THE UNIVERSITY OF CALGARY

DIPHENYLHYDANTOIN-PROTEIN BINDING IN CHRONIC RENAL FAILURE

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE

CALGARY, ALBERTA

FEBRUARY 1981

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

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ABSTRACT

The binding of many drugs to plasma proteins is decreased in patients with chronic renal failure. As a consequence, drug pharmacokinetics may be significantly altered in these patients, and it may be difficult to employ rational drug pharmacotherapy. The measurement of drug-protein binding may, therefore, be useful in the clinical management of patients with chronic renal failure.

At present there is no finite hypothesis for the defect in drug-protein binding that occurs in renal failure. Possible explanations for the defect in drug-protein binding include: 1) hypoproteinemia, 2) competitive inhibition, 3) non-competitive inhibition and 4) basic functional differences in the drug binding proteins.

The binding of diphenylhydantoin (DPHN) to plasma proteins was measured in normal and uremic plasma, using equilibrium dialysis and an ultrafiltration method. Both methods produced acceptable results, but equilibrium dialysis was preferred because of the effect of temperature.

Binding of DPHN to plasma proteins was decreased in patients with chronic renal failure, and the binding was not greatly improved by hemodialysis. Some patients with kidney transplants also had increased levels of unbound DPHN. The measurement of DPHN-protein binding would appear to be indicated in these clinical conditions.

Patients with chronic renal failure were not hypoalbuminemic. There were no significant differences in the

binding of DPHN to albumin isolated from uremic plasma versus albumin isolated from normal plasma. These observations suggest that the binding defect in chronic renal failure can not be attributed to hypoalbuminemia or to a basic functional defect in the primary drug binding protein albumin. Treatment of uremic plasma with charcoal normalized DPHN-protein binding, and substances capable of inhibiting DPHN-protein binding were extracted from the charcoal. The partial characterization of the inhibitory substances suggests that they are peptides (approximate molecular weight 1,000-2,000). Direct evidence is therefore presented to suggest that the defect in DPHN-protein binding observed in chronic renal failure is the result of competitive or non-competitive inhibition by peptide substances.

ACKNOWLEDGMENTS

I wish to thank the following people for their assistance during my graduate program. Without their co-operation this project would not have been possible.

Dr. E. Penikett, Dr. J. Todd, Dr. R. Baynton, Mr. G. Fettes and the staff of the Foothills Hospital Department of Laboratories. Dr. R. Hons, Dr. H. Mandin, and the patients and staff of the Renal Unit and the Renal Outpatient Clinic.

Dr. W. Veale, Dr. R. Hannah, Dr. H. Watanabe and Dr. D. Severson, past and present Associate and Assistant Deans of Research at the Medical School.

Edward Chan and Marwan Masri, for their assistance and for their friendship.

My supervisory committee, Dr. Lannigan, Dr. MacCannell and Dr. Yeager, for their guidance and suggestions.

In particular my supervisor, Dr. Nigel Boyd, for his knowledge, direction, concern and friendship.

This work was supported, in part, by grants from the Foothills Provincial General Hospital, the University of Calgary and the Alberta Heritage Foundation for Medical Research.

This thesis is dedicated to

my grandparents and my parents

and to

Lynne, Carmen and David

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PART I : INTRODUCTION

1.01 THERAPEUTIC MONITORING

The measurement of drugs and their metabolites in the tissues and fluids of the human body for the purpose of monitoring and regulating pharmacotherapy is an area of clinical laboratory medicine known as therapeutic monitoring. Recently therapeutic monitoring has experienced a most significant expansion both in terms of the variety of drug assays available and in the actual numbers of assays performed, such that the dicipline is now recognised as having a very major role in rational pharmacotherapy. Until very recently the measurement of drug concentrations was restricted to specialized analytical pharmacology research laboratories where the necessary analytical expertise was located. However, remarkable advances in technology have allowed the proliferation and dissemination of sophisticated instrumentation and analytical techniques such that virtually any clinical laboratory may now initiate procedures for the measurement of several clinically important therapeutic agents (Syva, 1980). Each month the literature grows with new methodologies, improved methodologies and clinical evidence to support the value of therapeutic monitoring for an increasing number of drugs. It would appear that as our society continues to utilize ever increasing numbers of therapeutic agents, and as the technology to measure these substances improves, therapeutic drug monitoring will become one of the most important areas of clinical laboratory medicine.

Therapeutic monitoring services are currently available in most large clinical laboratories. Table I lists those drugs which are measured routinely, as well as the methodologies that may be employed. In order to appreciate the rather sudden and large demand for therapeutic monitoring services, the rationale for the utilization of these services is examined:

The usefulness of therapeutic monitoring derives from the assumption that the estimation of the concentration of a drug in the blood may be useful in predicting the pharmacological action of that drug. Typically the interest is in those substances for which there is a wide variability in the patient's response to a given dose, and/or agents which have a narrow therapeutic margin (only a small difference between therapeutic and toxic doses, Pippenger, 1979). The distribution of blood drug concentrations in a large population after a specific drug dose is Gaussian, indicating that not all individuals respond to a drug dose in an identical manner. Moreover, certain drugs such as diphenylhydantoin (DPHN) obey zero order kinetics at therapeutic plasma concentrations of the drug. This means that the biotransformation pathway for DPHN may become saturated and the metabolism of DPHN may cease to be proportional to the total dose of the drug (first order kinetics). As a consequence a small increment in dose can produce a disproportionate and perhaps toxic increase in the blood concentration of the drug.

TABLE I

THERAPEUTIC MONITORING ASSAYS AVAILABLE

DRUG	ANALYTICAL TECHNIQUE
ACETYLSALICYLIC ACID	SPECTRO, COLOR, HPLC
AMITRIPYLINE	GLC, GLC/MS, HPLC
CARBAMAZEPINE	EMIT, HPLC, GLC
CHLORAMPHENICOL	HPLC, BIOASSAY
DESIPRAMINE	GLC, GLC/MS, HPLC
DIGOXIN	RIA, EMIT
DIPHENYLHYDANTOIN	EMIT, HPLC, GLC, RIA
DISOPYRAMIDE	GLC, SPECTRO
ETHOSUXIMIDE	EMIT, HPLC, GLC
GENTAMICIN	EMIT, RIA, HPLC
IMIPRAMINE	GLC, HPLC, RIA
LIDOCAINE	GLC, HPLC, EMIT, RIA
LITHIUM CARBONATE	FLAME EMISS, ATOMIC ABS
METHOTREXATE	GLC, RIA
NOTRIPTYLINE	GLC, GLC/MS, HPLC
PHENOBARBITAL	EMIT, HPLC, GLC
PRIMIDONE	EMIT, HPLC, GLC
PROCAINAMIDE	EMIT, HPLC, GLC
PROPRANOLOL	HPLC, GLC
THEOPHYLLINE	EMIT, HPLC, GLC, SPECTRO
TOBRAMYCIN	RIA, BIOASSAY
VALPROIC ACID	EMIT, GLC

SPECTRO spectrophotometry, COLOR colorometric,
HPLC high performance liquid chromatography,
GLC gas liquid chromatography, /MS mass spectrometry,
EMIT enzyme mediated immunoassay, RIA radioimmunoassay

In those patients who have demonstrated a constant and predictable response to a given drug dose the onset of some other disease process may induce considerable variation in the dose response equilibrium. Liver disease or renal disease, for example, may alter the biotransformation and elimination of many drugs such that conventional dosage schedules will not produce the desired pharmacological effects. Also, medications prescribed for one disease may interact with those prescribed for another disease, leading to an unpredictable response. Phenobarbital, for example, can induce hepatic microsomal enzymes and cause an increased biotransformation of warfarin and a reduced pharmacological effect (Cucinell et al, 1965). Conversely, dicoumarol can inhibit the metabolism of DPHN through a competitive mechanism resulting in a prolonged serum half life for DPHN and an increased pharmacological effect (Hansen et al, 1966).

Normal alterations in physiological state may also alter drug utilization patterns and cause drug related difficulties in a previously well controlled individual. Studies have shown that pregnancy may produce a dramatic fall in DPHN levels and an exacerbation of seizures (Strauss et al, 1979). Maturation can also induce significant changes in drug pharmacokinetics. The onset of puberty in epileptic children, for example, is often associated with the manifestation of toxic effects of anticonvulsants (Pipenger, 1978). Several authors have also reported that dose response may be altered in old age (Crooks et al, 1976; Richey and Bender, 1977).

Patients who respond favorably to a medication or those who experience a clinical problem only rarely may neglect to take their medication. This is known as non-compliance and has been identified as perhaps the most common reason for the failure of many therapeutic regimens. The seriousness of this problem is revealed in a recent study of epileptic patients, conducted by Mucklow and Dollery (1978). It was found that over 50% of the patients in the study failed to take their medication as prescribed. Leppik et al (1979) examined plasma DPHN concentrations in epileptic patients and concluded that a coefficient of variation in the drug level of greater than 20% was suggestive of non-compliance.

Without the benefit of therapeutic monitoring the clinician must attempt to adjust the drug dose according to the clinical response. Unfortunately, the clinical response may be unpleasant or even dangerous, the clinical problem may present itself only rarely and insidiously (for example some forms of epilepsy) or the patient's age, mental or physical status may limit the interpretation of the clinical response. A knowledge of the patient's blood level for a particular drug can allow the clinician to tailor the patient's drug dose towards the achievement of a blood level that is generally associated with a therapeutic effect and to avoid those levels that are associated with unwanted or toxic effects. Routine measurement of drug levels may also serve to encourage many patients to take their medications as

prescribed and may allow the physician to identify those patients who are not complying.

1.02 DRUG-PROTEIN BINDING AND PHARMACOKINETICS

The estimation of drug concentrations in the blood is usually a measure of the total drug concentration, that is the amount of drug in the free or unbound form plus the amount of drug that is bound to protein. The total drug concentration, however, is not equal to the concentration of the drug at the receptor sites, since it is only the free or unbound form of the drug which is able to reach the sites of action and exert a pharmacological effect, or be metabolized and excreted from the body (Levy, 1976). Drug-protein binding is therefore an important consideration in the pharmacokinetics of drugs, and the significance of these effects is briefly discussed here:

Only that fraction of the drug in the blood stream that is not attached to protein (primarily albumin) is free to diffuse through the capillary membranes and distribute throughout the extracellular water (Kurata and Wilkinson, 1974, Figure 1). As free drug leaves the circulation, bound drug is released to maintain the equilibrium and when distribution is complete the concentration of free drug in the plasma, not the total level, should equal the drug concentration in the extracellular water. The free drug concentration would thus be a more accurate estimate of the concentration of the drug affecting the tissue receptors.

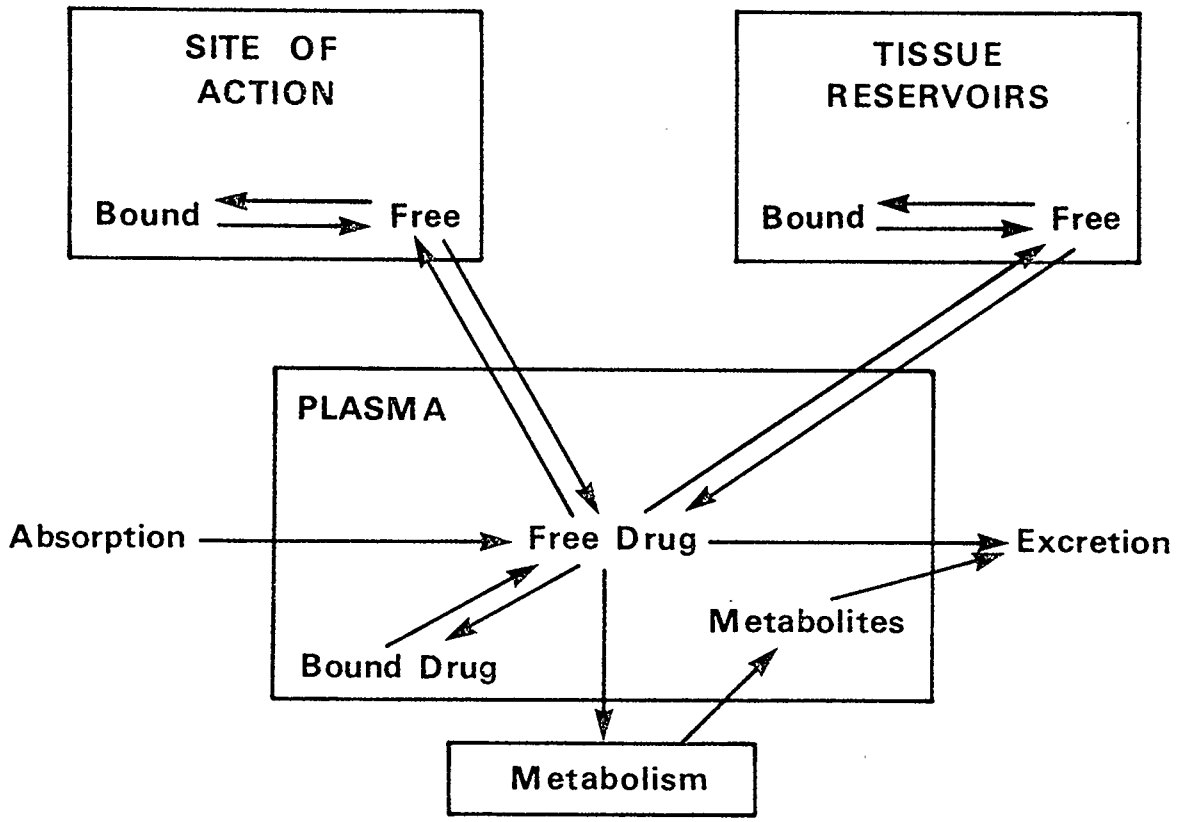


Figure 1. Distribution of unbound drug in various body compartments.

For highly albumin bound agents such as DPHN the unbound or free drug concentration in the blood may be only a fraction of the total level measured, and if binding to tissue proteins is a significant factor (as for the tricyclic antidepressants) then the pharmacologically active tissue concentrations may bear even less relationship to the total plasma levels measured.

Drug-protein binding may increase or decrease the half life of a drug, depending on the mechanism of biotransformation involved. Substances that enter the hepatocyte (the primary site of drug biotransformation) by simple diffusion are rate controlled by the extent to which they bind to plasma proteins. Coumarin anticoagulants, phenylbutazone and certain sulfonamides are examples of drugs whose rate of hepatic biotransformation has been shown to be inversely proportional to the degree of albumin binding (Koch-Weser and Sellers, 1976). Conversely drugs that enter the hepatocyte by specific transport mechanisms, such as propranolol, may not be restricted by binding to plasma protein (Evans and Shand, 1973). As free drug is transported into the hepatocyte more drug is released from protein to maintain the equilibrium and is available for transport into the cell. The greater efficiency of specific transport mechanisms, as compared to the simple diffusion process, results in the fact that drug-protein binding may not increase the serum half life for drugs that enter the hepatocyte by specific transport systems.

Drug-protein binding also affects the elimination of drugs by the kidney and again the effect is dependent upon the mechanism of elimination (Koch-Weser and Sellers, 1976). Drugs and their metabolities may enter the urine by glomerular filtration, a diffusion process, or by tubular excretion, an active transport process. Drugs that are bound to proteins with a molecular weight of approximately 66,000 or greater (molecular weight of albumin) are excluded from the glomerular filtrate, and thus highly protein bound drugs that are eliminated primarily by glomerular filtration (eg. diazoxide) may enjoy a long half life as a consequence of protein binding (Sellers and Koch-Weser, 1974). However, drug-protein interactions do not generally restrict the active transport mechanisms involved in the tubular elimination of drugs. In a manner analogous to the active transport of drugs into the hepatocyte, the renal cell may efficiently and rapidly remove the unbound drug from the plasma as the rapidly establishing equilibrium continues to make the free form available. For example penicillin compounds, which are greater that 90% bound to serum albumin, may be almost completely removed from the blood by tubular secretion during a single passage through the kidney (Keen, 1971). The opposing effects of drug-protein binding upon the biotransformation and elimination of drugs may be summarized as being either a storage effect, when cellular passage is primarily by diffusion, or as a transport effect, when active mechanisms are responsible for cellular passage of the substance.

1.03 FACTORS INFLUENCING DRUG-PROTEIN BINDING

There is considerable evidence to establish that certain drugs may have an impact on the binding of other drugs to protein. Displacement of drugs from protein is readily demonstrated *in vitro* by the addition of certain substances to whole serum or to albumin solutions containing some other protein bound drug (Lunde *et al*, 1970; Hooper *et al*, 1973a; Patsalos and Lascelles, 1977). The mechanisms of displacement may be described as competitive or noncompetitive interactions between the binding protein and the agents in question. Competitive interactions are those in which the drugs compete for a common binding site on the protein, and the drug with the greatest affinity for the protein, or the greatest concentration, will displace the other from the available binding sites. Non-competitive inhibition of drug-protein binding may occur when the inhibitor binds to the protein at some site other than the drug binding site and induces a conformational change in the tertiary structure of the protein. As a consequence, the number and/or the affinity of the drug-protein binding sites may be altered and less drug may be bound. Competitive displacement is illustrated by the effect of phenylbutazone or salicylates on tolbutamide (Koch-Weser and Sellers, 1976) and by the displacement of DPHN from albumin by valproic acid. Valproic acid may also inhibit the binding of DPHN to albumin in part by a non-competitive mechanism (Monks *et al*, 1978).

Certain disease states and physiological conditions

have also been shown to produce decreased drug-protein binding abilities. For example, individuals with hypoproteinemia, liver disease or renal disease as well as newborn infants and geriatric patients may have increased proportions of the unbound or free drug form as compared to control groups (Reidenberg and Affrime, 1973; Hooper et al, 1973b). Table II lists a number of drugs which have been reported to have altered protein binding in certain clinical conditions. At present no finite hypothesis has been presented to account for the defect in drug-protein binding observed in these conditions, and it may well be that a combination of factors are responsible, or that the etiology varies with the disease process and the drug in question. Four theories are currently postulated to explain decreased drug-protein binding in disease. These are:

1. Decreased binding protein concentrations
2. Competitive inhibition
3. Non-competitive inhibition
4. Alterations in the native binding protein

Endogenous and exogenous substances are bound to serum proteins, primarily to albumin, and decreases in protein concentration, especially in albumin, can produce significant increases in the proportion of the unbound and pharmacologically active drug form. Hypoalbuminemia as a consequence of gastrointestinal, hepatic or renal dysfunction has been associated with a decrease in the number of drug binding sites (Jusko and Gretch, 1976). Even moderate

TABLE II

SUBSTANCES WHOSE BINDING TO PROTEIN MAY BE ALTERED IN CERTAIN CLINICAL CONDITIONS

ACETYLSALICYLATE 2	PHENYTOIN 1,2,3,4	PHENYLBUTAZONE 2
AMINOSALICYLATE 4	FLUORESCEIN 1,2	PREDNISONE 3
AMOBARBITAL 1,3	FUROSEMIDE 1,2	QUINIDINE 1,3
ATROPINE 4	METHADONE 3	SALICYLATE 2,4
BENZYLPENICILLIN 2	METHYL ORANGE 2	SODIUM VALPROATE 2
CARBAMAZEPINE 2	METHICILLIN 4	SULFADIAZINE 2
CHLORAMPHENICOL 4	MORPHINE 1,2,3,4	SULFADIMETHOXINE 4
LIBRIUM 3,4	NITROFURANTOIN 4	SULFAMETHAZINE 2
CLOFIBRATE 2,3	OXYPHENBUTAZONE 4	SULFAMETHOXYDIAZINE 4
CONGO RED 2	PARACETAMOL 4	THIOPENTAL 2,4
DAPSONE 1,2	PENTOBARBITAL 2,4	THYROXINE 2
DESIPRAMINE 4	PHENACETIN 4	TRIAMTERENE 1,2,3
DIAZEPAM 1,3	PHENOBARBITAL 2,4	TRYPTOPHANE 2
DIGOXIN 2,4	PHENOL RED 2	

The numbers after the drug indicate the clinical conditions that may cause decreased protein binding of the drug.
(1) Hepatic (2) Renal (3) Hypoproteinemia (4) Newborn

hypoalbuminemia may lead to a significant increase in the free fraction of highly protein bound drugs such as DPHN (Porter and Layzer, 1975). Other studies have shown that adverse reactions to prednisone, DPHN and diazepam are more common in hypoalbuminemic patients (Boston Collaborative Drug Surveilance Program, 1973). The effect of other factors associated with these clinical conditions makes it difficult to identify clearly the significance of the hypoalbuminemia. However, in nephrotic disease, a condition that is characterized by the abnormal passage of protein into the urine (and otherwise relatively normal renal function), there is a very good correlation between albumin concentration and free drug concentration (Gugler and Azarnoff, 1976). The effect may also be readily demonstrated *in vitro* by the measurement of drug-protein binding in diluted normal sera (Lunde *et al*, 1970).

