INVESTIGATION OF ANTINATRIFERIC ACTIVITY IN HEMODIALYSIS FLUID, BLOOD, AND PLASMA ULTRAFILTRATE

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ABSTRACT

INVESTIGATION OF ANTINATRIFERIC ACTIVITY
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AND PLASMA ULTRAFILTRATE

By

James Murray Terris

An investigation of the literature of the last few years dealing with the renal excretion of sodium reveals that at the present time there are a number of theories available to describe the manner in which the excretion of this ion is regulated by the kidney. Most of the hypotheses deal with alterations in intrarenal hemodynamics. However, evidence since 1960 has been accumulating suggesting the existence of a presently unidentified natriuretic hormone which appears in the urine and/or blood of man, rats, cows, dogs, and cats, under certain conditions of extracellular fluid volume expansion. Plasma demonstrating natriuretic activity has also been reported to inhibit sodium transport across frog skin and toad bladder membranes (antinatriferic activity). Whether the material responsible for these activities is a single substance, or has a definite physiological function, has not yet been determined.

Antinatriferic and natriuretic activities have been reported to occur in the plasma of humans with chronic renal disease. Since the possibility exists that an antinatriferic substance might be dialyzable <u>in vivo</u> from humans, as appears to be the case with dogs, a study

was undertaken to determine if antinatriferic activity could be isolated from plasma ultrafiltrates and spent hemodialysis fluid following maintenance hemodialysis of patients with end-stage chronic renal failure. Methods are described for processing and concentrating these large volumes of fluid which can be as much as 180 liters in a given dialysis treatment. The assay employed to detect this substance utilized isolated ventral frog skins held in a Ussing-type chamber. Potential difference and short circuit current across the membranes were alternately monitored before and after addition of a test sample.

There was no reproducible antinatriferic activity observed in the specimens obtained from the patients in this study. All patients had been maintained by hemodialysis for 2 weeks or more, and none demonstrated a fluid retention greater than 5% of their body weight since the previous dialysis treatment. Plasma samples with and without trichloroacetic acid deproteinization were studied. In addition, dialysis fluid samples were concentrated 3600-fold by ultrafiltration and plasma ultrafiltrates obtained from the artificial kidney were similarly concentrated 8 to 27 fold and assayed for antinatriferic activity. It is concluded that patients who have a fluid retention which is less than 5% of their body weight, and who are undergoing chronic maintenance hemodialysis, do not possess measurable quantities of a previously described natriuretic hormone which others have suggested has antinatriferic activity.

In addition to the uremic human studies, dogs were expanded with saline to examine the antinatriferic activity of expanded plasma. No reproducible antinatriferic activity was demonstrated in expanded dog plasma with or without trichloroacetic acid deproteinization. Also, evidence is presented suggesting that direct application of trichloroacetic acid treated samples to frog skin membranes may yield erroneous results due to the presence of small amounts of residual trichloroacetate anion not removed by conventional extraction procedures. From these studies it is concluded that the natriuresis seen in acute expansion of the extracellular fluid volume with isotonic saline cannot be attributed to an antinatriferic substance.

INVESTIGATION OF ANTINATRIFERIC ACTIVITY IN HEMODIALYSIS FLUID, BLOOD, AND PLASMA ULTRAFILTRATE

By

James Murray Terris

A DISSERTATION

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DEDICATION

To my uncomplaining wife, whose patience, hard work, and unselfish dedication to her family during my years as a graduate student were my source of strength when times were tough; to Jimmy, John, and Jason, who make life worth its struggles.

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INTRODUCTION

In the maintenance of the volumes of the various fluid compartments of the body, the most important inorganic constituent is the sodium ion. The volume of the extracellular fluid compartment is determined primarily by the total amount of osmotically active solute which it contains. Since sodium and chloride are by far the most abundant osmotically active solutes in the extracellular fluid, and since changes in chloride are largely secondary to changes in sodium, the amount of sodium in the extracellular fluid is the most important determinant of the extracellular fluid volume. Therefore, the mechanisms that control sodium balance are the major mechanisms 'defending' this volume.

Prior to 1961 ideas concerning sodium excretion by the kidney were dominated by considerations of glomerular filtration rate (GRF or factor I) and the adrenal hormones, primarily aldosterone (factor II). Selkurt et al. (153), in 1949, showed that reduction of the GFR to 63% of the control value resulted in almost total sodium reabsorption, an observation confirmed more recently by others (22,101,107). Simpson and Tait (156), in 1952, isolated a new adrenal steroid which was to be called aldosterone and was shown to be the most potent of the naturally occurring sodium retaining steroids. With these observations it was felt that the primary factors concerned with sodium control by the kidney were established.

One of the first indications that there is a renal mechanism which operates independently of factor I and factor II for the excretion of excess sodium evolved during the period from 1941 to 1960. Investigations by several workers (6,63,75,143) demonstrated the existence of a phenomenon now referred to as 'mineralocorticoid escape'. When deoxycorticosterone acetate (DOCA) or aldosterone was chronically administered to humans (6,143), it was noted that there was a dramatic fall in sodium excretion. Following an initial period of sodium retention and weight gain (fluid retention), weight gain ceased and sodium excretion returned approximately to control levels despite continued administration of these compounds. Davis and Howell (63) demonstrated, in the dog, that even with an increased GFR there was only a transient retention of sodium with continued use of DOCA. DeWardener et al. (65) established another line of evidence for a sodium excreting factor not related to GFR or mineralocorticoids when they demonstrated that saline diuresis could occur in the presence of a reduced GFR during acute volume expansion with saline infusion in dogs treated with 9-alpha-fluorohydrocortisone.

With the findings that natriuresis could still occur in volume expanded states despite an appreciable fall in GFR and high mineralocorticoid levels, some other mechanism, or 'third factor', had to be sought which would presumably affect tubular reabsorption of sodium independently of factor I and factor II. That such a 'third factor' exists is not disputed. The nature of this factor, on the other hand, has been the subject of extensive studies, prompting one investigator

to ask "Which factor is third (16)"? The result of these studies has led to the conclusion that the renal handling of the sodium ion is a very complex interrelationship of many factors, both physical and hormonal, and that the 'third factor' natriuresis seen following extracellular fluid volume expansion may in fact be the result of changes in many factors.

During the early 1960's investigations began to suggest that this 'third factor' natriuresis observed in volume expanded states may in part be due to an unidentified 'natriuretic hormone' ('NH'). One study suggested that this 'NH' may induce natriuresis by inhibiting active reabsorption of sodium in the nephron by inhibiting Na-K-ATPase (99). However, this is not a consistent finding (97,166). Other studies have demonstrated that plasma obtained from volume expanded animals and humans not only induces natriuresis but also inhibits sodium transport across frog skin and toad bladder membranes (an antinatriferic activity) (19,20,21,32,33,35,49,53,137). In addition, using an Ussing-type short circuit current preparation, Nutbourne et al. (37) demonstrated that following volume expansion in the dog there was a reduction in sodium transport across frog skins incorporated into the dog's circulation. The expansion in these studies was performed with blood equilibrated with that of the dog undergoing expansion. Concomitant with the decreased sodium transport in the frog skin there was an increase in urinary excretion of sodium in the expanded animal. These observations, and others to be discussed in a later section, suggested that the natriuretic and antinatriferic activities observed in plasma following volume expansion may be due to a single substance.

The present studies were designed to further evaluate the contribution of a 'natriuretic hormone' (with antinatriferic activity) to the third factor natriuresis seen in volume expanded states. A natriuretic and antinatriferic activity has been demonstrated to be present in the serum of chronically uremic humans (19,20,21), who are characteristically volume expanded. Since an antinatriferic substance has been reported to be dialyzable in vivo from dogs exhibiting natriuresis as a result of saline expansion (36), the possibility that the material observed in humans with end-stage renal failure might also be dialyzable in vivo was considered. If the material is dialyzable from humans undergoing maintenance hemodialysis, plasma ultrafiltrates and spent dialysis fluid obtained from the artificial kidney could serve as an unlimited source for this substance. With such a source sufficient quantities of material might be obtained permitting its identification.

The newness of the concept of this 'natriuretic hormone', and the manner in which it was viewed as late as 1969, is perhaps best described by Cort and Lichardus (55) in reference to a symposium held in June of that year at Smolenice Castle in Czechoslovakia: "Most symposia tend to stress conceptual and theoretical advances in a delimited field of research. The present meeting had quite different goals--to discuss whether a given field exists or not and in detail the methods applied to try to find out."

The following discussion first reviews the literature which has provided evidence for the existence of this 'natriuretic hormone' in

situations of extracellular fluid volume expansion. This is followed by a summary of the information currently available which suggests that this same 'natriuretic hormone' may also be antinatriferic with regard to sodium transport across toad bladders and frog skins. Finally, a consideration of the source of this natriuretic-antinatriferic substance is given, in addition to a review of some of the physical factors which have been shown to contribute to the 'third factor' natriuresis seen with volume expansion.

REVIEW OF THE LITERATURE

A. Circulating Hormonal Factors in Volume Expanded States

The first evidence suggesting a circulating sodium excreting hormone in volume expanded states evolved from the experiments of DeWardener et al. (65). These workers demonstrated that natriuresis occurred not only in dogs volume expanded with isotonic saline, but also in an isolated kidney being perfused with blood from the expanded animal. This transmitted natriuresis also occurred in a cross perfused intact recipient animal. Administration of exogenous aldosterone ruled out mineralocorticoid dilution (which could cause natriuresis by decreasing sodium retention), and a complex system of perfusion pressure regulation was employed to keep the perfused intact animal at a constant body weight. There was thus no volume stimulus from cross circulation in the recipient animal. Changes in hematocrit, plasma protein oncotic pressure, and serum sodium were also not responsible for the observed natriuresis. Levinsky (103) and Levinsky and Lalone (104) later substantiated these findings.

Johnston and Davis (91) and Johnston et al. (92), with cross perfusion experiments performed on intact dogs, also provided evidence for the involvement of a hormonal substance in the natriuresis accompanying saline loading. In a control study blood was cross circulated

between DOCA escape donors and normal recipients. There was no significant increase in sodium excretion in the recipient dogs due to the cross circulation itself. When the donor dogs were expanded with 1 liter of 0.9% saline there was a significant increase in sodium excretion in the recipient dogs. In these experiments it was also noted that there was a significant increase in sodium excretion during periods of cross circulation when the filtered load of sodium in the recipient was significantly decreased by aortic constriction above both renal arteries.

Using a Ringer Locke solution containing canine red blood cells and 6% bovine albumin to expand a donor dog, Lichardus and Pearce (114) also demonstrated a natriuresis in a cross perfused recipient animal when the GFR was decreased by clamping the arterial perfusion line. This recipient natriuresis was not due to changes in hematocrit, oncotic pressure, or increased renal plasma flow. Similar results were reported by Bahlmann et al. (9) in cross circulation studies with dogs in which the donor dog was expanded with whole blood.

Martinez-Maldonado et al. (123) report a 38% decrease in proximal tubule reabsorption, as measured by micropuncture techniques, when plasma from saline expanded animals was perfused into isolated kidneys. Kaloyanides and Azer (94) demonstrated an increased sodium excretion in an isolated kidney being perfused with blood from a second dog undergoing volume expansion with equilibrated blood in a reservoir to which had been added 5% albumin in saline. The natriuresis in these studies occurred in spite of a decreased renal blood flow, decreased arterial

pressure, and in the absence of any change in plasma protein concentration or packed red cell volume. The donor animal had been pretreated with DOCA.

As a result of these cross circulation experiments the use of the term 'natriuretic activity' began to make an appearance in the literature in reference to a potential substance in the blood responsible for the natriuresis being observed. Rector et al. (142), in 1968, became one of the first to refer to this postulated circulating substance as 'natriuretic hormone' ('NH').

Stronger evidence for the existence of a 'natriuretic hormone' is provided by studies such as those of Sealey and Laragh (151) and Sealey et al. (150). Plasma and urine from salt-loaded humans and sheep, as well as patients with primary aldosteronism and essential hypertension, demonstrated an inhibitory effect on sodium reabsorption in rats. Similar results were obtained by Kruck (100) utilizing dialysates and ultrafiltrates prepared from urine of orally hydrated humans. Glomerular filtration rate, renal plasma flow, and blood pressure were not affected. Buckalew and Lancaster (33) demonstrated in dogs undergoing DOCA escape that a substance appeared in plasma ultrafiltrates which inhibited the short circuit current (SCC) in toad bladders. It was also noted that variations in urinary excretion of sodium coincided with oscillations in the inhibitory activity of the plasma samples. These authors suggested that a natriuretic hormone (with antinatriferic activity) may thus be involved in the day-to-day regulation of sodium balance.

Clarkson et al. (44), using whole blood to volume expand dogs, demonstrated that renal tubule fragments incubated in plasma obtained after expansion were less able to maintain a sodium gradient or accumulate para-aminohippurate (PAH) than when incubated in plasma obtained before expansion. PAH transport has been shown to be a sodium dependent process (19). Individuals carrying out the transport studies did not know the identity of the plasma samples. Extracts of urine were prepared by Clarkson and DeWardener (43) from salt-depleted and salt-loaded humans. The extracts from salt-loaded subjects inhibited sodium transport in tubules prepared from rabbit kidneys. Extracts from salt-depleted subjects had no effect.

Finally, Lichardus and Nizet (113) expanded the blood volume of dogs with whole blood from a donor dog in which the hematocrit and protein concentration were matched. Prior to the expansion, one of the dog's own kidneys was tied off, transplanted to the neck and anastomosed to the carotid artery and jugular vein in order to eliminate afferent and efferent renal nerves. Although there were no significant changes in GFR, renal blood flow, post-glomerular hematocrit, or plasma protein concentration following the transfusion, there were moderate but significant increases in urine output and renal sodium excretion in the transplanted kidney. The animals had been pretreated with DOCA and ADH prior to the experiment. The authors interpret the results as being consistent with the proposition that a specific factor ('NH') plays a role in the mechanism of natriuresis after blood volume expansion.

Although a considerable number of investigators have reported evidence in favor of a circulating salt excreting hormone during periods of blood volume expansion, a few have reported negative findings (7,10, 18,147,170). However, it might be of interest to note that with only two exceptions (147,170), all of these experiments reporting negative findings involved attempts to elicit the substance in rats. Wright et al. (170) failed to show the presence of a dialyzable inhibitor of proximal sodium reabsorption from plasma of dogs undergoing DOCA escape, and Schrier et al. (147) failed to demonstrate it with hypotonic volume expansion in dogs.

Lichardus and Ponec (116), commenting on their own negative findings in the rat, suggest that a species difference may exist and that the natriuretic response mechanism in the rat could be more dependent on a mutual interplay of the hormonal and physical factors than in the dog. They noted that diuresis due to blood volume expansion with isotonic or iso-oncotic blood in rats leads to a significant increase in plasma protein concentration. When the urine of a donor rat was returned intravascularly through a catheter connecting the bladder with the jugular vein, the protein concentration in the cross circulated blood to a recipient did not change. Under these conditions a significant diuresis and natriuresis took place in the recipient during donor expansion. Utilizing this procedure, Sonnenberg et al. (160) substantiated these findings in cross perfused rats.

Chemical Characteristics of Natriuretic-Antinatriferic Factors

Although the chemical nature of the natriuretic-antinatriferic factors studied is unknown, comparison of the available information regarding these factors reveals some interesting similarities. For example, a natriuretic activity has been reported to appear in the blood of animals during mineralocorticoid escape (32,33,35,137), volume expansion in dogs (37), carotid artery occlusion in cats (49,53), and humans with chronic renal disease (19,20,21). This same blood has also been shown to be antinatriferic when tested on frog skins and toad bladders in these experiments. Table 1 lists these various natriuretic-antinatriferic substances and their effects on renal sodium excretion (natriuretic-antinatriuretic activity) and sodium transport in frog skins and toad bladders (natriferic-antinatriferic activity). Also included are other known compounds or classes of compounds which have been considered as possible candidates for 'NH'.

From the table it can be seen that the only substances which demonstrate natriuretic as well as antinatriferic activity are the so-called natriuretic hormones. The possibility exists, of course, that there are actually two substances involved, one of which is natriuretic and the other antinatriferic. Further comparisons, however, tend to suggest that if the activities are not due to a single substance, they are at least due to similar substances. Summarized in Table 2 are the effects of various treatments on natriuretic and antinatriferic activities which appear in cats during carotid artery occlusion, dogs during mineralocorticoid escape and saline expansion, and in chronically

Table 1 Effects of various 'natriuretic hormones' and other known substances on renal sodium excretion

Table 1. Effects of various 'natriuretic hormones' and other known substances on renal sodium excretion and sodium transport across amphibian membranes.

	ACTIVITY			
SUBSTANCE	NATRIURETIC	ANTINATRIURETIC	NATRIFERIC	ANTINATRIFERIC
CAROTID ARTERY OCCLUSION 'NH'	Cort & Lichardus (53) Cort et al. (49)			Cort et al. (58) Cort et al. (49)
VOLUME EXPANSION 'NH'	Buckalew & Nelson (37)			Buckalew & Nelson (37) Buckalew & Lancaster (34)
MINERALOCORTICOID ESCAPE 'NH'	Nutbourne et al. (137) Buckalew & Lancaster (32,33,35)			Nutbourne et al. (137) Buckalew & Lancaster (32,33,35)
UREMIC HUMAN 'NH'	Bourgoignie et al. (19,21)			Bourgoignie et al. (20)
OXYTOCIN	Chan & DuVigneaud (41) Cort et al. (59)		Bastide et al. (13) Bastide & Jard (14) Leaf & Dempsey (102)	
ANTIDIURETIC HORMONE	Martinez-Maldonado et al. (122) Chan & DuVigneaud (41)		Bastide et al. (13) Leaf & Dempsey (102) Hong et al. (86)	
ANGIOTENSION II	Kaloyanides et al. (95) Lowitz et al. (119) Leyssac et al. (108) Leyssac (106)		NO EFFECT (61)	NO EFFECT (61)
CATECHOLAMINES		Jacobson et al. (89) Nickel et al. (134) Beck et al. (15)	Watlington (167) Bastide & Jard (14)	
STEROIDS		Cort (46)		Porter & Edelman (140) Crabbe (62) Nielson (135)
PROSTAGLANDIN E_1	Herzog et al. (82) Johnston et al. (93)		Fassina & Cantessa (74) Barry & Hall (12) Fassina et al. (73) Lipson & Sharp (118)	

Table 2. Summary of the effects of various treatments on natriuretic-antinatriferic activities obtained during carotid artery occlusion in cats and from humans with chronic renal disease. Only antinatriferic activity was studied in the mineralocorticoid escape and volume expansion experiments of Buckalew. Loss of activity is denoted by (-), retention of activity by (+). NA indicates that the treatment was not performed. Boiling of the plasma in the absence of proteins was performed for 2 hours or 10 minutes as indicated. Molecular weights were determined by: 1) H-TCA precipitation of proteins--no attempt was made to characterize further; 2) ultrafiltration through UM 2 membranes (Amicon Corp., molecular weight rejection of approximately 1000 to 3000 Daltons) and fractionation on Sephadex G25 resin; 3) ultrafiltration through UM 10 membranes (Amicon Corp., molecular weight rejection of approximately 10,000 Daltons)--no attempt was made to characterize further; 4) ultrafiltration through UM 05 membranes (molecular weight rejection of approximately 500 Daltons) and fractionation on Sephadex G10 resin.

REFERENCES	(47,49,58)	(27,29)	(20,28)	(19)	(35)	(36,37)
	CORT	BRICKER AND BOURGOIGNIE			BUCKALEW	
	CAROTID ARTERY	HUMAN UREMIC SPECIMENS			DOGS	
TREATMENT	OCCLUSION IN CATS	PAH STUDY	SCC STUDY	RAT STUDY	ESCAPE	EXPANSION
BOILING (-PROTEINS)	2 hours (+)	10 min.(+)	10 min.(+)	10 min.(+)	NA	NA
FREEZING	NA	(+)	(+)	(+)	(+)	(+)
CHYMOTRYPSIN INC.	(-)	(+)	(+)	(+)	NA	NA
TRYPSIN INC.	(-)	NA	NA	NA	NA	NA
AMINOPEPTIDASE INC.	some activity	NA	NA	NA	NA	NA
PRONASE INC.	NA	(+)	(+)	(+)	NA	NA
MOLECULAR WEIGHT	20,000 or less[1]	500-1000[2]	500-1000[2]	NA	10,000[3] or less	less than[4] 500-700
DIALYZABLE IN VIVO	NA	maybe	maybe	maybe	yes	NA

uremic humans. The only striking difference between the human and cat material is the apparent unresponsiveness of the human material to degradation by proteolytic enzymes. Bricker (27), however, does state that he is able to inactivate the substance with a specific peptidase, although the nature of this peptidase was not revealed. The human substance is not, therefore, completely immune to enzymic degradation.

Cort et al. (49,58) and Cort (47) have concluded that the substance released during carotid artery occlusion in cats is a peptide, small enough not to be precipitated by trichloroacetic acid (H-TCA). They suggest that the substance has at least one basic amino acid since both natriuretic and antinatriferic activities are destroyed by trypsin. The partial loss of both activities following incubation with aminopeptidase suggested a free terminal group, and the partial loss of both activities following incubation with chymotrypsin suggested one or more aromatic amino acid residues existed somewhere in the structure. Incubation of nondeproteinized plasma at $37^{o}C$ resulted in complete loss of both activities within 20 minutes. A similar incubation at $0^{o}C$ had no effect.

The fact that there were parallel changes in natriuretic and antinatriferic activities with the treatments described above and listed in Table 2, suggested that a single substance was responsible for both of the observed activities. Although no known purified naturally occurring substance possesses both activities, the observation that synthetic (4-leu)-oxytocin (152) does possess all the properties attributed to 'NH' provides evidence that such a naturally

occurring substance could exist. In cats, (4-leu)-arg-vasotocin is even more natriuretic than (4-leu)-oxytocin (60), Furthermore, oxytocin inhibitors (i.e., 2-O-methyltyrosine-oxytocin) inhibit not only the natriuretic action of oxytocin, but also the natriuretic response to carotid artery occlusion (144). This suggests that the 'NH' in cats may be similar in structure to oxytocin.

Comparing the natriferic (enhanced sodium transport in frog skin and toad bladder) effects of known concentrations of oxytocin with the antinatriferic activity of carotid artery occluded cat plasma, Cort et al. (58) estimate the maximum plasma concentration of this antinatriferic substance to be 10^{-11} to 10^{-15} molar. The assumption made in arriving at this conclusion, which is open to criticism, was that one molecule of oxytocin and antinatriferic material have equal but opposite effects on frog skin sodium transport. Finally, use of dibenzyline to counteract circulating catecholamines had no effect on the natriuretic response to carotid artery occlusion or 20% blood volume expansion with 6% dextran in saline (51). This suggested that the natriuretic substance is not a catecholamine.

Bricker et al. (29) observed that, following Sephadex fractionation of plasma from chronically uremic humans, one fraction was obtained that inhibited the accumulation of PAH by rabbit kidney cortical slices. A similar fraction from normal humans had no effect. Interestingly, the same fraction obtained from saline loaded dogs and cows also inhibited PAH transport whereas that from non-expanded animals did not. In subsequent studies, this same Sephadex fraction was shown to

inhibit the SCC in frog skin (20) and enhance sodium excretion in rat kidneys (19). These observations, and others listed in Table 2, suggest that the antinatriferic-natriuretic activities observed with acute volume expansion in animals and uremia in humans are due to substances which are at least similar.

Utilizing Sephadex chromatography and ultrafiltration studies, Bourgoignie et al. (20) estimated the molecular weight of this human, cow, and dog natriuretic-antinatriferic material to be approximately 500 to 1000 Daltons. Other studies demonstrated that the natriuretic activity was not destroyed by boiling or freeze drying (19,20,29), and, in contrast to Cort's findings in the cat, was also not susceptible to degradation by pronase and chymotrypsin. Boiling the active Sephadex fraction for 10 minutes at pH 10.5 destroyed the natriuretic activity in the rat assay (19). This treatment was not performed on the samples used in the frog skin and PAH studies. Also, these authors noted in the frog skin studies that the antinatriferic activity was stable for at least 4 weeks if stored frozen. If the specimen was kept at -80°C it could be stored for ten weeks before Sephadex fractionation without a detectable loss in antinatriferic activity.

Ultrafiltration studies by Buckalew et al. (36) led these authors to conclude that the molecular weight of an antinatriferic factor obtained from volume expanded dogs could not exceed 3000 Daltons. Fractionation on Sephadex G10 resin suggested that the minimum molecular weight was approximately 500 to 700 Daltons (34), although in further studies the molecular weight appeared to be less than 500 to 700 Daltons (37). Similar ultrafiltration studies with an antinatriferic

factor obtained during mineralocorticoid escape suggested that this material had a molecular weight less than 10,000 Daltons (35). No attempts were made to determine how much less. Of interest was the observation that the antinatriferic substance was dialyzable in vivo from dogs undergoing volume expansion with saline, as it suggests that it might also be dialyzable in vivo from other species including humans.

Martinez-Maldonado et al. (123) report that a natriuretic substance obtained by them from saline loaded rats and dogs was small enough to be dialyzable, could be stored at 4°C, but was destroyed when freeze dried. Cort et al. (56) describe a bovine material obtained by volume expansion of cows with dextran. From Sephadex chromatographic separation studies the molecular weight was estimated to be from 800 to 1000 Daltons. Sealey et al. (150) and Sealey and Laragh (151) report that a natriuretic substance obtained in the urine from salt-loaded humans appeared to have a molecular weight between 10,000 and 50,000 Daltons based on ultrafiltration and gel filtration studies. Attempts to demonstrate a lower molecular weight sodium transport inhibitor proved to be unsuccessful due to technical problems. The material was not destroyed by boiling, a finding consistent with Bourgoignie and Cort, but, in contrast to Cort, Sealey reports that her material is destroyed by H-TCA.

In summary, a few studies have attempted to characterize the substances responsible for the natriuretic and antinatriferic activities observed in plasma following various modes of extracellular fluid

volume expansion. These studies suggest that both of these activities may be due to a single substance, or at least to substances which are very similar chemically, irregardless of the type of expansion or species studied.

Source of Hormonal Natriuretic-Antinatriferic Factors

If a 'natriuretic-antinatriferic hormone' exists, and accumulating evidence would seem to suggest that it does, then it should be possible to identify the source of such a hormone. Lichardus (109) and Lichardus et al. (115) are of the opinion that the main cause of difficulties in the identification of such a substance is the lack of knowledge concerning this production site. Studies to date have involved body fluids in which the concentration of the 'hormone' may be very low. In efforts to overcome this difficulty cows have been used in an attempt to secure larger plasma volumes without significantly decreasing the total blood volume in expansion experiments (29,115). However, if the substance is dialyzable *in vivo* from humans, another alternative to secure larger quantities of material would be to isolate it from spent dialysis fluid from uremic patients undergoing maintenance hemodialysis.

A report which appeared May, 1971, by Sealey and Laragh (150) did not contribute to the clarification of this question. These authors found that, of ten different organs investigated in salt-loaded sheep, 8 possessed a natriuretic activity. It seems unlikely that all of these organs can be responsible for the secretion of such a factor. A more

probable explanation would be that blood circulated to these organs during imposed natriuresis contained the natriuretic factor which was then extracted by the investigators. Particularly disturbing in Sealey's study was the lack of activity found in the brain, since other investigators have found evidence indicating this organ to be the primary source of an antinatriferic and/or natriuretic factor.

One of the first indications that a sodium transport inhibiting substance may be elaborated from some area of the brain came from observations that jugular venous blood produced a greater sodium diuresis in the kidney and a greater inhibition of sodium transport across frog skins and toad bladders than blood from other areas of the body (36,47,54,57). Cort and Lichardus (54) observed that during bilateral common carotid artery occlusion in cats, jugular venous samples decreased frog skin short circuit current by 26.3%, while femoral arterial blood decreased it only 10.6%. Femoral venous blood and renal venous blood increased the short circuit current 10.8% and 5.8% respectively. All samples obtained from these anatomical locations before occlusion increased the SCC from 6.5 to 12.5%. Buckalew et al. (36) observed that ultrafiltrates prepared from jugular venous blood inhibited the SCC of toad bladders significantly more than did similar ultrafiltrates from femoral venous blood following saline expansion in dogs.

From 14 rats, a total of 8000 posterior, ventromedial, dorsomedial, and arcuate nuclei were histologically examined in 2 groups of animals by Lichardus et al. (112). One group of rats was given 2% saline to

drink ad libitum, and a second group drank tap water. A statistically significant decrease in nuclear volume was noted only in the posterior hypothalamic nuclei of the saline group. This was taken as evidence that the posterior nucleus has a neurosecretory function in the elimination of a saline load. In addition, it has been noted that electrolytic lesions of the posterior hypothalamus eliminate the natriuresis seen with carotid artery occlusion (52,53,59) and iso-oncotic blood volume expansion in cats (50).

Acute hypophysectomy markedly decreased sodium and urine output compared to nonhypophysectomized rats during blood volume expansion (110). Homogenates of anterior pituitary tissue were ineffective in restoring the ability of these hypophysectomized rats to excrete sodium after infusion of a saline load (117). Homogenates from the posterior pituitary, on the other hand, were effective.

Incubation of anterior and posterior bovine hypothalamic extracts with proteolytic enzymes such as chymotrypsin, trypsin, and swine kidney aminopeptidase, resulted in the appearance of an antinatriferic activity from posterior extracts (47,57,58). However, continued incubation with these enzymes resulted in a progressive loss of antinatriferic activity. The conclusion from these studies was that a small antinatriferic substance was first released from a larger 'hormonogen' form during the incubation. Further incubation resulted in a proteolytic degradation of the active material. Anterior hypothalamic material gave rise only to natriferic activity.

Since no differences were found in the natriuretic response to carotid occlusion in intact and adrenalectomized cats, Licardus and

Cort (111) concluded that the adrenals are not the source of a potential natriuretic hormone. Bourgoignie et al. (20) report that 2 of 3 anephric chronically uremic patients studied demonstrated in their serum an inhibitor of sodium transport in frog skin. Thus they concluded that the kidney was not responsible for the synthesis of this antinatriferic material. Evisceration and nephrectomy before saline loading in rats and dogs did not abolish the inhibitory activity of plasma on proximal tubule sodium reabsorption as measured by microperfusion techniques (123). Finally, Levinsky (103) found that the natriuresis observed in iso-oncotic blood volume expansion in dogs persisted after adrenalectomy and removal of the spleen, liver, and intestines. Elimination of the head and brain did not prevent a natriuresis. However, this is a rather drastic procedure, changing many parameters, and rendering rigid controls difficult.

Andersson et al. (1) noted that injection of 0.85 M NaCl into the third ventricle of unanesthetized goats resulted in a 5 to 10-fold increase in sodium excretion and a 3-fold increase in urine flow. Although depression of aldosterone secretion could not be ruled out in these experiments, further studies the following year (2) demonstrated that aldosterone administration did not prevent the increase in electrolyte excretion and that injections into the lateral ventricle had no effect.

In 1969 Dorn and Porter (68) perfused the third ventricle of rat brains with several substances and was able to induce natriuresis. When 0.85 M NaCl was perfused at a rate of 0.7 µl/min he noted an

insignificant increase in urine flow but a 10-fold increase in sodium excretion over the control rate. Infusion of 0.154 M NaCl and 1.7 M glucose into the ventricle and intravenous infusion of 0.85 M NaCl at the same rate produced no natriuresis. One explanation offered for the observed natriuresis was that hypertonic saline solution may pass from the ventricle into adjacent hypothalamic tissue and stimulate a neural or hormonal mechanism which mediated the natriuretic response.

Similar injections by Dorn et al. (67) into third ventricles of anesthetized dogs also induced a significant increase in urinary sodium excretion which was not related to alterations in renal plasma flow, GFR, or filtered load of sodium. The authors interpret all of these findings as being consistent with the action of a cerebral natriuretic hormone. They comment that "... the identicalness of an 'NH' whose secretion is stimulated by systemic volume loading and one whose release follows injection of the third ventricle with hypertonic saline is conjectural. It is conceivable that 2 hormones exist, one responsive to alterations in volume and one to changes in concentration of sodium. These hormones acting in concert could serve as a hormonal modulator of total body sodium."

It would seem, therefore, that a natriuretic-antinatriferic factor, or factors, has its origin in the posterior hypothalamus. This, however, remains to be proven unequivocally.

Possible Natriuretic Hormone Involvement with Reduced Nephron Populations and Renal Disease

Third factor hormonal activity has been demonstrated under situations of acute and massive expansion of extracellular fluid volume and

carotid artery occlusion. An interesting question which arises is whether or not this factor is available only as an emergency mechanism or does it play a role in the day-to-day regulation of sodium balance as has been suggested (33,35,150). This is difficult to ascertain in normal individuals with a full complement of nephrons because changes in salt excretion per nephron required by the usual range of salt ingestion are of such a magnitude that it is difficult to distinguish between glomerular and tubular factors. With a decreased number of nephrons, however, the distinction might be more readily determined since each nephron is required to excrete a greater fraction of the filtered load in situations of imposed saline loads. This type of assay system was used by Bourgoignie et al. (19), in rats, to test for a natriuretic substance in the plasma of uremic humans. These authors (19,149,157), and others (168), feel that the response to a given natriuretic stimulus is increased under these circumstances.

Schultz et al. (148) decreased the nephron population of one kidney of dogs approximately 80% by ligating terminal branches of the renal artery. The residual nephrons retained their normal blood supply. Prior to the removal of the contralateral kidney, the remnant kidney reabsorbed 99% of the filtered load of sodium. On the same salt intake, with the contralateral kidney removed, sodium excretion increased in the remnant kidney and fractional reabsorption decreased. Renal artery constriction ruled out hyperfiltration as the cause of the natriuresis. Since the dogs were on supramaximal doses of fluorohydrocortisone, mineralocorticoid insufficiency was also disregarded as the cause of the elevated saline diuresis. These authors conclude that the volume

control mechanism becomes more responsive in uremia, with the relative contribution of an 'NH' versus changes in intrarenal physical factors needing further investigation. Similar conclusions from experiments with dogs were reported by Wen et al. (168).

Hayslett et al. (80,81), in rats, reduced renal mass by progressively excising renal tissue. Following uninephrectomy these authors noted a 2-fold increase in sodium excretion per nephron with a 75% increase in GFR per nephron. With further removal of renal tissue, sodium excretion per nephron increased 5 to 6 times normal with no further rise in GFR per nephron. However, the half-time of fluid reabsorption in proximal tubules blocked with oil was unchanged by renal ablation. The authors therefore concluded that a 'third factor' hormone did not participate in the adjustment made to experimental renal insufficiency in these studies since this substance has been proposed to exert its effect in the proximal tubule. Here again is a negative finding with attempts to elicit 'NH' in the rat. This finding is in contrast to the observation of Bourgoignie and co-workers (19,20, 21,27,28,29), discussed previously, who have found evidence for a natriuretic and antinatriferic activity in the serum of patients with chronic renal disease. These individuals do have a reduced population of functional nephrons.

B. Physical Factors Affecting Tubular Reabsorption of Sodium Independently of Factor I and Factor II

In 1963 Blythe and Welt (17) infused 5% saline into dogs and decreased the GFR by inflation of a balloon inserted via the femoral vein

into the inferior vena cava to a point distal to the renal veins. Noting that urinary excretion of sodium could change independently of the filtered load of sodium, they concluded that the excretion of sodium is somehow related to the plasma levels of the ion and not necessarily to the filtered load. Dirks et al. (66), infusing isotonic and hypertonic saline into dogs, also noted a marked depression of proximal tubular fractional reabsorption which was independent of GFR and not blocked by reduction in GFR. Glabman et al. (76), however, concluded from micropuncture studies on proximal tubules of nonexpanded rats that filtered load of sodium is an important determinant of the rate of sodium reabsorption by the proximal tubule.

Compounding the difficulties of detecting decreased proximal sodium reabsorption by measuring the urinary excretion of sodium is reabsorption of the ion in the distal portions of the nephron. Higgins (84), for example, showed that infusions of 600 ml of 5% albumin or dextran in 5% glucose into normally hydrated dogs caused only moderate increases or even decreases in sodium excretion. Similar expansion with blockage of sodium reabsorption distal to the proximal tubule by ethacrynic acid and chlorothiazide resulted in large increases in sodium excretion. He concluded, therefore, that during plasma volume expansion the ultimate sodium excretion rate is determined by distal reabsorption in spite of a decreased proximal reabsorption. Similar conclusions were reached by Buckalew et al. (38) and Davis et al. (64) with acute saline loading in dogs and Sellman et al. (154) and Hayslett et al. (79) in the rat. During mineralocorticoid escape in normal man,

Martino and Earley (126) report that most of the proximally rejected sodium is recaptured by reabsorption at a site distal to the medullary loop of Henle, with Sonnenberg (158) placing it in the collecting duct during deoxycorticosterone escape in the rat.

