TRANSFER OF ASCORBIC ACID IN A HEMODIALYZER
POSSIBLE PROTEIN BOUND ASCORBIC ACID

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ABSTRACT

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By

Jean Mieczkowski Burge

A patient undergoing long-term renal dialysis may lose a number of nutrients from his blood. This is due to the fact that the dialysate solution contains only a limited number of nutrients. The constituents of the blood not present in this dialyzing solution may be removed from the blood during dialysis. According to other investigators folic acid is lost during hemodialysis whereas other B vitamins appear to be unaffected. Vitamin C has also been reported lost from the blood during hemodialysis (Sullivan et al., 1970).

The purpose of the present study was to evaluate the rates of transfer of ascorbic acid from blood to dialysate fluid across a dialysis membrane, and to determine whether some ascorbic acid is present in the blood as a non-diffusible complex. For the initial work a miniature parallel flow hemodialyzer, Babb-Grimsrud modification of the Kiil dialyzer using foam nickel metal membrane support rather than the original plastic support, was used. By this means it was possible to recycle small blood samples by

means of a pump. The results of this phase of the study showed that the permeability of ascorbic acid in this system was 0.0106 cm/min, fifty five percent of the ascorbic acid was lost after three complete recyclings of human plasma. Additional recyclings resulted in no greater loss of ascorbic acid which did not dialyze despite repeated recyclings. This may represent a protein bound form of the vitamin.

When anesthetized dogs were attached to a hemodialyzer the ascorbic acid in their blood dropped during the first 30 minutes of dialysis and remained stable thereafter. There was no indication that the ascorbic acid level of the blood was being restored when the dialysis was continued for four hours.

Results similar to the preceeding were obtained when blood samples were secured from patients undergoing hemodialysis at a local hospital. A rapid loss of ascorbic acid occurred during the first 25 minutes followed by a plateau, indicating a possible bound form of ascorbic acid in human plasma. Patients dialyzed 36 to 40 hours after the initial dialysis were not able to recuperate the levels of plasma ascorbic acid of the previous dialysis treatment when depending upon diet alone.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Pitts (1968) defines uremia as "a complex of symptoms and signs reflecting the dysfunction of all organ systems based on failure of renal regulation of the composition and volume of the body fluids." The term "Uremia", urine in the blood, was first used by Piorry in 1840, to describe the consequences of the abnormal retention of excretory products. The uremic syndrome manifests itself in alterations in every major organ system of the body. Apathy, anorexia, nausea, pulmonary edema, anemia and osteomalacia are frequent symptoms of the uremic patient.

According to Burton (1967) 50,000 people die each year from uremia. Nineteen percent of this population are between the ages of 15-54 years, 79% are over 55, and 2% are under 14 years of age. With the technology and economic resources available today 6,000-10,000 of these patients would be considered ideally suited for hemodialysis or kidney transplants; the two current modes of treatment for uremia (Leonard and Dedrick, 1968). Jones (1971) estimated that hemodialysis could prolong the lives of as many as 24,000 of these patients. In a situation where financial limits to treatment have been removed, the demand for hemodialysis will exceed 40,000 persons by the year 1976 (Leonard and Dedrick, 1968). This estimate would not be altered by even the most extensive transplant program.

The prognosis for the hemodialysis patient in the first year is good, with an 85% survival rate, however, by ten years this has declined to 30%. Organ transplants, with dialysis as a backup treatment if the transplant fails, boosts this ten year prognosis to 75% (Burton, 1968).

Knowledge regarding the effect of long term hemodialysis on the patient's nutrient stores becomes important with the ever increasing use of this type of maintenance; however, the research data available on the nutritional effect of hemodialysis are meager. Anemias due to iron and folic acid deficiencies have been reported in long term hemodialyzed patients (Comty et al., 1968). Jones (1971) has observed petechiae and hematomas suggestive of an ascorbic acid deficiency in some of his patients undergoing hemodialysis.

On the basis of the evidence associated with the relationship of ascorbic acid to iron and folic acid metabolism and since ascorbic acid is so soluble in water and has a relatively low molecular weight it seems justifiable to quantify the losses of ascorbic acid as a result of hemodialysis. The fact that ascorbic acid appears in the sweat only to a slight extent (Mitchell and Edman, 1951) may indicate that this vitamin exists at least to a certain extent in a non-diffusible form in body fluids.

REVIEW OF LITERATURE

Development of Hemodialysis

The hemodialyzer is a system in which circulating fluids are dialyzed across a semipermeable membrane against a countercurrent circulating dialysate fluid, the composition of which can be changed to favor the removal of specific substances. The development of the first experimental dialyzer in 1913 by Abel, Rowntree and Turner created the theoretical possibility of prolonging life in patients with acute renal insufficiency. Kolff (1965) changed this to a practical possibility with the development of the first successful artificial kidney (hemodialyzer) in 1944, for the relief of acute symptoms of uremia in human patients. Rehabilitation of patients with chronic renal insufficiency was hampered, however, by the limited number of blood vessel cannulation sites.

Quinton et al. (1960) overcame this problem with the development of the Teflon-Silastic shunt and expanded the use of the artificial kidney to chronic uremic patients. Teflon silastic tubes are inserted into a major artery and a superficial vein of the patient's arm or leg, a teflon silastic shunt is connected to these tubes for uninterrupted blood flow. Blood tubing connecting the patient's blood to the machine replace this shunt when the artificial

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kidney is in use. Although some problems do occur, many patients are able to maintain functional shunts for as long as two years, when another site of cannulation can be used. Brescia et al. (1966) developed a surgically created internal fistula between the radial artery and a superficial vein as a method for repeated access to the circulation in patients undergoing maintenance hemodialysis. This method solves many of the problems with infections, clotting and physical trauma inherent with the exterior arterio-venous shunt, and has remained functional for longer than two years. The use of this method is contra-indicated, however, for the patient over 40 years because of the increased incidence of stenosis in this age group.

Freeman et al.(1965) outlines seven characteristics of an ideal hemodialyzer: Removal of toxic products of metabolism in an efficient manner; removal of water from oliguric patients by ultrafiltration; a low internal volume requiring minimal blood priming and reducing blood loss; simplicity of assembly; safety; and finally low initial and maintenance costs. None of the hemodialyzers currently in use clinically meet all of these requirements.

The twin coil and the parallel flow hemodialyzer are the two major types of artificial kidney now used. The twin coil hemodialyzer was originally developed by Kolff in 1944. The dialyzing unit consists of two parallel cellophane tubes held in a fiberglass mesh and wrapped around a central core. The dialyzing surface and priming volume varies depending

upon the length of the cellophane tubing from $0.9m^2$ to $1.9m^2$ and with a volume capacity from 320 to 1200 ml respectively. Satisfactory dialysis can be achieved in as little as six hours with this dialyzer if it contains the longer length of tubing. The coil is prepackaged and presterilized reducing the assembly time. However, priming volume and high resistance in this system, when the optimum dialysis time is met, necessitates transfusions and the use of a pump increasing the dangers inherent with transfusions and the possibility of hemolysis from the pump.

Kiil in 1960 designed the parallel flow hemodialyzer; a low resistance system which eliminates the need for a pump and reduces the priming volume of blood to that which can be supplied by the patient's own blood system. The dialyzing unit consists of a blood layer between cellophane sheets supported by polypropylene boards. The dialysate solutions circulate outside the cellophane through grooves milled in the plastic boards. Its direction of flow is opposite to that of the blood (Cole et al., 1963). A two layer Kiil dialyzer has a surface area of $1.12m^2$ and can be assembled in 45 minutes. Kuruvila et al. (1969) compared the Kiil and the twin coil hemodialyzers in a clinical study of 38 patients and observed fewer incidences of hypertension and pulmonary edema, lower transfusion requirements and lower mortality associated with the Kiil hemodialyzer. The twin coil has, however, a greater efficiency in the removal of toxic solutes. This factor, along with its shorter assembly time,

makes this machine preferred for emergency treatments or where rapid removal of toxic substances is essential (Freeman et al., 1965).