Competitive inhibition has long been considered a possible cause of the increased free drug levels observed in certain disease states. It has been suggested that endogenous substances (nutrients, metabolic products and waste substances) that accumulate in conditions such as hepatic or renal insufficiency may interfere with the binding of drugs to serum proteins by competing for available drug binding sites and displacing bound drug. For example, Dromgoole (1973) suggested that the defect in drug-protein binding observed in uremia might be related to elevated serum concentrations of free fatty acids. Later, Dromgoole (1974)

concluded that free fatty acids might only be significantly elevated in uremic patients after regional heparinization in conjunction with hemodialysis. Odar-Cederlof and Borga (1976b) reported that total free fatty acid levels were not usually elevated in chronic renal failure, and the free fatty acid to albumin ratio was usually normal. They also reported that the free fraction of DPHN did not correlate with the serum concentration of free fatty acids, triglycerides or cholesterol. Increased concentrations of free fatty acids have been shown to produce decreased drug protein binding *in vitro* (Solomon *et al*, 1968) and *in vivo* in rats and dogs (Gugler *et al*, 1974). Fredholm *et al* (1975) found a correlation between DPHN-protein binding and the free fatty acid to albumin ratio in newborn cord serum, but substantial increases in free fatty acid concentrations (nonphysiological concentrations) were required to produce the *in vivo* effect. A molar ratio of free fatty acids to albumin of 3.5 to 1 or greater was associated with drug displacement from albumin. Patel and Levy (1979), however, reported that physiological concentrations of stearic, palmitic, oleic, and linoleic acids produced decreased drug-protein binding of valproic acid when added to defatted albumin solutions. The confusion as to the importance of free fatty acids in drug-protein binding, and the apparent contradictions of many of the reports in the literature may be explained by the nature of the displacement involved. Free fatty acids are bound to 2 high affinity sites on the albumin molecule and 2 or 3

secondary low affinity sites (Muller and Wollert, 1979). When the molar ratio of fatty acids to albumin is greater that about 3.5 to 1 (greater than physiological levels) competitive displacement of certain drugs from the low affinity sites may occur. The displacement of some drugs from albumin by low (physiological) concentrations of free fatty acids is thought to be due to non-competitive inhibition producing allosteric changes in the albumin molecule.

The number of substances that may accumulate in hepatic and renal dysfunction is large and incompletely defined, thus a great potential exists for the competitive inhibition theory. Thus far, however, the evidence available has not confirmed a competitive inhibition mechanism. Andreasen (1974) reported that _in vitro_ dialysis of plasma from patients with acute renal failure increased drug-protein binding and that the ultrafiltrate of the uremic plasma contained substances which inhibited drug-protein binding. This was not confirmed by Reidenberg _et al_ (1971) and Shoeman and Azarnoff (1972) in similar investigations of patients with chronic renal failure. Anton and Corey (1971) reported that _in vivo_ hemodialysis did not increase the binding of sulfonamides in chronic uremia, but Steele _et al_ (1979), in a study of one patient, found that DPHN-protein binding was increased after hemodialysis and suggested that the improvement was due to the removal of a small molecular weight inhibitor. Substances such as uric acid, urea and creatinine

(dialyzable substances) are known to accumulate in renal disease, but the correlation between the plasma concentrations of these substances and drug-protein binding is variable in the literature. Moreover, the addition of creatinine, urea, uric acid, sodium phosphate, methylguanidine, guanidinosuccinic acid and guanidoacetic acid to purified uremic albumin did not reproduce the abnormal binding of sulfamethoxazole observed in uremic plasma (Craig *et al*, 1976). Craig *et al* (1976) reported that the protein binding of sulfamethoxazole, dicloxacillin, salicylate, digoxin and DPHN in uremic plasma could be increased to normal after treatment with activated charcoal at pH 3. They suggested that the binding defect was caused by a substance that was irreversibly bound to albumin at physiological pH and thus was not removed by *in vitro* or *in vivo* dialysis, but was reversibly bound at pH 3. Sjoholm *et al* (1976) studied the binding affinities of normal and uremic serum proteins and concluded that a competitive action may be a contributory factor. They found that the affinity constants for salicylate and warfarin increased with dilution of normal and uremic sera. The fact that the affinity constants for uremic sera did not equal that for normal sera at infinite dilution would indicate the involvement of other factors in addition to competitive inhibition.

Substances accumulating in renal disease may act via a non-competitive mechanism, binding to albumin at some site other than the drug binding sites and producing a change

in the tertiary structure of the protein. A conformational change in the albumin molecule could reduce the affinity and/or the number of drug binding sites and result in an increased free drug concentration. Circumstantial evidence for this hypothesis derives from the observed changes in albumin structure upon the binding of substances such as bilirubin, free fatty acids and certain drugs (circular dichroism and fluorometry). Free fatty acids have such an effect on albumin, and valproic acid, which is similar in structure to the free fatty acids, has been shown to inhibit the binding of DPHN to albumin by a non-competitive mechanism (Monks et al, 1978).

The fourth hypothesis is that abnormal albumin molecules with decreased drug binding abilities may be synthesized in some disease states and physiological conditions. Shoeman and Azarnoff (1972) reported that albumin from normal and uremic plamsa produced two electrophoretic albumin bands and that the second band appeared to correlate with the binding capacity for digoxin and DPHN. Their electrophoretic studies also suggested the presence of a non-competitive inhibitor. Miyoshi et al (1966) reported heterogeneity in albumin fractions isolated from foetal serum and Boobis (1977) reported that albumin isolated from patients with renal disease had a different amino acid content compared to albumin from normal subjects. However, patients in renal failure, with abnormal drug-protein binding abilities, have been found to have improved drug-protein

binding after renal transplantation (Olsen et al, 1975; Odar-Cederlof, 1975 and Levy et al, 1976). Levy et al (1976) reported that the restoration of normal drug-protein binding occurred within two days of the renal transplant - a period of time that would appear to be too rapid to be compatible with the degradation of abnormal albumin and the synthesis of new protein, suggesting instead the removal of a competitive or non-competitive inhibitor.

1.04 CLINICAL SIGNIFICANCE OF DRUG-PROTEIN BINDING

It is well established that the therapeutic monitoring of certain therapeutic agents can contribute to the overall success of the drug therapy (Pippinger, 1979). However, it has been suggested that certain patients would benefit more from a measure of the free drug concentration rather than the total drug concentration, as is currently provided (Kurata and Wilkinson, 1974). Persons with renal or hepatic disease, or hypoproteinemia, as well as newborn infants, geriatric patients and persons receiving more than one drug are among those individuals for whom total drug concentrations may be misleading.

The suggested therapeutic range for DPHN is 10-20 ug/ml at steady state conditions (Ireland, 1980). In normal patients DPHN is about 90% bound to albumin and thus the therapeutic concentration of the unbound drug form is about 1-2 ug/ml. Patients with renal failure routinely have low blood levels (3-4 ug/ml or less) at the usual dose (about 300

mg per day), but still maintain seizure-free control of their epilepsy (Odar-Cederloff et al, 1970; Letteri et al, 1971). Renal failure causes a decreased binding of DPHN such that the unbound drug concentration may be 2 to 3 times normal (20-30% free). Consequently, an apparently subtherapeutic total level of 4 ug/ml in a patient with 70% binding would correspond to a free level of 1.2 ug/ml - which is similar to that expected in a normal individual. Gugler et al (1975) studied the pharmacokinetics of DPHN in patients with decreased drug-protein binding due to the nephrotic syndrome. They reported that the reduced protein binding of DPHN was associated with an increased plasma clearance of the drug, a decreased steady state plasma concentration of the drug, and greater fluctuations in the steady state total plasma concentration of DPHN. Gugler et al (1975) suggested that the regulatory pharmacokinetic mechanisms were dependent upon the unbound concentration of the drug, such that the absolute concentration of the unbound DPHN was not different from normal even though the total drug concentration was significantly lower and the relative percent unbound drug was significantly elevated. The authors concluded that the interpretation of routine plasma levels of highly protein-bound drugs in patients with reduced drug-protein could be a serious problem.

The concurrent administration of more than one therapeutic agent may also have an effect on drug-protein binding and a significant clinical impact, particularly,

although not exclusively, on persons with predisposing conditions that may produce decreased drug-protein binding. Table III lists some of the drugs which have been involved in displacement interactions. Not all drugs are significantly affected by protein binding changes. An example of an important drug displacement interaction is the displacement of bilirubin by sulfisoxazole (an antibacterial agent). A threefold increase in the incidence of bilirubin encephalopathy was found at autopsy in premature infants given prophylactic treatment with sulfisoxazole (Odell ,1973). Many clinically important drug interactions may occur due to drug displacement interactions involving anticoagulants. Phenylbutazone (an anti-inflammatory agent) can displace warfarin from albumin, causing increased concentrations of the pharmacologically active free drug form and serious bleeding problems in the patients. Chloral hydrate (an hypnotic agent) may produce a similar effect through its metabolite trichloracetic acid, which can also displace warfarin from albumin (Sellers and Koch-Wesser, 1970). Methotrexate, a folate metabolism antagonist used in the chemotherapy of certain cancers, can be displaced from plasma protein by sulfonamides and salicylate (Dixon et al, 1965). The extremely toxic effects of methotrexate make this a potentially lethal interaction. Displacement interactions involving DPHN are important to consider because of the wide ulilization of this agent as an anticonvulsant. Among those agents which may displace DPHN (see Table III), valproic acid

TABLE III

DRUG INTERACTIONS INVOLVING ALTERATIONS IN DRUG-PROTEIN BINDING

DPHN	PHENYLBUTAZONE, SULFISOXAZOLE, DIAZOXIDE, SALICYLATE, FATTY ACIDS, WARFARIN, TOLBUTAMIDE, VALPROIC ACID
WARFARIN, COUMARIN	PHENYLBUTAZONE, DIAZOXIDE, TRICHLORACETIC ACID, FATTY ACIDS, CLOFIBRATE
TOLBUTAMIDE	PHENYLBUTAZONE, SALICYLATE, SULFAPHENAZOLE
DIAZEPAM, PROPRANOLOL	FATTY ACIDS
METHOTREXATE	SULFONAMIDES, SALICYLATE
BILIRUBIN, URATE, THYROXINE, CORTICOSTEROIDS, L-TRYPTOPHAN	SULFONAMIDES, SALICYLATE, PHENYLBUTAZONE, PROBENECID, INDOMETHACIN
TRICYCLIC ANTIDEPRESSANTS	DPHN
PENTOBARBITAL	SODIUM ACETRIZOATE (RADIO CONTRAST MEDIA)

and salicylate are particularily worthy of mention. Valproic acid is also an anticonvulsant agent and is often used in conjunction with DPHN. At usual doses valproic acid has been shown to produce a significant displacement of DPHN (Patsalos and Lascelles, 1977; Monks et al, 1978; Dahlqvist et al, 1979). Salicylate is a metabolite of acetylsalicylic acid (aspirin), which is a common analgesic. In view of the extensive utilization of aspirin, the reported displacement of DPHN from albumin by salicylate (Fraser et al, 1980; Paxton, 1980) is an important drug displacement interaction. Typically those agents that are likely to be involved in clinically important displacement interactions are those which are highly bound to albumin at therapeutic concentrations (90% bound or greater), have a small apparent volume of distributiion and display a relatively narrow therapeutic range. Agents that satisfy these requirements are usually acidic drugs. A substance such as DPHN which is highly bound to albumin (90%) is subject to a significant (50%) change in the percent unbound drug concentration (pharmacologically active form) if the protein binding ability is reduced by only 5% (to 85%). By comparison, phenobarbital, which is only 30% protein bound, would only experience a 9% increase in percent unbound drug concentration for a similar decrease in drug binding. Obviously those therapeutic agents with a narrow therapeutic range are more likely to display toxic effects if changes in the free or pharmacologically active drug form occur. Semi-synthetic penicillins are highly

protein bound and subject to significant variation in free drug concentration. However, the range between therapeutic and toxic drug concentrations is large enough to accommodate the fluctuations in the free drug form and toxic effects are not observed.

1.05 METHODOLOGY

Drug-protein binding can be measured in plasma or serum by several methods (Table IV) involving either the physical separation of the unbound drug from the bound drug, or a homogenous spectroscopic analysis (Chingnell, 1971; Kurz et al, 1977; Rowland, 1980). The choice of methodology depends upon the intended application and the drug in question.

Equilibrium dialysis is the classical method for measuring drug-protein binding. The plasma or serum is dialyzed against a buffer solution until the concentration of the free drug is equal in both compartments (Figure 2). The drug concentration may then be measured in both compartments directly or by using a radioactive drug tracer. Errors may occur if the protein binding of the drug is not independent of the drug concentration, since the equilibration process effectively increases the volume of distribution for the unbound drug and results in a decreased drug concentration. In certain applications the Donnan effect may also interfere with the accurate estimation of drug-protein binding using the equilibrium dialysis technique. In this regard, the

TABLE IV

METHODS OF MEASURING DRUG-PROTEIN BINDING

EQUILIBRIUM DIALYSIS:	Classical reference method. Problems: dilution, Donnan effect, long analysis time.
ULTRAFILTRATION:	Relatively short analysis time. Problems: adsorption to membrane, protein leakage, manual manipulations.
ULTRACENTRIFUGATION:	Limited application. Problems: drug binding to tube or to lipoproteins, costly, long analysis time.
GEL FILTRATION:	Limited application. Problems: dissociation of drug-protein complex, large sample volumes, long analysis time.
SPECTROPHOTOMETRIC TECHNIQUES:	Limited application. Problems: costly, qualitative, more applicable for aqueous solutions than plasma.

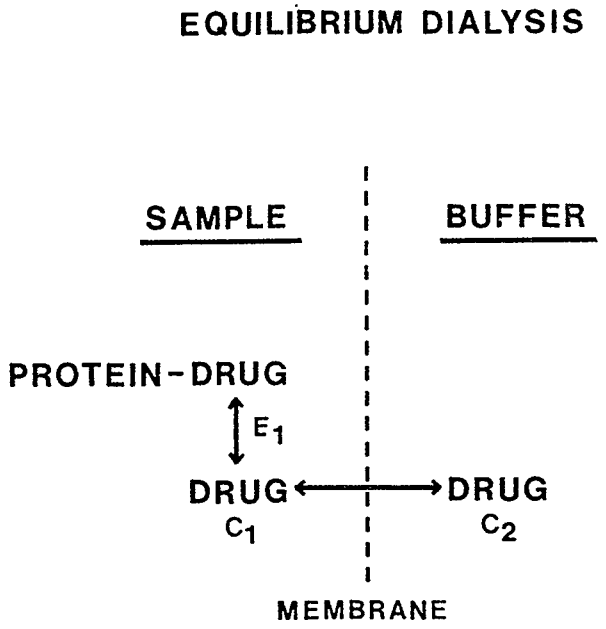


Figure 2. Equilibrium dialysis. The relative concentration of unbound drug in the sample is determined by the equilibrium E_1. Unbound drug is able to cross the membrane. As drug leaves the sample side more is released from protein to maintain the equilibrium E_1, until the concentration of free drug is equal in both compartments, $C_1 = C_2$.

maintenance of electrical neutrality across the membrane is complicated by the presence of non-dialyzable proteins that are restricted to one side of the membrane, and thus the migration of the ionic free drug form may be influenced by the migration of ions seeking to maintain electrical neutrality (Van Holde, 1971). However, the most significant limitation of this methodology in clinical applications is the lengthy equilibration times required, which tend to limit the rapid production of large numbers of test results.

Ultrafiltration of the plasma or serum through a semi-permeable membrane may be employed in order to produce a protein free ultrafiltrate containing the unbound form of the drug. This procedure may be complicated by adsorption of the drug to the membrane, protein leakage through the membrane, or the tedious manual manipulations required in the procedure.

The small unbound drug molecule may also be separated from the high molecular weight drug-protein complex by ultracentrifugation. However, the procedure is time consuming, costly, may require large sample volumes, and is not applicable for large molecular weight drugs. Problems may also arise if the drug is bound to the centrifugation tube or to low density lipoproteins which rise to the top of the tube during centrifugation.

Gel filtration procedures are based upon the more rapid passage of the drug-protein complex through a gel column, due to the exclusion of the bound drug, but not the

unbound drug, from the pores of the gel matrix. However, a significant disruption of the equilibrium between the bound and unbound forms of the drug may occur if the complex is readily dissociated on the column, causing inaccurate estimations of the drug binding. Frontal loading techniques (Morris and Brown, 1977) may be employed to overcome this problem but large sample volumes and lengthy analysis times are required.

Spectroscopic techniques such as ultraviolet and visible absorption, optical rotational dichroism, circular dichroism and fluorescence can also been used to study drug binding to protein. However, at present, these techniques are not generally employed in clinical quantitative applications, but rather in qualitative investigations of binding interactions in aqueous solution.

PART II : MATERIALS AND METHODS

2.01 METHODOLOGY

2.01.1 Radioactive Labelled Albumin

Iodinated (^{125}I) bovine serum albumin was prepared by the chloramine T oxidation procedure (Greenwood et al, 1963) Commercially labelled albumin (^{125}I) was obtained from New England Nuclear. Labelled albumin was stored at -20°C and was purified on a G 100 medium Sephadex column prior to use to remove high molecular weight aggregates and degradation products.

2.01.2 Equilibrium Dialysis

The binding of diphenylhydantoin (DPHN) to protein was measured in the whole plasma or serum samples and in the purified albumin preparations by equilibrium dialysis at 22°C and 37°C. Duplicate aliquots were incubated at room temperature for approximately 2 h with approximately 100,000 dpm/ml radioactive DPHN (5,5-diphenyl-[4-^{14}C hydantoin], 59.8 mCi per mmol, Amersham) and with various concentrations of unlabelled DPHN (Parke-Davis). The purity of the ^{14}C DPHN was verified by thin layer chromatography on plastic backed silica gel plates (BDH Chemicals) developed in chloroform-methanol (4:1) and visualized with mercuric sulfate. Whole serum or plasma was incubated with a physiological concentration of drug in a small volume of absolute ethanol. Typically 6 ul of DPHN in ethanol (stock 15 ug DPHN/2 ul ethanol) was added to the sample to achieve a final drug concentration of 15 ug/ml. Approximately 1.5 ml of sample was placed in the dialysis chamber (model 260 chamber with

model 299 membrane, Technilab Instruments, Figure 3) and dialyzed at 22°C or 37°C for 16-20 h against 0.1 M K_2HPO_4, pH 7.4. The dialysis cells were placed on a mechanical shaker to reduce the time required to reach equilibrium. At equilibrium the radioactivity in the dialyzed sample and buffer was determined by liquid scintillation and the percent free DPHN and DPHNprotein binding calculated according to the following formulas.

$$\% \text{ Free DPHN} = \frac{\text{dpm buffer (dialyzed)}}{\text{dpm sample (dialyzed)}}$$

$$\text{DPHN Binding} = 100\% - \% \text{ Free DPHN}$$

The effect of the sample pH, and the duration and temperature of the dialysis were investigated.

2.01.3 Membrane Ultrafiltration

The ultrafiltration procedure employed here (Figure 4) was similar to that used by Hooper et al (1973a). The membranes and assembly were obtained from Amicon (Centriflo XM 50, Table V, Figure 5). The membrane cones were soaked in distilled water for 2 h to remove a preservative and centrifuged for 12 min at 1,000 G to remove the excess water. Plasma or serum samples were prepared as described for the equilibrium dialysis procedure and 2 ml of sample was placed in the membrane cone and centrifuged for 12 min at 1,000 G. Two hundred ul of ultrafiltrate and 200 ul of the uncentrifuged sample were used for the liquid scintillation determination of the radioactivity in each fraction. The percent free DPHN and the DPHN-protein binding were calculated according to the following formulas.