Studies of McDonald et al. (128), in which dogs underwent volume expansion with Ringer's lactate solution or saline plus 6% dextran, and Martino and Earley (127) using isotonic saline or saline plus 5% bovine albumin, suggested that decreases in blood and plasma viscosity accompanying saline infusion may potentiate the natriuretic response by decreasing renal vascular resistance and increasing capillary hydrostatic pressure. This 'pressure natriuresis' was also observed by Kaloyanides et al. (96) when the arterial pressure was increased in an isolated kidney being perfused with blood from a nonexpanded intact dog. Similar observations were made by MacDonald and DeWardener (121) in isolated kidneys perfused at constant pressure with blood from an intact dog receiving an intravenous infusion of saline.

Lewy and Windhager (105) and Windhager et al. (169), from micropuncture studies in the rat, suggest that proximal tubular reabsorption is partly controlled by the rate of vascular removal of epithelial reabsorbate. Capillary removal of reabsorbate is influenced by the hydrostatic pressure gradient between renal interstitium and capillary lumen. Increased peritubular capillary hydrostatic pressure could lead to a decrease in the rate of capillary removal of reabsorbate. This would decrease the rate of sodium reabsorption by increasing renal interstitial pressure. Decreases in renal interstitial hydrostatic pressure would have the opposite effect (55, pp. 17-25).

Earley (69) demonstrated that vasodilatation of the kidney with infusions of acetylcholine into the renal artery resulted in an increased sodium excretion. Increasing the arterial pressure in this vasodilated kidney resulted in an additional increase in sodium excretion, which occurred even when renal blood flow and GFR were decreased. These observations provided additional evidence that changes in arterial pressure or vascular resistance may be involved in determining the natriuretic response to saline loading.

Other investigators have shown that postglomerular plasma protein concentration, either with (3,5,88,124,136) or with (4,23,25,105,161,165) blood volume expansion, is an important determinant in sodium excretion. Martino and Earley (125), for example, showed that when hyperoncotic albumin solutions (30%) were infused into animals previously loaded with isotonic saline, sodium excretion decreased despite an increase in GFR, renal blood flow, and arterial pressure. Spitzer and Windhager (161) found that perfusion of capillaries with colloid-free Ringers decreased reabsorption of sodium 49%, but inclusion of dextran (8%) in the Ringers produced results similar to those during normal blood perfusion. These observations support the Windhager theory that renal interstitial pressure affects sodium reabsorption.

Another factor which appeared to affect sodium reabsorption, both with (90,130,131,146) and without (24,26,39,42,98,132,133,145) blood volume expansion, is the hematocrit. Brenner et al. (26) and Brenner and Galla (24), as a result of micropuncture studies in rats, observed that although changes in hematocrit lead to corresponding changes in proximal sodium reabsorption, parallel changes in peritubular capillary

protein concentration also occur. It was concluded that this change in postglomerular protein concentration may in fact be responsible for the changes observed in sodium reabsorption which accompany changes in hematocrit. Substantiating this conclusion, Bahlmann et al. (8) isovolemically decreased the hematocrit in the dog 28% with Hartmann's solution containing bovine albumin and found no appreciable change in sodium excretion. A similar result was reported by Ponec and Lichardus (139) in the rat. Burke et al. (39), with recollection micropuncture techniques in the dog, suggest that changes in viscosity which alter capillary hydrostatic pressure may also account for hematocrit changes on proximal sodium reabsorption.

In addition to the physical factors previously discussed, others have been advanced to explain the mechanisms controlling sodium excretion. These include changes in renal plasma flow (70,71,72,169) and redistribution of blood flow from medullary to more superficial high sodium excreting nephrons both with (40,103) and without (11,85,87) saline loading. For example, Windhager et al. (169) demonstrated with micropuncture techniques in rat kidneys that the decreased proximal reabsorption of sodium seen with experimentally increased renal venous pressure was proportional to the decrease in renal plasma flow. With saline loading in the dog, Earley and Friedler (70,72) suggested that the increased renal plasma flow seen in their experiments may be one factor that contributes to the decreased tubular reabsorption of sodium.

Using X_e^{133} washout techniques, Hollenberg et al. (85) found that 67% of the total renal blood flow in humans on a 10 mEq/day salt intake

was cortical. In subjects on a 200 mEq/day salt diet 84% of the total renal blood flow was cortical. Barger (11) suggested a similar redistribution in dogs with right sided congestive heart failure. Other investigators, however, have reported that there is no significant redistribution of blood flow following saline loading (120,129,155).

In summary, it is apparent that the 'third factor' natriuresis which occurs following extracellular fluid volume expansion is a complicated interrelationship of many factors. In vivo experiments attempting to evaluate the role of a 'natriuretic hormone' must be carefully designed in order that the physical factors just described, which contribute to this 'third factor' natriuresis, do not change significantly.

PURPOSE OF INVESTIGATION

From the preceding review it is evident that the 'third factor' natriuresis observed with volume expansion may in fact prove to be multi-factoral, with the importance of individual physical and/or hormonal factors being dependent upon the mode of expansion. The current study was undertaken to 1) substantiate the observation that an antinatriferic substance exists in the plasma of expanded chronically uremic humans, and 2) to investigate the potential of utilizing spent hemodialysis fluid as a source of this substance. To substantiate Cort's finding that an antinatriferic substance remains in plasma following trichloroacetic acid deproteinization, the antinatriferic activity of H-TCA deproteinized and non H-TCA deproteinized plasma from uremic humans and moderately volume expanded dogs was also studied.

METHODS

A. Sodium and Potassium Determination

Sodium and potassium were determined by flame photometry using a lithium internal standard (double beam technique) which has a resonance line at 671 mμ. The resonance doublet employed for sodium occurs at 590 mμ and that for potassium at 767-769 mμ. In practice, the ratio of the emission intensity of the analysis line to that of the internal standard line is recorded and plotted against the concentration of the analysis element to prepare a calibration curve for a series of standards. Unknown concentrations of test element are then determined from the standard curve. Some advantages of this method over direct determination (single beam methods measuring absolute light intensities rather than ratios) in the flame are:

1. Better precision
2. Compensates for effects of irregularities of atomization
3. Compensates for variable loss of liquid in the spray chamber
4. Reduces systematic errors due to possible differences in viscosity and surface tension of samples (affects rate of delivery of sample into the flame)
5. Reduces errors due to some radiation interferences.

The instrument used was a Baird-Atomic Model KY-3 combination clinical and research filter flame photometer equipped with a gravity feed spray chamber aspirator (flow rate 3-6 ml/min) and Meker burner. A gas air mixture (air pressure 10 psi) yielding a low flame temperature of approximately 2100°K was utilized.

Reagents

I) 2.00 mMolar sodium chloride and potassium chloride stock solution: Weigh 11.688 mg of dried reagent grade NaCl and 14.912 mg of dried reagent grade KCl into a 100 ml volumetric flask. Dilute to volume with 90 ppm lithium water.

II) Stock lithium water: Dilute 5.0 ml of 18,000 ppm lithium solution to 1000 ml with deionized water. Final lithium ion concentration is 90 ppm. This solution is to be used to prepare all samples and standards to be analyzed.

Preparation of Samples

Pipette 0.020 ml of unknown sample to be analyzed into 10 ml of 90 ppm lithium water for the determination of sodium. For the determination of potassium, pipette 0.020 ml of unknown sample into 2 ml of 90 ppm lithium water. Mix by inversion or vortexing and read in flame photometer.

Preparation of Standards

In Table 3 is the procedure for the preparation of the sodium and potassium standard solutions utilized for the preparation of the standard curves. The designated volumes were pipetted into 15 ml Pyrex test tubes and mixed by inversion.

All solutions to be stored for indefinite periods should be stored in plastic bottles to prevent leaching of ions from the glass (especially new glassware), and refrigerated to minimize bacterial growth. The procedure for the preparation of the standards and samples was adapted from Terris (163).

Table 3. Procedure for the preparation of the sodium and potassium solutions utilized for the preparation of standard curves.

Standard Number	Reagent I (ml)	Reagent II (ml)	Na:K (mEq/l)
1	0.12	9.87	0.024
2	0.25	9.75	0.050
3	0.50	9.50	0.10
4	0.75	9.25	0.15
5	1.00	9.00	0.20
6	1.50	8.50	0.30

Calculation of Unknown Na and K Concentrations

$$\text{Na(mEq/l)} = \frac{\text{Instrument Reading}}{\text{Slope}} \times \text{Dilution Factor}$$

$$= \frac{\text{Instrument Reading}}{\text{Slope}} \times 500$$

$$\text{K(mEq/l)} = \frac{\text{Instrument Reading}}{\text{Slope}} \times \text{Dilution Factor}$$

$$= \frac{\text{Instrument Reading}}{\text{Slope}} \times 100$$

If the samples are prepared as described, the dilution factors employed in the calculations are as given above. The slope is obtained from a standard curve prepared by plotting the instrument reading (ordinate) versus the concentration of the standards (abscissa) in mEq/l. An example of such a curve is given by Figure 1.

Figure 1. Standard curves for sodium and potassium as determined with a Baird-Atomic Model KY-3 combination clinical and research filter flame photometer. Instrument reading (arbitrary units) is plotted on the ordinate, with known concentrations of sodium and potassium in mEq/l being plotted on the abscissa.

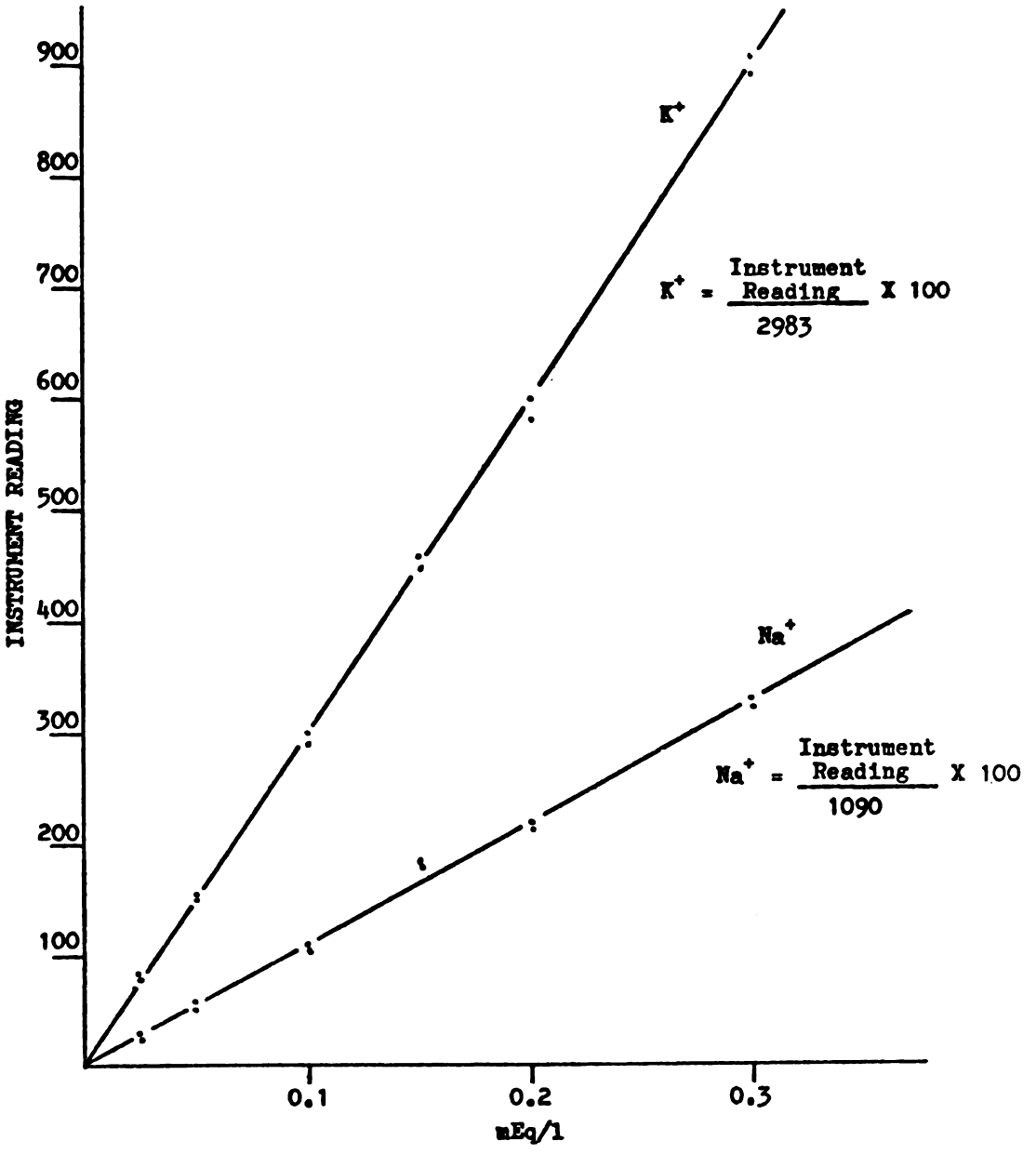
900
800
700
600
500
400
300
200
100
INSTRUMENT READING
K+
K+ = Instrument Reading / 2983 X 100
Na+
Na+ = Instrument Reading / 1090 X 100
0.1
0.2
0.3
mEq/l

Figure 1.

B. Chloride Determination

Chloride was determined by a coulometric-amperometric titration with silver ions utilizing a Buchler-Cotlove chloridometer. The method is based on the coulometric generation of reagent (silver ions) and amperometric indication of the end-point. A constant direct current is passed between a pair of silver generator electrodes in the generator (coulometric) circuit, causing release of silver ions into the titration solution at a constant rate. The end-point is indicated, after all chloride has been precipitated, by the increasing concentration of free silver ions which cause a rising current to flow through a pair of silver indicator electrodes. At a preset increment of indicator current a relay is actuated, stopping a timer which runs simultaneously with the generation of silver ions. Since the rate of generation of silver ion is constant, the amount of chloride precipitated is proportional to the elapsed time.

Reagents

I) Nitric-Acetic Acid Reagent (0.1 N HNO_3 and 10%, V/V, glacial acetic acid): To 900 ml of distilled water add 6.4 ml of concentrated reagent grade nitric acid and 10 ml of reagent grade glacial acetic acid. Store in glass container with a glass stopper.

II) Gelatin Reagent: To 6.2 grams of 60:1:1 dry mixture (gelatin: thymol blue (water soluble): thymol (reagent grade crystals)) add approximately 1 liter of hot water and stir gently until the solution is clear. Store refrigerated in glass tubes in volumes sufficient for a set of analyses. A new tube should be used for each day's analyses. To liquify the gelatin immerse the tube in hot water. Do not freeze (as this destroys the effectiveness of the gelatin). Do not use the gelatin if it has been at room temperature for more than a day or two.

III) Sodium Chloride Reagent (160 mEq/l): Dissolve 9.3520 grams of dried-reagent grade NaCl in distilled water and dilute to exactly 1 liter.

Preparation of Samples, Standards, and Blanks

A) Unknowns: To 0.1 ml of sample in a titration vial add 4 ml of nitric-acetic acid reagent and 4 drops of gelatin reagent.

B) Standard Sample: To 0.1 ml of NaCl standard solution (160 mEq/l) in a titration vial add 4 ml of the nitric-acetic acid reagent and 4 drops of the gelatin reagent.

C) Blank Sample: To approximately 4 ml of the nitric-acetic acid reagent in a titration vial add 4 drops of gelatin reagent.

Samples and reagents should not be stored in contact with rubber as sulfhydryl groups may be released which may combine with silver ions leading to inaccurate determinations. In addition, do not titrate a sample unless the solution is acid and the gelatin reagent is present (indicated by a red color of the thymol blue indicator).

Calculation of Chloride Ion Concentration

a) Gross seconds = timer reading

b) Average net seconds of standard = average gross seconds of standard minus average blank seconds

c) Calibration factor = K

$$K = \frac{(\text{ml of NaCl reagent})\ ([Cl^-] \text{ in mEq/l})}{\text{average net seconds of standard}}$$

d) Net seconds of unknown = gross seconds minus average blank seconds

$$\text{Unknown } Cl^- \text{ concentration} = \frac{K\ (\text{net seconds of unknown})}{\text{ml of unknown}}$$

If the samples, standards, and blanks are prepared as previously described, the above equation for the concentration of chloride ion in the unknown samples (in mEq/l) reduces to the following:

$$[Cl^-] = \frac{(160)\ (0.1)}{\text{net seconds for standard}} \times \frac{\text{net seconds for unknown}}{\text{ml of unknown}}$$

$$= \frac{160}{\text{net seconds for standard}} \times \text{net seconds for unknown}$$

$$= \frac{\text{net seconds for unknown}}{\text{net seconds for standard}} (160)\ \text{mEq/l}$$

C. Glucose Determination

Glucose was determined by a glucose oxidase method, which combines the following two reactions:

1) Glucose + O_2 + H_2O $\xrightarrow{\text{glucose oxidase}}$ H_2O_2 + gluconic acid

2) H_2O_2 + o-dianisidine (reduced) $\xrightarrow{\text{peroxidase}}$ o-dianisidine (oxidized) + H_2O

Glucostat, a prepared reagent for the quantitative colorimetric determination of glucose containing peroxidase, glucose oxidase, and reduced o-dianisidine, is available in a lyophilized form from Worthington Biochemical Corp., Freehold, N. J. This preparation was used in these analyses.

Glucose oxidase is highly specific for beta-D-glucose, and since glucose in solution is usually 36% alpha and 64% beta, complete oxidation requires mutarotation of the alpha to the beta form. Complete oxidation is not necessary for the success of the method. If, however, the glucose preparation does not contain mutarotase to accelerate this reaction, standard solutions prepared from dry glucose should stand at least 2 hours to insure that mutarotation has reached a state of equilibrium.

Reagents

I) Stock glucose: 200 mg percent--Dissolve 200 mg of reagent grade glucose in 100 ml of distilled water in a 100 ml volumetric flask. Store refrigerated.

II) Glucostat reagent: Dissolve 1 vial of 4X chromogen (o-dianisidine) and 1 vial of 4X enzyme in 200 ml of distilled water. Prepare fresh for each assay, or if storage of the prepared reagent is desired, place in an amber bottle and keep refrigerated for no longer than 1 month.

III) 4 N HCl: Dilute 33.33 ml of concentrated HCl (12 N) to 100 ml with distilled water.

Preparation of Standards

In Table 4 is the procedure for the preparation of the standard solutions utilized for the preparation of the standard curves. The designated volumes were pipetted into 15 ml Pyrex test tubes and mixed by inversion.

Table 4. Procedure for the preparation of the glucose standard solutions utilized for the preparation of standard curves.

Standard Number	Reagent I (ml)	H_2O (ml)	Glucose (mg/ml)	Glucose (mMolar)
1	0.25	10.00	0.49	0.27
2	0.50	10.00	0.98	0.54
3	0.75	10.00	0.14	0.78
4	1.00	10.00	0.18	1.01
5	1.25	10.00	0.22	1.22
6	1.50	10.00	0.26	1.45

Preparation of Samples and Standards for Analysis

Dilute 1/2 ml of unknown(s) to 10 ml with distilled water. Pipette 1/2 ml of this diluted sample to 2 ml with 1.5 ml of glucostat reagent (Reagent II above). To prepare the standards for analysis, pipette 1/2 ml of the above solutions to 2 ml with 1.5 ml of the glucostat reagent. Exactly 10 minutes after the addition of the glucostat reagent add 2 drops of the 4 N HCl to halt color development (the 10 minute incubation having been performed at room temperature). Following the addition of the acid, mix by inversion or vortexing immediately. Standards and unknowns should be analyzed simultaneously under conditions such that the rate of oxidation is proportional to the glucose concentration. In some methods the final mixture is acidified slightly to stop the reaction and the yellow color developed is measured at 400 mμ (as is the case here). In stronger acid, the color becomes pink with maximum absorption at 540 mμ (where both sensitivity and stability are improved). Introduction of the enzyme peroxidase and a chromogenic O_2 acceptor (reduced o-dianisidine) provides the color development.

Calculation of Unknown Glucose Concentration

Glucose (mg/ml) = (A/Slope) (dilution factor)

A = absorbance of unknown at 400 mμ

Slope = slope of standard curve, with absorbance of standards plotted on the ordinate and concentration of standards on the abscissa

Dilution Factor = 20 if the above procedures are followed.

A representative standard curve is given in Figure 2.

Figure 2. Standard curve for glucose as determined with a Beckman Model DB spectrophotometer at 400 mμ. Absorbance at 400 mμ is plotted on the ordinate against known concentrations of glucose in mg/ml on the abscissa.

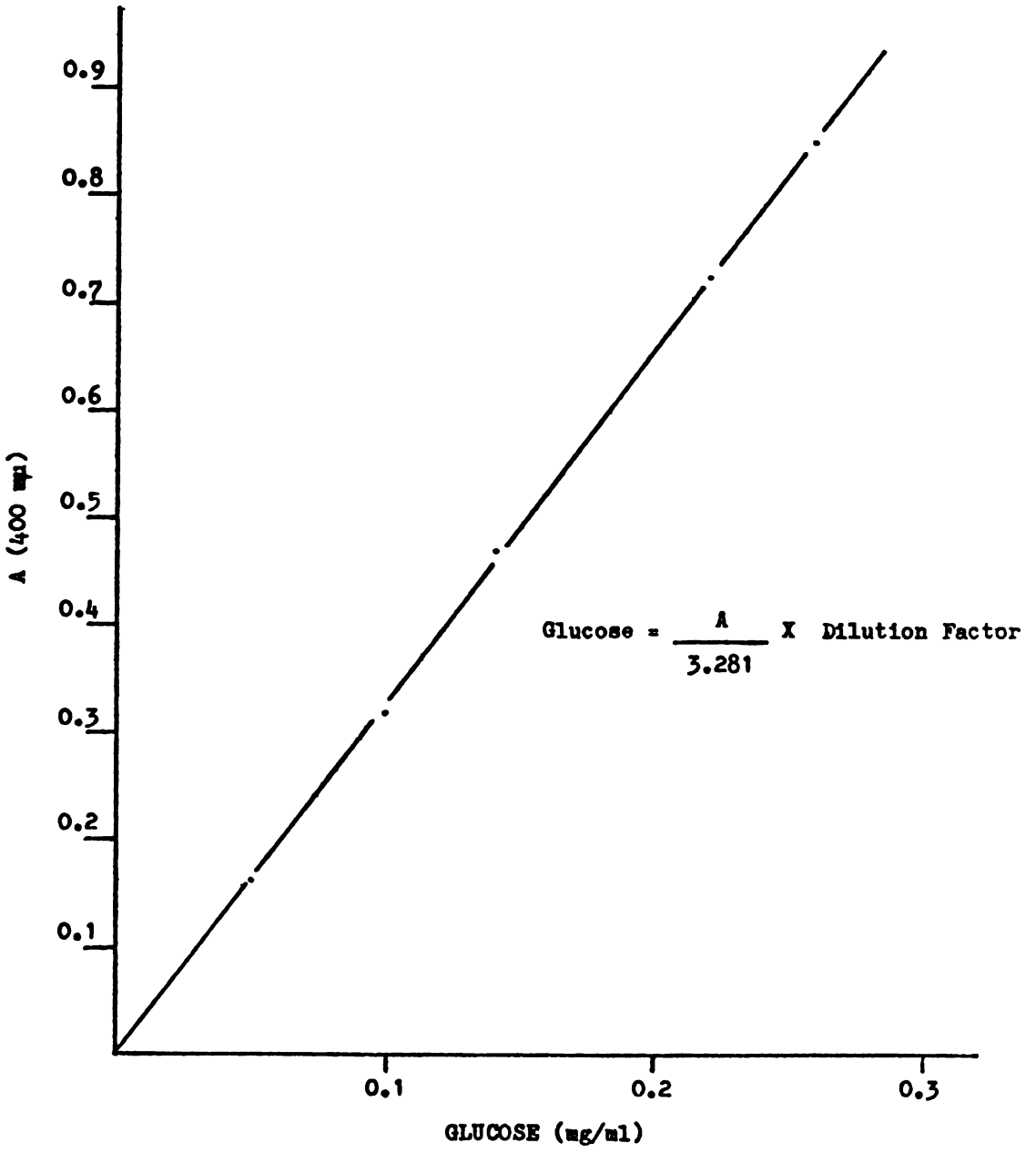
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A (400 mμ)
0.1
0.2
0.3
GLUCOSE (mg/ml)
Glucose = A / 3.281 X Dilution Factor

Figure 2.

D. Ammonium Ion Determination

The determination of ammonium ion as ammonia utilized the general principle of microdiffusion of volatile substances. A small aliquot of the sample to be tested is placed into a special microdiffusion apparatus originally described by Conway and Byrne (45). The sample is mixed with a concentrated alkali solution and the volatile ammonia thus liberated diffuses from an inner chamber to an inner well which contains an indicator solution. The concentration of alkali (sodium carbonate) in these studies was approximately 16%, adopted from Brown et al. (30). Titration of the absorbed ammonia with an acid of known concentration permits the quantitative determination of the ammonium ion contained in the original sample.

The apparatus employed for the determination of ammonium ion in these studies was a modified Conway unit described by O'Brink in 1955 (138). Figure 3 shows the construction of the unit.

Reagents

I) 20% sodium carbonate: weigh 20 grams of anhydrous sodium carbonate into a 100 ml volumetric flask and dilute to volume with distilled water. The final concentration in the diffusion unit will be approximately 16% if 0.2 ml of sample to be tested is used.

II) 0.004N HCl: dilute 0.40 ml of 1.0 N HCl (Acculute) to 100 ml with distilled water.

III) Tashiro's reagent (162): to 200 ml of a 0.1% alcoholic solution of methyl red add 50 ml of a 0.1% alcoholic solution of methylene blue. If stored in a brown bottle the solution will keep indefinitely.

IV) 1% boric acid: weigh 1 gram of boric acid into a 100 ml volumetric flask and dilute to volume with distilled water.

Figure 3. O'Brink modification of the Conway microdiffusion unit.

1) inner well
2) inner chamber
3) outer chamber

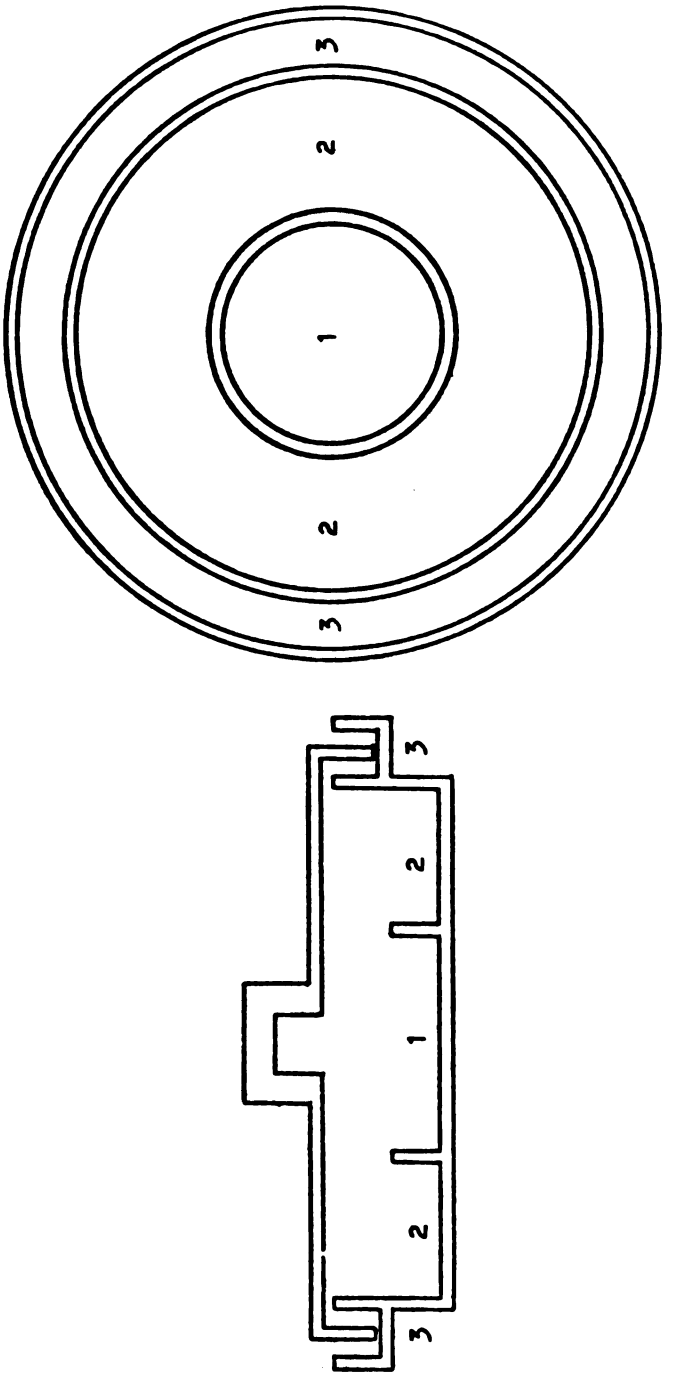

Figure 3.

V) Indicator solution: to 35 ml of distilled water add 15 ml of the 1% boric acid prepared as described above. To this mixture pipette 5 ml of Tashiro's reagent and mix thoroughly. For best results prepare this solution fresh just prior to use.

Procedure

Into the inner well of the microdiffusion apparatus pipette 1 ml of the indicator solution and place the lid onto the unit. Gently rotate the apparatus to completely wet and distribute the solution over the entire inner well. Observe the indicator color for 5-10 minutes to insure that the well is not contaminated (indicator will turn from violet to brown or green if a contaminant is present). Remove the lid, and into the inner and outer chambers pipette 1 ml of the 20% sodium carbonate solution. Into the inner chamber pipette 0.2 ml of sample to be tested in such a manner that it does not come into contact with the alkali. Replace and rotate the lid to completely distribute the alkali between the lid and outer chamber areas of contact. This must be done very carefully to insure a complete seal between the lid and diffusion apparatus. An incomplete seal will permit the ammonia liberated to diffuse out of the unit and lead to erroneous results.

With the establishment of a complete seal between the lid and outer chamber, the entire unit is then rotated several times to mix the test sample with the sodium carbonate in the inner chamber. Care must be taken not to allow spillage of the contents of the inner and outer chambers into the inner well. If this occurs the determination must be repeated. With the above volumes this danger is minimal.

Once the solutions have mixed, the unit is allowed to sit for 90-120 minutes to allow diffusion of the liberated ammonia into the inner well. An occasional swirling of the solution during this period will facilitate the process.

Following the incubation period the lid is very carefully removed. Care must be taken to avoid splashing of the sodium carbonate from the outer chamber and lid into the inner well. With gentle swirling the inner well is titrated with 0.004 N HCl to the original color of the indicator solution. For a blank, prepare the apparatus as described using 0.2 ml of water in place of the test solution. Titrate the blank and subtract this volume of HCl from that required to titrate the test solutions. As an alternative, known concentrations of ammonium ion can be titrated and standard curves established.

To clean the units after use do not use detergent. Allow them to soak in dilute acid overnight and rinse several times with tap water. Follow the tap water rinses with several distilled water rinses and allow the units to dry before reuse.

Calculation of the Unknown Ammonium Ion Concentration

At the equivalence point in any titration the number of mEq of standard is exactly equal to the number of mEq of substance being determined. To calculate the mEq of ammonia, therefore, one need only determine the mEq of HCl utilized during the titration. The final value can then be expressed as desired.

$$\text{mEq HCl utilized} = (\text{vol of HCl in ml})\ (\text{N of HCl})$$

$$NH_4^+\ (\text{mEq/l}) = \frac{(\text{ml HCl})\ (\text{N HCl})}{\text{ml of unknown}} \times 1000$$

E. Ether Extraction of Trichloroacetic Acid (H-TCA) from Samples

As previously discussed, Cort et al. (49,58) and Cort (47) have reported that plasma from carotid artery occluded cats which had been deproteinized with approximately 6% H-TCA exhibits both antinatriferic and natriuretic activities. The procedure which Cort describes for the removal of the added H-TCA involves repeated extractions with diethyl ether until the sample attains a pH between 5.0 and 6.0. At this point Cort et al. (58) report that the H-TCA is no longer an acid and that nearly all of the added acid has been removed. This procedure for the removal of plasma proteins was adopted in some of the studies reported here, since work by Cort and others suggests that rapid removal of proteins is desirable to retain maximum activity of any salt losing hormone which might be present in a given sample. Once proteins have been removed, samples have reportedly been stored up to 7 months (36) at -5°C with apparently no loss of activity.

To determine the number of ether extractions required to obtain a pH of 5.0 to 6.0 following addition of H-TCA to plasma, blood samples were obtained from several cats. To ten ml of plasma was added 5 ml of 20% H-TCA. Following the removal of the protein precipitate by centrifugation, the deproteinized plasma was decanted into a 60 ml separatory funnel and approximately 3 volumes (30 ml) of ether added to it. The separatory funnel was shaken for either 5 or 10 minutes, the layers allowed to separate, and the ether removed and discarded. A fresh 30 ml aliquot of ether was added to the aqueous layer

in the separatory funnel and the process repeated until a pH between 5.0 and 6.0 was obtained. Since there was no difference between the samples which had been extracted for 5 and 10 minutes, the results were combined and averaged. Figure 4 summarizes these results, and as can be seen at least 9 such extractions were required to obtain a pH of approximately 5.0, with several more being required if one is to obtain a pH of 6.0. This procedure is not only arduous, but as will be shown, apparently does not effectively remove all of the TCA^- anion. At pH 5.0 to 6.0 the TCA^- is perhaps no longer in the acid form, as Cort et al. (49) have stated, but in this form it is no longer ether soluble and may lead to erroneous results as it exhibits 'NH'-like activity on amphibian membranes.

Since TCA^- demonstrates antinatriferic activity on amphibian membranes (RESULTS--Section B), it became necessary to quantify the levels of this ion remaining in samples following ether extraction. A colorimetric procedure was developed in order to determine the efficiency with which ether extraction, as described by Cort et al. (49), removes H-TCA from plasma samples. As a result of these studies, Cort's procedure was modified to more effectively remove the H-TCA.

Figure 5 illustrates the ultraviolet absorbance spectrum of 1 mMolar H-TCA in water and 0.096 N HCl. In each case the H-TCA sample was referenced against the corresponding solvent. Samples were prepared by diluting 0.2 ml of 25 mMolar H-TCA to 5 ml with the appropriate solvent. There is an absorption maximum in the vicinity of 202-206 mμ when the diluting solvent is made acidic. In HCl at 205 mμ, however,

Figure 4. Summary of repeated ether extractions of H-TCA from cat plasma samples. 5 ml of 20% H-TCA was added to 10 ml of plasma and mixed by inversion. Following the removal of the protein precipitate by centrifugation, the deproteinized plasma was decanted into a 60 ml separatory funnel and approximately 3 volumes (30 ml) of ether added to it. The separatory funnel was shaken for 5 or 10 minutes, the layers allowed to separate, and the ether removed and discarded. This process was repeated until a pH of approximately 5.0 to 6.0 was obtained. Extraction points 1 to 10 are averages of 4 samples $\pm$ SEM, and extraction points 11 to 12 are averages of 3 samples $\pm$ SEM. pH is plotted on the ordinate against extraction number on the abscissa.

