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GRADUATE COLLEGE

DESIGN OF A DETOXIFICATION SYSTEM USING MACROMOLECULE-BOUND NADP

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

ROBERT ALAN WILLS

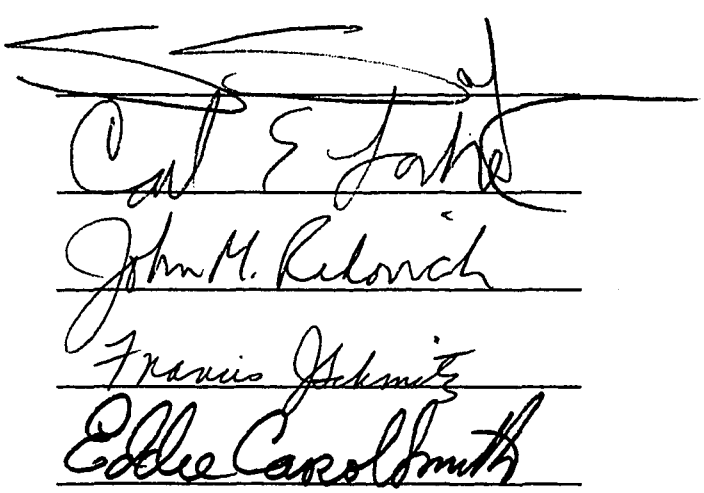
Norman, Oklahoma

1978

DESIGN OF A DETOXIFICATION SYSTEM

USING MACROMOLECULE-BOUND NADP

APPROVED BY



DISSERTATION COMMITTEE

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DESIGN OF A DETOXIFICATION SYSTEM USING MACROMOLECULE-BOUND NADP

CHAPTER I

BACKGROUND

Introduction

Today, there exists no effective treatment for hepatic failure. Current practices are for conservative and general treatment, usually providing only temporary, symptomatic relief or general support (1).

The need is not necessarily to replace the liver. The liver is the only organ, with the possible exception of the skin, which can completely regenerate into a functional organ. As an example, rat liver, when excised to 30% of its' original size, will completely regenerate in a few days (2). Because of the essential functions of the liver, a no-load period for regeneration after disease or damage is impossible (3). Therefore, the real need is in an assist device for the liver, to allow it adequate time to recover.

Detoxification

During liver failure, many of the substances typically produced by the liver can be substituted by intravenous injections. Many substances that provide a regulatory function

can be monitored and substituted for appropriately. Today, the only function of the liver that can neither be supported nor substituted is that of detoxification (4). As toxins build up in the body, they inhibit the enzymes which deal in energy, regeneration, synthesis, and so on. Thus, liver disease is often characterized by a descending spiral of liver efficiency, ending in hepatic coma and death (5). Because of the importance of detoxification, most attempted liver assists deal in the removal or inactivation of toxic substances.

Hemodialysis

Hemodialysis is probably the most acceptable manner of hepatic assist today (6,7). Toxins are removed from the blood by a concentration gradient across a membrane. This membrane could be synthetic or biological, such as the peritoneum. Use of hemodialysis as a means of hepatic assist was first performed by Kiley, et al. (8). The lack of selectivity they experienced using hemodialysis has been compensated for by many years of research. Still, this manner of detoxification falls short in removing higher molecular weight toxins. (The metabolites that accumulate in acute liver failure range up to 5000 in molecular weight (9,10)). Opolon, et al. (11,12) feel they have met this problem with a new polyacrylonitrile (PAN) dialyzing membrane that can handle higher molecular weight compounds. However, with what is known about detoxification, it is unlikely that hemodialysis will be effective

in any form against lipid soluble toxins or toxins bound tightly to proteins. Further, there are difficulties with the onset of severe hypotension (13-15), hypothermia (16), and changes in the blood picture (17-20).

Chemical Adsorbents

An area becoming increasingly more popular is that of chemical adsorbents. Rosenbaum (21-24) has noted selective removal of some compounds in lethal drug intoxication cases using a resin adsorption column. Willson (25-28) has experimented with charged and uncharged ion exchange resins on dogs. Resin hemoperfusion may produce, however, extreme hemolysis and lowering of blood platelet counts (29). Weston (29-31) found no appreciable lowering of blood platelet loss even when he perfused the resin with plasma from an Aminco continuous cell-separator in a dog.

Brünner (32) has used a column as the support for purified microsomal enzymes. He, however, uses no barrier between the enzymes and the blood. While more effective in reducing lipid-bound toxins, this system also is open to foreign protein attack.

Another very popular sorbent is charcoal. The first to use charcoal in a blood circulation device was Yatzidis (33). Many (17,18,19,34,36), including Williams at the Liver Unit of King's College Hospital in London (13,14), have reported reversal of hepatic coma using charcoal hemoperfusion. The greatest disadvantage of charcoal hemoperfusion is

that it is completely nonspecific. While adsorbing the toxins, charcoal will also, for example, adsorb some hormones in the blood (37). Another disadvantage is that it is not very biocompatible.

Microcells

In order to make the charcoal and other resins more biocompatible, Chang devised a method for polymer coating these sorbents (35,38-43). These "artificial cells", as they are referred to, still do not have a high degree of specificity. Recently, though, Chang has reported (44) the microencapsulation of an oxido-reductase enzyme. This offers a great potential in duplicating complex liver enzyme systems. The microcapsules may be injected intraveneously or peritoneally. They may be taken orally or perfused with blood in an extracorporeal device. If this is to be used with an enzyme system, it must be realized that the enzyme's cofactor and regeneration system must be encapsulated in the same cell with the enzyme to make an efficient system.

Biological Tissue

Utilization of biological tissue has been attempted by various investigators. The biological systems employed include healthy donor blood (45-49) as a transmitter of liver function, and liver tissue itself in various forms of preparation (50-55). These techniques have largely met with failure, due to preparation and procurement complexities,

difficulties of storage and preservation, and complications related to immunology. Although normal liver tissue will not replicate successfully *in vitro* (56,57), various methods of liver cell culturing and preservation do 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 (58-60).

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. 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. The concept of continuously exposing one's blood to a thriving, malignant cell culture may not be acceptable clinically.

A much more critical form of biological tissue use has been in the transplantation of whole livers. Starzl described this procedure in 1975 (61) and averages 10-20 such operations per year. There is also the technique of liver grafting (62-66). In both of these techniques, there is a critical matching of tissue to reduce rejection reactions. Because of the critical nature of these techniques, their use in treatment of hepatic failure is very limited.

Because of the inherent problem in the use of whole cells, a logical step would be to use only the catalytic

agent in the cell. This catalytic agent, or enzyme, is highly specific with respect to its action. However, it could not be injected directly into the body without possibly causing allergic reactions and anaphylactic shock (67,68). Further, the free enzyme in solution is subject to loss of activity due to its' accessability to destructive proteolytic enzymes. A method for enzyme utilization with more stability is immobilization (69). In addition to stability, the enzyme can be easily retrieved for repeated use or isolated in the hemoperfusion device. Many researchers (70-77) have attached hepatic detoxification enzymes to Dacron ^{R}, porous glass beads, sepharose, and other inert matrices. However, with most of these detoxification enzymes, there is an associated cofactor or cosubstrate such as nicotinamide adenine dinucleotide phosphate (NADP) or uridine diphosphoglucuronic acid (UDPGA). These must be maintained in high concentration around the enzyme to insure maximum detoxification. Hence, a membrane is usually incorporated to retain the cofactor and, additionally, repel blood proteins that would give a foreign body response to the immobilized enzymes. Even though a membrane is used, the cofactor often falls below acceptable reaction levels due to leakage.

The problem of supplying adequate amounts of cofactor remains dominant (78).

Hybrid Systems

A number of combinations of processes is possible

within a single hepatic assist device. For example, Yamazaki (79,80) has devised an automated system that uses a blood separator of hollow fibers, paired with an adsorbent charcoal column. A microfilter is incorporated to trap particles of charcoal in the plasma leaving this unit. A hollow fiber dialyzer is also used. Despite the complexity, the system still maintains its shortcomings. For example, the charcoal is nonspecific and will not detoxify lipotoxins or those toxins bound tightly to proteins.

Based on the information presented in this chapter, I have developed a similar hybrid system. It consists of a plasma separator, a matrix bound enzyme reactor, a mixer, a semi-permeable membrane for separation of reactor from plasma and, most important, a macromolecule bound cofactor. In the following chapters, the proposed system will be presented in more detail, as well as its advantages over other systems proposed up to now.

CHAPTER II

COFACTOR RETENTION

The most troublesome portion of any microsomal oxidative enzyme detoxification system, as mentioned in Chapter I, is the retention of the cofactors within the enzyme reactor. This retention serves two purposes: to prevent the cofactor from attaining potentially toxic levels in the body and to maintain the cofactor at a concentration that will allow optimum reaction conditions. It should be understood that not all cofactors are considered harmful at high concentrations. Uridine-5-diphosphoglucuronic acid (UDPGA) is a good example of this (81).

Nicotinamide adenine dinucleotide, NAD, and its phosphate, NADP, however, cause severe vasodepression in test animals at dosages as low as 50μg per kilogram body weight (82).

The manners in which these cofactors might be retained have been reviewed elsewhere (83), but three basic techniques may be noted:

1) retention by low molecular weight cut-off membranes
2) retention by the cofactor's ionic charge, and

3) retention by increased molecular weight of the cofactor.

Low molecular weight cut-off serves only to retard the permeation of the substrates and products of higher molecular weight. Therefore, we began studies to retain $NADP^+$ by an ionic charge. Ionic charge retention was investigated by Hare (82) of this laboratory and reinvestigated by Wills (83). Various membranes were used, including PVC. The charge was incorporated onto the membrane by alignment of the dipoles at a temperature above the glass transition point in an electric field (see Figure 1). This was followed by cooling the membrane while still in the field. This method proved to be ineffective. As seen in Figure 2, increased agitation decreases diffusional limitations (top curve), but also decreases effectiveness of the charge gradient and reduces the NADP retention (bottom curve). We then investigated the technique of retention by macromolecule attachment to the cofactor.

Many different macromolecules have been attached to the cofactor, (84-95); but for NADPH, these techniques have had only limited effectiveness. Attempts in this laboratory were made to couple succinylated NADP to polyethyleneimine (PEI) by a modification of the method of Wykes (86,93) for NAD. This proved unproductive and our reaction rates were very low, probably because of steric hindrances. Normally, a macromolecule of 40-50,000 MW is attached to a cofactor of 500-1000 MW. This complex is bulky, making diffusion a

FIGURE 1

ELECTRET FORMATION

Membrane electrets are formed by heating the polymer to a temperature above the glass transistion point and applying a field to the membrane as it cools. The heterocharge is the realignment of the dipoles. The homocharge is merely at the surface and drains off.

