it is proposed that this enzyme, in conjunction with its allied coenzymes, is capable of relatively long-term activity in an extracorporeal device with a minimum of harmful side effects. Only in such a "clean" system can the net effect of a particular therapy be followed in controlled experimentation on a wide data base. This paper is the result of a series of broad spectrum investigations that provide a basis on which such a device could be constructed.

Characterization of the Enzyme

The MFMF enzyme complex, from here on to be referred to as simply an "MFMF oxidase" is thought to be a relatively high molecular weight (~400,000) oligomeric complex containing multiple flavin functional groups tightly bound to allosteric sites on the enzyme which give it a characteristic yellow color. The FAD released on hydrolysis is a fortuitous method of determining enzyme concentrations spectrophotometrically in instances where protein analyses would be difficult or impossible. There are perhaps six monomers in the oligomer.

This particular oxidase is highly specific to secondary and tertiary amine containing substrates. A number of potential toxins in this category are: chlorpromazine, N-methylparaaminoazobenzene, ethylmorphine, brompheniramine, prochlorperazine, and 1-methyl-1-phenylhydrazine.

It catalyzes the conversion of secondary and tertiary amines to N-oxides with the consumption of one mole of reduced nicotinamide adenine dinucleotide phosphate (NADPH), and one mole of oxygen per mole of oxide formed. The reactions involve an external electron donating system as follows:



Typically, R_1 and R_2 are methyl or ethyl groups. They may, however, as in the case of prochlorperazine (94), be part of a ring system. A lipophilic alkyl or aryl group free from polar groups on the alpha carbon is indicated by "X". A functional group more polar than a hydroxyl within a two atom radius of the nitrogen prevents oxidation. Tertiary amines are converted to N-oxides, secondary amines to N-hydroxy amines, and thioureylenes are S-oxidized (84, 93,95).

Additionally, there is no indication that this oxidase resembles the recently isolated cytochrome P-450 system which acts as the terminal oxidase in a microsomal electron transport chain (164-167). Cytochrome P-450 is activated by phenobarbitol and inhibited by CO. The MFMF oxidase exhibits none of these properties (84,95).

The reaction is essentially irreversible. Octylamine is one of the few activators known. This enzyme may compose as much as 20% of all endoplasmic reticulum protein. All known enzyme reactions are controlled, turned on and off at the proper time, if you will, by a complex system of checks and balances such as feedback inhibition. The search for the control mechanism of this enzyme continues. All reactions with this enzyme will essentially go to 100% completion if given time.

Activation of the oxidase is believed to follow a distinct sequence: 1) molecular oxygen binds at the single active site; 2) like all oxido-reductases, a suitable soluble cofactor*, in this case, NADPH, (or NADH, with far less efficiency) binds at a specific allosteric site; 3) the substrate binds at the active site, and catalysis occurs; 4) the product and oxidized cofactor are released.

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* The term "cofactor" is, in the strictest sense, a misnomer. Cofactor implies a small, non-protein molecule necessary for the enzyme's catalytic activity, but which is not consumed in the reaction. Typically, transition metal ions fill this role. The hydrogen carrying "cofactor" in this context is consumed in the reaction, and is therefore more correctly defined as a cosubstrate. Indeed, in kinetic analyses, this entity must be treated as one substrate in a multiple substrate system. As a concession to avoid ambiguity with the current literature, which inexplicably refuses to acknowledge this fact, the terms cofactor, coenzyme, and cosubstrate will be used interchangeably to refer in context to the compounds NAD, NDAP, and FAD.

The cofactor necessary for this enzyme adds a significant complexity to its application:

1) It is quite expensive, yet must be available in relatively large quantities. On a molar basis, one must start out with at least as much cofactor as the amount of product desired.

2) It plays an essential role in the reaction mechanism, yet it cannot be duplicated by any other compound.

3) To perform its function, it must be bound to the enzyme at the proper time. At all other times it must be in free solution with the enzyme. This further complicates the issue of separating the products from the reaction medium.

The concept of irretrievably consuming a very expensive compound (NADPH, at $200,000/mole) (96) on a one-to-one molar basis with the product has provided a considerable incentive to develop a compatible system of regeneration so that the cofactor can be re-used.

The regeneration, in actuality a simple reduction of the oxidized $NADP^+$ to NADPH, may be achieved through treatment with dithionite (97,98). This is an effective method of preparation of NADPH from the less expensive $NADP^+$ in the laboratory, but the process is much too harsh for <u>in</u>

situ regeneration within an enzyme medium. Electrolytic reduction has also been reported, but with relatively low conversion and some decomposition of the parent compound (163). This lack of specificity and accompanying degredation becomes critical in a process incorporating a large number of turnovers. As Chambers (99) has pointed out, a regeneration process with 99% specificity would lose 63% of the starting cofactor after 100 regenerations.

An alternative is to reduce the cofactor enzymatically. Although the emphasis in the MFMF oxidase discussion has focused on an oxidation process in which NADPH is consumed, there is no reason to doubt the existence of an enzymatic reaction with the obligatory production of NADPH from $NADP^+$. Indeed, such is the case. Alcohol dehydrogenase, isocitrate dehydrogenase, and glucose-6-phosphate dehydrogenase are three examples of relatively inexpensive, commercially available, stable enzymes which possess this quality. Furthermore, all have shown the capability of regenerating $NADP^+$, with the consumption of an appropriate hydrogen donor, simultaneously with the MFMF oxidase reaction (84,100,101).

The key issue in the proposal of this thesis is to explore the varied ways in which this enzyme-cofactor complex, and eventually others resembling it, may be applied as a temporary hepatic support. Various hypothetical and largely speculative methods follow.

Methods of Application

Direct Oxidase Injection

The positive aspect of direct intravenous injection is its obvious simplicity. In addition, i.v. injection is normally considered a non-traumatic procedure offering minimal discomfort for the patient and a conventional mode of application with which most physicians are comfortable.

The negative aspects far outweigh these advantages. The oxidasewould quickly be identified as foreign protein in the blood and could easily lead to massive allergic reactions and anaphilactic shock. This has been proven time and again with other enzymes injected directly (102,103). Clinically effective levels of L-asparaginase, for instance, which has potential as an anti-cancer agent, cause a wide range of toxic effects on several organs including the liver, pancreas, kidney, and brain (104-106). The same has been shown for direct infusion of α-galactosidase in the treatment of Fabry's disease, regardless of the degree of purity of the extract (107). Furthermore, the MFMF oxidase has been shown to havea half life in buffer at 37 C of less than 5 minutes. The catabolic effects of the blood would further reduce its utility. Cost and availability of the oxidase must also be accounted for, even ignoring the recently developed tools of enzyme purification with affinity chromatography and advanced tissue culture methods. NADPH must also be present, yet its concentration in blood plasma

is not known. Within the red blood cell, concentrations of 30 μM have been reported (108,109). This may be adequate for some degree of conversion. It is unlikely, though, that such quantities will be found in the plasma in contact with the injected oxidase, because the free cofactor is apparently quickly metabolized through phagocytotic attack (110). Injection of the purified cofactor with the oxidase poses even more serious side effects. NAD and NADP, like other related adenosinic substances, possess potent vasodepressor action in dosages as low at 50 μg/kg body weight, depending on the experimental animal (111,112). The extent of its effect in man has not been reported. The response, however, is short lived -- usually on the order of 2-3 minutes -- which stresses its swift and thorough metabolic anihilation. Atropine sulfate, when injected intramuscularly, blocks the hypotensive effects of NAD and NADP. Based upon the degree of vasodepressor antagonism of atropine against these cofactors, it has been determined that 50 μg/kg of NADP or NAD is approximately equivalent to 1.0 μg/kg of acetylcholine (112). Additionally, increased levels of corticosteroids have been reported with NAD/NADP injection, yet this can be construed as a desirable side effect, supplementing the corticosteroid therapy frequently prescribed in hepatic disease (111).

Immobilized Enzyme in Direct Contact With Blood

Since the initial observations by Kidd (113) on the antitumor activity of guinea pig serum and the evidence

presented by Broome (114) that the active factor was L-asparginase, there has been a great deal of interest in this enzyme. As always, side effects were present when administered in large dosages, and supplies of the enzyme were small (104-106). As a result, a great deal of work has been done in the interest of immobilizing this enzyme to an insoluble support, while retaining catalytic activity.

Immobilization of enzymes for various purposes is well documented in the literature (115). In the context of this paper, immobilization is construed to mean the attachment, via an appropriate covalent linkage, of some portion of the enzyme to an insoluble support. The advantages are manyfold: 1) the enzyme can be retrieved for repeated use; 2) it can be isolated in a hemoperfusion device, greatly reducing side effects; and 3) often the stability of the enzyme is increased. The use of L-asparginase as a cancer cure has waned in recent years, but the knowledge gained during its utilization in extracorporeal devices can readily be applied to other enzymes, including the oxidase of this study.

MFMF oxidase has been immobilized by various researchers in this laboratory and at the University of Texas to nylon, porous glass, sepharose, alumina, and stainless steel (84, 100,101,116). The enzyme retains 50-80% of its activity in free solution, but the stability on glass beads is markedly increased (84).

Why this phenomenon occurs is not clearly understood.

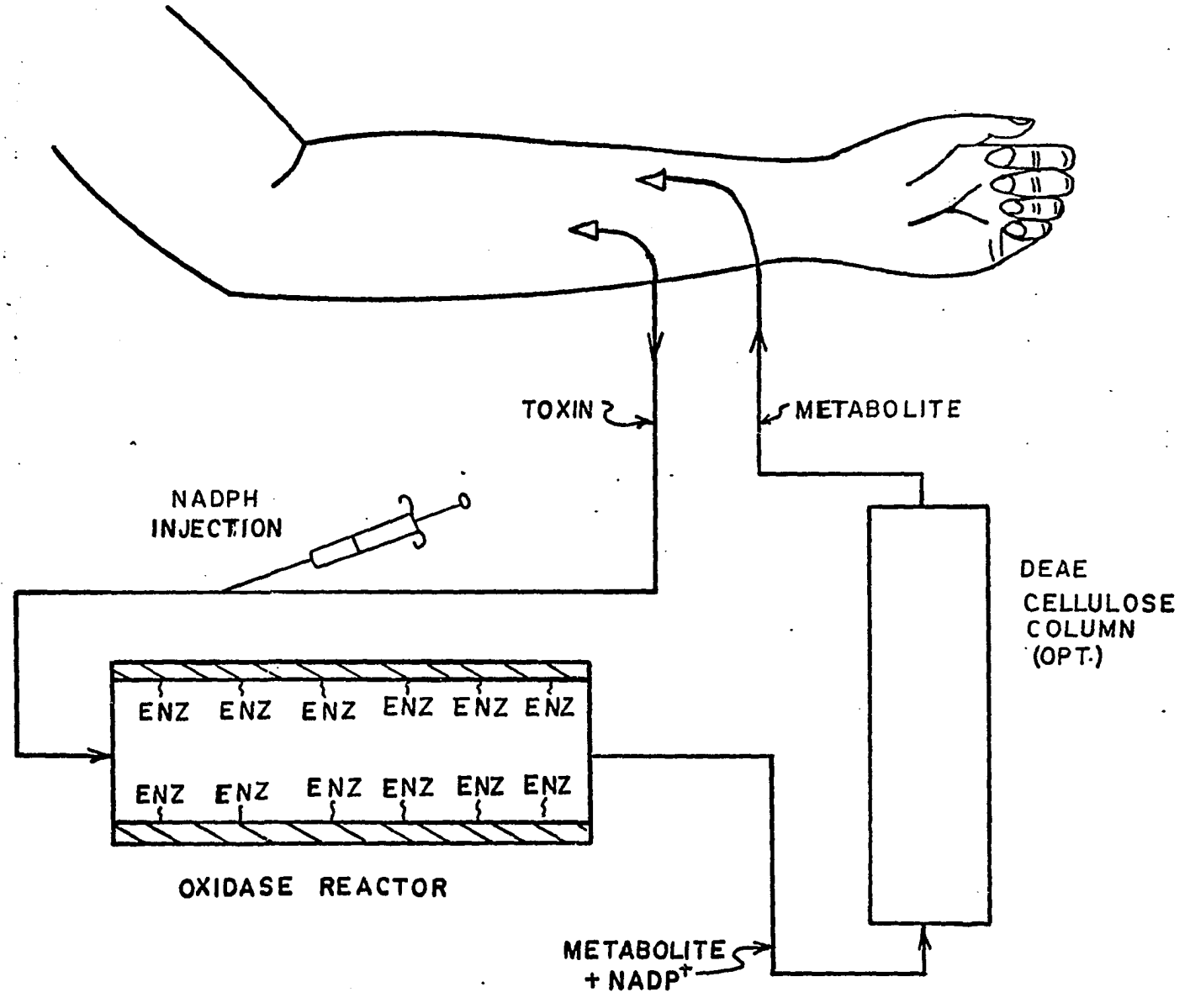
It is certainly the most striking example in the literature. Two hypotheses have been postulated. The first accounts for the fact that the oxidase in the liver is an immobilized enzyme in the sense that it is tightly bound to the wall of the smooth endoplasmic reticulum, and as such, in a teleological sense, is "happier" to be immobilized once again to a synthetic matrix. In a mechanistic sense, the enzyme may be in a strained, yet more stable configuration when immobilized. This is unlikely both from an entropy/free energy consideration and from the fact that the attachment sites on the enzyme are most likely not the same in the natural environment as they are in the synthetic. The second hypothesis posited is that despite the efforts of Ziegler and coworkers, the oxidase preparations are inherently impure, containing many isozymes prevalent in the liver in addition to the oxidase. Upon immobilization, a somewhat non-selective process which affects all enzymes to some degree, the degradative isozymes that may be attacking the oxidase in free solution are themselves isolated. This view is supported by recent data on ultrapure samples of the enzyme which have stabilities in solution approaching that of the immobilized enzyme (117). The latter analysis seems the more plausible, but the argument is largely academic at this point. The fact remains that MFMF oxidase immobilization may offer rewards too large to ignore, including the potential for using relatively impure hepatic extracts -- microsomes, possibly.

The stabilized enzyme has been studied in various types of reactors, and is currently being used to synthesize drug metabolites which are difficult or impossible to synthesize by standard organic techniques (100,101). It is not known what its specific properties are in direct contact with whole blood.

Figure 2 illustrates a hypothetical shunt containing the immobilized oxidase. As illustrated, the enzyme is attached to polymethylmethacrylate plates in a physical arrangement similar to one proposed by Hyden in an L-asparaginase shunt (118). Other researchers (119-124) have utilized similar devices for enzyme therapy with the respective enzymes attached to dacron, sepharose beads, porous glass beads, and glass plates. By the same token, the design in Figure 2 could incorporate a fixed-bed, packed tower, fluidized bed, or tubular reactor. In sharp contrast to all previous enzyme replacement therapies, the immobilized oxidase nevertheless requires the presence of the NADPH cofactor.

The immobilization of NADP has also been reviewed (125-128), but when isolated to an insoluble matrix it loses much of its activity to enzymes in free solution, and all activity is lost when both the enzyme and cofactor are immobilized. This fact will return time and again when dealing with labile cofactor dependent enzymes: at least one moiety (either the enzyme or the cofactor) must be in free solution. All efforts to date with the oxidase have

Figure 2. Simple Hemoperfusion Shunt. This is a modification of a design first proposed by Hyden for L-asparaginase therapy. A cofactor injection system has been included to meet the requirements of the MFMF oxidase, and a DEAE cellulose scrubber is included as an option, in the event the rate at which cofactor is supplied to the reactor exceeds the body's toxic limitations. This design has the bovious limitations described on page 34.


TOXIN
METABOLITE
NADPH
INJECTION
DEAE
CELLULOSE
COLUMN
(OPT.)
ENZ ENZ ENZ ENZ ENZ ENZ
ENZ ENZ ENZ ENZ ENZ ENZ
OXIDASE REACTOR
METABOLITE
+ NADP+

stressed the immobilization of the enzyme due to its increased stability and potential cost and toxicity and reductions as opposed to cofactor immobilization.

Mosbach, in a recent paper (129), has refuted such statements by demonstrating the catalytic activity of a simple oxido-reductase with no requirement for soluble cofactor. Basically, the technique he describes initially saturates the enzyme at all allosteric sites with the cofactor (NADH). The activated complex is then entrapped and covalently bound to a sepharose matrix. Due to the nature of the enzyme involved (alcohol dehydrogenase), the entrapped cofactor can alternately be oxidized and reduced, depending on the substrates encountered. Since the oxidase in this study does not have the bipartisan abilities of alcohol dehydrogenase, the entrapped cofactor would rapidly become depleted. Nevertheless, the possibility of regenerating the free cofactor in situ is not out of the question, as has been discussed earlier. A remaining drawback to Mosbach's novel system is that his substrates and metabolites are necessarily low molecular weight compounds that diffuse easily through the interstices of enzyme and sepharose. As was pointed out earlier, the real value of external enzymatic hepatic support lies in the metabolism of non-dialyzable higher molecular weight toxins.

Even faced with the eventuality of injecting soluble cofactor into the blood, one is not totally without recourse.

Pastore has developed a diethylaminoethyl (DEAE) cellulose chromatography column for the selective adsorption of reduced and oxidized pyridine nucleotides (130). Careful elution with a linear salt gradient resolves sharp peaks for each moiety. Ignoring its application as a laboratory separation technique, the essential issue is that the column is capable of removing nearly all pyridine nucleotides from a complex fluid in a single pass. Figure 2 illustrates how such a scrubber may be utilized downstream of the reactor. If desired, the column may be removed periodically, the cofactor eluted, regenerated by any convenient means, and re-injected upstream from the reactor. A number of critical questions remain unanswered regarding such a system. DEAE cellulose is known to be a powerful adsorbent for various blood proteins and salts. What effect would this have on the patient and on the performance of the scrubber? How quickly would the column become saturated? Could the spent cofactor be economically eluted? Would hemolysis be as serious a restriction with the DEAE cellulose column as it is with the various exchange resins being evaluated elsewhere? Many of these questions can be obviated, or at least de-emphasized, by simplifying the fluid being dealt with in the reactor and scrubber. There appear to be numerous advantages to such an approach, which will be discussed in the following section.

Enzyme Isolation Via Semi-Permeable Membrane Barrier

Figure 3 illustrates a generalized flow diagram that incorporates a membrane between the whole blood of the patient, and a dialysate side consisting mainly of bile salts, and small lipo proteins (<5000 M.W.). By selecting the permeability of the membrane, potential substrate molecules, as well as other small molecules are made to pass through the membrane due to the concentration gradient, while the protein and whole cell fraction is retained. This dialyzate solution then flows through an extracorporeal reactor containing the immobilized oxidase, where the substrate is converted into the corresponding metabolic oxide. This solution is then passed across a second* membrane, permitting re-equilibration with the blood, allowing the return of the small molecules previously removed and the net replacement of the toxic amine with its metabolic oxide. Thus, the net result is simply the selective metabolism of specific toxins.

Oreleja (131) has constructed an $NADP^+$ regeneration reactor using various types of immobilized oxidoreductases. Its use is also illustrated in Figure 3. The refinements

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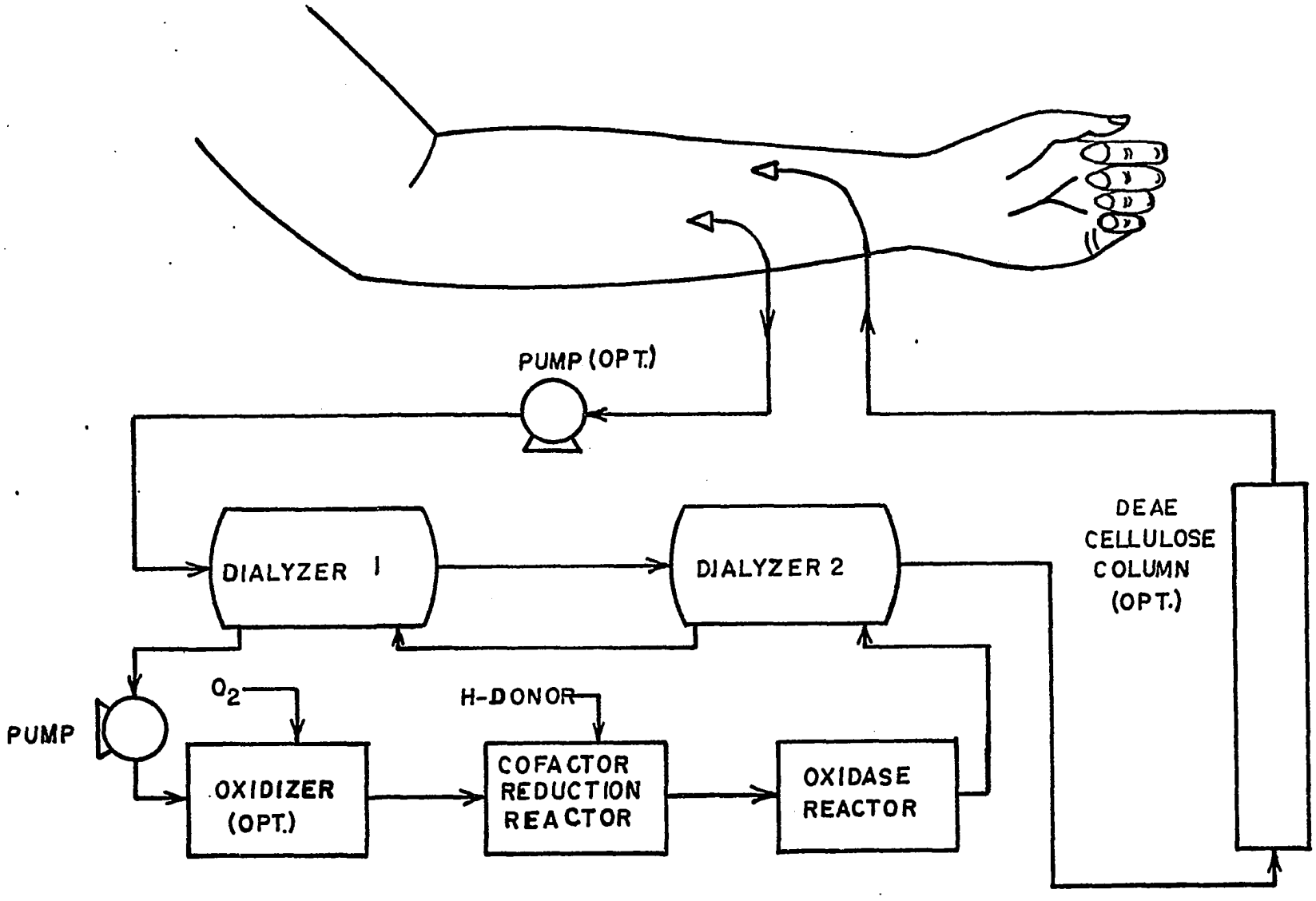
* The system as drawn employs two separate membranes. This feature is strictly for the purpose of facilitating the reader's visualization of the process; it offers no physical advantage. In truth, a single membrane compartment performs the same function due to the same concentration gradients.