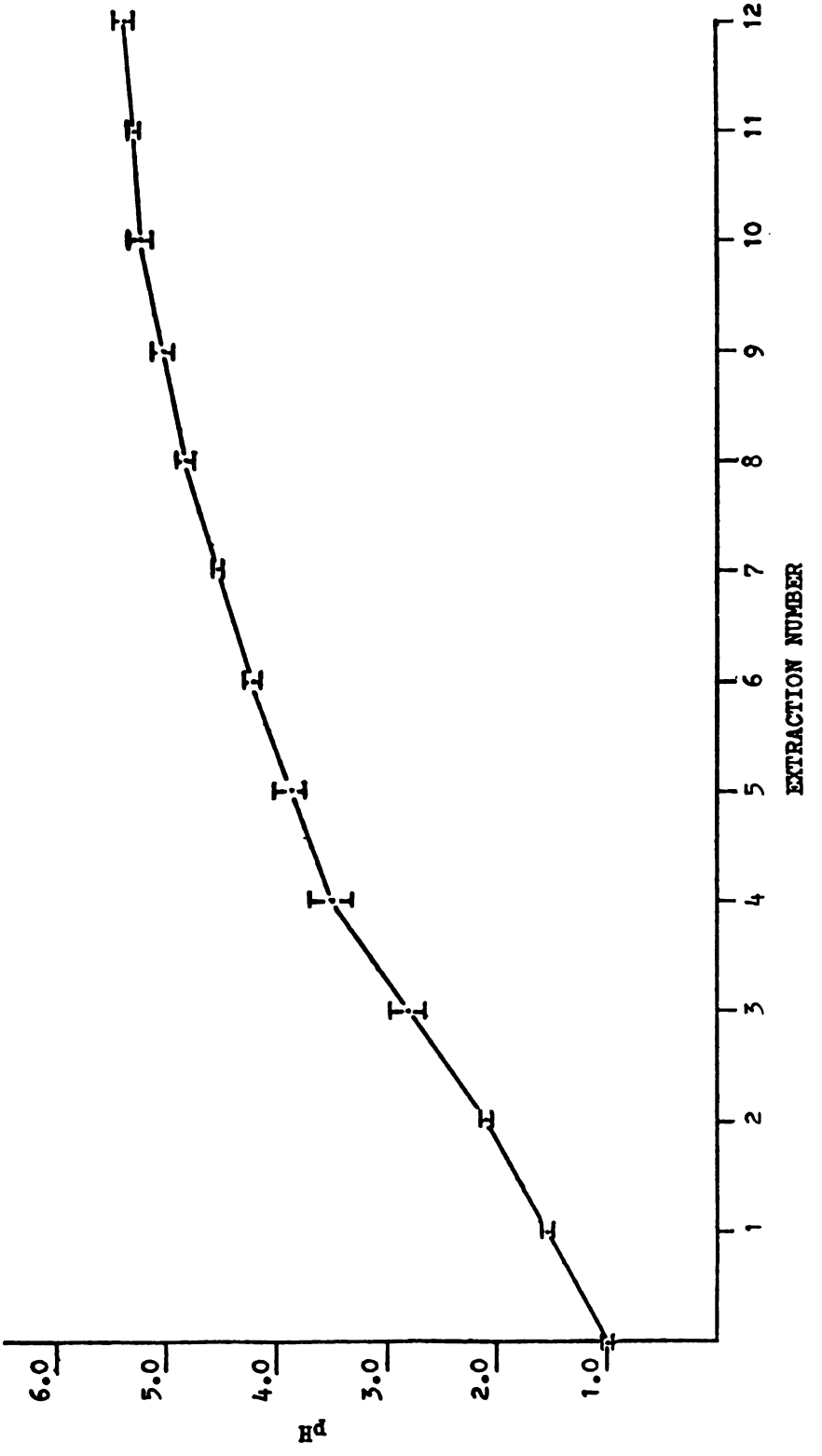

Figure 4.

Figure 5. Ultraviolet absorption spectrum of 1 mMolar H-TCA in distilled water (upper curve) and 0.09 N HCl (lower curve). Samples were blanked against water and 0.09 N HCl respectively. Absorbance is plotted on the ordinate against wavelength in mμ on the abscissa.

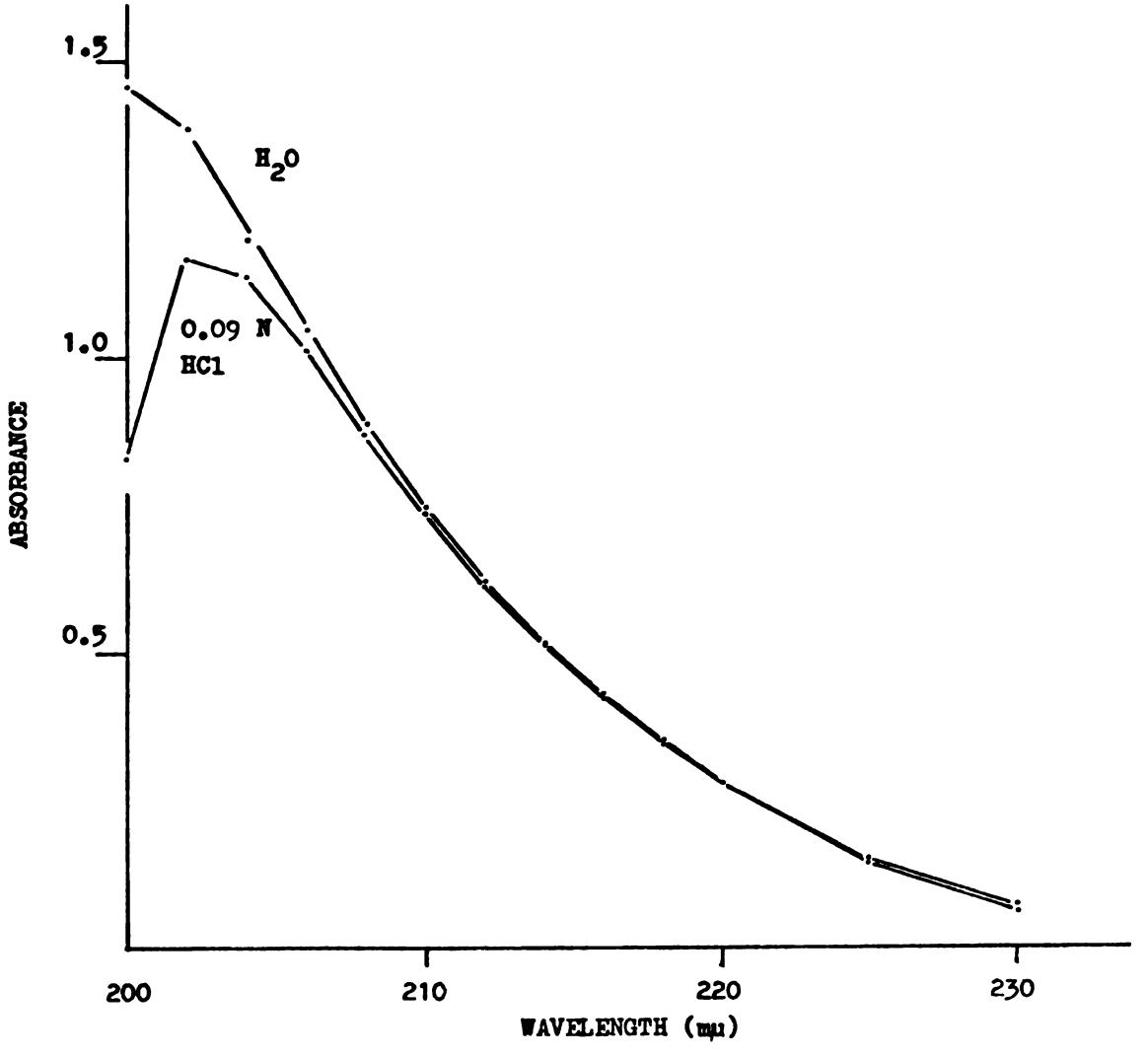
1.5
1.0
0.5
ABSORBANCE
H_2O
0.09 N
HCl
200
210
220
230
WAVELENGTH (mμ)

Figure 5.

there is a marked deviation from linearity above 1.0 mMolar as can be seen in the standard curve in Figure 6. At 210 mμ in an acidic environment the relationship between absorption and concentration does not deviate markedly from linearity until the concentration exceeds 2.0 mMolar. A similar situation exists when the diluting solvent is water (Figure 7).

Although there is no apparent absorption maximum in water for dilute solutions of H-TCA, it was chosen as the diluting solvent for purposes of convenience. The wavelength of choice was 210 mμ as the relationship between absorbance and concentration was linear over a greater range of concentrations than was true with 205 mμ. Verification of adherence to Beer's Law under a given set of conditions is necessary to justify the use of a single molar extinction coefficient (ε) if one is to be used when quantitative determinations are made. Non-adherence to Beer's Law would necessitate a standard curve or use of multiple molar extinction coefficients over limited concentration ranges. Table 5 summarizes the results of the determination of ε for H-TCA at 210 mμ with water as the diluting solvent.

With the molar extinction coefficient for H-TCA in water having been determined, it should theoretically be possible to determine the concentration of TCA^- anion remaining in a given sample following each of a series of ether extractions by observing the absorption at 210 mμ. In Figure 8 the results of the extraction of H-TCA from 9 dog plasma samples (upper curve) with ether are plotted with H-TCA concentration in mMoles/l against extraction number. It can be noted that after the

Figure 6. Standard curves for H-TCA in approximately 0.6 N HCl at 205 mμ (upper curve) and 210 mμ (lower curve). Absorbance is plotted on the ordinate against H-TCA concentration in mMoles/l on the abscissa.

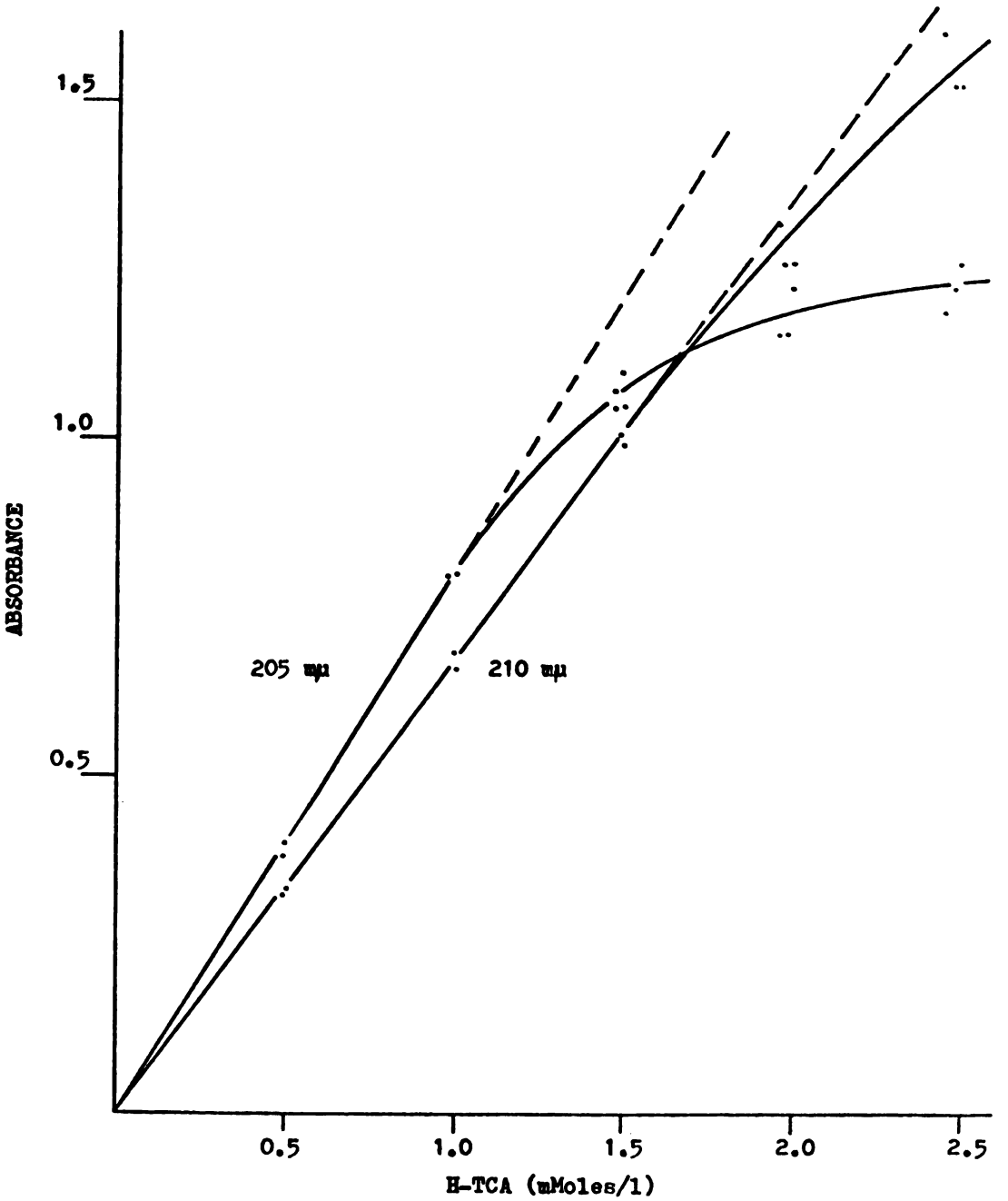

Figure 6.

Figure 7. Standard curves for H-TCA in distilled water at 205 mμ (upper curve) and 210 mμ (lower curve). Absorbance is plotted on the ordinate against H-TCA concentration in mMoles/l on the abscissa.

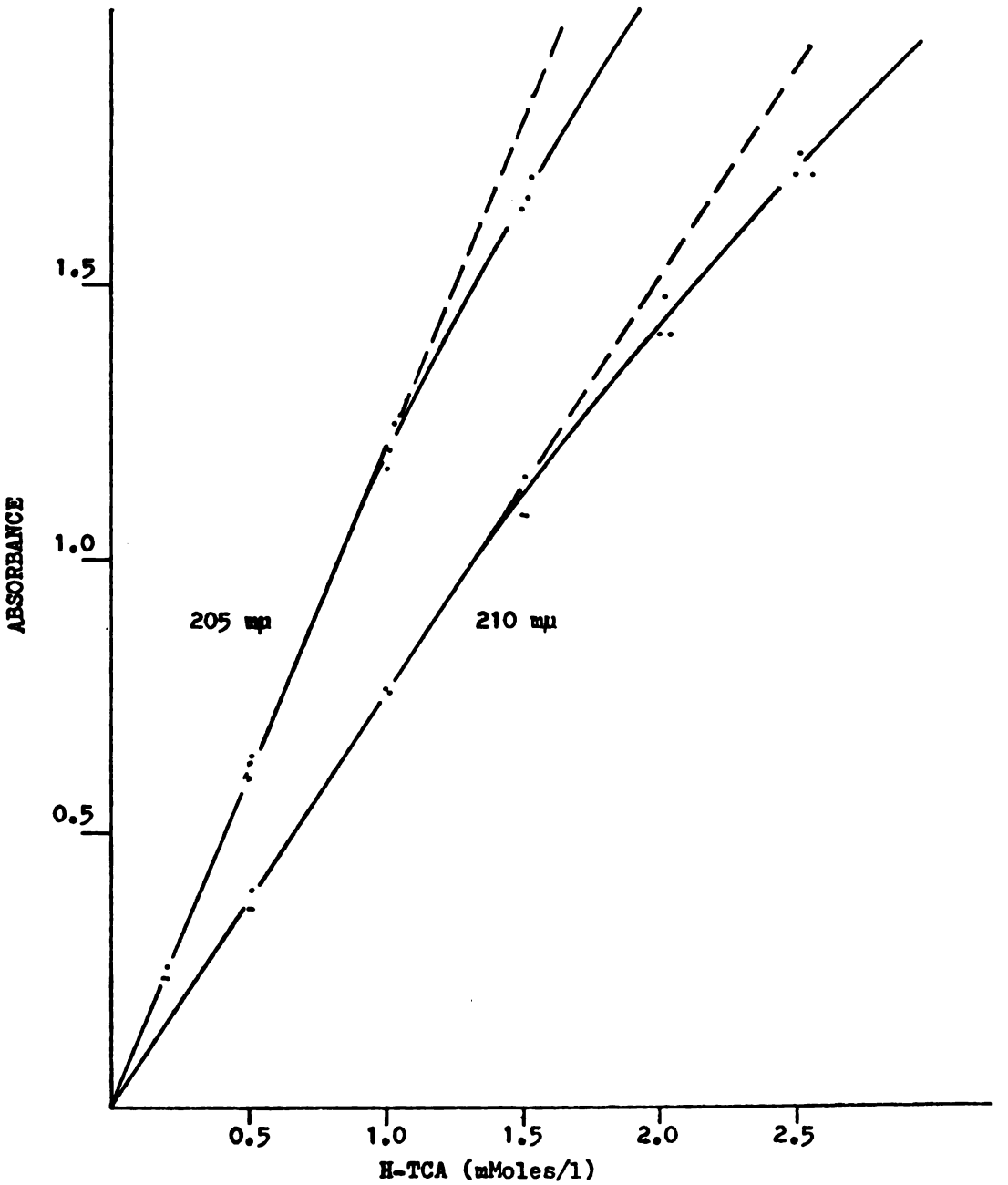

Figure 7.

Table 5. Determination of the molar extinction coefficient (ε) of H-TCA in distilled water at 210 mμ. A Beckman Model DB spectrophotometer was used for the measurements. Percent transmittance (%T) was determined directly and absorbance was calculated according to:

$$\text{Absorbance} = 2 - \log\%T$$

The extinction coefficient was calculated from the relationship $\varepsilon = A/C$, where A is the absorbance at 210 mμ and C is the H-TCA concentration in mEq/l. Samples I, II, and III, were independently prepared, and triplicate independent determinations were made with each. The average ε equals 732 ± 3 SEM.

SAMPLE	TCA CON. (mMoles/l)	ABSORBANCE (210 mμ)			ε		
I	1.00	0.721	0.728	0.773	721	728	733
II	1.00	0.726	0.728	0.735	720	722	728
III	1.00	0.762	0.762	0.757	746	746	741

Figure 8. Ether extraction of H-TCA from dog plasma with (lower curve) and without (upper curve) acidification with HCl after extraction number 2. TCA absorption at 210 mμ was not corrected for plasma ultrafiltrate absorption at 210 mμ. Data points are averages of 9 extractions (lower curve) and 8 extractions (upper curve) ± SEM. Samples were diluted with distilled water. Acidification following extraction number 2 was accomplished by adding 0.5 ml of 0.6 N HCl. H-TCA concentration remaining after each extraction in mMoles/l is plotted on the ordinate. Extraction number is plotted on the abscissa.

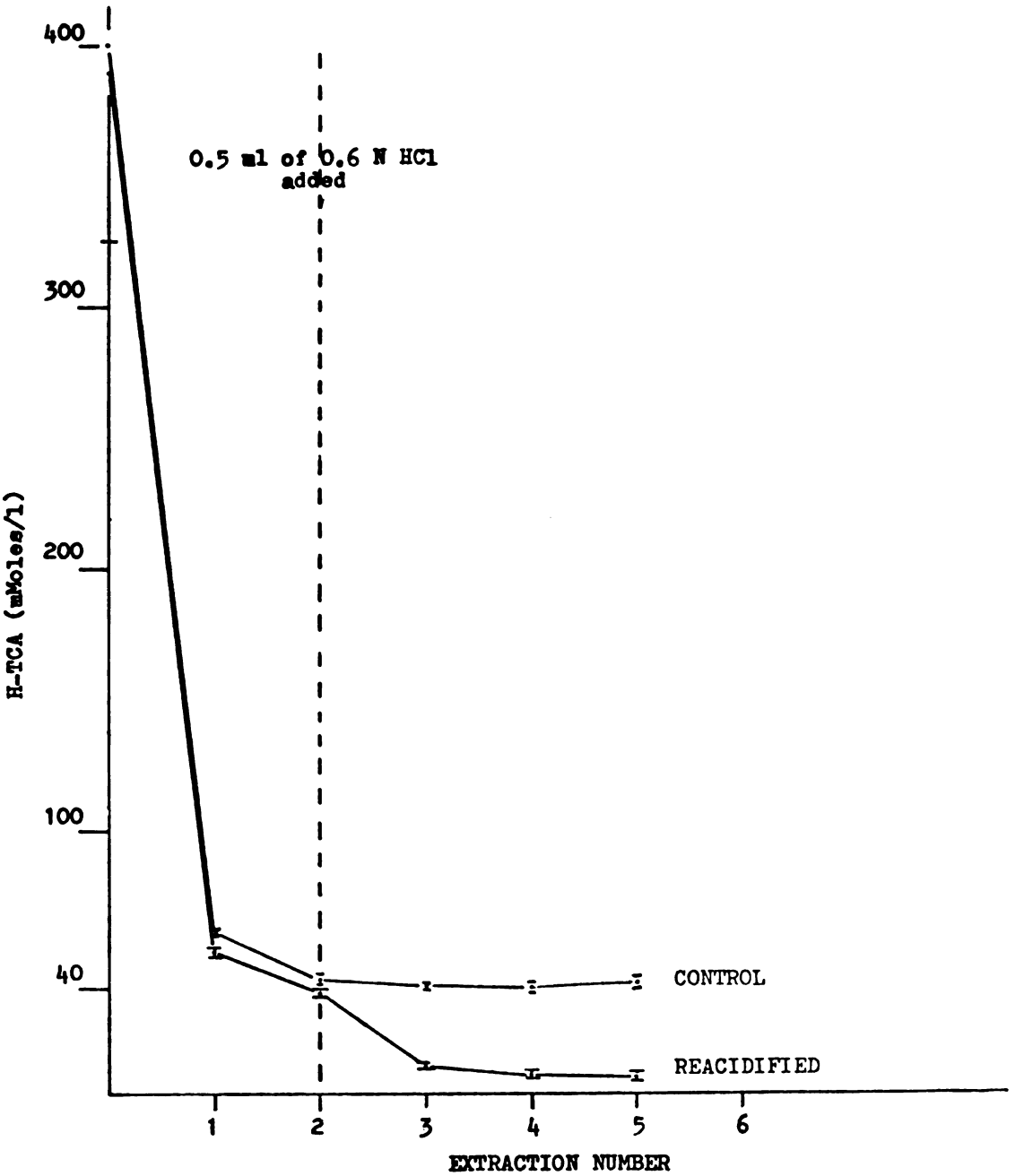
400
300
200
100
40
H-TCA (mMoles/l)
0.5 ml of 0.6 N HCl
added
CONTROL
REACIDIFIED
1
2
3
4
5
6
EXTRACTION NUMBER

Figure 8.

second extraction there is apparently very little change in the concentration of H-TCA with subsequent extractions. Following the second extraction the concentration does not fall below approximately 41 mMolar. If one acidifies the sample with HCl to a pH of 2.0 or less following the second extraction, further extractions lower the apparent TCA^- concentration to approximately 7 mMolar (lower curve). All extractions were performed by shaking the ether and aqueous layers for 30 seconds, allowing the layers to separate, and discarding the ether.

Figure 9 illustrates that if one starts with the sodium salt of trichloroacetic acid (Na-TCA), very little is extracted by the ether until the solution is acidified with HCl to a pH of approximately 2.0. When the pH rises above 3, extraction of the TCA^- anion decreases with further extraction until the solution once again is acidified to pH 2.0. This would indicate that the sodium salt of the acid is not ether soluble whereas the undissociated acid is ether soluble and thus ether extractable.

The preceding results suggested that the first 2 or 3 ether extractions are very efficient in removing the TCA^- anion in H-TCA solutions because as the undissociated acid is removed by the ether, there are sufficient H^+ and TCA^- ions available for a shift of the following equilibrium to the left:

$$\text{H-TCA} \rightleftharpoons H^+ + TCA^-$$

As the concentrations of H-TCA, H^+, and TCA^- decrease, and the pH rises, Na-TCA predominates because of the high concentration of plasma sodium

Figure 9. Ether extraction of Na-TCA added to dog plasma ultrafiltrate. TCA^- absorption at 210 mμ was not corrected for plasma ultrafiltrate absorption at 210 mμ. Samples were acidified with HCl to approximately pH 2 following extraction numbers 2 and 5. Data points are averages for 5 samples ± SEM. Samples were diluted in distilled water. Na-TCA^- concentration (left ordinate) in mMoles/l and pH (right ordinate) are plotted against extraction number (abscissa).

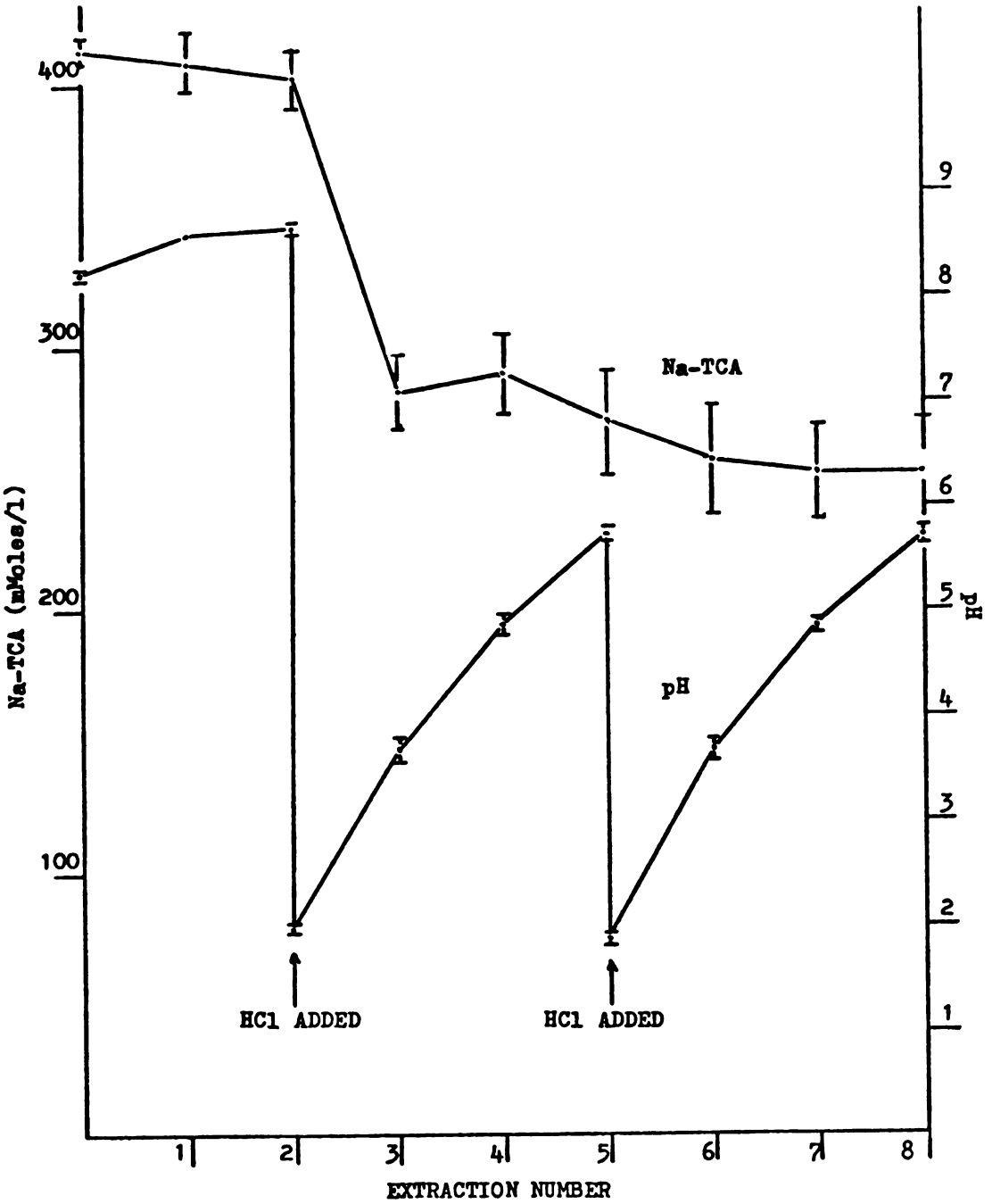
Na-TCA (mMoles/l)
400
300
200
100
Na-TCA
pH
9
8
7
6
5
4
3
2
1
HCl ADDED
HCl ADDED
1
2
3
4
5
6
7
8
EXTRACTION NUMBER

Figure 9.

relative to the H^+ concentration. Addition of sufficient acid (HCl) to lower the pH to approximately 2.0 and raise the hydrogen ion concentration, therefore, should be expected to enhance the extractability of the TCA^- as H-TCA. The results of such a procedure, summarized in Figure 8, suggest that this is the case. Addition of acid following the second extraction resulted in approximately a 6-fold decrease in the apparent final TCA^- concentration (lower curve). The results also suggest that there is a concentration of TCA^- of approximately 7 mMolar following a series of five 30-second extractions with 3 volumes of ether even with acidification with HCl.

In the preceding discussion the assumption made was that all of the optical density measured in the samples was due to TCA^- only. Therefore it is possible that all calculations of TCA^- concentrations in extracted plasma were overestimations. Thus it was desirable to determine if there is some optical density in protein free plasma per se, and to correct the absorbance of the H-TCA treated plasma samples for this background absorption. Such a procedure is described below.

To determine the background absorption of the plasma, a protein free filtrate was prepared by ultrafiltration of plasma through dialyzing tubing (Visking cellulose, Will Scientific, Inc.). A piece of 1½ inch diameter Visking tubing was cut as a single layer to fit the 300 ml capacity ultrafiltration unit used to concentrate the dialysis fluid and plasma ultrafiltrate in the human studies (to be discussed in METHODS--Section J). This membrane was used in place of the Amicon UM05 membrane (also to be discussed in METHODS--Section J). Plasma was pipetted into the unit and ultrafiltered at a pressure of 55 to 60

psi at 0 to 4°C. Addition of H-TCA to the filtrate thus obtained produced no precipitate, and optical density measurements at 210 mμ gave results identical to those of ether extracted H-TCA treated plasma which had been acidified with HCl after the second ether extraction (an apparent TCA^- concentration of approximately 7 mMolar--Figure 8). It was concluded, therefore, that deproteinization by H-TCA and ultrafiltration through Visking cellulose membranes give protein free plasmas which are identical in terms of substances which demonstrate an absorbance at 205 and 210 mμ. To correct the H-TCA treated plasma samples for the background absorption due to the plasma, the following procedure was employed:

1) ten ml of H-TCA treated plasma and approximately 3 volumes (30 ml) of ether were pipetted into a 60 ml separatory funnel,

2) into 10 ml of plasma ultrafiltrate was pipetted 5 ml of distilled water (recall that 5 ml of 20% H-TCA was added to the plasma in step 1. The water here is added to accomplish the same dilution of the plasma substituents that may demonstrate an absorption at 210 mμ). Ten ml of this diluted plasma and 30 ml of ether were pipetted into a second 60 ml separatory funnel,

3) both separatory funnels were shaken for 30 seconds,

4) after phase separation, the ether layers were discarded,

5) to the aqueous layers in the separatory funnels was added another 30 ml aliquot of ether. The funnels were shaken as before and the ether layers discarded,

6) 0.5 ml of 0.6 N HCl was pipetted into the aqueous layers, 30 ml of ether added, and the separatory funnels again shaken for 30 seconds. Again the ether layers were discarded, and

7) step 5 was repeated until a total of 8 extractions with ether had been performed.

Figure 10 depicts the results of 5 samples prepared and extracted as described above. Following each extraction the background optical

Figure 10. Ether extraction of H-TCA from dog plasma. TCA^- absorption at 210 mμ was corrected for plasma ultrafiltrate absorption at 210 mμ. Acidification following extraction number 2. Data points are averages of 5 independent extractions ± SEM for the H-TCA curve. H-TCA concentration (left ordinate) in mMoles/liter and pH (right ordinate) are plotted against extraction number (abscissa). Samples were diluted in distilled water.

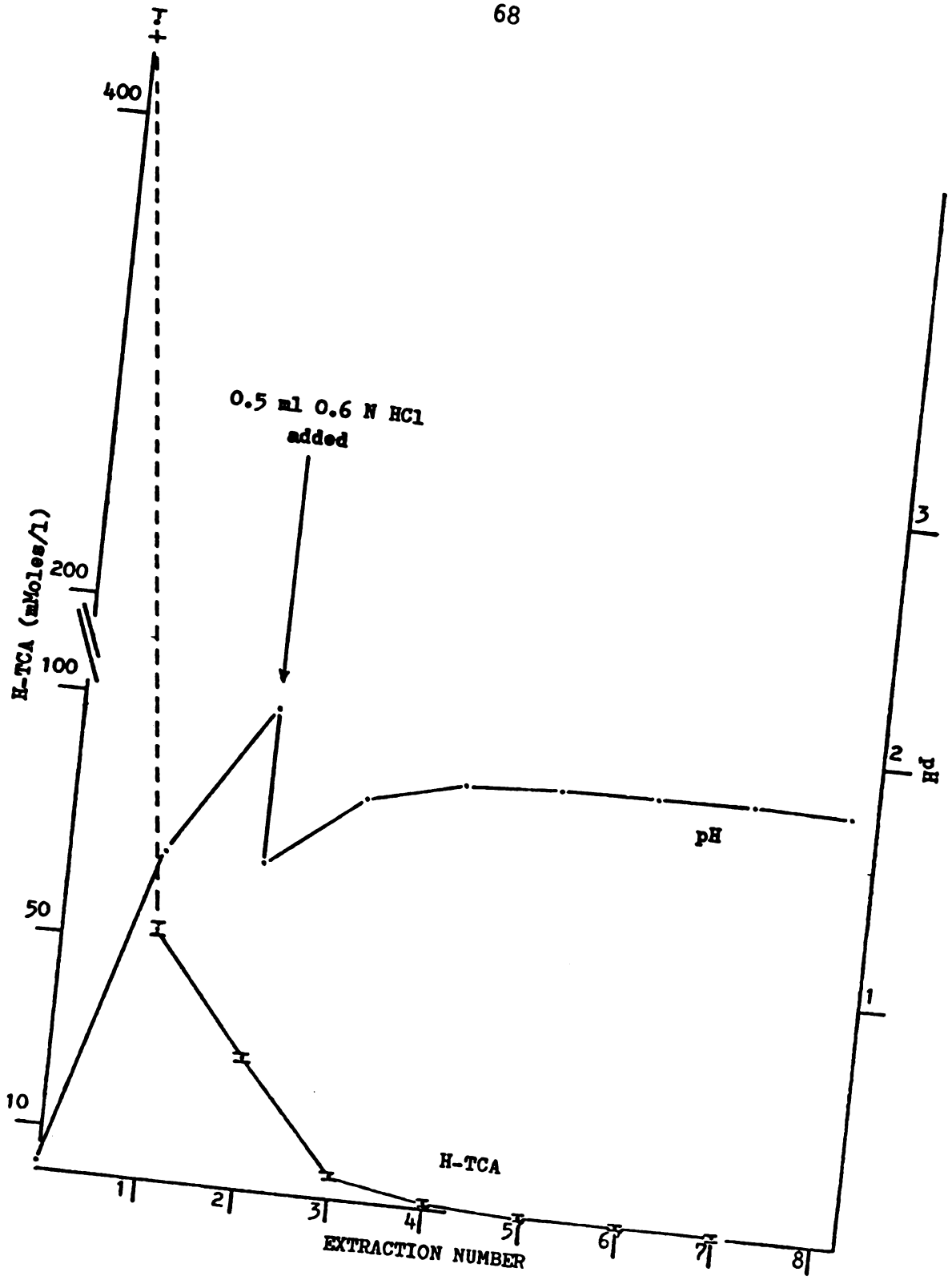
I
400
200
100
50
10
H-TCA (mMoles/l)
0.5 ml 0.6 N HCl
added
pH
3
2
1
pH
H-TCA
1
2
3
4
5
6
7
8
EXTRACTION NUMBER

Figure 10.

density obtained from the ultrafiltrate sample was subtracted from the H-TCA treated sample. The result of this subtraction equals A in the formula below. The concentration of TCA^- in the H-TCA sample is then calculated according to:

$A = \varepsilon C$, and $C = A/\varepsilon$, where ε for H-TCA in water is 732

(Table 5) and C equals the concentration of H-TCA in mEq/l. The concentration of H-TCA in the original sample then equals C times the appropriate dilution factor.

It can be seen from Figure 10 that after the second ether extraction the pH has risen to approximately 2.0, at which point addition of 0.5 ml of 0.6 N HCl lowers the pH to about 1.5 and it remains below 2.0 throughout the rest of the extraction procedure. It can also be seen that the resultant TCA^- concentration is in fact not 7 mMolar, but 0.4 or less following subtraction of non-TCA absorption at 210 mμ. Figure 11 is a similar diagram, except no acid was added following the second extraction, consistent with the procedure of Cort to prepare plasma samples for antinatriferic activity determinations. With subtraction of non-TCA absorption but no acidification, the resultant TCA^- concentration is on the order of 33 mMolar, which can significantly inhibit sodium transport (Figure 28).

F. Removal of TCA by Fractionation on Sephadex Resin

A second procedure for the removal of the TCA^- anion from the sample is fractionation of the sample on Sephadex resin. Figure 12 illustrates the results of the fractionation of a 10 ml sample of frog

Figure 11. Ether extraction of H-TCA from dog plasma. H-TCA absorption at 210 mμ was corrected for plasma ultrafiltrate absorption at 210 mμ. No acidification following extraction number 2. Data points are averages of 3 independent extractions ± SEM. Samples were diluted in distilled water. H-TCA concentration (left ordinate) and pH (right ordinate) are plotted against extraction number abscissa).