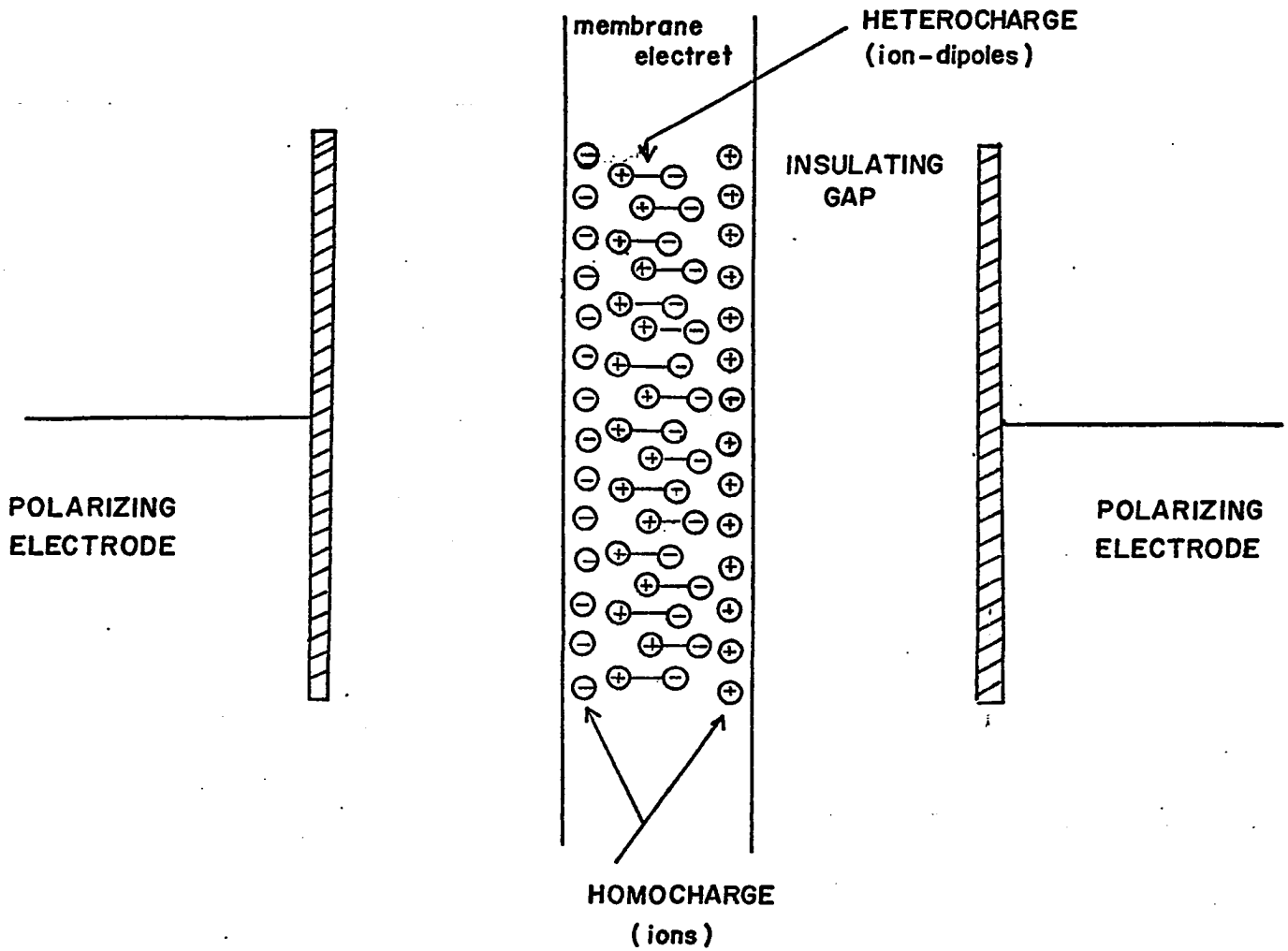
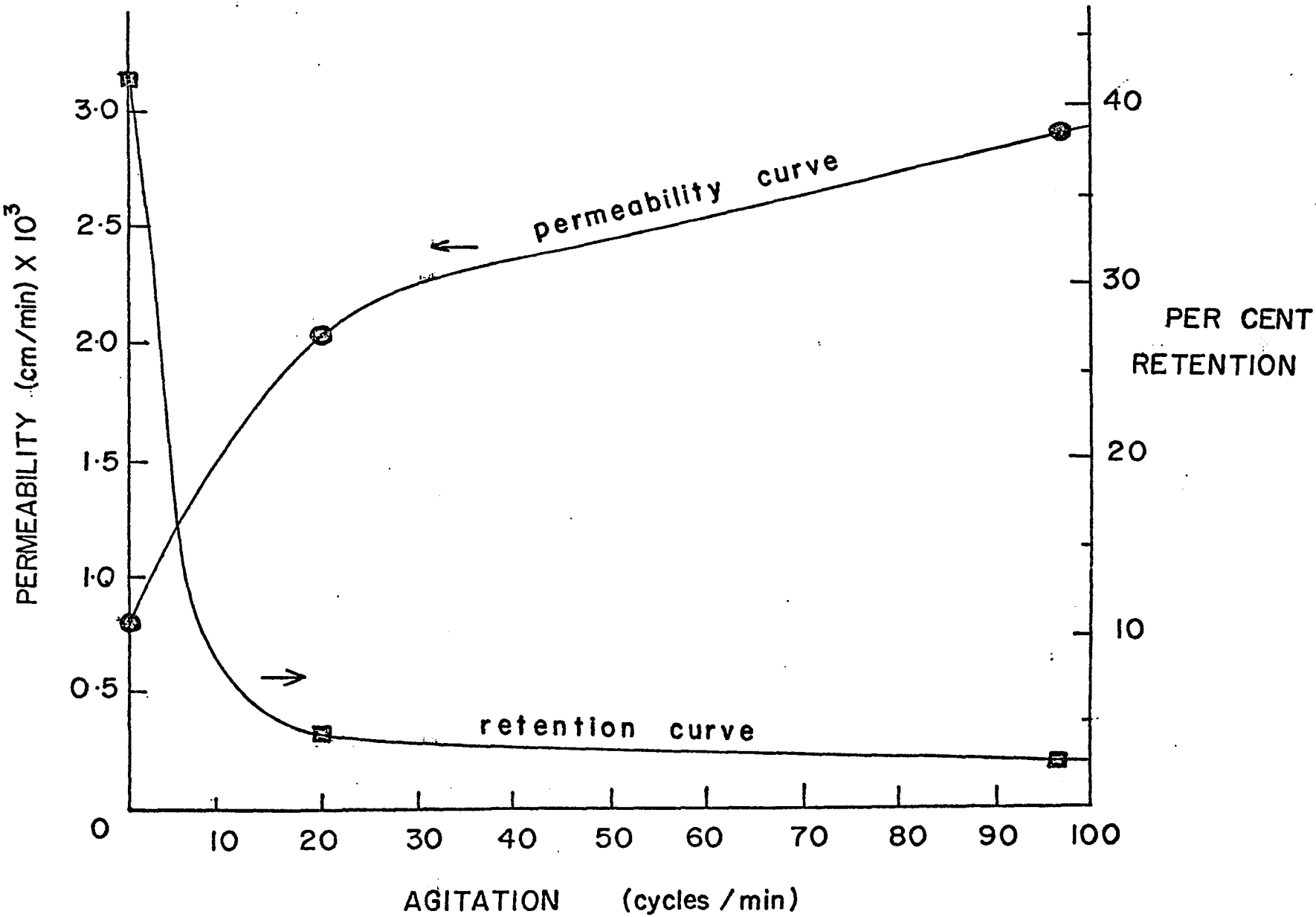

membrane
electret
HETEROCHARGE
(ion-dipoles)
INSULATING
GAP
POLARIZING
ELECTRODE
POLARIZING
ELECTRODE
HOMOCHARGE
(ions)

FIGURE 2

ELECTRET RETENTION OF $NADP^+$

The reduced effectiveness of the permanent charge on the membrane is shown as the agitation is increased.


PERMEABILITY (cm/min) X 10^3
3·0
2·5
2·0
1·5
1·0
0·5
0
permeability curve
retention curve
40
30
20
10
PER CENT
RETENTION
10
20
30
40
50
60
70
80
90
100
AGITATION (cycles / min)

limiting factor. Further, this spreads the "active" sites over a larger molecule. Therefore, we felt that perhaps a significantly smaller macromolecule might prove to be more effective. Following a modification of the procedure of Yamazaki, et al. (96), we attached poly-L-lysine hydrobromide (MW 2300) to NADP (MW 800) to form a complex of molecular weight approximately 3100, 10,000 if saturated.

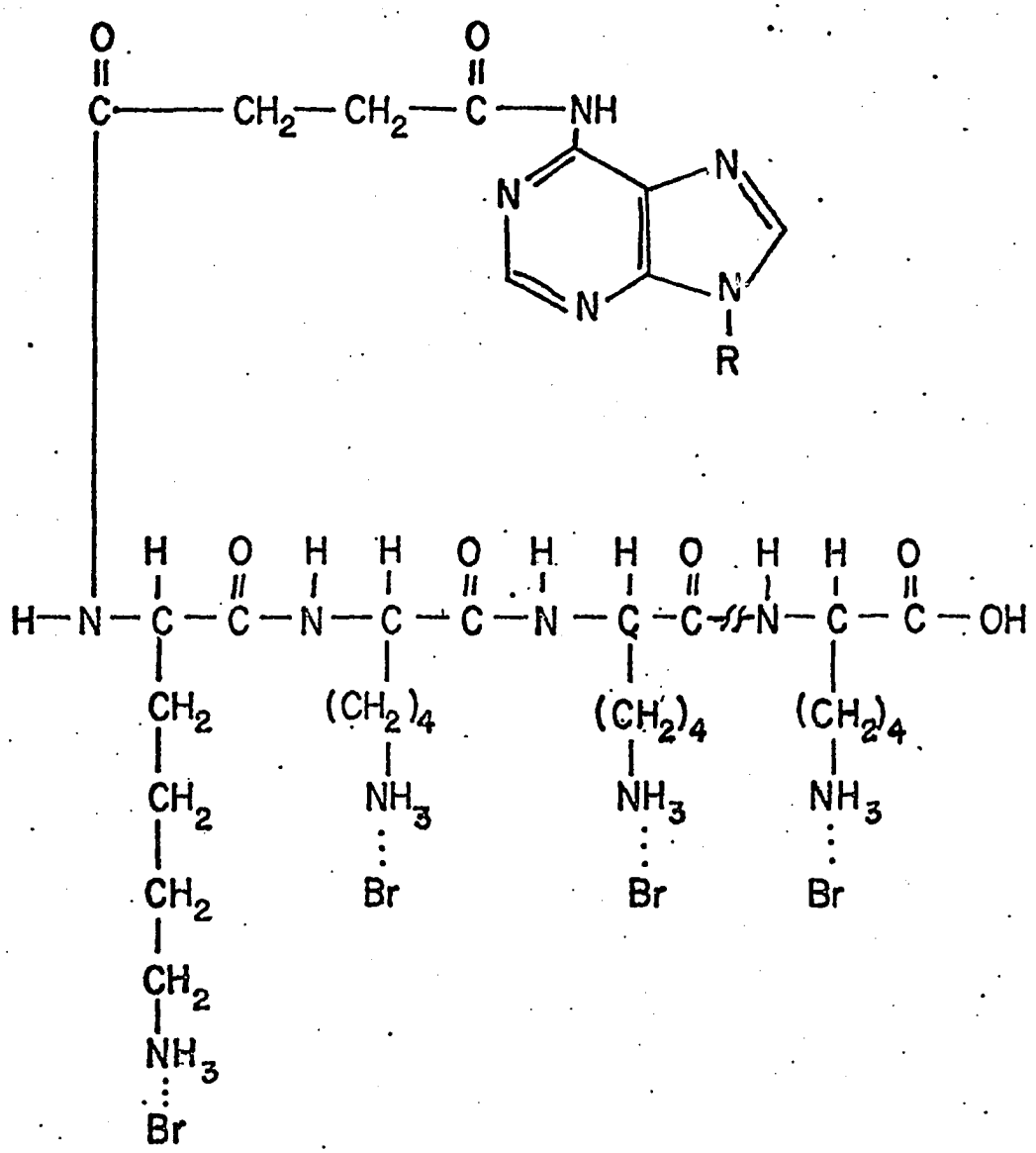
In Figure 3, the proposed structure of poly-L-lysine succinyl NADP (PL-SNP) is shown. The succinic anhydride is used as a spacer group, attached to the α amino end of the poly-amino acid. Note that possible binding at the ε positions is being investigated. Not only did this "enlarged" NADP work with the complex MFMF' oxidase enzyme (see Figure 4), it gave activities of up to 100% that of pure NADP. A graph of this is shown in Figure 5. The y axis represents the reaction rate of the PL-SNP divided by the reaction rate for a pre-set quantity of NADPH (60λ of a 0.01M solution). The x-axis denotes varying quantities of a 1.0 mg/ml H_2O solution of PL-SNP.

Therefore, some headway was made into the cofactor retention problem, at least for NADP/H. It then became logical to proceed with the design and evaluation of an hepatic assist device.

FIGURE 3

PROPOSED STRUCTURE OF PL-SNP

This is the proposed structure of poly-L-lysine succinyl amino group. Attachment at the ε positions is not shown but is assumed to be possible. The "R" group denotes the remainder of the NADP molecule.



R= rest of NADP/H moiety

FIGURE 4

PLSNP WITH MFMF OXIDASE

PLSNPH is shown reacting with N, N-dimethylaniline (model toxin) in the presence of MFMF oxidase enzyme. Also, a regeneration system is shown for maximizing PL-SNPH.

