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**DEVELOPMENT AND EVALUATION OF METHODS FOR THE
DETERMINATION OF CARBAMYLATED PROTEINS
IN HEMODIALYZED PATIENTS**

by

CYNTHIA M. BALION

A DISSERTATION

**Submitted to the Faculty of Graduate Studies and Research
through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor**

Windsor, Ontario, Canada

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Ode to Urea

Quietly unobtrusive, just doing your own thing
Maintaining the status quo for ammonium.
Who would have ever thought, though some had hinted
That someday your role would be heightened.
A small study here and a correlation there,
The idea was stuck and the fun was begun
Carbamylation is your new vocation.

Working through cyanate, isocyanate to be precise
Your're picked up by lonely electron pairs,
Though the environment should be right.
In vivo you prefer the company of amino residues,
Both α and ϵ , and sometimes sulfhydryls too.
Your nonpartisan character allows you to circulate
And without any haste form stable bonds
Proteins or lipoproteins, enzymes are neat,
Hemoglobin, membranes - just whomever you meet.

You don't mean to cause harm,
But what a ruckus you cause when you get a bit elevated!
Activities can increase, but are usually decreased
There are reductions, and preventions
Not to mention all those conformational changes
And sometimes the eyes get a bit cloudy
And the nerves get unsettled
An indicator of things gone amiss.

And now it is time for all to take action
To unravel the complexities of your disposition.
We may not be able to suppress you, but at least understand you
And monitor all of your moves.
The guise has been lifted and your new life has debut.

Cynthia Balion
1996

ABSTRACT

DEVELOPMENT AND EVALUATION OF METHODS FOR THE DETERMINATION OF CARBAMYLATED PROTEINS IN HEMODIALYZED PATIENTS

The carbamylation reaction *in vivo*, involves the nonenzymatic, covalent attachment of isocyanic acid, the spontaneous dissociation product of urea, to proteins. Carbamylated proteins have been proposed as markers of uremia and indicators of uremic control.

An enzyme-linked immunosorbant assay for carbamylated albumin, the major serum protein, was developed. Furthermore, an investigation was carried out to determine the relationship between carbamylated hemoglobin (CHb) and carbamylated total protein (CTP), and also their association with pre-dialysis urea and dialysis dose in hemodialyzed patients.

Polyclonal antibodies were made against *in vitro* prepared heavily carbamylated albumin in rabbits. The antisera were purified using protein A affinity columns and specific affinity columns (albumin, carbamylated albumin, and carbamylated hemoglobin). The albumin-affinity purified polyclonal antibody was found to be specific for carbamylated albumin, and did not react with albumin, carbamylated hemoglobin, carbamylated fibrinogen, homocitrulline or carbamylaspartate. Using *in vitro* carbamylated albumin as the standard, the competitive assay had a detection limit of 25 pmol of carbamyl groups and had a detection range of about 3 orders of magnitude. The sandwich assay, however, had a greater sensitivity (1 pmol of carbamyl groups), and a wider linear range (at least 5 orders of magnitude). Despite the sensitivity of these assays, they could not be used for the determination of minor degrees of carbamylation, as occurs *in vivo*.

A six-month longitudinal study of seven hemodialyzed patients showed that correlations of CHb and CTP concentrations with currently used uremic

indices were not significant. These data suggest that measurement of CHb or CTP may not be meaningful for hemodialyzed patients on maintenance dialysis.

Hemodialyzed patients were found to have significantly higher CHb ($157 \pm 40 \mu\text{g valine hydantion/g Hb}$) and CTP ($0.117 \pm 0.011 \text{ A/mg protein}$) concentrations as compared to normal individuals ($53 \pm 20 \mu\text{g valine hydantoin/g Hb}$ and $0.08 \pm 0.01 \text{ A/mg protein}$, respectively). A high correlation was found between CHb and CTP concentrations ($r = 0.87$, $p < 0.0001$), demonstrating a strong relationship between these two different half-lived proteins.

This study shows that the carbamylated proteins, CHb and CTP, are positively associated and reflect the degree of urea exposure in blood.

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LIST OF ABBREVIATIONS

A	absorbance
AAG	alpha ₁ -acid glycoprotein
AonCRF	acute on chronic renal failure
apo	apolipoprotein
ARF	acute renal failure
ATP	adenosine triphosphate
BCG	bromocresol green
BGP	bromocresol purple
CAPD	continuous ambulatory dialysis
CHb	carbamylated hemoglobin
CHSA	carbamylated human serum albumin
CNVal	carbamyl norvaline
CVal	carbamyl valine
CI	carbamylation index
CRF	chronic renal failure
CTP	carbamylated total protein
CV	coefficient of variation
CVal	carbamyl valine
CNVal	carbamyl norvaline
Da	daltons
DAM	diacetyl monoxime
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ESRD	end-stage renal disease
GC	gas chromatography
GHb	glycated hemoglobin
Hb	hemoglobin
HC	homocitrulline
HD	hemodialysis
HPLC	high pressure liquid chromatography
HSA	human serum albumin
IDDM	insulin-dependent diabetes mellitus
IEF	isoelectric focusing