Dialysis Membranes

> "A clinically acceptable membrane must be, all at once, a regulator of concentration - and - pressure - induced transport, a mechanical barrier and a surface which is compatible with the biological environment."
>
> (Wezmar and Leonard, 1970)

The essential function of a hemodialysis membrane is to control the degree of intercommunication between the permeable components in the blood and dialyzing solution. A membrane is also a mechanical barrier, fixing the geometry through which blood and dialysate fluid flow. Cellulosic membranes, commercial films currently in clinical use for extracorporeal dialysis, primarily operate by the diffusion of solute through microholes or pores (Klinkman et al., 1970). These membranes discriminate on the basis of size and shape, small ions and molecules permeating relatively easily while increasing size decreases the amount of transport. Alexander et al. (1965) has shown that molecules with molecular weights as high as 16,000 to 19,000 can be transferred in a dialyzer. Cellulose membranes are neutral and have negligible ion exchange capacity at the ionic concentrations employed in hemodialysis (Leonard and Dedrick, 1968).

Cuprophane PT 150* and Visking# membranes of regenerated cellulose are the two most widely used of the Cellulose membranes. Cuprophane membranes have an average wall thickness of approximately 16μ and show extreme evenness of pore size and distribution. The average pore size is from 10-15 Å (Klinkman et al., 1965). Several investigators have attempted to modify or develop new hemodialysis membranes to replace the commercial membranes. Although each has specific characteristics which may be superior to the cellulose membranes, as yet none of the newer membranes has proven to be superior in overall characteristics.

Dialysis Fluid

The dialysate fluid is the one component of hemodialysis which can readily be changed to favor the removal or addition of specific substances. The composition of the dialysate solution must include certain essential chemicals in sufficient concentrations to maintain normal levels of these solutes in the blood of the patient undergoing hemodialysis. Sodium, acetate, calcium, magnesium, chloride, potassium and glucose are the essential chemicals (Freeman et al., 1965). Table 1 gives a typical composition of a dialysate fluid. Potassium, one of the essential chemicals, may be retained by the patient with chronic renal failure and consequently,

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*Bremberg Corporation

#Dupont Corporation

Table 1. Composition of dialysate fluid: Hemotrate Formula 3[a]

Chemical	g/liter	Ion	meq/liter
NaCl	5.5	Na^+	130.0
Na Acetate	4.7	Acetate	35.0
$CaCl_2 \cdot 2H_2O$	0.18	Ca^{++}	2.5
$MgCl_2 \cdot 6H_2O$	0.15	Mg^{++}	1.5
KCl	0.15	K^+	2.0
Dextrose	2.0	Cl^-	101.0

[a]McGaw Laboratories hemodialysis concentrate (Cat. No. R-1625) after dilution 34:1.

this solute may at times be deleted from the dialysate solution in an attempt to lower the patient's elevated blood potassium. Other constituents of the dialysate solution may also be manipulated when clinical situations require it.

Applications for the Artificial Kidney

Treatment of acute and chronic uremia far surpasses all other uses for the artificial kidney. Merril et al. (1964) first demonstrated the feasibility of performing hemodialysis in the home. Failsafe systems have been developed which monitor the patient's blood pressure and the machine for blood-to-bath leaks. This system will awaken a patient should a problem arise, making unattended overnight dialysis

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possible. The training of the patient to perform home hemodialysis requires from four weeks to a year with many patients able to perform satisfactorily with an average of six weeks instruction (Pendras et al., 1970).

Although at present patient insurance will only cover those expenses incurred in the hospital, many insurance companies are now considering extending this coverage to the home dialysis patient. Presently the cost for home hemodialysis is $10,000 initially, followed by an annual cost of approximately $5,000 for supplies, maintenance and medical assistance. This figure would be increased by at least 3-5 fold had the patient remained hospitalized.

Acute poisoning is the second most frequent use of hemodialysis. Hemodialysis is effective in quickly removing many toxic substances after they have been absorbed into the blood stream. Barbituate, salicylate and glutethemide are the three most common compounds requiring dialysis and have been shown to be effectively cleared from the blood thereby (Kiley, 1969).

Walder and Faillace began treatment of acute alcoholism with the hemodialyzer in 1969 (Med. Wld. News, 1969). Preliminary observations revealed complete recovery in six hours and a tendency to remain sober for an undefined length of time. These investigators also showed that alcohol induced symptoms of CNS stimulation; nausea, headaches and delerium tremens were completely eliminated.

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Effect of Hemodialysis on the Nutritional Status of the Individual

Knowledge regarding the effect of long term hemodialysis on the patient's nutrient stores becomes important with the ever increasing use of this type of maintenance. Ginn et al., (1968) has shown that all the essential amino acids are permeable to the dialysis membrane and from 2.0 to 3.5g of amino acids were recovered from the dialysate after six hours of dialysis. Gulyassey et al., (1968) also observed several essential amino acids in the dialysate, at concentrations exceeding 50% of the minimal daily requirements in normal man. 0.75g/kg body weight (50g) per day of high biological value protein is necessary to maintain a neutral or slightly positive nitrogen balance and to maintain serum albumin concentrations in patients undergoing hemodialysis twice weekly. Sorensen and Kopple (1968) as well as many other investigators have reported poor dietary adherance to the 20 and 40g protein diets. Pendras (1968) observed that his patients would willingly undergo an additional dialysis treatment (3/week from 2/week) in exchange for an 80g protein diet.

The therapeutic effect of a sodium restriction in the treatment of renal disease has been established since the time of the Kempner rice diet (1944). Sodium may be restricted to as little as 500mg per day in a severely uremic patient, however, the average hemodialysis patient has a sodium restriction of from 1000 to 2000 mg sodium, (5,000mg is the normal adult intake, Frank and Mickelsen, 1969).

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More recently potassium has been restricted in the patient undergoing hemodialysis. Dangerously high levels can accumulate in these patients, especially those with no urinary output, between dialysis (Louis and Dolan, 1970).

Clearance of Vitamins by Dialysis

Anemias due to iron and folic acid deficiencies have been well documented in long term hemodialysis patients (Comty et al., 1968; Hampers et al., 1967). Hegstrum et al. (1961) reported development of perepheral neuropathy and symptoms of a pantothenic acid deficiency in chronic uremic patients undergoing hemodialysis. No plasma vitamin levels were measured. Lasker et al. (1963) studied the levels of the B vitamins in patients undergoing peritoneal dialysis and reported a decline in folic acid after dialysis. Low nicotinic acid levels were also observed in these patients, whereas, levels of vitamin B_{12}, thiamin, biotin and pantothenic acid were not altered by peritoneal dialysis. Whitehead et al., (1968) also reported a loss of folic acid during hemodialysis and was able to isolate this vitamin in the dialysate fluid. These investigators reported that folic acid loss occurred less rapidly than did urea and creatinine.

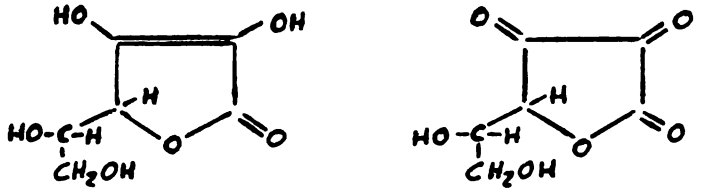
Sullivan et al. (1970) has reported a decline in the mean plasma ascorbic acid levels of patients undergoing dialysis with the twin coil hemodialyzer. Guskin et al. (1970) reported oral bleeding among 29 of 70 patients studied with uremic syndrome. Ascorbic acid deficiency was

not implicated as a possible cause by these authors. Jones (1970) has observed patechiae and hematomas in patients undergoing maintenance hemodialysis. Although blood levels of ascorbic acid were not measured, these symptoms were relieved by treatment with high doses of ascorbic acid (500-700/day).

Ascorbic acid

Zilva in 1923 did much of the early work on the isolation of L-ascorbic acid and established the general chemical properties of this vitamin. L-ascorbic acid (L-threo-hexono-1, 4-lactono-2-ene) is a white crystaline solid with the chemical formula $C_6H_8O_6$ and a molecular weight of 176.12g/mole. Figure 1 illustrates the chemical configuration of the reduced (L-ascorbic acid) and the oxidized (L-dehydroascorbic acid) forms of the vitamin (Sebrell and Harris, 1967).

Figure 1. Chemical configuration of L-ascorbic acid



L-ascorbic acid L-dehydroascorbic acid

Ascorbic acid is very water soluble, 1 gram dissolves in 3 ml of water. Urea is three times as soluble as ascorbic acid, 1 gram dissolves in 1 ml of water (Merck, 1960).