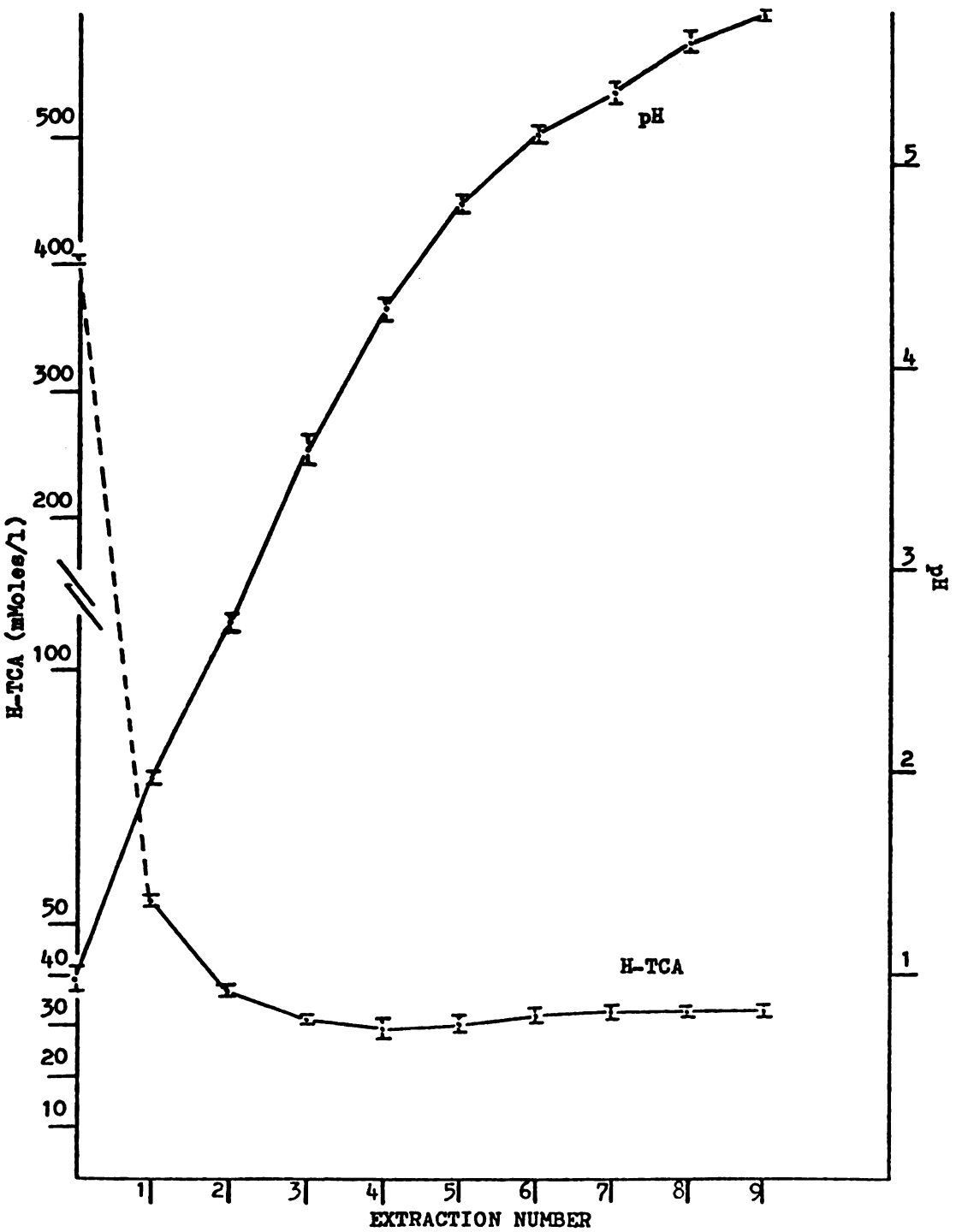
H-TCA (mMoles/l)
500
400
300
200
100
50
40
30
20
10
pH
5
4
3
2
1
H-TCA
1
2
3
4
5
6
7
8
9
EXTRACTION NUMBER

Figure 11.

Figure 12. Results of fractionation of 10 ml of Na-TCA frog buffer on Sephadex G25F resin at 4°C. The sample was eluted with 10 mMolar ammonium acetate from a 2.5 X 95 cm column. 10 ml aliquots were collected by fraction collector.

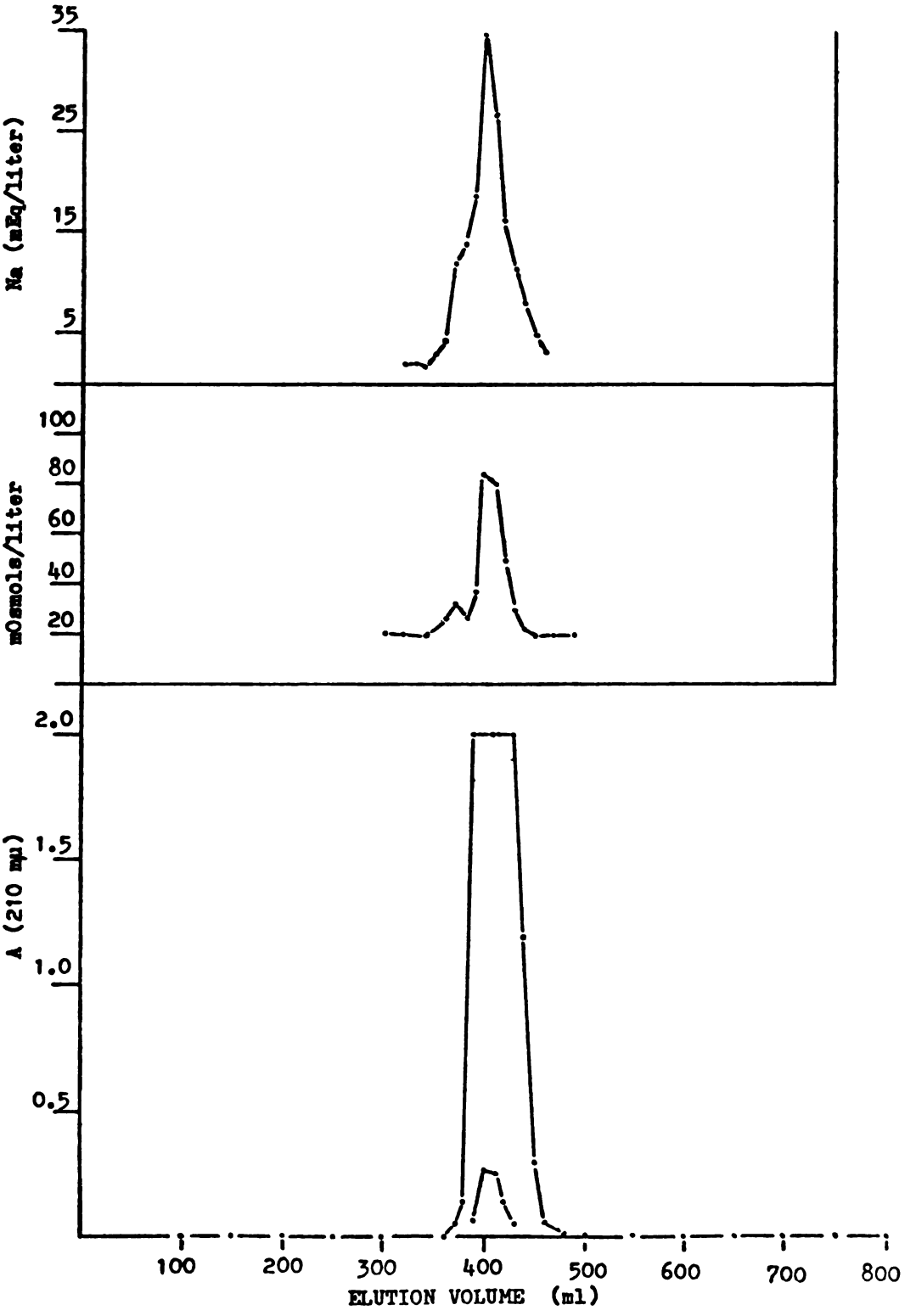
35
25
15
5
Na (mEq/liter)
100
80
60
40
20
mOsmole/liter
2.0
1.5
1.0
0.5
A (210 mμ)
100
200
300
400
500
600
700
800
ELUTION VOLUME (ml)

Figure 12.

buffer, the composition of which is described in METHODS--Section G, with NaCl being replaced by Na-TCA. The absorption at 210 mμ coincides with the appearance of the other osmotically active ions in an elution volume from approximately 350 to 500 ml. Therefore it can be concluded that the fraction reported to contain natriuretic-antinatriferic activity, which occurs in the elution volume from approximately 550-780 ml, does not contain the contamination TCA^- anion. Sodium ion and other osmotically active substances were separated as when blood, dialysis fluid, and plasma ultrafiltrate are fractionated as shown in RESULTS--Section A.

G. Short Circuit Current Determinations

Frogs, Rana Pipiens, were either purchased (Mogul Corp., Oshkosh, Wisc.; Wards Natural Science Est., Inc., Rochester, N. Y.) or captured from local ponds. In earlier studies the animals were kept in the cold in the presence of running water until the time of use. In later studies the animals were kept at room temperature in a large sink with running water. A portion of the sink was covered with sod to provide an area of dry terrain. From the time of purchase to the time of use the animals were provided with a diet of meal worms, crickets, and other live insects.

On the day of a run the frogs were sacrificed by double pithing or decapitation. A section of ventral unpigmented skin was immediately removed and mounted on the apparatus. The skins were rinsed several times with frog buffer prior to connecting a bubbler which

served both to aerate and stir the buffer in contact with the skin. The procedure from the killing of the frog to the start of the equilibration period usually required 2-4 minutes.

The Ussing type apparatus (164) employed is illustrated in Figure 13. It consists of 2 acrylic plastic chambers between which is clamped the frog skin membrane (M). The surface area exposed to the frog buffer measures 2.54 cm^2. The capacity of the chamber on each side of the membrane is approximately 4 ml.

A solution of agar and frog buffer was prepared by boiling gently until the agar became dissolved. It was then drawn into sections of polyethylene tubing (A) which served to establish contact between the 2 halves of the apparatus and the calomel electrodes (C). The calomel electrodes in turn were connected to a Grass Polygraph recorder (mV) which measured the membrane potential across the skin. The external current required to maintain the potential difference across the membrane (M) equal to zero was supplied by a 6V dry cell battery (B) and read from a Keithly Model 610C Electrometer (μA). The external resistance was adjusted to maintain the membrane potential equal to zero with a series of variable resistors (VR).

Once the skins had been mounted in the apparatus and rinsed several times with buffer, the chambers were connected to the aerating system at (D). The bubble rate per chamber was adjusted with an aquarium gang valve (AGV), and the air supplied by a small aquarium pump (AP) was saturated with water by bubbling it through a water bottle (WB) before entering the chamber at (O). Air bubbles were allowed to

Figure 13. Frog skin short circuit current set-up.

A = agar-KCl-frog buffer bridge in polyethylene tubing

AGV = aquarium gang valve to regulate bubble rates to chambers

AP = aquarium pump

B = 6 volt battery

C = calomel electrodes

D = 17 gauge needles used to connect chamber to bubbler (WB)

E = silver electrodes

G = recessed groove to prevent air bubbles from contacting skin

I = sample inlet and air outlet

M = frog skin membrane

mV = Grass Polygraph recorder

O = sample outlet and air inlet (small polyethylene tubing)

S = toggle switch

μA = Keithly Model 610C Electrometer

VR = variable resistors

WB = water bottle to saturate the air with water before entering the chambers

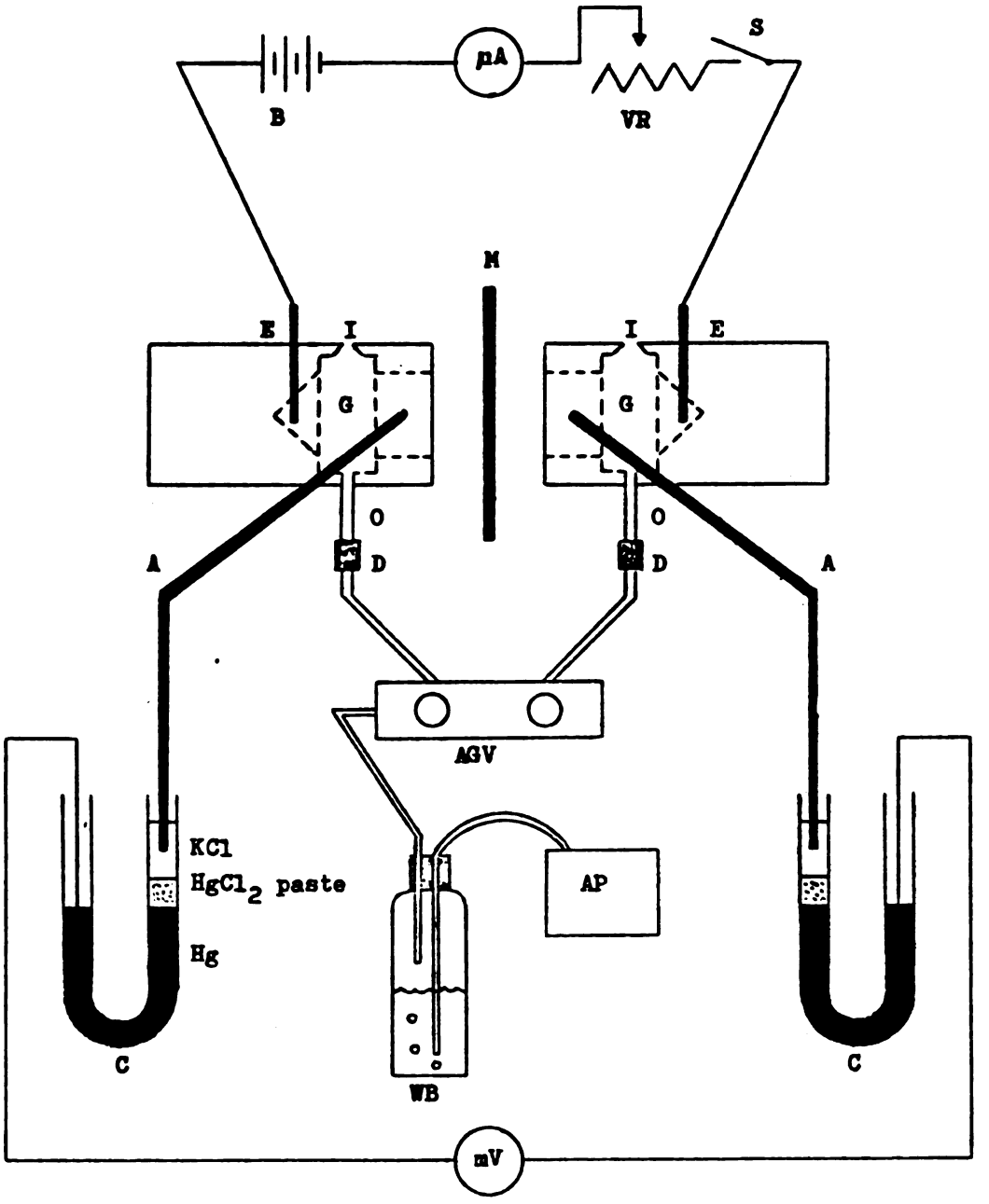

Figure 13.

escape at (I), and were prevented from contacting the skin surface by a recessed groove (G) which was cut into the inner circumference of each chamber.

The skins were continuously short-circuited according to Bourgoignie et al. (20), with membrane potential and short circuit current readings being taken every 5 minutes by turning off the external emf briefly with switch (S). Every 30-40 minutes the fluid from both sides of the skin was drained through the sample outlet (O) by disconnecting the system at (D). Fresh frog buffer was then added to both sides of the skin. This procedure was continued until an interval of at least 20 minutes was obtained during which the short circuit current was stable. When this occurred the unknown sample of interest was added to the serosal side of the skin. Readings at 5 minute intervals were obtained on the unknown sample for 30-50 minutes--after which it was removed; the skin flushed 3 times on both sides with fresh buffer; and measurements made every 5 minutes until a stable short circuit current was again obtained. At this point a new unknown was added to the serosal side of the skin. An attempt was made not to employ skins which had short circuit currents of less than 15 $\mu A/cm^2$, although this was not always possible.

Ussing et al. (164) demonstrated that under normal conditions, with frog buffer on both sides of the skin, the short circuit current is equal to the net active transport of sodium across the skin. Under these circumstances, therefore, changes in short circuit current would be indicative of changes in net active transport of sodium. This is the reason this type of system has been used to assay for 'NH', which

is thought to inhibit active transport of sodium in the kidney in a similar fashion.

The composition of the frog buffer utilized throughout the studies is given in Table 6. The pH of the buffer was adjusted to 7.8 ± 0.2 with approximately 0.45 ml of 1 N NaOH.

Table 6. Composition of frog buffer utilized through the studies concerned with short circuit current measurements on frog skins (20).

SALT	g/500 ml	Con. (mM/l)
Sodium chloride	3.214	110.0
Potassium chloride	0.093	2.5
Calcium chloride dihydrate	0.110	1.5
Magnesium chloride heptahydrate	0.204	2.0
Glucose	0.901	10.0
Tris-chloride	0.198	2.5

H. Column Chromatography

Column Preparation

Sephadex G25F resin (Pharmacia, Fine Chemicals Inc., Piscataway, N. J.) was allowed to swell in the elution solution, 10 mM ammonium acetate, for at least 3 hours at room temperature prior to column packing. During this period gentle stirring was accomplished with a stirring bar and magnetic stirrer (excessive stirring should be avoided as it may lead to rupture of the resin beads). The slurry was then deaerated under vacuum (trapped air bubbles must be removed to prevent

uneven sample flow through the packed column); the resin allowed to settle; and excess eluant decanted until a fairly thick slurry remained.

Once the resin had been allowed to swell, and the slurry deaerated and excess eluant removed, all of the slurry was poured at one time into a siliconized 2.5 X 95 cm column. Construction of the column is shown in Figure 14. Flow through the column was started as soon as possible in order to obtain an even sedimentation of the resin. Flow was allowed to continue for 12-24 hours in order to stabilize and equilibrate the gel bed. The quantity of dry resin required to pack a given column can readily be determined if the volume of the resin bed and water regain of the resin being used are known:

$$\text{Bed Volume} = \pi(r^2)H \qquad \begin{array}{l} r = \text{column inner diameter (cm)} \\ H = \text{column height (cm)} \end{array}$$

$$\text{Water Regain} = \text{g of water absorbed/g dry resin}$$

$$= \frac{\text{G-type number}}{10} \text{ of the resin being used}$$

For the column utilized in these studies the quantity of dry resin required would be approximately:

$$\text{g dry G25F resin} = \frac{\pi(1.25\ \text{cm})^2\ (95\ \text{cm})}{2.5\ \text{g/cm}^3}$$

$$= 186\ \text{g}$$

Before use, column performance and void volume were determined by allowing 10 ml of a dilute solution of Blue Dextran (Pharmacia) to pass through it. During extensive periods of no activity, 1 liter of 0.02% sodium azide in 10 mMolar ammonium acetate was passed through the

Figure 14. Construction of Sephadex resin column.

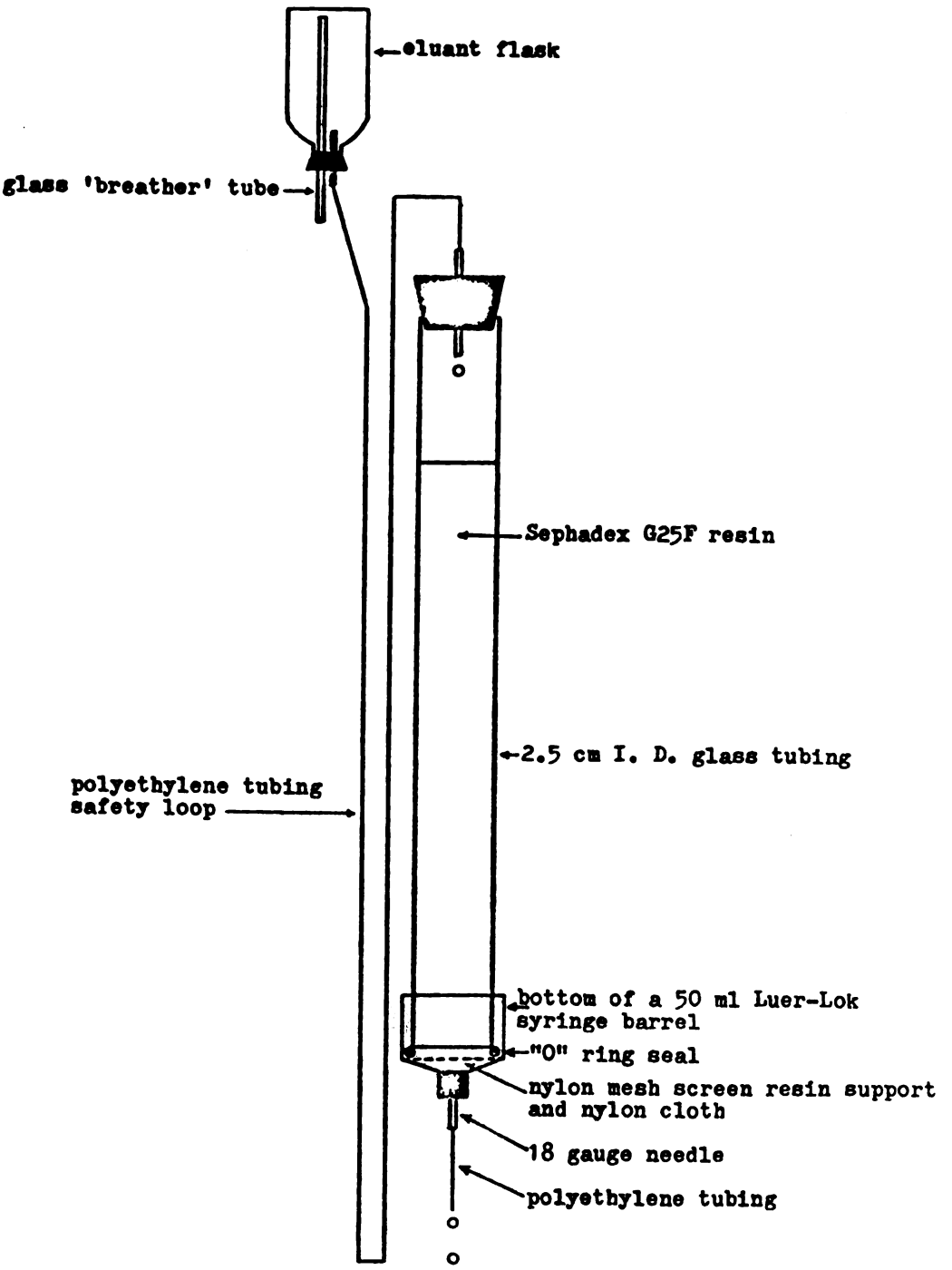
eluant flask
glass 'breather' tube
Sephadex G25F resin
2.5 cm I. D. glass tubing
polyethylene tubing
safety loop
bottom of a 50 ml Luer-Lok
syringe barrel
"O" ring seal
nylon mesh screen resin support
and nylon cloth
18 gauge needle
polyethylene tubing

Figure 14.

packed resin and allowed to remain there until the next run. In order to prevent the column from running dry when unattended during a fractionation, a polyethylene safety loop was employed as shown in Figure 14.

Sample Application

Most of the eluant above the gel surface was removed by suction. The column outlet was opened and the remaining eluant above the resin surface drained away. As soon as the eluant had drained to the surface of the resin, 8-25 ml of sample was very carefully applied with a pipette (the surface of the resin must not be disturbed). After the sample had drained into the bed, the gel surface and column wall in contact with the sample were washed with eluant 3 times. During these operations the eluant must not be allowed to run below the resin surface as air bubbles may become entrapped within the gel bed. Finally, the space above the resin in the column was partially filled with eluant and connected to the eluant flask. Fractionation was performed at 0-4°C in most cases, with 10 ml aliquots of eluant being collected by a fraction collector to a total elution volume of 1100 ml.

I. Collection and Handling of Uremic Human Specimens

Patients involved in this study had been undergoing regular maintenance hemodialysis for periods of 2 weeks or more at the Veteran's Administration or University hospitals in Ann Arbor, Michigan.

Specimens routinely collected from these patients included blood, plasma ultrafiltrate, and/or spent hemodialysis fluid. None of the patients studied was anephric, and all had at least some residual kidney function. Figure 15 illustrates one manner in which a patient can be attached to the artificial kidney or hemodialyzer. Blood passes from an arm or leg artery to the dialyzer unit. On passing through the dialyzer unit the blood is dialyzed and ultrafiltered and then returned to the patient via an arm or leg vein.

Fifteen to thirty ml of whole blood was obtained just prior to attachment of the patient to the hemodialyzer. The blood was centrifuged at room temperature to remove the red blood cells, and the plasma immediately placed into an ice bath or dry ice-acetone bath until application of the sample to the Sephadex resin column. The time from collection to application to the resin varied from approximately 2 to 4 hours. When plasma samples were deproteinized with H-TCA, 5 ml of 20% H-TCA was added to a 10 ml aliquot of plasma immediately after the removal of the red blood cells. The sample was shaken and placed into an ice bath until removal of the H-TCA by ether extraction.

To collect the plasma ultrafiltrate, the dialysis fluid inflow and outflow tubes were disconnected from the hemodialyzer at (X) and (Y) in Figure 15. The transmembrane pressure across the hemodialyzer was increased in order to increase the yield of the ultrafiltrate. This was accomplished by partially occluding the venous return tubing at (C) in Figure 15 and increasing the speed of the blood pump (Z). The transmembrane pressure usually obtained was 200-400 mm Hg, and the yield of

Figure 15. Diagrammatic representation of attachment of patient to hemodialyzer.

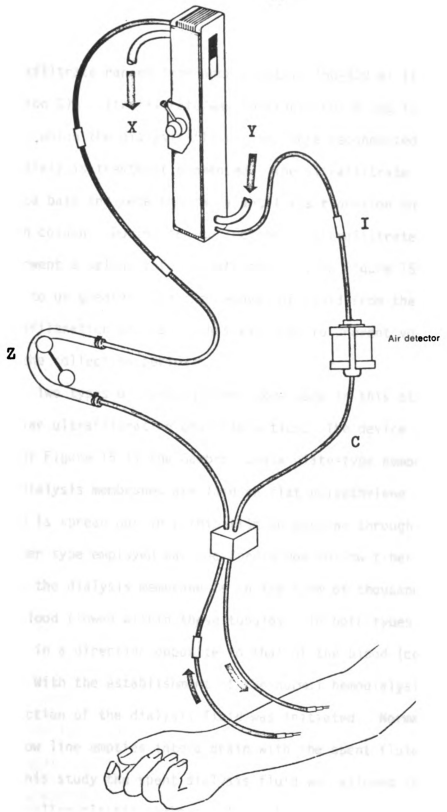

Figure 15.

ultrafiltrate ranged from approximately 150-400 ml (Table 16, RESULTS--Section C). Ultrafiltrate was routinely collected for 30 minutes, after which the dialysis fluid lines were reconnected and the normal hemodialysis treatment commenced. The ultrafiltrate was collected in an ice bath and remained there until fractionation on the Sephadex resin column. During the collection of ultrafiltrate, the patient underwent a saline infusion at point (I) in Figure 15 at a rate comparable to or greater than the removal of fluid from the blood by the ultrafiltration process. This was done to prevent volume depletion during the collection period.

Two types of hemodialyzers were used in this study, both having similar ultrafiltration characteristics. The device pictured at the top of Figure 15 is the Gambro-Lundia plate-type hemodialyzer, in which the dialysis membranes are laid on flat polyethylene supports. The blood is spread out in a thin film on passing through the unit. Another type employed was the Cordis Dow hollow fiber dialyzer, in which the dialysis membrane is in the form of thousands of small tubules. The blood flowed within these tubules. In both types the dialysis fluid flows in a direction opposite to that of the blood (countercurrent).

With the establishment of the normal hemodialysis period, the collection of the dialysis fluid was initiated. Normally the dialysis outflow line empties into a drain with the spent fluid being discarded. For this study the spent dialysis fluid was allowed to flow into 2 five-gallon plastic carboys. In most instances the dialysis fluid flow rate was about 500 ml/min, approximately 40 minutes being required to

fill each of the carboys. Since the artificial kidneys which were used in this study dialyzed and ultrafiltered by positive pressure, the dialysis fluid obtained was not diluted or contaminated by tap water which is often the case with negative pressure systems. In addition, the dialysate source was supplied on a single-pass basis, in that it ran by the membrane surface only once and was then discarded.

To retard bacterial growth in the dialysis fluid, 20 ml of 12 N HCl was added to each carboy resulting in a final concentration of approximately 0.01 N HCl and a pH of 5.5-6.0. The combination of low pH and cold storage was found to prevent bacterial growth during the period of ultrafiltration. Sodium azide at 0.02% was found not to be as effective. Antibiotics were judged to be undesirable, as problems of massive concentration of the drug might be encountered during the ultrafiltration process which was used to concentrate the dialysis fluid.

J. Ultrafiltration Procedure

The technique of ultrafiltration was employed in order to concentrate the large volumes of dialysis fluid and plasma ultrafiltrate which were obtained from the artificial kidney machines. This method was selected because of the availability of appropriately selective membranes, and because of the potential rapidity with which the process can be carried out. The apparatus used was constructed from acrylic plastic, and is depicted in Figure 16.

Figure 16. Construction of the ultrafiltration chambers employed to concentrate dialysis fluid and plasma ultrafiltrate and prepare protein free dog plasma ultrafiltrate.

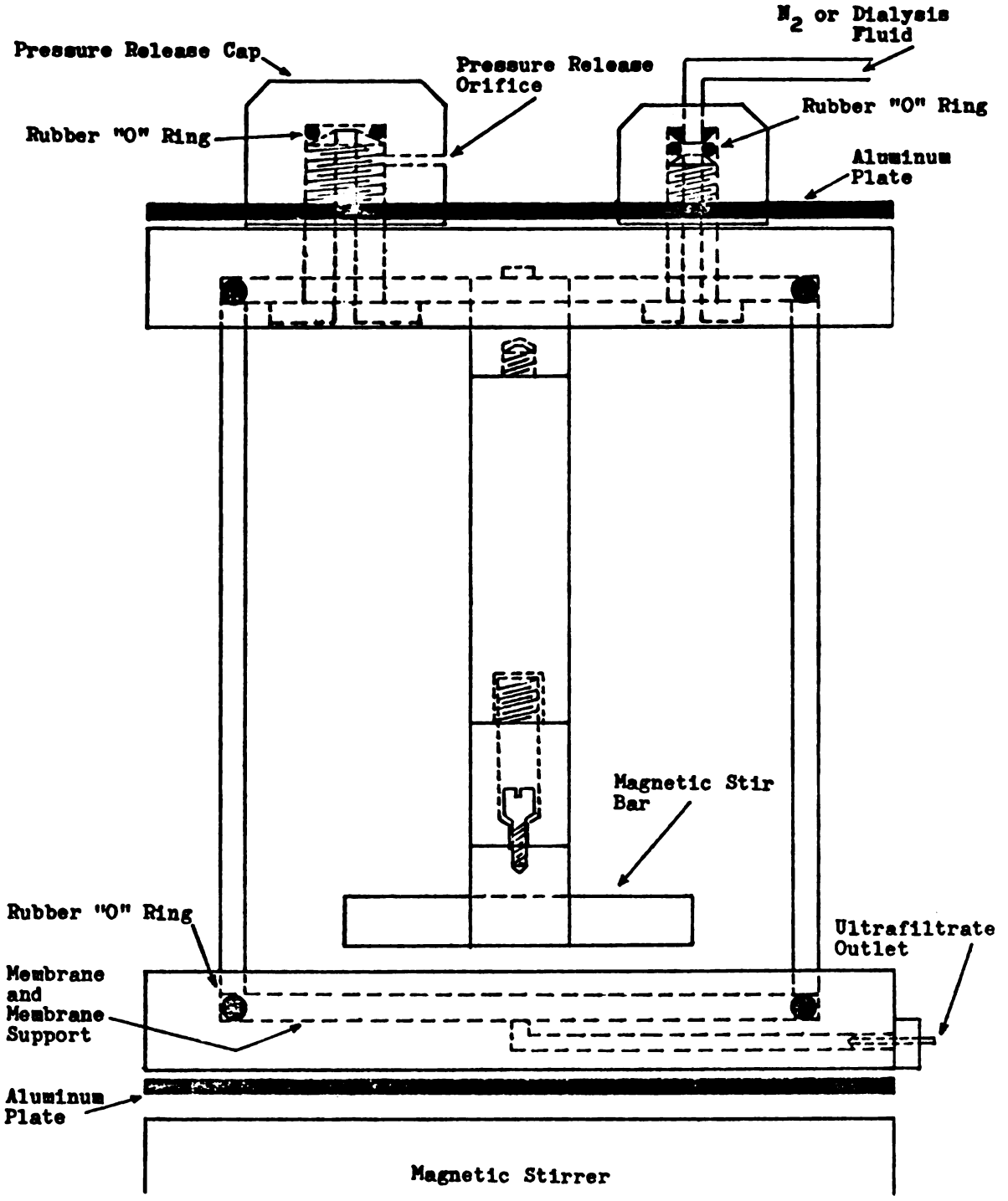
Pressure Release Cap
Pressure Release Orifice
N_2 or Dialysis Fluid
Rubber "O" Ring
Rubber "O" Ring
Aluminum Plate
Magnetic Stir Bar
Rubber "O" Ring
Ultrafiltrate Outlet
Membrane and Membrane Support
Aluminum Plate
Magnetic Stirrer

Figure 16.

For the ultrafiltration of the dialysis fluid two chambers were used in parallel, each with a capacity of approximately 1800 ml. A single fiberglas reservoir with a capacity of 12 liters was also employed in order to minimize the number of shut-downs required to replace the fluid which was being forced from the system. Stirred cells were used to minimize concentration polarization effects which occur at the membrane surface which affect both the permeability of the membranes and the rate of ultrafiltration.

The membrane employed in the units, supplied by Amicon Corporation, Lexington, Mass., was the UM05 type which has a molecular weight cut-off of approximately 500 Daltons. The chambers were constructed to withstand a pressure of 100 psi. For the ultrafiltration of the smaller volumes of uremic plasma ultrafiltrate, a smaller unit with a capacity of approximately 300 ml was constructed in a manner similar to the larger units. Dog plasma samples were also ultrafiltered in this smaller unit employing a dialysis membrane described elsewhere (pages 65-66). The larger units have a minimum stirred volume of approximately 150 ml, and for this reason the final concentration of the dialysis fluid was performed in the smaller unit which has a minimum stirred volume of approximately 15 ml.

Procedure

a) Dialysis Fluid

The 12 liter fiberglas reservoir was first filled with dialysis fluid and the inflow tube connected to the regulator of a large cylinder of nitrogen gas. The outflow tube was attached to the assembled

ultrafiltration chambers with the pressure relief caps removed. Sufficient pressure was applied from the nitrogen cylinder to the 12 liter reservoir to force the dialysis fluid into the ultrafiltration chambers. When the chambers were filled, the pressure relief caps were secured, the pressure adjusted to 55-60 psi, and the ultrafiltration allowed to proceed until the 12 liter reservoir became empty. At this time the nitrogen gas was shut off, the pressure relief valve on the reservoir opened, and the reservoir refilled with dialysis fluid. The pressure relief valve was then closed, the pressure again adjusted to 55-60 psi, and the ultrafiltration continued. Concentration of the dialysis fluid from an initial volume of 36 liters to a final volume of approximately 200 ml required about 4 days. The resulting 200 ml was transferred to the 300 ml capacity ultrafiltration unit and reduced to a volume of approximately 15-25 ml. This usually required an additional 8 hours. The entire procedure was carried out at 0-4°C. Stirring of the cells was accomplished by a magnetic stirrer and stir bar as shown in Figure 16.

b) Plasma Ultrafiltrate

The plasma ultrafiltrate obtained directly from the dialyser unit of the artificial kidney underwent a second ultrafiltration in the 300 ml capacity unit. The sample was syphoned into the unit which was then attached directly to the nitrogen cylinder. A reservoir was not required. Flow rate through the UM05 membrane in this unit at 55-60 psi was approximately 0.4 ml/minute. Flow rate through the larger units under the same conditions was approximately 4 ml/min. With extended

periods of use the flow rates were observed to decrease in both units.