Figure 3. Enzyme Isolation via Semipermeable Membrane Barrier. In such a system, potential substrates and metabolites are forced back and forth across the membrane by concentration gradients maintained by the oxidase. The advantages to this technique are increased enzyme stability, fewer undesirable side effects to the patient. The optional modules: reactor stream oxygenator, cofactor regeneration reactor, and DEAE trap may be included if desired. The major added dis advantage is an increase in mass transfer resistance.


PUMP (OPT.)
DIALYZER 1
DIALYZER 2
DEAE
CELLULOSE
COLUMN
(OPT.)
PUMP
O_2
H-DONOR
OXIDIZER
(OPT.)
COFACTOR
REDUCTION
REACTOR
OXIDASE
REACTOR

of this system over the previous use of soluble oxidoreductases for *in situ* regeneration allows a much cleaner, simplified reaction medium, and provides for strategic placement of the regeneration step in the MFMF oxidase reactor loop. Most significantly, it can maintain an artificially high concentration of NADPH necessary for high oxidase rates, yet the total cofactor concentration (both $NADP^+$ and NADPH), can be maintained at low catalytic levels. The latter benefit of *in situ* cofactor regeneration has not been mentioned in the literature.

Chambers has reported on a system similar to that depicted in Figure 3 (but without a cofactor intensive enzyme and its allied components) utilizing a soluble enzyme isolated by a cellulose hollow fiber dialyzer for the extracorporeal treatment of Fabry's disease (132). The use of hollow fiber dialyzer enzyme reactors for *in vitro* studies is also well documented (133).

Indeed, membranes are often used to immobilize an enzyme in free solution. Kinetic studies have been performed on various soluble enzymes entrapped within the fibers of a dialyzer which is then immersed in the solution containing the substrate. After a period of time, the enzyme will have converted the substrate to product, without contaminating the reaction medium with enzyme. Such a system lends itself to very stable catalysts which are not advantageously immobilized by covalent attachment to an insoluble matrix.

Microencapsulation by the methods of Chang is a novel method for achieving the same net effect. As mentioned earlier, Chang has suggested their use in an artificial liver and kidney, but due to the inavailability of a suitable enzyme, has been unable to produce a system capable of hepatic detoxification. Of interest in this study, however, are the numerous advances Chang has made with other enzyme systems. The enzymes catalase, asparginase, and urease have been stabilized to a degree without insolubilization by microencapsulation with a concentrated protein solution (134). This has been particularly effective with relatively crude enzyme preparations, yet half lives are seldom increased by more than 50% with this method. Recycling of cofactors, an issue critical to the application of the MFMF oxidase in microcapsules, has also been demonstrated (70). Although work is still progressing in this area, Chang and coworkers have been unsuccessful at developing microcell membranes with the same high molecular weight cutoffs possible with sheet and hollow fiber membranes. This may be the crucial factor if a microcell artificial liver were to be developed.

Chang's concepts have been extended recently in a paper by Updike (135) in which the author described the entrapment of L-asparaginase in bonafide red blood cells. As a strategy to avoid serious allergic reactions, the process requires gently lysing the RBC's in a hypotonic solution

containing the asparaginase, and then allowing the resealing of the autologous cells before injection. The possibilities are intriguing, especially when one considers that NADP occurs naturally in catalytic quantities in the RBC, but probably not in serum.

In any event, isolation of large blood groups from the reaction medium has numerous advantages.

1) Enzyme half life is demonstrably greater, presumably because isozymic and whole cell attack are obviated by excluding the large proteins and cells.

2) No foreign protein enters the patient's blood, reducing allergic reactions.

3) Cell and protein agglomeration on the catalyst surface are eliminated, hence maintaining the highest possible reaction rates.

The major drawback is simply the reduced overall reaction rate created by the diffusional limitations of the membrane. Yet, this has been shown to be not as significant as one might suppose when dealing with enzymes insolubilized on porous glass beads, the most successful support used today. Closset (136) has demonstrated in a mathematical model, and shown experimentally that the diffusional resistances at the membrane and at the glass bead may be of a comparable order of magnitude. Sofer (84) has shown in a non-membrane system that diffusional resistances at the bead are limit-

ing for the oxidase enzyme.

An issue that may be raised at this point deals with the state in which most foreign, lipophilic toxins exist in the plasma. They are primarily attached to large proteins and globulins. The binding equilibrium lies far in the favor of bound toxins. Chlorpromazine, for example, exists as a protein-bound complex with only 3% remaining in free solution (137). Membrane exclusion of such proteins also excludes the greatest percentage of toxin from the enzyme. This would seemingly be a mark against a membrane system, and a plus for the direct contact scheme of the previous section. This is not necessarily so. Evidence accumulated thus far indicates that the oxidase, whether in free solution, or insolubilized on glass, will be incapable of catalysis on a substrate bound to a large protein. It is possible that lipophilic toxins may be solubilized with bile salts, in which case the oxidase may be active. This facet has not been investigated. Nevertheless, it appears that the total catalytic processes of the detoxifying enzymes occur only on that portion in free solution -- 3% in the illustrative example of chlorpromazine. As this soluble fraction is consumed in the oxidation reaction, the equilibrium will continue to shift until all of the toxin has been metabolized. This is time consuming, but it is the path followed by a normal liver, and it does not appear that it can be improved upon if hepatic enzymes are the means

to the end. By this logic then, the artificial dialysis membrane compromises the potential efficiency of detoxification no more than the cell wall of an hepatocyte. In fact, one can most easily visualize the system of Figure 3 as a giant specialized liver cell.

Another valid concern is frequently raised concerning the wisdom of including any enzyme in a hemodialytic detoxification system. Assuming that the sole purpose of an extrahepatic support device is to remove toxins, then enzyme catalysis in combination with hemodialysis may indeed be redundant. After all, once the toxin is removed, why return it to the patient in a less toxic form? One can, however, view extrahepatic support as an enzyme replacement therapy. In this analysis, the enzyme becomes the primary necessity, and dialysis merely a necessary element. Also, bear in mind that detoxification has been selected for study here simply because it offers the best opportunity for a reasonably short-term evaluation of the project -- the 10-year goal having been stated earlier. This, of course, is a faltering first step in duplicating total liver function. Yet if this step is to have any validity in the future, we must begin now by duplicating biochemical pathways and not short-cutting to what may appear to be the more accessible goals of extractive detoxification. If as Fischer (59) believes, the normal liver synthesizes essential neurotransmitters, and lack of these compounds spawns the neuropathy of hepatic coma, then straight hemodialysis will never be

an effective therapy for reversal of level IV coma. This has been proven clinically (58). The beauty of an enzyme support system is that specific knowledge of the unknown neurotransmitters involved is unnecessary, if the extrahepatic device is capable of their synthesis. It is not known what role the oxidase of this study may play in such an eventuality.

More immediate advantages to enzyme catalysis in hemoperfusion devices may also be postulated. Currently, artificial kidney machines which may be used in liver detoxification are about the size of a washing machine, expensive, require at least four units of blood for priming and large volumes (200-300 ℓ) of sterile dialysate to maintain the necessary concentration gradients. Comparatively, a urease enzyme reactor coupled with a hemoperfusion device incorporating the same membrane area of the artificial kidney has been constructed within the volume of a soft drink can (138-140). Large volumes of dialysate are unnecessary because the concentration gradient is maintained by the enzymatic consumption of the substrate (urea). Furthermore, essential metabolites that may exist only in ppm concentrations in the blood would not be lost in a large dialysate volume. It is even plausible, depending on the compound(s) to be dialyzed and the rates of the enzyme system chosen, that higher dialysis rates could be achieved than with routine hemodilysis. This is especially true if the enzyme

is immobilized to the dialysate side of the membrane wall. Gregor (141) has collected some data to support this.

A third significant issue that has been glossed over until now is the recurring cofactor dilemma: it diffuses easily through most dialysis membranes. Chambers (142-144) has sidestepped this problem in an experimental reactor for converting ethanol to acetaldehyde by selecting a commercially available hollow fiber membrane with a particularly "tight" M.W. cutoff of 200. The substrates with which he dealt easily passed through, while the cofactor (approximate molecular weight of 800) was retained. Such a system may be adequate for treatment of some toxins in an hepatic support device. At this time, however, it is not known what other endotoxins may be responsible for the encephalopathy evident in hepatic coma, the one area that has not responded to any type of therapy to date. If these endotoxins are larger than the cofactor, a non-specific membrane would exclude them from the reaction medium, and hence from detoxification. It is felt, however, that some degree of optimization may be achieved between membrane cutoff and an acceptable cofactor flux into the blood. If such is the case, then an extrahepatic support device could be designed with a much wider potential for success in dealing with the unknown larger toxins.

Furthermore, a DEAE cellulose scrubber could be utilized in the blood return line in a manner analogous to

that described in the previous section. This adds the potential of further increasing the membrane cutoff, if desired, and/or increasing the concentration of cofactors in the reactor loop.

High Molecular Weight Cofactor Analogs. Several laboratories have taken a differing approach to cofactor retention within a membrane reactor: the synthesis of a soluble, macromolecular, enzymatically active cofactor.

Limited success has been obtained with the pyridine nucleotides. Wykes (145) coupled succinyl-NAD to polyethyleneimine (PEI), molecular weight 40 to 60,000. The resulting NAD-PEI complex contained 19-34 moles of NAD per gram of PEI. Of this, 45% was enzymatically reducible with alcohol dehydrogenase. Weibel (146) coupled C(6)-amino ethyl-NADH to a soluble dextran derivative yielding a cofactor loading of 200-300 moles NAD per gram of dextran, and was 80% enzymatically active. Chambers (144) has bound succinyl NAD to amino dextran (M.W. 500,000) with 55 moles NAD per gram of dextran remaining, of which approximately 50% was enzymatically reducible with alcohol dehydrogenase. More recently, Larsson and Mosbach (147) have coupled the N^6-(N-(6-aminohexyl)-acetamide) derivative of NAD to dextran through a long and complex synthesis. The product contained 65 moles NAD per gram of dextran, and was about 80% active with alcohol dehydrogenase.

Analogous polymer-NADP complexes have met with even

less success. In only one published example, Lowe and Mosbach (148) have attempted to couple their N^6-(N-(6-aminohexyl)-acetamide) derivative of NADP to dextran. The product showed a reasonable NADP loading of 85 moles NADP/gm dextran, but a disappointing activity of only 35% compared to the native NADP. Furthermore, stabilities of all of these cofactor analogues have been unsatisfactory, and the economics are difficult to justify when one considers that a large percentage of the costly cofactor is lost during the complex syntheses. As a result, there is extensive ongoing research to develop new methods of macromolecular cofactor production. Chambers (99) is currently evaluating various globular proteins such as albumin for soluble supports. Preliminary data have indicated very high loading and enzymatic activity with NAD-coupled albumins. Falb (99) has taken this one step further by complexing NAD directly to yeast alcohol dehydrogenase. His preliminary unpublished results indicate that a loading of approximately 50 NAD molecules per enzyme tetramer show some activity in a cycling system using a number of alcohols and aldehydes.

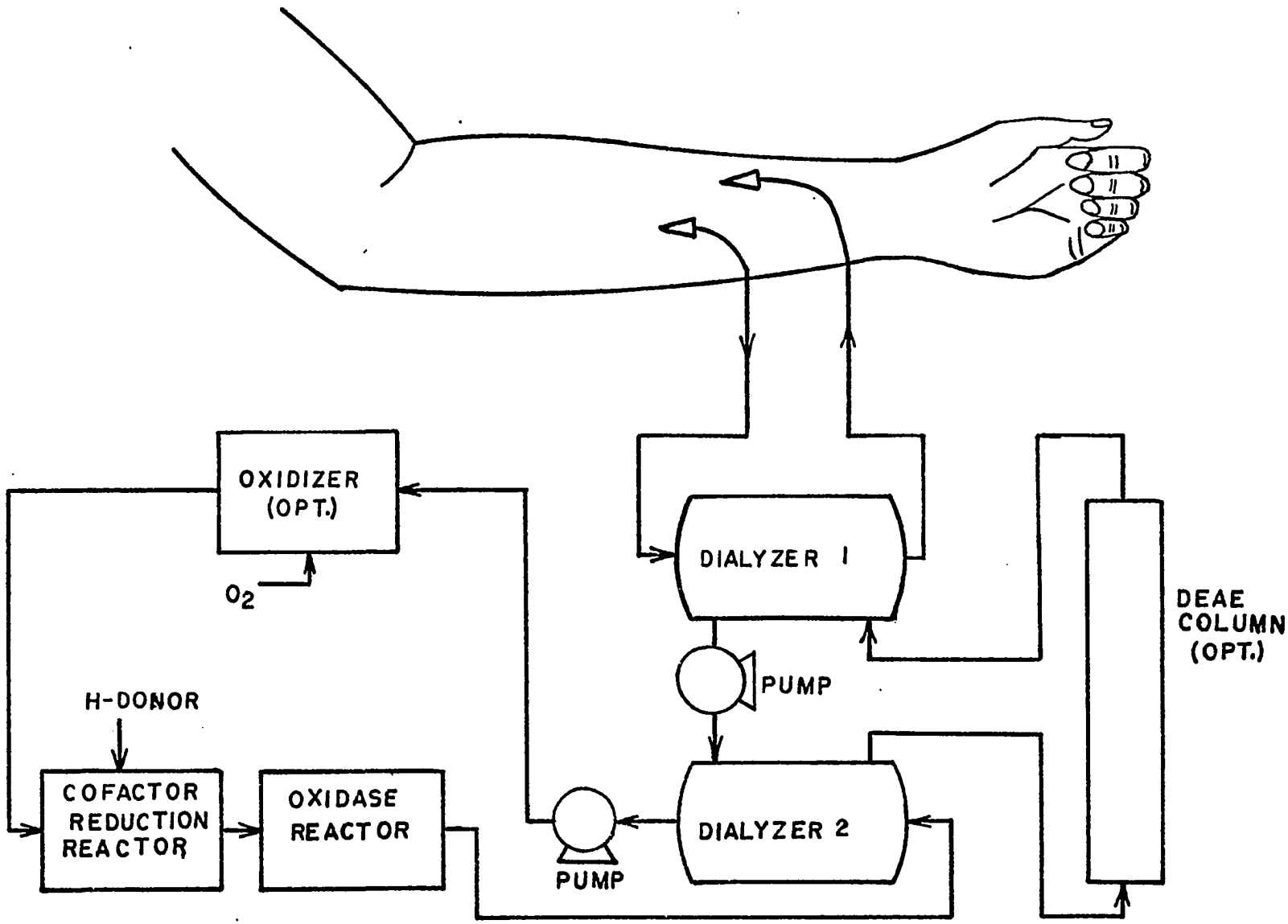
The successes of cofactor modification must, however, be viewed in perspective: 1) all analyses have been conducted with soluble enzymes; 2) these enzymes, usually yeast alcohol dehydrogenase, are typically simple, active, tough catalysts; and 3) only one case has demonstrated marginal activity of an NADP analogue, a considerably more sensitive

cofactor than NAD. The potential activity of an NADP analogue with the immobilized MFMF oxidase is not known, much less its capacity for regeneration. Wykes, Dunnill, and Lilly, who first postulated and synthesized high molecular weight cofactors in 1971, have recently all but written off this area of research by concluding that "the cost of immobilization of NAD cannot be justified unless a greater stability than that we have observed is achieved " (149).

Multiple Loop Dialysers. An outgrowth of the double loop system described in the previous section would be a system employing three or more loops, as illustrated in Figure 4. The penalties of multiple cycles and multiple membranes include the obvious rate limitations of such a design, and the necessity for an increased dialysate volume. As a result, such a flow scheme has not been suggested in the literature. It has been proposed here as the result of what may be serious design flaws in the double loop system coupled with the DEAE cellulose scrubber. The consensus regarding the scrubber is that all efforts should be made to remove it from the whole blood circuit because of the unknown, and potentially devastating effects it may have in salt and protein depletion, and hemolysis.

Wishing to accrue the benefits of dealing with a relatively clean dialysate in the DEAE cellulose scrubber for many of the same reasons enumerated for the enzyme reactor, a second dialysate loop was added. A comparison of Figure 4

Figure 4. Multiple Loop Dialyzers. This system is proposed in the event the DEAE cofactor trap is deemed necessary. The placement of the trap in Figure 3 may result in serious side effects when in direct onctact with the blood. To preclude protein agglomeration and hemolysis in the DEAE column, it too is placed behind a membrane.


OXIDIZER
(OPT.)
O_2
DIALYZER 1
DEAE
COLUMN
(OPT.)
PUMP
H-DONOR
COFACTOR
REDUCTION
REACTOR
OXIDASE
REACTOR
PUMP
DIALYZER 2

with Figure 3 clearly shows that the end result, conversion of substrate to metabolite, will occur and the toxic cofactor will be entrapped before reaching the whole blood. Although the absolute necessity of the scrubber has yet to be indicated, the foregoing is included as an exercise for this eventuality.

Selective Membranes

The bulk of the foregoing has been premised on the known availability of various isotropic membranes in multiple configurations and molecular weight range properties. Their selectivity relies, to a great extent, on the resolution of differing molecular weight species. Living membranes on the other hand, possess high specificity not only to molecular weight, but to charge, solubility, and configuration. Many living membranes are even capable of resolution of racemic mixtures (150). Significantly, they can, through an expension of energy known as active transport, transfer matter against the concentration gradient. The most widely known example is the selective uptake of sodium in the tubule of the kidney. It is believed that all cells are capable of active transport to some degree. Various theories have been hypothesized to explain this phenomenon. It is becoming apparent that several specialized systems are responsible for each case observed (151). Active transport has not been

demonstrated in a totally synthetic system*, yet work is now underway to develop a synthetic membrane containing fragments of living halobacter cell wall capable of active transport of hydronium ion with the consumption of solar energy (152).

The topic of membrane specificity is particularly timely to the development of an artificial liver. An hepatocyte cell wall is capable of transferring various compounds for metabolism, and retaining others necessary for maintenance of the organelle. In the design of the oxidase reactor, it would be desirable to duplicate some of these properties; specificially, to allow free passage of potential substrates and metabolites across the dialysis membrane, while retaining the compounds necessary for the net reaction (enzymes and cofactors). An understanding of the mechanism of transfer in various membranes that may find application in such a system is intrinsic to their selection in a model design.

Cellulosic Membranes

This type of membrane has found wide application in hemodialysis, desalinization, and other desalting operations which rely on the intrinsic selectivity of the polymer matrix. Cellulose, and cellulose acetate are highly organized polymers having groups capable of hydrogen bonding to water or other solvents subject to hydrogen bonding, like ammonia or

* Reverse osmosis, the simultaneous concentration of one solution and the dilution of the other across a membrane due to an external pressure gradient, and electrodialysis, involving the consumption of electric power, are the closest engineers have come so far.

alcohols. These hydrogen bonding solvents can complex with the carbonyl sites in the polymer matrix, but ions and non-hydrogen bonding substances are excluded to a greater degree than that observed in a non-interactive polymeric membrane (153) (Figure 5-A). The molecules that do enter the matrix as a result of hydrogen bonding then move from one bonding site to another and thus are transported through the polymer, provided there is a driving force to cause transfer.

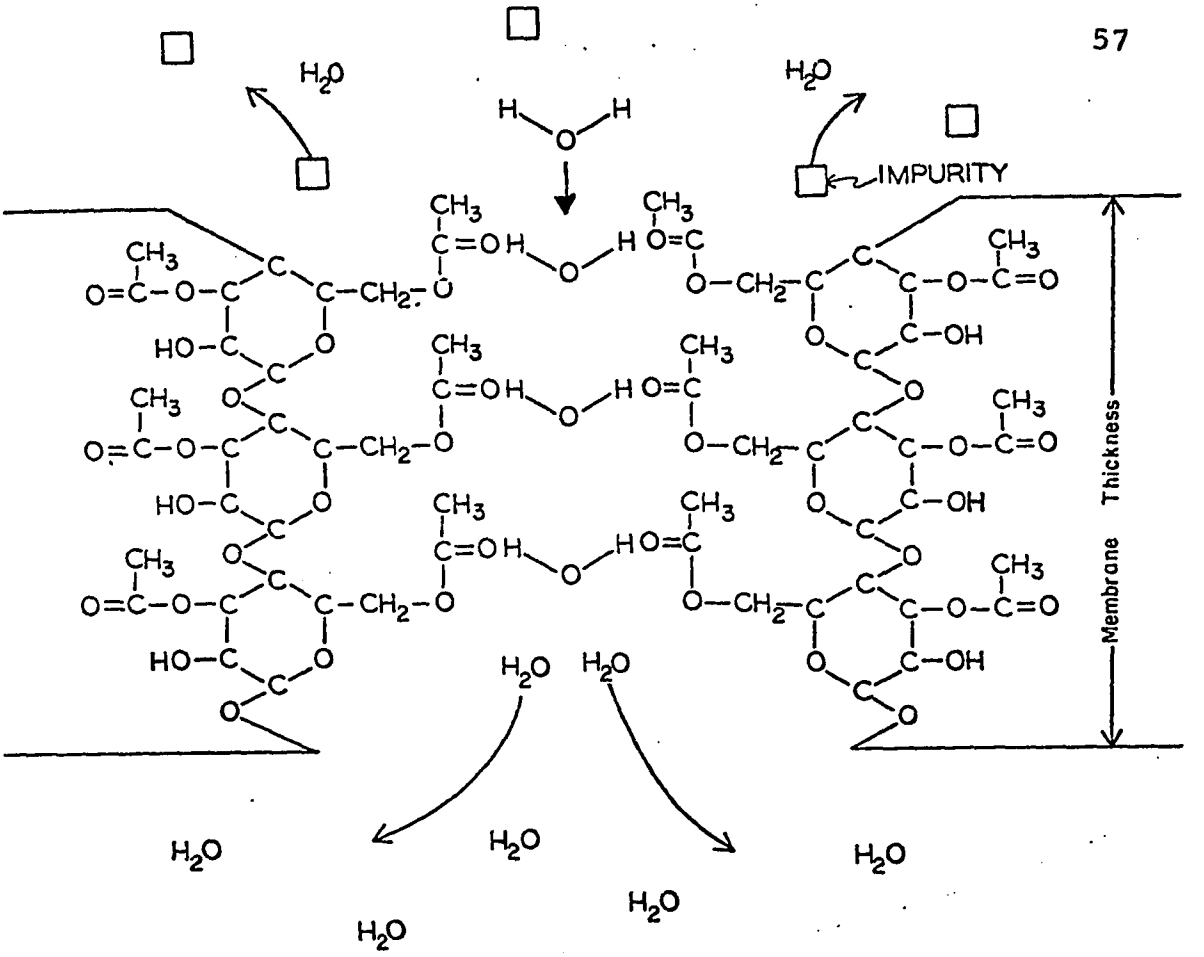
Such a mechanism of transfer lends itself quite readily to hemodialysis in which one wishes to retain most ionic salts, yet remove the hydrogen bond forming ammonia, urea, and some acid salts from the blood. Conversely, in an extrahepatic device the volume of dialysate will be very low, minimizing the problem of essential ion loss. More significantly, the lipophilic toxins of concern in hepatotoxicity are not well suited to tranport through a cellulosic membrane. Most enzyme reactors to date have utilized cellulosic hollow fiber membranes simply because of their wide availability.