Man and other primates, guinea pigs, the red vented Bulbul bird and the fruit eating bat are dependent upon an exogenous source of vitamin C (King, 1967). Recently evidence has established a requirement of this vitamin for the Rainbow trout,

and the Coho, Sockeyed, and Chinook salmon (S. Kitamura et al., 1965; Halver et al., 1962). Suggestive evidence may also establish a requirement for many other varieties of fish (Aoe et al., 1971).

> The characteristic features of a vitamin C deficiency, approximately in order of evidence, are: decreased urinary excretion, decreased plasma concentrations, decreased tissue and leucocyte concentration, weakness, lassitude, suppressed appetite and growth, anemia, heightened risk of infection, tenderness to touch, swollen and inflamed ankle joints, shortness of breath, fevers, patechial hemorrhages from the venules, beading or fracture of ribs at costochondral junctions, x-ray "scurvy lines" of tibia or femur, fracture of epiphysis, massive subcutaneous, joint, muscle, and intestinal hemorrhages.

The major physical changes observed in scurvy are associated with the failure to maintain collagen. Robertson (1961) reported that maintenance of preformed collagen does not generally require ascorbic acid. Small amounts of collagen are formed in the absence of ascorbic acid, however, rapid synthesis of this tissue requires ascorbic acid. Gould (1961) has shown that "growth" collagen is essentially ascorbic acid independent whereas "repair" collagen is ascorbic acid dependent. It has been fairly well established (Gould, 1961, Robertson, 1961) that ascorbic acid acts in the conversion of proline to hydroxyproline, a major amino acid constituent of collagen which must be hydroxylated after it is built into the polypeptide chain (Lehninger, 1970).

Ascorbic acid has been associated with the release of free folic acid from folic acid conjugates in food (Vinter, 1968), and Nicol et al., (1950) have shown that ascorbic acid

is involved in the metabolism of folic acid to its active form, citrovorum factor. Mazur (1961) has reported that ascorbic acid and adenosine triphosphate are involved with the biochemical mechanism for the transfer of plasma-bound iron to the liver and its subsequent incorporation into ferritin. McCurdy and Dern (1968) reported that ascorbic acid potentiates the absorption of iron from the intestinal tract. The potentiation increases with increasing doses of ascorbic acid with a cutoff point at 500mg and remains true with doses of iron up to 120mg.

Binding of Ascorbic Acid

Pal and Guda (1939) first reported evidence indicating the presence of a combined ascorbic acid "ascorbigen" in plant tissue. Ghosh and Guda (1939) further reported that this ascorbigen could be extracted from cabbage with chloroform and could be separated from free ascorbic acid.

The fact that symptoms of scurvy appear long after the plasma ascorbic acid levels have fallen to zero (Sargent, 1967) along with the observation that ascorbic acid appears in the sweat only to a slight extent (Mickelsen and Keys, 1943) may indicate that this vitamin exists in a non-diffusible form in the blood. Holtz et al., (1940) and Holtz and Walter (1940) reported the presence of a bound form of ascorbic acid which would not appear in the protein-free filtrate of human blood. The bound ascorbic acid was liberated by acid or enzymatic hydrolysis; Holtz reported bound ascorbic acid in

cell free organ extracts and milk as well as blood. Wachholder *et al.* (1940) were unable to find this bound ascorbic acid in blood or milk but did find considerable amounts in the heart muscle. Sargent and Golden (1950) using a diffusion technique failed to find a bound ascorbic acid in blood. However, further studies by this group (Golden and Sargent, 1951) revealed that ascorbic acid moves into the erythrocyte more quickly than it moves out, indicating that ascorbic acid is not transferred across the red cell membrane by simple diffusion and in fact ascorbic acid may be bound to the red blood cell.

OBJECTIVES OF THE PRESENT STUDY

The present study was designed to 1) determine the permeability of ascorbic acid in water through a semi-permeable hemodialysis membrane, 2) to study the rates of transfer of ascorbic acid from plasma, and 3) to determine whether some ascorbic acid is present in the blood as a non-diffusible complex.

The high water solubility of ascorbic acid and its low molecular weight makes a high permeability constant quite likely. No report of this permeability constant was found in the literature. The second objective is important since anemias due to deficiencies in iron and folic acid have been well documented in hemodialysis patients (Comty et al., 1968; Hampers et al., 1967). Previous workers have shown that ascorbic acid is associated with iron and folic acid metabolism. (Mazur, 1961; Nicol et al., 1950) It may be that losses in the dialysate solution contribute to these conditions. The fact that plasma levels of ascorbic acid seem to be independent of the levels in the sweat may indicate that this vitamin exists in the blood in a form which makes it non-diffusible at least as far as the sweat gland is concerned. (Mickelsen and Keys, 1943) This may also be true of the hemodialysis membrane.

METHODS

Apparatus

In vitro studies:

A small Babb-Grimsrud (1968) parallel flow hemodialyzer was used for this study in the determination of the permeability of ascorbic acid and its diffusion from plasma (Figure 2). This hemodialyzer is constructed of two 6 x 6 x 1 inch lucite plates each of which has a 4 x 4 x 1/8 inch rectangular depression milled in its center. A 4 x 4 x 1/8 inch piece of foam nickel metal sits in the depression flush with the lucite. The density of the foam nickel is about 3% of the solid nickel (Roth, 1970). In operation the plates are bolted together (Figure 2); blood headers, used to collect the blood, are attached to each end of the block with rubber gaskets to insure a tight fit. Stainless steel spacers were used to control the height of the blood channel. The dialysis area was approximately 200 cm^2. Ninty-five percent of this area equals the effective area which was approximately 190 cm^2.

Two variable flow pumps were used to deliver the fluids: the dialysate pump, a Maisch constant metering pump operated with a flow rate of approximately 800ml/min; the blood pump, a sigma hand pump, delivered at a rate ranging from 5 to 30 ml/min depending upon the experimental design. Figure 4 illustrates the complete system used in this study.

Cuprophane PT 150 membranes of regenerated cellulose were used throughout this study. These membranes have a dry thinkness of about 0.5×10^{-3} inch, and a wet thickness of about 1.0×10^{-3} inch (Roth, 1970). This is a common clinically used membrane and discriminates on the basis of size and shape, small ions and molecules permeating relatively easily, whereas increasing size decreases permeability.

Distilled water was used for the dialysate side of the permeability series of experiments. Hemotrate formula 3, (Table 1), was used as the dialysate solution for all the experiments utilizing blood plasma. This solution is isotonic with respect to blood plasma.

In vivo studies:

Commercial Kiil hemodialyzers in a local hospital were used for the in vivo studies with all subjects except one where a Travenol twin coil was substituted. Kiil hemodialyzers are parallel flow dialyzers. A larger Babb-Grimsrud hemodialyzer was used for the in vivo dog studies. Hemotrate formula 3 was used in the experiments with the human subjects. Isotonic Ringer's and potassium free Ringer's solutions were used in the canine experiments.

Ascorbic Acid and Potassium Chloride Solutions

Solutions of 0.176mg/ml and 0.01mg/ml of L-ascorbic acid in distilled water were used on the blood side for the determinations of permeability. Originally 0.176mg/ml ascorbic acid was used to decrease the possibility of any

losses of ascorbic acid due to oxidation in air. Concentrations of 0.01mg/ml, a concentration nearer to the physiological level in human blood, were then studied to determine if a 10 fold concentration difference would create a difference in the permeability of this membrane to ascorbic acid.

The permeability of ascorbic acid was determined by pushing a distilled water solution containing ascorbic acid through the blood side of the dialyzer at varying flow rates (7-22ml/min). This solution was dialyzed against distilled water delivered at a rate of 800ml/min, a rate great enough to reduce film resistance on the dialysate side to zero. The ascorbic acid was kept well mixed by an automatic stirrer. A closed loop system (Figure 4) was used. This meant that the ascorbic acid solution was returned from the dialyzer directly to the container from which it originally went to the dialyzer. There was, therefore, an uninterrupted flow of solution through the system at all times. A 5ml sample was taken from the inlet and outlet ports every three minutes in each run. Usually four sets of samples were taken each trial. Ascorbic acid was determined by the method of Roe and Kuether (1938) immediately after the trial was completed. A total of 8 trials were run.

The permeability of KCl through this membrane was determined using a solution of 20 mEq KCL in distilled water. Measurements of the KCl concentration of this solution was taken every 15 minutes. Exponential decay of the concentration of the KCL solution in this system was determined by a conductivity bridge.