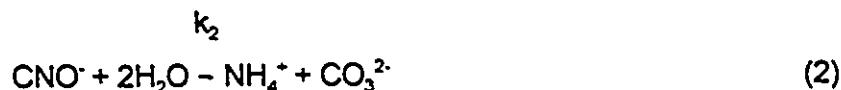
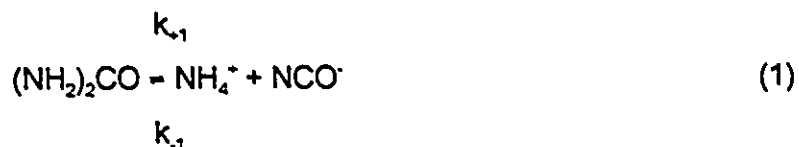
IgG	immunoglobulin G
K_a	acid dissociation constant
LDL	low-density lipoprotein
Lys	lysine
NIDDM	non-insulin-dependent diabetes mellitus
NSB	non-specific binding
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PCR	protein catabolic rate
pK_a	log acid dissociation constant
PMN	polymorphonuclear neutrophils
PRD	pre-renal dialysis
R.T.	room temperature
SDS	sodium dodecyl sulfate
TAC	time-averaged urea concentration
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
TNBS	2,4,6-trinitrobenzenesulfonic acid
TX	transplant
TX-RF	transplant-renal failure
Tyr	tyrosine
UKM	urea kinetic modelling
VH	valine hydantoin
VLDL	very-low-density lipoprotein

CHAPTER 1 INTRODUCTION

1.1 Chemistry of Carbamylation

1.1.1 Cyanate

The relationship between urea and cyanate has been known since the time of the historic synthesis of urea from ammonium cyanate by Wöhler in 1828. The spontaneous production of cyanate from urea is the net result of the reversible decomposition of urea to give ammonium ions and cyanate, and the decomposition of cyanate to yield ammonium and carbonate ions (1).



Kinetic data on the cyanate content of solutions of urea at different temperatures and pH's have been studied (2). A linear relationship exists between urea concentration and cyanate concentration. An increase in temperature causes an increase in k_{-1} , and therefore an increase in the decomposition of urea to cyanate (reaction 1). Similarly, a higher pH (3 - 7) also increases the decomposition rate. This effect is a combination of rate constant changes in the reverse reaction (k_{-1}) concomitant with rate constant changes in cyanate decomposition (k_2 , reaction 2). In one study, cyanate accumulation in a 6.66 M (pH~7, 85°C) solution of urea plateaued within 40 min (40 mM) with a corresponding rise in pH to 8.3 (1). The increase in pH is a result of the accumulation of ammonium ion, arising mainly from reaction 1, but also from reaction 2.

Aqueous incubation studies have shown that urea decomposes more and

at a faster rate at lower concentrations than at higher concentrations at the same temperature and pH (1, 3). Overall, a greater percentage of urea is decomposed at lower concentrations. However, this does not preclude the fact that quantitatively more cyanate is formed at higher urea concentrations than at lower urea concentrations. This contradiction is a function of the thermodynamic equilibrium of reaction 1 which favours the dissociated form of urea, *i.e.*, k_{-1} upon dilution and k_1 upon concentration (3). The incubates from this study (0.25 - 8.3 M urea, 38°C, 7 days), also showed the presence of carbonate which means cyanate was decomposed (reaction 2). When the incubations were done in buffer (0.1 M phosphate, pH 7.4) the same amount of cyanate was found; however, more carbonate was present indicating increased cyanate decomposition. Interpolation of the data gives a best fit curve with the equation $\text{cyanate (mM)} = 0.875 + 4.35(\text{urea}) - 0.246(\text{urea})^2$. In another study, the decomposition rate of cyanate at pH 7.0 and 25°C (reaction 2), was found to be $3.06 \times 10^{-5} \text{ min}^{-1}$ which corresponds to a $t_{1/2}$ of 378 sec (2).

A true estimate of cyanate production *in vivo* is extremely hard to come by. There are several factors which determine its production: time, pH, temperature, urea concentration and protein concentration. Protein concentration has a very important influence on the urea-cyanate equilibrium. The equilibrium will be shifted to the right because cyanate will be removed through the carbamylation reaction. Cyanate hydrolysis will therefore be reduced (reaction 2). In addition, biological dynamics will play a role in the carbamylation reaction. There have been no studies so far which address all these parameters. It is, therefore, limiting to try to make an estimate from the literature on cyanate concentration *in vivo* with limited aqueous *in vitro* data. If the assumptions are made that urea in plasma is at equilibrium and that the curve described above can be applied to urea concentrations below 0.25 M, then rough calculations can be made. A solution of urea (5 mM, physiological) would contain 0.90 mM of cyanate, and a solution of urea (50 mM, pathological) would contain 1.09 mM. No study has yet been able to quantitate plasma cyanate.

How much cyanate is really formed is probably not as important as the physiological effects it presents.

Another source of *in vivo* cyanate could come from the hydrolysis of carbamyl phosphate (4). The chemistry of carbamyl phosphate shows that at physiological pH, it is in the dianion form and decomposes yielding diphosphate ion and cyanate. The half-life of carbamyl phosphate is 45 min at 37°C at this pH. It has been proposed to be the more important physiological carbamylating agent as opposed to urea (5). However, the carbamyl phosphate exists in the mitochondria of liver cells (small amounts in other tissues), and when it is in excess it is shunted out into the cytosol. There carbamyl phosphate stimulates pyrimidine synthesis which makes it unlikely to be released into the plasma. There has been no report in the literature to my knowledge which has looked for or detected carbamyl phosphate in the plasma.