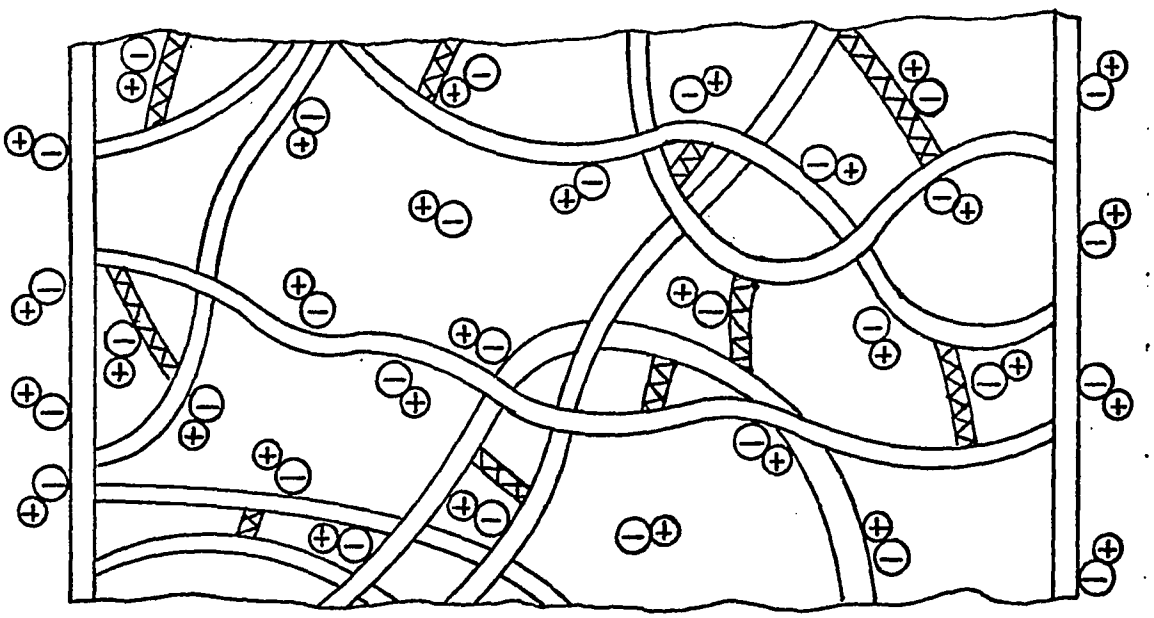
Ion Exchange Membranes

The selectivity here derives from the net charge of the dialyzing species. Some are permeable to positive ions (cationic membranes), while others allow passage of negative ions (anionic membranes). This is achieved by attaching charged groups throughout an inert polymer matrix. In the case of a cation exchanger, (Figure 5-B), positive ions are freely dispersed in the voids between crosslinked polymer

Figure 5-A. (153) Water Transfer in Cellulose Acetate Membrane. The characteristically high water fluxes observed in cellulosic membranes are due largely to a hydrogen bonding mechanism of the water. An impurity incapable of hydrogen bonding tends to be excluded by the membrane.

Figure 5-B. (153) Representation of Cation-Exchange Membrane. As a result of the fixed negatively charged sites with the matrix of the neutral polymer, negatively charged ions in solution tend to be repelled from the interstices of the polymer chains. The mobile positively charged groups, on the other hand, are free to move and be eluted from the membrane. They therefore offer no resistance to diffusion of positive groups in the dialysate. If positively fixed charges are attached to the polymer chains instead of negative fixed charges, the opposite type of selectivity is observed.





⊖ Fixed negatively charged exchange site, i.e. SO_3^-
⊕ Mobile positively charged exchangeable cation, i.e. Na^+
Polystyrene chain
Divinylbenzene crosslink

chains. The fixed negative charges repel negative ions that try to enter the polymer matrix, hence excluding them from the membrane. Positive ions, on the other hand, are free to pass, as are the unbound positive groups that were formerly associated with the fixed negative species during manufacture. If negative fixed charges are attached to the polymer chains instead of positive fixed charges, the opposite type of selectivity is achieved.

In an effort to increase the stability of ion exchange membranes, a high degree of crosslinking has been necessary to retain the fixed charges and to assure high charge densities within the polymer. As a result, fluxes of all but the smallest ions are low. A compromise must be struck between a loosely crosslinked structure offering little charge selectivity, and a tight structure with a very low molecular weight cutoff (153).

The reason for discussing charge selective membranes in this study stems from the observation that many cofactors, NAD and NADP included, possess a net positive charge in the oxidized state. Charge may possibly be used as a key to their selective retention within the reactor loop of Figures 3 and 4. For example, if the reactor in Figure 3 is operated under cofactor limiting conditions (high enzyme concentration), the effluent stream from the reactor will contain very little uncharged, reduced cofactor, and an anion exchange membrane may successfully retain the charged moiety.

It then returns to the regeneration reactor, where it is once again reduced.

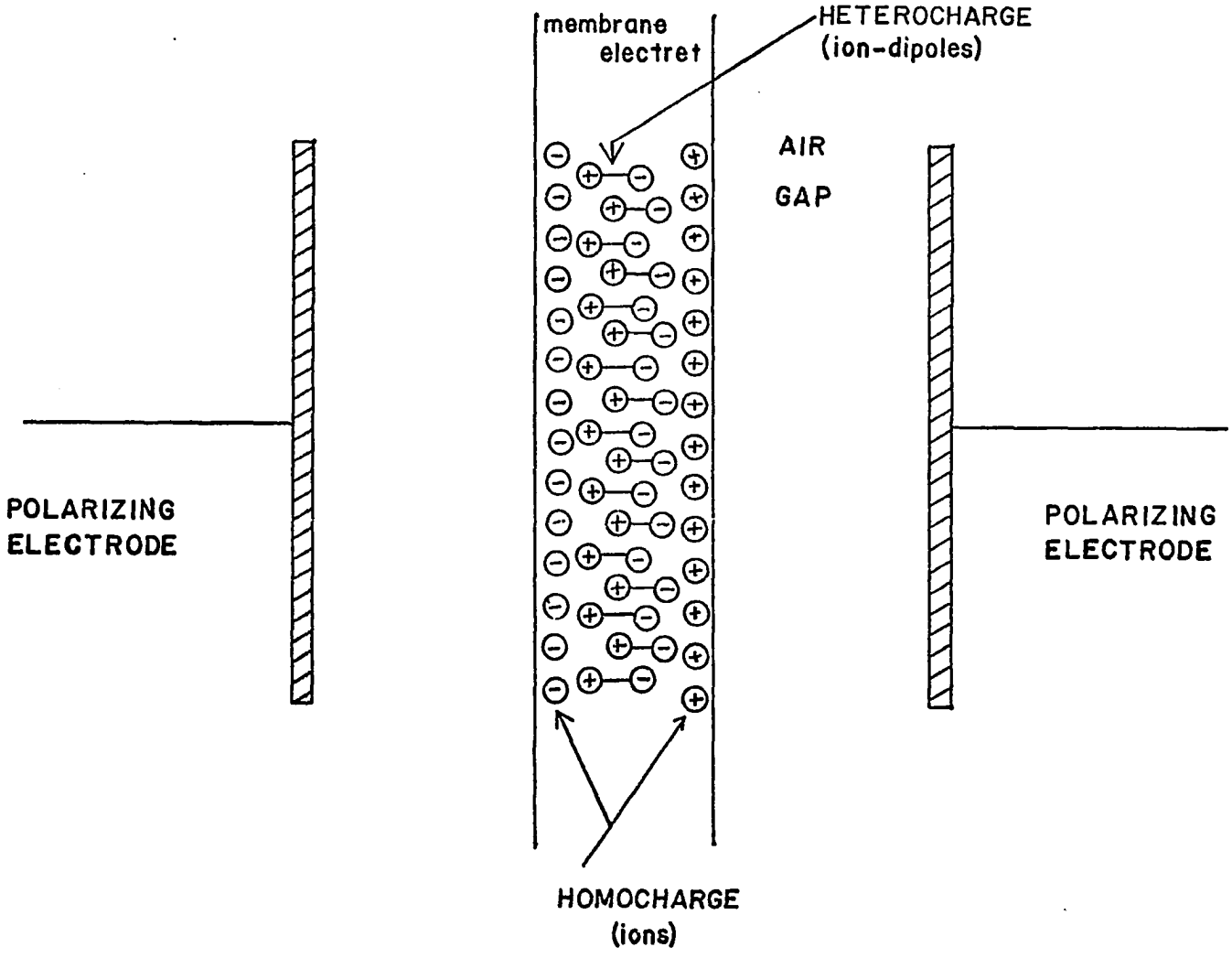
Polymeric Membrane Electrets

Althouth cofactor charge has not been reported in the literature as a means of retention, the possibility is nevertheless promising. For this reason, the survey of available membranes was extended to allied areas of polymer technology in search of a more porous, charge selective membrane. It was discovered that properly treated polymers are capable of maintaining a permanent dipole electrostatic charge at room temperature. In theory, at least, the charged cofactor would be repelled from the charged surface of an electret membrane in much the same way as in the ion exchange membrane.

Membrane electrets are polymeric films which exhibit a persistent internal electrical polarization after removal from an external polarizing DC field. Gross (154) has shown that electret polarization can be described by a two charge theory: homocharge and heterocharge. Homocharge is due to transfer of charge at the membrane/electrode interfact which results in the injection of ions into the surface of the electret. Heterocharge is due to volume polarization of the electret, i.e., dipolar alignment and space charge polarization. Figure 6 illustrates the probable mechanism for electret formation in a polymeric membrane.

Homocharge is typically the less persistent of the two. Results on cellulose acetate electrets indicate that

Figure 6. Representation of an Electret Formation. Membrane electrets are formed by heating the polymer to a polarization temperature and applying a high density field to the membrane as it cools. The formation of heterocharge deep within the membrane is the result of a volume polarization, or alignment of ion-dipoles. Homocharge is due to the injection of ions formed in the air gap by the high voltage applied. Heterocharge is stable at room temperature, whereas homocharge is a transient phenomenon which gradually drains off.


membrane
electret
HETEROCHARGE
(ion-dipoles)
AIR
GAP
POLARIZING
ELECTRODE
POLARIZING
ELECTRODE
HOMOCHARGE
(ions)

homocharge decays exponentially in dry air, approaching zero charge in 70 days (155). This decay can be accelerated by immersing the charged polymer in water. All homocharge character is lost within one hour of water immersion (155).

Heterocharge, conversely, appears to be a permanent phenomenon as long as the polymer is maintained below a critical depolarization temperature. This temperature is frequently near the glass transition point for amorphous polymers, presumably corresponding to a state in which the dipoles are free to align themselves in a more relaxed, non-aligned configuration (156).

The types of polymers that are conducive to being prepared as electret films must fit two criteria: 1) have adequate toughness at thicknesses of one micron and less, and 2) must posses polar (either mono or di-polar) groups capable of shifting alignment at some temperature before thermal decomposition occurs (156). It should be noted that the polymer need not be crystalline.

Polyvinylchloride (PVC) is an example of an amorphous polymer which can be made into a relatively strong electret. The repeat unit is polar with an effective dipole moment of 3.6×10^{-30} C·m (156). The usual form of PVC is amorphous because of the irregular positioning of the chloride. PVC is an equilibrium liquid above its glass transition temperature (about 80 C) although thermal decomposition is appreciable above this temperature without suitable stabilizers. Moreover,

PVC can be manufactured in tough films which have been evaluated as reverse osmosis membranes (150).

The objective in utilizing electrets (both PVC and cellulose acetate) in reverse osmosis systems has not been to exclude charged ions, but to inhibit the deposition of large charged colloids on the membrane surface. These contaminants, present in sea water, tend to reduce water fluxes in desalinization operations by several orders of magnitude within a short period of time. Membrane electrets have markedly reduced the rate at which this gel layer is deposited. Whether or not electrets can be equally effective at reducing $NADP^+$ fluxes is not known at this time. One advantage of polymer electrets has been reported in a biomedical application. Murphy (157) has reported that prosthetic arterial implants which possess a net negative charge on the surface exposed to the blood show excellent antithrombogenicity. Apparently, the large, negatively charged globulins in the blood that trigger clotting are repelled from the foreign surface, greatly reducing clotting and the deposition of megamolecular gels on the prosthesis. The advantages of a non-thrombogenic, self-cleaning membrane surface are quite alluring to extracorporeal devices. Allowing for the necessity of exposing the positively charged membrane surface to the reactor side containing the $NADP^+$ in Figure 3, the negatively charged surface is likewise exposed to the whole blood as Murphy suggested -- a serendipitous discovery to say the least.

Membrane Solubility

Charge is not the only criterion available for producing selective synthetic membranes. Solubility was indirectly touched on in the cellulosic membrane discussion. Its effect can be greatly accentuated through proper mating of membrane composition and the separation desired.

Rony has modified Amicon cellulose hollow fibers by treatment with organic solvents to render them highly selective (158-160). In his single fiber model system (with which scale up has proven difficult), ethanol is converted to acetaldehyde with an alcohol dehydrogenase isolated within the modified hollow fiber and, more significantly, the cofactor NAD is also retained. Rony describes the effect as the result of a stabilized liquid membrane formed in conjunction with the existing cellulose membrane. The water-soluble NAD is insoluble in the organic (iso-octane) liquid membrane, resulting in its retention by the membrane but simultaneously allowing transfer of both substrate and product, which are soluble in iso-octane and water.

Using a similar rationale, Dorson (161,162) has prepared selective membranes by forcing the deposition of a thin lipophilic protein gel on the surface of cellulose films. The resultant double membrane is reportedly selective to polar and non-polar compounds.

The application in an extrahepatic support device of a membrane which derives its selectivity from solubility

parameters warrants discussion at this point. To retain the cofactor (either NAD or NADP), a lipophilic barrier much like Rony's is called for. Now consider for a moment the typical substrate (toxin): it is most likely a fat soluble, middle molecular weight molecule. Its transfer into the reactor system would be permitted by the same lipophilic membrane. Once in the reactor though, recall that the primary mode of detoxification is to convert this substrate to a highly polar molecule, an N-oxide in the case of the MFMF oxidase acting on a tertiary amine. The flux of this new polar metabolite through a lipophilic barrier will be greatly obstructed, if not blocked altogether. It appears, then, that no matter how great the potential for solubility modified membranes in other enzyme reactor systems, their advantages will most likely never be realized in an enzymatic detoxification system. The foregoing is also applicable to a solvent extraction system in which the substrate is extracted in an organic layer, metabolized in an aqueous reactor, and returned to the patient via the donor solvent.

CONCLUSIONS

The salint feature to be gleaned from the foregoing potpourri is that sufficient information is currently available to launch a multifaceted attack on a problem that has failed to respond to the most advanced medical technology to date. The lethal certainty of hepatic failure allows wide

latitudes for one wishing to attack the problem. Furthermore, the topic offers the engineer a rare opportunity to test radical designs normally shunned by a conservative medical community. The specific goals outlined in Chapter I are but a stepping stone to define more clearly the direction a project of this potential must take.

CHAPTER III

MATERIALS

Purified Oxidase

The oxidase enzyme utilized in this study has been isolated through a separate research effort at the University of Texas. Although the technique of purification has been dealt with in detail elsewhere (93), a cursory review is included here.

The liver of mature hogs is the primary source for the enzyme. Although no effort has been made to increase the levels of this oxidase through drug treatment before slaughter, as has been done with the cytochrome P-450 system (164,165), experience has shown that MFMF availability is affected by factors such as sex, age, and even hair color. The pork liver tissue is homogenized and treated ultrasonically to promote cell and endoplasmic reticulum rupture. The microsomal fraction collected after an initial centrifugation is resuspended in 0.1M guanidine, pH 8.0, and spun at 78,000 g for 35 minutes. The pellet is resuspended in water to a final protein concentration of 20 mg/ml. The oxidase is solubilized from its natural matrix with the detergents Triton X-102 and X-45 at concentrations of 5 mg each per ml.

The detergent-protein solution is allowed to stand on ice for one hour, at which time 1 mg/ml protamine sulfate is added to precipitate surplus protein, which is removed by centrifugation at 78,000 g for 45 minutes. The supernatant fluid is immediately fractionated with an ammonium sulfate gradient. The fraction precipitating between 37 and 50% saturation is collected. This is dissolved in 0.1M acetate buffer, pH 4-8, and maintained at that pH with sulfuric acid during a second fractionation with saturated ammonium sulfate. The fraction collected between 45 and 50% saturation is dialyzed against a solution of 0.05M glycine and 0.05M histidine, pH 6.0. The dialysate is passed through a DEAE cellulose column equilibrated with the glycine-histidine buffer and eluted with the same buffer. The effluent is concentrated by ultrafiltration and then transferred to a Sephadex G-100-120 column equilibrated with 0.03M glycine, pH 6.0. Elution is continued with this buffer until the first bright yellow fraction, the purified oxidase appears.

Allied Enzymes, Cofactors, Supports, and Organic Chemicals

Table 1 summarizes the pertinent specifications.

Experimental Equipment and Commercial Membranes

Assay Microreactor. Figure 7 illustrates the reactor used for all kinetic studies with the oxidase enzyme. Features of this design include a liquid sealed injection port to insure a closed reaction system, a constant temperature

TABLE 1

Chemical Specifications and Distributors

Acetone, reagent grade, Fisher Scientific Co.

N,N-Dicycohexylacarbodiimide, 99%, Aldrich Chemicals

1,6-Diisocyanatohexane (DICH), Aldrich Chemicals

N,N-Dimethylaniline (DMA), Matheson Coleman and Bell

Dimethylsulphoxide (DMSO), tech grade, Mallinckrodt

Ethyl ether, anhydrous, Mallinckrodt

Glass bead supports, AlKyl Amine Zir-clad CPG-1350 pourous glass beads, Corning. Distributed by Pierce Chemical Co. Nominal surface area: 41 m^2/gm; particle diameter: 177-840 microns; nominal pore diameter: 1350Å.

D-glucose-6-phosphate, monosodium salt, Sigma Chemical Co.

D-glucose-6-phosphate dehydrogenase, type XV, Sigma Chemical Co.

Glutaraldehyde, 25%, practical grade, Matheson Coleman and Bell Manufacturing Chemists

Heparia, monosodium salt, injection, USP, 1000 units/ml, from beef lung, UpJohn Co.

Nicotinamide adenine dinucleotide (NAD), sodium salt from yeast extract, reduced and oxidized forms, Sigma Chemical Co.

TABLE 1 (Continued)

Nicotinamide adenine dinucleotide phosphate NADP), sodium salt, from yeast extract, reduced and oxidized forms, Sigma Chemical Co.

Potassium phosphate, mono and di-basic, anhydrous, Fisher Scientific Co.

Poly (ethylenimine), 30% solution in water, Aldrich Chemical Co.

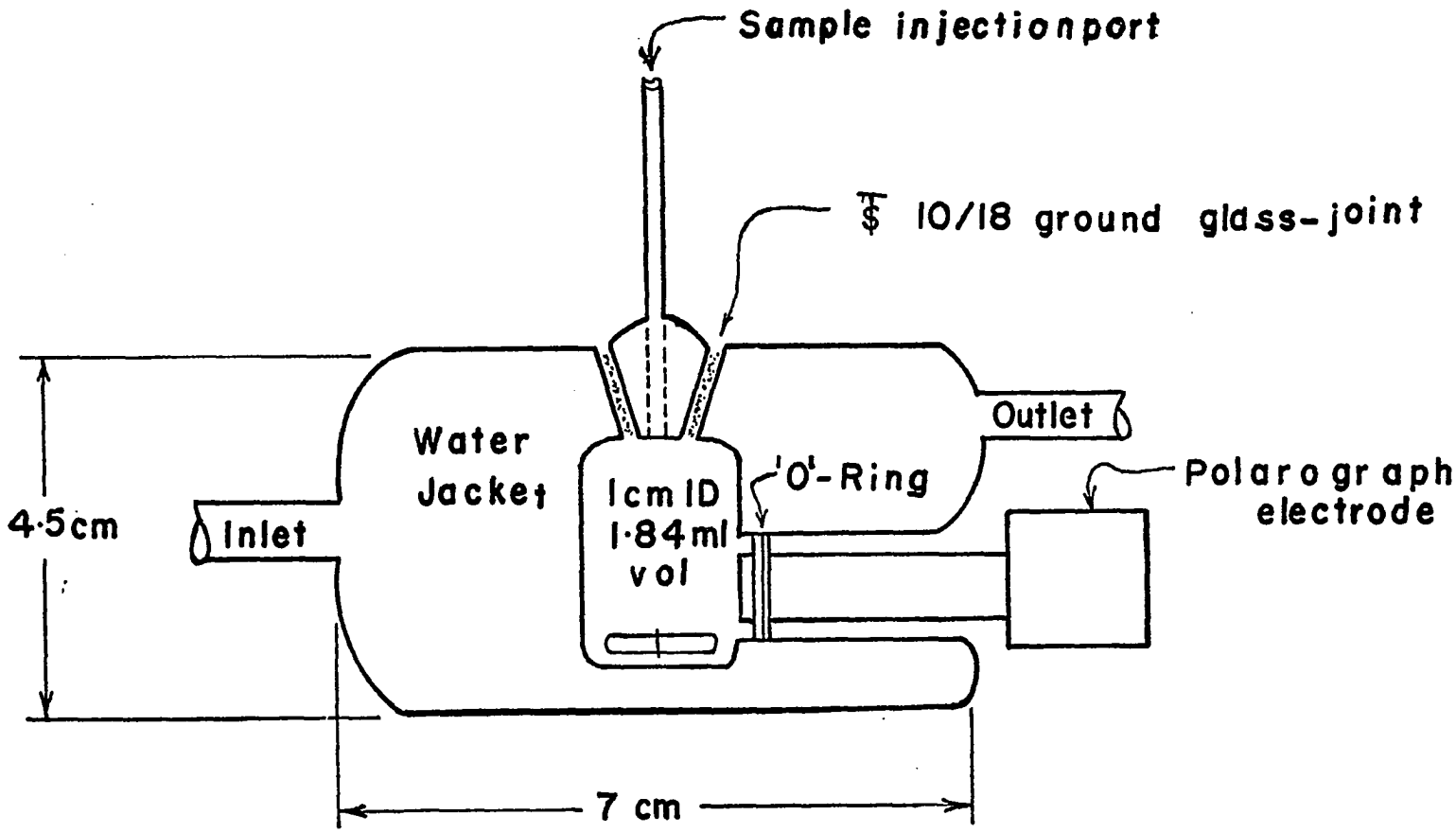
Polyvinylchloride, (PVC) powder from suspension polymerization, gift of Union Carbide.