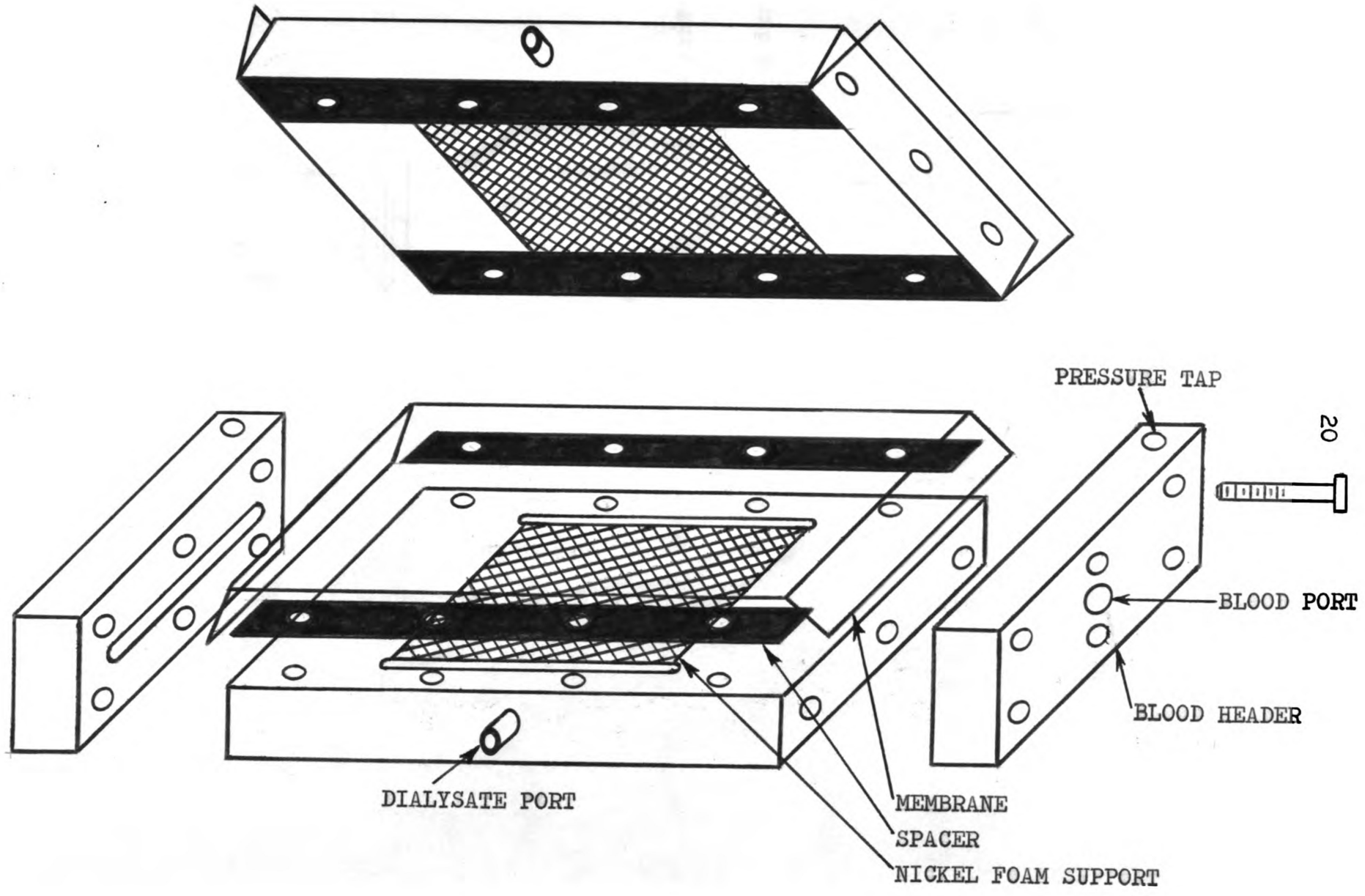


Figure 2. Exploded View of Hemodialyzer (Roth, 1970)

Figure 3. Open loop system for diffusion of ascorbic acid from plasma

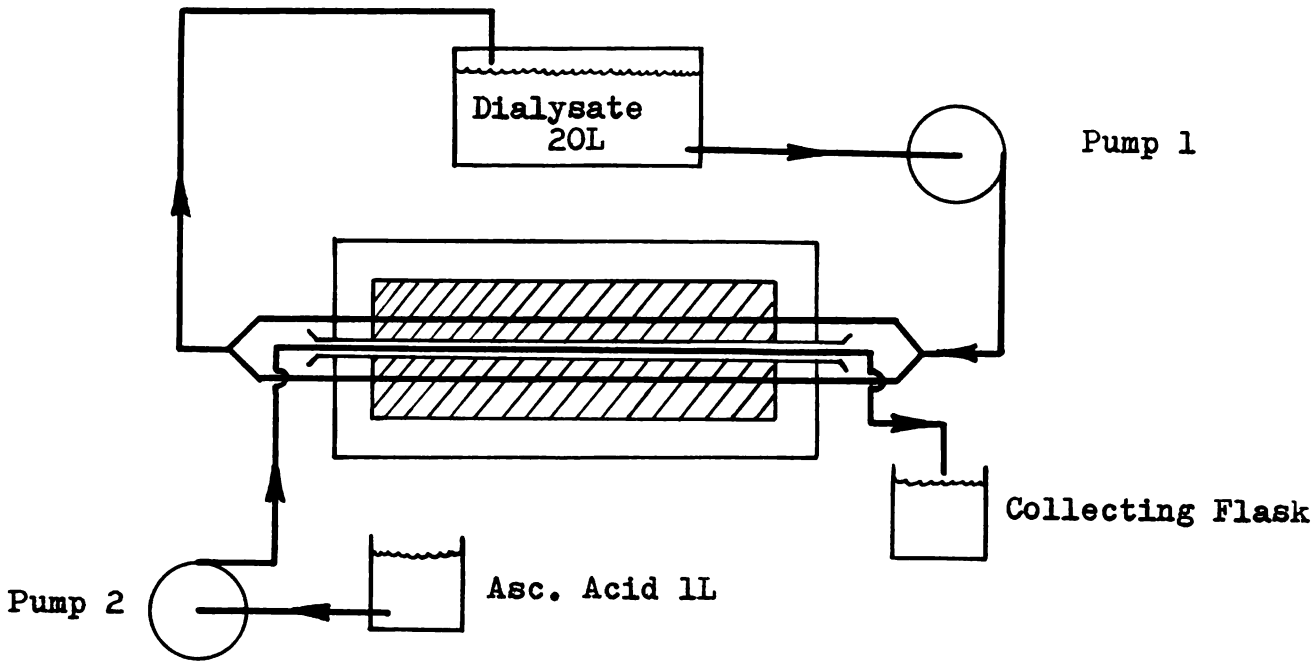
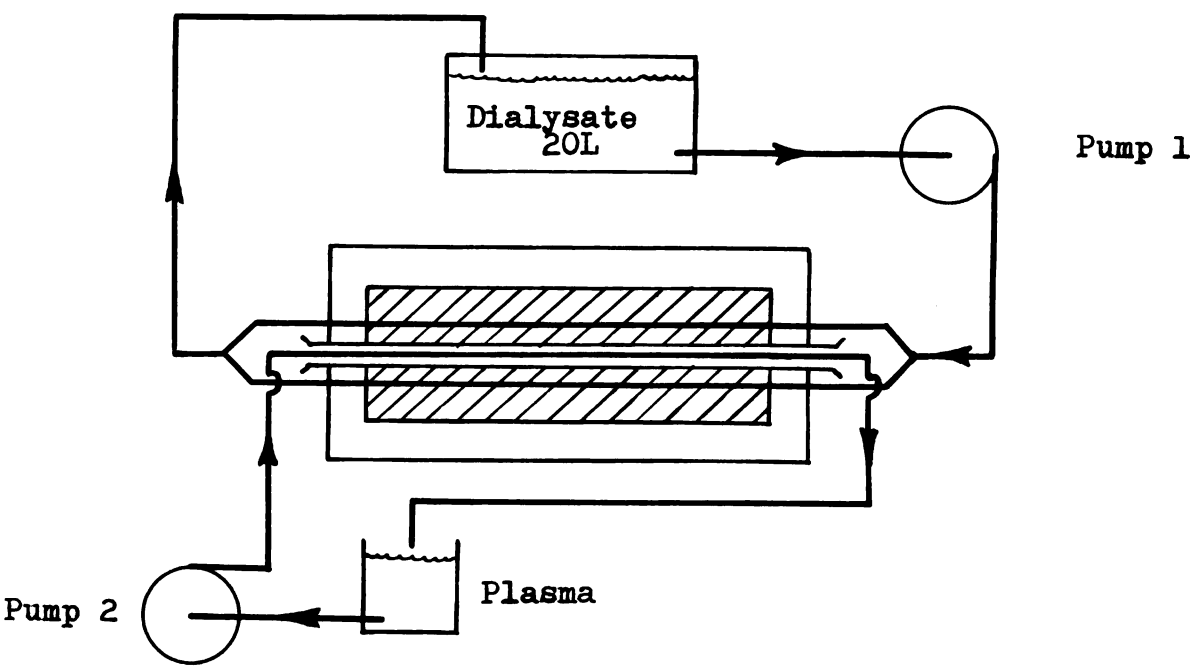