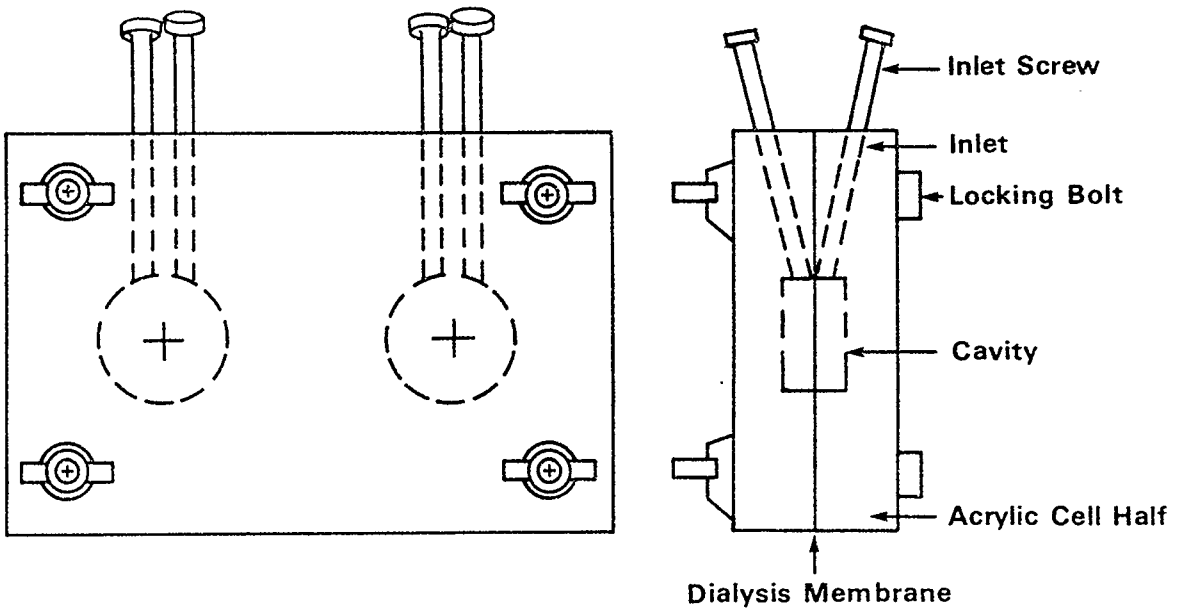


Figure 3. Technilab equilibrium dialysis cell.

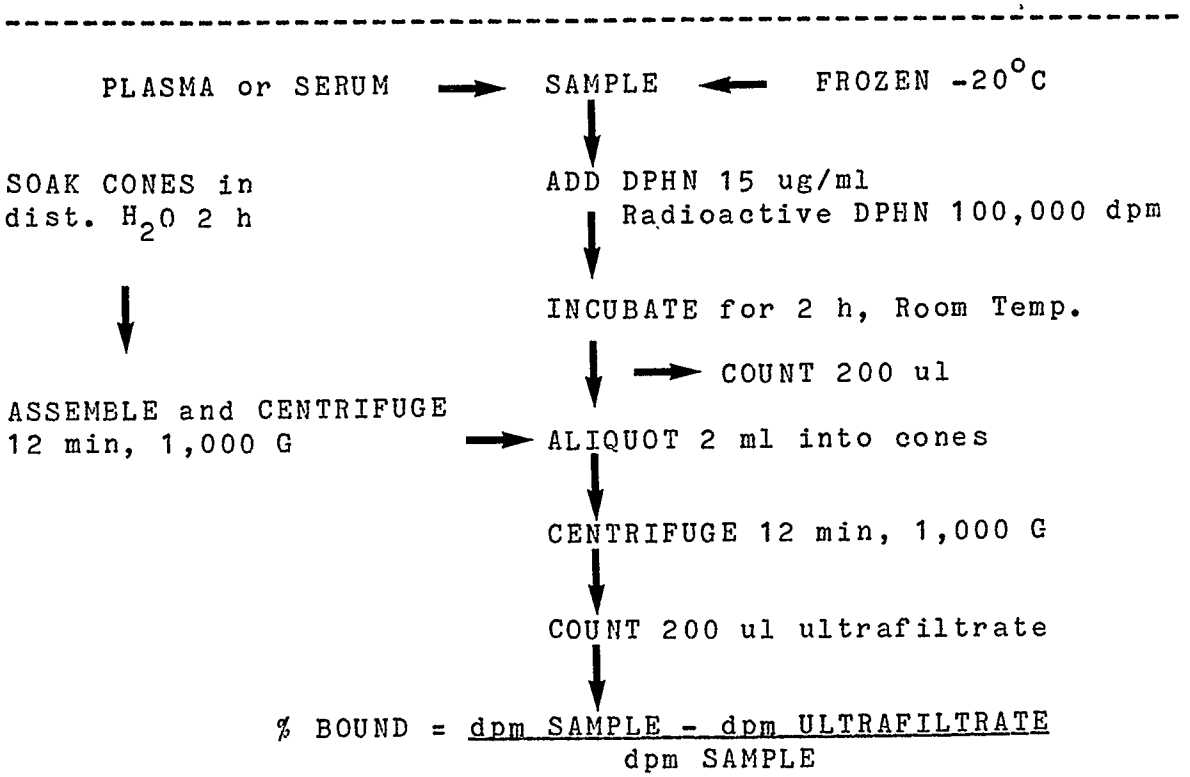


Figure 4. Cone ultrafiltration procedure for measuring DPHN-Protein binding

TABLE V

AMICON CENTRIFLO MEMBRANE CONE SYSTEM

TYPE CF 25	RETENTION: 25,000 daltons
TYPE CF 50A	RETENTION: 50,000 daltons
MEMBRANE CONE:	non cellulose polymer laminated on an inert backing.
CONICAL SUPPORT:	polyethylene
COLLECTION TUBE:	polycarbonate
SAMPLE VOLUME:	max 7 ml with horizontal swing head rotor.
:	max 3.5 to 5 ml with fixed angle head rotor.
OPERATION:	max RCF of 1,000 G.

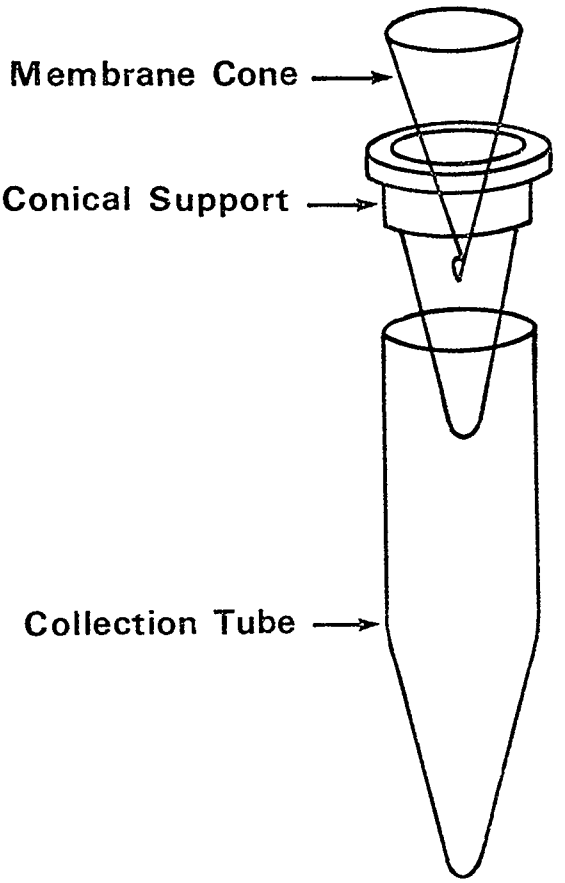


Figure 5. Amicon cone ultrafiltration assembly.

$$\% \text{ Free DPHN} = \frac{\text{dpm ultrafiltrate}}{\text{dpm uncentrifuged sample}}$$

DPHN Binding = 100% - % Free DPHN

Protein leakage through the membrane cone was determined by using ^{125}I labelled albumin, as well as by direct Lowry protein determinations (Lowry *et al*, 1951). Interaction between DPHN and the membrane cone was investigated by recovery studies with ^{14}C labelled DPHN in 0.1 M K_2HPO_4 buffer, pH 7.4. The effect of centrifugation time and the resultant ultrafiltrate volume on the measurement of DPHN-protein binding were also studied.

2.01.4 Measurement of Total DPHN Concentrations

Total DPHN concentrations were measured by a gas liquid chromatographic procedure. One ml of plasma or serum, buffered with 1 ml of 0.1 M phosphate buffer, pH 6.9, containing pentobarbital and methohexital internal standards, was extracted with 20 ml of chloroform (spectrograde, Fisher Chemicals). After mechanical shaking for 10 min and centrifugation for 10 min (1,000 G) the aqueous layer was aspirated off and the solvent filtered through solid sodium sulfate. The solvent was dried under air at 37°C and the residue redissolved in 50 ul of a methylating reagent (0.2 M trimethylanilinium hydroxide, Pierce Chemical Co.). Two ul were injected onto a 6 foot glass column packed with 10% DC 560 on Chrom Q (Chromatographic Specialities). The initial oven temperature was 170°C, isothermal for 5 min then programmed at 8°C per min to 250°C; inlet temperature was 300°C; flame ionization detector temperature was 300°C. DPHN concen-

tration was determined from the peak area relative to that of the internal standards. The linearity (0-30 mg/dl), sensitivity (0.1 mg/dl) and precision (within-day coefficient of variation of 10%) of the method were previously established.

2.02 CLINICAL SIGNIFICANCE OF DPHN-PROTEIN BINDING

2.02.1 DPHN-Protein Binding in Control Samples

Heparinized plasma or serum was obtained from healthy, non-hospitalized volunteers and from hospitalized individuals not having renal or hepatic dysfunction (normal or control samples). These were stored as individual samples (15 subjects) or as pools, each containing approximately 10-20 individual samples (control pools 1-5). Samples were frozen at -20°C until required, or assayed immediately. Biochemistry profiles including creatinine (Worthington, 1976) and albumin (Instrumentation Laboratory, 1978) were obtained. DPHN-protein binding was measured using cone membrane ultrafiltration and/or equilibrium dialysis at 22°C or 37°C. DPHN was added exogenously to the samples.

2.02.2 DPHN-Protein Binding in Renal Failure

Heparinized plasma (uremic plasma) was obtained from patients with severe chronic renal failure requiring hemoperfusion dialysis (Gambro Lundia Dialyzer, 3-5 h dialysis, 3 times a week). Samples were collected as pools, each containing the remaining plasma from approximately 20-30 individual patient samples which had been sent to the laboratory for other biochemical analyses (uremic pools 1-8),

as randomly timed individual samples (12 patients) and as pre- and post-dialysis samples (18 patients). Plasma samples were also obtained from 20 patients who had received a kidney transplant. No other restriction on patient selection, such as age, sex, type of renal disease or medication was applied. All samples were frozen at -20°C until required, or analyzed immediately. Creatinine (Worthington, 1976) and albumin (Instrumentation Laboratory, 1978) concentrations were obtained for each sample. DPHN-protein binding was measured using cone membrane ultrafiltration and/or equilibrium dialysis at 22°C or 37°C. DPHN was added exogenously to the samples.

2.02.3 Protein Concentration and DPHN-Protein Binding

Aliquots of serum or plasma from control individuals and from patients with renal failure were diluted with 0.1 M K_2HPO_4 buffer pH 7.4, to achieve 1 in 2, 1 in 4 and 1 in 8 dilutions. DPHN-protein binding was measured in these solutions by equilibrium dialysis at 37°C.

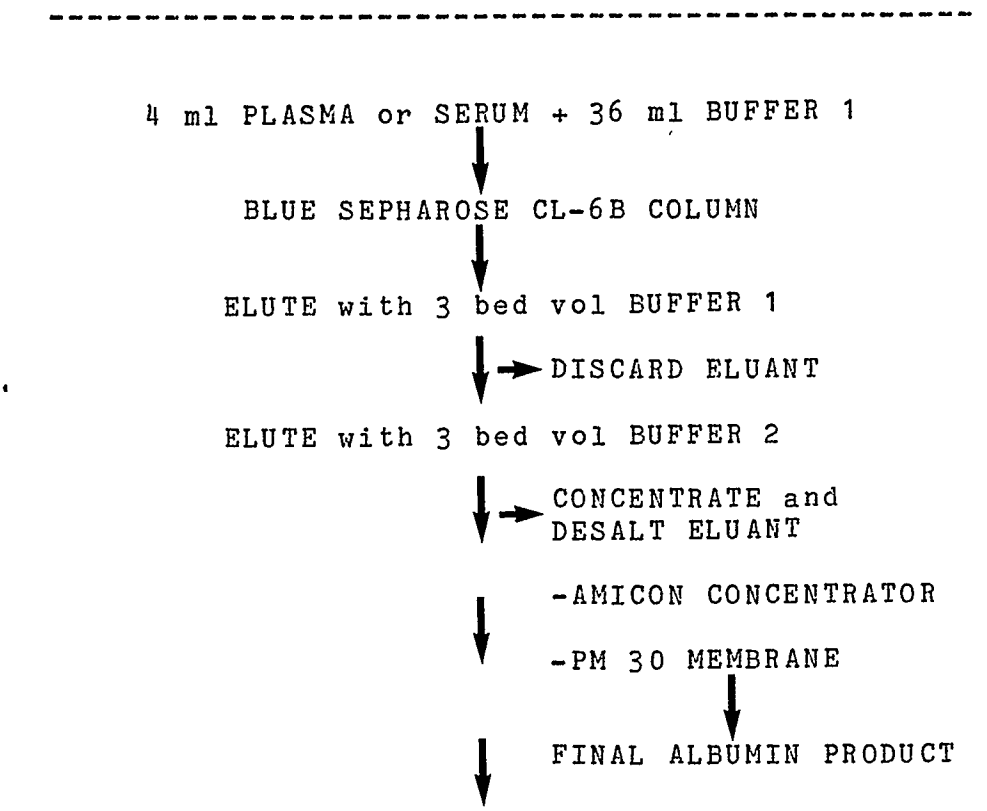
Pooled plasma from patients with chronic renal failure, collected prior to hemoperfusion dialysis, was concentrated using the cone membrane ultrafiltration assembly (CF 50A cone). Three ml aliquots were centrifuged at 1,000 G for 45 min. Albumin (Instrumentation Laboratory, 1978) and DPHN-protein binding (equilibrium dialysis at 37°C) were measured before and after the ultrafiltration.

2.03 CHARACTERIZATION OF DPHN-PROTEIN BINDING INHIBITION

2.03.1 Isolation of Albumin

Albumin was isolated from serum or plasma using Blue Sepharose CL-6B (Pharmacia), a column packing with specific affinity for nucleotide requiring enzymes and for albumin. The freeze dried powder was swollen in distilled water, the fines removed by sedimentation, and washed on a sintered glass filter with 2,000 ml of distilled water. The gel was packed on a 1.5 by 20 cm column, equilibrated with 0.05 M Tris-0.1 M KCl, pH 7.5, washed with 3 bed volumes of 0.05 M Tris-1.5 M KCl, pH 7.5, then reequilibrated with 0.05 M Tris-0.1 M KCl, pH 7.5. The flow rate was approximately 30 ml per hr.

To isolate the albumin fraction (Figure 6) 4 ml of plasma or serum was diluted to 40 ml with 0.05 M Tris-0.1 M KCl, pH 7.5 and loaded onto the column. The column was washed with 3 bed volumes of the 0.05 M Tris-0.1 M KCl, pH 7.5 and the albumin was then eluted with 3 bed volumes of 0.05 M Tris-1.5 M KCl, pH 7.5. The albumin was concentrated on an Amicon stirred cell concentrator with a PM 30 membrane and desalted by washing with 0.05 M Tris-0.1 M KCl, pH 7.5. The final volume of the albumin preparation was approximately 9 ml. Albumin concentrations were measured by a bromocresol green method (Instrumentation Laboratory, 1978) and the purity of the preparation was determined by protein electrophoresis on cellulose acetate (Gebott, 1975).



BUFFER 1: 0.05 M Tris-0.1 M KCl, pH 7.5
BUFFER 2: 0.05 M Tris-1.5 M KCl, pH 7.5

Figure 6. Isolation of albumin

2.03.2 DPHN-Protein Binding Kinetics

DPHN-protein binding was measured by equilibrium dialysis at 22°C or 37°C. Whole serum or plasma was incubated with 30-75 ug per ml of drug and the purified albumin preparations were incubated with 5-15 ug per ml of drug. A reduced drug concentration was employed in the purified albumin preparations because of their lower albumin concentrations. The ratio of drug to albumin was thus similar to that used in the studies of whole serum or plasma. The binding data were analyzed using Scatchard (1949) and Lineweaver-Burk double reciprocal (Anton and Corey, 1971) techniques. Scatchard graphs were prepared by plotting R/D versus R where R is the molar concentration of drug bound per mole of albumin and D is the molar concentration of unbound drug. The apparent affinity constant (K_{app}) for the binding of drug to protein was obtained from the slope of the graph (-k) and the number of binding sites (n) was obtained from the y intercept (nK_{app}). Double reciprocal graphs were prepared by plotting 1/R versus 1/D. The apparent affinity constant was obtained from the slope of the line ($-1/nK_{app}$) and the number of binding sites from the y intercept (1/n).

2.03.3 Circular Dichroism Studies

Circular dichroism measurements were made on a Jasco J-500 A spectropolarimeter, calibrated with D-camphor-sulphonic acid prior to use. Purified albumin from normal and uremic plasma was diluted to 20 mg/ml in 0.1M phosphate buffer, pH 7.4, and the spectra measured from 200-250 nm

using 25 mm cells. The molar ellipticity (0) in degrees x cm^2 x $dmole^{-1}$ was calculated according to the following equation.

$$(0) = \frac{O \times M}{C \times L \times 10}$$

O is the observed ellipiticity in degrees, M is the molecular weight in g (66,200 for albumin), C is the concentration in g/ml and L is the path length in cm.

2.03.4 Cross-Over Experiments

Seven ml aliquots of pooled plasma from normal subjects and from patients with chronic renal failure were centrifuged in duplicate in Amicon Centriflo (CF 50A) membrane cones at 1,000 G for 1.5 h, producing approximately 4 ml of ultrafiltrate. The protein and ultrafiltrate from one of the normal samples were remixed, as were the protein and ultrafiltrate from one of the uremic samples. Protein and ultrafiltrate from the remaining tubes were interchanged such that normal protein was mixed with uremic ultrafiltrate and uremic protein was mixed with normal ultrafiltrate. The four samples were thoroughly mixed and allowed to equilibrate for 1 h, then aliquots were taken for the determination of albumin (Instrumentation Laboratory, 1978) and creatinine (Worthington, 1976). DPHN-protein binding was measured by cone membrane ultrafiltration.