Polyvinylchloride/Polyvinylacetate, (PVC/PVA), copolymer, 83% PVC, 17% PVA, powder from suspension polymerization gift of Union Carbide.

Pyridine, Matheson Coleman and Bell

Succinic anhydride, Matheson Coleman and Bell

Figure 7. Assay Microreactor. This reactor was used for all kinetics studies with the immobilized MFMF oxidase. The magnetic stirrer provides adequate mixing to suspend the finely divided catalyst, while the oxygen probe accurately measures the changing oxygen concentration in the closed system during reaction. Low catalyst requirements and quick turnaround time for initiation of a new run are two advantages of this design. The reactor is emptied by vacuum aspiration. Typical reaction mixtures are described on page 9 8.



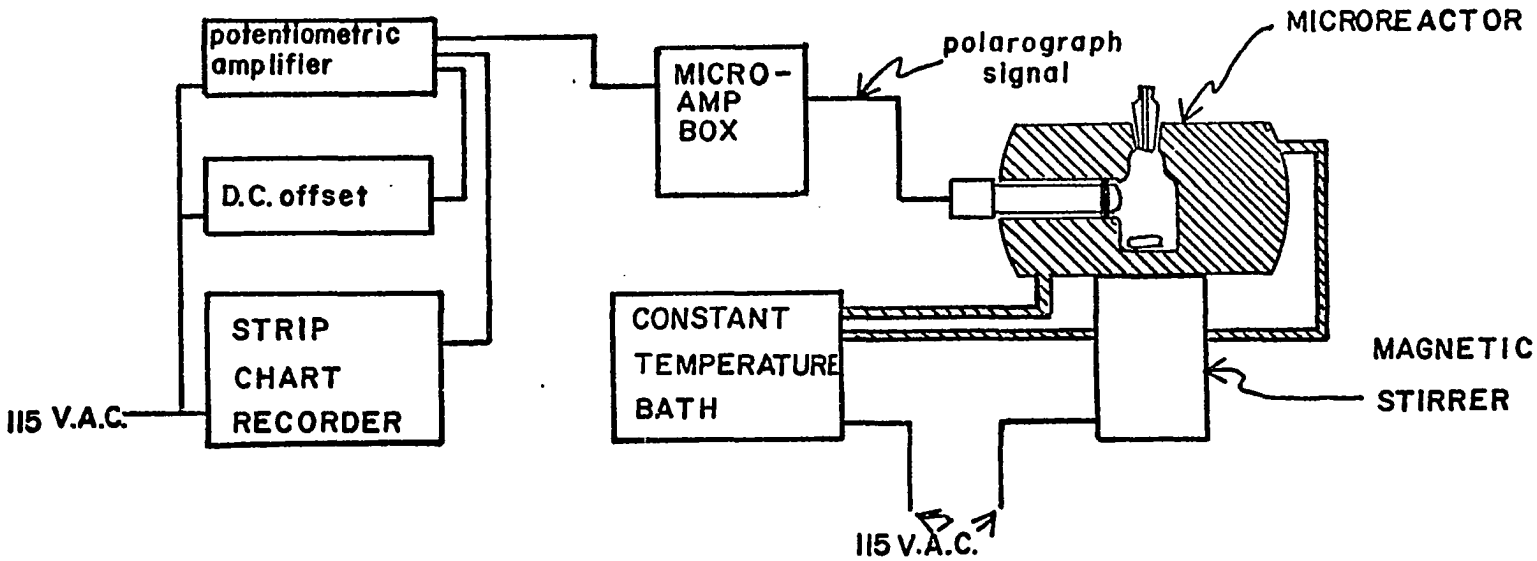
ASSAY MICROREACTOR

water jacket, coupled to a Haake constant temperature bath maintained at 37 ± 0.1 C, an oxygen electrode port (see below), and economical consumption of catalyst and substrate. Sofer has shown that agitation rate is critical to reaction rate as well as to electrode performance(101). A magnetic stirrer has been added to preclude any error due to the above.

Oxygen Polarograph. The course of the oxidase reaction was observed by monitoring the decreasing oxygen tension in the closed system microreactor. A Yellow Springs Instrument Co. Model No. 4004 Clark-type oxygen electorde was coupled to a Heath/Schlumberger model EU-205-11 strip chart recorder via a potentiometric trimming circuit built in this laboratory. A schematic of the entire system appears in Figure 8. The decision to monitor the consumption of oxygen as a substrate (as opposed to other substrate amines and cofactors) proved to be particularly convenient. The microreactor in conjunction with the oxygen polarograph allowed turnaround of less than one minute between successive runs, without complex quantitative analyses and the error induced by non-precision reaction termination required by other techniques. Reaction mixtures are described on page 98.

Heated Press. A Carver model C laboratory press capable of exerting a 24,000 lb. load over a 6" x 6" area coupled with two Carver model 2101 independently controlled heaters top and bottom (room temperature to 500 ± 5°F) was used in the preparation of the electret membranes (see Methods).

Figure 8. Schematic of Polarograph Input to Strip Chart Recorder via the Potentiometric Trimming Circuit. Due to the varying properties of the oxygen electrode with time, and membrane thickness, a micro-amp box is used as a coarse adjustment for input to the recorder. Final adjustments and zeroing may be made at the recorder.


potentiometric amplifier
D.C. offset
STRIP CHART RECORDER
115 V.A.C.
MICRO-AMP BOX
polarograph signal
MICROREACTOR
CONSTANT TEMPERATURE BATH
MAGNETIC STIRRER
115 V.A.C.

Other researchers have reported the necessity of applying evaporated silver electrodes to the membrane surfaces to eliminate air gaps and the resulting heterocharge effects (155,156,168). The heated press allowed the use of easily assembled electrode "sandwiches"; air gaps were successfully eliminated by applying loads in the range of 10,000 to 20,000 lbs., greatly simplifying the electret preparation procedure.

Membranes. Several commercially available membranes were evaluated, in addition to those prepared in the laboratory. The family of Bio-Fiber hollow fiber cellulose and cellulose acetate membranes, manufactured by Dow Chemical Co. and distributed by Bio-Rad Laboratories, Inc. have found numerous applications in experimental enzyme reactor systems. Cordis Labs, Miami, Florida, offers the only example of a semi-commercial biomedical application in the Cordis-Dow artificial kidney. Although transport properties of this family of hollow fiber membranes have proven difficult to control, the advantages of laminar tube-side flow and a high ratio of membrane area to jacket volume mandate their evaluation in a survey study. The unit chosen for testing is the Bio-Fiber 50 miniplant dialyzer, lot No. 342. This unit incorporates cellulose fibers with a nominal molecular weight cutoff of 5,000 in a generalized shell and tube configuration. Within a shell volume of 120 ml and a fiber volume of 150 ml, this 10-inch dialyzer packs nominally

15,000 cm^2 of surface area. In comparison, the Kiil dialyzer, using sheet membranes, is approximately ten times larger, with 30% less membrane surface area. Operation of all flow-type membrane cells, including the miniplant, were performed with Cole Parmer variable speed paristaltic pumps through silicon tubing.

Cellulose sheet membranes were also evaluated, due to the ease with which they could be modified by the electret process. These are simply single layers of common dialysis tubing, available through VWR Scientific as well as numerous other suppliers. The tubing, prepared of regenerated viscose cellulose, is split and peeled apart to a single thickness, averaging between 0.5 and 0.8 mil. In this condition, the membrane exhibits excellent toughness both wet and dry, and excellent transport properties for water and relatively small, water soluble molecules.

Two types of ion exchange membranes were obtained from a commercial supplier. Both a cation transfer (no. 61-CZL-183) and an anion transfer (no. 103-QZL-183) were gifts of Ionics, Inc., Watertown, Massachusetts. These are examples of membranes currently being used in selected industrial applications, most notably in electrodialysis. The 103 series are copolymers of vinyl compounds containing quarternary ammonium groups and tertiary amine groups. The 61 series consist of sulfonated copolymers of vinyl compounds. Both types of membrane are homogeneous films,

cast in sheet form on a synthetic cloth backing (169,170).

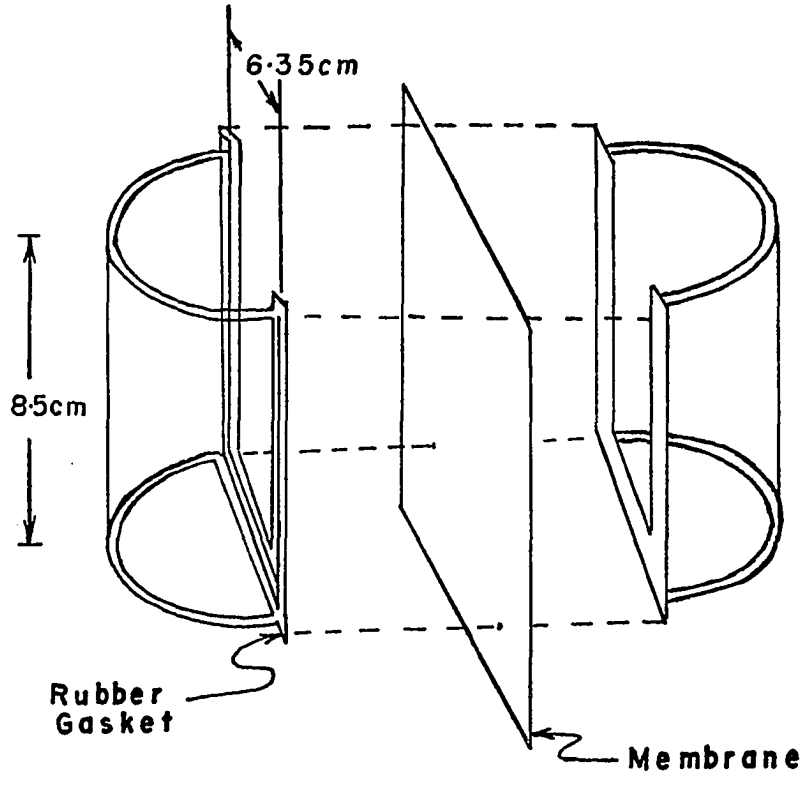
Power Supply. A Harrison Model 6522 DC power supply with output ranges of 0 to 2000 V.D.C. and 0 to 100 mA, provided a high quality direct current source for generating high field potentials for the preparation of electret membranes and for studying the behavior of charged cofactors within an electric field. An overload feature allows the power supply to be operated either within a current limiting or a voltage limiting mode. If the current selected is beyond the power capacity, the circuit automatically reverts to the voltage limiting mode, and vice-versa. This anomaly is apparent in Figure18, but is not believed to grossly affect the properties of the electret being formed. The volt and ammeters within the power supply were deemed sufficiently accurate when compared to an RCA VTVM/multimeter to allow direct collection of voltage/current data for studies requiring this information.

Ultrafiltration Membranes. Although not used directly in any of the flow schemes proposed, Diaflo 10 xM50 ultrafiltration membranes inan Amicon model 52 cell were used in various syntheses for protein and polymer concentration.

Test Cells. Two types of membrane test cells were designed and constructed of Plexiglass (Reg. trademark, Rohm and Haas Corp., for polymethylmethacrylate). Figure 9 illustrates exploded views of the two types. Type 1 is a simple, non-flow, two compartment cell, in which a small

Figure 9-A. The Type 1 Micro Membrane Evaluation Cell. This type of cell was used for all sheet-type membrane analyses. Media of various concentrations may be placed on either side of the membrane, and concentration measurements taken at timely intervals. In addition, several of these cells may be placed in a shaker bath to provide intimate mixing on both sides of the membrane. A rubber gasket, when properly fitted, afforded a leak-proof design for multiple membrane evaluations in a short period of time.

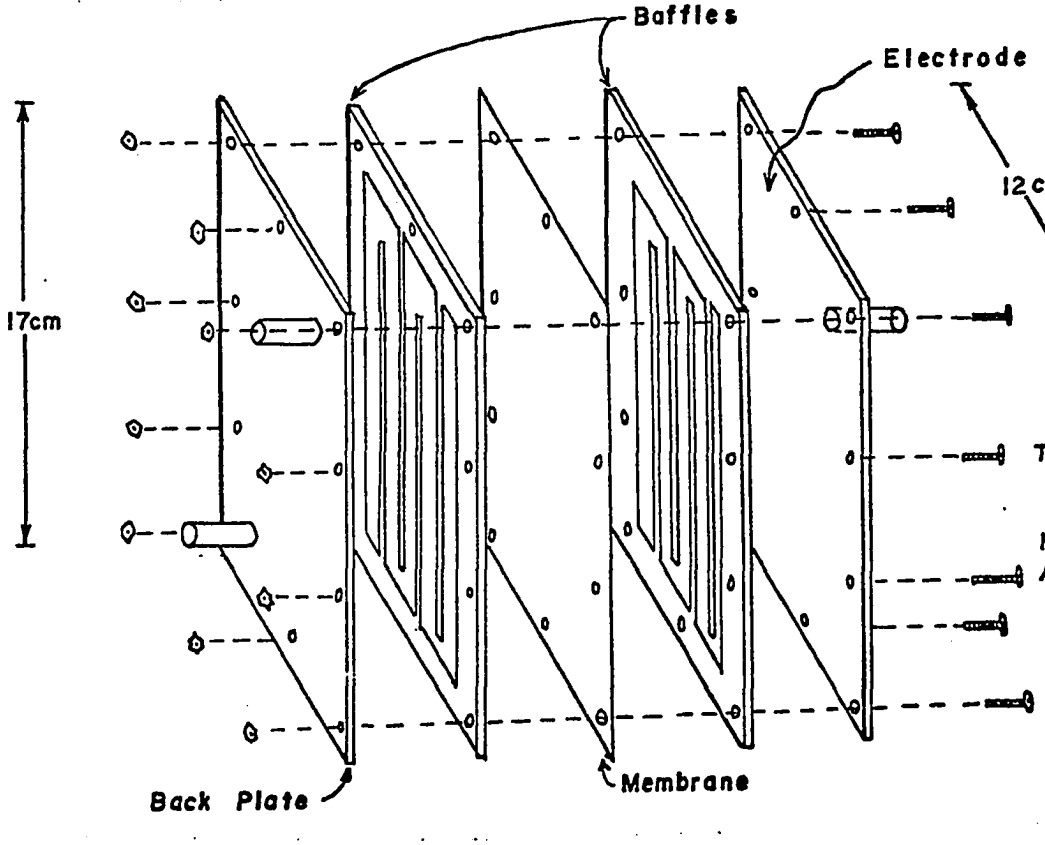
Figure 9-B. Exploded View of Tortuous Path Flow Cell. The type-2 cell was constructed to observe the effects of impressed current on net flux behavior of charged cofactors. It features replaceable electrodes and membranes. A water seal was attained by applying a light coating of vacuum grease to all mating surfaces. The overall thin construction allows high current and field densities through the dialyzing solutions.



TYPE I CELL

Total Volume: 106cm^3

Membrane Area: 26cm^2



TYPE 2 CELL

Total Volume: 129cm^3

Membrane Area: 135cm^2

sample membrane may be sandwiched between two rubber gaskets. Four to five rubber bands served to hold the assmebly together. The cell can be placed in a shaker bath and agitated at various speeds, or allowed to stagnate. Type 1 cells proved so effective for quick and dirty analyses of membrane properties that over 15 were constructed for mass screenings. This generally reduced the evaluation time of a new membrane from several days to a matter of hours.

The Type 2 cell was constructed for two reasons: 1) to further investigate membrane properties in a more complex flow process; and 2) to study the effects of charged cofactors in a flow process both when exposed to an external field, and to an impressed current. Features of the Type 2 cell include replaceable membranes and electrodes, and a reduced thickness to acentuate the effects of induced electric fields.

Spectrophotometer. An Hitachi model H-191 single beam spectrophotometer was used for all assays in which a compound exhibited sharp absorption peaks in the 200 to 600 nm wavelength range. For the ultraviolet regions (<340 nm), Beckman 1307 quartz cells of 0.1 ml capacity and 1.0 cm path length were used. For visible colorimetric tests, Hitachi type 0120 1.0 ml, 1.0 cm path length cells were substituted, when available.

CHAPTER IV

METHODS

Insolubilization of Enzyme via Covalent Attachment to Pourous Glass Beads

Theory

Enzyme attachment to insoluble substrates through various linkages is well documented in the literature (115). The oxidase extract of this study has been shown to be particularly well suited to immobilization by many of these techniques (84,100,101).

In general, the chemical methods for covalent attachment draw heavily from experience gained in ligand chromatography in which the matrix surface is activated, a nonfunctional derivative may then be grafted onto the activated surface, and the organic functional group (the enzyme) is then attached to this "arm". The first example of a synthetically produced, water insoluble, polymer bound enzyme derivative was described by Grubhofer and Schleith in 1953 (171,172). In 1969, the use of glass as a support for the covalent attachment of enzymes was propounded by Weetal (173,174). Small glass particle supports offer advantages of high protein binding efficiency, good stability and

inertness, as well as providing wide flexibility in the reactor design. Sofer (84,100,101) and Osinowo (116) have conducted extensive evaluations of the oxidase in several reactor configurations using various insolubilizing supports, including glass. For small scale kinetic studies, the use of Corning porous glass beads (40-80 mesh) was determined to be the method of choice. They exhibit highly stable activities (over 100 times that of soluble enzyme), and may be conveniently stored at 4 C over buffer for indefinite periods. Additionally, the small particles are easily weighed and poured into the microassay reactor. The foregoing is not intended to be an unequivocal commitment to porous glass bead catalyst supports in a hemoperfusion device. Indeed, such supports may prove quite unsatisfactory in regard to hemolysis and particle embolism. They simply offer a convenient and economical means of applying the enzyme for the time being.

If desired, enzyme binding efficiency may be determined by hydrolyzing the flavin groups on the oxidase, and observing the intensity of the resulting yellow solution at 400 nm. It is not, however, the purpose of this study to investigate extensive methods of immobilizing the oxidase. Catalytically active glass heads were prepared by the standardized techniques that follow, and all kinetic studies were performed with excess catalyst.

Bead Preparation

The following is an adaptation of a method supplied by Pierce Chemical Company for enzyme immobilization on zirconia-clad porous glass particles (175).

Twenty-five ml of a 2.5% glutaraldehyde solution was poured over 3 gms of Corning GAO 7920 glass beads. In addition to its role as an "arm" extension to reduce stearic hindrances to catalyst activity, the glutaraldehyde acts as a carbonyl intermediate on which to attach the enzyme via a Schiff base exchange. The mixture was then evacuated under vacuum aspiration at room temperature for 30 minutes to remove air bubbles and assure penetration of the glutaraldehyde deep within the particle pores. The vacuum was released and the reaction was then allowed to continue at room temperature and atmospheric pressure for an additional 40 minutes. The glutaraldehyde solution was decanted, and the beads were repeatedly washed in glass distilled water until the characteristic sweet aldehyde odor was no longer perceptible -- usually at least ten washes. The activated beads are now ready for chemical coupling. The processed enzyme solution (see below) is now added to the flask containing the treated beads such that all particles are covered in the enzyme liquor. This corresponds roughly to a protein concentration of 30-40 mg enzyme per gram dry glass beads. Care should be exercised at this point to assure that the mixture remains chilled over an ice bath. The bead and enzyme mixture, on

the ice slurry constantly, is once again placed under vacuum aspiration for two hours. The reaction may then be allowed to continue at atmospheric pressure overnight in the refrigerator, with occasional shaking when convenient. The following day the oxidase supernatant liquor is decanted and re-frozen. It may be used again in subsequent preparations, with marginal loss in activity. The catalyst is then washed three to five times with 30 ml aliquots of 0.025 M chilled K-phosphate buffer, pH 7.6, and stored in this buffer at 4 C until ready for use.

Enzyme Processing

The purified oxidase is shipped on dry ice from the Clayton Foundation Biochemistry Labs in Austin, Texas, to the Norman Campus via air freight. As received, the extract has a protein concentration of from 5-20 mg/ml and assayed activity of from 200-1000 n moles/min-mg. An 8-10 ml sample is dialyzed in 0.01 M K-phosphate buffer, pH 7.6, at 0 C to remove glycine buffers. 500 ml aliquots of fresh, chilled buffer are changed three times during the two-hour dialysis procedure. The dialyzed solution is ultrafiltered at 0 C to a final volume of 4-6 ml. This results in a final protein concentration of approximately 10 mg/ml.

Preparation of a Soluble, High Molecular Weight Poly(ethyleneimine) NADPH Complex

Theory

A method has been described in the literature for the production of a high molecular weight (~40,000) Dextran/NADP complex (129). In an effort to avoid duplication of this work and to sidestep some of the shortcomings of the procedure, an alternate method was developed in this laboratory. The method, a modification of a synthesis described by Wykes (145) involves the attachment of a soluble poly(ethyleneimine) (PEI) chain to a single activated group on the NADP molecule.

Procedure

Eight gm of succinic anhydride were added to 40 ml of dimethylsulphoxide (DMSO). 224 mg of either $NADP^+$ or NADPH was added, and after 48 hours at room temperature, the NADP components were precipitated with 10 volumes of cold acetone. The precipitate was recovered by centrifugation and washed three times with acetone. The white powder was then dried under vacuum aspiration. The dried succinyl derivative (at least 200 mg) was then dissolved in 1.0 ml of water and 1.0 ml of pyridine was added. This was followed by 50 mg of dicyclohexylcarbodiimide dissolved in 0.2 ml of pyridine. The mixture was incubated at room temperature overnight. 4.0 ml of a 30% PEI solution adjusted to pH 6.0 with concentrated HCl were added and allowed to react for three hours

at room temperature. The suspension was adjusted to 50 ml with chilled water and PEI-succinyl-NADP precipitated by the addition of an equal volume of chilled 1.0 M K-phosphate buffer, pH 6.0. Chilling of the cloudy suspension aids in the agglomeration of the polymer to yield a bright yellow, gooey pellet on centrifucation at 1000 g for five minutes.