Figure 4. Closed loop system for ascorbic acid permeability



Plasma

Canine plasma from live donors and human plasma from the Regional American Red Cross were used for the plasma diffusion studies. Plasma was delivered through the blood side of the dialyzer at the rate of 23ml/min or 40ml/min. An open loop system was used (Figure 3) so that the ascorbic acid removed from a single pass could be determined.* Samples of plasma were taken initially and then at the half way point of each complete pass. A complete pass through the dialyzer constituted a cycle, six cycles made up each trial. Concentration of ascorbic acid was determined immediately after the completion of a trial by the method of Farmer and Abt (1936).

Two samples of human plasma were held overnight after a complete dialysis trial and subjected to the same procedure with a fresh membrane and dialysate solution.

Subjects

Large mongrel dogs were anesthetized by intravenous injection of sodium pentobarbital (30mg/Kg body weight) and ventilated with an artificial respirator. These dogs were

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*After the plasma passed through the dialyzer, it was collected in a separate receptacle. This permitted evaluation of plasma ascorbic acid concentrations of blood samples which had made complete passes through the dialyzer. Problems with clotting of plasma prevented any other method which would have assured complete mixing.

being hemodialyzed as a part of another project; samples of blood were obtained before dialysis, 15 minutes after the start of the dialysis and every 30 minutes thereafter until the end of the dialysis. All samples were analyzed for plasma ascorbic acid by the Farmer and Abt method (1936).

Eight hemodialysis patients from a local hospital were selected for this study (Table 8, p. 36). The patients were both male and female and ranged in age from 22 to 52 years. The length of time on a dialysis program varied from 6-40 months. Samples of blood were drawn before the onset of dialysis, 30 minutes into dialysis and at the end of dialysis for each patient. Arterial blood samples (blood entering the dialyzer) were drawn into heparinized tubes, plasma was separated and then analyzed immediately for ascorbic acid by the method of Farmer and Abt (1936). In four patients additional samples were obtained at four hours, or the half way point of dialysis.

Twenty four hour diet recalls were obtained for each patient for the day prior to dialysis and the day on which dialysis was performed. These were calculated for protein, ascorbic acid, potassium and sodium with the values reported in Handbook #8 (Watt and Merrill, 1963).

RESULTS AND DISCUSSION

Permeability of the Cuprophane PT 150 Membrane to Ascorbic Acid

Results of the ascorbic acid permeability study are shown in Tables 2 and 3. The permeability of ascorbic acid through the Cuprophane PT 150 membrane, a cellulose semipermeable membrane, at 25°C was 0.0106cm/min or when expressed as a function of resistance 1/98.3cm/min. The permeability value for ascorbic acid in this system is consistent with its size and shape. Permeability was determined using the formula, flux (J moles/cn^2/sec) = permeability (cn/sec) x change in concentration (moles/cn^3).

It has been established (Leonard and Dedrick, 1968) that the Cuprophane PT 150 membrane discriminates on the basis of size and shape; small molecules permeate with relative ease, whereas, increasing size of the molecule decreases the amount of transport until eventually it will not pass through the membrane at all. The permeability of this membrane to potassium chloride, a compound with a molecular weight approximately half that of ascorbic acid, was studied to determine the validity of the ascorbic acid data. Potassium chloride with a molecular weight of 75.56 g/g-mole has a permeability in this system of 0.0246cm/min or 1/40.7 cm/min. The ratio of molecular weights of ascorbic acid to that of KCl is 2.33:1; an inverse ratio of permeabilities is 2.41:1. The six percent differences in these two ratios

Table 2. Permeability of Cuprophane PT 150 Membrane to Ascorbic Acid and Potassium Chloride at 25°C

	Permeability cm/min	g-Molecular weight
Ascorbic Acid	0.0106 1/98.3	176.0 g/g-mole
Potassium Chloride	0.0246 1/40.7	75.56 g/g-mole

Ratio of Molecular Wts = AA/KCL 2.33:1

INVERSE RATIO OF PERMEABILITIES $\frac{1}{\text{AA/KCL}} = 2.41:1$

Table 3. Permeability of ascorbic acid[a] through a Cuprophane PT 150 Membrane

Trial	Flow rate ml/min	Initial Conc. mg/ml	Permeability at 25°C cm/sec
17a	7	0.176	0.1797×10^{-3}
17b	13	0.176	0.1637×10^{-3}
17c	17	0.176	0.1638×10^{-3}
18	10	0.176	0.1752×10^{-3}
19	10	0.176	0.1705×10^{-3}
20	14	0.176	0.1902×10^{-3}
21	10	0.010	0.1797×10^{-3}
22	10	0.010	0.1625×10^{-3}
Average			$.1731 \times 10^{-3} \pm .0099 \times 10^{-3}$ [b]

a. ascorbic acid solution - L-ascorbic acid in distilled H_2O

b. standard error

is within experimental error, and supports the validity of the ascorbic acid permeability constant. Flow rate and initial concentration had no effect on the ascorbic acid permeability through this membrane (Table 3).

The work with a pure solution of ascorbic acid provides a theoretical basis for evaluation of ascorbic acid transport in plasma. The theoretical permeability is dependent upon the physical properties of the ascorbic acid molecule and the physical characteristics of the membrane. A change in this permeability can be attributed to changes in the ascorbic acid molecule such as binding to a larger molecular weight substance, if the physical properties of the membrane are kept constant.

Diffusion of Ascorbic Acid from Plasma: In Vitro

Since Cuprophane PT 150 is permeable to the ascorbic acid molecule it was necessary to determine if there were any factors in human plasma which would alter the degree of permeability of the membrane to ascorbic acid. The results of diffusion data for human and canine plasma were similar and therefore will be considered together (Tables 4, 5; Figures 3, 4). Ascorbic acid diffused freely through the membrane during the first three passes through the hemodialyzer; 55% of the ascorbic acid was lost after 3 complete passes. The calculated permeability of plasma ascorbic acid in the first pass through the dialyzer, regardless of whether human or canine, was 0.0102 cm/min, a value quite similar to that

Table 4. Ascorbic acid concentration (mg%) in canine plasma with each pass through the hemodialyzer

Pass through hemodialyzer	Ascorbic acid in canine plasma (mg%)[a]		
	23ml/min.[b]		
0	0.40	0.37	0.29
1	0.24	0.28	0.18
2	0.16	0.21	0.11
3	0.18	0.07	0.09
4	0.16	0.07	0.09
5	0.16	0.07	0.09
6	0.16	0.07	0.09

[a]Values refer to amount of ascorbic acid in canine plasma following one complete pass through the hemodialyzer and are expressed for 3 trials wherein each trial consisted of 6 passes.

[b]Blood flow rate-ml/min.

Table 5. Ascorbic acid concentration (mg%) in human plasma with each pass through the hemodialyzer

Pass through hemodialyzer	Ascorbic acid in human plasma (mg%)[a]			
	23ml/min[b]		40ml/min[b]	
0	0.37	0.45	0.52	0.50
1	0.22	0.33	0.34	0.38
2	0.15	0.30	0.26	0.32
3	0.15	0.22	0.24	0.28
4	0.11	0.19	0.24	0.26
5	0.11	0.19	0.22	0.26
6	0.11	0.19	0.24	0.26

[a]Values refer to amount of ascorbic acid in human plasma following one complete pass through the hemodialyzer and are expressed for 4 trials wherein each trial consisted of 6 passes.

[b]Blood flow rate-ml/min.