A study in the literature in 1948 reported a new enzyme "cyanase" from crude tissue extracts which could catalyze the hydrolysis of cyanate into ammonia and CO₂ (6). In 1977 this phenomenon was reanalyzed and found to be false. Rather, carbamyl phosphate is formed from cyanate and phosphate and it is this compound which decomposes to yield ammonia and CO₂ (7). However, current literature still quotes the 1948 study which is misleading. An addendum to this is that there is a cyanase, but it is only produced in bacteria (8, 9). The potential importance of this enzyme will be discussed later.

1.1.2 Carbamylation

Schütz in 1949 reported that plasma proteins were able to "catch" cyanate (10). He hypothesized that the reaction may be similar to that of isocyanates with proteins. It was not however shown that cyanate was attached to proteins. It was in an investigation of the effects of denaturing agents on ribonuclease A by Stark *et al.* a decade later (11) who first identified the carbamylation reaction. They found that when ribonuclease A was treated with urea there was a decrease in enzyme activity, a decrease in lysine content and the presence of a new amino acid. This new amino acid was later identified as homocitrulline, the

epsilon carbamylated derivative of lysine.

Stark *et al.* in the early 1960's detailed the reactions of cyanate with proteins. Cyanate was shown to undergo carbamylation reactions with amino (12), sulfhydryl (13), phenolic (14), hydroxyl (15), carboxyl (12) and imidazole (16) groups on proteins (11, 17). The most stable of the carbamyl derivatives were with amino groups. The other carbamyl derivatives become unstable with dilution or varying pH.

So far, no literature has investigated the reaction mechanism of isocyanate ($\text{HN}=\text{C}=\text{O}$) with amino groups on proteins. However, this reaction may not be much different from the classic reaction of carbon dioxide ($\text{O}=\text{C}=\text{O}$) with proteins, particularly with hemoglobin, forming carbamino compounds (18, 19). Carbon dioxide reacts with the amine groups of blood proteins and requires no enzyme. Similarly, isocyanate also reacts with amine groups except it produces an uncharged species whereas the carbamino compounds are mostly negatively charged. The amount of carbon dioxide which binds to hemoglobin depends upon the pH of the media. This is consistent with what has been found with isocyanate and proteins (19). The amino group must be in the neutral form (NH_2) for binding to occur.

The proposed reaction mechanism for carbamylation is based on the reaction of alkyl isocyanates with amines and is detailed in Figure 1.1. The reaction involves the nucleophilic attack of the unprotonated amino nitrogen to isocyanic acid. The unprotonated form of the nitrogen has been shown to be important and that pH changes, which affect the protonation of the nitrogen, will therefore, affect the rate of carbamylation (14). Isocyanic acid is the stable and reactive form of cyanate (4).

The reaction rate varies inversely and linearly with the pK_a values of the amino groups (12). The rate of carbamylation of several peptides and amino acids (2 - 4 mM) was determined using KCNO (0.2 - 2.0 M) at pH 8 and 30°C . The graph of pK_a versus $\log k$ gave a line with the equation $k (\times 10^3 \text{ M}^{-1} \text{ min}^{-1}) = 7.94 (\pm 0.08) - 0.71 (\pm 0.02)\text{pK}_a$. Accordingly, at neutral pH α -amino groups can

FIGURE 1.1**The Carbamylation Reaction with Amino Groups***Legend*

Urea, through a spontaneous decomposition reaction, is converted to isocyanic acid. Isocyanic acid reacts with free amino groups through a nucleophilic attack mechanism. The reaction is non-reversible.

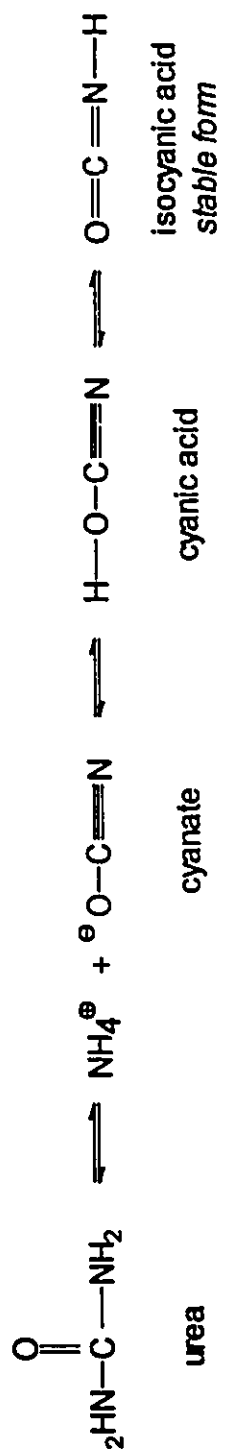
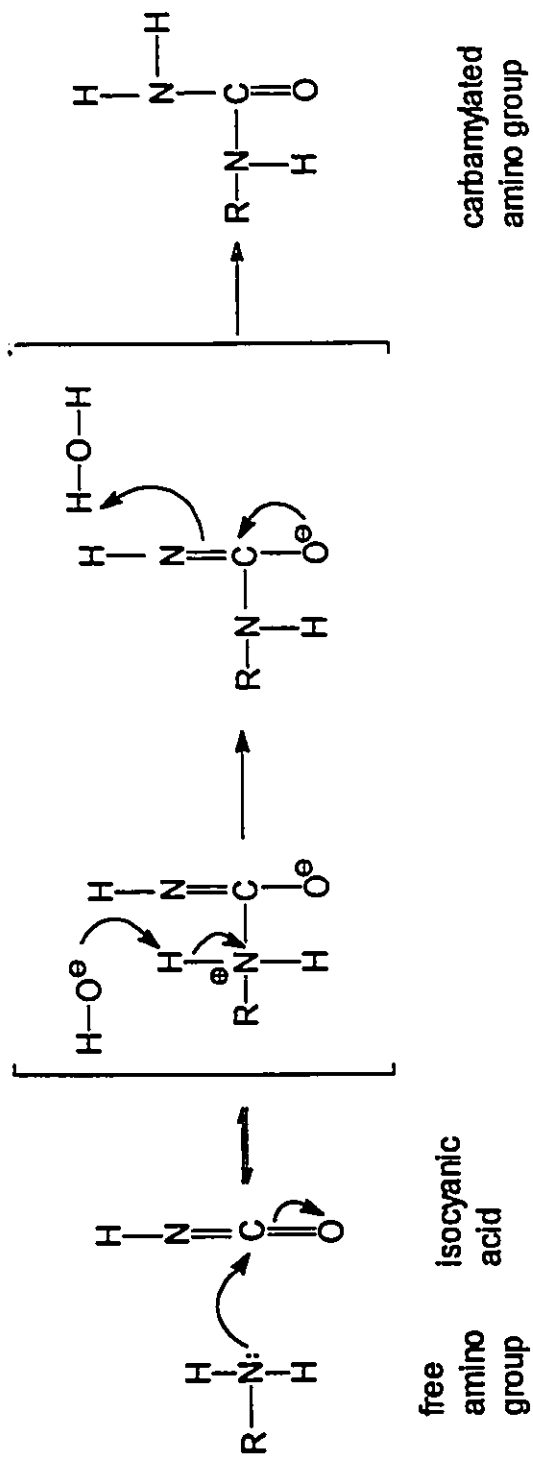