2.03.5 In Vitro Dialysis of Uremic Plasma

Six ml aliquots of pooled plasma from patients with chronic renal failure and from normal subjects were dialyzed for 48 h against 2,000 ml of deionized water with at least 4

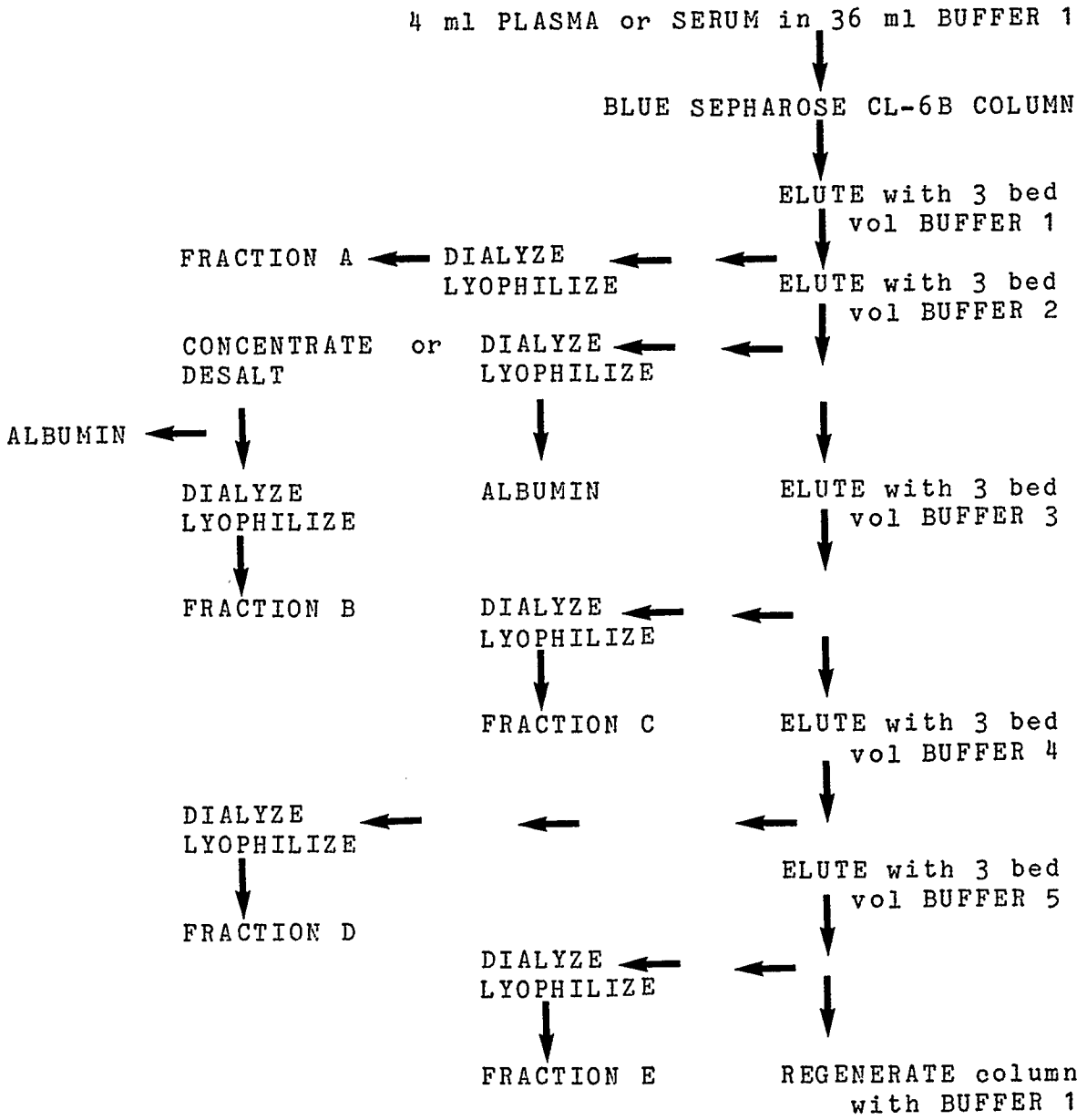
changes of the dialysis fluid. Albumin (Instrumentation Laboratory, 1978), creatinine (Worthington, 1976) and DPHN-protein binding (equilibrium dialysis at 37°C) were measured in the samples before and after dialysis.

2.03.6 Elution by Ultrafiltration

Four ml aliquots of pooled plasma from patients with renal failure and from normal subjects were diluted with 100 ml of buffer. Buffers used were 0.05 M Tris-0.1 M KCl, pH 7.5, 0.05 M Tris-1.5 M KCl, pH 7.5, 0.05 M Tris-1.5 M KCl, pH 8.7 and 0.05 sodium citrate-0.1 M KCl, pH 3.0. The diluted plasma was concentrated and desalted using an Amicon stirred cell concentrator with an XM 50 membrane to a final volume of 4 ml. The final concentrates were analyzed for albumin (Instrumentation Laboratory, 1978) and creatinine (Worthington, 1976), and the DPHN-protein binding was measured by equilibrium dialysis at 37°C.

2.03.7 Blue Sepharose CL-6B

Plasma from patients with chronic renal disease was fractionated using Blue Sepharose CL-6B and the various fractions were tested for their inhibitory effect on DPHN-protein binding. The column procedure (Figure 7) was as described in section 2.08 with the following additions. After elution with the 0.1 M and the 1.5 M KCl Tris buffers, pH 7.5, the column was eluted with 100 ml of 0.05 M Tris-1.5 M KCl, pH 8.7, 100 ml of 0.05 M sodium citrate-1.5 M KCl, pH 3.0 and 100 ml of 25% ethylene glycol-0.05 M Tris-1.5 M KCl, pH 7.5. The 0.05 M Tris-1.5 M KCl, pH 7.5 eluant was



BUFFER 1 0.05 M Tris-0.05 M KCl, pH 7.5
BUFFER 2 0.05 M Tris-1.5 M KCl, pH 7.5
BUFFER 3 0.05 M Tris-1.5 M KCl, pH 8.7
BUFFER 4 0.05 M sodium citrate-1.5 M KCl, pH 3.0
BUFFER 5 25% ethylene glycol-0.05 M Tris-1.5 M KCl, pH 7.5

Figure 7. Blue sepharose CL-6B procedure for the isolation of ingibitor substances

concentrated and desalted on a PM 30 membrane to recover the albumin fraction. The resultant ultrafiltrate (fraction A), the 0.05 M Tris-0.1 M KCl, pH 7.5 eluant (fraction B), the 0.05 M Tris-1.5 M KCl, pH 8.7 eluant (fraction C), the 0.05 M sodium citrate-1.5 M KCl, pH 3.0 eluant (fraction D) and the 25% ethylene glycol 0.05 M Tris-1.5 M KCl, pH 7.5 eluant (fraction E) were dialyzed against 40 volumes of deionized water for 48 h (4 changes of the dialysis bath) and then lyophilized. The lyophilized residues were redissolved in 4 ml 0.05 M Tris-0.1 M KCl, pH 7.4 and the absorbance of each was measured over 230 to 300 nm. Dialysis was performed using conventional cellulose tubing (Fisher Chemicals) and benzyolated cellulose tubing (Sigma Chemical Co.).

One ml volumes of normal plasma were mixed with equal volumes of fractions A, B, C, D and E. Normal plasma diluted with equal volumes of phosphate buffer served as the reference containing no inhibitor. Plasma from normal subjects, plasma from patients with chronic renal failure and albumin isolated from uremic plasma were diluted to equal albumin concentrations with phosphate buffer, to verify that the inhibitior had been removed by the column procedure. DPHN-protein binding was measured using equilibrium dialysis at 37°C.

2.03.8 Extraction of Nonesterified Fatty Acids

Plasma from patients with chronic renal failure, and plasma from normal subjects was extracted with an isopropanol-heptane-H_2SO_4 mixture, used in the determination

of nonesterified fatty acids (Kelley, 1965). Ten ml of extraction mixture (40 vol isopropanol, 10 vol heptane, 1 vol 1 N H_2SO_4) was added to 2 ml plasma in a glass stoppered tube. After vigorous shaking for a few seconds the tube was left standing for 10 min or longer. Three ml each of water and heptane were then added to separate the mixture into 2 phases. The top layer was transferred into a glass tube and the contents evaporated to dryness under a stream of air, in a 37°C water bath. The fatty acids were redissolved in ethanol and 0.1 M K_2HPO_4 and mixed with normal plasma. DPHN-protein binding was measured by equilibrium dialysis at 37°C.

2.03.9 Charcoal Adsorption of Uremic Plasma

Six ml aliquots of plasma from patients with chronic renal failure and from normal subjects were treated with activated charcoal (Norit A, Fisher Chemicals) using a modification of the method described by Craig et al (1976). Fifty mg charcoal was added for each ml of plasma, the pH adjusted to 7.4 if necessary, the mixture vortexed frequently over 4 h at room temperature, and then incubated at 4°C overnight. The charcoal was removed by centrifugation at 25,000 G for 60 min. Albumin (Instrumentation Laboratory, 1978), creatinine (Worthington, 1976) and DPHN-protein binding (equilibrium dialysis at 37°C) were measured before and after charcoal treatment. Charcoal adsorption was also examined at pH 3.0 and 8.7. Charcoal used in the treatment of uremic plasma was termed "uremic" charcoal, and charcoal

used in the treatment of normal plasma was termed "normal" charcoal.

The charcoal pellet was suspended in 3 ml of ethanol:water (1:1 vol), the pH adjusted to 7.4 if necessary, the mixture vortexed frequently over 4 h incubation at 37°C, then incubated at 4°C overnight. The charcoal was removed by centrifugation at 25,000 G for 30 min. A second extraction of the charcoal was performed, omitting the overnight incubation at 4°C. The ethanol:water extracts were combined, dried at 37°C under a stream of air, and the residue dissolved in 0.2 ml of 0.1 M K_2HPO_4, pH 7.4. Extracts obtained from charcoal used in the adsorption of uremic plasma were termed "uremic" extracts, and extracts obtained from charcoal used in the adsorption of normal plasma were termed "normal" extracts. Ultraviolet spectra were examined and the effect upon DPHN-protein binding was measured by adding the redissolved extracts to 1.5 ml normal plasma and measuring DPHN-protein binding by equilibrium dialysis at 37°C.

The extracts, redissolved in 0.1 M K_2HPO_4 were subjected to ultrafiltration through Amicon membranes of specific molecular weight retention, using distilled water as the eluant. Membranes used included YM 5 (5,000 M.W. retention), UM 2 (2,000 M.W. retention) and UM 05 (500 M.W. retention). The concentrated residue retained by each membrane and the lyophilized ultrafiltrate (reconstituted in 0.2 ml H_2O) were added to normal plasma and the effect upon

DPHN-protein binding was measured by equilibrium dialysis at 37°C.

The extracts were also applied to a Sephadex G 25 column (Pharmacia superfine, 50 ml bed volume, flow rate 3 ml/min, 5 mM K_2HPO_4 eluant). The absorbance at 280 nm was monitored and 4 ml fractions were collected. These were lyophilized and tested for inhibitory effect on DPHN binding to normal plasma proteins.

2.04 DATA TREATMENT

Analysis of data was performed using a PDP 11/45 computer and programs written in Basic-plus language. Statistical analysis involved mean and standard deviation, Student's t-test (by group or paired data, as applicable), and correlation coefficient (Dixon and Massey, 1969). Shelby's (1969) standard mathematical tables were used.

Computer programs were written to calculate the best straight line (least squares fit), and the apparent affinity constant and number of binding sites from the Scatchard and double reciprocal studies.

PART III : RESULTS

3.01 METHODOLOGY

3.01.1 Equilibrium Dialysis

The equilibrium dialysis procedure using the acrylic cells was easily performed as compared to conventional procedures employing dialysis tubing. The exogenous DPHN was found to equilibrate in the sample within 30 min at room temperature, as verified by measurements of DPHN-protein binding after various sample incubation times. However, a two h sample incubation was chosen to insure sample equilibration in all instances. The ^{14}C DPHN showed no significant deterioration when stored at $-4^{o}C$ for up to 12 months. The concentration of exogenous DPHN was verified to be 15 ug/ml by gas liquid chromatography. Measurement of DPHN-protein binding in commercial albumin solutions, buffered to pH 6.4, 7.4 and 8.4, showed that reasonable fluctuations in the pH of the sample did not affect the analysis of binding. Figure 8 shows the percent free DPHN measured in a normal sample with various dialysis times. Equilibrium appeared to be complete in 12 h. However, the procedure was routinely allowed to proceed overnight for 16-20 h to insure equilibrium conditions in all instances. The measured DPHN-protein binding in 23 healthy volunteer subjects was 93.1 ± 0.4% by equilibrium dialysis at $22^{o}C$ (Table VI).

3.01.2 Membrane Ultrafiltration

The membrane cone ultrafiltration procedure described here was easily performed as compared to others utilizing knotted dialysis tubing. The time required for the

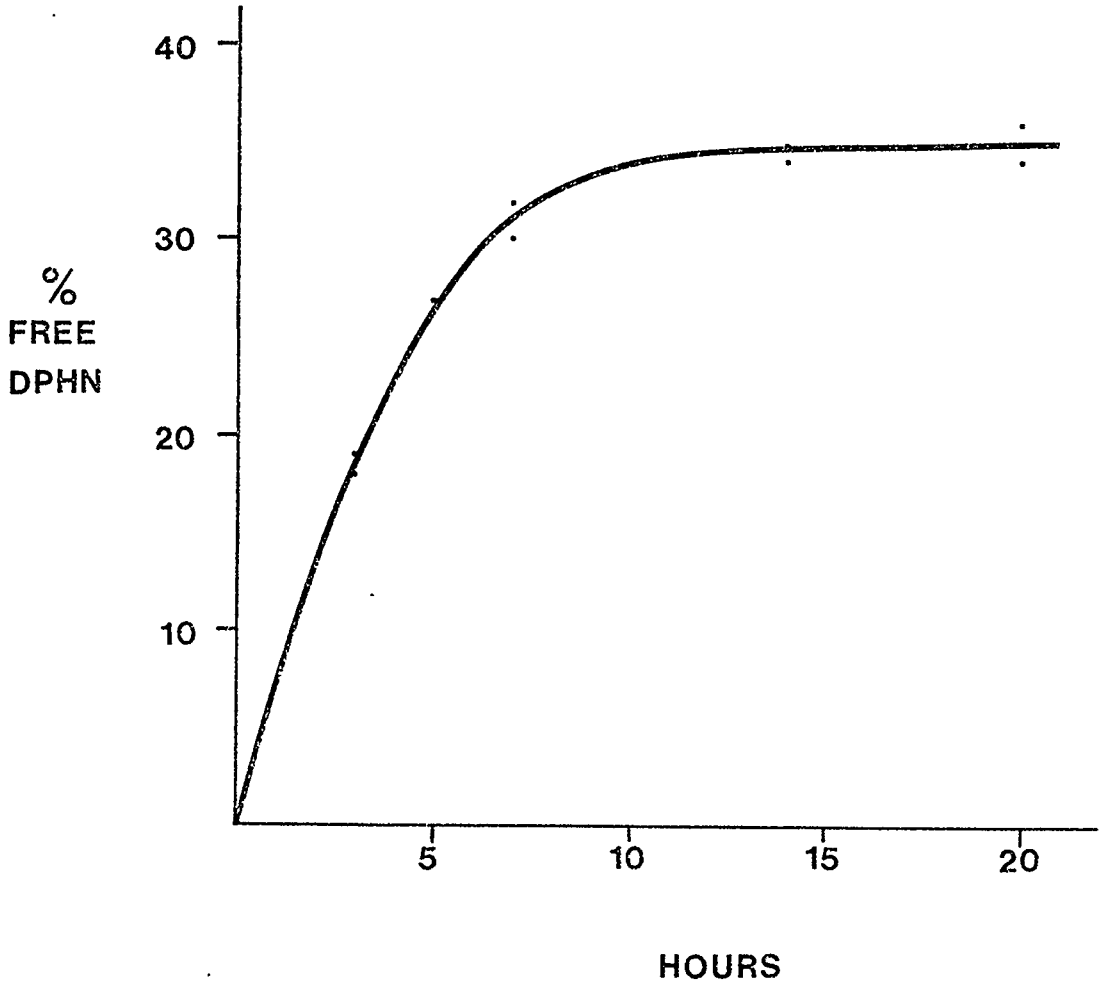


Figure 8. The effect of dialysis time on the measurement of DPHN-protein binding. The points are the results of duplicate measurements.

TABLE VI

DPHN-PROTEIN BINDING IN CONTROL SUBJECTS
CONE ULTRAFILTRATION vs EQUILIBRIUM DIALYSIS

	% PROTEIN BOUND DPHN
CONE ULTRAFILTRATION	94.5 ± 0.4
EQUILIBRIUM DIALYSIS	93.1 ± 0.4

DPHN-protein binding was measured in plasma or serum from 23 control patients using cone ultrafiltration and equilibrium dialysis at 22°C. Samples were spiked with DPHN (15 ug/ml).

analysis of a sample in duplicate was less than 3 h including the sample preparation and the determination of the radioactivity. As many as 36 duplicate estimations could be made in one day. In 36 estimations of a prepared sample, over 5 separate assays, the mean protein binding of DPHN was 94.4 ±0.5% at 22°C (Table VII). The measured DPHN-protein binding in 23 healthy volunteer subjects was 94.5 ± 0.4% by cone ultrafiltration at 22°C (Table VI).

Initially a significant problem with protein leakage into the ultrafiltrate was experienced. However, it was discovered that the protein leakage was not via the membrane pores but rather via leaks at the adhesive seam in the cone. When the cones were handled with greater care, especially in the seating of the cone into the support, the problem was resolved. As determined by the passage of ^{125}I albumin into the ultrafiltrate and from Lowry protein estimations (Lowry et al, 1951), the protein leakage through the membrane cones was less than 1% in almost all cases. As a precaution, all estimations by cone ultrafiltration were performed in duplicate and any sample with a greater than 10% variation in duplicate measurements was reanalyzed.

Binding of DPHN to the membrane cone was observed upon ultrafiltration of phosphate buffer containing the drug. An apparent binding of as much as 20% was observed, which suggested that the cones would be unsuitable for the measurement of drug-protein binding. However, when plasma or serum containing DPHN was ultrafiltered there was no

TABLE VII

BETWEEN RUN REPRODUCIBILITY OF
CONE ULTRAFILTRATION METHOD

	PERCENT BOUND DPHN				
ASSAY	1	2	3	4	5
	94.5	93.7	94.6	93.9	94.5
	94.8	94.3	94.4	94.2	94.5
	95.0	94.6	94.3	94.3	95.2
	94.6	94.9	94.0	94.1	95.0
	94.7	94.4	93.6	94.4	95.0
	95.1	94.2	93.6	93.9	94.8
	94.4		93.9		95.1
	94.3		93.7		95.1
	MEAN = 94.4 $\pm$ 0.5 % BOUND DPHN				

DPHN-protein binding was measured in a control sample, using cone ultrafiltration at room temperature. 36 estimations were made over 5 assays. The sample was spiked with DPHN (15 ug/ml).

significant binding of the drug to the cone. This effect may be explained by the presence of large concentrations of salts and proteins in the plasma and serum, which also bind to the membrane cone and inhibit the binding of DPHN.

The effect of the centrifugation time and the resultant ultrafiltrate volume on the measured percent free DPHN is shown in Figure 9. At shorter centrifugation times and at smaller ultrafiltrate volumes there was a sharp rise in the observed measurement of unbound DPHN, probably as a result of dilution of the ultrafiltrate by water trapped within the membrane. The binding of drug to the membrane may also be a significant factor at low ultrafiltrate volumes and tend to decrease the measured free drug level at shorter centrifugation times. Beyond the plateau region, at prolonged centrifugation times producing ultrafiltrate volumes of greater than 50% of the original sample volume, there was an increase in the measured percent free DPHN and a greater scatter of the estimates about the mean. Excessive concentration of the protein in the sample and protein leakage through the cone occurred in this region of the graph. A centrifugation time of 12 min was chosen to achieve the plateau region as soon as possible and to avoid the increased variance of the estimates at longer centrifugation times.