Figure 5. Ascorbic Acid Concentrations of Human Plasma: In Vitro Passages Through a Parallel Flow Hemodialyzer

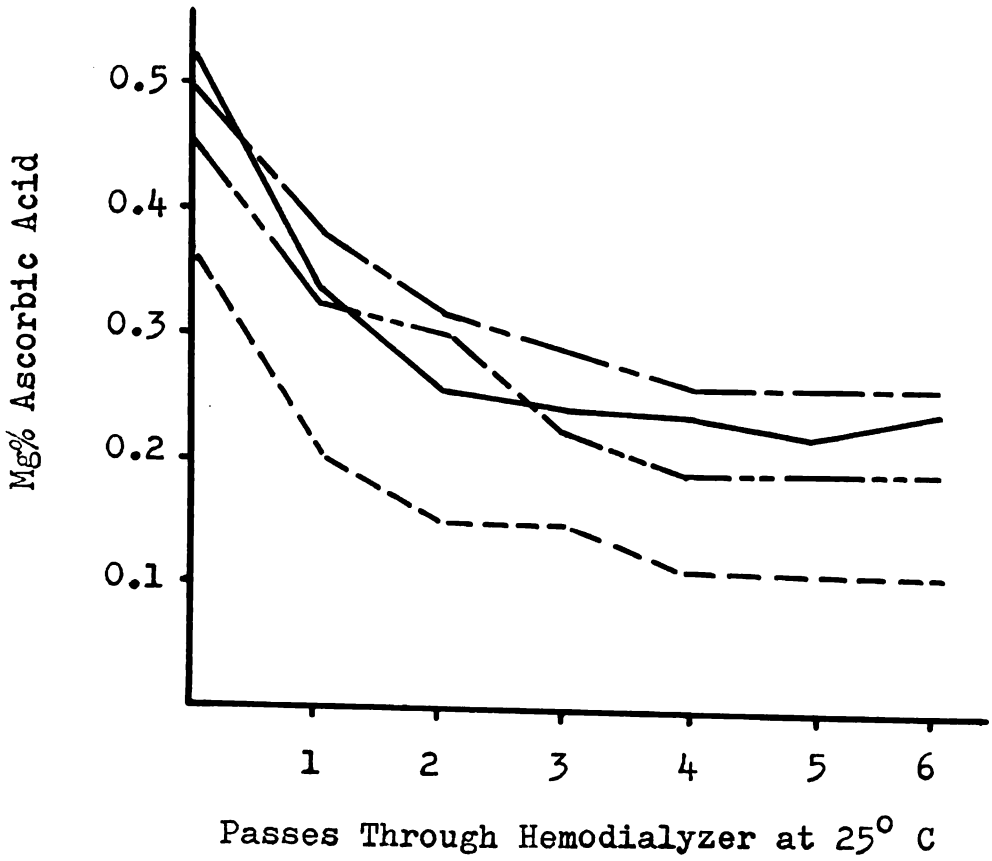
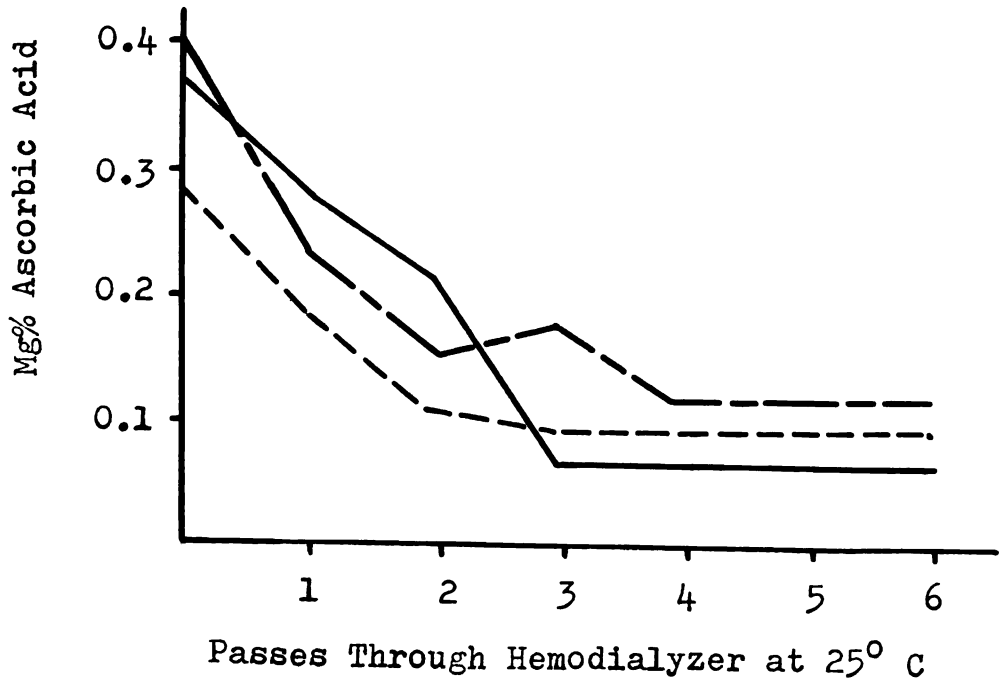


Figure 6. Ascorbic Acid Concentrations of Canine Plasma: In Vitro Passages Through a Parallel Flow Hemodialyzer



obtained from the ascorbic acid/distilled water solution. After the third pass, no more ascorbic acid passed through the membrane. This would suggest that the remainder of the ascorbic acid was bound to a molecule of greater molecular weight. Despite repeated recyclings through the hemodialyzer plasma ascorbic acid was maintained at a mean constant level of 0.15mg%. Analysis of the dialysate fluid showed levels of ascorbic acid (0.0003mg/ml) far below a level needed to establish an equilibrium.

The plateau seen in the diffusion studies could be explained by the fact that proteins or other large molecules may accummulate on the membrane and thereby block the diffusion of the ascorbic acid. A dialyzed sample of human plasma was refrigerated overnight and then subjected to the same procedure with a fresh membrane and dialyzing solution. In this case free diffusion occurred in the first pass (drop from 0.15mg% to 0.07mg%) followed by another plateau again above zero. The fresh membrane would rule out any blockage by protein and since a new plateau occurred after only one pass through the dialyzer there was not time for proteins or any other large molecules to block a significant number of pores.

The diffusion of ascorbic acid from the plasma held overnight followed by another plateau may indicate that an equilibrium exists between the free vitamin and the vitamin bound to a non-diffusible complex in the blood.

Sargent and Golden (1950) also found free diffusion of ascorbic acid in the first 30 minutes followed by a plateau which remained stable for the remainder of their experiment (90 minutes). However, it was not until later studies, completed in 1951, that they considered this to be an indication of a bound form of ascorbic acid. These investigators believed that ascorbic acid may be bound to the circulating red blood cell.

Analysis of the plasma samples after dialysis by two different methods was done to establish values above zero. Table 6 gives the comparison of the values for these two methods. The Roe and Kuether (1938) method depends upon

Table 6. Comparison of Two Methods of Ascorbic Acid Analysis in Human and Canine Plasma

	2, 4-dinitrophenyl hydrazine	2, 4-dichlorophenol indophenol
	mg%	mg%
Canine		
Sample 1	0.07	0.10
Sample 2	0.08	0.09
Human		
Sample 3	0.20	0.20
Sample 4	0.20	0.24

the formation of an osazone between the ascorbic acid molecule and the phenylhydrazine. This osazone has a color which is proportional to the concentration of ascorbic acid in the sample. The Farmer and Abt (1936) method is a micro method which depends upon the reduction of a dye, 2, 4-dichlorophenolindophenol. It seems unlikely that the agreement between these two methods would be good if the low concentrations of ascorbic acid were artifacts.

In vivo Studies - Canine

The results of plasma ascorbic acid diffusion from anesthetized dogs undergoing hemodialysis paralleled those obtained from plasma. Loss of ascorbic acid occurred in the first 15 minutes, plasma ascorbic acid levels of 0.31mg% and 0.30mg% fell to 0.15mg% and 0.16mg% respectively (Table 7). No further losses occurred after the first 15 minutes. There was no indication of a restoration of the lost ascorbic acid even after four hours of dialysis. Thus the mechanisms restoring plasma ascorbic acid levels do not appear to be functional during the first few hours after depletion in the dog.