FIGURE 1.1



be expected to react ~100x more rapidly, due to their lower pK_a (~ 8), as compared to ϵ -amino groups (pK_a ~ 10.7) on proteins. Some sulfhydryl carbamylation also occurs in this pH range, the pK_a for cysteine being 8.3. However, the carbamylmercaptan is not very stable ($t_{1/2}$ ~ 6 - 11min).

The carbamylation (or carbamoylation) reaction in general refers to the nonenzymatic, covalent attachment of isocyanic acid, the spontaneous dissociation product of urea, with species bearing a free electron pair. *In vivo*, free amino groups of proteins are the major donors, however, sulfhydryl groups of proteins can also be carbamylated. Carbamylation is sometimes misappropriately applied to the reaction of CO_2 with amino groups (e.g., carbamino compounds). Other carbamylating agents include carbamylphosphate, alkyl-isocyanates (e.g., 2-chloroethylisocyanate), phenyl-isocyanates (e.g., diphenylmethane diisocyanate), and carbamyl esters (e.g., N-methyl-7-dimethylcarboxamoxyquinolium). Carbamylation in this Dissertation refers to the reaction with cyanate only.

1.2 Effects of Carbamylation

The probability for a protein, or other compound, to become carbamylated and then have its function altered depends upon several factors. As mentioned before, the pH and the k_a of the group to be carbamylated are important, but so is the position of the susceptible group on the protein (20), the presence of ligands around the site (21-23), and the extent of prior carbamylation (24) or other types of derivatizations (25, 26). In one study, glucose derivatization was found to influence carbamylation (25). Furthermore, additional considerations are necessary for *in vivo* systems such as the location of the protein (intra- or extracellular and tissue type), and its half-life.

1.2.1 *In Vitro* Studies

There are numerous studies in the literature which have used carbamylation as a means to study proteins. A comprehensive listing of all these

studies up to the mid 1970's can be found in a review on protein carbamylation (4). Table 1.1 is a subsequent listing of proteins that have been carbamylated since then. It also serves to highlight the effects that carbamylation has on proteins. In addition to these effects, carbamylation has been shown to stabilize proteins against heat, heavy metal salts and alcohol, and aid in the dissolution of denatured and precipitated proteins (27). Furthermore, the formation of carbamylated products in electrophoresis and chromatography when urea has been used (4), has been exploited to increase the anodicity of various proteins for separation purposes (28, 29).

Most carbamylation occurs on amino groups of proteins, probably because of the quantity of amino groups present, and secondly because it was usually only carbamylated NH_2 -terminal amino acids or lysine residue that were looked for. However, carbamylated sulfhydryl groups have also been shown to occur in proteins such as glutathione (30), β -lactoglobulin, alkaline phosphatase, papain (4), histone H3 (31), and hemoglobin (32).

Hemoglobin carbamylation has been studied to a large extent because of its potential use as a desickling agent (described later). It aids in the dispersion and solubilization of hemoglobin derivatives, increases oxygen affinity, and causes a shift in its absorption band (27). Hemoglobin synthesis is also affected by cyanate (33). In addition, the carbamylation of red cell membranes has been shown to increase Na^+ permeability and to a lesser extent increase K^+ permeability when carbamylated (34).

Carbamylation has even been found to occur in non-mammalian species such as in a nodulation factor from wild type *Rhizobium etle* (58).