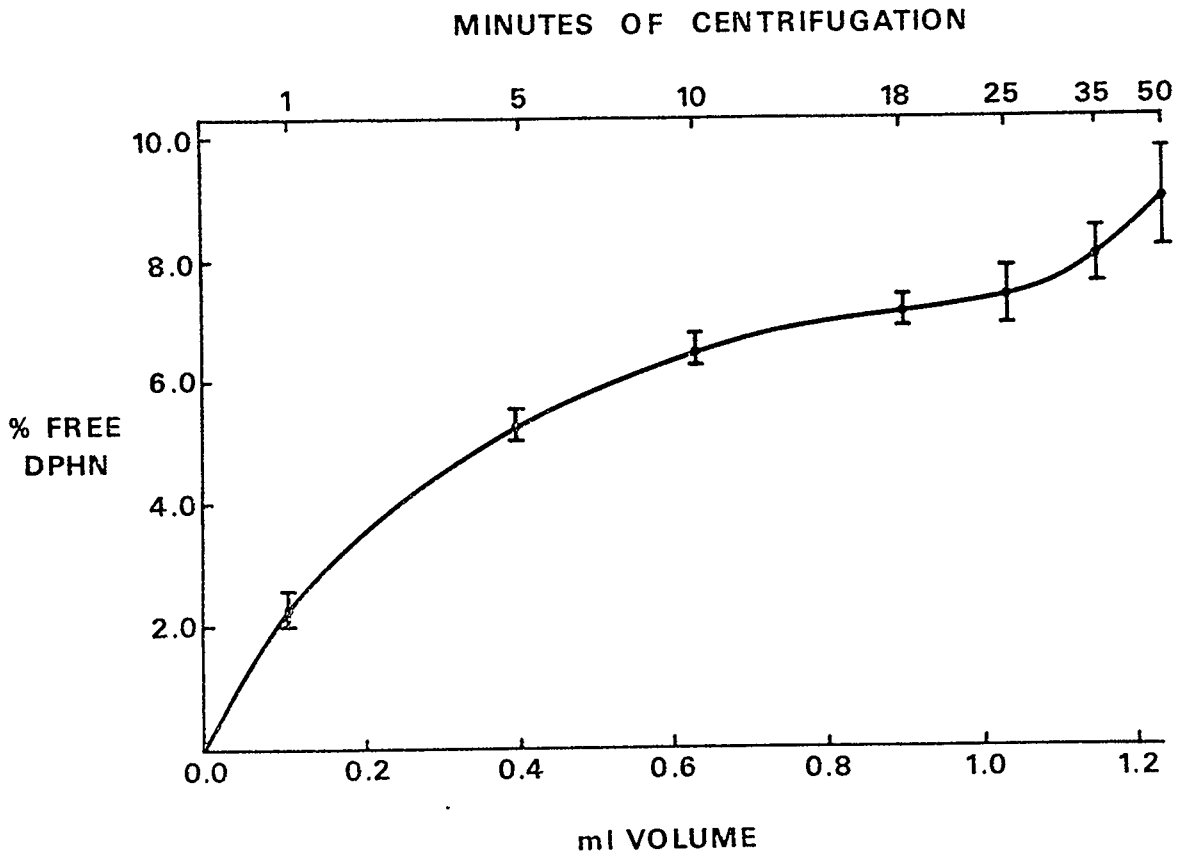


Figure 9. The effect of centrifugation time and the resultant ultrafiltrate volume on DPHN-protein binding.

TABLE VIII

DPHN-PROTEIN BINDING AT 22°C
IN CONTROL AND UEMIC PLASMA POOLS

	CONE ULTRAFILTRATION % UNBOUND	EQUILIBRIUM DIALYSIS % UNBOUND	CRE mg/dl	ALB g/dl
CONTROL POOL 1	7.0	8.2	1.0	3.7
CONTROL POOL 2	6.8	7.7	1.1	3.5
UREMIC POOL 1	13.1	15.7	9.4	3.6
UREMIC POOL 2	14.4	17.8	7.9	3.5
UREMIC POOL 3	13.6	17.5	7.7	3.7
UREMIC POOL 4	13.1	15.8	10.0	3.7

DPHN-protein binding was measured in uremic and normal samples using cone ultrafiltration and equilibrium dialysis at 22°C. Samples were spiked with DPHN (15 ug/ml).

3.02 CLINICAL SIGNIFICANCE OF DPHN-PROTEIN BINDING

3.02.1 DPHN-Protein Binding in Renal Failure

Table VIII shows the levels of free DPHN in pooled serum or plasma from control subjects and from persons with renal failure, measured by equilibrium dialysis and membrane cone ultrafiltration at 22°C. Estimations of DPHN-protein binding made by the classical method of equilibrium dialysis were found to correlate well with those obtained by the cone ultrafiltration method. The level of unbound DPHN in patients with renal failure was almost twice that of the control pools. Albumin concentrations were not significantly different between the two groups.

3.02.2 Temperature and DPHN-Protein Binding

Table IX further illustrates the defect in DPHN-protein binding in renal disease and shows the effect of temperature on the estimation of DPHN-protein binding. The table compares the unbound DPHN levels in control pools versus uremic pools (uremia verified by creatinine) as measured by equilibrium dialysis at 37°C. Hypoproteinemia was not present in either group. DPHN-protein binding was reduced at 37°C as compared to measurements at 22°C such that the unbound DPHN at 37°C was approximately twice as great as that measured at 22°C. Membrane cone ultrafiltration was not performed at 37°C since a suitable temperature-controlled centrifuge was not available.

3.02.3 Protein Concentration and DPHN-Protein Binding

Figure 10 shows the effect of dilution upon free

TABLE IX

DPHN-PROTEIN BINDING AT 37°C
IN CONTROL AND UREMIC PLASMA POOLS

	EQUILIBRIUM DIALYSIS % UNBOUND	CRE mg/dl	ALB g/dl
CONTROL POOL 3	14.1	1.0	4.4
CONTROL POOL 4	15.1	1.0	4.3
UREMIC POOL 4	24.3	10.0	3.7
UREMIC POOL 5	23.5	10.0	3.7
UREMIC POOL 6	21.1	8.2	4.1

DPHN-protein binding was measured in uremic and normal serum and plasma using equilibrium dialysis at 37°C. Samples were spiked with DPHN (15 ug/ml).

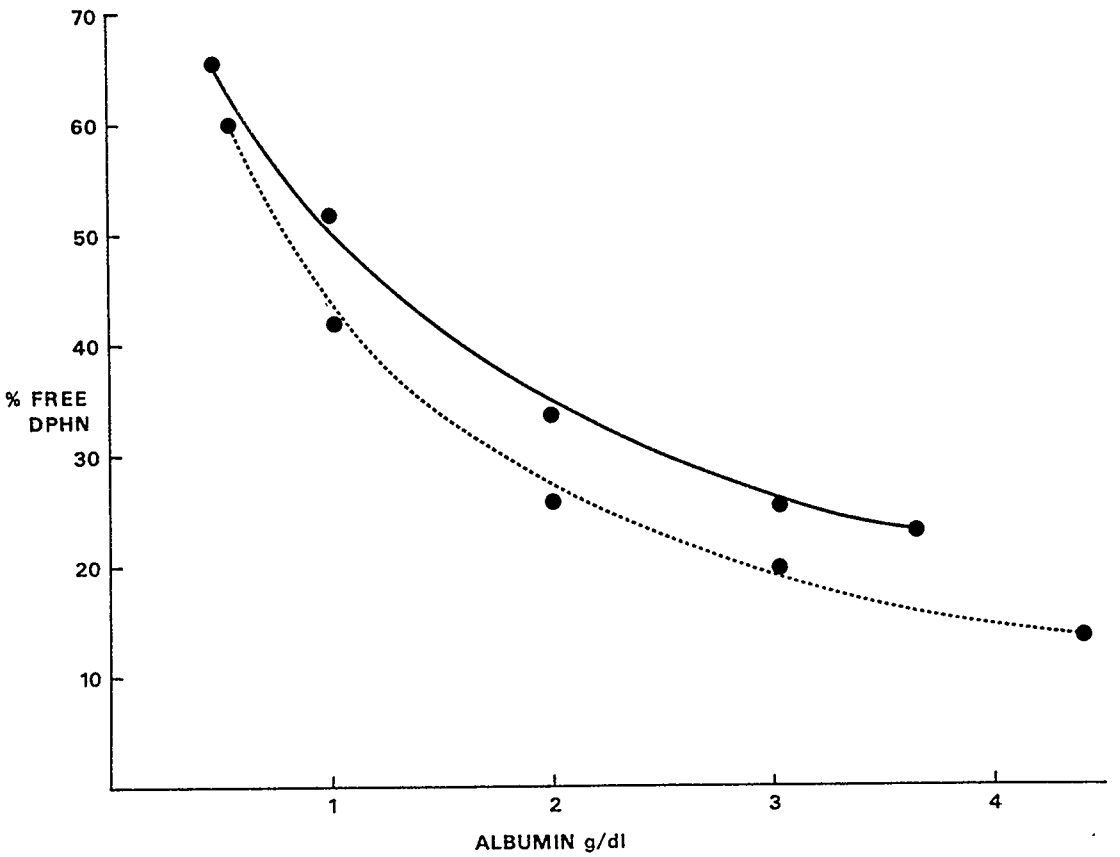


Figure 10. Effect of dilution on DPHN-protein binding in normal plasma (...) and in uremic plasma (____).

DPHN levels measured at 37°C in control plasma and in plasma from patients with renal failure. The resulting curves indicate a greater percent free DPHN concentration for patients with renal failure at all dilutions studied (0.5 - 4.0 g/dl).

3.02.4 DPHN-Protein Binding in Specific Individuals

Table X shows the percent unbound DPHN levels in 12 individuals with chronic renal failure requiring hemoperfusion dialysis and in 15 healthy volunteers. DPHN-protein binding was measured by equilibrium dialysis at 37°C. The unbound DPHN ranged from 17.1% to 26.7% (mean 21.3 ± 2.5%) in the patients with chronic renal failure and did not correlate with the plasma albumin or creatinine concentration. The unbound DPHN ranged from 12.2% to 13.9% (mean 13.1 ± 0.6%) in the healthy volunteers and did not correlate with the plasma albumin or creatinine concentration. When all the samples were considered there was no correlation between the unbound DPHN and the albumin concentration, but the unbound DPHN did correlate with the creatinine concentration ($p < .001$).

3.02.5 Hemodialysis and DPHN-Protein Binding

Table XI shows the mean percent unbound DPHN and the mean albumin concentration in 18 patients with chronic renal failure, before and after *in vivo* hemoperfusion dialysis. DPHN-protein binding was measured by equilibrium dialysis at 37°C. The effectiveness of the dialysis treatment was verified by plasma creatinine concentrations, which were decreased after dialysis. The unbound DPHN was

TABLE X

DPHN-PROTEIN BINDING AT 37°C IN CONTROL SUBJECTS vs PATIENTS WITH RENAL FAILURE

PATIENT		CRE mg/dl	ALB g/dl	% UNBOUND DPHN
RENAL	421	15.8	4.4	22.1
	345	11.5	4.0	21.7
	325	6.6	3.2	21.4
	423	17.8	4.2	21.1
	344	10.4	4.2	21.2
	323	8.6	3.0	26.7
	346	16.6	3.6	22.9
	324	2.4	2.9	18.3
	495	5.3	4.4	18.4
	422	12.9	3.9	22.8
	343	14.0	4.2	21.3
	424	9.1	5.2	17.1
			MEAN	21.3 ±2.5
CONTROL	1	1.0	4.5	13.8
	2	1.0	4.4	12.2
	3	1.1	4.0	12.7
	4	1.0	3.9	13.3
	5	1.2	4.1	13.9
	6	0.9	4.1	13.2
	7	1.0	3.7	12.5
	8	1.2	4.6	13.7
	9	0.8	4.1	12.9
	10	1.0	4.3	13.1
	11	0.9	4.4	13.8
	12	0.9	4.5	13.0
	13	1.0	4.5	12.2
	14	1.2	4.6	12.5
	15	1.0	4.0	13.3
			MEAN	13.1 ±0.6

DPHN-protein binding was measured in individual patients with chronic renal failure and in normal subjects, using equilibrium dialysis. Samples were spiked with DPHN (15 ug/ml).

TABLE XI

DPHN-PROTEIN BINDING
BEFORE AND AFTER HEMODIALYSIS

PATIENT	PRE-DIALYSIS ALB g/dl	PRE-DIALYSIS CRE mg/dl	PRE-DIALYSIS DPHN % free	POST-DIALYSIS ALB g/dl	POST-DIALYSIS CRE mg/dl	POST-DIALYSIS DPHN % free
W.I.	4.2	9.7	21.5	4.0	5.2	23.0
P.A.	3.4	14.7	24.9	4.0	9.7	19.6
D.E.	3.9	13.4	25.2	5.0	7.7	16.8
S.C.	2.7	13.0	22.1	2.6	6.6	23.9
C.A.	2.7	10.8	24.6	3.9	7.3	21.5
K.I.	3.4	10.6	23.7	3.8	6.4	19.3
C.H.	3.1	10.4	29.5	3.8	4.4	23.0
S.P.	3.5	10.7	27.9	4.6	7.5	19.7
L.A.	2.5	9.7	24.3	4.8	7.1	20.6
S.T.	3.6	13.3	22.7	4.1	8.9	24.4
A.T.	3.3	12.8	24.0	3.6	6.8	22.9
S.C.	2.9	10.5	30.6	3.0	3.7	27.6
A.G.	3.7	9.2	19.8	4.0	4.5	18.7
D.A.	3.8	11.3	23.0	4.0	7.0	23.2
H.I.	3.2	15.6	25.4	4.6	8.9	19.9
R.E.	4.1	10.2	28.1	3.2	5.7	28.9
K.E.	3.6	13.1	27.0	4.0	7.6	28.2
N.J.	2.9	9.5	32.4	2.9	6.9	30.6
MEANS	3.4 ±0.5	11.6 ±1.9	25.4 ±3.3	3.9 ±0.7	6.8 ±1.6	22.9 ±3.9

DPHN-protein binding was measured in 18 patients before and after hemoperfusion dialysis, using equilibrium dialysis at 37°C. Samples spiked with DPHN (15 ug/ml).

significantly decreased in the post-dialysis samples (22.9% post, 25.4% pre, $p < .01$), and the albumin concentration was significantly increased in the post-dialysis samples (3.9 g/dl post, 3.4 g/dl pre, $p < .01$). The unbound DPHN did not correlate with the plasma albumin or creatinine concentration in either the pre-dialysis or the post-dialysis group. There was a significant correlation ($p < .01$) between the unbound DPHN and the albumin concentration for the entire group, pre-dialysis and post-dialysis.

3.02.6 Kidney Transplantation and DPHN-Protein Binding

Figure 11 shows the plasma albumin concentration, the plasma creatinine concentration and the DPHN-protein binding ability, measured in a patient before and after a kidney transplant. The creatinine level was greatly elevated prior to the transplant (18.0 mg/dl) and fell to near normal (2.0 mg/dl) within 2 days after the transplant. The unbound DPHN was elevated prior to the surgery (18.5%) and gradually decreased over the post operative period. Twenty-three days after the transplant the percent free DPHN had not yet reached the normal range. The creatinine and the albumin concentration were within normal limits and the transplanted kidney was functioning well with no signs of rejection.

Table XII shows the percent unbound DPHN, the plasma creatinine concentration and the plasma albumin concentration measured in 20 patients who had received kidney transplants. Half of the patients examined had free DPHN levels greater than that measured in normal controls (greater

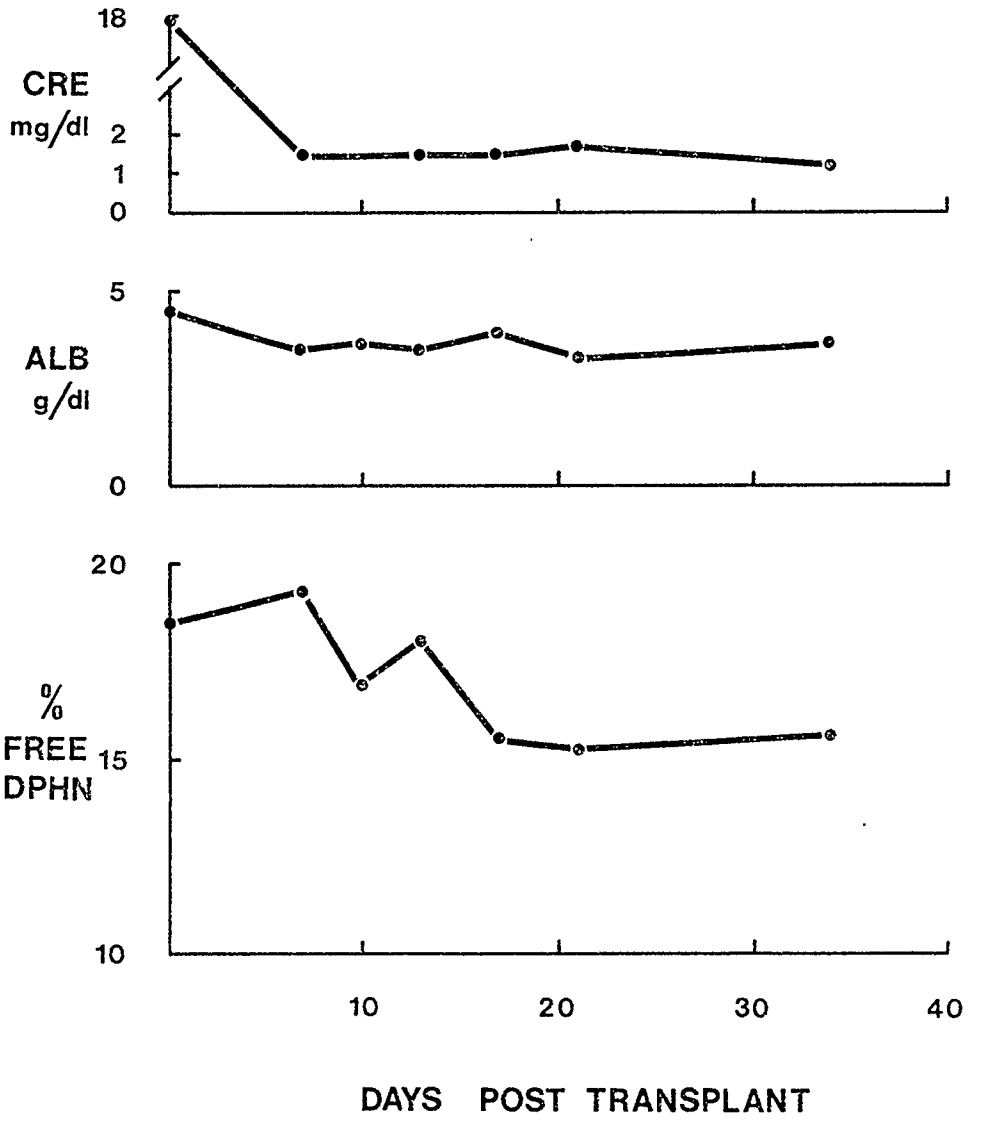


Figure 11. The effect of kidney transplantation upon DPHN-protein binding. DPHN-protein binding was measured by equilibrium dialysis at 37°C. Samples were spiked with DPHN (15 ug/ml).

TABLE XII

DPHN-PROTEIN BINDING
AFTER RENAL TRANSPLANTATION

PATIENT	POST TRANSPLANT months	CRE mg/dl	ALB g/dl	% UNBOUND DPHN
M.L.	6	1.9	4.2	16.1
R.H.	30	1.2	4.2	13.5
G.T.	95	2.6	4.1	15.2
W.C.	34	1.4	4.4	14.9
J.C.	10	1.6	4.0	16.8
S.C.	35	3.0	4.7	13.9
C.D.	42	1.1	4.3	15.2
D.L.	31	1.6	4.5	13.5
B.S.	44	1.4	4.3	13.4
L.D.	34	1.0	3.8	14.8
D.S.	53	2.0	4.5	12.5
D.H.	2	1.2	3.6	15.6
J.M.	137	1.4	4.6	12.7
P.W.	15	1.4	4.3	13.3
D.M.	96	2.1	3.9	15.8
M.C.	44	1.1	5.0	10.7
A.E.	55	1.3	4.3	12.0
K.M.	73	1.7	4.4	13.9
W.D.	13	1.4	4.0	15.6
A.W.	41	.6	4.3	15.6

DPHN-protein binding was measured (equilibrium dialysis 37^{o}C) in patients who had received a kidney transplant. Samples were spiked with DPHN (15 ug/ml).

than 14.0%, mean plus 2 S.D.), and one patient had an unbound DPHN level that was less than normal (less than 11.9%, mean minus 2 S.D.). The percent unbound DPHN did not correlate with the plasma creatinine concentration or the time since transplantation. The percent unbound DPHN did correlate with the albumin concentration ($p < .001$). The medications taken by each patient were reviewed. These included azathioprine and prednisone for all patients, as well as hydrochlorothiazide, propranolol, methyldopa, prazosin, clonidine, calcium, potassium, estrogenic preparations and antacid preparations, taken by some of the patients. No correlation between medications and DPHN-protein binding could be identified. Neither was there any correlation between DPHN-protein binding and the physician's assessment of the patient's clinical progress.