In vivo Studies - Human

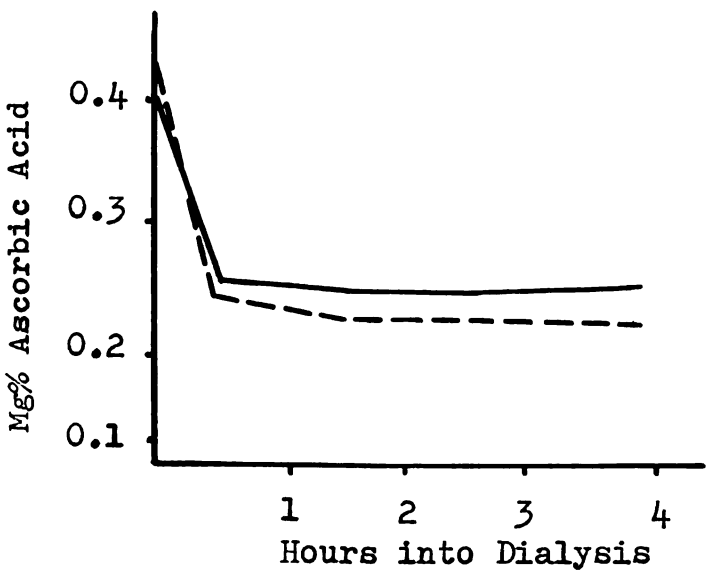
Results of the data obtained from hemodialysis patients were similar to the canine experiments. A period of rapid diffusion occurs in the first 30 minutes of dialysis followed by a slower diffusion for the remaining 5½ to 7½

Table 7. Ascorbic Acid Concentrations of Canine Plasma: In Vivo Passages Through a Parallel Flow Hemodialyzer

Time into Dialysis Min	Ascorbic Acid Concentration (mg%) 25ml/min[a]	
0	0.31	0.30
30	0.15	0.16
90	0.13	----
150	0.12	----
180	0.12	0.14
240	----	0.14

[a]Blood Flow Rate through Hemodialyzer

Figure 7. Ascorbic Acid Concentrations of Canine Plasma: In Vivo Passages Through a Parallel Flow Hemodialyzer



hours (Figures 8, 9). Those patients with initial levels of ascorbic acid below 0.80mg% (Figure 8) reached a plateau within the first 30 minutes. Those patients with plasma ascorbic acid levels above 0.80mg% did not reach a stable state until much later in the dialysis period (Figure 9). In four subjects additional samples were obtained at four hours into dialysis, in each case this sample had a level of plasma ascorbic acid equal to the last sample. The fact that in all cases a cessation or a decline in this rate of diffusion occurs may be another indication of a bound form of this vitamin.

During the entire period of dialysis, hemodialysis patients undergo a total loss of 15mg ascorbic acid from the plasma alone. However, losses equaling 133mg ascorbic acid could be calculated if losses from the extracellular fluids was also considered. Predialysis levels of this vitamin (Table 8) followed closely the total levels of ascorbic acid intakes of these patients. Patients undergoing hemodialysis are restricted in terms of their intakes of protein, sodium and potassium. High potassium foods, citrus fruits and vegetables, are also high in ascorbic acid. Those patients with the greatest restriction of potassium also had the lowest levels of ascorbic acid in their diets.

Recuperation of predialysis levels of ascorbic acid did not occur in any of the eight subjects in the 36 to 40 hours between dialysis treatment. This was true even though in one case a level of 116mg ascorbic acid was consumed on the day

Table 8. Medical History and Dietary Intakes of Patients Undergoing Hemodialysis

Patient	Age Yrs	Sex	Months on Dialysis	Level of As. Acid Supplement
				mg/day
RJ	48	M	29	0
JJ	47	M	6	100
AJ[b]	50	F	21	100
PW	52	M	13	400
EM[b]	33	F	5	100
DW	22	M	30	300
JB	33	M	20	300
MS	49	F	41	300

Table 8 (cont'd)

Diet Record[a]			
Protein	Ascorbic Acid	Potassium	Sodium
g	mg	mEq.	mEq.
31	20	20	38
36	36	27	44
41	55	37	29
29	34	35	48
19	7	19	35
67	111	61	42
24	27	24	38
63	111	44	52

[a]Average of four 24 hr. diet recalls.

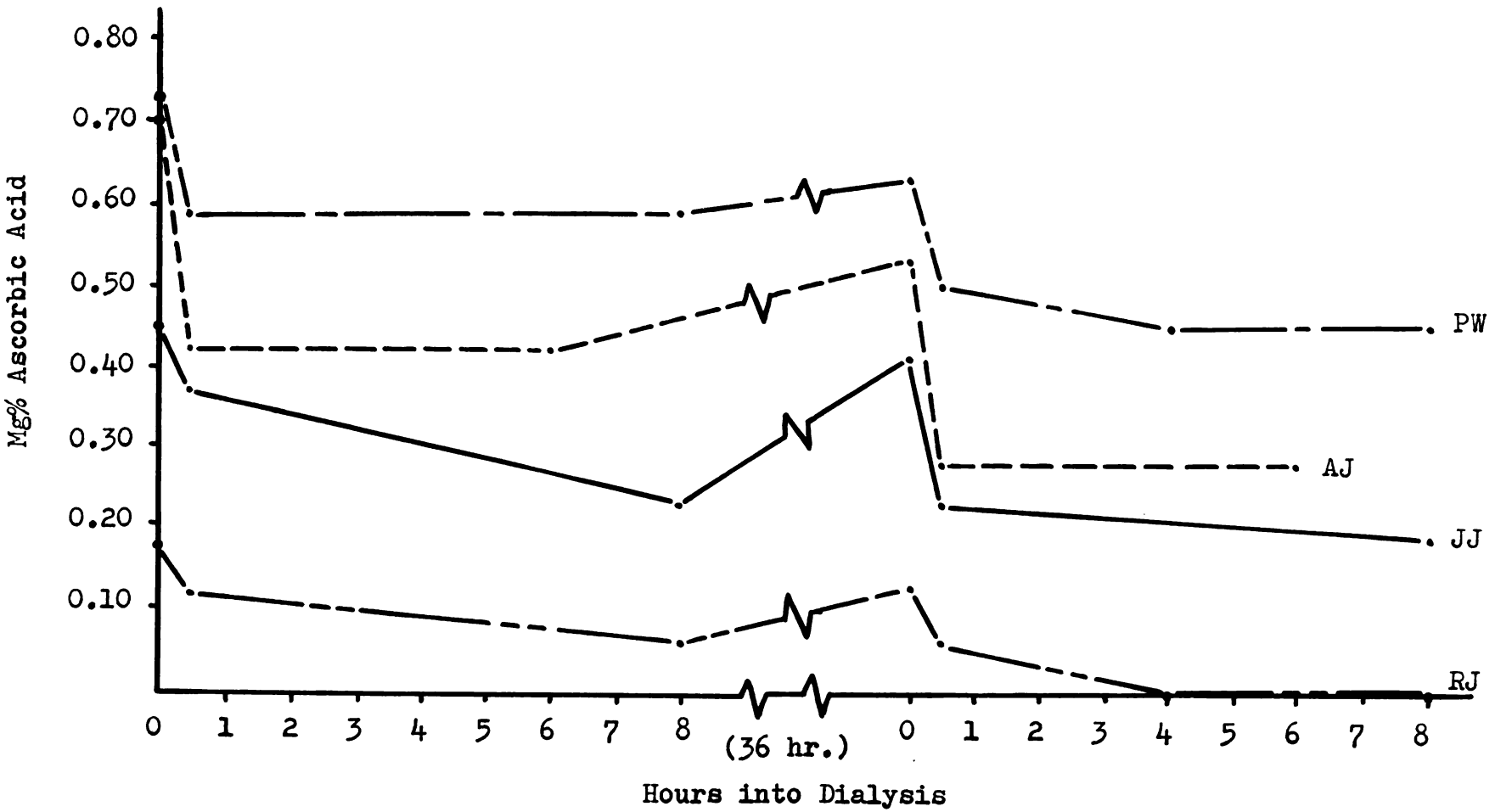
[b]E.m. and A.J. were dialyzed 6 hours. All other patients were dialyzed 8 hrs.