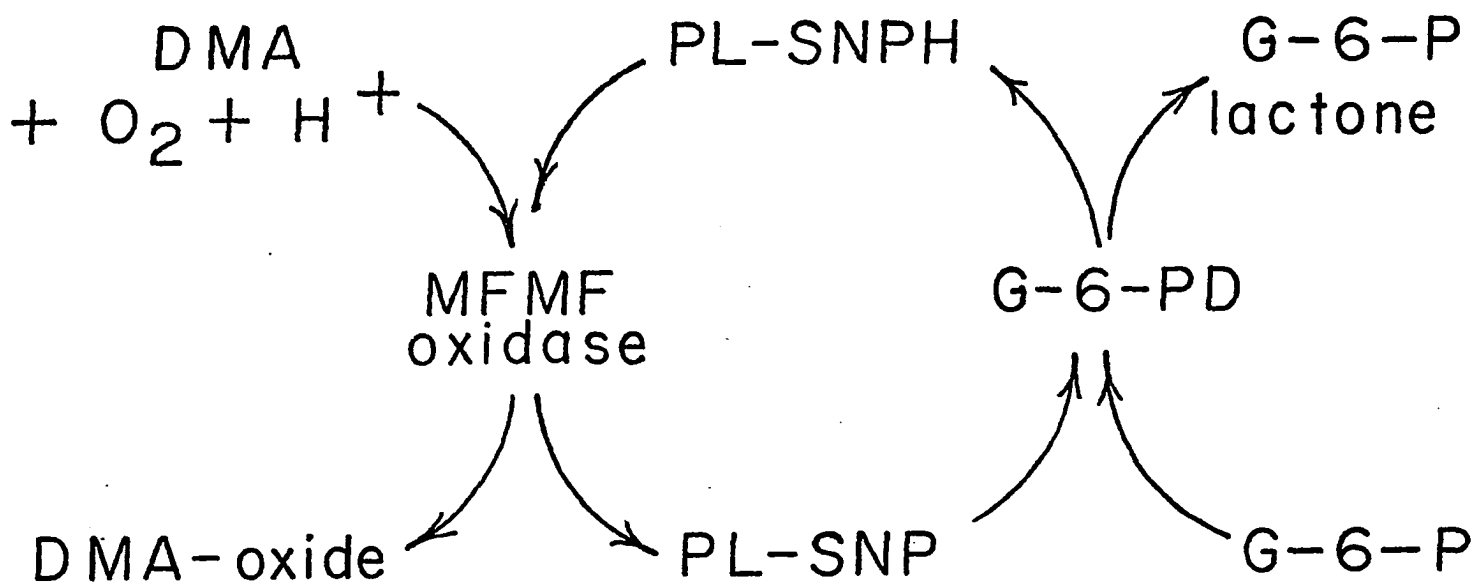
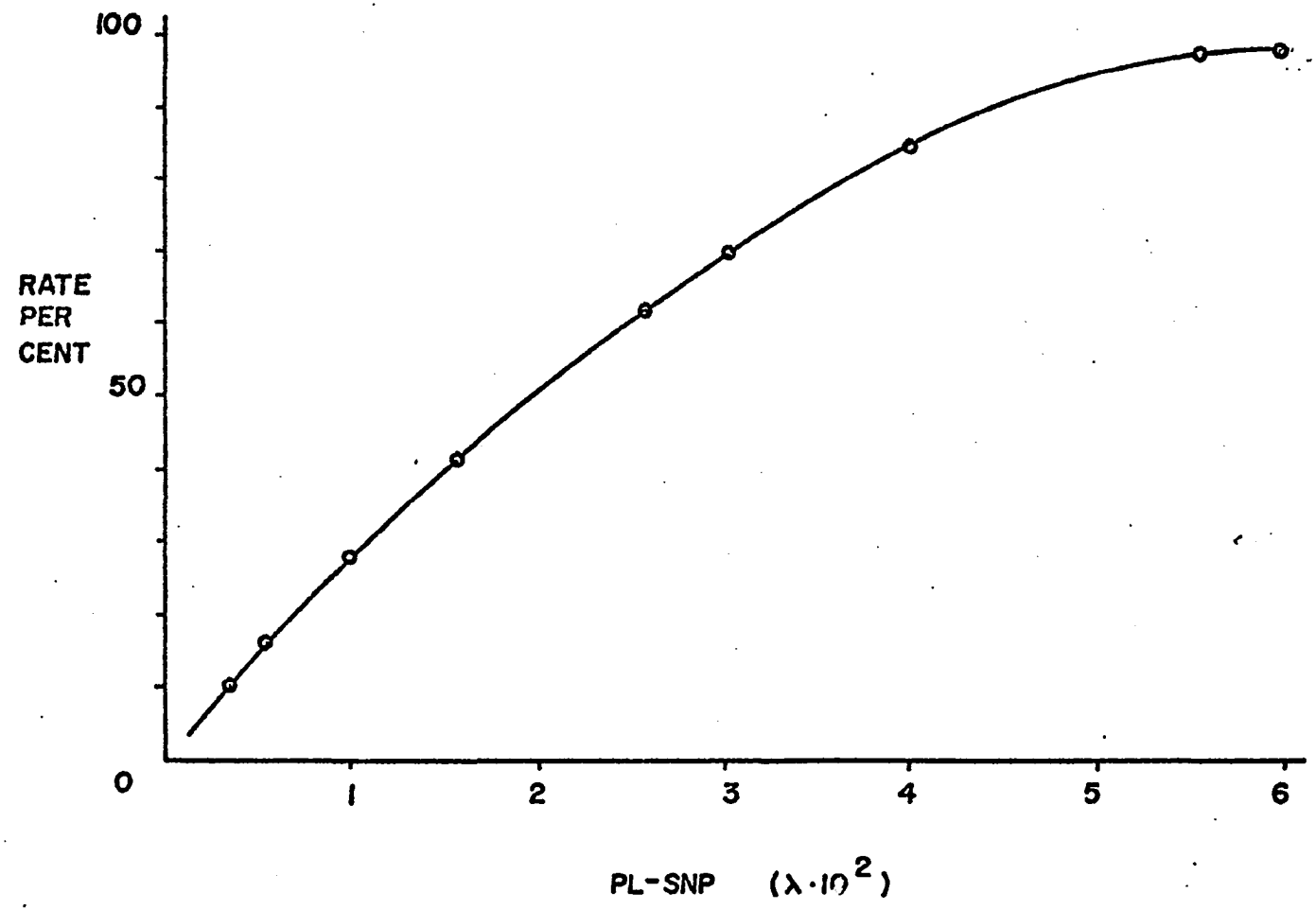

DMA
+ O_2 + H^+
PL-SNPH
G-6-P
lactone
MFMF
oxidase
G-6-PD
DMA-oxide
PL-SNP
G-6-P

FIGURE 5

RELATIVE RATE OF PL-SNP ACTIVITY

The initial reaction rate for PL-SNP is divided by the rate for pure NADPH in the same reaction. The PL-SNP was capable of giving in excess of 95% the activity of pure NADPH. The λ unit at the base of the figure represents microliters of a 1 mg/ml solution of the PL-SNP analogue. This is used as the exact molecular weight of the cofactor is still unknown.


100
50
0
RATE
PER
CENT
1
2
3
4
5
6
PL-SNP (λ·10²)

RESULTS

Equilibrium

An attempt was made to evaluate where the equilibrium point for formation of reduced PL-SNP lay. The enzyme, 6-phosphogluconolactonase, was used to drive the reaction of glucose-6-phosphate (G6P), PL-SNP, and glucose-6-phosphate dehydrogenase (G6PD) to completion as shown below:

$$\text{PL-SNP} + \text{G6P} \xrightarrow{\text{G6PD}} \text{PL-SNPH} + \text{6-phosphogluconolactone}$$

$$\xrightarrow{\text{lactonase}} \text{PL-SNPH} + \text{6-phosphogluconate}$$

Adsorption at 340 nanometers was observed as lactonase was added to the reaction mix (Figure 6). This did not work as either the equilibrium was already far to completion or the lactonase was impure. Further study was discontinued.

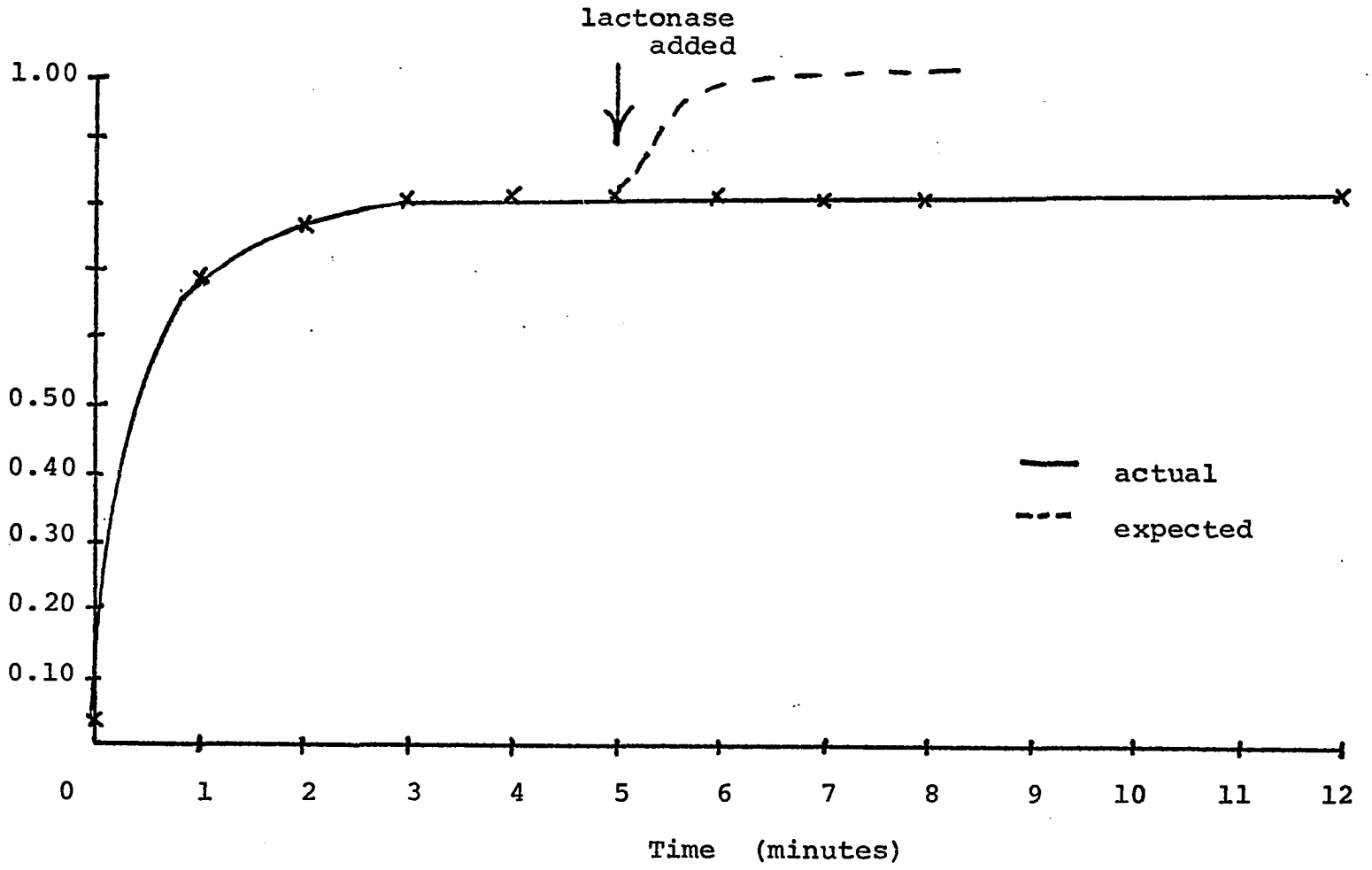
Binding

Several different techniques were attempted to determine the degree of binding. As shown in the outline that follows, the first attempts were made using known quantities of polylysine hydrobromide and NADP. The mixture from the synthesis reaction (see Chapter IV) was dialyzed. The amount of NADP that was in the dialysate was calculated by its' extinction coefficient. This gave ratios of binding of NADP to PL-HBr of 3.9 and 2.6.