The clear supernatant was decanted, and the precipitate was re-dissolved in 10 ml of 2 M NaCl in 0.05 M acetate buffer, pH 5.5. 40 ml of water were then added, followed by 50 ml of chilled 1.0 M K-phosphate buffer, pH 6.0. The washing procedure was repeated four times after the initial precipitation. The final precipitate was dissolved in acetate buffer as before, and dialyzed against water overnight at room temperature. 1.0 l aliquots were changed every 30 minutes for the first two hours, and a fifth aliquot remained unchanged until morning. The dialyzed PEI-succinyl-NADP solution was concentrated to a final volume of ~10 ml in a high pressure ultrafilter. The ultrafiltrate is retained for later analysis of free NADP by testing for catalytic activity in the microreactor. NADP loading on the polymer was determined spectrophotometrically at 260 and 340 nm.

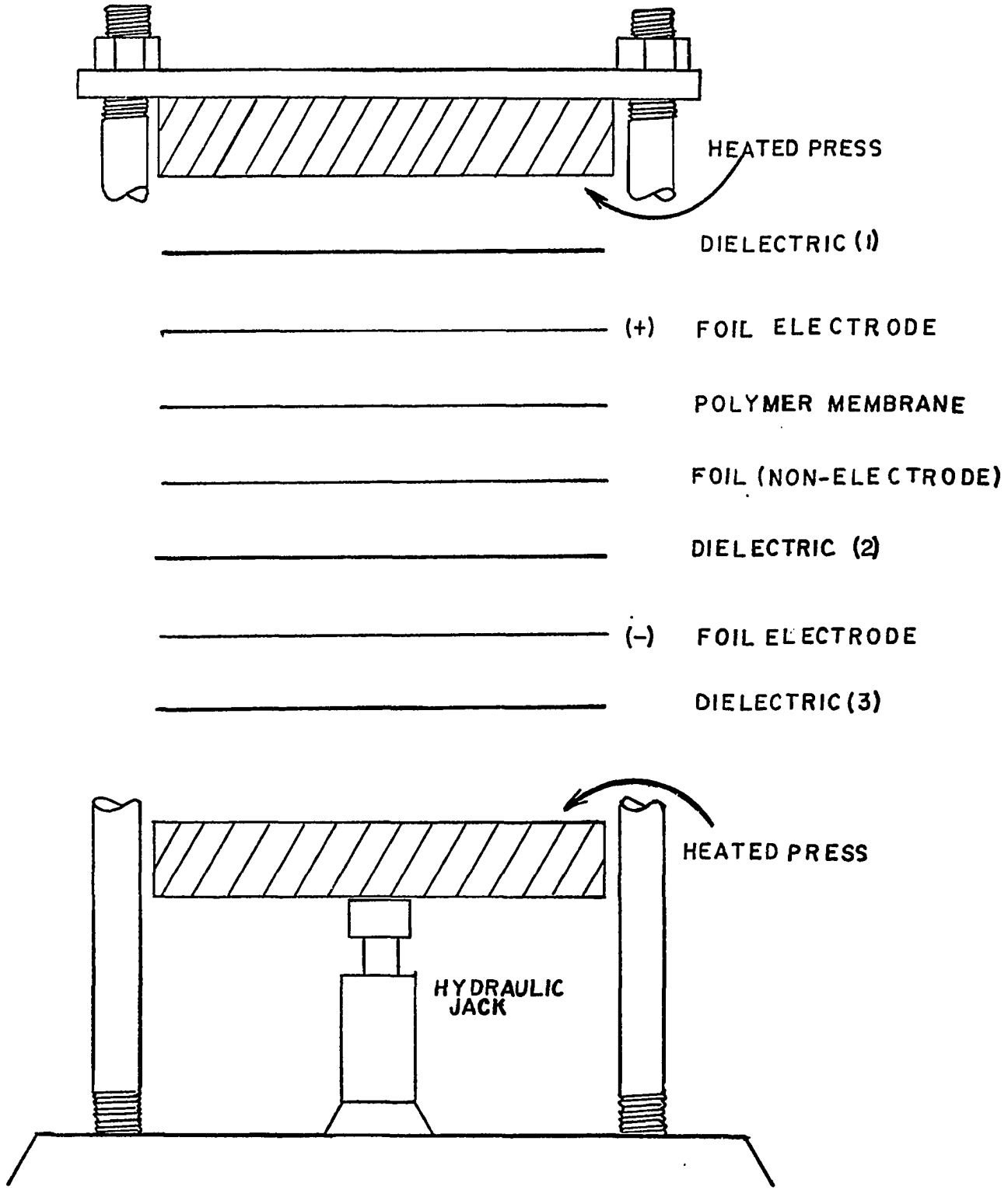
Preparation of Polymer Film Electrets

Other workers reporting on the preparation of electret films have in every case either evaporated electrodes or applied conductive paint to the film surfaces. This in itself is a time consuming step, and necessitates the subsequent

removal of the conductive layer after polarization with acid washes, organic solvents,and the comensurate risks of membrane damage. The desire to eliminate air gaps creating undesirable homocharge polarization is nevertheless recognized. As a result, a procedure was developed in which an electrode sandwich could be quickly prepared, using aluminum foil electrodes. The sandwich was compressed at up to 700 lbs/in^2 in a heated press to eliminate air gaps. After polarization was complete, the sandwich could be removed from the press and the electrodes peeled off and discarded. This allowed a more rapid evaluation of a great number of polymer electrets. A typical procedure appears below.

An isotropic polymer film, 6.5" x 6.5" was sandwiched between successive layers of aluminum foil electrodes and dielectric sheets as shown in Figure 10. Dielectric sheets, also trimmed to 6.5" x 6.5" consisted of dense, high grade paper, 8 mils thick. Dielectrics 1 and 3 serve to insulate the electrodes from the press faces. Dielectric 2 was necessitated by arcing which occurred due to minor imperfections in the polymer film and electrodes accentuated by the high compressive loads. A third, uncharged aluminum sheet was also included to assure a smooth mating surface for both sides of the film. The net effect of the paper dielectric and foil is to reduce the effective field intensity through the polymer by approximately 10 times. This was more than offset by the capacity of the power supply and by the higher

Figure 10. Electrode Sandwich for Electret Manufacture. The multi-layered composite illustrated, when placed between the faces of the heated press under high pressure, is a significant improvement over previous methods in which silver electrodes were evaporated onto the surface of the membrane. Dielectrics 1 and 3 insulate the charged aluminum foil electrodes from the press. Dielectric 2 prevents arcing across the extremely thin (0.5 mil) membranes. The center, uncharged layer of foil is strictly to assure a smooth mating surface for the membrane.


HEATED PRESS
DIELECTRIC (1)
(+) FOIL ELECTRODE
POLYMER MEMBRANE
FOIL (NON-ELECTRODE)
DIELECTRIC (2)
(-) FOIL ELECTRODE
DIELECTRIC (3)
HEATED PRESS
HYDRAULIC JACK

potentials that can be tolerated in this design.

The sandwich of Figure 10 was than placed between the cool faces of the press, and loads varying from 100 to 700 lb/in^2 applied. The power supply was set in the voltage limiting mode at 1.0 to 1.5 Kv, (greater potentials than 1.7 Kv caused arcing through the film and dielectric sheets), and the electrodes connected to the respective foil tabs on the sandwich. At this point, it should be noted which side of the membrane will have the desired charge. The potential is allowed to accumulate to the set limit, and the individually controlled thermostatic heaters are set at the appropriate polarization temperature (80 C for PVC, 60 C for cellulose acetate). As the temperature increases, data are collected at timely intervals on current, voltage, and temperature. During initial stages of polarization, data should be taken at 15-second intervals to observe the rapid changes occurring. The interval can be gradually increased to several minutes until current and temperature stabilize after one to two hours. At this point, all potential dipole alignment has probably taken place, yet the field and heating are usually maintained for a total of three hours to assure a maximum persistent charge. After this period, the heaters are turned off, and forced convection cooling from a large blower bring the press faces to room temperature within two hours. It is critical that the high voltage potential be maintained uninterrupted during this cooling period, as the

electret will relax in a matter of seconds at high temperatures. Furthermore, removal of the electret from the press even at modest temperatures of 45-50 C results in appreciable charge decay. Once the press has cooled completely, the sandwich is removed and the electrodes carefully peeled from the film. The polarity of the film may be marked directly on the film at this time. To assure that no destructive homocharge behavior will be present during membrane evaluation, the film is immersed in water overnight.

Film Casting Technique

Cellulose and cellulose acetate films are widely available commercially. Demand for PVC film, however, in either cast, extruded, or calendered sheets of 1 mil thickness and less is limited strictly to plasticized PVC. It was felt that a plasticized PVC electret would not allow adequate persistence of the charge. Plasticization reduces the rigidity of the polymer, in effect lowering the glass transition temperature, and allowing stress relaxations to proceed at room temperature. Due to the unavailability of non-plasticized PVC films, a solution casting technique was developed. A PVC film of good clarity, toughness, and uniformity can be prepared by the following technique.

PVC powder, the direct product from suspension polymerization, was dissolved without further purification in tetrahydrofuran (THF). THF is undesirable as a solvent for solution casting because of its flammability and tendency to

form explosive peroxides when evaporated to dryness. It is believed nevertheless that any peroxides formed within the PVC matrix during casting would quickly be neutralized by charge transfer or even by limited crosslinking. No other suitable solvents for PVC were found. The THF/PVC solution was poured on a chilled (0°C) 7" x 7" glass plate and evenly distributed. The glass plate was placed within a dessicator containing an adjustable stand to support the plate above several pieces of dry ice. The dessicator was partially sealed, and reduced pressure was applied with a vacuum aspirator. A small hole in the dessicator allowed a gentle scavenging of the evaporating THF. The noxious fumes were scrubbed out in the water venturi of the aspirator and flushed down the drain. Evaporation rate, critical to membrane quality, was controlled by temperature, and rate of scavenging of the solvent.

After two hours in the dessicator, the clear, tough, uniform PVC film was removed from the dessicator, still attached to the glass plate, and allowed to air dry at room temperature for two more hours. The isotropic membrane, carefully peeled from the plate is now ready for evaluation as is, or after conversion to an electret. While PVC films prepared by this process are noticeably more brittle than their plasticized counterparts, they do exhibit adequate toughness.

Measurement of Membrane Permeability

For a dialysis system composed of two closed compartments with constant volumes V_1 and V_2 separated by the dialyzer membrane, Ruiz (180) has determined that the change in concentration of one component is given by:

$$V_1(dC_1/dt) = -PA(C_1 - C_2) \quad (1)$$

where: A is the membrane area

P is the membrane permeability

C is the concentration of the moiety under study

The constant volume assumption was shown to be valid when working with the extremely dilute solutions typical of this study. Since the system is closed, the mass is constant:

$$m = C_1V_1 + C_2V_2 \quad (2)$$

Substituting (2) into (1) and integrating, we obtain:

$$-\ln \frac{C_1 - C_2}{C_{10} - C_{20}} = PAt(V_1 + V_2)/V_1V_2 \quad (3)$$

where: C_{10} is the initial concentration in side 1

C_{20} is the initial concentration in side 2

P, the permeability may be determined from equation (3) by plotting $-\ln(C_1 - C_2)/(C_{10} - C_{20})$ vs. t, and multiplying the slope of the resulting line by $V_1V_2/A(V_1 + V_2)$. The resulting permeability is most accurately described as an effective permeability, including bulk diffusion and film resistances. Its use should be limited to a quantitative comparison of the membranes tested in this study. Its interpretation as an absolute membrane permeability in a

mathematical model or to systems of differing geometries than those represented here is not recommended.

Cofactor Permeability Flux Determinations

The cofactors NAD(H) and NADP(H) are both optically opaque at specific frequencies, exhibiting good linearity in the 0-200 μM concentration range. An ultraviolet spectrophotometer with quartz cells was used to directly read NAD and NADP concentrations in all assays. The reduced nicotinamide moiety absorbs strongly at 340 nm, whereas the adenine moiety of both the oxidized and reduced nucleotides resonates at 260 nm. This allows resolution of both the reduced and oxidized moieties of either NAD or NADP in the same solution. The inability to distinguish between NAD and NADP spectrophotometrically did not pose a problem because they were never used jointly in the same medium.

Microcell evaluation (Figure 5-A) was performed by placing a standardized cofactor concentration on one side, glass distilled water on the other. Initial readings were taken, the cells agitated at room temperature in a shaker bath for one hour, and final concentration readings were taken. The error induced by taking only two data points was counteracted by evaluating several membranes simultaneously. Membrane permeability was determined by direct substitution into Equation (3) and an average taken.

Since in most cases the concentration of cofactor on the side being dialysed did not measurably change during

the course of the one-hour runs, and the rise in cofactor concentration on the dialysate side is quite linear in this time span, total flux of cofactor as a function of concentration is simple determined as follows:

$$F_{C_1} = C_2V_2/tA \qquad (4)$$

where: F_{C_1} = mass flux per unit area from a concentration C_1 to a pure dialysate fluid

C_2 = concentration of dialysate at t

V_2 = constant volume of dialysate

t = time

A = membrane area

Hollow fiber membrane evaluation was accomplished by recirculating the standardized solution in the fibers against a recirculating dialysate fluid in the shell initially containing a zero concentration of the compound under study. Flow rates were held constant throughout the run. Data taken at 30-minute intervals was plotted according to Equation (3) and a graphical solution obtained for P, the permeability.

The tortuous path type-2 cell of Figure 5-B was evaluated in a manner similar to the hollow fiber cell above, when operated as a straight dialysis unit. Recall that an added feature of this cell design is the ability to investigate the retention of charged cofactors in an impressed electric field or in an impressed current with electrodes in direct contact with the solution. In this case, data reduction was performed as before, yet a new variable, P*, was substituted

for P in Equation (3). This notation is meant to emphasize that P* is even less a function of membrane properties than P.

Instantaneous fluxes as a function of concentration difference for a type-2 cell and the hollow fiber cell were obtained from a rearrangement of Equation (1):

$$F_{\Delta c} = -P(C_1 - C_2) = \frac{V_1}{A}(dC_1/dt) \qquad (5)$$

where: $F_{\Delta c}$ = mass flux/unit area across a concentration difference of $(C_1 - C_2)$.

DMA Permeability and Flux Determinations

Analogous data reduction techniques as above were also utilized in determining DMA membrane behavior. An assay procedure for the detection of nanomolar quantities of DMA was developed. It is essentially a modification of a colorimetric method described earlier (176).

To a 0.9 ml, protein-free sample, 0.08 ml of 3 M trichloroacetic acid (TCA) and 5.0 ml of 1.0 M glycine were added, and mixed well. Following the addition of 0.1 ml of 1% sodium nitrate, the solution was heated in a water bath at 60 C for six minutes, and then cooled on an ice bath. When kept cool, the resulting bright yellow nitroso-amine was stable for up to thirty minutes, during which time its optical density was read at 420 nm. (A Δ OD of 1.0 = 7.4 μM/ml nitroso-DMA at 420 nm.) This procedure proved adequate for a DMA concentration range of 0.05 to 1.5 mM. Nanomolar quantities may be detected by reducing the volume of buffer

initially added.

Reaction Rate Assays

MFMF oxidation rates were determined by observing the uptake of dissolved molecular oxygen in a closed system with an oxygen electrode during the course of the reaction. This method was first demonstrated by Poulsen, and later refined for a continuous flow reactor system by Sofer (84). A typical aqueous reaction medium consisted of the following:

1) 0.054 M K-phosphate buffer, pH 7.4
2) 0.11 u/ml glucose-6-phosphate dehydrogenase
3) 5.0 mM glucose-6-phosphate
4) 0.5 mM N,N-dimethylanaline
5) 0.0 - 0.22 mM $NADP^+$
6) 10 mg of catalyst (MFMF oxidase on glass beads)

For reactions without the cofactor regeneration system, items 2 and 3 were omitted, and an equivalent amount of NADPH substituted in item 5. For runs in ultrafiltered serum, 0.2 ml of 0.5 M K-phosphate buffer was added as before, but the make-up water normally needed to fill the reactor (~1.4 ml) was replaced with ultrafiltered serum. This corresponds to a solution of 75% blood/serum. For runs in whole blood and serum, item 1 was omitted. The reaction was initiated by injection of DMA following a five-minute equilibration of the above mixture in the reactor.

The solid catalyst was prepared in pre-weighed samples, generally 20 mg each. To avoid deactivation of the enzyme

upon drying, the 20 mg sample was gingerly wicked dry with a tissue, and weighed moist. As a quality control check, several 20 mg samples prepared in this way were dried with acetone, and reweighed. Their dry weights averaged 8.0 ± 0.8 mg, a reasonable error when the alternative -- collection, drying and weighing of individual samples -- was considered. Furthermore, a quantitative collection of the catalyst after a run proved to be difficult. In some cases, a single aliquot of catalyst was allowed to remain in the reactor, washed twice in situ, and the next run begun with a new reaction medium. No detectable catalyst decay was noted within the range of a maximum of 3 to 4 five-minute runs with the same catalyst charge. All reactions were carried out at 37 ± 0.01 C, and all buffered reactions at pH 7.4. The blood systems were naturally buffered at a physiological pH 7.2.

The strip chart recorder was adjusted to give a full scale reading corresponding to a dissolved oxygen concentration of 200 nM, an accurate and invariant figure at 37 C, and air saturation. The change in slope observed when an amount of cofactor was injected to the reaction medium was directly proportional to the rate of oxygen consumption, which is itself equal to the rate of product synthesis. A chart speed of 60 sec/in proved adequate for elucidating all slope changes associated with the reaction rates in this study.

Blood Preparation

Fresh whole blood was drawn from two donors on an as-needed basis in 60 ml aliquots in the labs of the Goddard Student Health Center. The blood, collected by venupuncture in EDTA treated evacuated vials was immediately refrigerated. All reactions in blood and serum were initiated within two hours of collection. No clotting was noted either during or after the experimental period. In the whole blood studies, a 10 ml sample in the vial was warmed to room temperature, and vigorously aerated for 1-2 minutes. It was then injected directly into the reactor. A pseudo-serum was prepared by spinning the EDTA treated whole blood in a clinical centrifuge to precipitate the platelets, and the supernatant was used as the reaction fluid. It was not further deliberately re-oxygenated. Finally, serum prepared by the above method was ultrafiltered in a nitrogen pressurized ultrafiltration cell. This blood fraction took the longest to prepare (approximately one hour per reaction sample), and was provided the greatest chance of thermal and mechanical degradation in the agitated cell. It was intended to be a facsimile of a blood dialysate fraction.

CHAPTER V

RESULTS

The results of this work may be divided into two broad categories. The first deals with the kinetic studies of the enzyme in various reaction media and conditions. The second category pertains to membrane diffusional studies of the compounds of interest in this study. The two fields are necessarily dependent. For example, the maximum allowable cofactor concentration in the reactor depends on the expected permeability of the membrane to the cofactor. For the purpose of this investigation, however, the membrane and kinetics problems have been attacked independently in an effort to reduce the number of variables involved with each experiment, and to simplify the computational techniques involved. A case in point is the considerable increase in complexity of a membrane evaluation when one must account for chemical reaction at the same time.

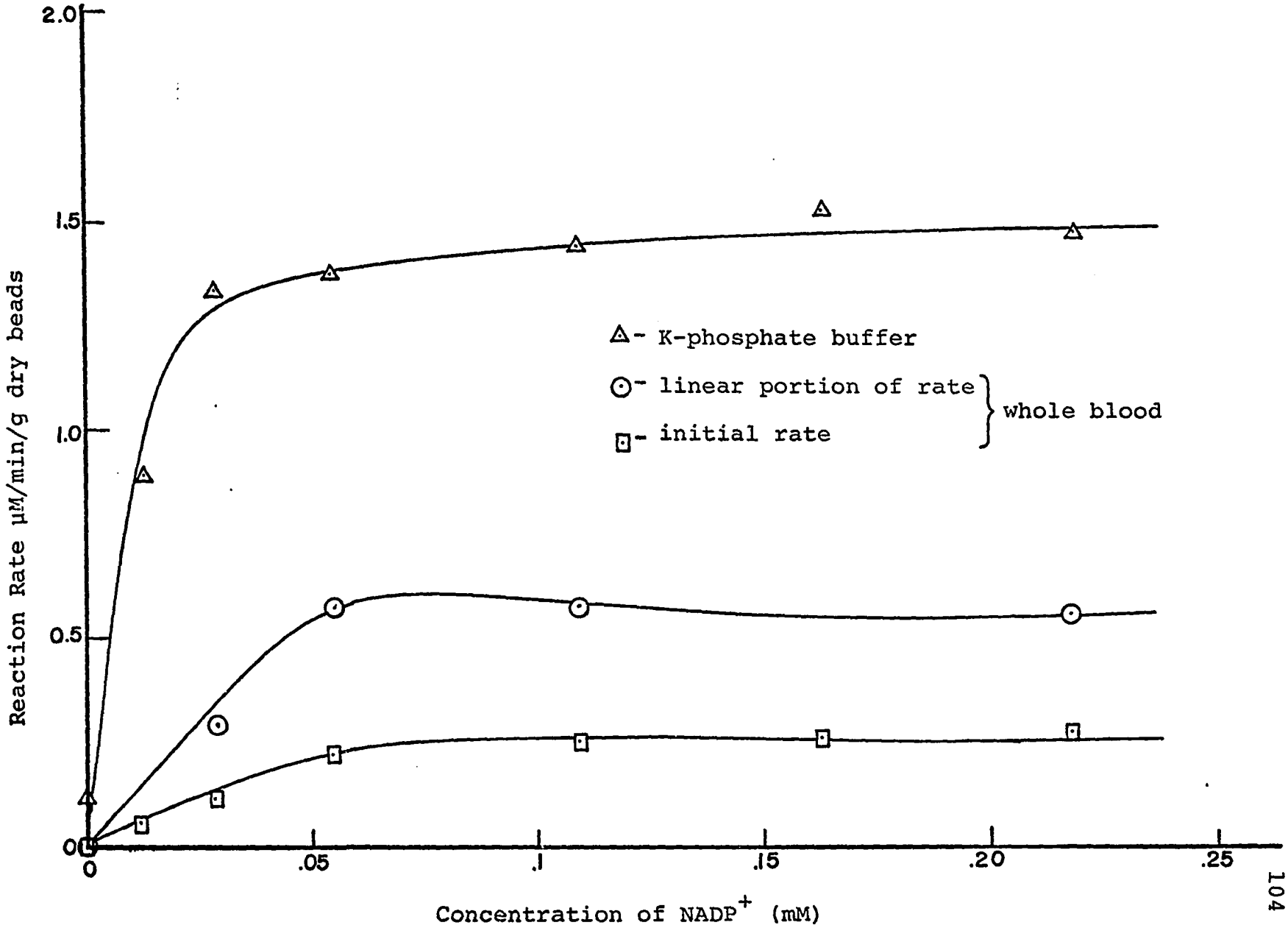
Enzyme Kinetics

First priority was the determination of reaction rates to be expected in various blood components. Irrespective of the eventual method of administering the MFMF oxidase

therapeutically, some degree of activity must be shown in blood and/or blood fractions. The success with which the enzyme has been immobilized and used in in vitro work at optimum temperatures and buffered pH's has led to an almost cavalier attitude toward the sensitivities of the catalyst. Many enzymes, however, show dramatic inhibition in highly concentrated protein solutions, and as a result, demonstration of activity in blood was of primary importance.