At the completion of a given ultrafiltration, the membranes can be rinsed and allowed to soak in distilled water for 1 or 2 days. This is usually adequate to restore the membrane so that it can be reused. Storage in 25% alcohol will prevent bacterial growth on the membrane between uses.

K. Acute Volume Expansion with Dogs

Fourteen dogs, of both sexes, ranging in weight from 7.3-12 Kg, were volume expanded with 0.154 M saline. With the exception of 2 dogs (numbers 13 and 14), all were expanded via a cannula inserted into the brachial vein. Dogs 13 and 14 were expanded by infusion into the femoral vein. All blood samples were collected with a cannula which was inserted into one external jugular vein.

Dogs numbered 2-8 received an initial expansion equivalent to 3% of the total body weight at a rate of 3 ml/Kg/min. Sustaining infusion rates ranged from 0.44 to 0.72 ml/Kg/min and continued until the collection of the final blood sample. When the final sample was collected the total volume of fluid added ranged from 3.4% to 6.2% of the total body weight (Table 7). In all dogs 20 ml samples of whole blood were collected in ice and the red blood cells (RBC) immediately removed by centrifugation at approximately -10°C. Five ml of 20% H-TCA was added to 10 ml of the resultant plasma (final H-TCA concentration 6.67% or approximately 400 mMolar), mixed by inversion, and centrifuged at room

temperature to remove the precipitated proteins. The H-TCA was extracted with ether as described in METHODS--Section E. Figure 17 summarizes the procedure followed for dogs 2-8 receiving this initial 3% expansion, with Table 7 summarizing the experimental conditions of each of the 3% expansion studies.

In addition to the 3% expansion experiments just described, 6 animals were initially expanded to 6% of their total body weight. The initial infusion rate was 6 ml/Kg/min with sustaining rates ranging from 0.30 to 1.56 ml/Kg/min. In these experiments the total expansion at the time of collection of the final sample ranged from 7.1% to 10.6% of the total body weight (Table 8). Figure 17 summarizes the procedure for the first such experiment, dog 9, where samples were collected and treated as previously described. In this experiment samples were obtained at 0, 15, 30, and 45 minutes following the completion of the initial expansion. Following the collection of the 15 and 30 minute samples, the blood removed was replaced with 0.154 M NaCl.

Figure 18 summarizes the procedure for dogs numbered 10, 11, and 12. In these experiments 100 ml of whole blood was obtained for the control period as well as the experimental period. Following the removal of the control sample, the red blood cells were resuspended to 100 ml with 0.154 M NaCl and reinfused into the animal. With both the control and experimental samples, 3 aliquots of plasma were obtained. One aliquot was treated with H-TCA to render it protein free as in the 3% experiments; one aliquot was ultrafiltered as described previously to remove the proteins; and the third aliquot was applied directly to a

Figure 17. Protocol for dogs 2-8 receiving an initial 3% (total body weight) volume expansion with 0.154 M NaCl. Also included is the protocol for dog 9 receiving a 6% (total body weight) volume expansion with 0.154 M NaCl. (-) indicates removal of substance by centrifugation (cent.).

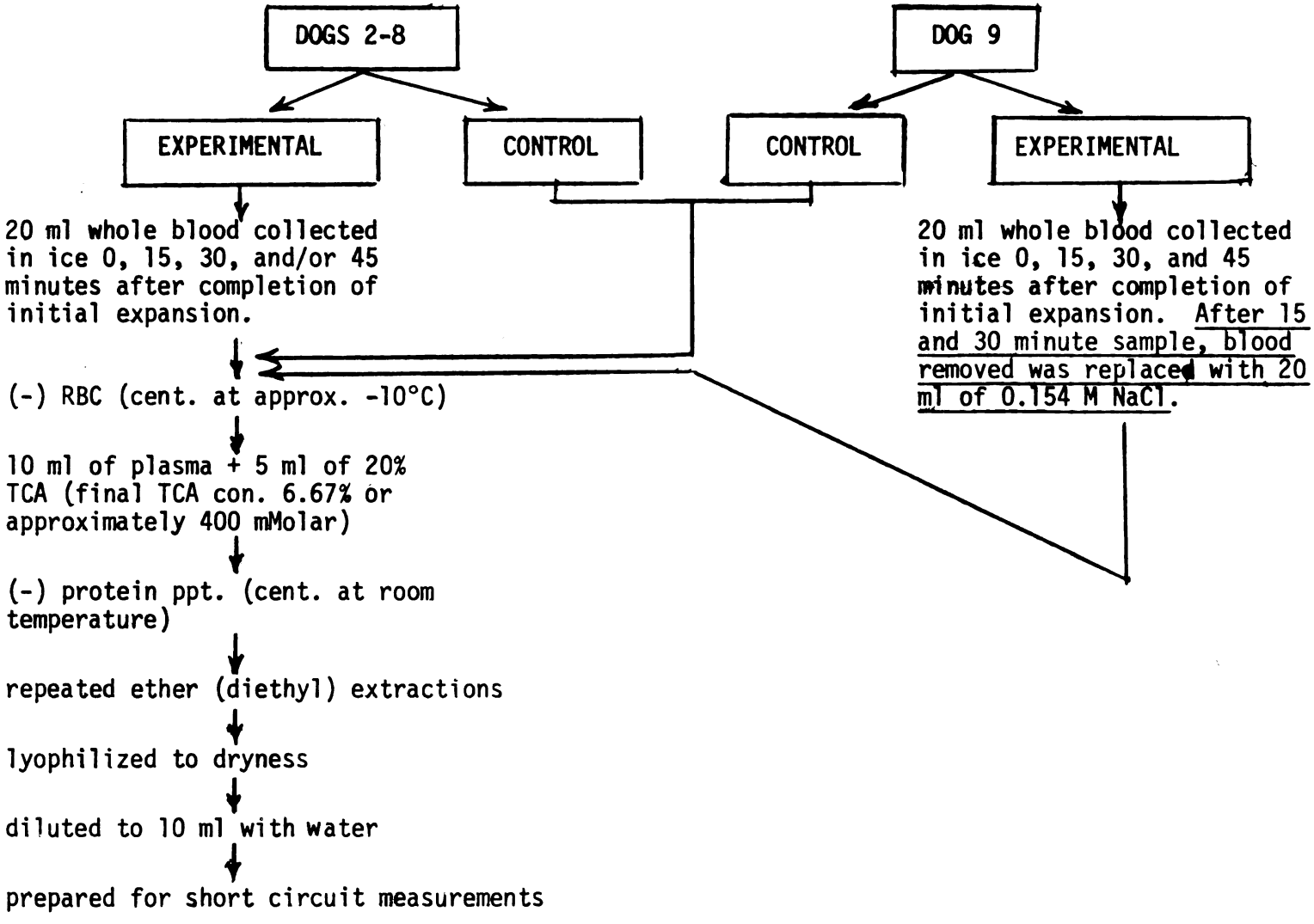
DOGS 2-8
DOG 9
EXPERIMENTAL
CONTROL
CONTROL
EXPERIMENTAL
20 ml whole blood collected in ice 0, 15, 30, and/or 45 minutes after completion of initial expansion.
20 ml whole blood collected in ice 0, 15, 30, and 45 minutes after completion of initial expansion. After 15 and 30 minute sample, blood removed was replaced with 20 ml of 0.154 M NaCl.
(-) RBC (cent. at approx. -10°C)
10 ml of plasma + 5 ml of 20% TCA (final TCA con. 6.67% or approximately 400 mMolar)
(-) protein ppt. (cent. at room temperature)
repeated ether (diethyl) extractions
lyophilized to dryness
diluted to 10 ml with water
prepared for short circuit measurements

Figure 17.

Table 7. Summary of the experimental conditions under which the 3% (total body weight) 0.154 M NaCl volume expansion experiments on dogs were carried out.

DOG	WEIGHT (Kg)	INITIAL EXPANSION VOLUME (ml)	INITIAL INFUSION RATE (ml/kg/min)	SUSTAINING INFUSION RATE (ml/kg/min)	SAMPLE TIMES (min)	TOTAL VOLUME INFUSED (ml)	TOTAL FLUID ADDED (% TOTAL BODY WEIGHT)
2	8	240	3	0.62	*C,0,15	275	3.4
3	9	270	3	0.52	C,0,15	315	3.5
4	11	330	3	0.72	C,15,30	444	4.0
5	10	300	3	0.54	C,15,30	411	4.1
6	10	300	3	0.44	C,15,45	448	4.5
7	10.5	315	3	0.50	C,15,45	500	4.8
8	8	240	3	0.72	C,30,45	500	6.2

*C refers to control sample. Other times refer to times when experimental samples were taken after completion of the initial expansion.

Figure 18. Protocol for dogs 10-12 receiving an initial 6% (total body weight) volume expansion with 0.154 M NaCl. (-) signifies removal of substance by centrifugation (cent.).

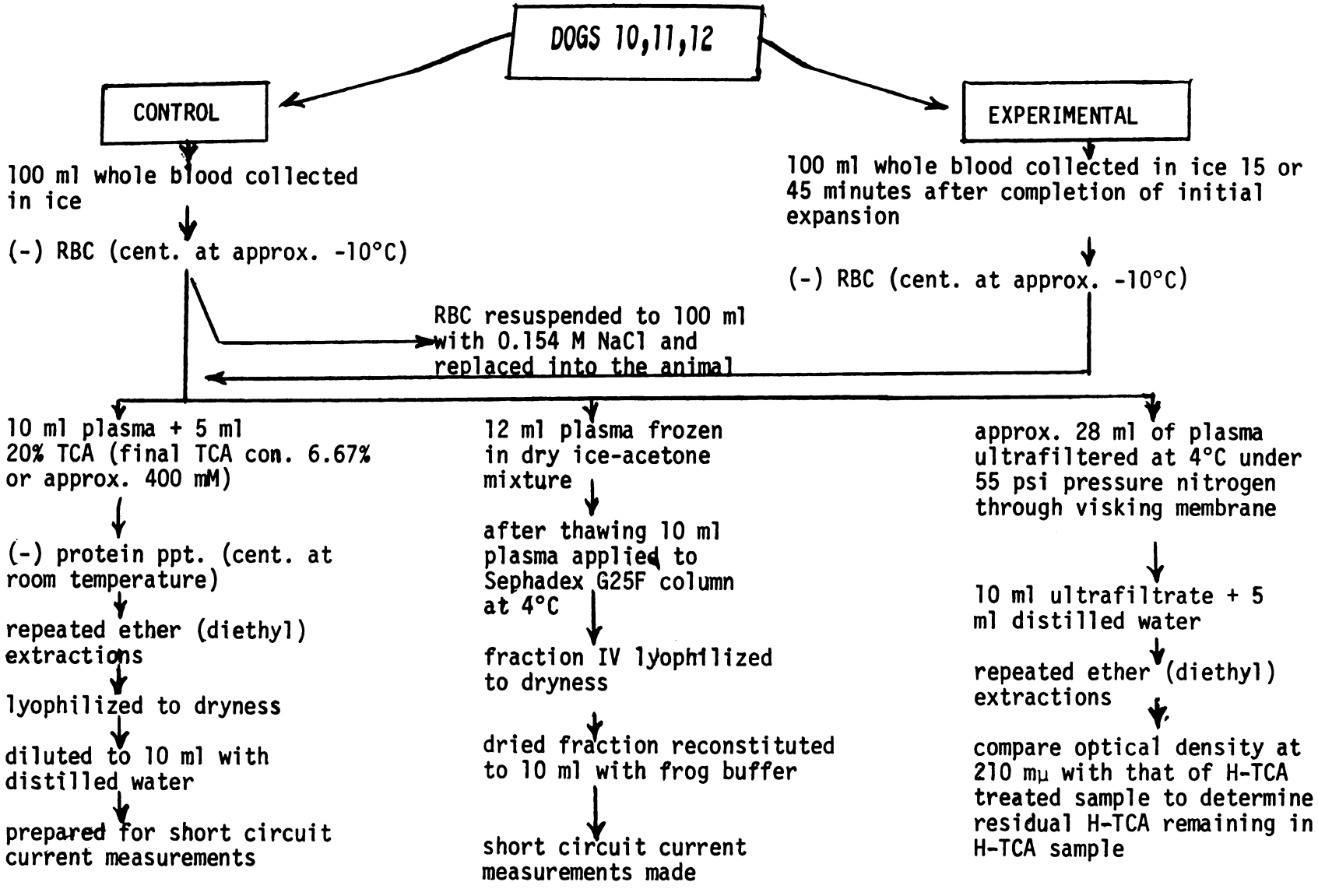
DOGS 10,11,12
CONTROL
EXPERIMENTAL
100 ml whole blood collected in ice
(-) RBC (cent. at approx. -10°C)
100 ml whole blood collected in ice 15 or 45 minutes after completion of initial expansion
(-) RBC (cent. at approx. -10°C)
RBC resuspended to 100 ml with 0.154 M NaCl and replaced into the animal
10 ml plasma + 5 ml 20% TCA (final TCA con. 6.67% or approx. 400 mM)
(-) protein ppt. (cent. at room temperature)
repeated ether (diethyl) extractions
lyophilized to dryness
diluted to 10 ml with distilled water
prepared for short circuit current measurements
12 ml plasma frozen in dry ice-acetone mixture
after thawing 10 ml plasma applied to Sephadex G25F column at 4°C
fraction IV lyophilized to dryness
dried fraction reconstituted to 10 ml with frog buffer
short circuit current measurements made
approx. 28 ml of plasma ultrafiltered at 4°C under 55 psi pressure nitrogen through visking membrane
10 ml ultrafiltrate + 5 ml distilled water
repeated ether (diethyl) extractions
compare optical density at 210 mμ with that of H-TCA treated sample to determine residual H-TCA remaining in H-TCA sample

Figure 18.

Sephadex G25F resin column. The ultrafiltered aliquot was used merely as a control which was extracted with ether to attempt to quantitate the effectiveness of removal of H-TCA from the first aliquot. The second aliquot served to compare activities on the frog skins of samples which were and were not treated with H-TCA.

Figure 19 summarizes the procedure for 3 experiments in which H-TCA was not utilized. All samples in these experiments were applied directly to the Sephadex resin column. Table 8 summarizes the experimental conditions of each of the 6% expansion studies.

L. Sample Preparation for Short Circuit Current Measurements

All plasma samples deproteinized with H-TCA first underwent removal of the added H-TCA with ether as described in METHODS--Section E. These deproteinized plasma samples were then either fractionated on Sephadex G25F resin or immediately lyophilized to dryness. The preparation of fractionated specimens for SCC measurements is described below (METHOD I AND METHOD II). Lyophilized samples were diluted to the original plasma volume with distilled water containing 2.5 mMolar tris-Cl and 2.0 mMolar magnesium chloride. The pH was adjusted to approximately 7.8 with 1 N NaOH and the sodium chloride and potassium chloride concentrations adjusted to conform with the frog buffer (Table 6, METHODS--Section G). If the milliosmolality of the sample was equivalent to that of the frog buffer no further adjustments were made. Samples which were hypo-osmotic were made isosmotic with the addition of glucose.

Figure 19. Protocol for dogs 13-15 receiving an initial 6% (total body weight) expansion with 0.154 M NaCl. (-) signifies removal of substance by centrifugation (cent.).

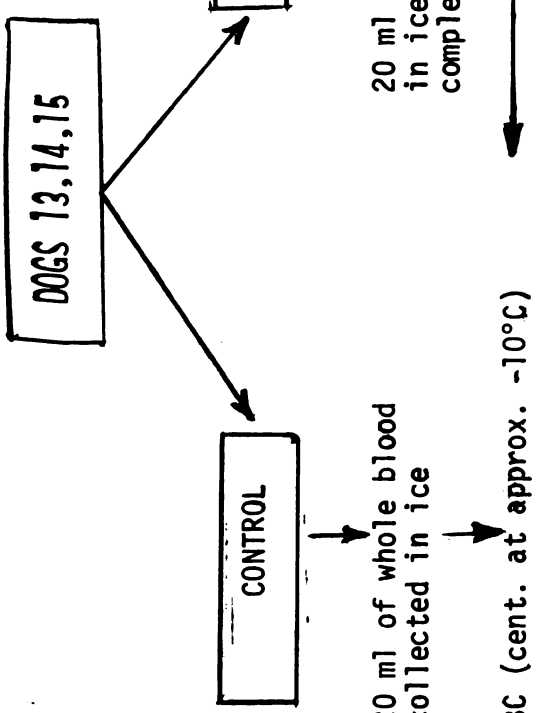
DOGS 13,14,15
CONTROL
EXPERIMENTAL
20 ml of whole blood collected in ice
20 ml whole blood collected in ice 15 or 20 minutes after completing initial expansion
(-) RBC (cent. at approx. -10°C)
10 ml plasma applied to Sephadex G25F column at 4°C
Fraction IV lyophilized to dryness
dried fraction reconstituted to 10 ml with frog buffer
short circuit current measurements made

Figure 19.

Table 8. Summary of the experimental conditions under which the 6% (total body weight) 0.154 M NaCl volume expansion experiments on dogs were carried out.

DOG	WEIGHT (Kg)	INITIAL EXPANSION VOLUME (ml)	INITIAL INFUSION RATE (ml/kg/min)	SUSTAINING INFUSION RATE (ml/kg/min)	SAMPLE TIMES (min)	TOTAL VOLUME INFUSED (ml)	TOTAL FLUID ADDED (% TOTAL BODY WEIGHT
9	9.5	570	6	0.65	*C,0,15, 30,45	850	8.9
10	9.5	570	6	1.12	C,15	700	7.4
11	9.0	540	6	0.74	C,15	640	7.1
12	12.0	720	6	0.38	C,45	925	7.7
13	9.0	540	6	1.52	C,30	950	10.6
14	7.3	438	6	1.20	C,30	700	9.6
15	12.0	720	6	1.56	C,15	1000	8.3

*C refers to control sample. Other times refer to times when experimental samples were taken after completion of the initial expansion.

The individual 10 ml aliquots obtained from samples which were fractionated on Sephadex G25F resin were pooled into 5 fractions as shown in Figure 23. These pooled fractions were lyophilized to dryness and fraction IV prepared for short circuit current measurements by one of the following methods:

Method I (20):

Plasma samples, both H-TCA treated and non-H-TCA treated, were diluted with distilled water to 1/10 the volume which was applied to the resin column. For short circuit current determinations 0.3 ml of this concentrated sample was mixed with 0.15 ml of a 3-fold concentrated frog buffer. Removal of 0.45 ml of frog buffer from the serosal side of the frog skin and injection of the 0.45 ml of sample resulted in an approximate 10-fold dilution of the reconstituted sample. Thus the concentration of any plasma antinatriferic substance in contact with the frog skin would be nearly equal to that of the original sample obtained. Plasma ultrafiltrate and dialysis fluid concentrates were handled in a similar fashion, except that the concentration of an antinatriferic material in these samples relative to plasma was unknown. Ammonium ion concentration determinations were performed on all samples, as it has been shown that low levels of this ion have an antinatriferic effect on amphibian membranes (78)--0.5 mMolar ammonium ion decreasing the short circuit current by 12%, 1.0 mMolar 22%, and 2.0 mMolar 30%.

Method II (20,36):

Following lyophilization to dryness, fraction IV samples were diluted with frog buffer to a volume equivalent to the volume of sample

applied to the resin column. Ammonium ion concentration, milliosmolality, and pH of the sample were measured before short circuit current measurements were made.

RESULTS

A. Fractionation of Specimens on Sephadex Resin

Elution of a sample of plasma (dog or human) with ammonium acetate (10 mMolar) from Sephadex G25F resin produced an optical density pattern of 280 mμ similar to that observed by Bourgoignie et al. (20). The pattern obtained from patient GS is illustrated in Figure 20, showing the 3 major absorption peaks used by Bourgoignie et al. (20) to localize the region of natriuretic-antinatriferic activity. This region, believed to contain these activities, occurs between the latter two peaks in Figure 20 in an approximate elution volume of 500 to 780 ml.

Figure 21 compares the fractionation of a plasma sample from patient CH with that of an aliquot of plasma ultrafiltrate obtained from the artificial kidney before and after an 8-fold concentration by ultrafiltration at 0-4°C. It can be noted that the latter 2 peaks of the concentrated plasma ultrafiltrate have an increase in absorbance at 280 mμ. Conspicuously absent from these ultrafiltrate patterns is the absorbance due to protein in the region of approximately 150 to 300 ml. This demonstrates the effectiveness with which the hemodialysis membranes used in the hemodialyzer retain these large molecular weight substances. Figure 22 depicts the elution pattern of plasma ultrafiltrate after a 15-fold concentration from patient GS.

Figure 20. Elution pattern at 280 mμ of a sample of plasma from patient GS. Sample was eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. 10 ml aliquots were collected by fraction collector.

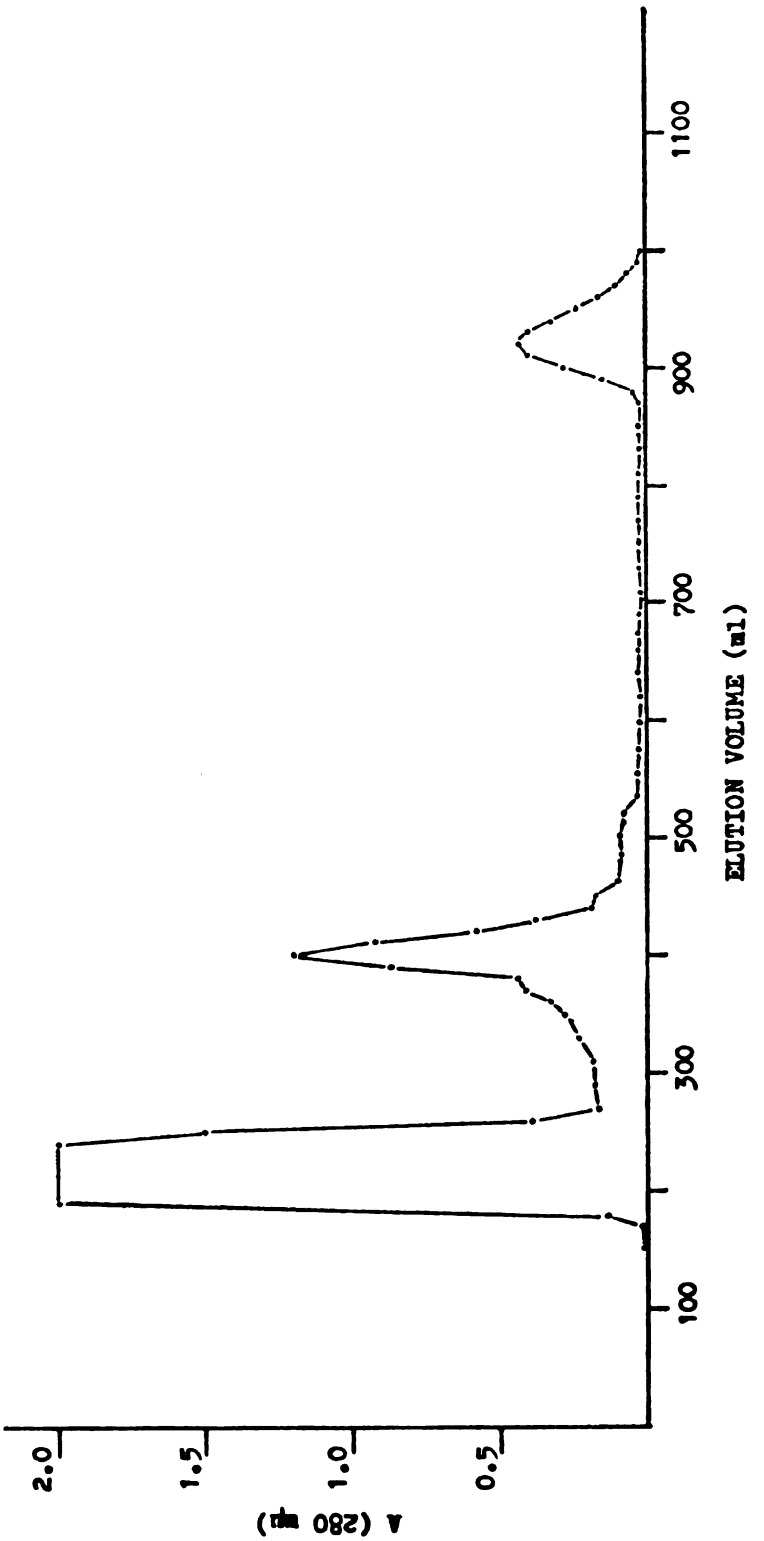

Figure 20.

Figure 21. Elution pattern at 280 mμ of a sample of plasma from patient CH. Also included is the elution pattern of plasma ultrafiltrate, obtained from the artificial kidney, before and after an 8-fold concentration by ultrafiltration. Ultrafiltration was performed at 4°C and fractionation at 13°C. Samples were eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. 10 ml aliquots were collected by fraction collector.

- ● Plasma ultrafiltrate before concentration
- ■ Plasma ultrafiltrate after an 8-fold concentration
- ▲ Plasma

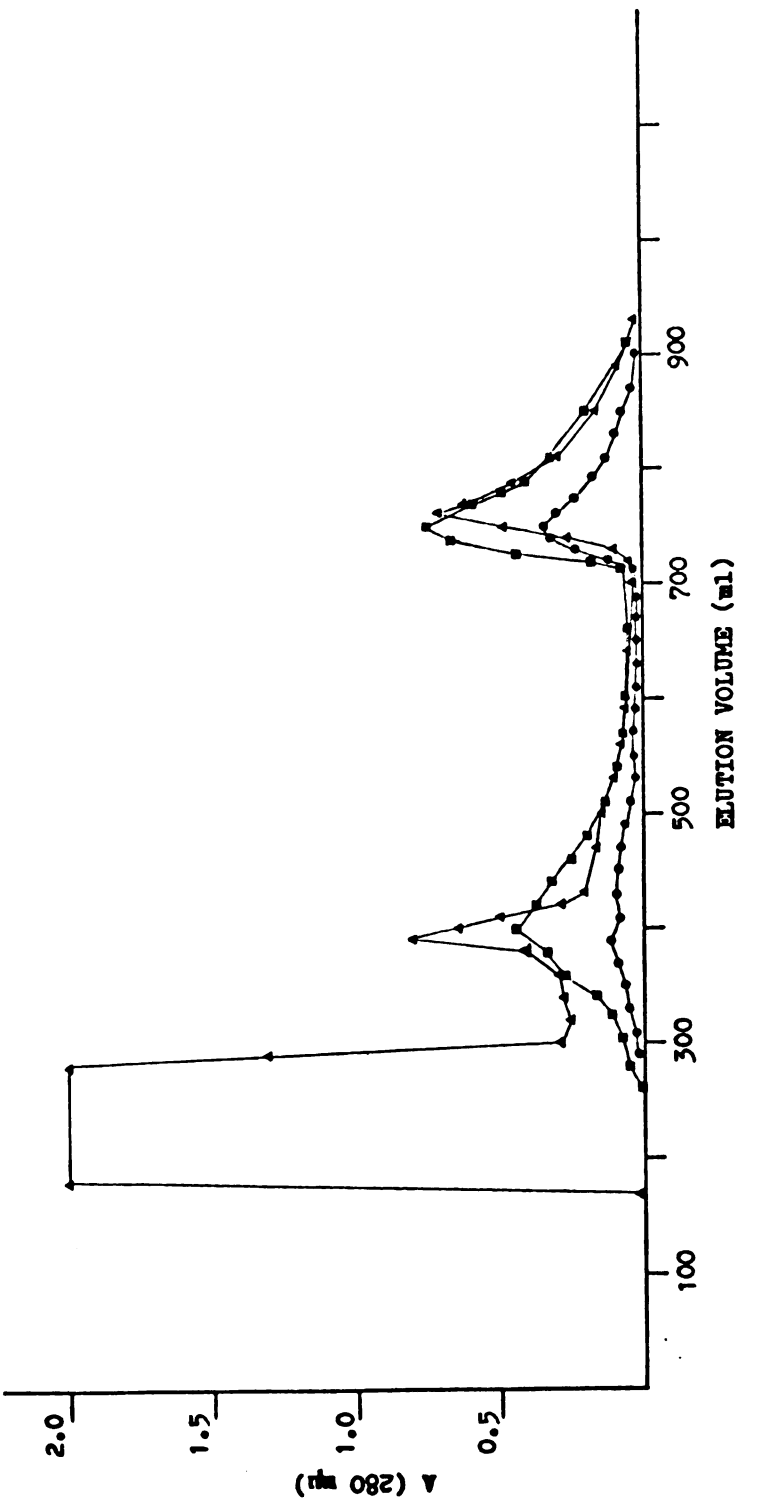

Figure 21.

Figure 22. Elution pattern at 280 mμ of plasma ultrafiltrate from patient GS after a 15-fold concentration by ultrafiltration. Sample was eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. 10 ml aliquots were collected by fraction collector.

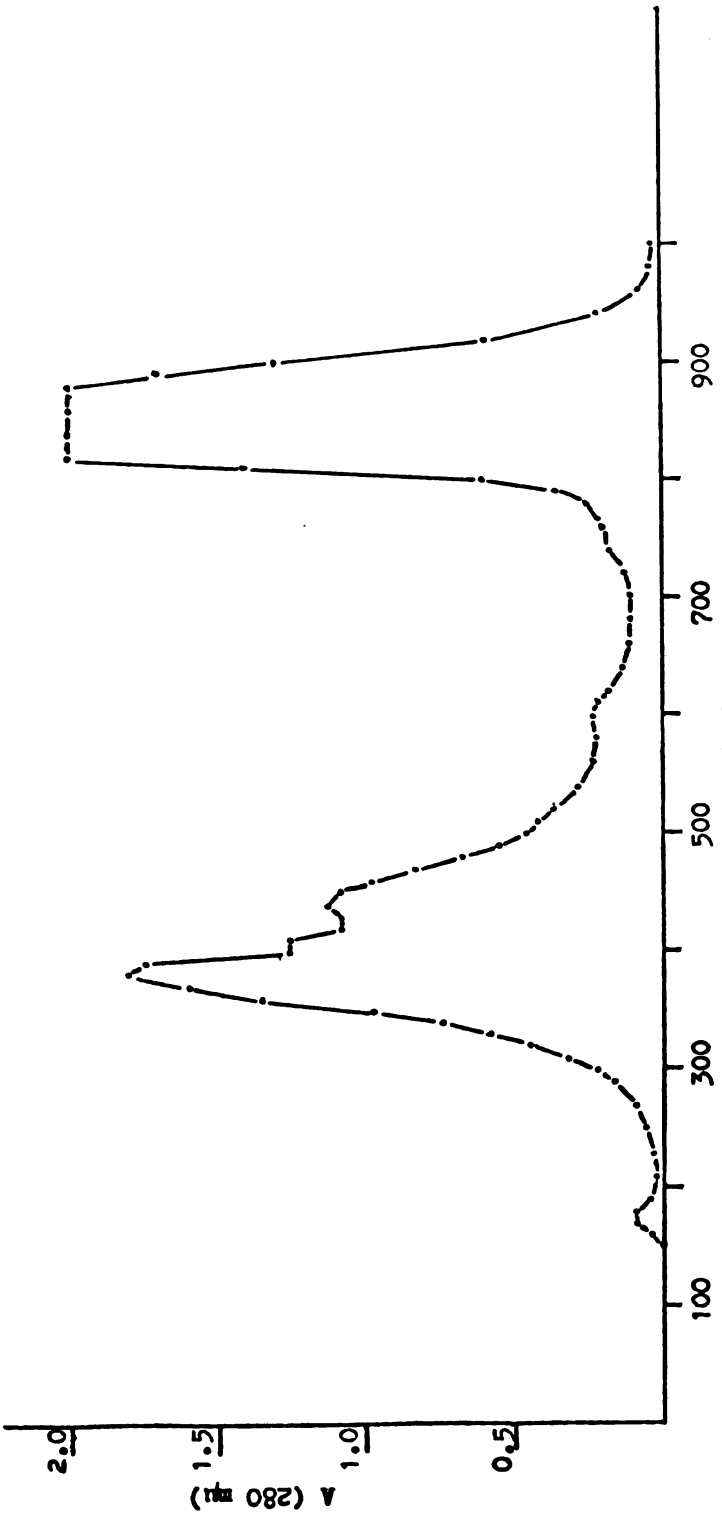

Figure 22.

Figure 23 indicates the absorbance at 280 and 360 mμ of the individual 10 ml fraction collector samples obtained following elution of an aliquot of concentrated dialysis fluid obtained from patient GS. To be noted first is the presence again of the 2 major absorbance peaks at 280 mμ and the absence of a protein peak at about 180 ml. During the concentration of dialysis fluid and plasma ultrafiltrate, the specimens become progressively pigmented. The nature of the pigment is at present not known. The color varies from a light yellow to brown and can occasionally be noted to be present in as much as the first 900 ml of the elution volume (depicted in Figure 23 by the horizontal line above the elution volume axis). An absorbance spectrum of the pigment demonstrated a maximum absorbance at approximately 360 mμ. The individual 10 ml fraction collector samples exhibited 3 absorbance peaks at 360 mμ, the latter 2 corresponding to the maximum visible coloration as indicated by the darkened area on the horizontal pigment line.

In addition to the absorbance at 280 and 360 mμ, the pH and milliosmolality of the individual 10 ml fraction collector samples are also shown in Figure 23. The pH of the eluant is approximately 6.5. As noted earlier, the 36 liters of dialysis fluid is acidified with HCl to retard bacterial growth over the period of 4 days required for concentration by ultrafiltration. This added hydrogen ion can be seen to be eluted in a volume very closely correlated with the major osmotically active substances, the lowest pH occurring at a volume of approximately 400 ml. Plasma, and plasma ultrafiltrates obtained

Figure 23. Fractionation of dialysis fluid concentrate from patient GS. Plotted against elution volume are absorbance at 280 and 360 mμ, pH, and milliosmolality. Also shown are the elution volumes pooled to give fractions I, II, III, IV, and V (vertical lines). Fraction IV was routinely assayed in the frog skin short circuit current apparatus. The horizontal line just above the elution volume axis depicts the volumes in which a noticeable pigmentation existed. The darkened portions on the line show the volumes in which the darkest visible coloration occurred. The sample was eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. Ten ml aliquots were collected by fraction collector.

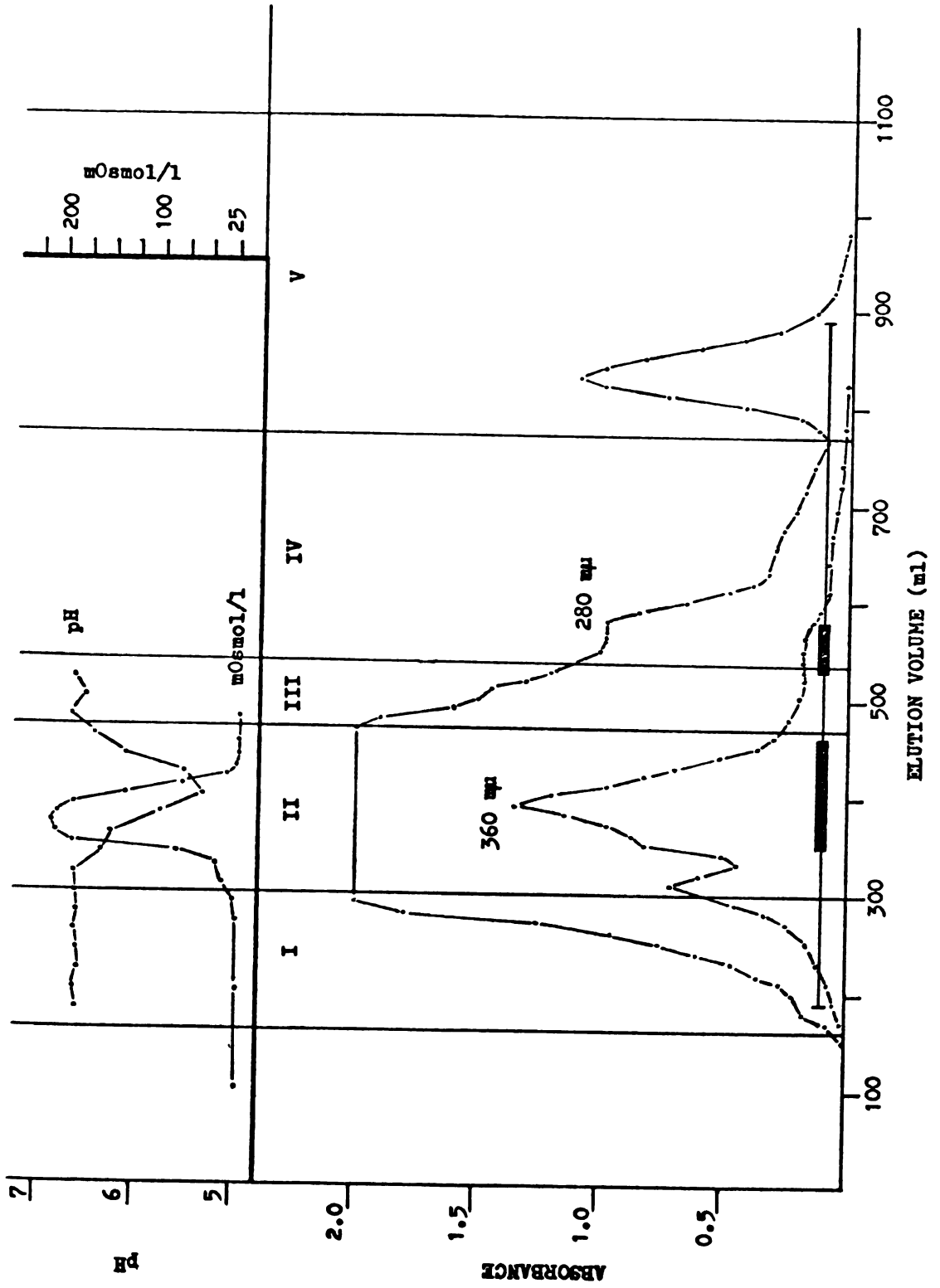
pH
mOsmol/l
200
100
25
I
II
III
IV
V
280 mμ
360 mμ
7
6
5
2.0
1.5
1.0
0.5
100
300
500
700
900
1100
ELUTION VOLUME (ml)
ABSORBANCE

Figure 23.

directly from the artificial kidney, are not acidified with HCl. As shown in Figure 24 these specimens exhibit no decrease in pH in this region but rather an increase. The presence of proteins in the plasma imparts an additional pH peak in an elution volume of approximately 220 to 300 ml (protein buffering of the eluant perhaps) not seen in the ultrafiltrate samples.