FIGURE 6

ADSORPTION AT 340 nm USING LACTONASE

The adsorption curve at 340 nanometers showed no increase, indicating no further production of PL-SNPH, upon addition of lactonase. See text for reaction equation.


lactonase
added
1.00
0.50
0.40
0.30
0.20
0.10
Absorption
0
1
2
3
4
5
6
7
8
9
10
11
12
Time (minutes)
actual
expected

The next attempt was to purify the PL-SNP formed using a liquid chromatography column. The sample was collected and the PL-HBr moiety was analyzed by the biuret method. The NADP moiety was analyzed spectrophotometrically at 260 nanometers. This gave a ratio of 5.2. In the final technique, the sample was column purified as above. A dry weight was taken. The sample was brought up in a known quantity of distilled water and its NADP quantity determined. This weight was subtracted from the total dry weight. The remaining weight was assumed to be all PL-HBr. This last method yielded 1.67 moles of NADP per mole of PL-HBr.

It is possible that these variations were a function of small differences in technique for each preparation. The likelihood exists that this polyamino acid will not be used as the ultimate macromolecule for binding because of its potential antigenic properties. Therefore, further studies to determine the NADPH/macromolecule ratio were postponed.

OUTLINE

A. Dialysis Purified

1. known quantities

a) 25 mg removed of NADP (ext. coeff.)
53 mg remaining of NADP

$$\frac{53 \text{ mg}}{743 \text{ mg/mmole}} = 7.13 \times 10^{-5} \text{ moles NADP}$$

b) 42.2 mg PL-HBr starting

$$\frac{42.2}{2300 \text{ mg/mmole}} = 1.84 \times 10^{-5} \text{ moles PL-HBr}$$

c) $$\frac{7.13}{1.84} = 3.9 \frac{\text{moles NADP}}{\text{mole PL-HBr}}$$

2. known quantites

a) 35.16 mg NADP removed
67.21 mg NADP remaining

$$\frac{67.21}{743} = 9.05 \times 10^{-5} \text{ moles NADP}$$

b) 80 mg PL-HBr starting

$$\frac{80}{2300} = 3.48 \times 10^{-5} \text{ moles PL-HBr}$$

c) $$\frac{9.05}{3.48} = 2.6 \frac{\text{moles NADP}}{\text{moles PL-HBr}}$$

B. Column Purified

1. protein and ext. coeff.

a) 0.375 mg protein (PL-HBr) (from biuret)

$$\frac{0.375 \text{ mg}}{2300 \text{ mg/mmole}} = 1.63 \times 10^{-7} \text{ moles PL-HBr}$$

b) 0.625 mg NADP (ext. coeff.)

$$\frac{0.625}{743} = 8.41 \times 10^{-7} \text{ moles NADP}$$

c) $$\frac{8.41}{1.63} = 5.2 \frac{\text{moles NADP}}{\text{moles PL-HBr}}$$

2. dry weight and ext. coeff.

a) conc. $= \frac{\text{abs.}}{(\epsilon)(L)} = \frac{0.612}{(2.6 \times 10^4)} = 23.5 \frac{\mu \text{ mole}}{\text{liter}}$

$$23.5 \ \frac{\mu mole}{liter} \times 10^{-3} \text{ liter} = 23.5 \times 10^{-9} \text{ moles NADP}$$

b) $23.5 \text{ n moles} \times 743 \ \frac{ngm}{nmole} = 17.46 \times 10^{-6} \text{ gm NADP}$

$$50.0 - 17.46 = 32.54 \times 10^{-6} \text{ gm PL-HBr remaining}$$

c) $\frac{32.54 \ \mu gm}{2300 \ \mu gm/\mu mole} = 14.15 \text{ nmoles PL-HBr}$

d) $\frac{23.5}{14.15} = 1.67 \ \frac{\text{moles NADP}}{\text{mole PL-HBr}}$

CHAPTER III

OVERALL DESIGN

The design that I propose consists of 3 units: the blood separator, the detoxification device, including a semi-permeable membrane, and the mixer. In this chapter, I would like to first briefly describe how the overall system works. I will then give more detail for each unit.

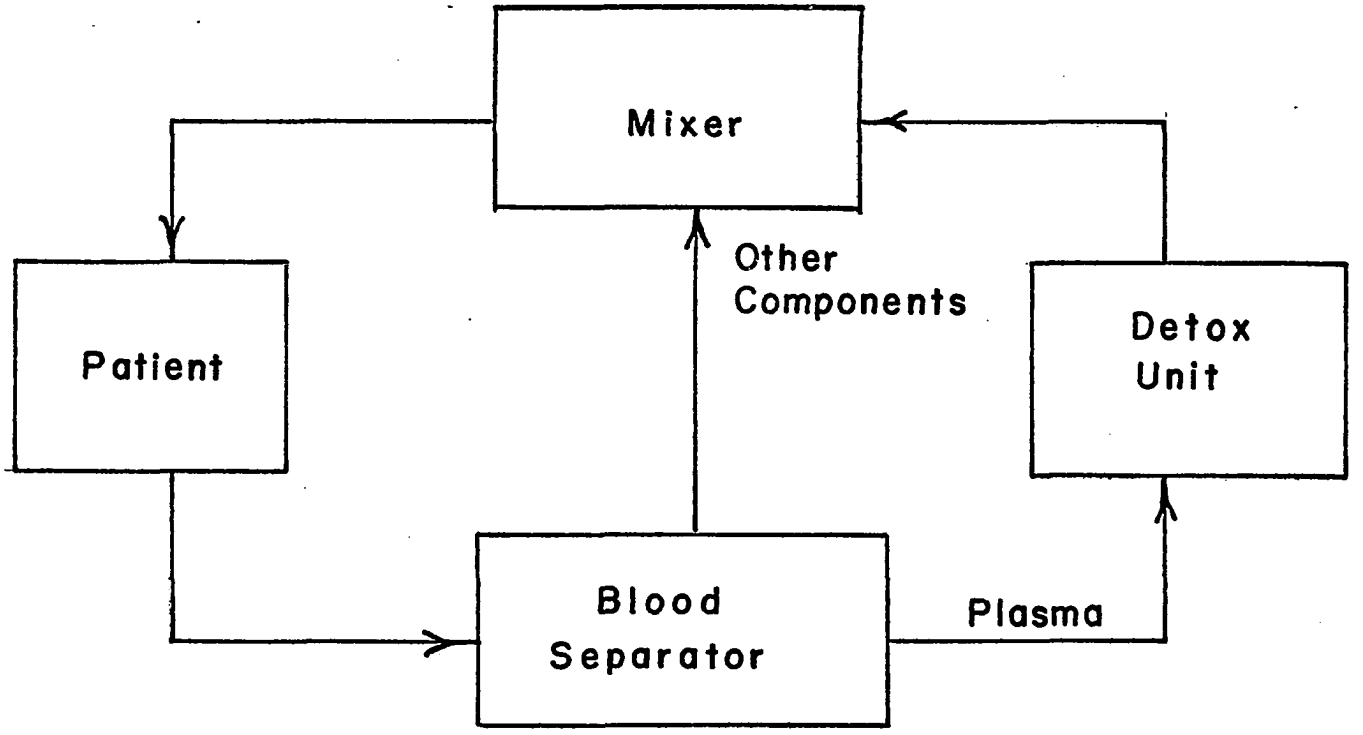
In Figure 7, we see the proposed overall design. Blood containing toxins is continuously taken from the patient and fed to a blood separator. This device essentially splits the whole blood into two fractions: plasma, about 50% by volume, and the remaining components, about 50% by volume. The plasma with the toxins is continuously shunted to a detoxification unit. Here the toxins are inactivated and, together with the plasma, sent to the mixer, then back to the patient.

This design can also incorporate additional units, for instance, a charcoal perfusion system for adsorbing endotoxins, such as lipopolysaccharides (LPS)(97). This unit might be added after the detoxification unit and would remove LPS and some of the more water-soluble metabolites formed in the detoxification unit. This is cited only as an example.

FIGURE 7

OVERALL DESIGN

Blood containing toxins is continuously taken from the patient and fed to a blood centrifuge. This device essentially splits the blood into plasma and RBC's. The plasma with toxin is continuously shunted to the detoxification unit. Here the toxins are inactivated and together with the plasma, are sent to the mixer, then back to the patient.


Mixer
Other
Components
Patient
Detox
Unit
Blood
Separator
Plasma

In fact, if the patient were suffering endotoxin shock, the removal of chemical toxins would probably have little effect on survival.

Continuous flow rates for this design will vary, depending on a number of parameters related to its application. All of the available area of the semi-permeable membrane will not be used for compound transfer if the flow is too slow. Very high flow rates will cause shearing and/or stressing of the blood and may cause coagulation problems (98). The flow rate limits will be in the area of 60-200 milliliters/minute.

In order to avoid unnecessary stresses to the body, the environment within the assist device should approximate that of the body. The pH should be 7.2-7.4. The system should be primed with physiological saline and the temperature kept between 34 and 38°C. For such a system in use, it would be advisable to have frequent hematocrit and blood chemistry checks.

Blood Separator

The importance of the removal of blood formed elements in an hepatic assist device has been demonstrated with the use of micropore filters. Further, the advantages of working with blood plasma rather than whole blood has been outlined very well by Yamazaki, et al. (79,80). Briefly, there are four distinct advantages to working with plasma:

1) the toxins are going to be in the plasma fraction,

2) use of only the plasma lessens deposition on the membrane surface of other blood components,
3) the working fluid volume is reduced, on the average, by a minimum of 40%, and
4) allowances do not have to be made for shear on blood elements such as red and white blood cells.

If the system is to be continuous, as in dialysis, it must contain a continuous blood separator. This may be a blood filter with a semipermeable membrane or a centrifuge. In the past, continuous centrifugation has always required that the components flow past rotating seals while being pumped in or out. Even the best of these rotating seals tend to lyse the red blood cells (RBC's). Just recently, however, a continuous flow centrifuge has been designed which contains uninterrupted tubing with an anti-twisting mechanism, instead of rotating seals (99). When applied to blood separation, continuous seal-less centrifuges have demonstrated constant plasma flow rates with negligible platelet injury and little or no evidence of hemolysis for 12-hour periods (99).

Some considerations for choosing between continuous seal-less centrifuges and blood filters are:

1) The centrifuge gives 80 ml/min. of plasma while the blood filter gives only 40 ml/min. (99,100).
2) The filter undergoes filtrate flux reduction due to platelet deposition on the membrane (101).

3) The latest spiral designs in seal-less centrifuges, allow other selected blood fractions to be available for alteration. It is projected that not only will the white blood cells (WBC's) be available, but selected leucocytes, such as granulocytes, may be chosen.
4) Seal-less blood centrifuges are expensive and not readily available.
5) Blood filters are simple, inexpensive and readily available.

The blood separator is a good example of how the design of an hepatic assist device can change with application. If higher plasma flow rates or separation of other blood fractions are not necessary for detoxification, then a blood filter might be advisable over a blood centrifuge.

Mixer

The need for a mixer has not been established, and further study is suggested in Chapter 5. The mixer is responsible for blending all separated parts of the blood into a homogeneous mixture. This is a relatively simple process, but there must be a minimization of shear on the cellular components. A typical mode of mixing is to discharge the components into a holding tank of small volume, approximately 100 ml, and stir with paddles. Baffles may be placed in the tank to increase turbulence, which assists in mixing. With paddles, or any moving object within the

tank, there will be shear. The amount of shear will depend on the configuration of the paddles and also on the speed at which they are turned.

Another, less complicated, mixing tank is shown in Figure 8. As shown, the plasma, RBC's, and remaining fractions are discharged into the top of the tank but below the liquid level. The plasma is discharged vertically while the RBC's are discharged horizontally. Note that the horizontal nozzle is flared and mid-way between the center and the side of the tank. The momentum of the fractions as they are pumped into the tank is used to create mixing. The flared nozzle also creates a back-mix. The mixture is swirled as gravity, a small force, actually, settles the mixture. At or near the bottom of the tank, the homogeneous mixture is removed to the patient. The second configuration contains fewer shear-producing parts and converts the energy from pumping into mixing energy for the components.

Detoxification Unit

The most complicated unit of the entire design is the detoxification system. As can be seen in Figure 9, it consists of three chambers.

"Walk-through" of the unit. How this unit works might best be explained by following the reaction of a model compound, N,N-dimethylaniline (DMA), through the system at steady state conditions.