The fact that physiological pH favours carbamylation of amino groups, and that the reaction is indiscriminate leads to the conjecture that any biological molecule that has a free amino group could be carbamylated. This would include not only amino acids, proteins and hormones (dopamine, serotonin, thyroxine, γ -amino-butyrate), but also lipids with amino groups, creatine, histamine, porphobilinogen, ATP, and even DNA (adenine, guanine, cytosine). The only

TABLE 1.1

Types of Effects Carbamylation has on Biological Compounds

Compound	Effect	Ref.
Acetylcholinesterase	Conformational changes	(35)
Albumin	Decreased acidic drug binding	(17)
	Decreased binding to BCG	(36)
Alpha ₁ acid glycoprotein	Decreased basic drug binding	(37)
Aspartate aminotransferase	Apo form is inactivated	(21)
Canaline	Unknown	(38)
Carbonic anhydrase	Decreased CO ₂ hydratase activity	(39)
Chondrosarcoma proteoglycan	Increases the number of sites For chymotrypsin digestion	(40)
Crystallins	Aggregation of crystallins	(41)
	Conformational changes	(42)
C-Reactive protein	Structural changes	(43)
	New antigenic reactivity	
Erythropoietin	Loss of activity	(44)
Fibrin	Resistance to fibrin degradation	(45)
Histone H3	Loss of gel retardation by Triton	(31)
Lactoferrin	Reduced uptake	(46)
Lactate dehydrogenase	Freeze-stabilizes activity	(47)
Leukocytes	Unknown	(48)
Low-density lipoprotein	Reduced binding to B/E receptors	(49)
	New antigenic sites	(50)
Pepsinogen	Loss of potential pepsin activity	(51)
Procaine esterase	No effect on hydrolysis	(52)
Phospholipase A ₂	Reduced binding	(53)
	Reduced lethal potency	
Protein P	Destroys anion and Phosphate binding sites	(54)
Superoxide dismutase	Decreased activity	(55)
Tropomyosin	Loss of binding to actin	(56)
Tyrosine	Unknown	(57)

determinants for carbamylation are the pK_a of the group and its location (ability of cyanate or urea to reach those sites and the amino acid environment). In addition, sulfhydryl groups on biological molecules (e.g., coenzyme A) should not be overlooked since these groups too can be carbamylated at physiological pH.

Some evidence showing that these reactions can occur includes a recent *in vitro* study that showed phospholipids (phosphatidylethanolamine and phosphatidylserine) from red cell membranes could be carbamylated (59). In a study that involved cyanate treatment of murine P388 leukemia cells, it was found that ATP was slightly lower in the treated cells versus the control cells (60). This decrease in ATP may have been due to the inability of the enzyme assay to detect carbamylated ATP. Investigation of the effect of nitrosourea with leukemia cells showed some support for potential DNA carbamylation (61).

1.2.1.1 Carbamylated hemoglobin and glycated hemoglobin

The relationship between carbamylated hemoglobin and glycated hemoglobin has been studied *in vitro* (62). The study showed that when cyanate and glucose were incubated with whole blood, glycated hemoglobin was produced at a higher rate as compared to carbamylated hemoglobin. High glucose concentrations reduced the formation of carbamylated hemoglobin, but high concentrations of cyanate had no effect on the formation of glycated hemoglobin. One point to keep in mind, though, is that only carbamylated valine residues were measured. However, lysine residues on hemoglobin are also carbamylated (63, 64). Therefore, the apparent loss of carbamylated hemoglobin with increased glucose concentration may only reflect loss of valine carbamylation and total carbamylation which would need to include lysine carbamylation. Furthermore, the simplicity of this *in vitro* system may not represent the dynamics present in an *in vivo* system.

In patient studies, urea concentrations (>25 mmol/L) were found not to affect glycated hemoglobin (GHb) concentrations in poorly controlled diabetics (glucose > 12 mmol/L); however, falsely elevated GHb concentrations were found in non-diabetics. If GHb were measured by a specific immunoassay, then

non-diabetics with renal failure had a lower concentrations of GHb compared to the reference population (26). The inference that can be made from both these studies is that carbamylation and glycation are not mutually exclusive reactions - one influences the other. Furthermore, it is conceivable for a protein to have both carbamyl and glucose groups on it.

1.2.2 *In Vivo* Studies

1.2.2.1 Distribution of cyanate

Injection of [^{14}C]cyanate into mice showed that most of the cyanate undergoes rapid pulmonary excretion ($t_x = 43$ min) (65). The t_x for [^{14}C]CO₂ formation was found to be 96 min which is 15-fold longer than the aqueous *in vitro* decomposition t_x for [^{14}C]cyanate (6.3 min at pH 7.0, 25°C) (2). Approximately 16% of the [^{14}C]cyanate was bound after 5 min and 57% was bound after 180 min (65). In another study, the rate of carbamylation (bound cyanate) was also high, reaching a steady state within about 4 hours (66). These hydrolysis and carbamylation kinetics seem to explain why cyanate has not been detected in plasma. It looks as though cyanate formed from urea would be very quickly hydrolyzed or bound.

In another study, 24 hours after a single injection of [^{14}C] cyanate (10 $\mu\text{mol/g}$) into mice, 72% of the dose was excreted as CO₂, 7% was found in the urine, and the remainder was bound to plasma and tissue proteins (67). The bound cyanate was highest in erythrocytes (7.5%), then in bones (3.3%), muscle (2.1%) and other organs (<3%). A study in rats (1.25 μmol [^{14}C]cyanate/g) showed a slightly different pattern (66). The amount of bound [^{14}C]cyanate (nmol/mg protein) was highest in plasma proteins (2.0), followed by hemoglobin (1.31), liver (0.79) and brain (0.43). However, this may be because measurements in this later study were made only after six hours versus 24 hours in mice.