Table 9. Plasma Ascorbic Acid Concentration of Hemodialysis Patients

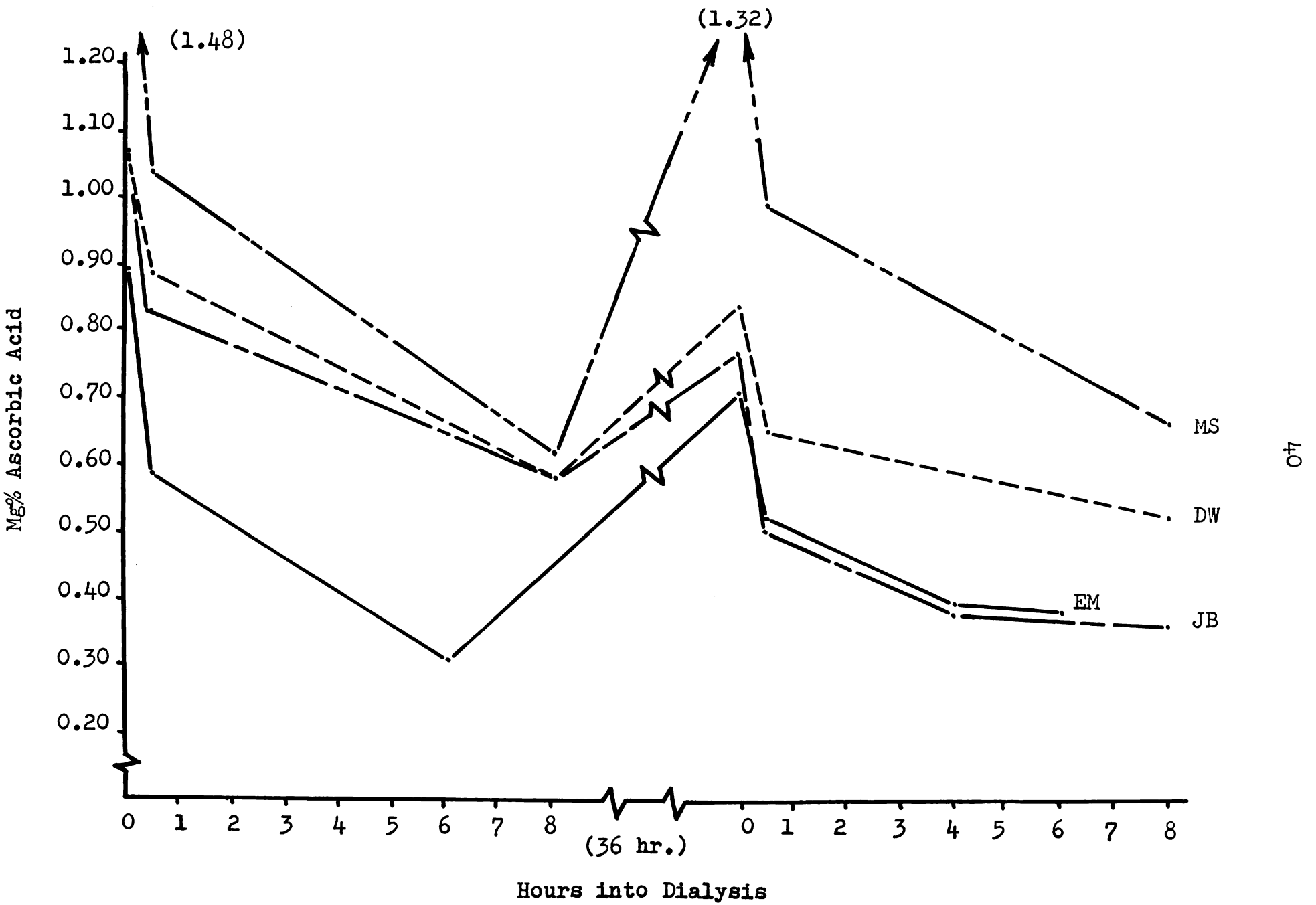
Patient	First Dialysis				Second Dialysis[a]				
	Time into dialysis (hrs.)				Time into dialysis (hrs.)				
	0	0.5	6	8	0	0.5	4	6	8
	mg%				mg%				
RJ	0.18	0.12	----	0.06	0.13	0.06	0.00	----	0.00
JJ	0.45	0.37	----	0.23	0.41	0.23	----	----	0.19
AJ	0.70	0.42	0.42	----	0.58	0.28	----	0.28	----
PW	0.73	0.59	----	0.59	0.63	0.50	0.45	----	0.45
EM	0.88	0.59	0.31	----	0.71	0.52	0.39	0.38	----
DW	1.06	0.89	----	0.58	0.84	0.65	----	----	0.52
JB	1.07	0.83	----	0.58	0.77	0.50	0.38	----	0.36
MS	1.48	1.14	----	0.61	1.32	0.99	----	----	0.66

[a]Second dialysis occurred 36 hrs. after the end of the first dialysis.

Figure 8. Plasma Ascorbic Acid Concentrations of Hemodialysis Patients During Dialysis: I



Dialysis: II


(1.48)
(1.32)
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
Mg% Ascorbic Acid
MS
DW
EM
JB
0 1 2 3 4 5 6 7 8
(36 hr.)
0 1 2 3 4 5 6 7 8
Hours into Dialysis

prior to dialysis. Studies with the absorption of vitamin D in chronic hemodialysis patients indicate that this vitamin may be poorly absorbed from the digestive tract of these patients. Similar problems may also occur with the absorption of ascorbic acid in hemodialyzed patients. Repeated dialysis over extended periods of time will result in the eventual lowering of the plasma ascorbic acid concentration to zero (patient R.J.). The non-diffusible form of the ascorbic acid in blood seems to be in an equilibrium state with its free form. Therefore, a percentage of bound ascorbic acid (probably as much as 55%) can be lost with every dialysis.

SUMMARY

The Cuprophane PT 150 membrane has been shown to be permeable to ascorbic acid molecule. Theoretically this membrane will also be permeable to the ascorbic acid in blood. Although a fraction of the ascorbic acid present in the blood diffuses freely, and has the same permeability constant 0.0102cm/min, 45% of this ascorbic acid is in a non-diffusible state and has a permeability constant of zero. This non-diffusible form of ascorbic acid does equilibrate over time with the free ascorbic acid. Therefore, repeated dialysis over many months with no additional vitamin C supplement will result in zero levels of this vitamin in the blood of hemodialysis patients. Supplementation of ascorbic acid at levels great enough to produce a predialysis level of 1.5mg% ascorbic acid are desirable if levels of this vitamin are to be kept above low normal during dialysis.

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APPENDIX

APPENDIX

Calculations for Permeability

The mathematical evaluation of the permeability was done under the guidance of Dr. Donald K. Anderson.

The equations of Babb *et al.* (1968) were used for the calculation of permeability of ascorbic acid in this system. Table 10 illustrates a representative example of these calculations.

Table 10. The determination of the permeability of ascorbic acid through a membrane

$$h_o = \frac{Q_B \ln(C_{Bi}/C_{Bo})}{A}, \quad Q_B = 10ml/min$$

$$h_o = (10ml/min)\ln(0.1043/0.0870)/190.1cm^2$$
$$= (10ml/min)\ln(1.1989)/190.1cm^2$$
$$= (10cm^3/min)\ (0.1814)/190.1cm^2)\ (60sec/min)$$
$$h_o = 1.590x10^{-4}cm/sec$$

Using Eq. (9),

$$1/h_o = R_o = R_B + R_M + R_D \quad , R_D = 0$$

But $R_M = 1/P$, and $R_B = 0.5a/D$, giving Eq. (10)

$$1/h_o = 1/P + 0.5a/D$$

a, the half-channel height is known to be $0.55x10^{-2}cm$ at 25^oC[4]

D, the diffusivity of ascorbic acid, was theoretically calculated to be $0.65x10^{-5}cm^2/sec$

Therefore,

$$1/h_o = 1/P + (0.5)\ (0.55x10^{-2}cm)/(0.65x10^{-5}cm^2/sec)$$

$$1/P = 1/h_o - 0.423x10^3(cm/sec)^{-1}$$
$$= 1/1.590x10^4(cm/sec)^{-1} - 0.423x10^3(cm/sec)^{-1}$$
$$= (0.6289x10^4 - 0.423x10^3)\ (cm/sec)^{-1}$$
$$= 0.5866x10^4(cm/sec)^{-1}$$
$$P = 0.1705x10^{-3}cm/sec$$

QB = Blood Flow Rate

D = Dialysate

M = Membrane

R = Resistance

B = Blood

D = Diffusivity

a = Half channel height

A = Area