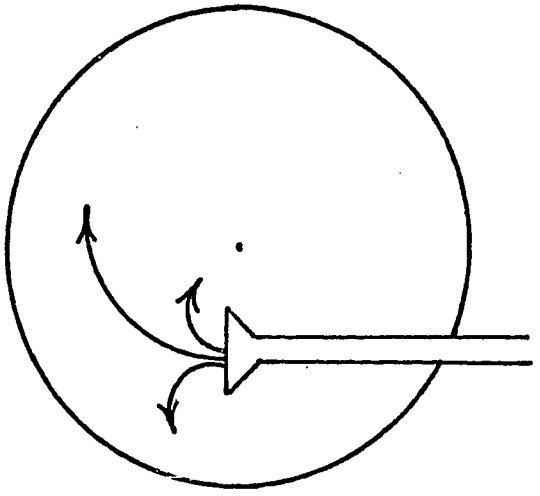
The DMA toxin enters chamber 1 with the blood plasma. Here it encounters a concentration gradient of DMA between

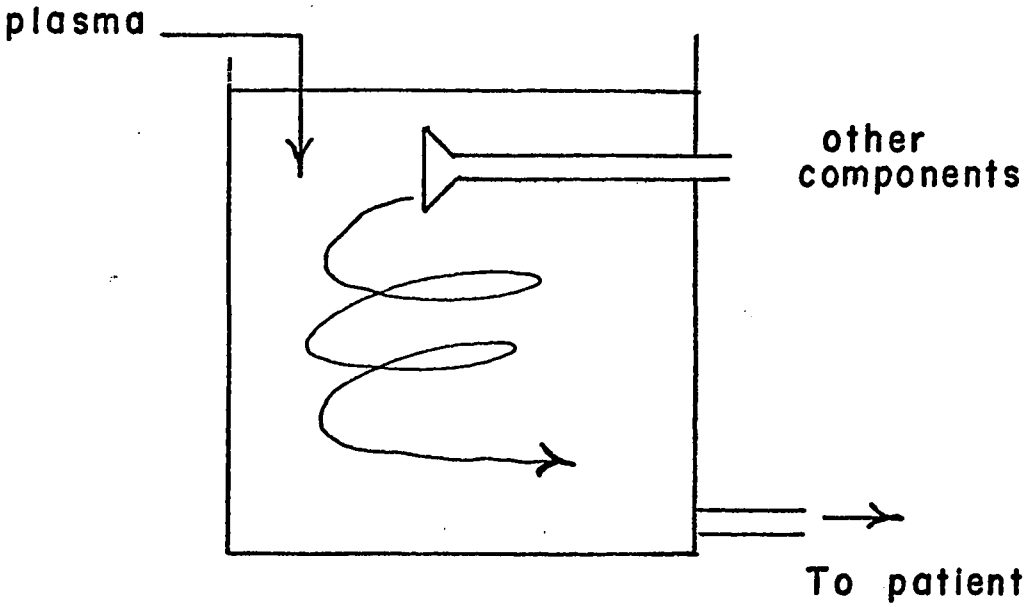
FIGURE 8

THE MIXER

As shown, the plasma, RBC's, and remaining fractions are discharged into the top of the tank but below the liquid level. The plasma is discharged vertically while the RBC's are discharged horizontally. Note that the horizontal nozzle is flared and mid-way between the center and the side of the tank. The momentum of the fractions as they are pumped into the tank is used to create mixing. The flared nozzle also creates a back-mix. The mixture is swirled as gravity, a small force, actually, settles the mixture. At or near the bottom of the tank, the homogeous mixture is removed to the patient.

TOP



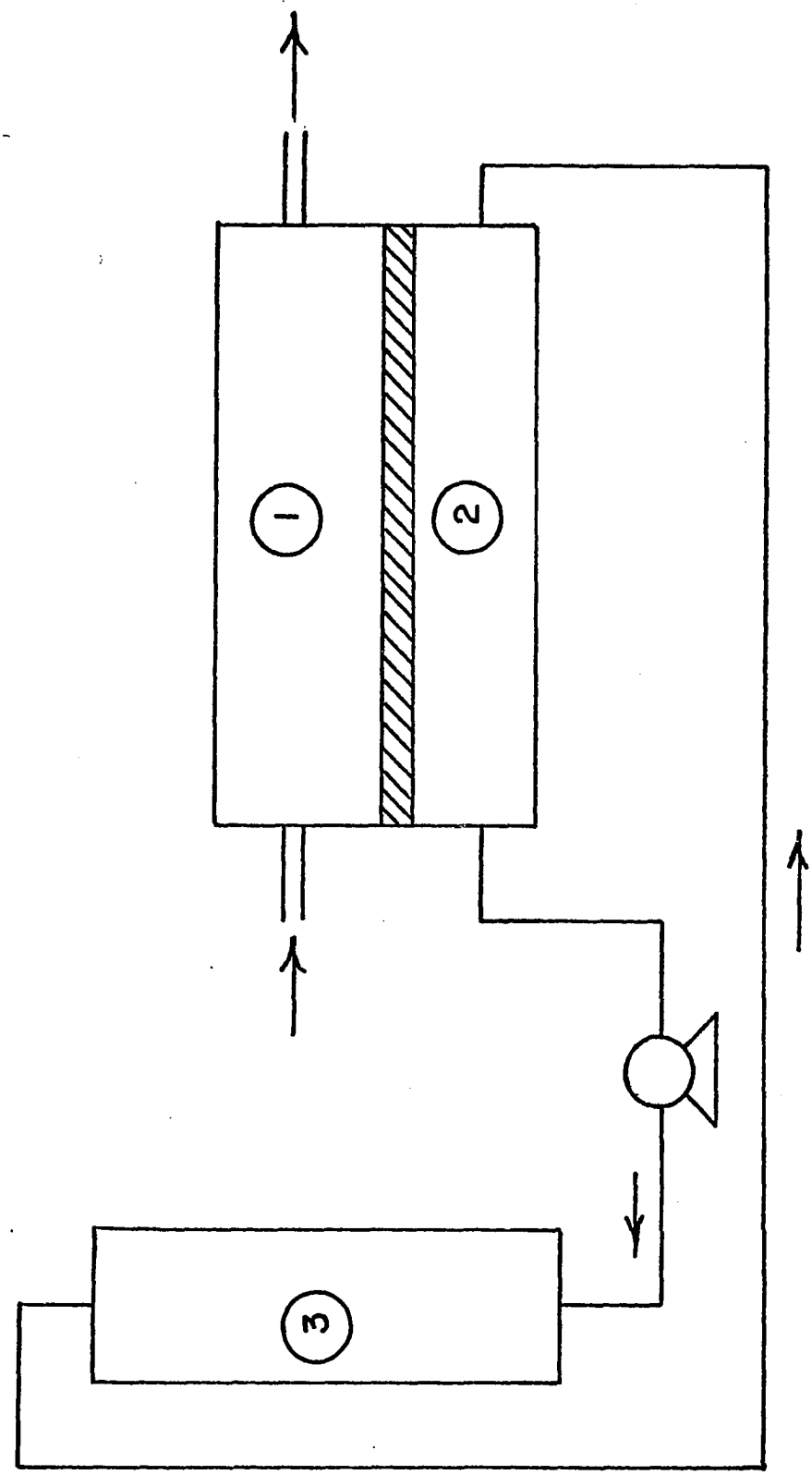


SIDE

FIGURE 9

THE DETOXIFICATION UNIT

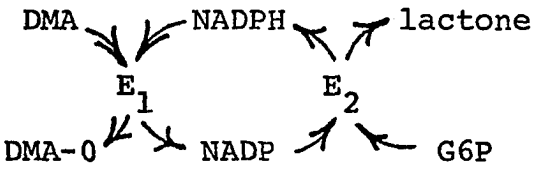
Toxin enters with the plasma into chamber 1. Here it contacts a concentration gradient of toxin between chambers 1 and 2 across the membrane. In chamber 2, the toxin is pumped to the fluidized bed reactor, chamber 3. Here, the toxin is inactivated by the catalytic action of the enzymes. The inactivated compound is brought back to chamber 2 where a gradient of inactivated compound exists opposite to the original toxin. The inactivated toxin is taken away by the plasma once it enters chamber 1.


1
2
3

chambers 1 and 2 . High DMA concentration in chamber 1 forces the DMA across the semi-permeable membrane into chamber 2 , which contains low concentrations of DMA. From chamber 2 , the DMA in plasma is pumped to chamber 3, which is a fluidized bed reactor. In the reactor, there is the potential for many different reactions. However, only one reaction will be discussed. An explanation of why there is such a potential for other reactions will be discussed later in the paper. The reaction is:

$$\text{NADPH} + \text{DMA} + \text{O}_2 + \text{H}^+ \xrightarrow{\text{enzyme}} \text{NADP}^+ + \text{DMA-oxide} + \text{H}_2\text{O} \qquad [1]$$

The specific enzyme to catalyze this reaction is mixed function microsomal flavoprotein oxidase, MFMFO, (E.C.1.14.13.8). The cofactor involved, nicotinamide adenine dinucleotide phosphate (NADP), is very expensive and has therefore been regenerated by an additional enzyme system as shown in equation [2].



[2]

where: E_1 = MFMFO ; E_2 = G6PD

The DMA has now been changed to DMA-oxide in the reactor and is returned to chamber 2 . A concentration gradient of DMA-0 is set up in the opposite direction of that of DMA. The DMA-0 permeates across the semi-permeable membrane, to the plasma in chamber 1. From there it exits the unit to the mixer.

Chamber 1 . Chamber 1 must be of a biocompatible material because it contacts the plasma. There is a variety of materials to choose from: silicone rubber, Teflon, polyacrylamides, and so on (102, 103). The most commercially available is silicon rubber, which goes by the trade name Silastic ^{R} . A fairly new material, Hydron ^{R} , is rapidly becoming popular. It is part of a class of materials known as hydrogels. These have shown exceptional biocompatibility, particularly when applied to large surface areas (104). The actual chamber would probably best be made of glass or aluminum coated with the biomaterial on the plasma-contacting surfaces.

Semipermeable membrane. The membrane separating chambers 1 and 2 will vary in its specifications as the application changes. The function of the membrane is to allow the toxins to permeate but retard passage of large molecules, such as the blood proteins, which include fibrinogen and albumin, into the reaction chamber. It has an additional function, retaining the cofactor, which is NADPH in this case, on the reaction side of the unit. Retention of the cofactor tends to set an upper limit on the molecular weight cut-off of the

semi-permeable membrane. This limit will be discussed further in the cofactor section of the paper. Part of the aim of the detoxification unit is to inactivate "middle molecular weight" toxins. These molecules are often not dealt with in dialysis (9,10). Two membranes that have worked fairly well in our system are Cuprophan (RP-514) and cellulose acetate (AN-69). They are also known for acceptable biocompatibility (105). Care must be taken, however, in using these membranes in different environments. The flux rate across the membrane for a compound in distilled water can differ greatly from the same compound in saline. This difference is, in part, due to different degrees of swelling of the membrane. This indicates another design parameter. Consideration of the concentration of the dissolved solutes and the flux of the subject toxic compound should influence the selection of the membrane used in the unit.

Chamber 2 , and the fluidized bed reactor. Chamber 2 acts as a small reservoir for the fluidized bed reactor. The purpose of the reactor is to provide a catalytic bed for the reactants involved. The catalytic action is provided by specially prepared hepatic enzymes. While not the only way to detoxify a compound, these enzymes are the most specific (106,107). Because of high specificity, side reactions are minimized and the potential for creating a new compound that is even more toxic than the original toxin is greatly reduced. The enzymes may be used in one of two forms: 1)

free in solution or 2) immobilized on an inert matrix. Which form is used again depends on the application of the whole unit. In dealing with the MFMFO enzyme, Sofer (106-108) found that half-life and thermal stability were increased by over a factor of 100 when this enzyme was immobilized on inert glass beads. At the same time, however, mass transfer limitations to the surface reduced the reaction rate by 10 fold. This same trend was found true by Wills (109) when immobilizing liver microsomes. However, there is a disadvantage to using free microsomes in solution when proteolytic enzymes are available. These proteolytic enzymes destroy the detoxifying enzymes. Caldwell of this lab (110) has recently compared the reaction rates of MFMFO and microsomal enzymes, both immobilized and free (Table 1-5). The procedure we used to acquire all rates is given in Chapter IV. The immobilization was performed by Schiff base likage of the amine-coated glass with glutaraldehyde, followed by Schiff base linkage of the bound glutaraldehyde with free enzymes or microsomes (109). The upper activity limit is 284 nanomoles (n moles) of DMA metabolized per minute per milligram of enzyme protein, using a semi-pure MFMFO free in solution. The half-life for the enzyme in this reaction scheme is approximately 10 minutes. The lower activity end is 0.07 n moles of DMA/minute/mg of glass beads, using microsomes immobilized as described above, but with a half-life of approximately 5+ days. The MFMFO is not available commercially and is very expensive. It becomes even more

TABLE 1

REACTION RATES FOR DIFFERENT ENZYME FORMS

(See Reference 110)

Enzyme Form	Maximum Rate n moles/min/mg*
Oxidase in Solution	284.2
Hog microsomes in solution	6.9
Dog microsomes in solution	4.2
Dog microsomes immobilized on glass beads	0.07+

*mg of protein

+This reaction rate is on a per milligram of dry bead basis. Determining the mg of protein per mg of dry beads is very difficult with microsomes because of the low amount of protein immobilized on the glass beads. It is estimated at 0.02 mg protein/mg beads (109).