3.03 CHARACTERIZATION OF DPHN-PROTEIN BINDING INHIBITION

3.03.1 Isolation of Albumin

The purification of albumin was easily accomplished using the affinity column procedure. Figure 12 shows a scan of the protein electrophoresis analysis performed on one of the purified albumin preparations. It is representative of the results for all of the preparations. A scan of whole serum shows the usual pattern of proteins. The purity of the albumin preparations, as calculated from protein electrophoresis, was greater than 90%. The recovery of albumin, as calculated from the albumin concentrations measured before

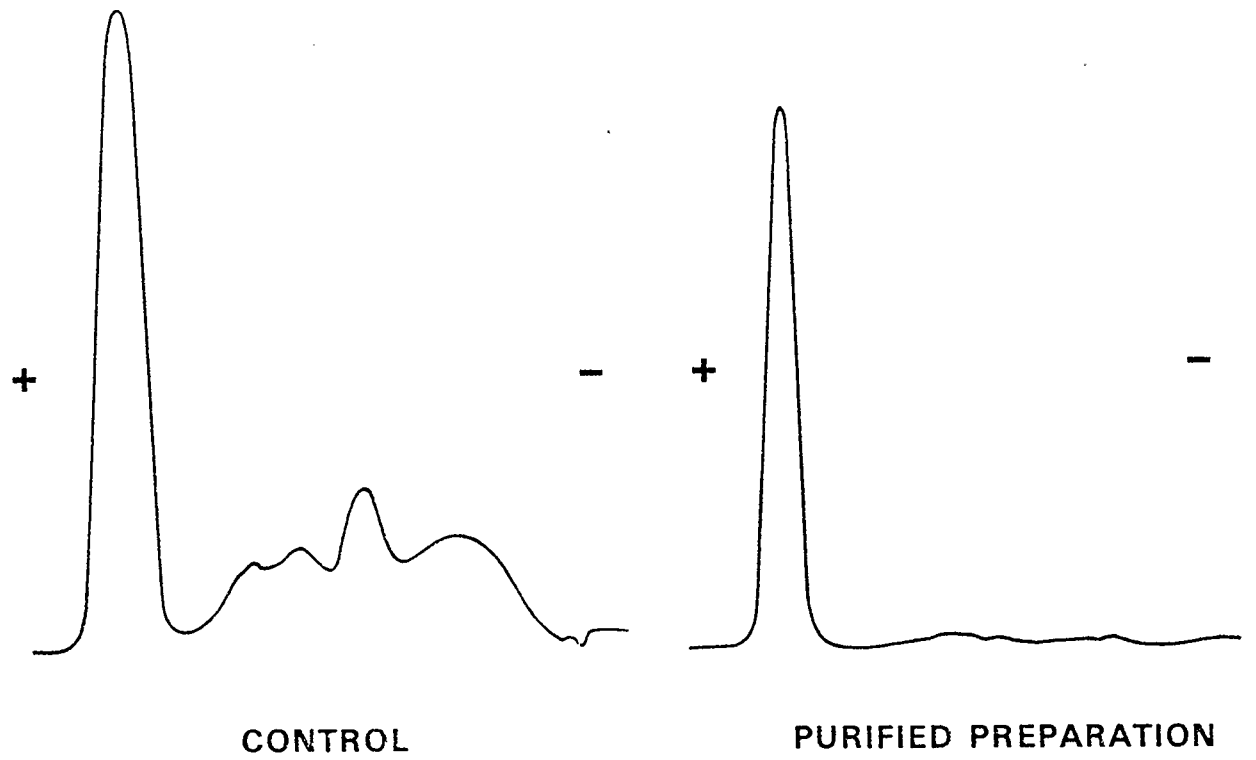


Figure 12. Protein electrophoresis of albumin isolated from serum using Blue Sepharose CL-6B. The control shows the normal pattern.

and after purification, averaged 80%.

3.03.2 Circular Dichroism

Circular dichroism spectra were recorded in the far ultraviolet region (200-250 nm) for the albumin preparations isolated from normal and uuremic plasma (Figure 13). The ellipiticity at this wavelength range is indicative of the secondary and tertiary protein structure. Figure 13 shows no significant difference between the circular dichroism spectra for the albumin preparations isolated from both sources.

3.03.3 DPHN-Protein Binding to Purified Albumin

No significant difference was observed in the binding of DPHN to albumin isolated from the control pools versus the pools collected from the patients with renal disease. Table XIII, which shows the binding of DPHN to various preparations of purified albumin, illustrates this observation. Comparison of the DPHN binding between the albumin isolated from the two sources was facilitated by the fact that the albumin concentrations between pairs of preparations were made equal.

3.03.3 Scatchard and Double Reciprocal Analysis

Figure 14 shows the double reciprocal graphs for the measurement of DPHN binding at 22°C and 37°C. Drug binding studies performed at 37°C produced a significantly greater slope than those performed at 22°C. All other measurements of DPHN binding were performed at 37°C unless otherwise stated.

Figure 15 shows the double reciprocal graphs for

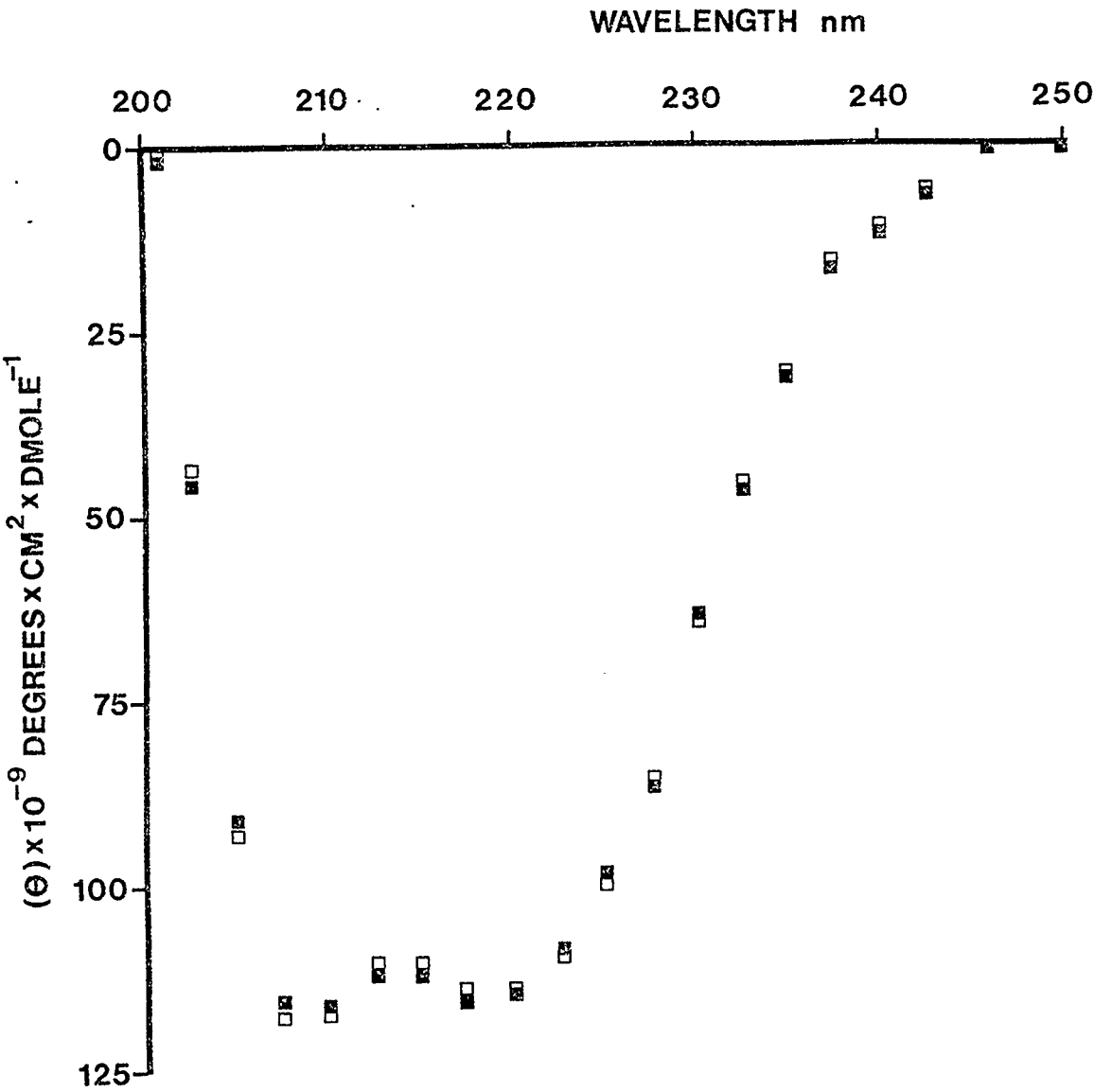


Figure 13: Circular dichroism spectra for albumin preparations from normal (□) and uremic plasma (■).

TABLE XIII

DPHN BINDING TO PURIFIED ALBUMIN

	ALB g/dl	% FREE DPHN
CONTROL POOL 4	0.82	49.1
UREMIC POOL 5	0.82	47.4
CONTROL POOL 3	1.04	41.2
UREMIC POOL 7	1.04	41.9
CONTROL POOL 5	0.97	45.6
UREMIC POOL 8	0.97	46.3

DPHN-protein binding was measured in albumin isolated from control and uremic pooled plasma, using equilibrium dialysis at 37°C. Samples were spiked with DPHN (3 ug/100 mg albumin).

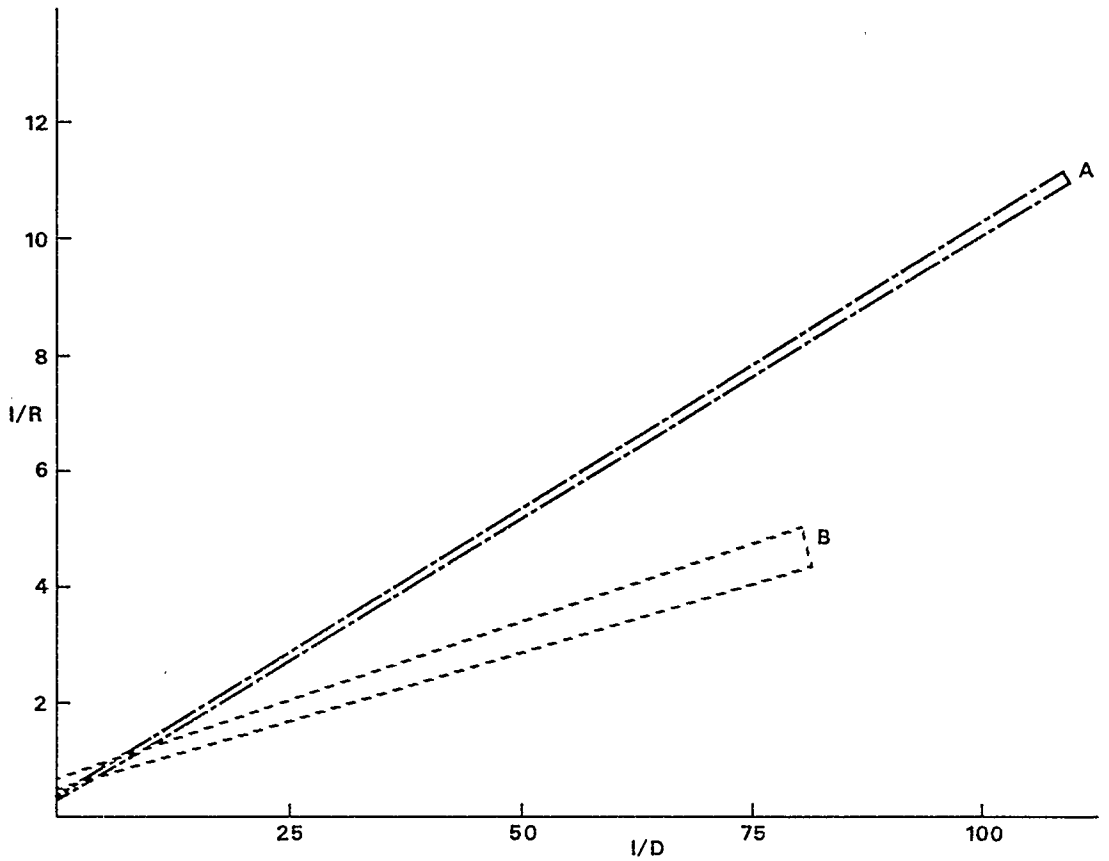


Figure 14. Double reciprocal graphs from the measurement of DPHN-protein binding at 22°C (B) and 37°C (A). R is the molar ratio of drug bound to albumin; D is the molar concentration of free drug.

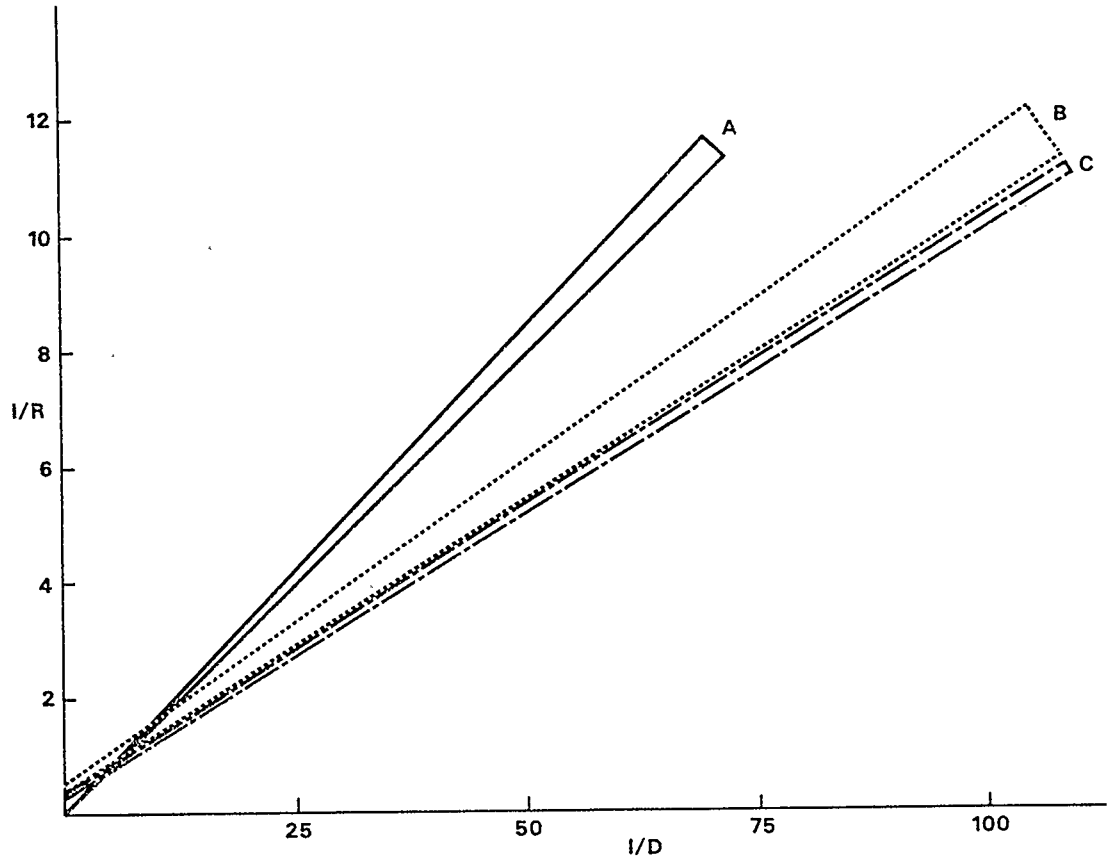


Figure 15. Double reciprocal graphs for DPHN-protein binding in whole serum or plasma from controls (C), from patients with renal disease (A), and in albumin isolated from both groups (B). R is the molar ratio of drug bound to albumin. D is the molar concentration of free drug.

the DPHN-protein binding in whole serum or plasma from controls and from patients with renal failure and to purified albumin from both groups. The graph shows the range of results for assays of at least three pools of plasma or serum. The decreased drug-binding ability of persons with renal disease is evident from the Figure. Whole plasma from patients with renal disease produced a family of lines with a significantly greater slope than those for whole plasma or serum from control groups. The range of results for DPHN-binding to purified albumin, from controls and from persons with renal disease, produced a family of lines with a slope somewhat greater than that for whole serum or plasma from control pools.

Table XIV gives the calculated apparent affinity constant and the number of binding sites as well as the product of the two, as determined by the Scatchard and Lineweaver-Burk double reciprocal analysis for the whole serum or plasma and purified albumin studies. Control whole serum or plasma had a higher apparent affinity (4.49×10^3 M^{-1}, Scatchard; 4.37×10^3 M^{-1}, double reciprocal) and slightly fewer binding sites (2.3, Scatchard; 2.4, double reciprocal) than the apparent affinity (2.03×10^3 M^{-1}, Scatchard; 2.21×10^3 M^{-1}, double reciprocal) and the number of binding sites (3.3, Scatchard; 3.0, double reciprocal) calculated for whole plasma from patients with renal disease. The apparent affinity and the number of binding sites as calculated for the purified albumin preparations were

TABLE XIV

SCATCHARD AND DOUBLE RECIPROCAL
ANALYSIS OF DPHN-PROTEIN BINDING

	K_{app}	n	nK_{app}
WHOLE SERUM OR PLASMA			
CONTROL			
SCATCHARD	4.49×10^3	2.3	10.3×10^3
DOUBLE RECIPROCAL	4.37×10^3	2.4	10.5×10^3
UREMIC			
SCATCHARD	2.03×10^3	3.3	6.7×10^3
DOUBLE RECIPROCAL	2.21×10^3	3.0	6.6×10^3
PURIFIED ALBUMIN			
CONTROL			
SCATCHARD	2.41×10^3	3.8	9.1×10^3
DOUBLE RECIPROCAL	2.18×10^3	4.2	9.1×10^3
UREMIC			
SCATCHARD	3.03×10^3	3.2	9.5×10^3
DOUBLE RECIPROCAL	3.10×10^3	3.1	9.4×10^3

Binding was determined by equilibrium dialysis at 37°C. Samples were spiked with DPHN (3 ug/100 mg alb.). Number of binding sites (n), apparent affinity constant (K_{app}), and the binding capacity (nK_{app}).

somewhat different for the control samples versus the samples from patients with renal disease. However, the nK_{app} products were nearly equal for both groups. The apparent affinity and the number of binding sites for the purified albumin preparations did not equal those obtained for the control whole serum or plasma but the nK_{app} products were again similar.

3.03.5 Cross-Over Experiments

Table XV illustrates the results of the cross-over experiments, in which protein and ultrafiltrate from normal and uremic plasma was interchanged to observe the effect on DPHN-protein binding. The ultrafiltration procedure itself did not alter the binding, as shown by the unbound DPHN levels measured in the remixed normal and uremic controls (normal: 8.2%, 7.6%; uremic: 12.3%, 12.5%). Similarly, the creatinine and albumin concentrations were not changed by the ultrafiltration (normal: cre 0.8 mg/dl, alb 4.3 g/dl; uremic: cre >10 mg/dl, alb 4.7 g/dl). Protein from normal plasma mixed with ultrafiltrate from uremic plasma did not have a defect in DPHN-protein binding (7.8%, 7.8% unbound DPHN), as compared to the remixed control samples; the albumin concentration (4.3 g/dl) was within the usual limits, but the creatinine concentration was elevated (4.2 mg/dl). Protein from uremic plasma mixed with ultrafiltrate from normal plasma did display a defect in the binding of DPHN (12.1%, 12.3% unbound DPHN) similar to that observed in the remixed renal control; the albumin concentration was only slightly

TABLE XV

EFFECT ON DPHN-PROTEIN BINDING OF INTERCHANGING PROTEIN AND ULTRAFILTRATE FROM NORMAL AND UREMIC PLASMA

	ALB g/dl	CRE mg/dl	% UNBOUND DPHN
NORMAL REMIX	4.3	0.8	8.2, 7.6
UREMIC REMIX	4.7	>10	12.3, 12.5
NORMAL PROTEIN	4.3	4.2	7.8, 7.8
UREMIC PROTEIN	4.3	7.6	12.1, 12.3

DPHN-protein binding was measured (equilibrium dialysis at 37oC) in samples of normal protein mixed with uremic ultrafiltrate, and in uremic protein mixed with normal ultrafiltrate. These were compared to controls prepared by remixing normal protein with normal ultrafiltrate, and uremic protein with uremic ultrafiltrate.

less than in the control, and the creatinine level was reduced to 7.6 mg/dl.