Figure 11 compares the activity of the immobilized MFMF oxidase utilizing a simultaneous G-6-P-D cofactor regeneration system in a pH 7.4 K-phosphate buffered solution with the activity observed polarographically in whole blood treated with EDTA. Both reactions were run at air saturated oxygen tensions and 37 C, with the same amount of catalyst and regeneration system. In contrast to the clean and sharp slope changes customarily observed in K-phosphate buffer, the whole blood studies repeatedly resulted in poor data and highly non-linear rate of dissolved oxygen concentration. By this criterion, the enzyme is only capable of an activity roughly 1/5 of that observed in a synthetic medium based on initial rates, and approximately 1/3 when the rate of dissolved oxygen consumption is measured after 2-3 minutes, at which point the slope appears to be linearizing somewhat. This does not necessarily offer concrete support for the percentage of activity lost in whole blood. Hemoglobin is known to be an excellent buffer for blood oxygen tension. Oxygen

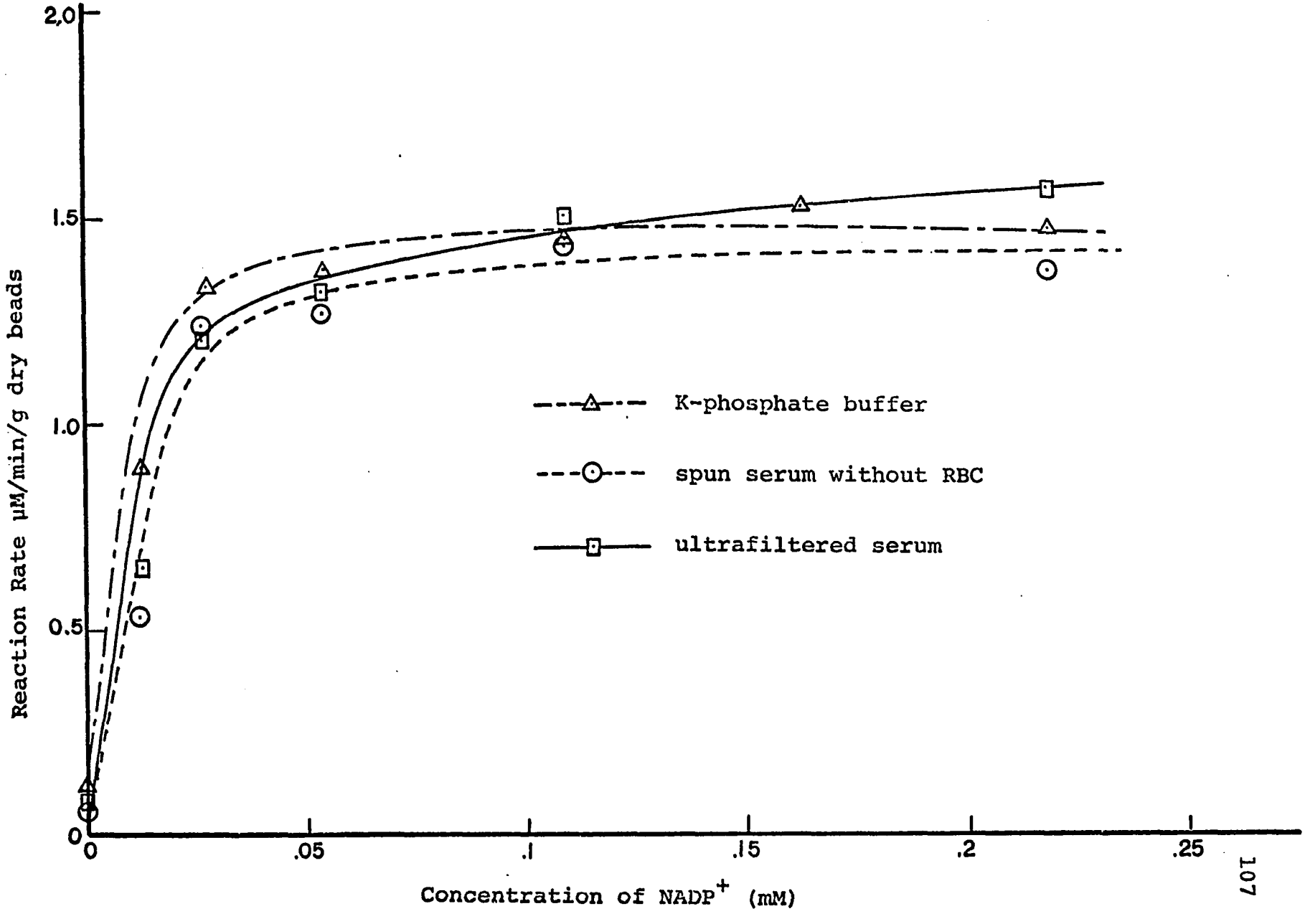
Figure 11. Verification of MFMF Oxidase Activity in Blood. The activity of the oxidase in K-phosphate buffer (upper curve) is compared to the initial rate observed by the oxygen polarograph (lower curve) and again at a point 2-3 minutes after initiation (middle curve) at which time the rate of changing oxygen concentration began to linearize somewhat. It is believed that the oxygen buffering of the hemoglobin is masking an appreciable reaction rate, and is therefore deemed an unsatisfactory method for monitoring oxidase activity in blood. The reaction media (see Chapter III) in all cases contained a soluble G-6-P-D cofactor regeneration system.


2.0
1.5
1.0
0.5
0
Reaction Rate µM/min/g dry beads
0
.05
.1
.15
.20
.25
Concentration of NADP+ (mM)
Δ- K-phosphate buffer
⊙- linear portion of rate
⊡- initial rate
whole blood

uptake studies indicate that whole blood is capable of an oxygen loading at least five times greater than that expected by a simple dissolution model, which has been normalized at 200 nM oxygen tension in aqueous buffer for the purposes of this project. As a result, the monitoring of dissolved oxygen concentration may not be a valid method of determining enzyme rates in a system containing an oxygen buffer like hemoglobin. Not only is the initial oxygen equilibrium constant, a complicating factor necessary for instrument calibration, but the rate at which hemoglobin unloads oxygen relative to a largely unknown MFMF oxidase rate further clouds the issue. This would also explain the uncharacteristic nonlinear behavior of the polarograph during initial rates.

Figure 12 strongly supports this conclusion. The reaction rates in ultrafiltered whole blood, and unclotted blood from which the platelets have been removed by centrifugation, both illustrate excellent reaction rates comparable in all cases with K-phosphate buffer under the same concentrations. Because the spun, unclotted supernatant presumably still contains all serum proteins necessary for acid/base buffering at an observed pH 7.2-7.4, additional K-phosphate buffer, pH 7.5, was not added. It is noteworthy that the slight decrease in reaction rate at saturated cofactor concentrations between the K-phosphate buffered and the serum protein buffered solutions can be totally accounted for by the marginal difference in pH (7.3 vs. 7.5). Sofer (84) has

Figure 12. MFMF Oxidase Activity in Selected Blood Fractions. When the hemoglobin is removed from the whole blood by spinning out the platelets its activity is nearly that of the K-phosphate buffered reaction medium. Ultrafiltered serum, a facsimile of a blood dialysate fraction, also shows excellent activity with the oxidase. As before, the reaction media all contain the G-6-D-P regeneration system.


2.0
1.5
1.0
0.5
0
Reaction Rate µM/min/g dry beads
0
.05
.1
.15
.2
.25
Concentration of NADP+ (mM)
K-phosphate buffer
spun serum without RBC
ultrafiltered serum

shown that a difference in pH of this order of magnitude will result in a 7% decrease in reaction rate for the lower pH. The buffering action of the ultrafiltered serum, on the other hand, was supplemented by an amount of K-phosphate buffer (pH 7.5) equal to that added for the totally aqueous studies. Since the normal buffering action of the ultrafiltered serum was believed to have been lost, the extra buffer is believed to have raised the resulting pH from approximately 7.3 to 7.5. The almost identical reaction rates for the totally aqueous and serum reaction media bear this out.

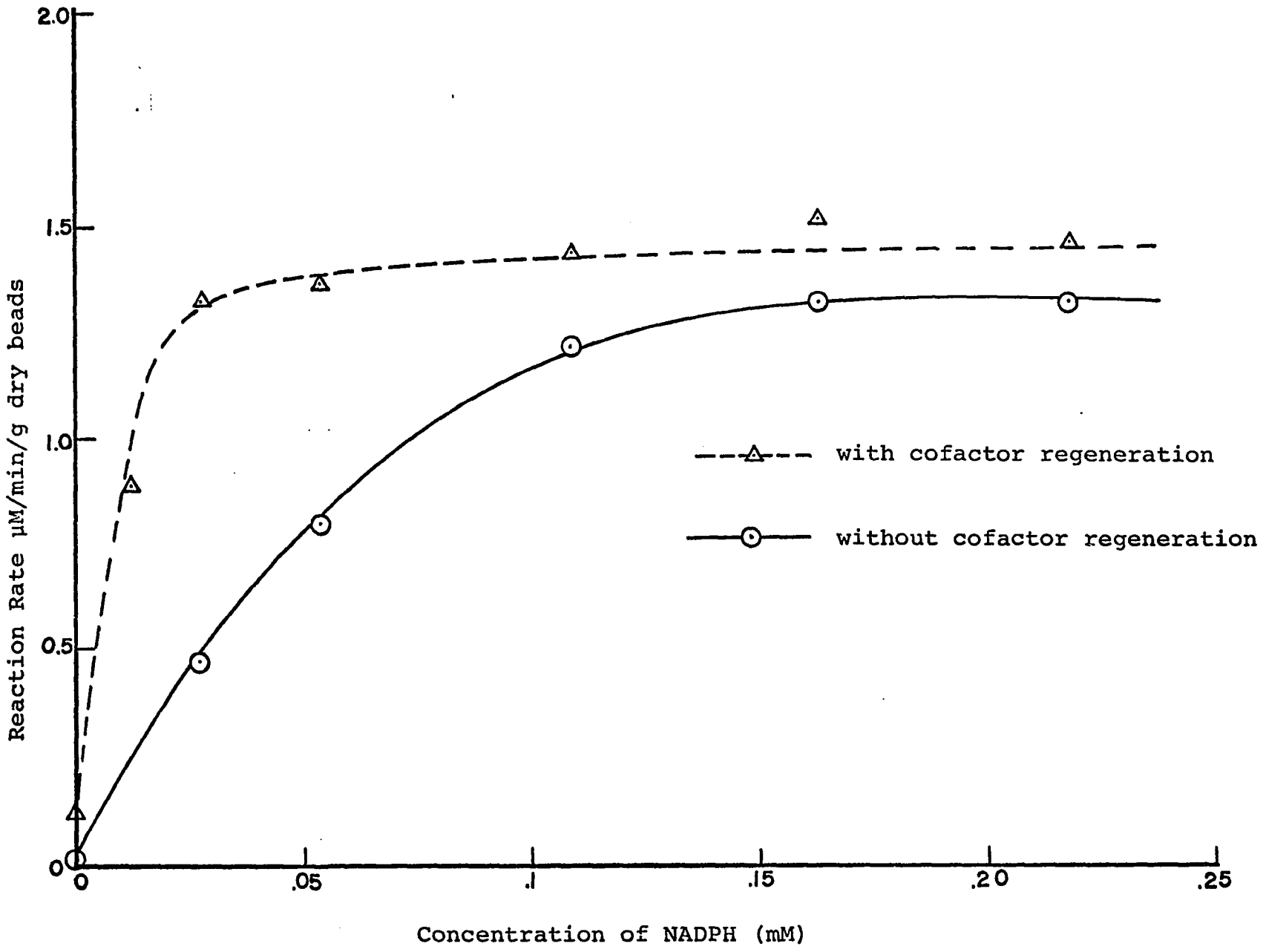
Ultrafiltered serum has been included in this study to represent an approximate composition of a blood dialysate stream for a membrane equipped reactor. In most examples of hemodialysis (e.g. artificial kidney), the blood dialysate is nowhere nearly as concentrated as has been represented here. Recall, however, that we wish to deal with only a very small dialysate volume (200-300 ml maximum) in the ultimate application. It is expected that the equilibration of all blood fractions capable of membrane transfer (with the obvious exception of the toxin(s) to be metabolized) will occur in a relatively short time, resulting in a concentrated dialysate comparable in composition to the ultrafiltrate of this study.

A second feature apparent in Figures 11 and 12 is the expected decrease in reaction rate with decreasing cofactor concentration. This is to be expected, yet the shape of the V-[s] curves plotted in Figures 11 and 12 appears to represent

a differing type of reaction kinetics than is expected when the cofactor is properly treated as a cosubstrate. The sharp break in reaction velocity at a cofactor concentration of 0.025 mM in all cases indicates that the NADP is behaving as a "true" cofactor, necessary only in catalytic concentrations, and is apparently not consumed at a rate proportional to reaction rate. Recall, however, that the reaction media of Figures 11 and 12 contain an efficient cofactor regeneration system which is capable of reducing the spent $NADP^+$ at a rate greater than that in which it is consumed in the oxidase reaction. The result is an uncharacteristic behavior of the cofactor NADPH at low concentrations. Recent data by Ziegler (179) on the soluble oxidase indicate that this phenomenon is due to oxidase activation by the oxidized cofactor ($NADP^+$). In the regeneration system illustrated by the upper curve of Figure 13, $NADP^+$ is present in abundant quantities due to the equilibrium between the reduced and oxidized cofactor moieties established by the G-6-P/G-6-P-D regeneration system. In the non-regenerative case, represented by the lower curve of Figure 13, $NADP^+$ is not present initially, and the oxidase is not activated by its presence. This is the first demonstration of $NADP^+$ activation on the immobilized oxidase. Note that the lower curve shows a more gradual change in rate with changing concentration characteristic of a typical V-[s] plot, which indeed it is.

Cofactor regeneration, then, proves to be a great asset

Figure 13. Cofactor Regeneration as a Means of Reducing Total Required Cofactor Concentration. The MFMF oxidase reaction responds well to *in situ* enzymatic cofactor regeneration. An equivalent reaction rate with regeneration (top curve) can maintain a saturated reaction rate with a cofactor concentration one-fourth that required without regeneration (lower curve). The minimum concentration of cofactor allowed without compromising reaction rate is 0.025 mM. Both reactions were run in K-phosphate buffer at 37 C.


2.0
1.5
1.0
0.5
0
Reaction Rate µM/min/g dry beads
0
.05
.1
.15
.20
.25
Concentration of NADPH (mM)
with cofactor regeneration
without cofactor regeneration

not only in terms of reducing cofactor costs, but in the ability to lower the total concentration of pyridine nucleotide in the reaction medium by maintaining an artificially high concentration of the reduced moiety, while retaining the advantages of oxidase activation from the equilibrium concentration of $NADP^+$. Figure 13 indicates that cofactor regeneration allows as much as an 80% reduction in total pyridine nucleotide concentration without sacrificing a high reaction rate. This may prove to be a stronger argument for cofactor regeneration than the economic implications.

Cofactor Analogues

As originally envisioned, this project was to involve the design of a membrane-bound reaction utilizing the various newly-discovered techniques of Wykes, Mosbach and Chambers (see Chapter I) for the synthesis of active, high molecular weight cofactors which would be retained by the membrane. At that time (later 1974) the literature was still painting a rosy picture for such methods, despite the disappointing data that had been collected to date. In an equally optimistic fashion, this laboratory jumped onto the bandwagon to explore the various advantages of macromolecular cofactors.

Preliminary data indicated that a macromolecular derivative of NADPH was synthesized, but at great cofactor expense, and with a marginal activity. A maximum reaction rate of approximately 10% that of the soluble native cofactor with the MFMF oxidase was observed, based on a cofactor loading

determined spectrophotometrically to be 50-80 μ moles NADP per gram of polymer. The improved activity expected with improving lab techniques did not materialize, but his was not the reason for discontinuing the project. Two more critical factors became apparent: 1) the high molecular weight cofactor showed activity only with the soluble MFMF oxidase; and 2) the cofactor analogue lost all activity with either the soluble or insolubilized enzyme after 36 hours, irrespective of storage temperature.

The first point may be explained by various mechanisms. If the enzyme lost as much as 50% of its activity upon immobilization to glass beads (this is the maximum loss observed) and the cofactor lost 90% of its activity upon attachment to the polymer (as determined by studies with the soluble enzyme), this results in a net activity for the system of 5% that expected under optimum conditions. This rate of reaction may be too low to be detected with the apparatus available. A second hypothesis explains the decrease in activity in a more mechanistic sense. Sofer (84) has shown that the reaction on glass bead catalyst and soluble native cofactor is diffusion limiting, presumable because of the location of the catalytic sites within the well protected bead pores. Indeed, the method of bead preparation stresses the activation and binding of the enzyme within these pores (see Chapter IV). It is quite locigal, then, to assume that a cofactor molecule experiences the same diffusion limitations in attempting to bind at its

allosteric site. A macromolecular cofactor, furthermore, representing a molecular weight as much as 75 times that of its native counterpart, would experience greatly accentuated diffusion limitations. Perhaps an enzyme immobilization technique that offered lower diffusion limitations (nylon tubing) would retain some net oxidase activity with the cofactor analogue.

As far as the second compromising factor is concerned, macromolecular cofactor instability was determined by this laboratory to be the major, and unacceptable constraint on the technique. Six months later, Wykes, Dunnill and Lilly published their sobering paper, which reached the same conclusion (149). Apparently, the problem of functional stability is one of pH, the reduced analogue being stable at high pH, and the oxidized analogue at low pH (177). Shelf life also posed severe limitations, regardless of storage temperature and medium. Freezing, or the accompanying freeze-thaw cycle, resulted in complete loss of activity, obviating lyophilization as a means of long-term storage. Vacuum dessication, however, seemed feasible, but the resulting pellet was very hard and resisted resolublization. No increase in stability upon drying was noted in comparison to that portion stored at 4 C in a buffered solution (pH 7.9).

Membrane Evaluations

Table 2 summarizes the permeability data collected on all the membranes studied. All membranes were tested in the

TABLE 2-A

Membrane Permeabilities to $NADP^+$

Membrane, thickness (mil)	Avg. Permeability (cm/min) x 10^3
PVC, (1)	2.98
PVC electret, (1)	3.09
Cellulose, (0.5)	1.40
Cellulose electret (0.5)	2.84
Anion exchange, (54)	0.233
Cation exchange, (54	0.206
Bio-Fiber - 50, (0.3)	1.27
Induced field flow cell (0.5)	0.3 (P*)

TABLE 2-B

Membrane Permeabilities to DMA

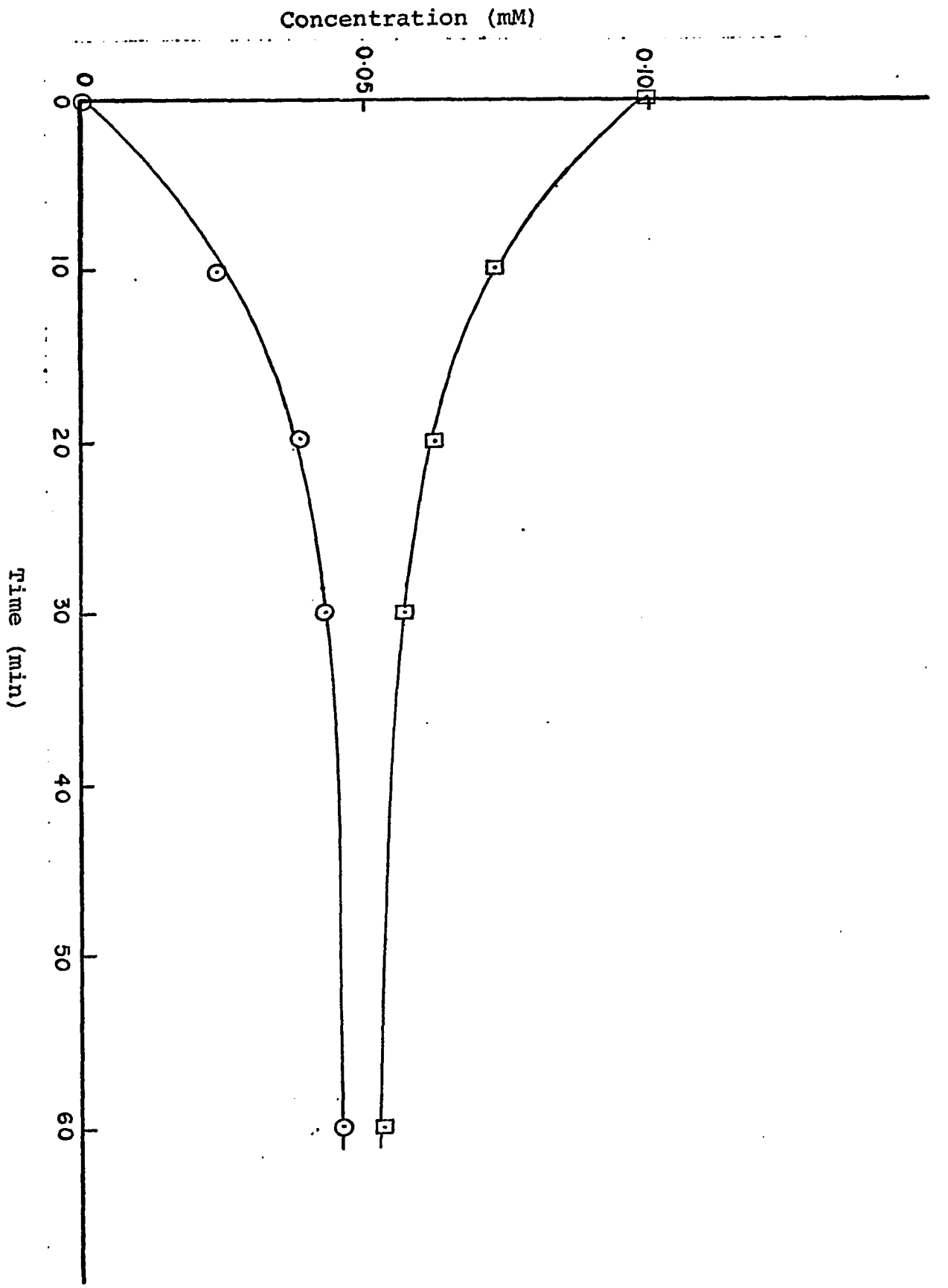
Membrane, thickness (mil)	Avg. Permeability (cm/min) x 10^3
Bio-Fiber - 50, (0.3)	1.95 at pH 6.5
PVC electret, (1.0)	6.06 at pH 6.5
Anion exchange, (54)	.295 at pH 6.5

type-1 cell (Chapter II), with the exception of the Bio-Fiber 50 cell, which was evaluated in its own jacketed container with recirculating fluids. The induced field tests were performed in the specialized type-2 cell. A discussion of the individual membranes follows.