TABLE 2

OXIDASE ACTIVITY*

λTPN	Rate $\frac{\text{nmoles}}{\text{sec ml}}$	Rate $\frac{\text{nmoles}}{\text{min mg}}$	Avg. Rate For Conc.
20	.8	208.70	208.70
20	.8	208.70	
40	.8667	226.70	
40	.8	208.70	223.39
40	.9	234.78	
60	1.145	298.81	284.19
60	1.033	269.56	
80	1.12	292.17	276.52
80	1.00	260.87	

*The activity is based on 9.2 mg protein per ml of liquid. The experimental method is given in Chapter IV.

TABLE 3

PIG MICROSOME ACTIVITY*

λTPN	Rate $\frac{\text{nmoles}}{\text{sec ml}}$	Rate $\frac{\text{nmoles}}{\text{min mg}}$	Avg. Rate For Conc.
20	.3135	5.860	
20	.3251	6.077	5.968
20	.3135	5.862	
20	.3251	6.077	
40	.3200	5.982	
40	.2743	5.127	5.984
40	.3657	6.836	
60	.3840	7.118	
60	.3442	6.434	6.894
60	.4006	7.488	
60	.3446	6.477	
80	.3572	6.677	
80	.3505	5.982	6.404
80	.3251	6.552	
100	.3251	6.077	
100	.3429	6.409	6.265
100	.3376	6.310	

*The activity is based on 51.36 mg of protein per ml liquid. The experimental method is given in Chapter IV.

TABLE 4

DOG MICROSOME ACTIVITY*

λTPN	Rate $\frac{\text{nmoles}}{\text{sec ml}}$	Rate $\frac{\text{nmoles}}{\text{min mg}}$	Avg. Rate For Conc.
20	.209	3.028	
20	.293	4.239	2.908
20	.193	2.789	
40	.223	3.238	3.208
40	.219	3.179	
60	.244	3.532	
60	.266	3.853	3.786
60	.274	3.974	
80	.244	3.532	
80	.299	4.335	3.932
80	.268	3.885	
80	.274	3.974	
100	.299	4.335	
100	.293	4.239	4.183
100	.274	3.974	

*The activity is based on 66.27 mg of protein per ml of liquid. The experimental method is given in Chapter IV.

TABLE 5

DOG MICROSOMES IMMOBILIZED ON BEADS*

λTPN	Rate $\frac{\text{nmoles}}{\text{sec ml}}$	Wt Beads (mg)	Rate $\frac{\text{nmoles}}{\text{min mg}}$
40	.0375	54.90	.06557
60	.0180	53.74	.03219
80	.0250	52.42	.04578
80	.0240	33.80	.06816
100	.0178	33.21	.05139
120	.0250	37.02	.06483

*The activity is based on per mg of glass beads basis. The amount of protein per mg of glass bead is difficult to calculate and is estimated at 20 mg/gm (109). Experimental procedure for the above rates is given in Chapter IV.

expensive if immobilized. The liver microsomes are fairly inexpensive and relatively easy to prepare (109). The microsomes also allow for a flexibility in reaction control. That is, the particular reaction necessary may be dictated by merely specifying which cofactor will be available, in solution, for reaction. This reduces the need to have a specific homogeneous enzyme available at any given moment or application. The reaction rate is a critical design parameter when considered in conjunction with the semi-permeable membrane. Even though the reaction rate may be 160 n moles/min, the flux across the membrane (for a set area) may be only 100 n moles/min. Obviously, there is variability in design to correct such a problem. This again reinforces the philosophy that there is no one set design for an hepatic assist device. The design will be dictated, for the most part, by its use.

Cofactors. The cofactor problem has already been discussed in some detail in Chapter II. The reader is referred to that discussion.

Substrates (Toxins). Whether toxins can be detoxified with this system will depend on:

1) whether they are small enough to permeate the membrane;
2) their solubility in the plasma;
3) if there is a proper cofactor available to allow the reaction to take place.

(Note: If liver microsomes are used as the enzyme source,

there is a fairly good chance that the proper enzyme will be available to toxify the particular toxin.) To date, four model toxins have been tested. Para-nitrophenol (p-Nϕ) and 7-hydroxychlorpromazine (7-OHCPZ) have both been tested using microsomes, both free and immobilized, with the cofactor UDPGA (109). The specific catalyzing enzyme was UDP-glucuronyl-transferase. DMA and ethylmorphine have been tested, using free and immobilized oxidase (111), and free and immobilized microsomes (110). Again, these are only model toxins used to acquire data about the system and are not to be construed as the only toxins capable of inactivation. A word should also be made here about the term "inactivation". In some cases a toxin, particularly a drug toxin, may be inactivated by covering the molecule with other chemical groups. These groups, such as glucuronic acid from UDPGA, also make the molecule more water-soluble, thus making it easier to be eliminated from the body with the urine. There are some compounds, however, that are not inactivated when run through this metabolic scheme. For instance, β-naphthylamine is an active carcinogen when N-hydroxylated with MFMFO and NADPH (112). This example is the exception rather than the rule and is presented only to emphasize the need for careful selection of the particular detoxifying system.

Critical Design Parameters

The application of the hepatic assist device will in part dictate its design. The limiting factors and how they

interact will be discussed next. Some of these parameters have already been mentioned. Perhaps, it would be best to list them all and discuss each.

Flow Rate. In dealing with flow rate in the design, the limiting factors will be the blood separators.

The greater the flow the more membrane surface area is used and thus the greater the flux. Of the two available sources for blood separation, the seal-less centrifuge will provide the highest flow rate. Again, this unit is less available and more complicated than the blood filter, but can provide approximately twice the flow rate, 80 ml/min (100).

Membrane. The semi-permeable membrane plays one of the most important roles in the assist device. It must retain the cofactors on the reactor side and the blood proteins and macromolecules on the body side. At the same time it should provide maximum permeability for the toxin and its counter-part the inactivated form. The molecular weight cut-off, and hence the permeability, will be determined for two reasons. One is to maximize the reaction conditions in the reactor. The second reason is to keep those cofactors that can be harmful out of the body. This becomes a very difficult situation when the toxin and the cofactor are approximately the same size. One answer, as mentioned previously, is to make the cofactor larger. This has been done with NADP while still maintaining reactions rates above 95% (113).

As with chamber 1 from Figure 9, the membrane will also have to be biocompatible, with blood plasma.

Reaction Rate. Two things will dictate which enzyme form is to be used: 1) how long the device will be in use, and 2) which detoxification reaction is to be duplicated. Referring to Table 1, if for example, one were to detoxify 18 mg of DMA in 20 minutes, it would be necessary to use 10 mg of pure oxidase with a reaction rate of 284 n moles of DMA/min/mg of enzyme. This, however, would be very expensive, require very permeable membranes, and high blood plasma flow rates. On the other hand, if the situation is less critical, inexpensive immobilized dog microsomes with a reaction rate of 0.07 n moles/min/mg of glass bead could be used. If 10 grams of beads were used it would take about 13 hours to detoxify the same 18 mg of DMA.

Interrelationship of Parameters. One fundamental relationship for calculating the membrane area is:

$$A = \frac{F}{P(C_B - C_R)} \qquad [3]$$

where: A = Area (cm^2)

F = Flux of toxin (mg/min)

P = Permeability of toxin (cm/min) (Assuming perfect mixing on both sides of the membrane and only a concentration gradient)

C_B = Body toxin concentration (mg/cm^3)

C_R = Toxin concentration in the reactor

Using this relationship, a balance can be set up around the detoxification unit. Referring to Figure 10, the change with time in the mg quantity of ethylmorphine, (EM, model toxin) in the reactor is:

$$\frac{d(EM_R)}{dt} = k_1\left(\frac{EM_B}{V_B} - \frac{EM_R}{V_R}\right) - k_3\, EM_R \qquad [4]$$

The change with time of the mg quantity of EM in the blood is:

$$\frac{d(EM_B)}{dt} = -k_1\left(\frac{EM_B}{V_B} - \frac{EM_R}{V_R}\right) \qquad [5]$$

where: $k_1 = P\ A$ [6]

$k_3 = (Act)\ (Wt_{beads})$ [7]

$V_R = 5\ (Wt_{beads})$ [8]

The total change in EM with time is:

$$\frac{d(EM)}{dt} = -k_3(EM_R) \qquad [9]$$

where: V_R = volume of reactor (mls)

V_B = volume of blood detoxified (mls)

P = permeability (as before)

A = area (as before)

Wt_{beads} = quantity of beads used (gms)

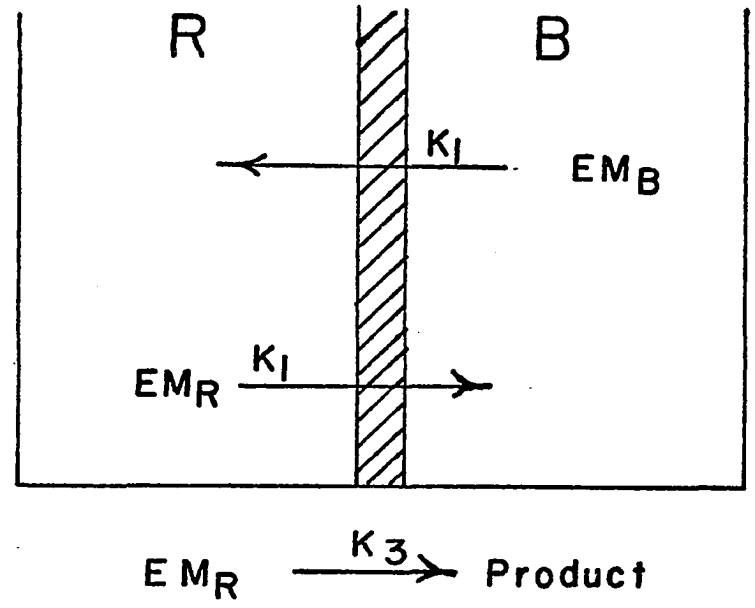
Act = activity for dog microsomes immobilized on glass beads ($min^{-1}\ gm^{-1}$)

FIGURE 10

PARTIAL KINETIC MODEL OF

DETOXIFICATION UNIT

A model toxin, ethylmorphine (EM) is shown permeating from the body (B) into the reactor (R) at a rate k_1. Once in the reactor, the EM can either react to product at rate k_3 or permeate back to the body at rate k_1.