3.03.6 In Vitro Dialysis of Uremic Plasma

Aliquots of plasma from patients with chronic renal failure were dialyzed *in vitro* to determine if DPHN-protein binding inhibition might be due to a dialyzable substance. The procedure employed here was more extensive than that employed in *in vivo* hemoperfusion dialysis treatments and as such may be more effective in removing small molecular weight substances. Comparison of DPHN-protein binding before (29.5% unbound) and after (28.4% unbound) the dialysis did not indicate a significant improvement in drug binding, as compared to a normal control sample diluted to an equal albumin concentration (24.2% unbound). Measurement of the creatinine concentration before (12.2 mg/dl) and after (less than 1.0 mg/dl) the procedure did confirm the efficiency of the dialysis.

3.03.7 Elution by Ultrafiltration

Ultrafiltration of uremic plasma was performed in order to determine if an inhibitor substance might be removed by a more efficient elution process (compared to simple dialysis) employing a membrane with a permeability for substances less than 50,000 M.W. Buffers of various pH and ionic strength were used in an effort to overcome strong binding interactions between the inhibitor and albumin that may have prevented removal of the inhibition by simple dialysis. Table XVI shows the effects of the various

TABLE XVI

ULTRAFILTRATION OF UREMIC PLASMA

ELUANT	% UNBOUND DPHN PRE	POST	CONTROL
H_2O	20.3	21.2	12.5
.05 M TRIS-.1M KCl pH 7.4	21.0	19.3	12.5
.05 M TRIS-.1 M KCl pH 7.5 .05 M TRIS-1.5 M KCl pH 7.5	21.0	17.5	12.5
.05 M SODIUM CITRATE-.1 M KCl pH 3.0, .05 M TRIS-.1 M KCl pH 7.5	31.4	25.2	17.7
.05 M TRIS-1.5 M KCl pH 8.7, .05 M TRIS-.1 M KCl pH 7.5	31.4	26.4	17.7

DPHN-protein binding (equilibrium dialysis at 37°C) was measured in plasma from patients with chronic renal failure before and after ultrafiltration elution with various buffers. These were compared to a normal control plasma diluted to equal albumin concentration.

ultrafiltration procedures on DPHN-protein binding in plasma from patients with chronic renal failure. Drug binding to plasma proteins was measured before and after the ultrafiltration and compared to a normal control sample diluted to the same albumin concentration. From Table XVI it can be seen that while some improvement did occur with all eluants except distilled water, DPHN-protein binding was not improved to equal that observed for normal control samples of the same albumin concentration.

3.03.8 Blue Sepharose CL-6B

Albumin isolated from plasma obtained from patients with chronic renal failure had a normalized DPHN-protein binding as compared to albumin isolated from normal control plasma (Table XIII). For this reason considerable effort was made to recover an inhibitor substance from the Blue Sepharose CL-6B column. The various fractions required dialysis to remove the high concentrations of salts, prior to lyophilization and addition to normal plasma, in order to test for an inhibitory effect on DPHN-protein binding. Ultraviolet absorption measurements (200-250 nm) and Lowry et al (1951) protein estimations, performed on the reconstituted samples, showed that protein was eluted from the column. However, none of the fractions (after dialysis and lyophilization) conferred an inhibitory effect on the binding of DPHN to normal plasma proteins.

3.03.9 Effect of Nonesterified Fatty Acids

Nonesterified fatty acids extractions were per-

formed on plasma from patients with chronic renal failure in order to isolate non-polar substances which might have an effect on DPHN-protein binding. However, the extracts obtained from uremic plasma did not produce significantly different binding of DPHN to normal plasma proteins (25.2 % unbound), as compared to extracts from normal plasma (24.2 % unbound), and control normal samples diluted to the same albumin concentration (26.3 % unbound).

3.03.10 Charcoal Adsorption of Renal Plasma

Table XVII shows the DPHN-protein binding in plasma from patients with chronic renal failure and from normal controls, before and after charcoal treatment at pH 3.0 and at 7.4. Comparison of unbound DPHN levels was facilitated by measuring drug binding at equal albumin concentrations. DPHN-protein binding in uremic plasma was improved by charcoal treatment at pH 3.0, but not normalized. Charcoal treatment at pH 7.4, however, did improve the DPHN binding to uremic plasma proteins such that unbound DPHN levels after charcoal treatment were equal to that in control plasma.

Table XVIII shows the inhibitory effect associated with various ethanol:water extracts of charcoal, previously used in the adsorption of normal and uremic plasma. The table compares the efficiency of the extraction process at pH 3.0, 7.4 and 8.7, as reflected in the degree of drug binding inhibition conferred to normal plasma by each extract. The results show that minimal inhibition of DPHN-protein binding occurred with extractions performed at pH 3.0.

TABLE XVII

EFFECT OF CHARCOAL ADSORPTION ON DPHN-PROTEIN BINDING

	pH	% FREE DPHN PRE CHAR	% FREE DPHN POST CHAR
UREMIC	3.0	22.0	19.0
NORMAL	3.0	16.0	15.0
UREMIC	7.4	22.0	15.0
NORMAL	7.4	16.0	15.0

DPHN-protein binding was measured (equilibrium dialysis at 37oC) before and after charcoal adsorption at pH 3.0 and 7.4.

TABLE XVIII

EFFECT OF PH ON THE EXTRACTION OF DPHN-PROTEIN BINDING INHIBITORS FROM CHARCOAL

EXTRACTION PH	SOURCE OF CHARCOAL RESIDUE	EFFECT ON NORMAL PLASMA: %FREE DPHN
3.0	NORMAL PLAMSA	17.2
3.0	UREMIC PLASMA	16.7
7.4	NORMAL PLASMA	16.7
7.4	UREMIC PLASMA	21.8
8.7	UREMIC PLASMA	21.0

DPHN-protein binding was measured (equilibrium dialysis at 37oC) in normal plasma containing ethanol:water extracts from charcoal used in the adsorption of uremic and normal plasma.

However, at pH 7.4 and 8.7 ethanol:water extracts of charcoal, previously used in the adsorption of uremic plasma, conferred a significant degree of inhibition toward the binding of DPHN to normal plasma proteins, as compared to control extracts of charcoal previously used in the adsorption of normal plasma. Ethanol:water extractions at pH 7.4 were also preferable to extractions at pH 3.0 or 8.7 because visibly cleaner extracts occurred at pH 7.4.

Biochemical analysis (SMAC Technicon Corporation) of the normal and uremic plasma was performed before and after the charcoal treatment at pH 7.4 to observe the effect of the procedure on commonly measured substances in plasma (Table XIX). Little or no change occurred in sodium (Na), potassium (K), chloride (Cl), phosphate (PO_4), aspartate transaminase (AST), lactate dehydrogenase (LD), alkaline phosphatase (ALP) and cholesterol (CHOL). Moderate to large reductions in concentration were observed for total protein (TP), albumin (ALB), blood urea nitrogen (BUN), uric acid (URA), creatinine (CRE), bilirubin (BILI), calcium (Ca), and triglyceride (TRIG). These results are in agreement with those obtained by others (Craig et al, 1976).

Figure 16a shows the ultraviolet absorption spectra for the ethanol:water extracts (from the charcoal adsorption of normal and uremic plasma), measured over 250-325 nm, the region where maximum absorption occurs for peptitdes and proteins. The Figure indicates that a significantly greater amount of material (absorbing 250-325 nm) was adsorbed from

TABLE XIX

EFFECT OF CHARCOAL ADSORPTION ON BIOCHEMICAL PARAMETERS

TEST	RENAL PLASMA PRE CHAR	RENAL PLASMA POST CHAR	NORMAL PLASMA PRE CHAR	NORMAL PLASMA POST CHAR
Na	142	137	141	140
K	4.7	4.4	4.0	3.9
Cl	103	100	104	101
PO_4	4.6	4.7	2.7	2.7
AST	20	21	27	28
LD	183	175	203	189
ALP	136	125	53	52
CHOL	n/a	n/a	202	201
TP	6.4	5.6	7.0	6.5
ALB	3.8	3.5	4.5	4.3
BUN	75	62	16	11
URA	6.4	0.1	6.0	0.1
CRE	10.2	0.1	1.2	0.1
BILI	1.2	0.1	0.8	0.1
Ca	9.3	3.4	9.6	4.5
TRIG	144	98	141	124

Biochemical estimations (SMAC Technicon Corporation) were made on normal and uremic plasma, before and after charcoal adsorption at pH 7.4. Explanation of abbreviations in text.

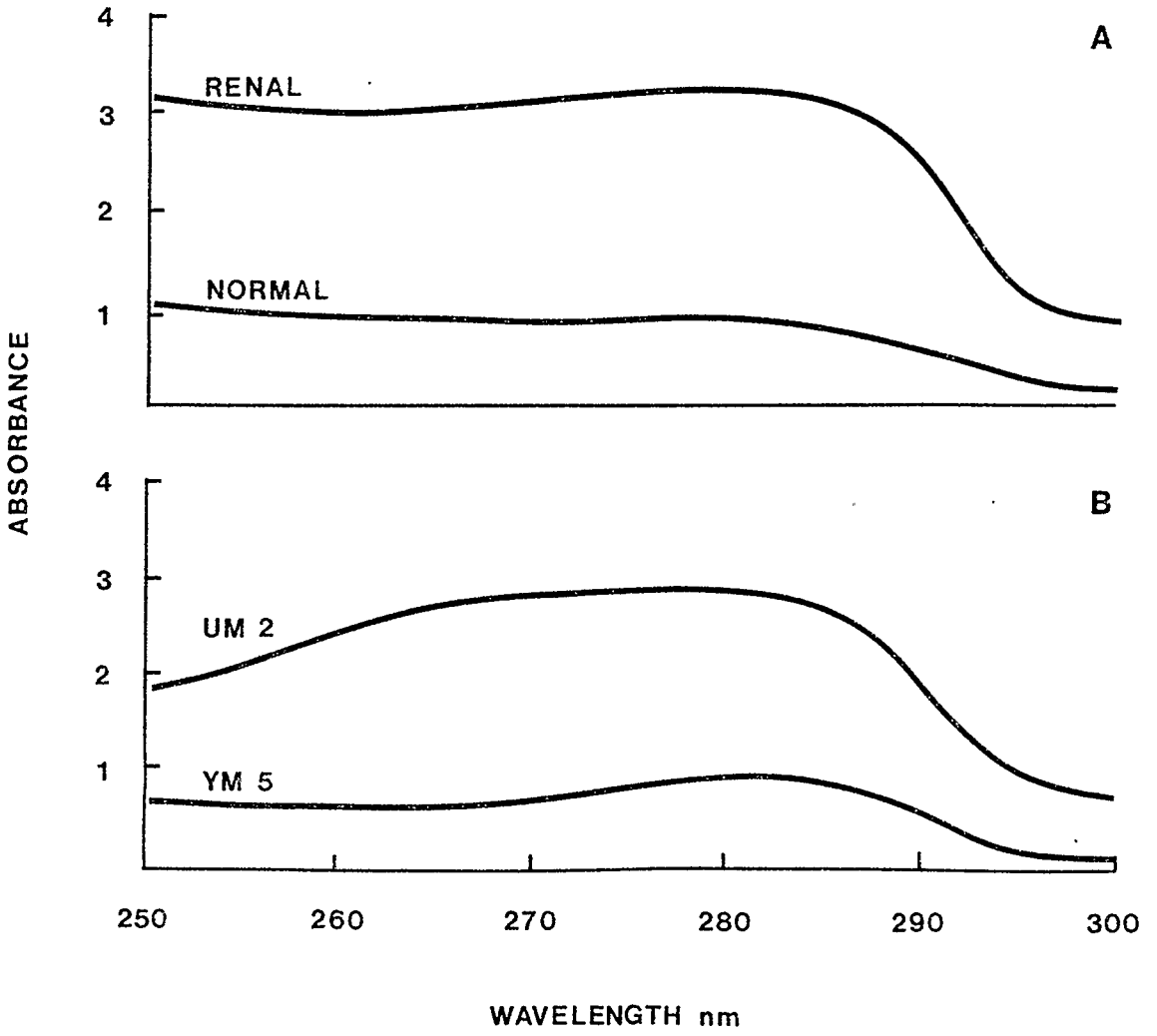


Figure 16. Absorption spectra for ethanol:water extracts of "normal" and "uremic" charcoal (A), and in "uremic" charcoal after ultrafiltration through UM 2 and YM 5 membranes (B).

the uremic plasma and subsequently extracted into the ethanol:water. Lowry et al (1951) protein measurements revealed that approximately 5.5 mg/ml protein was recovered in the ethanol:water extracts from "uremic" charcoal compared to 0.7 mg/ml protein from "normal" charcoal. Figure 16b further shows the absorption spectra for the "uremic" ethanol:water extracts after ultrafiltration through YM 5 (5.000 M.W. retention) and UM 2 (2,000 M.W. retention) membranes. The ultraviolet absorption of the extracts was decreased slightly by ultrafiltration through the UM 2 membrane, and reduced significantly by ultrafiltration through the YM 5 membrane.

Measurement of the DPHN-protein binding in normal plasma mixed with the fractions obtained by ultrafiltration of the ethanol:water extracts through the molecular sieve membranes showed that the inhibitory effect was associated with the 500-2,000 M.W. fraction (Table XX).

Figure 17 shows the elution pattern (absorption at 280 nm) obtained for "normal" and "uremic" extracts applied to the G 25 column. Variable amounts of protein appeared in the void volume for both extracts (possibly albumin), but a much larger series of peaks (perhaps 3) appeared at fraction 42-60 in the extracts from charcoal used in the adsorption of uremic plasma. Fraction 42-60 was positive for protein, using a fluorometric procedure (Robrish et al, 1978). Measurement of DPHN-protein binding in normal plasma mixed with fractions obtained from the G 25 column showed that the

TABLE XX

EFFECT OF ULTRAFILTRATION OF ETHANOL:WATER EXTRACTS ON DPHN BINDING TO NORMAL PLASMA PROTEINS

ETOH:WATER EXTRACT FROM	% UNBOUND DPHN
NORMAL PLASMA	12.8
- YM 5 RESIDUE	13.3
- YM 5 ULTRAFILTRATE	13.7
UREMIC PLASMA	19.4
- YM 5 RESIDUE	12.9
- YM 5 ULTRAFILTRATE	17.4
- UM 2 RESIDUE	13.2
- UM 2 ULTRAFILTRATE	16.2
- UM 05 RESIDUE	15.1
- UM 05 ULTRAFILTRATE	13.7

DPHN-protein binding was measured in normal plasma (equilibrium dialysis 37°C) mixed with residue and ultrafiltrate from ultrafiltration of ethanol:water extracts of charcoal, previously used in the adsorption of normal and uremic plasma. Membranes used were YM 5, UM 2, and UM 05.

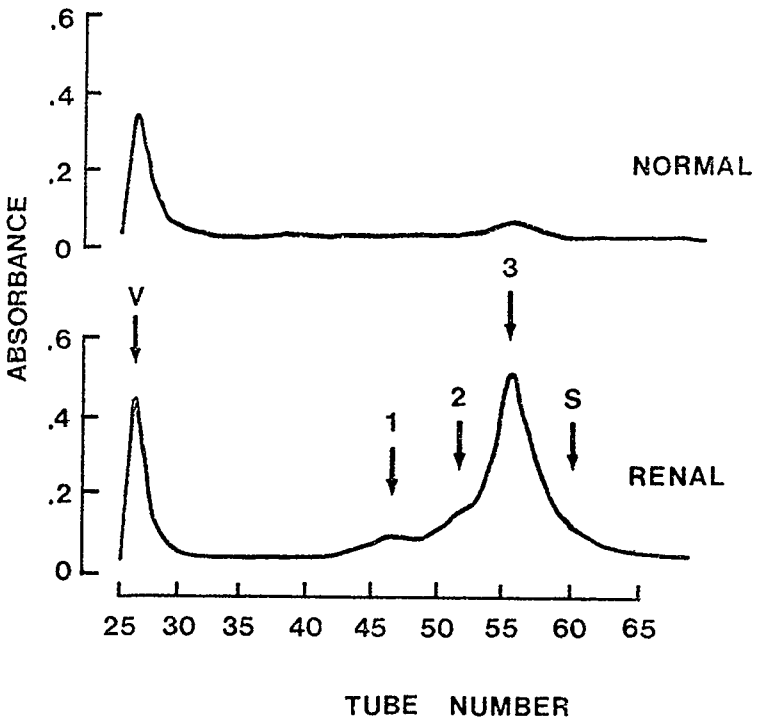


Figure 17. The G 25 elution pattern for ethanol:water extracts of charcoal, used in the adsorption of normal and uremic plasma. Void volume, V; peptide peaks, 1,2,3; salt retention volume, S.

inhibitory effect was associated with fractions 42-60 (Table XXI).

TABLE XXI

THE INHIBITORY EFFECT ON DPHN-PROTEIN BINDING ASSOCIATED WITH FRACTIONS OBTAINED BY G 25 GEL FILTRATION OF INHIBITOR SUBSTANCES

FRACTION NUMBER	% UNBOUND DPHN
27-42	14.4
43-53	15.9
54-64	15.9
65-85	13.6
NORMAL CONTROL	12.8
UREMIC CONTROL	16.2

Phenytoin-protein binding was measured in normal plasma mixed with fractions from the G 25 gel filtration of the ethanol: water extract obtained from "uremic" charcoal. Normal control was normal plasma with 200 ul buffer. Uremic control was normal plasma with "uremic" extract.

PART IV : DISCUSSION

4.01 METHODOLOGY

Equilibrium dialysis is perhaps the most popular method for the measurement of drug binding to proteins. By virtue of the accuracy and reliability of the results obtained, the method is considered by many to be the reference method. However, as discussed in section 1.03, there are certain limitations to the technique which must be recognized in order to achieve the required performance.

Dilution of the DPHN concentration, due to passage of unbound drug into the buffer, and by the osmotic action of the sample proteins, was not a problem in the estimation of DPHN-protein binding in this investigation. Passage of unbound DPHN into the buffer would not be expected to change the drug concentration by more than 15%, and the rigid design of the dialysis cell and membrane would tend to reduce osmotic expansion of the sample volume. Moreover, the binding of DPHN is constant over a wide drug concentration range and thus is not affected by changes in the drug concentration during equilibrium dialysis. In the study of binding kinetics (section 3.07) the binding of DPHN was found to be relatively unchanged over DPHN concentrations of 5-75 ug/ml, which supports the findings of others (Hooper *et al*, 1973b; Lecomte *et al*, 1979). In most of the DPHN-protein binding experiments performed here DPHN was added exogenously to the sample to achieve a final concentration of 15 ug/ml, which approximates the ideal therapeutic concentration of the drug for the control of epileptic seizures (therapeutic range

10-20 ug/ml, Ireland 1980).

A second source of error that may be encountered when using equilibrium dialysis, the Donnan effect, also did not appear to be a significant problem in this investigation. This may be inferred from the results of the binding kinetics studies (section 3.07), which showed that dilution of the sample did not alter the estimated apparent binding affinity constant or the number of binding sites. It is likely that the buffering capacity of the plasma proteins and that of the 0.1 M K_2HPO_4, pH 7.5 buffer, used in the analysis, were sufficient to overcome any error due to the Donnan effect.

The equilibrium dialysis estimation of DPHN-protein binding in plasma samples required overnight (16-20 h) incubation. The lengthy incubation time required in the procedure was a problem with respect to the rapid production of results and the analysis of large numbers of samples. In clinical applications this factor would likely be even more significant, due to the demand for rapid analytical procedures in the clinical laboratory. In an effort to avoid the lengthy analysis times required for equilibrium dialysis, and for comparative purposes, DPHN-protein binding was also measured by ultrafiltration, using Amicon Centriflo membranes. Ultrafiltration through knotted dialysis tubing has been used by many investigators (Lunde et al, 1970; Barth et al, 1976; Monks et al, 1978) and the technique is reported to be accurate and reliable for the determination of drug-protein binding in whole plasma. Amicon membrane cones

were employed here to avoid the manual manipulations required for the dialysis tubing procedures. Booker and Darcey (1973) utilized Amicon Centriflo membranes in the investigation of DPHN-protein binding in 1973, but their results were criticized by Porter and Layzer (1975), who reported that protein leakage through the cones and adsorption of drug to the membrane caused erroneous results. In this investigation protein leakage and drug adsorption to the cone were not significant problems in the estimation of DPHN-protein binding in whole plasma. Estimations of DPHN-protein binding in normal and uremic plasma, using the ultrafiltration procedure, were highly reproducible (Table VIII) and agreed with estimations made by equilibrium dialysis (Table VI). Careful attention to the handling of the somewhat fragile membrane cones, and the determination of optimal operating conditions (ie. centrifugation time and ultrafiltrate volume) may account for the satisfactory performance of the Amicon cones in this investigation.