The turnover of carbamylated protein in various tissues (erythrocytes, bones, muscle, skin, liver, serum proteins, intestine, brain, heart, stomach, kidneys, lungs, spleen and other), has been monitored over time (67). The

amount of radioactivity in different tissues was found to plateau at different times even when animals were injected daily, reflecting an equilibrium between carbamylation and catabolism. The loss of radioactivity also declined at different rates in different tissues. After chronic administration of [¹⁴C]cyanate (78 injections), there was still substantial radioactivity left in several tissues such as bones (74%) and muscle (43%), 130 days post-treatment.

1.2.2.2 Pharmacological effects

In the late 1940's a series of classical studies on the pharmacology of cyanate in animals and humans were carried out by Schütz and colleagues (3, 10, 27). Table 1.2 lists a number of pharmacologic effects of protein carbamylation. These effects are usually reversed when cyanate is removed.

1.3 Measurement of Carbamylated Compounds

There are several direct methods to measure carbamylated proteins (63, 73-75). The first method was developed by Stark and Smyth after the observation that cyanate could react with proteins to produce carbamyl derivatives (73). They took advantage of the fact that carbamylated NH₂-terminal amino acids could cyclize under strong acid conditions to form hydantoins. Hydantoins were separated using ion-exchange chromatography, decarbamylated by hydrolysis, and then identified by amino acid analysis. The drawback of this method, in addition to the complexity, was the fact that homocitrulline residues are not stable under the hydrolysis conditions, and depending on the protein, between 17 to 30% of homocitrulline are converted back to lysine. One could take the mean conversion percent and estimate the amount of homocitrulline converted back to lysine, but this is only valid when the protein is completely carbamylated.

Shortly thereafter, a modified assay for urea was found that could detect carbamyl groups (76, 77). The reagent diacetyl monoxime was found to bind to carbamyl groups and the product detected colorimetrically. The assay requires

TABLE 1.2

Pharmacologic Effects of Cyanate (Protein Carbamylation)

Tumors

- Alteration of tumor growth
 - Inhibition of protein synthesis (60, 65, 68)
- Inhibition of DNA synthesis (69)
 - Decrease in tumor growth (70)
 - Decrease in uptake of compounds into hepatomas (70)

Animals (10, 67)

- Anorexia (high dose)
- Cataract formation (42)
- Diuresis
- Drowsiness (seizures and death in large doses)
- Hypnotic effect
- Inhibition of growth (antimitotic agent)
- Memory loss

Humans

- Cataract formation (42)
- Diuretic effect (mild) (10)
- Drowsiness
- Dim vision
- Nausea (71, 72)
- Neuropathies (69)
- Vomiting (69)

Hemoglobin

- Increased the oxygen affinity
- Increased cell survival

Referenced from (10) unless otherwise indicated.

urea and other urea-like compounds to be removed beforehand since they also react with the reagent. Quantitation of carbamyl groups is done by comparison to a standard curve of [^{14}C]cyanate labelled protein (absorbance vs radioactivity). However, in this case it is assumed that there are no carbamyl groups on the native protein. This is difficult to imagine, since as in other forms of derivatizations, such as in glycation, there is always some normal derivatization. Therefore, quantitation by this method is only relative. Homocitrulline can also be quantitated by this method (78). Carbamylated protein is first hydrolyzed and then homocitrulline is separated by cation-exchange chromatography.

Another colorimetric procedure was developed which was based on the permanganate oxidation of radioactive carbamyl groups on proteins to form urea (79). Urea is then quantitated by the release of [^{14}C]CO₂ with urease. This method could not be applied to the measurement of nonradioactive carbamylated proteins because the assay used 5 M urea during its oxidation/hydrolysis step. The urea derived from carbamyl groups would not be distinguishable from the urea reagent.

Later, a gas chromatography (GC) technique (74) was developed to measure the amount of carbamyl groups on hemoglobin. This method involved an acid-acetone procedure for heme removal and then hydrolysis of the globin to give free carbamylated NH₂-terminal valine residues. The hydrolysis step also converted these residues to valine hydantoins (VH) which were extracted and detected by GC. Valine hydantoin can also be detected by an HPLC procedure (75).

High-pressure liquid chromatography (HPLC) has also been used to detect carbamylated amino acids. In some procedures the carbamylated protein is acid hydrolyzed first to release the amino acids (44, 57, 80-82). Quantitation is then done by comparison to the noncarbamylated protein or by detection of a specific carbamylated residue such as homocitrulline. Since hydrolysis also causes some homocitrulline to be converted back to lysine, some procedures did involve a quasi correction factor to account for this reversion. However, this

correction factor was based on experiments that Stark and coworkers had done with fully carbamylated proteins (73). In another modification (55), protein was pretreated with nitrous acid which destroys unprotected lysine residues (not carbamylated), before hydrolysis. Therefore, the sum of lysine and homocitrulline after HPLC would be a true measure of the number of carbamyl groups.

In lieu of the hydrolysis procedure to separate the amino acids of a protein, a method has been developed to remove the carbamyl groups off the protein (all sites) (32, 83, 84). Carbamyl groups are converted to cyanate by reaction with nitrite to form nitroso-urea. Nitroso-urea is then degraded to cyanate in alkali solution and separated by ion-exchange HPLC. Cyanate is hydrolyzed and ammonium ion is detected by the indophenol reaction.

Other methodologies include mass spectrophotometric techniques (30, 85) which are not as harsh on the modified protein and therefore prevents loss of carbamyl groups on lysine and cysteine residues. Indirect measurements such as loss of free amino groups can be measured by reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (86). Also, a gel electrophoresis technique with polyacrylamide gels containing acetic acid, ammonium hydroxide, urea, and Triton X-100 has been found to be able to separate one-charge differences into discrete bands (31).