R
B
K_1
EM_B
EM_R
K_1
EM_R
K_3
Product

EM = total quantity of ethylmorphine (mg)

EM_B = quantity of ethylmorphine in the body only (mg)

EM_R = quantity of ethylmorphine in the reactor only (mg)

$$V_R = \frac{5 \text{ mls liquid}}{1 \text{ gm beads}} \times \# \text{ gm beads (rule of thumb)}$$

With such a scheme, the values of EM_R, EM_B, and EM can be found by solving equations 4-8 simultaneously. Of course, there are certain major assumptions with this such as:

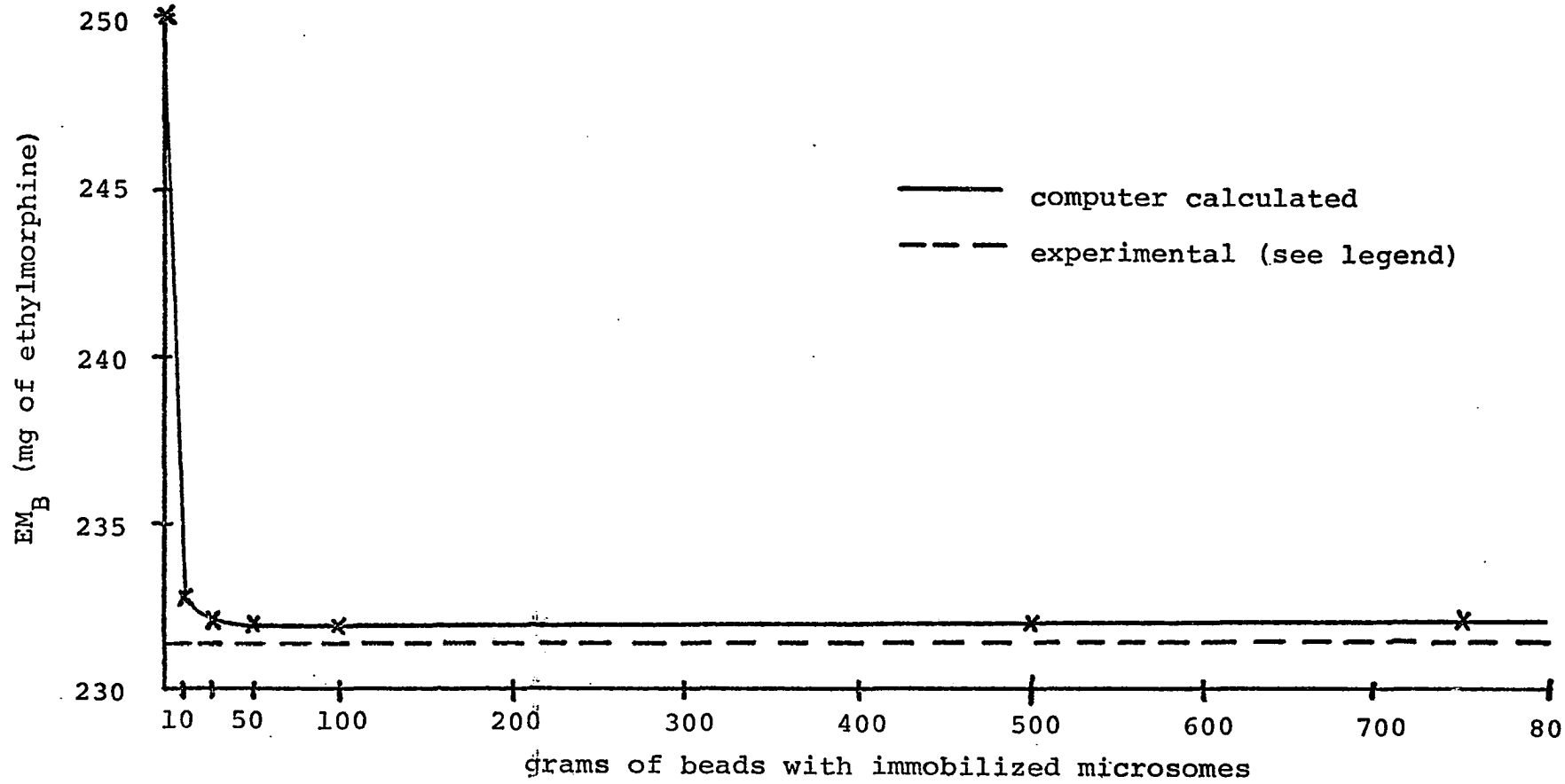
1) a two compartment model only
2) perfect mixing on both sides of the membrane
3) an average reaction rate (in actuality for long periods this would be adjusted to an exponential decay)
4) that there is no liver function to assist or inhibit the detoxification rate.

However, when programmed correctly on a computer, they can be modeled very nicely by varying one parameter while the others are fixed. Because of the complex nature of the design, it is not feasible to present all the possible configurations and their mathematical models here. An example is given in Figure 11 illustrating how the toxin level varies when different amounts of immobilized microsomes are used. Reaction time is 600 minutes. It is clear that a tool exists for the design and evaluation of the hepatic assist device.

FIGURE 11

ETHYLMORPHINE CONCENTRATION VS.
GRAMS OF IMMOBILIZED MICROSOMES

The difference in ethylmorphine concentration is shown using different quantities of immobilized microsomes. This plot was generated by the computer model on page 51. The reaction time is 600 minutes. For 100 grams of beads, the calculated quantity of EM passed to the reactor is 17.97 mg. The experimental value, assuming continuous maximum concentration difference (0 concentration in the reactor and 250 mg of EM in the body) is 18.65 mg. This value should be achieved at the asymptote as shown.


250
245
240
235
230
EM_B (mg of ethylmorphine)
computer calculated
experimental (see legend)
10
50
100
200
300
400
500
600
700
800
grams of beads with immobilized microsomes

CHAPTER IV

METHODS

Preparation of Liver

The procedure for preparation of the liver used as an enzyme source is taken directly from another work by the author (109).

The homogeneous MFMF oxidase enzyme and the hog liver microsomes were the gift of Dr. D.M. Ziegler of the Clayton Foundation, University of Texas at Austin. They came to our laboratories already prepared for use. The exact procedure for preparation of hog liver and MFMFO is published elsewhere (107).

Dog liver was obtained through the cooperation of the Oklahoma City Animal Shelter. The shelter would inform this lab in advance when animals were to be euthanized. This allowed proper holding solutions to be prepared and allowed staff to be on hand to perform surgery immediately after the animals had been euthanized. Animals were placed in a high altitude chamber (the equivalent of 50,000 ft) for 15-20 min. After animals had been pronounced dead by shelter technicians, the livers were removed, sliced into thin sections, and dropped into a cold solution of 0.15 M sucrose/.001 M EDTA. The liver,

in solution, was then transported to our lab for further preparation.

The liver was drained of buffer and put through a food and meat chopper (Universal Chopper Division, Union Manufacturing Co., Model #1551) at its finest setting, twice. During this grinding procedure, chopper and liver remain cold (approximately 10°C). The ground liver is then homogenized with a tissue grinder (pyrex, Corning #7725), utilizing an electric drive (A.H. Thomas Co.). During this homogenizing process, the grinding tubes are immersed in an ice bath to maintain the liver sample below 10°C. The homogenized liver sample is then spun in a Beckman model L2-65B ultracentrifuge at 20,000 g for 20 minutes. The pellet, which contained connective tissue, cell wall, nuclei, lysosomes, and so on, was discarded. The supernatant was respun at 100,000 g for 1 hour. This time the supernatant, which contained the cytoplasma, was discarded. The pellets were brought up in approximately 50 ml total of cold, 0.01 M potassium phosphate buffer (Kphos) pH 7.5. This fraction contained the microsomes and ribosomes. The mixture was then pulse sonicated (0.20 sec on, 0.80 sec off) for a total of 300 sec at 200 watts by a Sonic cell disrupter (model W-350 from Heat Systems-Ultrasonics, Inc.). This procedure was carried out in a stirred ice bath. The combination of the ice bath and pulse sonication kept the mixture temperature from going over 10°C. The sonicated microsome solution was then dialyzed in 15-20 ml

quantities using #20 Dialyzer Tubing (VWR Scientific) in 0.01 M Kphos buffer pH 7.5. The dialysis was performed 3 times for 45 minutes each, using fresh buffer each time. The microsomes were then respun at 70,000 g for 1 hour. The pellets were brought up in a total of approximately 15 ml of cold 0.01 M Kphos buffer pH 7.5, giving around 20-30 mg/ml protein, and stored in the refrigerator. The supernatant was concentrated to 60-70 mg/ml protein in an Amicon Ultrafiltration cell, model 52, using a PM10 membrane, all of which were submerged in an ice bath. The supernatant was also stored at approximately 4°C.

Preparation of Immobilized Microsomes

The liver microsomes were used in two forms, bound and unbound to glass beads. The unbound microsomes were stored on a long-term basis in quantities of 3-5 ml in the freezer. Frozen microsomes were slowly thawed and rehomogenized in an ice bath before use.

The matrix used for insolubilizing the microsomes was glass beads with an alkylamine coating (Pierce Chemical Co. Biomaterial Support No. 23650). The technique is quite simple (114). One gram of beads is placed in 10 mls of a 2.5% glutaraldehyde solution. This mixture is then placed under vacuum, at room temperature, for 30 minutes. The mixture is removed from the vacuum and allowed to sit an additional 30 minutes at room temperature and pressure. The glutaraldehyde has now undergone Schiff base linkage with the amine groups on the

surface of the beads. The beads are washed several times with glass distilled water until the odor of the glutaraldehyde is no longer detectable. Two mls of the microsomes are added to the beads, a quantity sufficient to cover the surface. This mixture is placed in an ice bath and subjected to a vacuum again for 30 minutes. The mixture is removed from the vacuum and refrigerated at approximately 10°C at room pressure for 4 to 6 hours. The excess microsomes are decanted, and the beads are washed 5 or 6 times, with 50 mls each time of 0.05 M Kphos buffer, pH 7.4. The beads are then stored at 5-10°C under 50 mls of the same buffer.

PL-SNP Preparation

(As taken from a paper by Wills, et al. (113)).

Preparation of the PL-SNP was according to a modification of the technique of Yamazaki, et al. (96) for NAD.

Two grams of succinic anhydride were dissolved in 10 ml of dimethylsulfoxide, followed by the addition of 50 mg of NADP. The reaction vessel was covered with Parafilm, magnetically stirred, and placed in a 30° water bath for 65 hours. As performed by Wykes, et al. (86,93), for NAD, the succinylated NADP (SN) was then precipitated with acetone. The precipitate, after being washed throughly with acetone, was set aside to dry. The dried SN was brought up in approximately 10 ml of H_2O. Concurrently, 80 mg of poly-L-lysine hydrobromide was brought up in approximately 3 ml of H_2O, then added to the SN. The pH was adjusted to 4.8, followed by

addition of 300 mg of ethyldimethylaminopropylcarbodiimide hydrochloride (EDC). The pH was then readjusted to 4.8. The vessel was covered with parafilm and again magnetically stirred in a water bath, only this time at 40°C. After 8 hours, another 300 mg of EDC was added and the pH adjusted to 5.3. The reaction was returned to the bath and allowed to run an additional 12 hours. The poly-L-lysine-succinyl-NADP (PL-SNP) reaction solution was purified by column chromatography using an anion exchange packing. The resin used was Dowex-1, 1X2-400, chloride form, 2% cross-linked in a column 21 x 1.5 cm. The column was eluted with an exponential gradient from 0.0 to 0.1 M HCl at a flow rate of approximately 2 ml/min. The final peak, with a retention time of 1 hour and 20 minutes was identified as PL-SNP. The PL-SNP mixture was detected at 254 nm. The proper peak for PL-SNP was determined by an evaluation of polypeptide content and ability to form a detectable peak at 340 nm (reduced NADP).

The purified PL-SNP was lyophilized, brought up in a few ml of H_2O, then re-lyophilized. The yield was approximately 10 mg of a slightly yellow, feathery compound. The PL-SNP was stored at 8°C. Concentration for the assays was 1.0 mg/ml.