Closer inspection of the composition of the osmotic peak reveals that sodium, chloride, potassium, and glucose are the major contributors. These substances do not appear in any other region in measurable quantities. Elution of glucose with the inorganic ions was unexpected. Based on its molecular weight (180 Daltons) one would have expected it to appear in the volume containing small amino acids (approximately 850 ml). Figure 25 illustrates the elution pattern of these substances from a sample of plasma ultrafiltrate concentrate obtained from patient GS. The importance of these observations is the demonstration that there is no sodium, potassium, chloride, glucose, inorganic acid or base, or any other osmotically active substance in the fraction reported to contain antinatriferic-natriuretic activity (Fraction IV). There also is an absence of substances with a measurable absorbance at 280 mμ in Fraction IV.

To estimate the approximate molecular weights of substances in the various fractions obtained, compounds of known molecular weight were applied to the resin column and eluted with 10 mMolar ammonium acetate. Substances employed were Blue Dextran (M.W. 2 X 10^6 Daltons), bovine albumin (M.W. 69,000 Daltons), inulin (M.W. 5000 Daltons),

Figure 24. pH elution pattern of plasma and plasma ultrafiltrate obtained from the artificial kidney, before and after an 8-fold concentration, from patient CH. Fractionation was performed at 13°C and ultrafiltration at 4°C. The samples were eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. 10 ml aliquots were collected by fraction collector.

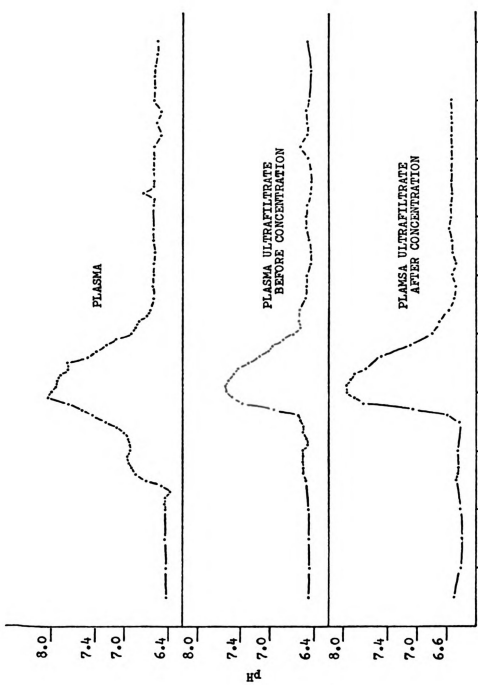
PLASMA
PLASMA ULTRAFILTRATE
BEFORE CONCENTRATION
PLAMSA ULTRAFILTRATE
AFTER CONCENTRATION
pH
8.0
7.4
7.0
6.4
6.6
100
300
500
700
900
ELUTION VOLUME (ml)

Figure 24.

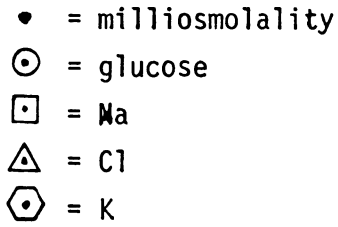

Figure 25. Major components of resin eluate samples demonstrating osmotic activity following fractionation of plasma ultrafiltrate concentrate (concentrated 15-fold by ultrafiltration) obtained from patient GS. The sample was eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. 10 ml aliquots were collected by fraction collector.

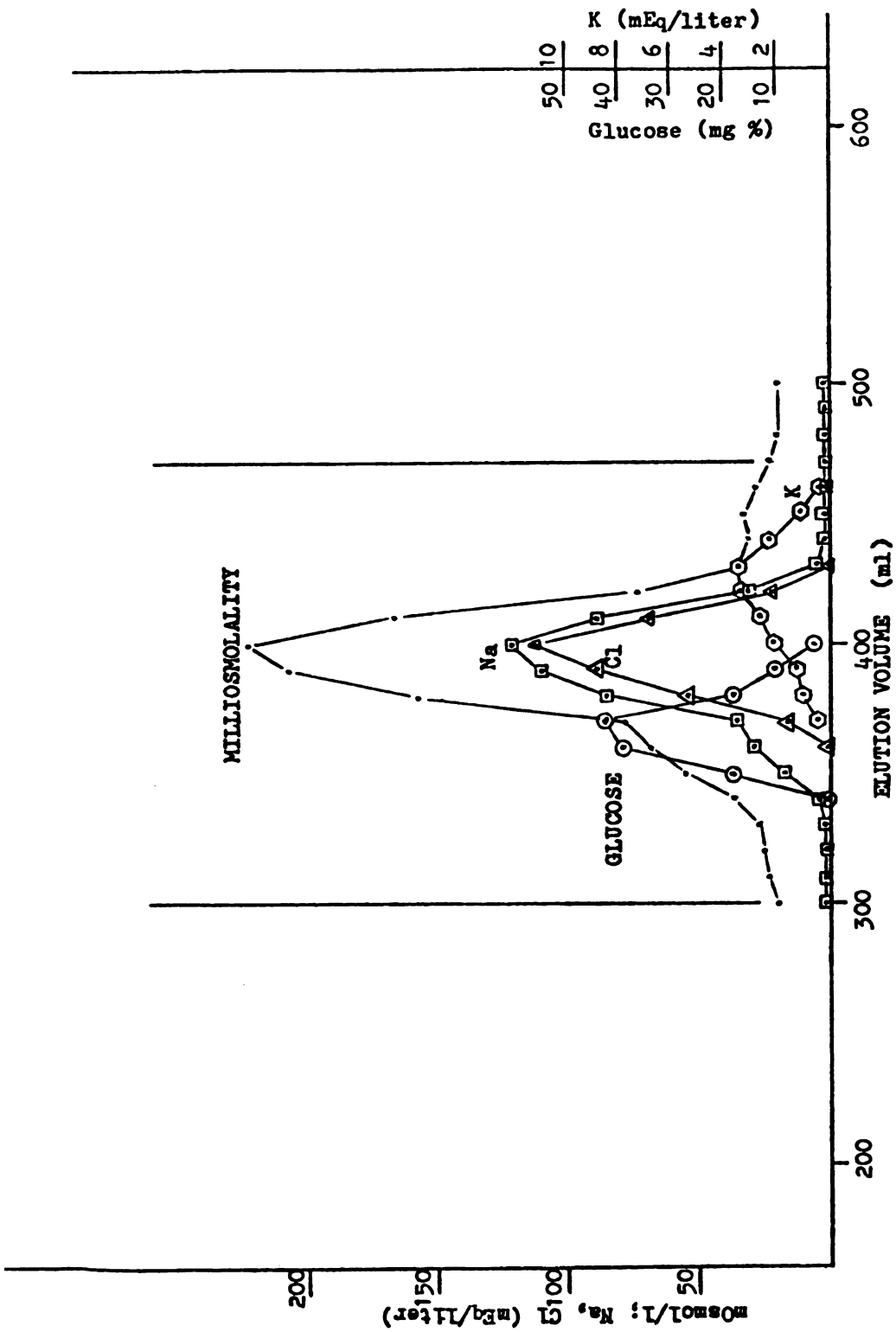
K (mEq/liter)
2
4
6
8
10
Glucose (mg %)
10
20
30
40
50
MILLIOSMOLALITY
Na
Cl
K
GLUCOSE
ELUTION VOLUME (ml)
200
300
400
500
600
mOsmol/l; Na, Cl (mEq/liter)
50
100
150
200

Figure 25.

bacitracin (M.W. 1400 Daltons), and tryptophan (M.W. 204 Daltons). Figure 26 demonstrates that Blue Dextran, albumin, and inulin are all eluted with the column void volume of approximately 180 ml. Bacitracin is eluted with a volume of approximately 300 ml, and tryptophan at approximately 850 ml. Substances eluted with volumes between 300 and 850 ml (Fraction IV) might therefore be expected to have molecular weights on the order of 1400 to 200 Daltons, a molecular weight range consistent with that reported for 'NH'.

It should be stressed, however, that although there may be a correlation between molecular weight and elution volume, a more accurate relationship exists between the three-dimensional configuration of a molecule and its elution volume. Non-linear substances, for example, are excluded from the resin particles to a greater extent than are linear molecules of similar molecular weight and are therefore eluted from the column sooner than would have been expected. Heterocyclic and aromatic substances are often abnormally retarded in their passage through the resin and therefore are eluted later than would have been expected based on their molecular weight. This phenomenon is affected by such parameters as ionic strength and pH of the medium being employed. Column calibration must be cautiously interpreted therefore.

B. Effects of ADH, TCA^-, and Ammonium Ion on Frog Skin Short Circuit Current, Membrane Potential, and Resistance

As mentioned in METHODS, Sections E and L, ammonium ion and TCA^- exhibit antinatriferic activity with frog skin preparations. As these

Figure 26. Calibration of the 2.5 X 95 cm column packed with Sephadex G25F resin utilized in the studies described in this thesis.

● = albumin

■ = Blue Dextran

▲ = inulin

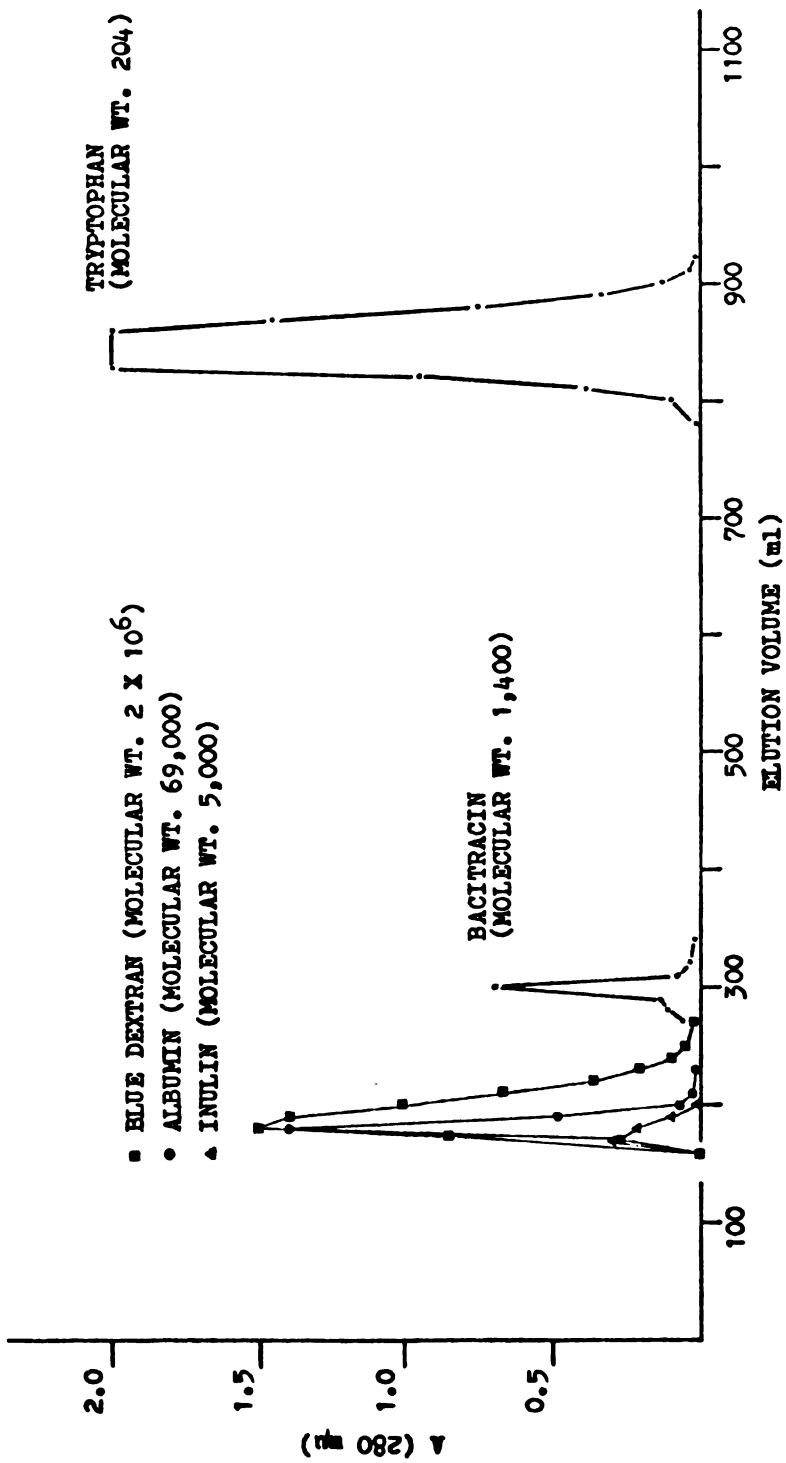
TRYPTOPHAN
(MOLECULAR WT. 204)
BLUE DEXTRAN (MOLECULAR WT. 2 X 10⁶)
ALBUMIN (MOLECULAR WT. 69,000)
INULIN (MOLECULAR WT. 5,000)
BACITRACIN
(MOLECULAR WT. 1,400)
A (280 mμ)
2.0
1.5
1.0
0.5
100
300
500
700
900
1100
ELUTION VOLUME (ml)

Figure 26.

substances were employed in the separation procedures, it was desirable to estimate quantitatively the magnitude of their effect. Several determinations were made with these substances on the skins of several different frogs. For purposes of comparison, several determinations of the effects of ADH were also made. These results are discussed below.

Following a period of equilibration, during which the short circuit current was stable for approximately 20-30 minutes, 0.02 ml of Pitressin (4 units /ml) was pipetted into the buffer on the serosal side of the frog skin. Measurements were carried out for 40-50 minutes, after which the medium was drained from both sides of the skin and fresh buffer added. The effects of ADH on frog skin short circuit current, membrane potential, and resistance are summarized in Table 9, and illustrated in Figure 27. These observations of increased SCC and decreased resistance with ADH are similar to those made by others (13,86,102) and therefore indicate that techniques and frogs used were adequate.

Table 9. Effect of ADH on frog skin short circuit current, membrane potential, and resistance. The percent change in the parameter was determined by averaging the difference between the last 3 measurements during the control and experimental periods. A total of 11 determinations were made on skins from 9 frogs. Averages are reported as ± SEM. Resistance was calculated by:

$$R(\text{ohms}) = \frac{MP(mV)}{SCC(\mu A)} \times 10^3$$

PARAMETER	AVERAGE PERCENT CHANGE AFTER ADDITION OF ADH
Short Circuit Current (μA)	+156 ± 37
Membrane Potential (mV)	+128 ± 31
Resistance (ohms)	− 23 ± 6

Figure 27. Effect of ADH on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Following a 30 minute control period, 0.02 ml (final concentration of ADH equals 0.02 units/ml) of a commercial preparation of Pitressin was injected into the serosal side of the chamber (time zero). Fifty minutes after the addition of ADH, the medium from both sides of the membrane was drained and the chambers flushed 3 times with fresh frog buffer. Fresh buffer was added to both sides of the membrane and measurements continued for another forty minutes. At this time the chambers were again drained and fresh buffer added. The skins were continuously short circuited, with MP and SCC being recorded at 5 minute intervals throughout the control and experimental periods.

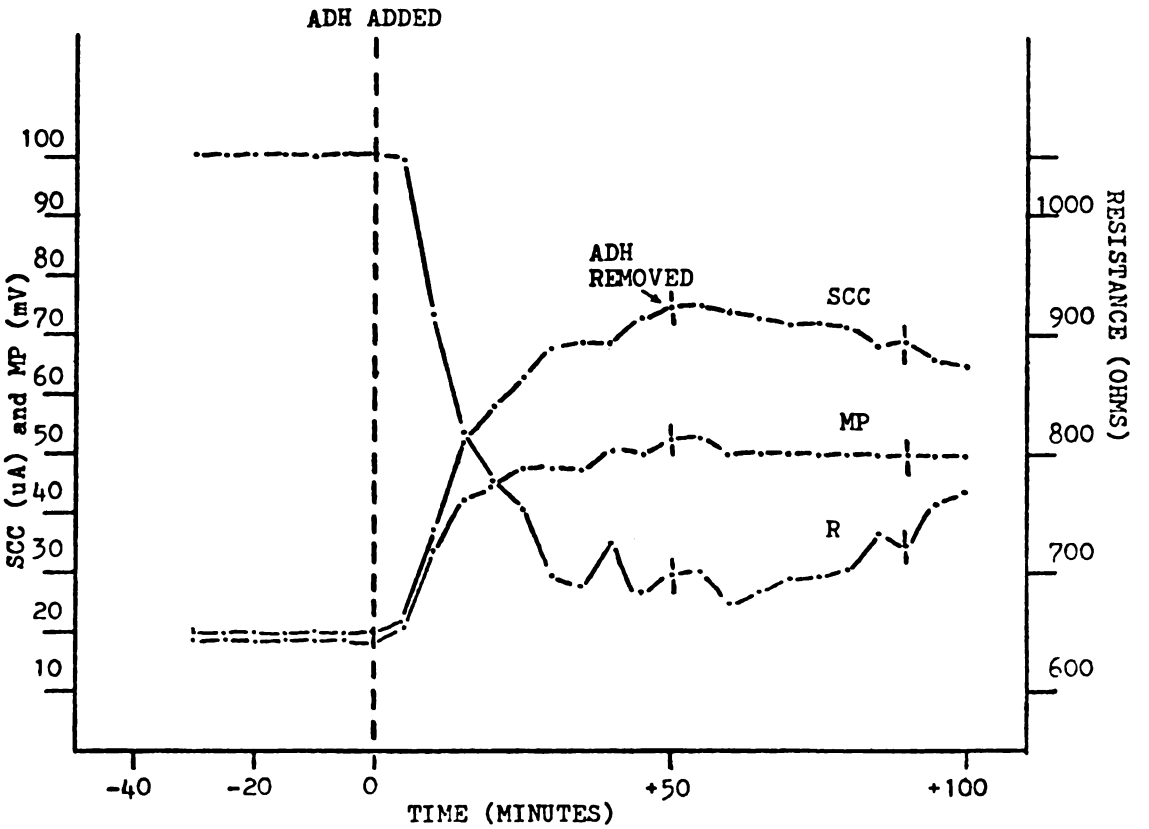
ADH ADDED
ADH
REMOVED
SCC
MP
R
SCC (uA) and MP (mV)
RESISTANCE (OHMS)
100
90
80
70
60
50
40
30
20
10
1000
900
800
700
600
-40
-20
0
+50
+100
TIME (MINUTES)

Figure 27.

To ascertain the effects of the trichloroacetate anion (TCA^-) on the frog skin, the sodium salt of the acid (Fischer Scientific) was utilized. Two buffer solutions were prepared, one as usual (METHODS--Section G), and the second with Na-TCA replacing NaCl. Buffers with various concentrations of Na-TCA were then prepared according to Table 10. Following a period of equilibration, one of the Na-TCA buffer solutions was applied to the serosal side of the skin; measurements obtained for 40 minutes; and the Na-TCA buffer then replaced with the normal NaCl buffer. Figure 28 summarizes the results of the TCA^- anion on the short circuit current and resistance of the frog skin, with Figure 29 depicting the effects on the membrane potential. Figure 30 illustrates the time course of the effects of 0.9 mMolar TCA^- on these parameters.

Table 10. Preparation of Na-TCA samples employed to determine the effects of TCA^- on frog skin short circuit current, membrane potential, and resistance. Buffers were prepared as described in METHODS--Section G. The designated volumes were pipetted into 15 ml Pyrex test tubes and mixed by vortexing.

TCA^- CONCENTRATION (mMoles/liter)	ml Na-TCA BUFFER	ml NaCl BUFFER
0.10	0.009	9.99
0.20	0.018	9.98
0.30	0.027	9.97
0.40	0.036	9.96
0.50	0.045	9.96
0.60	0.054	9.95
0.70	0.064	9.94
0.80	0.073	9.93
0.90	0.082	9.92
1.10	0.100	9.90
1.50	0.136	9.86
2.00	0.182	9.82

Figure 28. Effects of TCA^- on frog skin short circuit current and resistance.

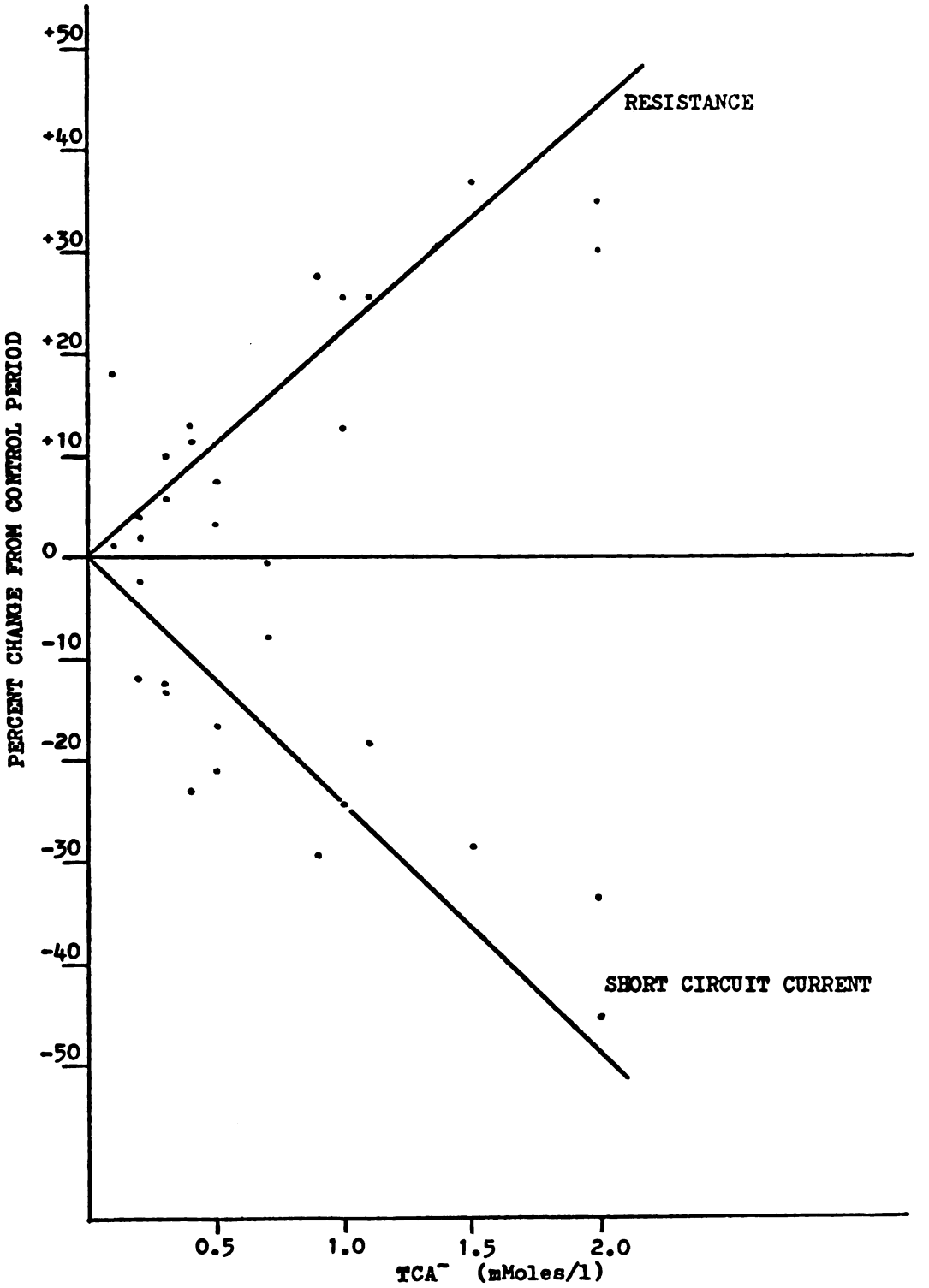
+50
+40
+30
+20
+10
0
-10
-20
-30
-40
-50
PERCENT CHANGE FROM CONTROL PERIOD
RESISTANCE
SHORT CIRCUIT CURRENT
0.5
1.0
1.5
2.0
TCA⁻ (mMoles/l)

Figure 28.

Figure 29. Effects of TCA^- on frog skin membrane potential.

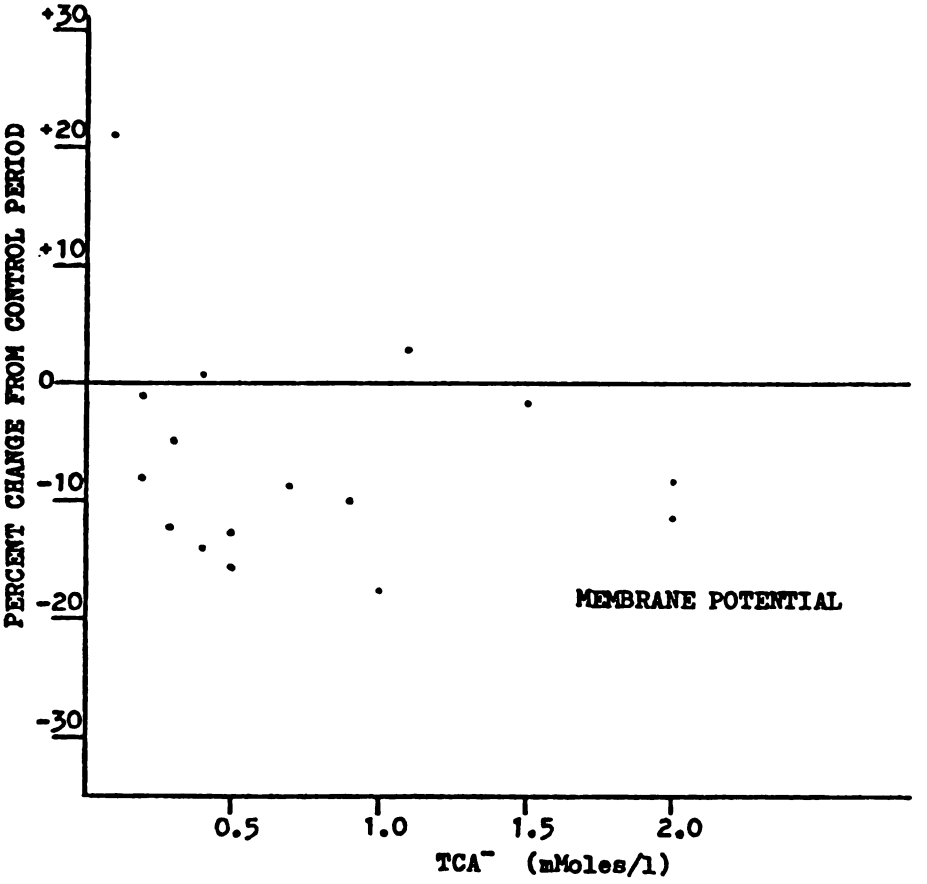
+30
+20
+10
0
-10
-20
-30
PERCENT CHANGE FROM CONTROL PERIOD
MEMBRANE POTENTIAL
0.5
1.0
1.5
2.0
TCA⁻ (mMoles/l)

Figure 29.

Figure 30. Effect of 0.9 mMolar Na-TCA on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Following a 25 minute control period, the frog buffer was drained from both sides of the membrane and replaced by regular frog buffer on the mucosal side of the skin and frog buffer containing 0.9 mMolar Na-TCA on the serosal side. After 40 minutes the medium was drained from both sides of the membrane and flushed 3 times with fresh frog buffer. Fresh frog buffer was added to both sides of the membrane and measurements continued for another 30 minutes. The skins were continuously short circuited, with MP and SCC being recorded at 5 minute intervals throughout the control and experimental periods.

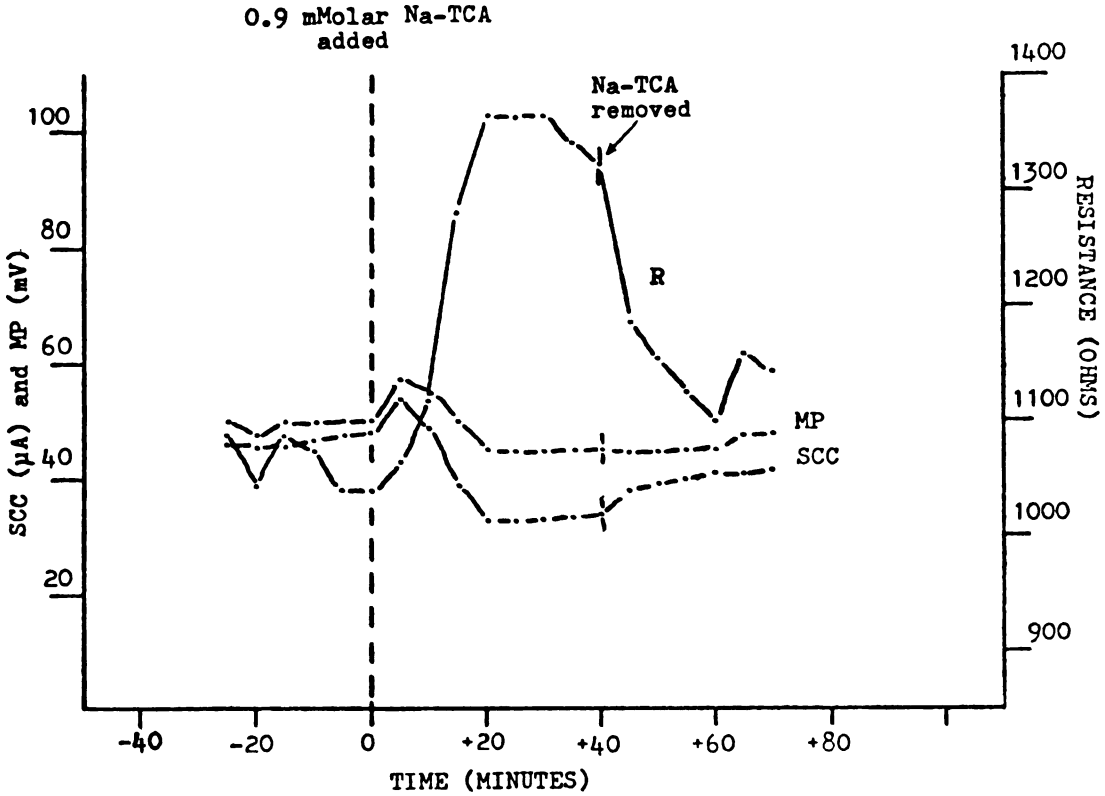
0.9 mMolar Na-TCA
added
Na-TCA
removed
R
MP
SCC
100
80
60
40
20
SCC (μA) and MP (mV)
1400
1300
1200
1100
1000
900
RESISTANCE (OHMS)
-40
-20
0
+20
+40
+60
+80
TIME (MINUTES)

Figure 30.

From these figures it can be seen that the TCA^- produces an inhibition of the SCC and MP which is reversible. The effects of the TCA^- on the SCC and MP result in a reversible increase in the calculated resistance.

To determine the effects of ammonium ion on the frog skin, a stock solution of 20 mMolar ammonium acetate was prepared by dissolving 77 mg in 50 ml of frog buffer. Solutions to be tested on the skins were prepared according to Table 11.

Table 11. Preparation of samples employed to determine the effects of ammonium ion on frog skin short circuit current, membrane potential, and resistance. Buffers were prepared as described in METHODS--Section G. The designated volumes were pipetted into 15 ml Pyrex test tubes and mixed by vortexing.

ml AMMONIUM FREE BUFFER	ml 20 mMolar AMMONIUM BUFFER	AMMONIUM ION (mMoles/l)
9.80	0.20	0.40
9.60	0.40	0.80
9.00	1.00	2.00

The results of 2.0 mMolar ammonium ion on the frog skin are illustrated in Figure 31. Figure 32 summarizes the percent change in short circuit current, membrane potential, and resistance produced by 0.4, 0.8, and 2.0 mMolar ammonium ion. Comparison of Figures 27, 30, and 31 illustrates that in contrast to ADH, TCA^- and ammonium ion result in a decrease in short circuit current and membrane potential with an increase in resistance.

Figure 31. Effect of 2.0 mMolar ammonium ion on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Following a 25 minute control period, the frog buffer was drained from both sides of the membrane and replaced by regular frog buffer on the mucosal side of the skin and frog buffer containing 2.0 mMolar ammonium acetate on the serosal side. After 40 minutes the medium was drained from both sides of the membrane and flushed 3 times with fresh frog buffer. Fresh buffer was added to both sides of the membrane and measurements continued for another 30 minutes. The skins were continuously short circuited, with MP and SCC being recorded at 5 minute intervals throughout the control and experimental periods.

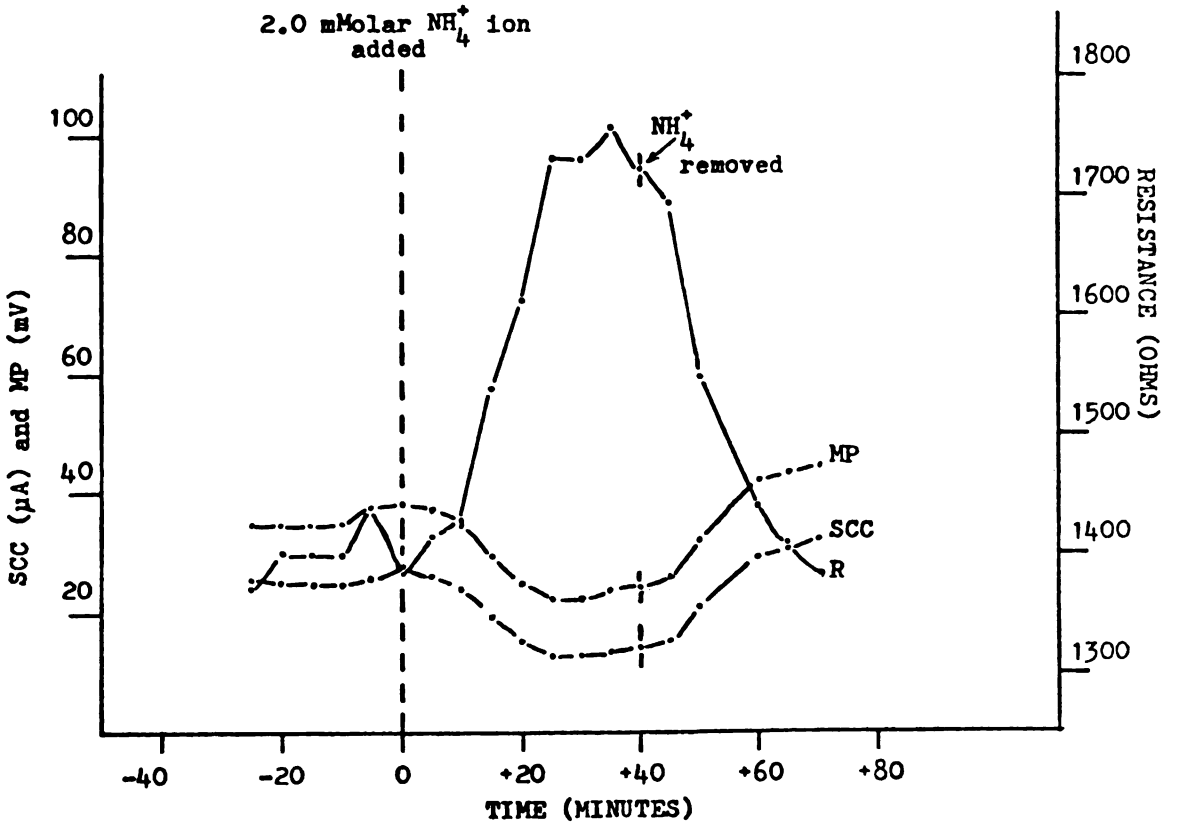
2.0 mMolar NH_4^+ ion added
NH_4^+ removed
MP
SCC
R
SCC (μA) and MP (mV)
RESISTANCE (OHMS)
100
80
60
40
20
1800
1700
1600
1500
1400
1300
-40
-20
0
+20
+40
+60
+80
TIME (MINUTES)

Figure 31.