The most recent method (63) is an immunoassay method which uses rabbit polyclonal antibody against carbamylated low-density lipoprotein (LDL) (see Section 1.5.2.3). This antibody is reported to be specific only for carbamyl groups on lysine residues *i.e.*, homocitrulline residues.

1.4 Causes of Elevated Urea in Plasma

Extreme elevations of urea are seen not only in renal disease, but in other conditions as well (87). The main causes of elevated urea can be grouped as increased urea load (dietary or tissue metabolism), decreased glomerular

filtration rate, or increased tubular resorption. The non-renal causes of elevated urea are prerenal azotemia which includes gastrointestinal hemorrhage, gastrointestinal obstruction, shock, tissue necrosis, third degree burns, fever (protracted), dehydration, diarrhea, diabetic coma, congestive heart failure. Elevations are also seen in Addison's disease, steroid therapy, and high protein diets..

The duration of increased urea levels will determine the extent to which cyanate is produced and therefore the amount of carbamylation. In the short-term, carbamylation in episodic urea increases will be short-lived and the pathophysiologic significance will be limited. However, in the long-term, in tissues with a very slow turnover, carbamylation will be cumulative and could have a profound pathophysiologic effect. From animal studies it has been shown that some tissues retain [^{14}C]cyanate longer than others (see Section 1.2.2.1). In studies with lens crystallins, which have an extremely low turnover rate, carbamylated crystallins have been associated with cataracts (41, 80, 85). Furthermore, the results of one study suggested that carbamylation of lens proteins occurred during cataractogenesis in Pakistan and not in England (Oxford) (42). The hypothesis was that diarrhea is more prevalent in some populations and therefore the potential for more carbamylation events is increased.

Fibrin degradation by plasmin is reduced when fibrin is carbamylated (45). This could lead to fibrin accumulation in such tissues as the kidney, retina, nerve and artery. Therefore carbamylation of fibrin may play a role in disease pathogenesis.

The physiological and pathological significance of carbamylation is therefore far-reaching. Considering the magnitude of molecules that could be carbamylated and the omnipresence of urea, elevated in several situations, there is a real foundation for more studies to be done to investigate this phenomenon. The real crux will be to see if the extent of their carbamylation (also dependent on half-life) causes an effect (good or bad) *in vivo*.

1.4.1 Uremia

Uremia is a syndrome that is caused by renal failure. The characteristics of this syndrome are variable and inconsistent (88). Biochemically, it is a failure to excrete waste products and conserve needed substances. Clinically, it presents with one or more of the following (89): weakness; fatigue; lethargy; insomnia; malaise; cardiovascular symptoms (hypertension); gastrointestinal symptoms (nausea, vomiting, diarrhea); neurological problems (mental clouding, convulsions, peripheral neuropathy); anemia; infection; metabolic acidosis; renal osteodystrophy; and hyperkalemia.

The acidosis of uremic patients may influence the rate of carbamylation slightly. This is due to the effect pH has on the urea-cyanate equilibrium (see Section 1.1.1).

1.4.2 Adequacy of Dialysis

There is no accurate method to determine the efficiency of dialysis (90). The currently accepted method to quantify dialysis therapy and define its adequacy is through a mathematical description of urea balance called urea kinetic modelling (UKM). It is based on urea removal on the blood-side (91, 92). The parameters used in this model include dialyzer urea clearance (K_D), residual renal function (K_R), number of treatments per week, duration of dialysis (t), urea generation rate (G), volume of distribution (V), and change in body weight during dialysis (β) (92). The calculation requires solving two simultaneous equations to obtain G and V . The normalized dialysis dose (Kt/V) and protein catabolic rate (PCR) can then be determined. The value of Kt/V is used for quantitation of dialysis therapy. There are several variations of this model, the more complicated ones being better at predicting blood urea (93-96). The "gold" standard measurement is by direct quantitation of urea in the dialysate (92). However, the cost and technical complexity of this measurement limit its routine use.

The mathematical model (91) describing urea kinetics is based on the premise that urea follows single pool kinetics during and after dialysis. However,

this does not accurately reflect the urea content of the body because dialysis causes disequilibrium between the cell compartments and the blood (93). What is really needed is some sort of urea record that extends over the previous several weeks. This would be more meaningful in determining how effective dialysis treatment or renal management has been and what future course of action should be taken.

Carbamylated protein could therefore provide the clinical information needed as an indicator of uremic control. The benefit of using carbamylated hemoglobin as a marker is that it can be used as an alternative to time-averaged urea concentration (TAC). It only requires a single measurement compared with accurately timed urea measurements to be taken at three occasions for TAC. In fact, it may provide an even better indication of uremic exposure than TAC does because of its inherent characteristics.

Carbamylated protein could be used as a better index of chronic uremic exposure than a single measurement of urea, which is subjected to day-to-day dietary and metabolic influences (97). Carbamylated protein could be used in differentiating acute from chronic renal failure (98), monitoring the deterioration of renal function, assessing compliance to dietary therapy, or evaluating the adequacy of dialysis treatment (99).