The effect of temperature on measured DPHN-protein binding is an important consideration, regardless of the method of analysis employed. Figure 13 clearly demonstrates the reduced DPHN-protein binding at 37°C, as compared to 22°C. This effect is significant and merits careful attention in establishing an experimental protocol, as well as in the interpretation of results. Theoretically, drug binding to plasma proteins may be measured at any temperature if suitable control measures are included to ensure the

reliability and reproducibility of the results. Ultrafiltration analysis was performed only at room temperature (22°C) in this investigation because of the unavailability of a temperature controlled centrifuge. Equilibrium dialysis was performed at 22°C and at 37°C. Measurement at 37°C was preferred because a) results at this temperature may more accurately reflect physiological conditions, b) unbound levels were greater at 37°C and thus sensitivity was improved, and c) fluctuation in room temperature (22°C) can be significant and unpredictable. Regardless of the temperature chosen for the assay, accurate regulation of that temperature would appear to be an important consideration in order to avoid undue variation of the measured drug-protein binding.

In conclusion, an equilibrium dialysis procedure and an ultrafiltration procedure were used for the measurement of DPHN-protein binding in normal and uremic plasma. It would appear that both techniques, as described here, are suitable for the measurement of DPHN binding to plasma proteins, in health and in chronic renal failure. However, because of the significant effect of temperature on the analysis, and the difficulty of performing the ultrafiltration technique at 37°C (the preferred temperature), equilibrium dialysis may be the method of choice.

4.02 DPHN-PROTEIN BINDING IN CHRONIC RENAL FAILURE

There are many reports in the literature confirming the fact that the binding of various drugs to plasma proteins may be greatly reduced in renal disease, as well as in other conditions. It is also well documented that, as a result of impaired drug-protein binding, the pharmacokinetics and physiological effects of a drug may be significantly altered (Koch-Weser and Sellers, 1976). Reduced binding of drugs to plasma proteins may cause increased levels of the unbound or pharmacologically active form of the drug. In turn, this may lead to an increased volume of distribution, an increased concentration of the drug at the receptor site, increased pharmacological effects, increased toxic effects, increased concentrations of the drug at sites of metabolism, increased biotransformation, a decreased half life, decreased plasma concentrations of the drug and a change in the correlation between total drug concentrations and unbound concentrations.

This information, however, appears to have had little impact upon the clinical management of patients with impaired drug-protein binding, particularly in the utilization of therapeutic drug monitoring services. Many patients would likely benefit from the measurement of unbound or free drug levels. Indeed, the measurement of total drug concentrations only, may be misleading in patients with drug-protein binding defects.

DPHN-protein binding was measured in patients with chronic renal failure requiring hemoperfusion dialysis, and

compared to that of individuals having normal renal function. The results of the DPHN-protein binding measurements (Tables VIII, IX, X and Figure 10), obtained by two analytical methods, clearly confirm that chronic renal failure can produce a decreased ability to bind DPHN, and are in general agreement with the results of others obtained under similar experimental conditions (Hooper et al, 1973a, 1973b; Odar-Cederlof and Borga, 1976). Using equilibrium dialysis at 37°C, the unbound DPHN was 21.3 ± 0.6% in uremic plasma compared to 13.1 ± 0.6% in normal plasma. It would appear that the usual therapeutic range for DPHN (bound plus unbound DPHN), which is 10-20 ug/ml, is too high for patients with chronic renal failure. To achieve a comparable level of the unbound drug (the physiologically active form) a total concentration range of 6-12 ug/ml would be more realistic. Furthermore, the range of the measured unbound DPHN levels in chronic renal failure was 17.1-26.7% (Table X). Thus, patient 424 (17.1% unbound) might achieve a therapeutic concentration of unbound DPHN (1.3 ug/ml) at a total DPHN concentration of 7-8 ug/ml, while patient 323 (26.7% unbound) could achieve the same unbound level of DPHN at a total concentration of less than 5 ug/ml. The wide range of values observed for DPHN-protein binding in chronic renal failure would further support the argument for measuring DPHN-protein binding in these patients.

The unbound level of DPHN, measured before and after hemoperfusion dialysis in patients with chronic renal

failure, was significantly decreased in the post dialysis samples (22.9 % post, 25.4% pre). However, there was also a significant increase in the albumin concentration after hemodialysis (3.9 g/dl post, 3.4 g/dl pre), sufficient to account for the improvement observed in DPHN-protein binding. There was a good correlation between the albumin concentration and the unbound DPHN when all samples (pre and post dialysis) were examined. The *in vitro* dialysis of uremic plasma also failed to improve DPHN-protein binding (section 3.08.02). Anton and Corey (1971), in a study of sulfonamide binding in chronic uremia, did not find an increase in drug-protein binding after hemodialysis, and neither did Reidenberg *et al* (1971) and Shoeman and Azarnoff (1972), who examined the effect of *in vitro* dialysis on DPHN-protein binding. Conversely, Andreasen (1974) reported that *in vitro* dialysis did improve the binding of several drugs, including DPHN, to plasma proteins, but the study involved patients with acute renal failure, as opposed to patients with chronic renal failure studied here. More recently, Steele *et al* (1979) reported that *in vitro* hemodialysis did reduce the unbound DPHN level from 20.05% to 7.91% in one patient with dialysis encephalopathy. DPHN-protein binding was measured by continuous ultrafiltration at 37°C and radioimmunoassay, but the measured unbound DPHN in the three normals studied was only 2.1%. This is much less than was observed in this study (13.1%) and is not consistent with the expected protein binding of DPHN at 37°C. The authors found no change in the

albumin concentration after hemodialysis and thus concluded that an endogenous dialyzable inhibitor had been removed. However, before such a conclusion could be accepted, a greater number of individuals (normal and hemodialyzed) would have to be examined, using a second more established technique, such as equilibrium dialysis, for measuring drug-protein binding.

In this investigation the level of unbound DPHN was greater than normal (greater than 14.3%, mean plus 2 S.D.) in 10 of the 20 patients who had received a kidney transplant (Table XII). The results obtained by other investigators do not entirely agree with the results of this investigation, but the other studies involved very small sample populations and the results obtained may be due to the variation in DPHN-protein binding observed in this study. Dromgoole (1974) reported that the plasma protein binding of methyl orange in patients who had received a kidney transplant was lower than normal, but the investigation was performed on a pool of serum collected from 10 patients, 1 to 812 days after transplantation, not on individual samples as in this study. Olsen et al (1975) found the protein binding of DPHN to be normal 3 months after transplantation, but they studied only one patient. Odar-Cederlof (1975) reported that DPHN-protein binding was almost normal 10-14 days after renal transplantation, but the study involved only 2 patients. Similarly, Levy et al (1976), who reported that the binding of sulfisoxazole was normalized within 2 days, subsequently increased

for 50 days and normal again after 2 months post transplantation, studied only one patient.

In this investigation, none of the patients had unbound DPHN levels in the range observed for the chronic renal failure patients (17.1-26.7%). However, in terms of clinical effect the increase may be significant. For example, one might expect J.C. (Table XII), with an unbound DPHN of 16.8%, to realize a therapeutic effect at a total concentration of 7-8 ug/ml DPHN instead of at 10 ug/ml, or to experience toxic effects from DPHN at 15-16 ug/ml instead of at 20 ug/ml DPHN.

Figure 11, which shows the % unbound DPHN, albumin and creatinine concentrations for one patient over 34 days post transplantation, is interesting. The slow decrease in the % unbound DPHN, as compared to the rapid decrease in creatinine (rapidly filtered by the glomeruli), is consistent with the hypothesis that the inhibition of DPHN-protein binding is not caused by a readily dialyzable substance.

In conclusion, it would appear that the binding of DPHN to plasma proteins is significantly altered in chronic renal failure, and that binding is not substantially improved by *in vivo* or *in vitro* dialysis. The small decrease in unbound DPHN after hemodialysis may be explained by the increased albumin concentration. Patients who have had a kidney transplant may also have clinically significant defects in DPHN-protein binding. The measurement of DPHN-protein binding would, therefore, appear to be recommended

for patients with chronic renal failure and for patients with renal transplants, when total DPHN concentrations are required.

4.03 CHARACTERIZATION OF DPHN-PROTEIN BINDING INHIBITION

Four hypothesis are proposed to explain the defect in the binding of drugs to plasma proteins from patients with renal failure (section 1.03). These are: 1) hypoproteinemia, 2) differences in the native structure of the binding protein, 3) competitive inhibition and 4) non-competitive inhibition. Figure 10 confirms that DPHN-protein binding in normal and uremic plasma may be influenced by albumin concentration, but shows that, at equal albumin concentrations, binding is less in the uremic plasma. Furthermore, the pools of uremic plasma studied in Tables VIII and IX were not significantly different from the normal pools with respect to albumin concentration. Similarly, in Table X, while some uremic individuals were hypoalbuminemic, the mean albumin concentration (3.9 g/dl) was not abnormal or greatly different from the mean albumin concentration for the control subjects (4.2 g/dl). It would appear that albumin concentration may be an important factor in DPHN-protein binding, but the defect in DPHN-protein binding observed in chronic renal failure can not be attributed to hypoalbuminemia. The data might also be used to argue that the measurement of DPHN-protein binding would be of value in any condition producing hypoalbuminemia, since in hypoalbuminemia the measured total DPHN concentration would tend to

underestimate the unbound or physiological concentration of the drug in the plasma.

DPHN is bound primarily to albumin (Lecomte *et al*, 1979). Albumin was, therefore, isolated from normal and uremic plasma and the DPHN-protein binding compared to that measured in whole plasma (normal and uremic). Circular dichroism spectra for the albumin isolated from both groups did not indicate protein structural differences. The binding of DPHN was also not significantly different in albumin isolated from control groups compared to albumin from patients with chronic renal failure (Table XIII). It is important to note that the preparations were compared at equal albumin concentrations to overcome the significant effect of protein concentration on drug-protein binding. The concentrations of the albumin preparations were less than usually found in plasma. However, the concentration of DPHN was reduced to compensate for this and to maintain the ratio of drug to albumin that might be expected *in vivo*. From Figure 10 it is apparent that DPHN binding was significantly different in patients with renal disease versus control plasma, when measured over a wide range of albumin concentrations. Therefore, the normalization of drug-protein binding in the purified albumin from the renal pools was not simply the result of dilution of a competitive inhibitor. Sjoholm *et al* (1976) also investigated the binding of purified albumin from patients with renal disease. They reported that albumin from uremic serum showed lower binding

affinity for salicylic acid and warfarin as compared to albumin isolated from control sera, but that a further treatment of the albumin (isolated from uremic sera) with charcoal normalized the affinity. The fact that Sjoholm *et al* studied different drugs in a different population of individuals, as compared to this study, may be a factor in this discrepancy and cannot be discounted. However, Sojholm *et al* used ammonium sulfate precipitation and DEAE affinity chromatography to isolate albumin from whole serum, and a further treatment of the preparation with charcoal at pH 3 to normalize the drug-protein binding in the albumin from uremic sera. In this study affinity chromatography using Blue Sepharose CL-6B was employed to isolated albumin. The procedure was more easily performed than ammonium sulfate precipitation, provided a product of acceptable purity and yield, and normalized DPHN-protein binding without charcoal treatment. Also, because of the mild conditions employed and the limited number of sample manipulations required, the method used here would not be expected to produce artifactual changes in the final albumin product. Boobis (1977) reported the isolation of albumin from patients with renal disease using an affinity chromatography procedure similar to the one employed here. However, the author did not measure DPHN-protein binding in the purified albumin samples.

The double reciprocal graphs in Figure 14 further document the difference in DPHN-protein binding between the control and the renal failure populations studied in this

investigation. The greater slope of the family of lines for the renal whole plasma as compared to the control whole plasma or serum corresponds to a reduced apparent affinity for DPHN and a somewhat greater number of binding sites (Table XIV). Odar-Cederlof and Borga (1976b) measured DPHN binding in renal disease using experimental procedures similar to those used here and found similar resulty found for control patient whole serum an apparent affinity of $4.01 \times 10^3\ M^{-1}$ and a number of binding sites equal to 2.48, as compared to an apparent affinity of $1.76 \times 10^3\ M^{-1}$ and a number of binding sites equal to 3.35 for renal patient whole serum. Monks et al (1978) reported a greater apparent affinity ($K_{app} = 1.86 \times 10^4\ M^{-1}$) and fewer binding sites (n = 1.04) for control sera, and Lecomte et al (1979) found a lower apparent affinity ($K_{app} = 7.45 \times 10^2\ M^{-1}$) and a greater number of binding sites (n = 8) for DPHN-protein binding to human serum albumin. It is difficult to make many valid comparisons of the data from this investigation with that of others in the literature because of differences in the experimental conditions employed. Monks et al (1978) measured DPHN-protein binding at room temperature and Lecomte et al (1979) measured DPHN-protein binding in commercial human serum albumin using a rapidly equilibrating (4 hours) dialysis system at 37°C. The effect of temperature on the binding parameters is evident from Figure 13. In this investigation the apparent affinity at 22°C was $1.0 \times 10^4\ M^{-1}$ and the number of binding sites equal to 2.0. It is

therefore emphasized that the technique used to study drug-protein binding, as well as the conditions (the temperature, pH, time of equilibrium, buffer, and the concentrations of the ligand and the binding protein) may have a significant effect on the binding kinetics, and these factors must be considered in an interpretation of the data generated.

The results of the Scatchard and double reciprocal analysis of the DPHN binding in purified albumin preparations from the control and uremic populations (Table XIV) are somewhat ambiguous and should be interpreted with caution. The total drug concentrations and albumin concentrations were considerably lower in the purified albumin preparations than in the whole plasma or serum. Indeed the entire matrix of the purified albumin preparations was significantly altered and subtle changes in DPHN binding and/or experimental error may have been magnified under these conditions. The calculated apparent affinity constant and the number of binding sites for the purified albumin preparations from the two sources were somewhat different. However, in view of the close agreement of the % unbound levels of DPHN in the purified preparations (Table XIII) this discrepancy suggests only a minor difference in the manner of DPHN binding between the control and uremic preparations.

The cross-over mixture of uremic proteins with normal ultrafiltrate, and normal proteins with uremic ultrafiltrate indicated that inhibition of DPHN-protein binding was not associated with small molecular weight

substances able to pass into the ultrafiltrate. The inhibition was associated with the uremic protein fraction, suggesting that the inhibitor substances were either bound to high molecular proteins, or had a molecular weight of greater than 50,000. The results of the *in vitro* dialysis and ultrafiltration elution support this hypothesis.

Purification of uremic plasma, using Blue Sepharose CL-6B, normalized DPHN-protein binding as compared to control plasma. However, no inhibitor substances could be eluted from the column by the various buffers used. It may be that the inhibitor substances were very tightly bound to the column, lost their inhibitory effect in the elution procedure, or were lost in the ultrafiltration or dialysis performed to desalt the fractions. Because benzoylated tubing was used in the dialysis, the inhibitor substances would have had to be less than 2,000 M.W. to pass through the membrane.

Craig *et al* (1976) and Grafnetterova *et al* (1979) reported that the charcoal adsorption of uremic plasma at pH 3.0 normalized drug-protein binding. In this study charcoal adsorption was found to normalize DPHN-protein binding, but the effect was most pronounced at pH 7.4. The difference in the optimal pH for the extraction may be due to differences in experimental conditions, such as the drug studied, the charcoal used, the time and temperature of the procedure.

Chen (1967) first used charcoal adsorption at pH 3.0 to remove fatty acids from serum albumin, suggesting that

the inhibitor removed by Craig et al (1976) may have been a fatty acid. The observation that total free fatty acids were not elevated in renal failure led Craig et al (1976) to discount this possibility. In this investigation free fatty acids, extracted from uremic plasma, did not inhibit DPHN-protein binding, as compared to free fatty acids extracted from normal plasma. However, elevations in individual fatty acids or metabolities may occur and have an inhibitory effect on the protein binding of certain drugs.

Grafnetterova et al (1979) reported that substances capable of inhibiting the binding of chloramphenicol to normal plasma proteins could be recovered from charcoal previously used in the adsorption of uremic plasma. The extraction involved ethanol:0.1 N HCl (1:1). The authors examined the plasma levels of cholesterol, triglycerides, free fatty acids and free amino acids, before and after the charcoal treatment, but could not identify the inhibitor substances. They did speculate, without experimental evidence, that the substances might be peptides.

In this study substances which inhibited the binding of DPHN to normal plasma proteins were extracted from "uremic" charcoal, using ethanol:water at pH 7.4. The ethanol:water extraction of charcoal at pH 7.4 was more efficient and produced visibly cleaner extracts than extractions performed at pH 3.0, and would not be expected to contain significant amounts of free fatty acids. The solubility of the dried extracts in phosphate buffer is

further evidence suggesting that lipids were not extracted. Absorption at 280 nm showed significant amounts of protein material in the extracts, greater in the extracts from "uremic" charcoal, and fractionation of the extracts using Amicon membranes indicated that the inhibitor substances had a molecular weight of less than 2,000. However, the demonstration of an inhibitor effect in fractions obtained from the G 25 column is the most convincing evidence that the inhibitor substances in the ethanol:water extracts are peptides. The G 25 column was not calibrated (except for void and salt volumes), but the molecular weight of the inhibitor peptides would be estimated as 1,000-2,000 on the basis of the suggested separation range for G 25 (500-5,000 M.W.).

Abnormal amounts of peptides have been discovered in the plasma and urine of patients with renal disease (Bergstrom and Furst, 1976) and these substances, termed middle molecules, have been implicated in the inhibition of glucose utilization, fibroblast proliferation, phagocytic activity of leukocytes and proliferation of lymphocytes. The isolation of middle molecules from plasma usually involves a preliminary ultrafiltration to remove substances of greater than 50,000 M.W. (Gordon et al, 1975). Thus, the peptides isolated in this investigation, because they are strongly bound to albumin, would not be expected to have been previously isolated in middle molecule studies of uremic plasma. The isolation, here, of peptides from uremic plasma

that inhibit the binding of DPHN to plasma proteins is perhaps the first substantiated link with the middle molecule studies, and raises the question of what other activity the peptides isolated here may have.

In summary, the binding of DPHN to plasma proteins was significantly impaired in chronic renal failure. However, the binding of DPHN to albumin isolated from patients with renal failure was not significantly different from that observed for albumin from control groups. Subtle differences were observed in the apparent binding constant and the number of binding sites for the albumin isolated from the two sources, but the difference was not significant enough to account for the extent of the binding defect observed in whole plasma. These results suggest that the defect in DPHN-protein binding that occurs in renal disease can not be attributed to a basic functional defect in the primary drug-binding protein albumin. The normalization of DPHN-protein binding in uremic plasma by charcoal adsorption at pH 7.4 is indirect evidence that the binding defect is caused by an inhibitor. However, the isolation and partial characterization of peptide substances (molecular weight 1,000-2,000) with an inhibitory effect on DPHN-protein binding is direct evidence of a competitve or non-competitive inhibitor substance in chronic renal failure that causes decreased binding of DPHN to albumin.

4.04 FUTURE STUDIES

The examination of drug-protein binding appears to be an important addition to the therapeutic monitoring of certain drugs in certain clinical conditions. However, well controlled clinical studies, involving several drugs, would be required to determine the benefit of using drug-protein binding measurements in conjunction with therapeutic monitoring programs. The investigation of more rapid means of measuring drug-protein binding at 37°C, to facilitate efficient large scale laboratory analysis, would also be indicated.

The isolation of peptides which appear to be responsible for the inhibition of DPHN-protein binding in chronic renal failure creates the opportunity for a great deal of future research. The further purification and characterization of the peptides would be a major priority. In addition a study of the effect of the purified inhibitor on normal plasma proteins and purified albumin, using Scatchard and double reciprocal analysis, and circular dichroism, may indicate the nature of the inhibition (competitive or non-competitive). The possibility that the peptides isolated from uremic plasma may be different from middle molecules previously studies would suggest the investigation of other possible actions of the peptides, such as inhibition of glucose utilization, fibroblast proliferation, phagocytic activity of leukocytes and proliferation of lymphocytes.

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