Figure 32. Effect of 0.4, 0.8, and 2.0 mMolar ammonium ion on frog skin short circuit current, membrane potential, and resistance. Points are averages of 5 observations ± SEM for 0.4 mMolar and 6 observations ± SEM for 0.8 and 2.0 mMolar.

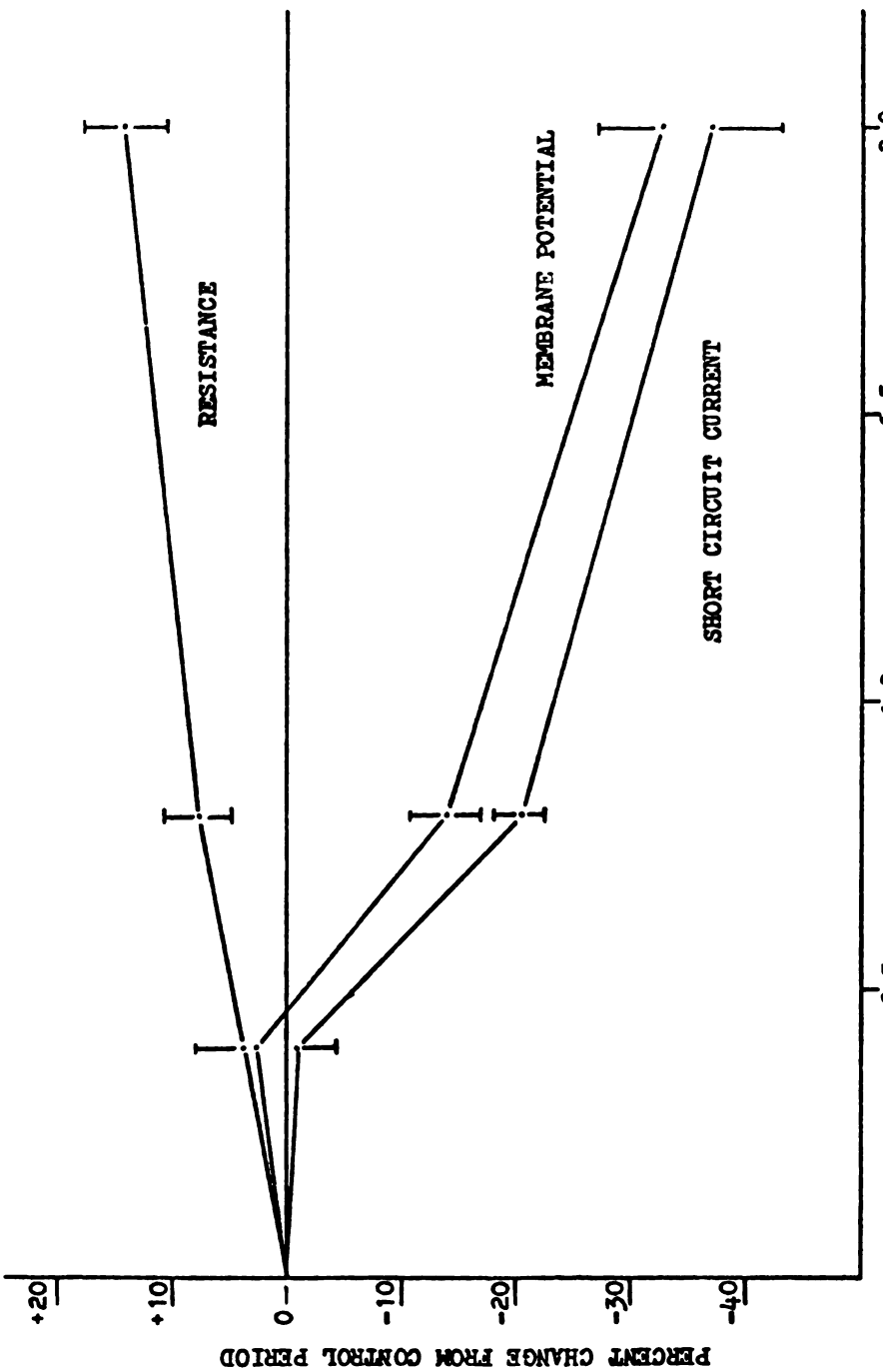
RESISTANCE
MEMBRANE POTENTIAL
SHORT CIRCUIT CURRENT
PERCENT CHANGE FROM CONTROL PERIOD
+20
+10
0
-10
-20
-30
-40
0.5
1.0
1.5
2.0
(mMoles/l)

Figure 32.

C. Human Uremic Studies

Patients involved in this study were selected on the basis of a change in their dry body weight following the previous dialysis treatment, an increase being indicative of fluid retention and possible volume expansion. The dry body weight for a given individual is defined as the total body weight at which the patient develops hypotension (a decrease in diastolic pressure of 30 mm Hg or orthostatic hypotension). This change in dry body weight for the patients studied, as shown in Table 12, ranged from -0.54 to +4.17%.

Table 13 presents a summary of the types of specimens (plasma, plasma ultrafiltrate from the artificial kidney, and/or dialysis fluid) obtained from each of the individuals studied. Non H-TCA treated plasma specimens were obtained from 9 patients, with H-TCA being used to precipitate proteins from the plasma of 4 patients. Dialysis fluid was obtained from 5 patients, and plasma ultrafiltrate from the artificial kidney was obtained from 8 patients. Non-TCA treated plasma from patients JS2, CdeB, and DH was frozen in a dry ice-acetone bath until fractionated on Sephadex G25F resin. All other specimens, excluding dialysis fluid, were placed in an ice bath until Sephadex fractionation.

Table 13 also summarizes the effects of lyophilized fraction IV from each sample on frog skin short circuit current. This fraction (elution volume of approximately 550 to 780 ml) has been reported to contain natriuretic and antinatriferic activities following elution of human uremic plasma and plasma of volume expanded dogs and cows (19,20,21,29).

Table 12. Blood pressure data and change in dry body weight of patients participating in this study. For definition of dry body weight see text, page 139. JS and JSe were different patients. JS2 was a second study with patient JS.

PATIENT	USUAL BLOOD PRESSURE	BLOOD PRESSURE ON DAY OF TREATMENT	PERCENT CHANGE IN DRY BODY WEIGHT SINCE PREVIOUS TREATMENT
JK	120/60	unknown	-0.54
CZ	160/90	160/80	+1.09
CH	150/70	160/90	+1.65
HM	120/60	105/60	+0.64
CO	160/80	158/84	+3.09
ML	160/90	100/50	+1.28
JS	160/100	unknown	+0.60
FP	130/50	150/60	+3.55
JSe	140/70	150/70	+1.85
GS	130/70	140/80	+4.17
JS2	160/90	164/82	+2.21
CdeB	160/100	150/90	+2.86
DH	140/100	145/80	+3.88

Table 13. Summary of the values of the short circuit current during the control period (C), following addition of lyophilized fraction IV from uremic samples (E), and during the recovery period (R) after replacement of the test sample by fresh frog buffer. Values were obtained by averaging the last 3 measurements during each period. Recovery values were from 20-40 minutes after replacement of the test sample by frog buffer. Plasma ultrafiltrate concentrate samples from patients JSe and GS contained high levels of ammonium ion (*). Samples were tested on 1 or 2 skins (from different frogs) as indicated in the table.

		SHORT CIRCUIT CURRENT (μA)														
		DIALYSIS FLUID			PLASMA ULTRAFILTRATE						PLASMA					
					(UNCON.)			(CON.)			NO H-TCA			H-TCA		
PATIENT	SKIN	C	E	R	C	E	R	C	E	R	C	E	R	C	E	R
JK	1	50.5	50.5	51.5												
	2	100	106	100												
CZ	1	31.0	27.0	33.2												
	2	54.0	53.3	49.0												
CH	1				31.7	30.0	28.3	69.0	70.5	67.3	61.3	60.0	58.0			
	2							58.0	48.7	48.0	78.0	80.7	84.3			
HM	1				22.0	24.0	22.7	23.7	23.3	19.7	24.0	22.2	22.0			
	2				29.7	30.7	26.7				20.0	18.0	11.7			
CO	1				19.5	19.4	17.5	32.3	29.7	29.5	51.0	50.3	**40.0**			
	2				40.0	39.7	36.6	57.5	51.5	56.3	21.0	18.8	**16.4**			

ML	1	38.5	35.7	40.5											48.0	48.0	54.0
	2	41.0	41.0	44.0											25.3	26.0	43.5
JS	1										34.0	49.0	53.2	29.3	45.0	30.8	
	2										15.3	16.0	13.7	53.2	89.0	48.8	
FP	1										23.8	23.8	20.7	20.7	23.0	29.0	
	2										37.7	37.0	36.0	38.5	38.3	37.0	
JSe	1							33.3	9.2*	32.3				47.5	65.0	67.3	
	2							32.2	12.5	24.3				20.0	27.0	22.8	
GS	1	67.3	69.0	68.3				149	93.3*	143	57.8	58.3	54.3				
	2	35.0	35.5	33.6				34.0	31.2	59.8	33.7	36.5	52.9				
JS2	1	98.6	87.0	86.3				110	96.3	98.7	29.5	33.3	38.2				
	2	62.0	54.2	51.3				69.0	66.3	64.5	88.3	92.0	85.0				
CdeB	1							23.2	36.7	24.3	39.0	42.7	45.3				
	2							57.0	66.0	54.0	27.8	32.8	25.0				
DH	1							52.0	48.0	46.4	52.0	48.3	58.3				
	2							22.0	21.3	16.8	27.7	24.0	26.3				

The short circuit current during the control period (C), following addition of the fraction IV sample (E), and during the recovery period (R) after the sample was replaced with fresh frog buffer are recorded. Inspection of the table indicates that only with the plasma ultrafiltrate concentrate samples from patients JSe and GS was there a large, reproducible and reversible inhibition of the short circuit current. These specimens, however, contained levels of ammonium ion (Table 15) sufficient to account for this inhibition (Figure 32--RESULTS). Table 14 and Appendixes I-III, which summarize the effects of the fraction IV samples on short circuit current, membrane potential, and resistance, further demonstrate that only these 2 specimens resulted in a reproducible decrease in membrane potential and an increase in skin resistance.

Table 15 summarizes the results of ammonium ion determinations on lyophilized fraction IV following specimen elution from Sephadex G25F resin with 10 mMolar ammonium acetate. Values obtained ranged from 0.00 to 4.40 mEq/liter. It has been shown that concentrations of ammonium ion greater than 0.4-0.5 mEq/l exhibit antinatriferic activity with frog skin preparations (78, and this thesis). Therefore specimens containing ammonium concentrations of 0.4-0.5 mEq/l or higher will produce an artifactitious decrease in SCC.

Table 16 summarizes the initial volumes of plasma ultrafiltrate which were obtained from each of 9 patients. These specimens were concentrated by ultrafiltration and then fractionated on Sephadex G25F resin with 10 mMolar ammonium acetate. Fraction IV was lyophilized to dryness and diluted in frog buffer.

Table 14. Summary of the effects of lyophilized fraction IV from uremic specimens on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Values are percent change in parameter after addition of test sample to the frog skin preparation. Also included in the table are the group means ± SEM of the membrane potential, short circuit current, and resistance for each type of specimen. *Since the plasma ultrafiltrate concentrate for patients JSe and GS contained ammonium ion at a concentration of 4.4 and 1.1 mEq/liter, respectively, they were not included in the computation of the means.

		DIALYSIS FLUID			PLASMA ULTRAFILTRATE (UNCON.)			PLASMA ULTRAFILTRATE (CON.)			PLASMA NO H-TCA			PLASMA H-TCA		
PATIENT	SKIN	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R
JK	1	+ 7	0	+ 6												
	2	+ 8	+ 6	+ 2												
CZ	1	-11	-13	- 2												
	2	- 2	- 1	0												
CH	1				- 1	- 5	+ 5	0	+ 2	- 2	- 4	- 2	- 2			
	2							- 8	-16	+10	- 5	+ 4	+ 7			
HM	1				+29	+ 9	+18	+34	- 2	+35	0	- 8	+ 9			
	2				0	+ 3	- 3				- 5	-10	+ 6			
CO	1				+ 4	- 1	+ 4	-14	- 9	- 7	+ 9	- 1	+11			
	2				0	- 1	+ 1	0	-10	+12	+ 8	-11	+20			
ML	1	+ 3	- 7	+11										- 5	0	- 4
	2	- 2	0	- 2										- 7	+ 3	-10

JS	1 2															
FP	1 2										+28 + 6	+44 + 5	-11 -19	+21 +25	+54 +67	-21 -25
*JSe	1 2										+13 - 1	0 - 2	+14 + 1	+15 - 4	+11 - 1	- 3 + 5
*GS	1 2	+ 5 0	+ 3 + 1	+ 3 - 1				-42 -60	-72 -61	+111 + 3				+20 +29	+37 +35	-12 - 4
JS2	1 2	- 2 -25	-12 -13	+11 -14				- 6 -12	-37 - 8	+50 + 4	+ 1 - 3	+ 1 + 8	0 -12			
CdeB	1 2							- 4 - 7	-12 - 4	+10 - 3	+27 +12	+13 + 4	+12 + 8			
DH	1 2							+39 +20	+58 +16	+12 + 4	+ 8 +33	+10 +18	- 1 +12			
								+12 + 1	- 8 - 3	+22 + 4	- 5 +15	- 7 -13	+ 2 +33			
MEAN		- 2	- 4	+ 1	+ 5	+ 1	+ 4	+ 7	+ 1	+ 9	+ 8	+ 3	+ 5	+12	+26	- 9
+SEM		3	2	2	6	2	1	5	6	4	3	3	3	5	9	4

Table 15. Results of ammonium ion determinations on lyophilized fraction IV samples obtained from human uremic specimens following elution of specimen from Sephadex G25F resin with 10 mMolar ammonium acetate.

AMMONIUM ION CONCENTRATION (mEq/l)					
		ULTRAFILTRATE		PLASMA	
PATIENT	DIALYSIS FLUID	UNCONCENTRATED	CONCENTRATED	NO H-TCA	H-TCA TREATED
JK	0.08				
CZ	0.16				
CH		0.17	0.12	0.26	
HM		0.18	0.23	0.18	
CO		0.48	0.30	0.20	
ML	0.50				0.03
JS				0.30	0.03
FP				0.35	0.35
JSe			4.40		0.35
GS	0.20		1.10	0.40	
JS2	0.48		0.30	0.28	
CdeB			0.00	0.00	
DH			0.00	0.00	

Table 16. Initial volumes and magnitude of concentration of plasma ultrafiltrates obtained from the artificial kidney. The initial volumes of plasma ultrafiltrate were reduced and concentrated by ultrafiltration at 0-4°C using an Amicon UM05 ultrafiltration membrane. Following ultrafiltration the specimen remaining in the ultrafiltration cell was eluted from Sephadex G25F resin with 10 mMolar ammonium acetate, fraction IV lyophilized to dryness, and diluted in frog buffer.

PATIENT	INITIAL VOLUME OF PLASMA ULTRAFILTRATE OBTAINED FROM ARTIFICIAL KIDNEY (ml)	MAGNITUDE OF CONCENTRATION INCREASE AFTER ULTRAFILTRATION AND SEPHADEX FRACTIONATION
CH	400	8
HM	240	9
CO	180	18
JSe	275	27
JS2	265	26
CdeB	250	25
DH	150	15
GS	150	15

These procedures resulted in an 8- to 27-fold concentration of the original sample. From Tables 13 and 14 and Appendix III it can be seen that, even after these concentration procedures, the plasma ultrafiltrates did not contain marked reversible antinatriferic activity in the absence of ammonium ion. A similar result was obtained with dialysis fluid specimens which had been concentrated 3600-fold.

As previously described (METHODS--Section L), samples for frog skin measurements were prepared in two ways. Samples from patients CZ, JK, CH, ML, and CO were diluted in distilled water to approximately

1/10 of the volume applied to the Sephadex column. A 0.3 ml aliquot was then diluted to 0.45 ml with a 3-fold concentrated frog buffer; 0.45 ml of frog buffer removed from the serosal side of the frog skin; and the 0.45 ml of sample added (Method I, Section L--METHODS). All other specimens were assayed on the frog skins according to Method II described in Section L of METHODS.

D. Acute Volume Expansion Experiments with Dogs

Dogs numbered 2 through 8 underwent an initial volume expansion with 0.154 M NaCl equivalent to 3% of their total body weight. All plasma samples obtained were deproteinized with H-TCA, the H-TCA being removed by ether extractions as previously described in METHODS--Section E. Samples from dogs 2, 3, 7, and 8, were then prepared for short circuit current assay before fractionation on Sephadex G25F resin. The results of chemical analyses of these samples are tabulated in Table 17. Sodium in these samples averaged 110 $\pm$ 0.3 SEM mEq/liter; potassium, 2.9 $\pm$ 0.0 SEM mEq/liter; and milliosmolality, 228 $\pm$ 0.3 SEM mOsmol/liter. Samples from dogs 2-8, following elution from Sephadex resin and lyophilization, exhibited a range of ammonium ion concentration from 0.00 to 62.0 mEq/liter (Table 17).

Table 19 and Appendix IV summarize the effects of the unfractionated 3% expansion samples from dogs 2, 3, 7, and 8, on frog skin short circuit current, membrane potential, and resistance. With the exception of a plasma sample from dog 7, obtained 30-minutes after expansion, there is no evidence for a reproducible antinatriferic substance.

Table 17. Summary of chemical analyses of plasma samples obtained from dogs prior to (CONTROL) and following a 3% total body weight volume expansion with 0.154 M NaCl. Experimental samples were obtained from 0 to 45 minutes after the completion of the initial expansion. All samples had undergone TCA^- removal by ether extraction, lyophilization, and preparation for frog skin assay.

		BEFORE FRACTIONATION			AFTER FRACTIONATION
		Na (mEq/l)	K (mEq/l)	mOSmol/l	NH_4^+ (mEq/l)
CONTROL:	Dog 2	110	2.8	227	0.00
	Dog 3	110	2.8	228	0.04
	Dog 4	---	---	---	0.00
	Dog 5	---	---	---	0.00
	Dog 6	---	---	---	0.05
	Dog 7	111	3.2	228	0.00
	Dog 8	110	2.6	228	1.70
0 MINUTES:	Dog 2	110	2.8	226	0.00
	Dog 3	109	2.8	228	0.24
15 MINUTES:	Dog 2	108	2.6	228	0.00
	Dog 3	111	2.9	230	0.79
	Dog 4	---	---	---	0.00
	Dog 5	---	---	---	0.00
30 MINUTES:	Dog 4	---	---	---	0.00
	Dog 5	---	---	---	0.06
	Dog 6	---	---	---	0.00
	Dog 7	110	3.4	227	0.00
	Dog 8	110	3.0	228	0.00
45 MINUTES:	Dog 6	---	---	---	0.00
	Dog 7	110	3.4	227	0.00
	Dog 8	110	2.7	228	62.00

Table 18. Summary of the values of the short circuit current during the control period (C), following the addition of unfractionated H-TCA treated plasma samples from the 3% expanded dogs (E), and 20-30 minutes after replacing the test sample by fresh frog buffer (R). Values were obtained by averaging the last 3 measurements during each period.

		SHORT CIRCUIT CURRENT (μA)														
		SAMPLE TIMES														
		CONTROL			0 MINUTES			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	C	E	R	C	E	R	C	E	R	C	E	R	C	E	R
DOG 2	1	18.0	32.3	17.2	38.3	54.5	25.7	25.3	46.8	22.5						
	2	8.0	28.2	14.3	19.2	19.5	8.2	6.3	11.0	8.0						
Dog 3	1	19.0	27.0	6.4	15.3	24.3	9.0	16.8	14.0	15.3						
	2	11.3	15.2	12.8	8.3	20.7	9.5	23.8	24.0	20.3						
Dog 7	1	32.7	45.7	36.5							34.7	15.8	23.7			
	2	30.3	29.0	27.6							29.8	13.3	26.8			
Dog 8	1	46.0	45.0	37.0							37.3	53.3	45.8	46.5	44.0	45.2

Table 19. Summary of the effects of unfractionated H-TCA treated plasma samples from the 3% expanded dogs on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Values are percent change in the parameter after addition of test sample.

		SAMPLE TIMES														
		CONTROL			0 MINUTES			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R
DOG 2	1	+103	+ 79	+13	+41	+42	- 2	+ 65	+85	-11						
	2	+ 78	+252	+29	-17	+ 2	-18	+117	+75	+24						
DOG 3	1	+ 46	+42	+ 1	+60	+59	+ 1	- 7	-17	+12						
	2	+ 53	+34	+14	+171	+149	+10	+ 5	+ 1	+ 4						
DOG 7	1	+ 26	+40	-10							-78	-54	-51			
	2	+ 1	- 4	+ 6							-48	-55	+18			
DOG 8	1	+ 1	- 2	+ 4							+31	+43	- 8	+ 5	- 5	+10
MEAN		+ 44	+63	+ 8	+64	+63	- 2	+45	+36	+ 7	-32	-22	-14	+ 5	- 5	+10
±SEM		14	33	5	39	31	6	29	26	7	32	21	20			

Table 18 summarizes the values of the short circuit current during the control (C), experimental (E), and recovery periods (R). The results in this table demonstrate that the effects of nearly all the specimens, whether inhibitory or stimulatory, were reversible.

Table 20 and Appendix V summarize the effects of lyophilized fraction IV from samples obtained during the 3% expansion studies. All plasmas were eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. Table 21 summarizes the values of the short circuit current during the control period (C), following addition of the test samples (E), and 20-30 minutes after replacement of these samples by fresh frog buffer. Inspection of these tables reveals that although there are several instances of a reversible increase in short circuit current, evidence for a reversible inhibitory activity is lacking.

Since there was no evidence for an antinatriferic substance in dog plasma following a 3% total body weight expansion, dogs numbered 9-15 were expanded with a volume of 0.154 M NaCl equivalent to 6% of their total body weight. Ten plasma samples were deproteinized with H-TCA and prepared for assay on the frog skin. Results of chemical analysis of these samples is given in Table 22. For these samples sodium averaged 111 $\pm$ 2.2 SEM mEq/liter, potassium 2.8 $\pm$ 0.1 SEM mEq/liter, and milliosmolality 229 $\pm$ 1.6 SEM mOsmol/liter. Following Sephadex fractionation, ammonium ion concentration in lyophilized fraction IV ranged from 0.00 to 0.40 mEq/liter (Table 22).

Table 23 summarizes the values of the short circuit current during the control period (C), following the addition of unfractionated

Table 20. Summary of the effects of lyophilized fraction IV from H-TCA treated plasma samples from the 3% expanded dogs on short circuit current (SCC), membrane potential (MP), and resistance (R). Values are percent change in the parameter after the addition of test sample to the frog skin preparation. Also included in the table are the group means ± SEM.

		SAMPLE TIMES														
		CONTROL			0 MINUTES			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R
DOG 2	1	+37	+26	+ 8	-17	-16	- 2	+ 4	- 8	+13						
	2	+27	+37	- 7	- 9	- 5	- 2	-14	- 5	- 8						
DOG 3	1	+12	- 8	+23	+24	- 1	+25									
	2	0	- 6	+ 8	+10	- 4	+16									
DOG 4	1	+16	- 4	+21				+11	+33	-17	-33	+15	-42			
	2	- 3	- 9	+ 6				+17	+ 6	+10	+12	+ 8	+ 3			
DOG 5	1	+27	+16	+ 9				+93	+53	+26	+26	+10	+15			
	2	+19	+17	+ 2				+14	+20	+46	+11	+ 4	- 7			
DOG 6	1	+ 4	+ 4	0							+ 5	-10	+16	- 2	- 9	+ 7
	2	+45	- 3	+49							-12	+ 4	-15	+36	- 3	+40
DOG 7	1	+ 1	- 1	+ 2							- 6	-15	+15	+26	+ 3	+22
	2	+19	+10	+12							- 6	-13	+ 8	-12	-10	- 2
DOG 8	1										+ 1	- 3	- 4			
	2										+20	0	+20			
MEAN		+17	+ 6	+11	+ 2	- 6	+ 9	+21	+16	+12	+ 2	0	1	+12	- 5	+17
+SEM		4	4	4	9	3	7	15	10	9	5	3	6	11	3	9

Table 21. Summary of the values of the short circuit current during the control period (C), following the addition of lyophilized fraction IV from H-TCA treated plasma samples from the 3% expanded dogs (E), and 20-30 minutes after replacing the test samples by fresh frog buffer (R). Values were obtained by averaging the last 3 measurements during each period.

		SHORT CIRCUIT CURRENT (μA)														
		SAMPLE TIMES														
		CONTROL			0 MINUTES			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	C	E	R	C	E	R	C	E	R	C	E	R	C	E	R
DOG 2	1	34.4	43.0	39.8	25.2	21.2	19.5	21.3	19.5	23.8						
	2	25.5	35.0	31.8	31.7	30.0	20.1	37.3	35.3	34.0						
DOG 3	1	25.0	23.0	21.9	38.5	38.3	36.5									
	2	50.3	47.2	47.6	49.7	47.7	38.7									
DOG 4	1	65.0	62.3	60.0				57.3	76.3	64.5	34.0	39.0	43.7			
	2	46.0	42.0	40.0				63.0	67.0	65.3	39.7	43.0	41.7			
DOG 5	1	65.0	75.3	59.0				43.7	66.7	64.0	47.7	52.5	51.8			
	2	44.3	51.7	41.0				47.7	57.3	53.0	62.0	64.3	51.6			
DOG 6	1	50.7	52.7	55.0							80.3	72.7	72.6	59.0	53.7	53.3
	2	36.0	34.8	28.5							51.7	53.7	45.7	20.8	20.2	16.3
DOG 7	1	14.2	14.0	13.3							18.5	15.8	16.5	46.7	48.0	50.5
	2	21.2	23.3	17.7							77.3	67.3	62.3	37.0	33.3	30.0
DOG 8	1										29.8	29.0	24.7			
	2										38.2	38.3	31.3			

Table 22. Summary of chemical analyses of plasma samples obtained from dogs prior to (CONTROL) and following a 6% total body weight volume expansion with 0.154 M NaCl. Experimental samples were obtained from 0 to 45 minutes after the completion of the initial expansion. All samples had undergone TCA^- removal by ether extraction, lyophilization, and preparation for frog skin assay.

		BEFORE FRACTIONATION			AFTER FRACTIONATION
		Na (mEq/l)	K (mEq/l)	mOsmol/l	NH_4^+ (mEq/l)
CONTROL:	Dog 9	114	2.3	232	0.04
	Dog 10	110	3.6	227	0.00
	Dog 11	110	2.9	228	0.25
	Dog 12	109	3.2	230	0.00
	Dog 13	---	---	---	0.00
	Dog 14	---	---	---	0.00
	Dog 15	---	---	---	0.00
0 MINUTES:	Dog 9	114	2.2	232	0.00
15 MINUTES:	Dog 9	113	2.7	228	0.00
	Dog 10	106	2.9	229	0.00
	Dog 11	lost	lost	lost	----
	Dog 15	---	---	---	0.00
30 MINUTES:	Dog 9	114	2.0	228	0.40
	Dog 13	---	---	---	0.00
	Dog 14	---	---	---	0.00
45 MINUTES:	Dog 9	112	2.6	226	0.00
	Dog 12	110	3.4	232	0.00

Table 23. Summary of the values of the short circuit current during the control period (C), following the addition of unfractionated H-TCA treated plasma samples from the 6% expanded dogs (E), and 20-30 minutes after replacing the test samples by fresh frog buffer (R). Values were obtained by averaging the last 3 measurements during each period. All samples from dogs 10-12 were shown to contain elevated levels of TCA^- (see text).

		SHORT CIRCUIT CURRENT (μA)														
		SAMPLE TIMES														
		CONTROL			0 MINUTES			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	C	E	R	C	E	R	C	E	R	C	E	R	C	E	R
DOG 9	1	23.7	17.3	17.0	21.5	18.3	27.3	32.0	34.8	26.5	43.7	37.0	20.3			
	2	26.2	22.0	27.7							17.2	16.3	32.0			
DOG 10	1	59.3	44.0	50.0				27.7	29.0	28.3						
	2	30.7	28.3	30.5				43.2	38.8	38.5						
DOG 11	1	30.5	30.0	27.7												
	2	47.0	47.0	48.8												
DOG 12	1	32.0	29.0	30.7										48.8	47.0	61.8
														49.7	43.2	43.0

H-TCA treated plasma samples from the 6% expanded dogs (E), and 20-30 minutes after replacing the test samples by fresh frog buffer (R). The control samples from dogs 10, 11, and 12, contained TCA^- at a concentration of 2.9, 1.2, and 1.4 mEq/liter, respectively, following ether extraction. The 45 minute sample for dog 12 contained TCA^- at a concentration of approximately 1.3 mEq/liter, and the 15 minute sample from dog 10 contained 0.82 mEq/liter. Quantitative data on the levels of TCA^- in the plasma samples from dog 9 are not available. The TCA^- concentration in each of the above samples would be expected to be somewhat less in the final sample following lyophilization. This is because there is some sample loss during the ether extraction procedure and the specimens become slightly overdiluted when made up in the frog buffer. Nonetheless, the reversible inhibition of the short circuit current seen with these samples was probably due to TCA^- contamination. Table 24 and Appendix VI summarize the effects of these samples on frog skin short circuit current, membrane potential, and resistance. As can be seen the effects were variable.

Table 25 summarizes the values of the short circuit current during the control period (C), following addition of lyophilized fraction IV from H-TCA treated 6% expansion plasma samples from dog 9 (E), and 20-30 minutes after replacing the sample with fresh frog buffer (R). Only with the 45 minute sample is there an indication of a reproducible reversible inhibition of short circuit current. Also in this table (and Appendix VII) is a summary of the effects of these samples on short circuit current, membrane potential, and resistance, and it can be seen that the 45 minute sample had a variable effect on the membrane potential.

Table 24. Summary of the effects of unfractionated H-TCA treated plasma samples from the 6% expanded dogs on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Values are percent change in the parameter after the addition of test sample. All samples from dogs 10 to 12 were shown to contain high levels of TCA^- (see text). Also included in the table are the group means ± SEM.

		SAMPLE TIMES														
		CONTROL			0 MINUTES			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R
DOG 9	1	-16	-27	+16	-10	-15	+ 5	+ 6	+ 9	- 2	-18	-15	+ 1			
	2	- 8	-16	+ 9							- 3	- 5	+ 2			
DOG 10	1	-17	-26	+12				+ 4	+ 5	- 1						
	2	-11	- 8	- 4				- 1	-10	+10						
DOG 11	1	- 4	- 2	- 6												
	2	0	0	0												
DOG 12	1	+ 2	- 9	+12										-13	- 4	-10
														- 4	-13	+10
MEAN		- 8	-13	+ 6	-10	-15	+ 5	+ 3	+ 1	+ 2	-10	-10	+ 2	- 9	- 9	0
±SEM		3	4	3				2	6	4						

Table 25. Summary of the values of the short circuit current during the control period (C), following the addition of lyophilized fraction IV from the H-TCA treated plasma samples from dog 9 (E), and 20-30 minutes after replacing the test samples with fresh frog buffer (R). Values were obtained by averaging the last 3 measurements during each period. Also in the table is a summary of the effects of lyophilized fraction IV from these samples on frog skin short circuit current (SCC), membrane potential (MP), and resistance (RES). Values are percent change in the parameter after the addition of test sample to the frog skin preparation.

		DOG 9								
		SCC (μA)			PERCENT CHANGE IN PARAMETER					
SAMPLE	SKIN	C	E	R	MP	MEAN	SCC	MEAN	RES.	MEAN
CONTROL	1	35.0	39.8	38.0	+23	+48	+14	+40	+ 8	+ 7
	2	48.7	80.7	73.0	+72		+66		+ 5	
0 MINUTES	1	48.7	39.0	38.7	- 3	- 7	-20	-20	+21	+16
	2	28.3	23.0	22.7	-11		-19		+10	
15 MINUTES	1	43.2	59.3	49.6	+105	+71	+37	+26	+48	+36
	2	59.3	68.0	60.0	+38		+15		+24	
30 MINUTES	1	46.3	55.3	60.0	+16	+15	+19	+16	- 3	- 1
	2	68.0	76.7	62.7	+14		+13		+ 1	
45 MINUTES	1	42.7	34.7	39.3	- 4	+ 1	-19	-13	+17	+16
	2	27.7	25.8	26.3	+ 6		- 7		+14	

H-TCA treated plasma samples from dogs 10, 11, and 12, were not fractionated

Table 26 summarizes the values of the short circuit current during the control period (C), following the addition of lyophilized fraction IV from non H-TCA treated plasma samples from dogs 10-15 (E), and 20-30 minutes after replacing the test samples by fresh frog buffer (R). Table 27 and Appendix VIII summarize the effects of these samples on frog skin short circuit current, membrane potential, and resistance. Inspection of these tables again reveals no consistent evidence for a reversible inhibition of short circuit current which is accompanied by a reproducible decrease in membrane potential and increase in resistance.

Table 26. Summary of the values of the short circuit current during the control period (C), following the addition of lyophilized fraction IV from dogs 10-15 (non H-TCA treated plasmas) (E), and 20-30 minutes after replacing the test samples by fresh frog buffer (R). Values were obtained by averaging the last 3 measurements during each period.

		SHORT CIRCUIT CURRENT (μA)											
		SAMPLE TIMES											
		CONTROL			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	C	E	R	C	E	R	C	E	R	C	E	R
DOG 10	1	36.3	57.3	52.7	29.7	27.8	22.0						
	2	30.7	59.3	55.0	80.7	77.0	69.5						
DOG 11	1	45.7	60.3	50.7	61.0	113	99.2						
	2	38.3	39.7	23.0	22.3	35.3	31.0						
DOG 12	1	72.0	72.8	67.0							54.3	77.0	66.3
	2	34.7	42.7	31.5							27.8	67.3	36.0
DOG 13	1	38.5	42.7	48.8				39.7	55.3	54.3			
	2	64.0	56.2	48.0				50.0	46.0	49.0			
DOG 14	1	52.0	50.0	48.0				52.7	66.8	69.0			
	2	27.0	26.3	23.7				49.0	49.3	43.0			
DOG 15	1	30.7	30.5	29.3	27.0	19.0	15.3						
	2	59.3	57.1	54.2	103.0	88.3	83.7						

Table 27. Summary of the effects of lyophilized fraction IV from dogs 10-15 (non H-TCA treated plasmas) on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Values are percent change in the parameter after the addition of test sample. Also included in the table are the group means ± SEM.