Reaction Rate Assays

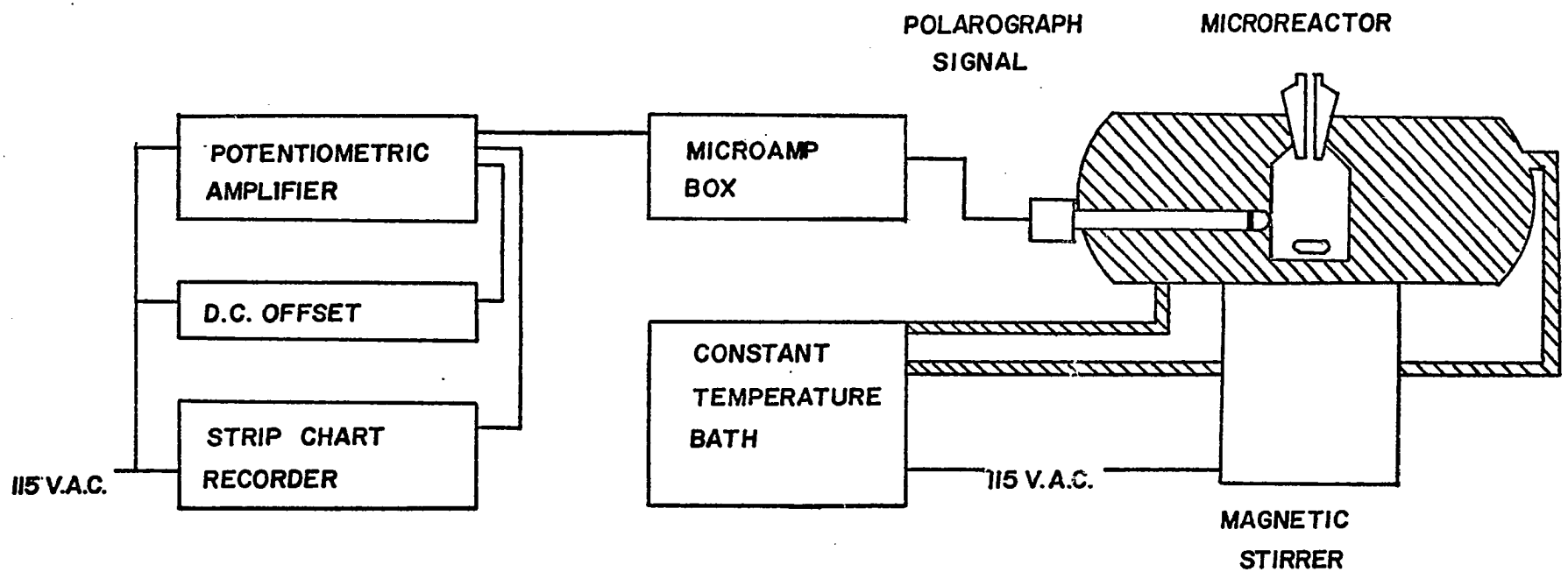
(Taken from a paper coauthored by Wills (83))

Reaction rates were determined by observing the uptake of dissolved molecular oxygen in a closed system with a typical water-jacketed polarograph equipped with a Clark-type oxygen

FIGURE 12

POLAROGRAPHIC ASSAY APPARATUS

Small scale batch reactions were studied with the polarograph. The water jacketed, closed system microreactor uses a Clark-type oxygen electrode to measure initial reaction rates in the nanomole (oxygen) region.


POLAROGRAPH
SIGNAL
MICROREACTOR
POTENTIOMETRIC
AMPLIFIER
MICROAMP
BOX
D.C. OFFSET
CONSTANT
TEMPERATURE
BATH
STRIP CHART
RECORDER
115 V.A.C.
115 V.A.C.
MAGNETIC
STIRRER

electrode (see Figure 12). A typical aqueous reaction medium consists of the following: 0.054 M K-phosphate buffer, pH 7.4, enough glucose-6-phosphate dehydrogenase to regenerate a minimum of 400 mmoles/min $NADP^+$, 0.5 mM N,N-diemthylaniline, 0.0-0.22 mM $NADP^+$, and 10 mg of catalyst (MFMF oxidase on glass beads). Total reactor volume was 1.6 ml. The solid catalyst was prepared in pre-weighed samples of 20 mg each. All reactions were carried out at 37 $\pm$ 0.01°C, and all buffered reactions at pH 7.4.

Reaction rate was determined by observing the rate of dissolved oxygen uptake in the closed system, which is itself equal to the rate of product synthesis. The rate of oxygen consumption was measured by the electrode coupled to a strip chart recorder.

Permeability Studies

Permeabilities of various compounds through test membranes were measured in specially built reactors (115). The reactors were a sandwich of 2 plexiglass pieces and a membrane. Each plexiglass piece had a recessed chamber 5 cm in diameter and 6 mm deep, with an outer sealing ring of rubber. Each chamber contained 2 access ports, 1.5 mm in diameter. During testing the reactors were placed in a shaker bath at 37°C. The shaker bath was a Dubnoff model, set to agitate at 100 cycles/min. The membranes of Cuprophan ^{R}, RP-514, cellulose acetate, and AN-69, were a gift of HOSPAL, Inc. (Salt Lake City, Utah USA).

In Table 6, permeabilities for the RP-514 and the AN-69 membranes were calculated from experimental data using equation [3]. Other permeabilities were found in the literature (115,116).

Membrane Preparation

(As taken from a paper coauthored by Wills (83))

An isotropic PVC film 6.5 x 6.5 inches is sandwiched between successive layers of aluminum foil electrodes and dielectric sheets (Figure 13). Dielectric sheets, also trimmed to 6.5 x 6.5 inches, consisted of dense, high grade paper, 8 mls thick. Two dielectrics serve to insulate the electrodes from the press faces. Another dielectric has been necessary because of arching which may occur due to minor imperfections in the polymer film and electrodes accentuated by the high compressive loads. A third, uncharged aluminum sheet is 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 potentials that can be tolerated in this design.

The sandwich is then placed between the cool faces of the press, and loads varying from 100 to 700 lb/in^2 applied. The power supply is set in a 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,

TABLE 6

Permeability x 10^4 (cm/sec)

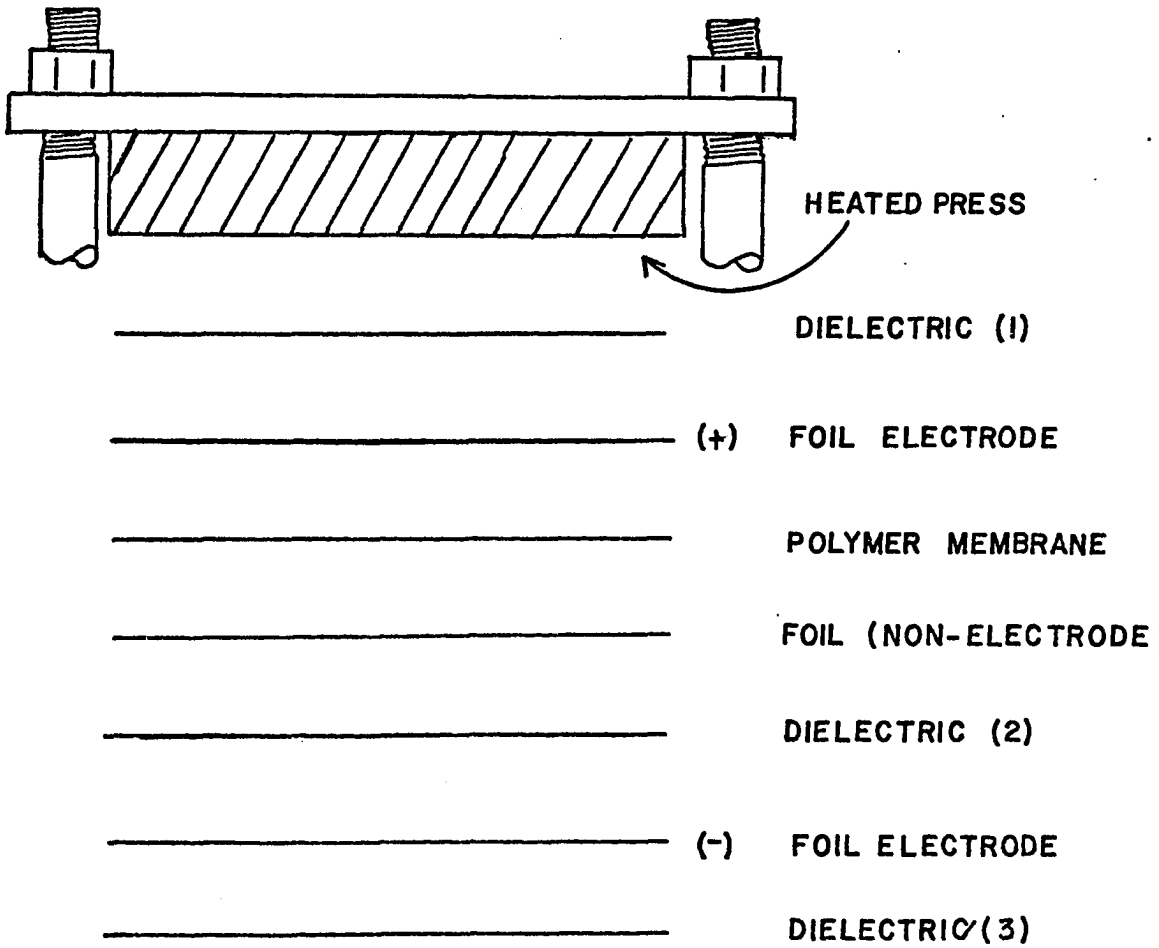
Solute	Membrane		
	PT-150	RP-514	AN-69
NADP	(.79)	.66	1.07 (2.7)
PLSNP MW 3500	(.17)	.13	1.55 (1.50)
PLSNP MW 10,000	(.08)		(.62)
EM	(1.8)	1.9	3.1 (5.0)
DMA	(3.9)		(7.9)

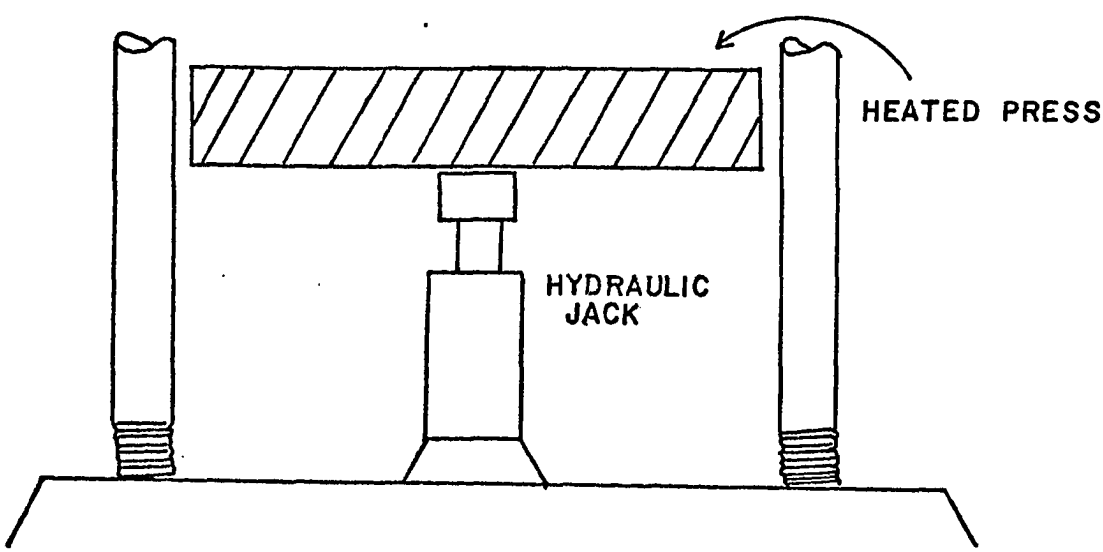
Permeabilities of solutes NADP, PLSNP, EM and DMA at 37°C are listed for cuprophans PT-150 and RP-514, and for acrylonitrile RP-AN-69. Permeabilities listed in parenthesis for the PT-150 and the AN-69 are based on membrane resistance plots found in the literature (115,116), while others for the RP-514 and AN-69 are typical values determined in experimental studies in our lab. Experimental values were found to vary with ionic concentration of the solvent as well as with the degree of agitation of the model membrane reactors.

FIGURE 13

ELECTRET SANDWICH

The polymer membrane is shown sandwiched between two electrodes. The electrodes are isolated from the heated press by dielectrics. The center foil provides a dampering effect to prevent arcing through the membrane.


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


HEATED PRESS
HYDRAULIC JACK

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 intervals on current voltage, and temperature. During initial stages of polarization, data should be taken at 15-second intervals to observe rapid changes. 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 carfully 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.

CHAPTER V

RECOMMENDATIONS

The design of an hepatic assist and a mathematical model have been presented. There remains, however, much work before the unit may be tested on an animal. Therefore, I submit the following recommendations for further research:

1) An evaluation is needed of whether the mixer is necessary. Can the two streams be adequately mixed if they are simply reinfused into the body?
2) A more rigorous study needs to be made of the vasodepressant effects of NADP/H. Will the slow infusion of NADP/H be a problem?
3) While suggesting a 50/50 split on the blood plasma in the separator, the problem of pumping an 80 hemtocrit could be very difficult. Therefore, a study should be made of pumping high hematocrit blood.
4) More parametric data is needed, particularly on a larger size unit. A scale-up from the 2-5 ml reactors and study is in order.
5) The mathematical model suggested needs to be expanded to the whole design and then utilized

to eliminate the extreme designs which would not be feasible to build due to size or cost.

6) Multi-enzyme reactions should be investigated for incorporation into the proposed hepatic assist design.

With the completion of the above studies, the testing of the device on an animal should be a reality.

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