Bio-Fiber - 50. As originally envisioned, a non-specific, high molecular weight cutoff membrane would be the method of choice for isolating the whole blood from the reaction loop, as discussed in Chapter II. Since cofactor flux was expected to be the limiting factor in the design, its evaluation was undertaken first, and DMA evaluated last. There were no modifications of membrane properties ascertainable as a result of this sequencing.

As received from the manufacturer, the fibers have been stabilized with a glycerine solution which must be flushed from the fibers before use. Afterward, the membranes must be kept wet in an antiseptic formaline solution. Figure 14 illustrates a typical run. The apparent loss of solute from the closed system was at first inexplicable. Leaks were never observed throughout the course of a run, and all 0.1 ml samples were returned to their respective reservoirs after analysis (with the exception of the nitroso-DMA samples, which were discarded). With repeated experimentation, however, the mass balance discrepencies tended to decline and the problem was temporarily forgotten. After several runs, it was necessary to install new silicone tubing in the pump head, and

Figure 14. Sample Data for Determination of Membrane Permeabilities and Fluxes in Bio-Fiber - 50 Flow Cell. Preliminary results with this system gave poor results, until it was discovered that $NADP^+$ and DMA were soluble in the silicone tubing used. This is an example of the superior data obtained after saturating the silicone tubing with the appropriate solute before use. The upper curve represents the decreasing concentration of $NADP^+$ in the tube side. The lower curve illustrates the resultant increase in cofactor concentration in the shell side. Good mass conservation is noted after 60 minutes.


Concentration (mM)
0
0·05
0·10
0
10
20
30
40
50
60
Time (min)

the non-conservative mass balance once again appared. DMA, it was discovered, and NADP, to a lesser degree, are soluble in the silicone tubing, and possibly in the cellulose membrane and polycarbonate case. Therefore, an overnight soaking period was instituted before each run with new tubing or a new solute. The results in Figure 14 represent the nearly optimum results from such an experiment. Figure 15 illustrates the excellent linear correlation attainable on two typical runs on $NADP^+$ and DMA. Total $NADP^+$ and DMA fluxes are given in Figures 16 and 17 respectively. Details of the data reduction techniques are covered in Chapter III.

Ion Exchange Membranes. It was initially observed that an impressed current across a cellulose membrane in a Type-1 cell was capable of maintaining a 10-fold concentration difference of $NADP^+$ across the membrane. This was investigated further in the type-2 cell, but the results were not as striking, and electrolysis was an undesirable side effect. Gold foil electrodes were more stable than aluminum, particularly at the anode, despite the fact that the "gold" leaf was mostly copper. In addition, electrolytic reduction and decomposition, and $NADP^+$ attachment to the anode proved troublesome. Nevertheless, charge does appear to be a valid method for the differential separation of charged cofactors from neutral solutions. The method of choice at present for such a process is through the use of ion exchange membranes. The "tight" characteristics of these membranes were well known

Figure 15. Sample Data Correlation Used in Graphical Solution of Membrane Permeabilities. The lower curve is the data of Figure 14 plotted in the form suggested in Chapter I.V for the graphical determination of permeability.

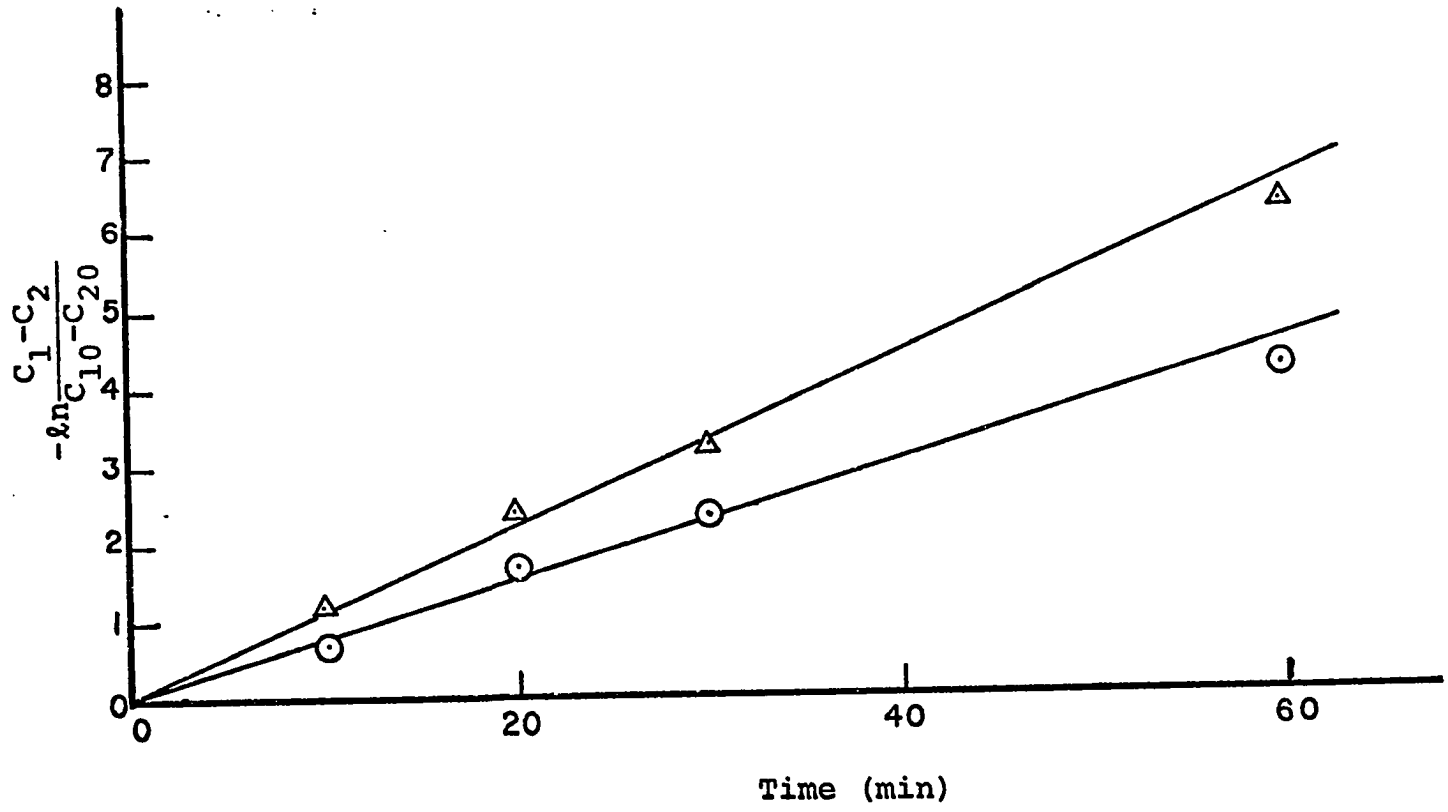

$-\ln\frac{C_1-C_2}{C_{10}-C_{20}}$
0
1
2
3
4
5
6
7
8
0
20
40
60
Time (min)

Figure 16. Net $NADP^+$ Fluxes of Each Membrane Evaluated. Assuming a constant membrane permeability, the best straight line was drawn to represent the cofactor flux to be expected of the patient for a given concentration in the reactor. Recall from Figure 13 that the kinetically optimum cofactor concentration is 0.025 M.

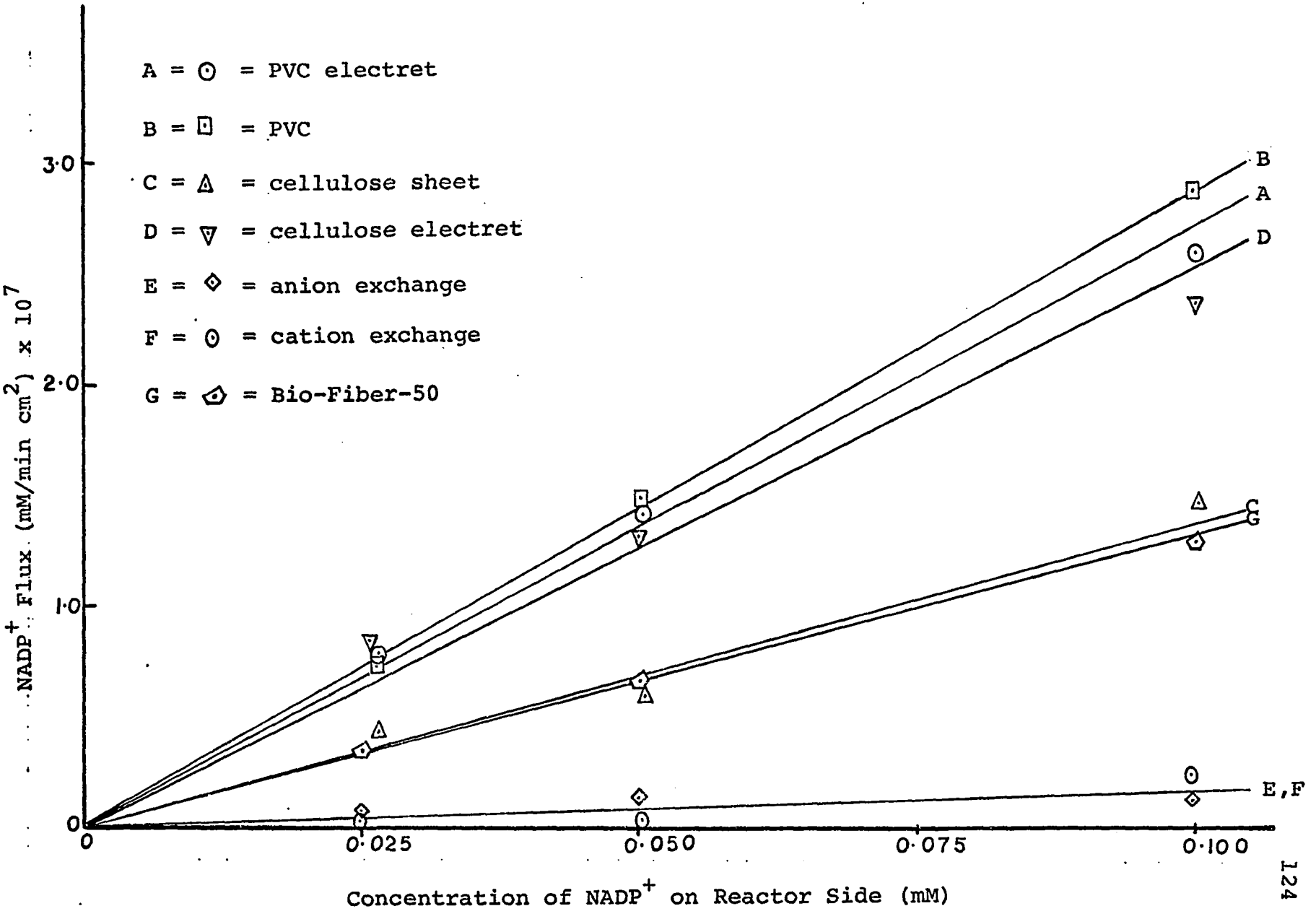
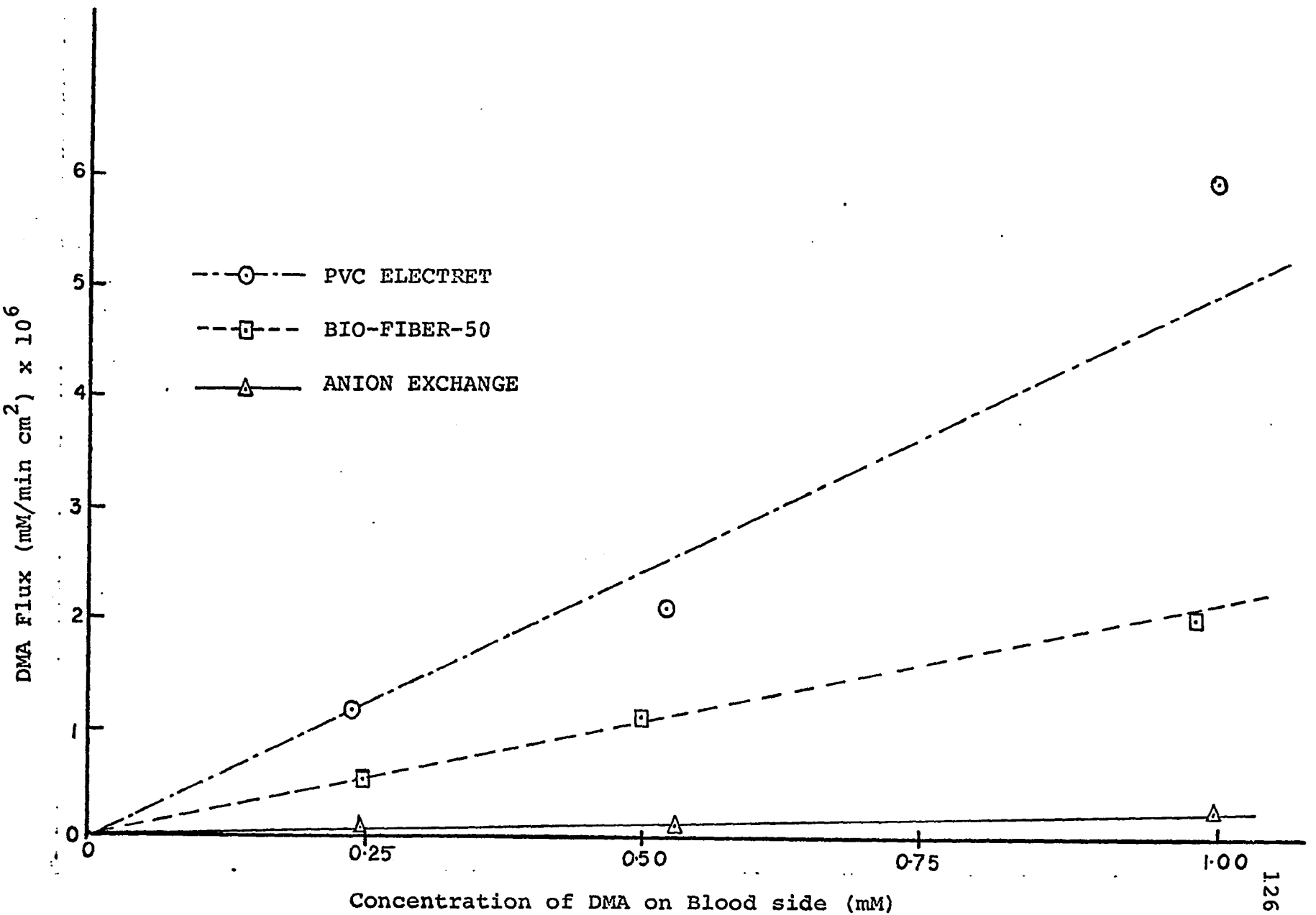

A = ⊙ = PVC electret
B = ⊡ = PVC
C = △ = cellulose sheet
D = ▽ = cellulose electret
E = ◇ = anion exchange
F = ⊙ = cation exchange
G = ⬠ = Bio-Fiber-50
NADP+ Flux (mM/min cm2) x 10^7
3·0
2·0
1·0
0
0·025
0·050
0·075
0·100
B
A
D
C
G
E,F
Concentration of NADP+ on Reactor Side (mM)

Figure 17. Net DMA Fluxes of Each Membrane Evaluated. Several of the membranes initially evaluated in the NADP studies were eliminated due to poor properties. The remaining membranes that showed some promise were evaluated with DMA. PVC showed a surprisingly high permeability to DMA. This is believed to be a solubility phenomenon that would not benefit the DMA-N-oxide product, due to its increased hydrophilic character. Bio-Fiber - 50 exhibited adequate fluxes, especially when the large surface area is accounted for. The anion exchange membrane appears to be totally unacceptable both in regard to $NADP^+$ and DMA fluxes.


PVC ELECTRET
BIO-FIBER-50
ANION EXCHANGE
DMA Flux (mM/min cm^2) x 10^6
0
1
2
3
4
5
6
0
0·25
0·50
0·75
1·00
Concentration of DMA on Blood side (mM)

ahead of time, and the data confirms they are only capable of low DMA and $NADP^+$ fluxes. Surprisingly, within the range of experimental error, there appears to be no substantial differentiation between charged moieties; $NADP^+$ flux through the anion exchanger is comparable to that through the cation exchange membrane.

There is a possibility that the DMA data contains a systematic error the result of an anomaly in the construction of the anion-selective membrane. The fixed charges contain quarternary ammonium groups and tertiary amine groups. The possibility exists that the tertiary amines in the membrane are slowly leaching into the dialysate side and being identified in the nitroso-DMA test as DMA itself. It is doubtful that this comprises a significant error, especially when one considers that DMA fluxes are still quite low.

PVC and PVC-Electret Membranes. All PVC membranes were cast from the THF solution as described in Chapter I V. A simplified casting algorithm was developed for the predictive determination of membrane thickness:

$$(0.02)(T)(A) = W$$

where: T = desired membrane thickness (thousandths of an inch)

A = desired membrane area (in^2)

W = grams of PVC which must be in casting solution

Generally, a 5% w/v solution of PVC in THF has adequate casting properties. Membrane thicknesses less than 0.5 mil

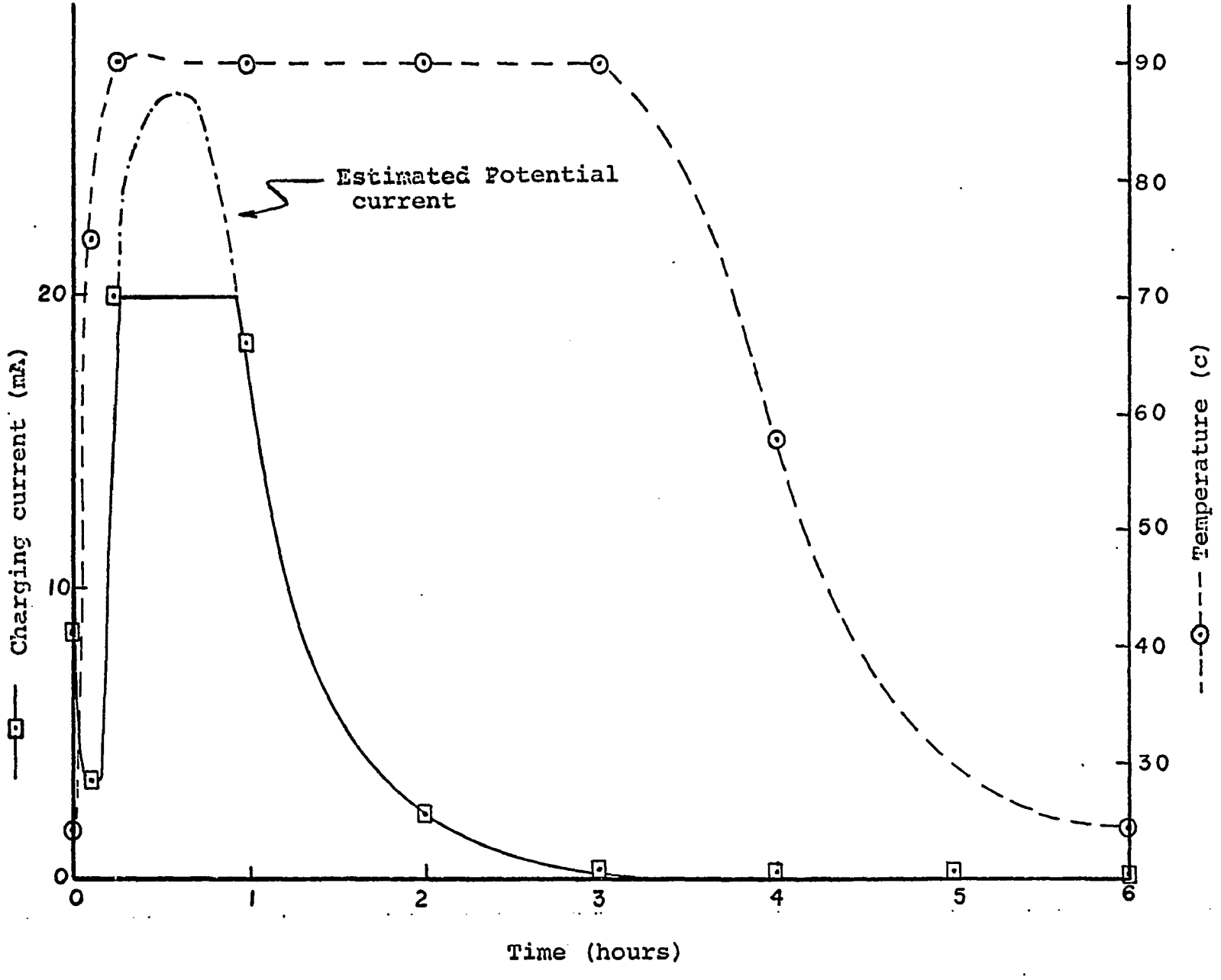
ruptured easily.

The electret process was greatly simplified with the advent of the heated press and disposable electrode sandwiches. Still, this laboratory lacked the sophisticated equipment necessary (a vibrating probe electrometer) needed to measure charges on the order of 10^{-10} coulombs/cm^2. Instead, Figure 18 is presented as evidence of the formation of an electret. The shape of the time/temperature and time/charge curves closely resemble those obtained by others (155-157) in the production of verified electrets. Initially, the charging current decays as it normally would at room temperature. Later, the trend reverses and the current starts to increase in response to rising current. This is believed to be associated with the initial disruption of the polymer lattice in which free electrons are more abundant. The current experiences a peak at approximately the same time as the temperature reaches its controlled value. After that, it gradually decays as the dipoles in the polymer align, creating a more resistive dielectric.