		SAMPLE TIMES											
		CONTROL			15 MINUTES			30 MINUTES			45 MINUTES		
	SKIN	MP	SCC	R	MP	SCC	R	MP	SCC	R	MP	SCC	R
DOG 10	1	+150	+58	- 5	-14	- 6	- 9						
	2	+52	+93	-22	0	- 5	+ 1						
DOG 11	1	+33	+32	+ 2	+76	+85	- 4						
	2	+ 3	+ 4	0	+36	+58	-13						
DOG 12	1	+ 3	+ 1	+ 2							+13	+42	-20
	2	+14	+23	- 8							+30	+140	-47
DOG 13	1	+24	+11	+12				+ 3	+39	-26			
	2	- 6	-12	+ 7				- 2	- 3	+ 6			
DOG 14	1	- 6	- 4	- 2				+16	+18	- 9			
	2	+14	- 3	-17				0	1	- 1			
DOG 15	1	+16	- 1	+16	+11	-30	+58						
	2	+ 1	- 4	+ 4	+10	-14	+29						
MEAN		+25	+17	- 1	+20	+15	+10	+ 4	+14	- 8	+22	+91	-34
±SEM		12	9	3	13	19	11	4	10	7			

DISCUSSION

It has been reported that an antinatriferic-natriuretic substance, which appears to be similar to that found in experimental acute and chronic volume expansion, occurs in the serum of patients with end-stage renal disease (19,20,21). This observation, plus the finding that an antinatriferic substance is dialyzable *in vivo* from acutely volume expanded dogs (36), prompted an investigation for this substance in spent hemodialysis fluid and plasma ultrafiltrates from patients with chronic renal failure undergoing maintenance hemodialysis. Studies were also conducted to measure the antinatriferic activity of plasma, with and without deproteinization with trichloroacetic acid, obtained from these patients and saline expanded dogs.

The membranes currently employed in artificial kidney hemodialyzers have been shown to have solute clearances of approximately 30 ml/min for substances with molecular weights of 500 and approximately 10 ml/min for substances with molecular weights of 3000 (77) (and personal communication with Cordis Dow Corp., Walnut Creek, Calif.). In addition, approximately 3 to 13 ml/min of plasma ultrafiltrate appear in the dialysis fluid depending on the transmembrane pressure gradient developed between the blood and dialysis fluid line (Gambro, Inc., Wheeling, Ill.). This plasma ultrafiltrate would increase substantially the yield of any antinatriferic substance which might be dialyzable.

In the present studies the yield of plasma ultrafiltrate was from 150 to 400 ml during a 30 minute collection period.

It seems, therefore, that if a small molecular weight antinatriferic material is present in uremic serum it should appear in the dialysis fluid and/or plasma ultrafiltrate in vivo. However, evidence that this substance is dialyzable from humans can only be inferred from the work of others. Bourgoignie et al. (20) observed, in non-dialyzed patients, that a fraction obtained following elution of serum from Sephadex G25F resin produced a 24.9% (N=18) inhibition of sodium transport across frog skins. The same fraction from dialyzed patients produced a significantly smaller inhibition of 16.2% (N=13), and that from normal subjects 5.3% (N=11).

In the studies of this thesis a 2.5 X 95 cm column, packed with Sephadex G25F resin, was employed to fractionate the uremic specimens and samples obtained in the expansion studies performed with dogs. This column was similar to that employed by Bourgoignie et al. (20). Comparison of elution patterns at 280 mμ suggests that the column used by Bourgoignie was similar to the one used in the present study. Further evidence for the similarity of these systems was obtained with electrolyte determinations in the individual 10 ml fraction collector samples. Electrolytes in the present studies were found to be eluted in a volume similar to that in which Bourgoignie et al., above, noted a high specific conductance. Therefore, a similar elution volume (fraction IV--approximately 550-780 ml) would be expected to contain any antinatriferic material which might be present in the specimens of this investigation.

To be noted in the elution patterns of plasma ultrafiltrate and dialysis fluid (Figures 21, 22, and 23) is the absence of a protein absorbance at 280 mμ in the region of approximately 150 to 300 ml. As proteins are not dialyzable or ultrafilterable through the artificial kidney hemodialyzer membranes, this is to be expected. However, in these fluids there is an absorbance maximum at approximately 400 and 850 ml which is seen with elution of plasma. Both of these regions show an increased absorbance following concentration. These observations are important because they indicate that 1) substances with molecular weights larger than the postulated antinatriferic substance are being dialyzed and ultrafiltered from the patient's blood (absorbance peak at 400 ml), and 2) that it may be being retained and concentrated by the ultrafiltration procedure (greater absorbance at 850 ml than is seen with unconcentrated plasma).

The absence of measurable amounts of sodium, potassium, chloride, osmotically active substances, and no unusual pH's beyond an elution volume of 500 ml, indicates an efficient desalting by the resin column of fraction IV (Figures 23, 24, and 25). Elution of substances with known molecular weights from the column suggested that materials with elution volumes from 300 to 850 ml might be expected to have molecular weights from approximately 1400 to 200 Daltons (Figure 26), a range consistent with that reported by others for natriuretic and antinatriferic substances (20,27,28,29,36,37,56). This conclusion must be viewed with caution, however, since as pointed out in RESULTS--Section A, some substances may be abnormally retained by or excluded from the

resin particles during elution. Glucose appeared to be an example of such a substance in these studies since it was eluted from the resin much earlier than would be predicted on the basis of its molecular weight (Figure 25).

In addition to decreasing the short circuit current, fraction IV from uremic serum was also reported to decrease the membrane potential and increase the resistance in frog skin preparations (20). These effects were reversible when the test sample was removed and replaced with fresh frog buffer. Similar findings were reported by Buckalew et al. (36) using plasma dialysates and ultrafiltrates from volume expanded dogs. In the present studies there were no uremic samples, either plasma with or without H-TCA deproteinization, dialysis fluid, or plasma ultrafiltrates, that reproducibly demonstrated these effects that could not be accounted for by ammonium ion contamination (Tables 13 and 14).

The dialysis fluid sample from patient CZ produced a 13% decrease in short circuit current which was reversible, a second determination producing a 1% decrease which was not reversible. Plasma ultrafiltrate from patient CO, which had been concentrated 18-fold, produced a 10% reversible decrease in one frog skin run but a non-reversible 9% decrease in a second determination. Dialysis fluid from patient ML inhibited the short circuit current 7% reversibly in one determination, but had no effect in a second determination. The effect of these specimens on membrane potential and resistance were variable. H-TCA treated plasma from patient ML was without effect. Although non

H-TCA treated plasma from patient DH reversibly inhibited the SCC 7% and 13%, the membrane potential decreased 5% and increased 15%, respectively. The plasma ultrafiltrate from this patient, which had been concentrated 15-fold, inhibited the SCC an average of 6% non-reversibly.

The only examples of a marked reversible inhibition of both the SCC and MP, which were accompanied by an increase in resistance, occurred with plasma ultrafiltrates from patients JSe and GS. However, the ammonium ion concentrations in these samples were 4.40 and 1.10 mEq/liter, respectively. This level of ammonium ion is sufficient to account for all of the inhibition seen (refer to Figure 32--RESULTS).

In the experiments of this study, in which dogs underwent an acute volume expansion with 0.154 M sodium chloride equivalent to 3% of their total body weight, all plasma samples were quickly deproteinized with H-TCA to eliminate possible degradation of antinatriferic activity. Blood samples were obtained prior to the expansion and 0, 15, 30, and 45 minutes after completion of the expansion. Before being fractionated on Sephadex G25F resin, only 2 samples (Table 19) demonstrated an inhibitory effect of frog skin short circuit current and membrane potential. One determination with H-TCA deproteinized plasma taken 15 minutes after expansion from dog 3 decreased the SCC 17%, the membrane potential 7%, and increased the resistance 12%. As can be seen in Table 18 this effect was partially reversible upon sample removal. A second determination with this sample on another skin was without effect. Plasma sampled from dog 7, 30 minutes after expansion, reversibly inhibited the SCC and MP but caused both an increase and a decrease

in resistance in two separate runs (Table 19).

Following fractionation of the 3% expansion samples on Sephadex G25F resin, several exhibited inhibitory effects on the short circuit current (Table 20). However, the effects on membrane potential and resistance were variable. Only one determination with a plasma sample from dog 2, taken 15 minutes following expansion, exhibited a reversible (Table 21) inhibition of SCC of 8% (Table 20). Since most samples did not inhibit the SCC prior to Sephadex fractionation, it is concluded that the levels of TCA^- obtained following ether extraction were low enough not to interfere with the determinations. It is also concluded that there was very little, if any, antinatriferic material recovered as a result of the expansion followed by H-TCA deproteinization of the plasma at any of the sample times. This is in contrast to the findings of Cort (47) and Cort et al. (49,58) with H-TCA deproteinized plasma from carotid artery occluded cats. Although the inhibition of SCC seen by these authors following occlusion could have been the result of TCA^- contamination, it is difficult to explain why other samples treated similarly were not inhibitory.

In addition to the 3% total body weight expansion experiments, several animals were expanded with a volume of 0.154 M saline equivalent to 6% of the total body weight. Blood samples were obtained prior to the expansion and 0, 15, 30, and 45 minutes after the expansion. There were several examples of inhibition of SCC with the samples before fractionation on Sephadex resin, including inhibition with control samples (Table 24). However, the effects on membrane potential and

resistance were variable. Only with the control samples from dogs 9, 10, and 12, and the 45 minute sample from dog 12, are found examples of reversibility of the effect (Table 23). Since H-TCA was found to be present in the samples from dogs 10 and 12 (0.82 to 2.90 mEq/liter), inhibition due to H-TCA cannot be discounted. Quantitative determinations of H-TCA could not be made for the samples from dog 9.

Following fractionation of the samples from dog 9 on Sephadex resin, inhibitory activity was found with fraction IV plasma samples obtained immediately following (0 minutes) and 45 minutes after completion of the expansion (Table 25). Only with the 45 minute sample was the inhibition reversible (Table 25). With both determinations there was an increase in resistance, but the effect of the specimen on MP was variable.

In addition to the samples from dogs 10, 11, and 12, which were deproteinized with H-TCA, samples were also obtained which were not treated with H-TCA but were deproteinized by fractionation on Sephadex G25F resin. Although there were several samples which resulted in a decrease in frog skin short circuit current (Table 27), none were reversible (Table 26). The effects on the membrane potential and resistance were variable. Similar samples from dogs 13, 14, and 15, including the control samples, also exhibited inhibitory activity on the SCC (Table 27). However, in only one instance (30 minute sample from dog 13) was the effect a reversible one (Table 26). A second determination with this sample produced an increase in the SCC. Effects on MP and R were variable.

These studies indicate that hemodialyzed uremic patients, who exhibit a fluid retention which is less than 5% of their dry body weight, possess no reproducible evidence of an antinatriferic activity in their plasma. Plasma ultrafiltrates which were concentrated from 8- to 27-fold, and dialysis fluids concentrated 3600-fold, also did not demonstrate an antinatriferic activity previously described by others. Dogs which were acutely volume expanded with saline, equivalent to 3% and 6% of their total body weight, also exhibited no antinatriferic activity which was similar to that reported by others. If a natriuretic hormone exists which is also antinatriferic, it is concluded that such a substance was not present in the specimens of this investigation.

The lack of antinatriferic activity in the uremic plasma samples might have been due to the long delay between sample collection and fractionation. Cort (47) and Cort et al. (49,58) report that an antinatriferic substance from carotid artery occluded cats is destroyed by a 30 minute incubation in the presence of plasma protein at 37°C. A similar incubation at 0°C resulted in no loss of active material (as measured by its effect on frog skin SCC). Although the material from cats is stable at 0°C for at least 30 minutes in nondeproteinized plasma, the human material in the present studies may not have been stable in an ice bath for the 2 to 4 hours which elapsed between collection and fractionation. However, plasmas from JSe, CdeB, and DH were frozen in a dry ice-acetone bath immediately after removal of the red blood cells by centrifugation. Only fraction IV from the plasma

from DH resulted in a reproducible reduction in short circuit current, but a variable effect on membrane potential was obtained. Also, none of the plasmas deproteinized with H-TCA was inhibitory.

A lack of antinatriferic activity in the dialysis fluid and plasma ultrafiltrate samples could indicate that the substance is not dialyzable from humans in vivo. Bourgoignie et al. (20), for example, observed that when whole uremic serum was ultrafiltered through a membrane with a molecular weight rejection of 50,000 Daltons, no antinatriferic activity was present in the ultrafiltrate. On the other hand, ultrafiltration of an active fraction (fraction IV) from Sephadex G25F resin through the same membrane did result in antinatriferic activity in the ultrafiltrate. It was suggested that perhaps the active material was released from a larger molecule (i.e., bound to a plasma protein) on passage through the resin.

Nondeproteinized uremic plasma samples, as discussed previously, have been shown to retain an antinatriferic activity. Also, plasma samples from carotid artery occluded cats have been shown to possess an antinatriferic activity following H-TCA deproteinization. Uremic plasma samples in the studies reported here, which were similarly treated, exhibited no such activity. This observation further substantiates the conclusion that the patients studied did not possess an antinatriferic material in their plasma, which would also account for the lack of this activity in the concentrated dialysis fluid and plasma ultrafiltrate specimens.

One possible explanation for the lack of antinatriferic activity in the volume expansion experiments may be that none of the animals

studied were prehydrated, pretreated with mineralocorticoids, or given excess salt in their diet prior to the studies. These maneuvers are frequently done in experiments of this type (9,32,65,71,91,94,104,131, 147,148,165). As a result there may not have been an adequate expansion of the extracellular fluid volume with isotonic saline to require the release of an antinatriferic substance. Other factors previously discussed (REVIEW OF THE LITERATURE--Section B) may have been sufficient to eliminate the imposed saline load.

Higgins (83), for example, observed that dogs which were in a state of positive sodium balance, as a result of DOCA administration and a high salt diet, showed a more rapid rate of sodium excretion during saline loading than did dogs that were salt depleted. It was postulated that the interstitial fluid volume may play an important role in the control of sodium excretion. In salt depleted dogs, with a plasma volume significantly below normal, influsion of 6% dextran in 5% glucose expanded the plasma volume from 3.5% to 5.4% but failed to increase the rate of sodium excretion (84). Even in dogs on a normal salt diet, infusion of up to 600 ml of albumin or dextran caused only moderate increases (and also decreases) in sodium excretion.

In support of Higgens' observations, Schrier et al. (147) observed that the stimulus to natriuresis in the dog seemed to be an increase in the total extracellular fluid volume, including the interstitial space, rather than an increase in just the intravascular volume. Also, Sonnenberg and Pearce (159), investigating the natriuretic response to measured blood volume expansion in differently hydrated dogs,

observed that there was a significantly greater response in animals prehydrated with saline than was true in normally hydrated or dehydrated animals. These authors suggested that the renal regulation of the extracellular fluid volume in the dog in response to intravascular expansion is determined by the existing extravascular volume.

SUMMARY AND CONCLUSIONS

The possibility of using spent hemodialysis fluid and plasma ultrafiltrates from uremic humans undergoing maintenance hemodialysis as a source of antinatriferic activity was investigated. Uremic plasma, from the patients studied, either with or without H-TCA deproteinization, demonstrated very little if any reversible antinatriferic activity in fraction IV following Sephadex fractionation. Fraction IV from spent hemodialysis fluid and plasma ultrafiltrate concentrates also demonstrated no reversible antinatriferic activity which could not be attributed to ammonium ion.

It is concluded from these studies that patients who have a fluid retention which is less than 5% of their dry body weight, who are being maintained by chronic hemodialysis, do not possess measurable quantities of a previously described antinatriferic material. It is also concluded that the spent hemodialysis fluid and plasma ultrafiltrates from these patients do not contain measurable amounts of this substance as determined by the methods used in this investigation.

Plasma samples from acutely volume expanded dogs, with or without H-TCA deproteinization, demonstrated no reversible antinatriferic activity in fraction IV following Sephadex fractionation. Plasma samples from these dogs which had been deproteinized with H-TCA, but had not been fractionated on Sephadex resin, demonstrated no reversible

antinatriferic activity that could not be attributed to TCA^- anion contamination. From these results it is concluded that the natriuresis seen in situations of acute expansion of the extracellular fluid volume with isotonic saline cannot be attributed to an antinatriferic substance in the plasma. If a natriuretic hormone possesses antinatriferic activity, as previously described by others, it is also concluded that there was no natriuretic hormone present in any of the uremic or dog specimens of this study.

APPENDICES

APPENDIX I

Effects of lyophilized uremic fraction IV plasma samples, with and without H-TCA deproteinization, on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Samples were obtained from patients undergoing maintenance hemodialysis and were eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. Duplicate determinations on separate skins were made with each sample. The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 8 measurements taken after addition of the sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. The resistance was calculated as shown in the table.

		PLASMA (NO H-TCA)						PLASMA (DEPROTEINIZED WITH H-TCA)					
		MP (mV)		SCC (μA)		*R (ohms)		MP (mV)		SCC (μA)		*R (ohms)	
PATIENT	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
CH	1	67.0	65.0 (-4%)	61.3	60.0 (-2%)	1100	1080 (-2%)						
	2	100	95.0 (-5%)	78.0	80.7 (+4%)	780	835 (+7%)						
HM	1	4.0	4.0 (0%)	24.0	22.2 (-8%)	167	182 (+9%)						
	2	21.0	20.0 (-5%)	20.0	18.0 (-10%)	1050	1110 (+6%)						
CO	1	27.7	30.0 (+9%)	51.0	50.3 (-1%)	542	603 (+11%)						
	2	8.8	9.5 (+8%)	21.0	18.8 (-11%)	421	505 (+20%)						
ML	1							61.0	58.2 (-5%)	48.0	48.0 (0%)	1270	1220 (-4%)
	2							48.0	44.5 (-7%)	25.3	26.0 (+3%)	1900	1710 (-10%)
JS	1	32.0	41.0 (+28%)	34.0	49.0 (+44%)	941	838 (-11%)	48.3	58.3 (+21%)	29.3	45.0 (+54%)	1650	1300 (-21%)
	2	17.0	18.0 (+6%)	15.3	16.0 (+5%)	1120	914 (-19%)	42.5	53.3 (+25%)	53.2	89.0 (+67%)	800	600 (-25%)
FP	1	38.0	43.0 (+13%)	23.8	23.8 (0%)	1590	1810 (+14%)	40.0	43.3 (+15%)	20.7	23.0 (+11%)	1940	1880 (-3%)
	2	27.7	27.3 (-1%)	37.7	37.0 (-2%)	735	739 (+1%)	18.5	19.3 (-4%)	38.5	38.3 (-1%)	481	504 (+5%)

$$*R\ (\text{ohms}) = \frac{MP\ (10^{-3}\ \text{volts})}{SCC\ (10^{-6}\ \text{amps})}$$

$$= \frac{MP}{SCC}\ (10^{3})$$

continued

APPENDIX I--continued

		PLASMA (NO H-TCA)						PLASMA (DEPROTEINIZED WITH H-TCA)					
		MP (mV)		SCC (μA)		*R (ohms)		MP (mV)		SCC (μA)		*R (ohms)	
PATIENT	SKIN	C	E	C	E	C	E	C	E	C	E	E	E
JSe	1							33.3	40.0 +20%	47.5	65.0 +37%	703	616 -12%
	2							22.7	29.3 +29%	20.0	27.0 +35%	1130	1090 -4%
GS	1	31.7	32.0 +1%	57.8	58.3 +1%	548	549 0%						
	2	53.5	51.7 -3%	33.7	36.5 +8%	1580	1400 -12%						
JS2	1	10.0	12.7 +27%	29.5	33.3 +13%	339	380 +12%						
	2	30.7	34.5 +12%	88.3	92.0 +4%	347	375 +8%						
CdeB	1	16.0	17.3 +8%	39.0	42.7 +10%	410	406 -1%						
	2	18.3	24.3 +33%	27.8	32.8 +18%	659	741 +12%						
DH	1	38.0	36.0 -5%	52.0	48.3 -7%	731	744 +2%						
	2	15.0	17.3 +15%	27.7	24.0 -13%	542	727 +33%						

APPENDIX II

Effects of lyophilized uremic fraction IV from dialysis fluid concentrates on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Samples were obtained from patients undergoing maintenance hemodialysis and were eluted from Sephadex G25F resin with 10 mMolar ammonium acetate after being concentrated by ultrafiltration at 4°C. Duplicate determinations on separate skins were made with each sample. The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 3 measurements taken after addition of the sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. The resistance was calculated as shown in Appendix I.

		DIALYSIS FLUID (CONCENTRATED)					
		MP (mV)		SCC (μA)		R (ohms)	
PATIENT	SKIN	C	E	C	E	C	E
CZ	1	37.0	33.0	31.0	27.0	1190	1220
			-11%		-13%		-2%
	2	49.0	48.0	54.0	53.3	907	906
			-2%		-1%		0%
JK	1	26.0	27.7	50.5	50.5	515	548
			+7%		0%		+6%
	2	59.0	63.5	100	106	590	600
			+8%		+6%		+2%
ML	1	43.0	44.3	38.5	35.7	1120	1240
			+3%		-7%		+11%
	2	20.5	20.0	41.0	41.0	500	488
			-2%		0%		-2%
GS	1	67.5	77.1	67.3	69.0	1000	1030
			+5%		+3%		+3%
	2	56.2	56.2	35.0	35.5	1600	1580
			0%		+1%		-1%
JS2	1	74.2	72.5	98.6	87.0	752	833
			-2%		-12%		+11%
	2	24.5	18.5	62.0	54.2	395	341
			-25%		-13%		-14%

APPENDIX III

Effects of lyophilized uremic fraction IV from plasma ultrafiltrates, obtained from the artificial kidney, before and after concentration by ultrafiltration at 4°C on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R). Samples were obtained from patients undergoing maintenance hemodialysis and were eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. Duplicate determinations on separate skins were made with each sample (except patient CH before concentration and patient HM after concentration). The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 3 measurements taken after addition of the sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. The resistance was calculated as shown in Appendix I. *Sample contained high ammonium ion concentration (see RESULTS--Table XV).

		ULTRAFILTRATE (BEFORE CONCENTRATION)						ULTRAFILTRATE (AFTER CONCENTRATION)					
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
PATIENT	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
CH	1	38.3	38.0	31.7	30.0	1210	1270	55.0	55.0	69.0	70.5	797	785
		-1%		-5%		+5%		0%		+2%		-2%	
	2							65.0	60.0	58.0	48.7	1120	1230
								-8%		-16%		+10%	
HM	1	4.0	5.2	22.0	24.0	182	215	5.0	6.7	23.7	23.3	211	286
		+29%		+9%		+18%		+34%		-2%		+35%	
	2	7.0	7.0	29.7	30.7	236	229						
		0%		+3%		-3%							

CO	1	14.2 | 14.7 +4%	19.5 | 19.4 -1%	727 | 755 +4%	17.5 | 15.0 -14%	32.3 | 29.7 -9%	541 | 506 -7%
	2	26.0 | 26.0 0%	40.0 | 39.7 -1%	650 | 656 +1%	17.0 | 17.0 0%	57.5 | 51.5 -10%	296 | 331 +12%
*JSe	1				26.7 | 15.4 -42%	33.3 | 9.2 -72%	800 1690 +111%
	2				45.0 | 17.9 -60%	32.2 | 12.5 -61%	1400 1440 +3%
*GS	1				108 | 101 -6%	149 | 93.3 -37%	724 1090 +50%
	2				60.0 | 52.8 -12%	34.0 | 31.2 -8%	567 590 +4%
JS2	1				73.3 | 70.4 -4%	110 | 96.3 -12%	667 | 731 +10%
	2				28.2 | 26.2 -7%	69.0 | 66.3 -4%	408 | 395 -3%
CdeB	1				17.3 | 24.0 +39%	23.2 | 36.7 +58%	748 | 655 +12%
	2				26.2 | 31.3 +20%	57.0 | 66.0 +16%	459 | 475 +4%
DH	1				24.5 | 27.5 +12%	52.0 | 48.0 -8%	471 | 573 +22%
	2				26.7 | 27.0 +1%	22.0 | 21.3 -3%	1210 1270 +4%

APPENDIX IV

Effects of H-TCA deproteinized dog plasma samples from four of the 3% total body weight expansion experiments on frog skin short circuit current (SCC), membrane potential (MP), and resistance (R) before fractionation on Sephadex G25F resin. Duplicate determinations on separate skins were made with each sample (in most cases). The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 3 measurements taken after addition of the sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. The resistance was calculated as shown in Appendix I.

		EXPERIMENTAL SAMPLE TIMES									
		CONTROL						0 MINUTES			
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
DOG 2	1	16.0	32.9	18.0	32.3	898	1010	26.8	37.5	38.3	54.5	700	689
			+103%		+79%		+13%		+41%		+42%		-2%
	2	7.3	12.0	8.0	28.2	917	1180	13.3	11.0	19.2	19.5	689	562
			+78%		+252%		+29%		-17%		+2%		-18%
DOG 3	1	12.0	17.5	19.0	27.0	632	638	10.0	16.0	15.3	24.3	653	656
			+46%		+42%		+1%		+60%		+59%		+1%
	2	19.3	29.5	11.3	15.2	1700	1940	14.7	39.8	8.3	20.7	1760	1930
			+53%		+34%		+14%		+171%		+149%		+10%
DOG 7	1	28.3	35.7	32.7	45.7	867	782						
			+26%		+40%		-10%						
	2	27.0	27.3	30.3	29.0	890	943						
			+1%		-4%		+6%						
DOG 8	1	35.5	36.0	46.0	45.0	772	800						
			+1%		-2%		+4%						

		15 MINUTES						30 MINUTES					
DOG 2	1	24.3	40.0	25.3	46.8	961	856						
			+65%		+85%		-11%						
	2	7.5	16.3	6.3	11.0	1190	1480						
			+117%		+75%		+24%						
DOG 3	1	9.7	9.0	16.8	14.0	574	643						
			-7%		-17%		+12%						
	2	10.8	11.3	23.8	24.0	454	472						
			+5%		+1%		+4%						
DOG 7	1							27.7	6.2	34.7	15.8	798	393
									-78%		-54%		-51%
	2							42.5	22.3	29.8	13.3	1420	1680
									-48%		-55%		+18%
DOG 8	1							28.8	37.7	37.3	53.3	772	707
									+31%		+43%		-8%
	2												
								45 MINUTES					
DOG 8	1							28.7	30.0	46.5	44.0	617	682
									+5%		-5%		+10%

APPENDIX V

Effects of lyophilized fraction IV from H-TCA deproteinized dog plasma samples obtained in the 3% total body weight expansion experiments on frog skin membrane potential (MP), short circuit current (SCC), and resistance (R). All samples had been eluted from Sephadex G25F resin with 10 mMolar ammonium acetate. Duplicate determinations on separate skins were made with each sample. The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 3 measurements taken after addition of the sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. Resistance was calculated as shown in Appendix I.

		EXPERIMENTAL SAMPLE TIMES											
		CONTROL						0 MINUTES					
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
DOG 2	1	12.3	16.8	34.4	43.0	363	391	23.0	19.0	25.2	21.2	914	898
		+37%		+26%		+8%		-17%		-16%		-2%	
	2	39.3	50.0	25.5	35.0	1590	1430	34.3	31.2	31.7	30.0	1080	1060
		+27%		+37%		-7%		-9%		-5%		-2%	
DOG 3	1	10.0	11.2	25.0	23.0	400	493	27.3	34.0	38.5	38.3	710	887
		+12%		-8%		+23%		+24%		-1%		+25%	
	2	20.0	20.0	50.3	47.2	397	427	10.0	11.0	49.7	47.7	202	231
		0%		-6%		+8%		+10%		-4%		+16%	
DOG 4	1	19.0	22.0	65.0	62.3	292	353						
		+16%		-4%		+21%							
	2	31.0	30.0	46.0	42.0	674	714						
		-3%		-9%		+6%							
DOG 5	1	29.7	37.7	65.0	75.3	457	500						
		+27%		+16%		+9%							
	2	41.2	49.2	44.3	51.7	931	952						
		+19%		+17%		+2%							
DOG 6	1	62.5	65.0	50.7	52.7	1230	1230						
		+4%		+4%		0%							
	2	23.5	34.0	36.0	34.8	653	976						
		+45%		-3%		+49%							
DOG 7	1	23.0	23.2	14.2	14.0	1620	1650						
		+1%		-1%		+2%							
	2	30.3	37.3	21.2	23.3	1430	1600						
		+19%		+10%		+12%							

continued

APPENDIX V--continued

		EXPERIMENTAL SAMPLE TIMES											
		15 MINUTES						30 MINUTES					
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
DOG 2	1	9.8	10.2	21.3	19.5	461	521						
			+4%		-8%		+13%						
	2	16.0	13.8	37.3	35.3	428	392						
			-14%		-5%		-8%						
DOG 4	1	21.3	23.7	57.3	76.3	372	310	6.0	4.0	34.0	39.0	176	102
			+11%		+33%		-17%		-33%		+15%		-42%
	2	30.0	35.0	63.0	67.0	476	522	42.5	47.5	39.7	43.0	1070	1100
			+17%		+6%		+10%		+12%		+8%		+3%
DOG 5	1	4.8	9.3	43.7	66.7	111	140	5.8	7.3	47.7	52.5	122	140
			+93%		+53%		+26%		+26%		+10%		+15%
	2	40.0	45.0	47.7	57.3	839	800	31.1	34.5	62.0	64.3	618	574
			+14%		+20%		+46%		+11%		+4%		-7%
DOG 6	1							32.0	33.5	80.3	72.7	398	461
									+5%		-10%		+16%
	2							11.3	10.0	51.7	53.7	2190	1860
									-12%		+4%		-15%
DOG 7	1							8.0	7.5	18.5	15.8	432	496
									-6%		-15%		+15%
	2							40.0	37.7	77.3	67.3	517	559
									-6%		-13%		+8%
DOG 8	1							35.0	35.5	29.8	29.0	1170	1220
									+1%		-3%		-4%
	2							17.7	21.3	38.2	38.3	463	557
									+20%		0%		+20%
		45 MINUTES											
DOG 6	1	55.0	53.8	59.0	53.7	932	000						
			-2%		-9%		+7%						
	2	7.0	9.5	20.8	20.2	336	471						
			+36%		-3%		+40%						
DOG 7	1	23.0	29.0	46.7	48.0	493	604						
			+26%		+3%		+22%						
	2	23.0	20.3	37.0	33.3	622	610						
			-12%		-10%		-2%						

APPENDIX VI

Effects of H-TCA deproteinized dog plasma samples from the 6% total body weight expansion experiments on frog skin membrane potential (MP), short circuit current (SCC), and resistance (R) before fractionation on Sephadex G25F resin. Where duplicate determinations were made, different skins were used. The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 3 measurements taken after addition of sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. The resistance was calculated as shown in Appendix I. *Samples were shown to contain high levels of TCA^-. Quantitative determinations on the level of TCA^- in samples from dog 9 are not available.

		EXPERIMENTAL SAMPLE TIMES									
		CONTROL						0 MINUTES			
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
DOG 9	1	7.5	6.3	23.7	17.3	316	366	24.5	22.0	21.5	18.3	1140	1200
		-16%		-27%		+16%		-10%		-15%		+5%	
	2	14.2	13.0	26.2	22.0	541	591						
		-8%		-16%		+9%							
*DOG 10	1	42.0	35.0	59.3	44.0	708	796						
		-17%		-26%		+12%							
	2	10.3	9.2	30.7	28.3	336	324						
		-11%		-8%		-4%							
*DOG 11	1	11.0	10.2	30.5	30.0	361	339						
		-4%		-2%		-6%							
	2	43.0	43.0	47.0	47.0	915	915						
		0%		0%		0%							
*DOG 12	1	8.8	9.0	32.0	29.0	276	310	42.3	36.7	48.8	47.0	867	780
		+2%		-9%		+12%		-13%		-4%		-10%	
	2							38.3	36.7	49.7	43.2	772	849
								-4%		-13%		+10%	

45 MINUTES

continued

APPENDIX VI--continued

		EXPERIMENTAL SAMPLE TIMES											
		15 MINUTES						30 MINUTES					
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
DOG 9	1	19.0	20.2	32.0	34.8	594	579	18.0	15.3	43.7	37.0	412	414
		+6%		+9%		-2%		-18%		-15%		+1%	
	2							16.7	16.2	17.2	16.3	971	990
								-3%		-5%		+2%	
*DOG 10	1	9.7	10.0	27.7	29.0	349	345						
		+3%		+5%		-1%							
	2	37.5	37.0	43.2	38.8	869	953						
		-1%		-10%		+10%							

APPENDIX VII

Effects of lyophilized fraction IV from H-TCA deproteinized plasma samples from dog 9 (6% total body weight expansion) following fractionation on Sephadex G25F resin. Duplicate determinations were made using different skins. The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 3 measurements taken after addition of sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. The resistance was calculated as shown in Appendix I. H-TCA deproteinized plasma samples from dogs 10-12 were not fractionated.

		MP (mV)		SCC (μA)		R (ohms)	
DOG 9	SKIN	C	E	C	E	C	E
CONTROL	1	22.0	27.0	35.0	39.8	628	678
		+23%		+14%		+8%	
	2	6.5	11.2	48.7	80.7	133	140
		+72%		+66%		+5%	
0 MINUTES	1	25.7	25.0	48.7	39.0	528	641
		-3%		-20%		+21%	
	2	47.5	42.2	28.3	23.0	1670	1840
		-11%		-19%		+10%	
15 MINUTES	1	7.8	16.0	43.2	59.3	182	269
		+105%		+37%		+48%	
	2	21.0	29.0	59.3	68.0	345	426
		+38%		+15%		+24%	
30 MINUTES	1	19.5	22.7	46.3	55.3	421	410
		+16%		+19%		-3%	
	2	8.8	10.0	68.0	76.7	130	131
		+14%		+13%		+1%	
45 MINUTES	1	7.3	7.0	42.7	34.7	171	202
		-4%		-19%		+17%	
	2	13.2	14.0	27.7	25.8	476	542
		+6%		-7%		+14%	

APPENDIX VIII

Effects of lyophilized fraction IV from non H-TCA deproteinized dog plasma samples from the 6% total body weight expansion experiments on frog skin membrane potential (MP), short circuit current (SCC), and resistance (R). Duplicate determinations were made using different skins. The average of the last 3 measurements obtained during the control period (C) is followed by the average of the last 3 measurements taken after addition of sample to the membrane (E). Immediately below these figures is the percent change from control obtained during the experimental period. Frog skins were continuously short circuited, with SCC and MP being recorded at 5 minute intervals throughout the control and experimental periods. Resistance was calculated as shown in Appendix I.

		EXPERIMENTAL SAMPLE TIMES											
		CONTROL						15 MINUTES					
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
DOG 10	1	31.0	46.7	36.3	57.3	853	814	25.7	22.0	29.7	27.8	866	790
			+150%		+58%		-5%		-14%		-6%		-9%
	2	44.4	67.1	30.7	59.3	1440	1130	50.0	50.0	80.7	77.0	641	649
			+52%		+93%		-22%		0%		-5%		+1%
DOG 11	1	6.0	8.0	45.7	60.3	131	133	7.6	13.5	61.0	113	125	120
			+33%		+32%		+2%		+76%		+85%		-4%
	2	45.0	46.2	38.3	39.7	1170	1170	34.8	47.5	22.3	35.3	1540	1340
			+3%		+4%		0%		+36%		+58%		-13%
DOG 12	1	38.3	39.3	72.0	72.8	532	540						
			+3%		+1%		+2%						
	2	24.3	27.7	34.7	42.7	702	649						
			+14%		+23%		-8%						
DOG 13	1	21.7	27.0	38.5	42.7	563	633						
			+24%		+11%		+12%						
	2	52.5	49.3	64.0	56.2	820	878						
			-6%		-12%		+7%						
DOG 14	1	33.3	31.2	52.0	50.0	641	625						
			-6%		-4%		-2%						
	2	10.5	12.0	27.0	26.3	389	456						
			14%		-3%		-17%						
DOG 15	1	8.3	9.7	30.7	30.5	272	317	6.0	6.7	27.0	19.0	222	351
			+16%		-1%		+16%		+11%		-30%		+58%
	2	29.0	29.3	59.3	57.1	489	513	26.3	29.0	103	88.3	255	328
			+1%		-4%		+4%		+10%		-14%		+29%

continued

APPENDIX VIII--continued

		EXPERIMENTAL SAMPLE TIMES											
		30 MINUTES						45 MINUTES					
		MP (mV)		SCC (μA)		R (ohms)		MP (mV)		SCC (μA)		R (ohms)	
	SKIN	C	E	C	E	C	E	C	E	C	E	C	E
DOG 12	1							61.8	69.6	54.3	77.0	1140	904
								+13%		+42%		-20%	
	2							32.7	42.5	27.8	67.3	1180	631
								+30%		+140%		-47%	
DOG 13	1	44.6	45.8	39.7	55.3	1120	829						
		+3%		+39%		-26%							
	2	14.3	14.0	50.0	46.0	287	304						
		-2%		-3%		+6%							
DOG 14	1	31.0	35.8	52.7	66.8	589	537						
		+16%		+18%		-9%							
	2	25.3	25.3	49.0	49.3	517	514						
		0%		+1%		-1%							

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