Permeabilities and fluxes of DMA and $NADP^+$ through PVC membranes appear in Figures 16 and 17, and in Table 2.

Cellulose Sheet and Cellulose Electret Membranes. Cellulose, in the form of regenerated viscose cellulose dialysis tubing, was re-evaluated in the Type-1 cell strictly as a comparison to its evaluation as an electret in much the same way as the PVC membranes. Unlike the PVC membranes, however,

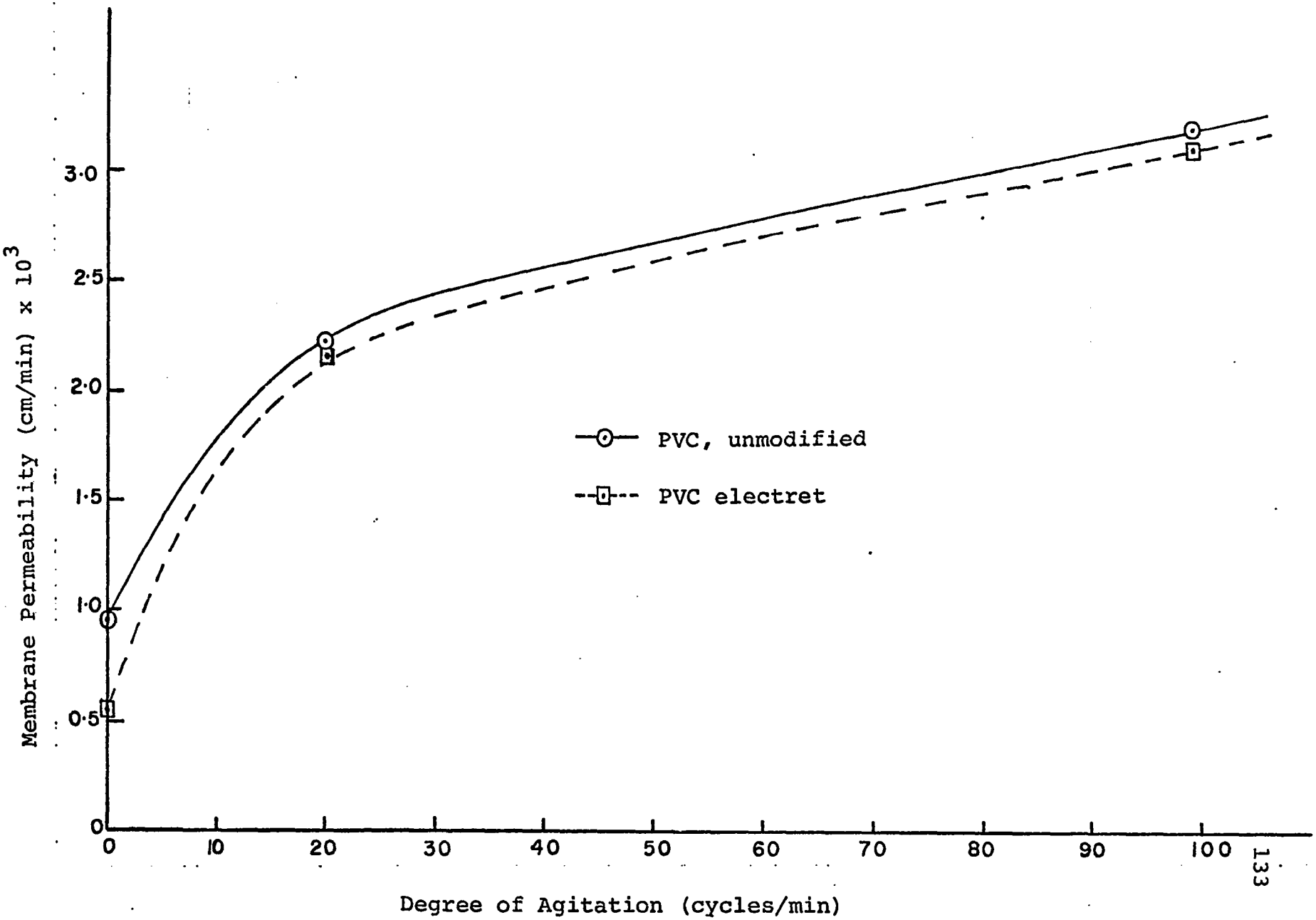
Figure 18. Evidence for the Formation of an Electret. The time/temperature and time/current curves superimposed in this plot contain features identical to those observedby others during electret preparation. This sample data from a typical experiment is offered as proof that an electret has indeed been formed.


Estimated Potential current
Charging current (mA)
Temperature (c)
Time (hours)
0
10
20
30
40
50
60
70
80
90
0
1
2
3
4
5
6

cellulose experienced considerable degredation in visual appearance and toughness during the electret-forming process. in addition, membrane permeabilities to all moieties increased. This property alone made it undesirable as a charge selective membrane, despite the fact that it displayed a similar charging curve to that for PVC in Figure 19. Permeabilities and fluxes for all the cellulose sheet membranes are given in Figure 17 and Table 2.

Agitation as a Function of $NADP^+$ Permeability in PVC Electret Membranes. Purely by accident, a series of Type-1 cells containing PVC electrets and $NADP^+$ solutions were not agitated in the shaker bath, as was the customary procedure to assure good mixing on each side of the membrane. Data was nevertheless collected, and it was immediately apparent that some selectivity had been obtained. The experiment was repeated with other prepared PVC electrets in other Type-1 cells, with consistent results. The permanent charge on the membrane surface, however weak, was being overpowered by the turbulence generated on shaking. Figure 19 demonstrates a quantitative representation of the effect of agitation on membrane permeability. Although the differences are minimal at high degrees of agitation, at stagnation there is a marked 44% reduction in flux of the charged $NADP^+$ across a charged PVC membrane with the positive face exposed to the high co-factor concentration. This clearly suggests that a laminar flow cell, in which the boundary layer at the membrane is

Figure 19. PVC Electret Permeability as a Function of Agitation. Previous data indicated no significant decrease in $NADP^+$ flux when either the positive or negative sides were exposed to the side of the cell containing the cofactor. It appears from this data, however, that the slightest turbulence disturbs the static repulsive boundary layer near the surface of the membrane. At stagnation, a significant 44% decrease in $NADP^+$ flux is observed. This suggests that laminar flow across the membrane face is necessary to preserve this boundary layer.


Membrane Permeability (cm/min) x 10^3
3·0
2·5
2·0
1·5
1·0
0·5
0
0
10
20
30
40
50
60
70
80
90
100
Degree of Agitation (cycles/min)
PVC, unmodified
PVC electret

essentially undisturbed, and cross diffusion through the bulk liquid is comparable to a stagnate fluid, may provide the answer to significantly lower toxic $NADP^+$ fluxes into the blood. Many clinical dialyzers today are capable of maintaining laminar flow across sheet membranes, the Kiil dialyzer being the most common.

Membrane overview. A comparison of the data in Figures 16, 17, and 18, and Table 2 suggests several things. First, the Bio-Fiber membrane is not a bad unit when one considers the overall convenience and high surface area available. It does fall short, in absolute terms, of the cellulose sheet, probably due to poor flow distribution in the shell side of the dialyzer. This could no doubt be optimized with baffles and increased flow rates. A flow rate of 200 ml/min in both the tubes and the shell was selected merely because this is a typical flow rate through a comparably sized extracorporeal hemoperfusion device, the artificial kidney.

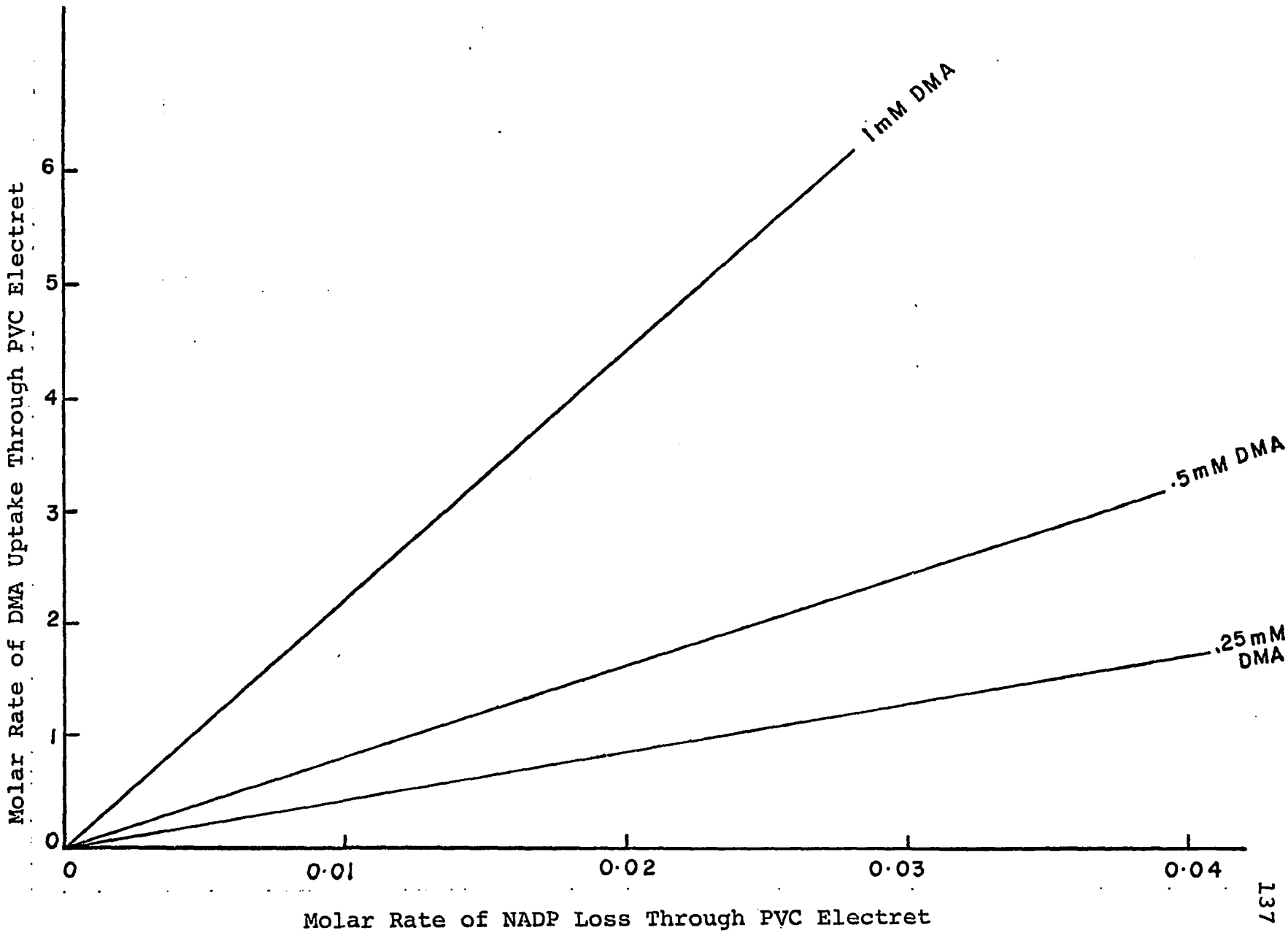
Secondly, the family of PVC membranes show excellent permeabilities to DMA, and the potential for decreasing the $NADP^+$ permeability (Figure 19). If one uses a criterion such as (DMA permeability)/($NADP^+$ permeability) for the selection of a membrane, PVC again excells. The ratio is 12 for the PVC electret, 1.5 for Bio-Fiber - 50, and 1.3 for the anion exchange membrane. This, however, may be a deceptive advantage. The effect is believed to be due to a solu-

bility phenomenon, the DMA being much more soluble in PVC than $NADP^+$. The effect, evidently, is not as pronounced in the other membranes. As was discussed earlier in Chapter I, specificity based on solubility is not desirable in a detoxification system because the hydrophilic product of the reaction, DMA-N-oxide in this example, may have a more difficult time getting back across the membrane than the precursor did initially.

The above argument should not be construed to be an argument against the PVC electret. The evidence to date indicates that the PVC membrane is still the preferred method, simply because it can be prepared as an electret. The Bio-Fiber - 50 system, purchased off the shelf, is not amenable to modification, especially by the electret process. It is quite possible, however, that the manufacturer can produce charged hollow fiber membranes in which the electret is formed during extrusion of the fiber by suitably charging the die. This, of course, would require a close alliance between the user and manufacturer, and currently a demand for such a product has not been established.

As a result, Figure 20, generalized representation of the countercurrent fluxes of DMA and cofactor to be expected for a given membrane area, is premised on the use of a PVC electret membrane in a Kiil dialyzer. This plot, together with the kinetic studies of Figures 11 and 12, should prove to be particularly useful in sizing membrane and reactor systems given the constraint of maximum cofactor flux desired.

Figure 20. A Generalized Comparison of Counter-Current Fluxes of Cofactor and Substrates for a Given Membrane Area. These curves represent a collation of the results in Figures 17 and 19 for the PVC electret membrane in an idealized laminar flow dialyzer. Since this membrane offers the most advantageous permeabilities, it has been selected as the current method of choice.


Molar Rate of DMA Uptake Through PVC Electret
0
1
2
3
4
5
6
1mM DMA
.5mM DMA
.25mM DMA
0
0·01
0·02
0·03
0·04
Molar Rate of NADP Loss Through PVC Electret

CHAPTER V I

RECOMMENDATIONS

The lethal certainty of hepatic failure allows wide latitudes for one wishing to attack the problem of extra-hepatic support in a synthetic system. As a result, the topic offers the engineer a rare opportunity to test radical designs normally shunned by the conservative medical community. This paper has attempted to focus on those wide latitudes somewhat by addressing four previously unexplored areas: 1) oxidase activity in blood with a model compound; 2) membrane modification; 3) significantly reducing required cofactor concentrations; and 4) exploring macromolecular cofactors for this system. In addition to these, several other avenues for potential research have emerged. For the time being they are best grouped into four discrete projects: 1) animal testing; 2) computer modeling; 3) cofactor retention, phase II; and 4) isolation of various other hepatic extracts. Each of these projects appeals to a different type of researcher, underscoring the need for a unified team effort.

Animal testing can offer immediate results. Several

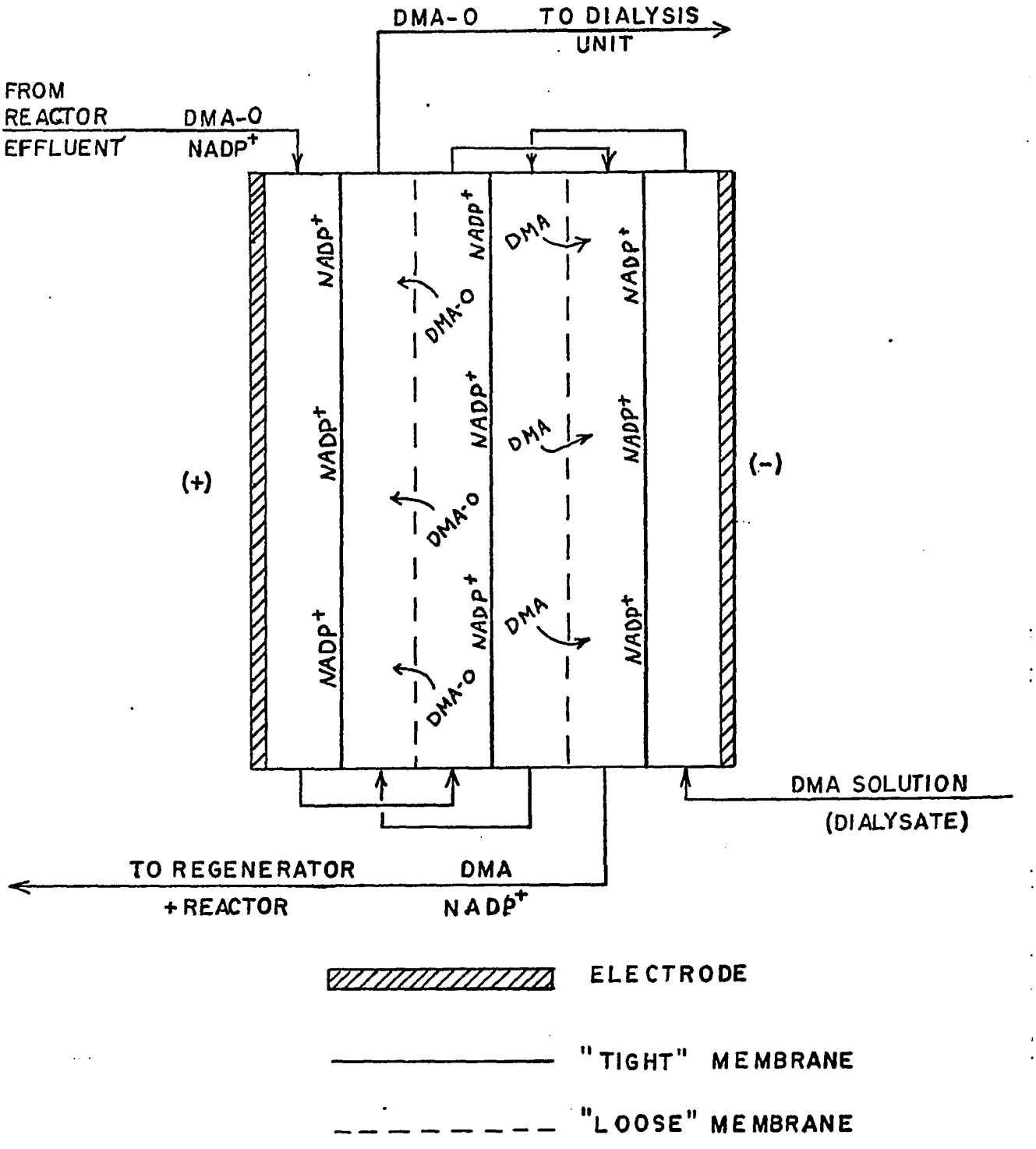
issues have haunted this project since its inception: What effect does a slow infusion of NADP have on a large animal? Can it be effectively counteracted with intramuscular injection of atropine sulphate? Once these questions have been answered, a device could be built overnight. The materials for constructing an extracorporeal shunt reactor are available in this laboratory today. At this point, the use of common components is advisable. A commercially available hollow fiber dialyzer, coupled to a nylon tube oxidase reactor, both known quantities, would be sufficient for animal testing. The knowledge that a PVC electret is capable of reducing cofactor flux is largely academic at this point. Instead, a crude, pragmatic approach to the problem is called for. The effectiveness of the shunt may then be evaluated in a series of animals representing normal, CCl_4 induced cirrhosis, and possibly anhepatic test specimens. A more realistic model compound is also needed, chlorpromazine, for example. This study should be begun immediately because it will necessarily involve time consuming liaisons with other researchers.

The second front to be explored, a computer simulation, is a highly theoretical issue, yet one that totally sidesteps the laboratory. It will be used as a predictive tool and a means of fine tuning the animal testing study. It is far easier to modify two or three parameters in a computer program than it is to cannulate a strung-out dog.

Sufficient experimental data currently exists to determine all kinetic and membrane parameters for this system. The solution will involve the straightforward solution of a set of simultaneous differential equations, eminently suited to IBM's CSMP program. The program should be capable of predicting concentrations of all essential moieties on each side of the membrane, as the reaction progresses with time.

The third project has far reaching ramifications in all areas of cofactor intensive enzyme research, and especially that concerned with the artificial liver. It has been demonstrated that $NADP^+$ experiences a marked electrolytic migration in solution under an impressed current. Electrolysis has obviated this technique for cofactor separation and/or retention in this study, yet the technique need not be dropped. Industrially, when one is faced with this problem, electrode area is reduced, and the anode and cathode are isolated by an appropriate electrolyte. Furthermore, the current resulting from the two electrodes is distributed across as many membranes as possible. The result of such logic is commonly referred to as electrodialysis, a method being explored today for desalinization of sea water. An analogous electrodialytic cell can be proposed for the retention of $NADP^+$ (or any charged cofactor) within the enzyme reactor of an artificial liver. The flow scheme for a single unit is illustrated in Figure 21. The alternating "tight" and "loose" membranes would allow current flow, but the $NADP^+$ would

Figure 21. A Proposed Method for the Retention/Separation of Charged Cofactors. The alternating "tight" and "loose" membranes allow current flow, but the $NADP^+$ is continually attracted to a membrane through which it cannot pass. The uncharged substrate (DMA) and metabolite (DMA-O), on the other hand, will be free to diffuse in any direction, specifically through the loose membranes.


DMA-O
TO DIALYSIS UNIT
FROM REACTOR EFFLUENT
DMA-O
NADP+
(+)
(-)
NADP+
DMA
DMA-O
DMA SOLUTION (DIALYSATE)
TO REGENERATOR + REACTOR
DMA
NADP+
ELECTRODE
"TIGHT" MEMBRANE
"LOOSE" MEMBRANE

continually be attracted to a membrane through which it could not pass. The uncharged substrate and metabolites, on the other hand, would be free to diffuse in any direction, specifically through the loose membrane. Numerous compromises must still be made for such a design. pH control will be a continuing problem, and electrode material will be critical. Ion exchange membranes could play a vital role here, where their poor permeability to middle molecules is immaterial. It nevertheless appears to be a potential breakthrough in cofactor separation and containment.

The fourth and final area of artificial liver research to be investigated during this phase of development is concerned with generalizing the relatively specific system that currently exists. The single enzyme reactor incorporating the MFMF oxidase is, despite the versatility of the oxidase, still representing only one detoxification pathway (albeit a highly significant one). The utility of an extrahepatic support device incorporating several detoxification enzymes would offer a quantum jump in clinical significance, with only a marginal increase in complexity. Alas, hepatic oxidases, transferases, mutases, etc., are, like the MFMF oxidase, difficult or impossible to isolate, and quite expensive. As an alternative, work has begun in this laboratory, and will no doubt continue, in an effort to immobilize microsomes on glass beads in a manner analogous to that used for enzymes. The microsomes, which contain all the membrane-

bound enzymes of the endoplasmic reticulum, are also greatly benefited by insolubilization. They are easily and cheaply prepared from various hepatocyte sources, and respond well to long periods of storage. There is no doubt now that the extrahepatic support reactor that will see clinical use will employ immobilized enzymes and microsomes in its role of liver detoxification.

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