1.5 Clinical Significance of Carbamylated Proteins

1.5.1 Sickle Cell Anemia

Hemoglobin carbamylation has been investigated in detail because it was once used in the therapy of sickle cell anemia. Sickle cell disease is a hereditary disorder of the blood in which a mutant gene codes for a valine residue instead of a glutamate residue in the β -chain of hemoglobin (HbS). Hemoglobin S exhibits the same solubility as HbA in the oxygenated state, but has a lower solubility in the deoxygenated state (100). Due to this characteristic, HbS will aggregate within the erythrocytes into long polymers that distort the membrane (sickle

shape). A critical level of deoxygenated hemoglobin must be reached before sickling occurs. Therefore, shifting the oxygen binding capacity will lessen the capacity for HbS to aggregate. Treatment of HbS with sodium cyanate increases oxygen affinity, doubles the survival time, prevents gelling of deoxygenated hemoglobin and the sickling of the erythrocytes. Carbamylation was found to occur on the terminal valine residues (α and β chains) of HbS (64). There is also carbamylation of ϵ -amino groups, but the reaction is slower by two orders of magnitude (101). However, this form of treatment was stopped when clinical trials showed patients experienced nausea, vomiting, lethargy, peripheral neuropathy (97), and even cataracts (102). The adverse clinical consequences seen were probably due to the carbamylation of other proteins which affected their structure and/or function. These symptoms are also similar to those seen in advanced uremia (see Section 1.4.1).

1.5.2 Renal Failure

The supposition that cyanate could be responsible for some of the symptoms seen in uremia was first proposed by Schütz in the late 1940's (10). However, it was not until 1964, when Gilboe and Javid (103) in a simple study with nephrectomized dogs were able to show that uremic symptoms could be induced by dialyzing with either urea or isocyanate.

1.5.2.1 Hemoglobin

It was almost two decades later before the relationship between uremia and urea was rediscovered. Flückiger *et al.* in 1981 noticed that when hemoglobin from nondiabetic uremic patients (dialyzed and nondialyzed) was chromatographed, the HbA_{1c} values were significantly elevated (104). This indicated the presence of another co-eluting species of Hb, possibly the product of hemoglobin and the urea-derived reactant cyanate. In further experiments, the amount of carbamylation was quantitated and it correlated to the time-averaged urea concentration ($r = 0.57$, $p < 0.05$, $n = 11$). Carbamylated hemoglobin has been found to interfere with the measurement of HbA_{1c} based on charge separation (cation-exchange chromatography including HPLC), but not on

specific detection methods (immunoassay, nitroblue tetrazolium colorimetric methods, and affinity chromatography) (25, 26).

A few years later Oimomi *et al.* investigated the possibility of using HbA_{1c} as an indicator of control in patients with renal failure (105). When HbA_{1c} values were compared to urea concentrations in nondiabetic patients with renal failure a correlation of 0.59 ($p < 0.001$, $n = 32$) was found. Furthermore, a time-course relationship between urea and HbA_{1c} demonstrated a correlation with HbA_{1c} and urea concentrations measured one to two weeks earlier. They concluded that HbA_{1c} could be used as an indicator of the state of renal failure.

However, there have been no further reports on this subject except for an indirect comment pertaining to measurement of HbA_{1c} (106). It was suggested that the degree of interference in HbA_{1c} caused by CHb in patients with diabetic nephropathy, could be accounted for by the difference between measurements made using an immunoassay method, which was not interfered by CHb, and an electrophoresis method which measured CHb and GHb. A corollary to this is that all carbamylated hemoglobin measurements could be made by using this difference type of analysis. This type of methodology is simpler than what is currently available, albeit it does not specifically measure carbamyl groups.

It was not until 1988 that an in-depth study was done to evaluate CHb as a potential marker in renal disease (97). Carbamylated hemoglobin (ng VH/ mg globin) was evaluated in 167 subjects and included controls, patients with primary renal disease (PRD), chronic renal failure (CRF), patients on continuous ambulatory dialysis (CAPD), hemodialysis (HD), renal transplant patients with good grafts (TX), and transplants with renal failure (TX-RF). There were significant differences between CHb in the control group and all groups except the PRD and TX groups. Fair to good correlations were also found between CHb and renal function (urea and creatinine) for all groups except the HD (creatinine), PRD, and TX groups. Table 1.3 lists the values of carbamylated hemoglobin found in this study along with subsequent studies.

Several other researchers have investigated the usefulness of

TABLE 1.3

Carbamylated Hemoglobin Values in Normal Individuals and Patients with Renal Failure

	CHb* ($\mu\text{g CVal/g Hb}$)	Patients (n)	Ref.
Normal			
(Normal renal function)	26 (2)	20	(97)
	28	20	(98)
	41 (12)	25	(75)
	37 (7)	-	(109)
<i>Hospital personnel</i>	37 (7)	30	(107)
<i>Unselected in-patient</i>	38 (12)	33	(107)
<i>Diabetic</i>			
<i>IDDM</i>	38 (11)	24	(75)
<i>IDDM and NIDDM</i>	38 (11)	29	(107)
Renal Failure			
Primary renal disease	29 (2)	22	(97)
Acute renal failure			
(<i><10 day</i>)	29 (27-35)	11	(98)
(<i>>10 days</i>)	72 (60-83)	9	(98)
Acute on chronic renal failure	42 (31-67)	22	(107)
Chronic renal failure			
	137 (10)	40	(97)
	164 (88)	30	(75)
	186 (92)	43	(98)
	148 (122-210)	24	(107)
Continuous ambulatory peritoneal dialysis			
	127 (8)	24	(97)
	143 (32)	18	(107)
Hemodialysis			
	57 (18)†	10	(104)
	100 (5)	31	(97)
	131 (42)	27	(107)
<i>Adequate dialysis, adequate PN‡</i>	142 (29)	11	(99)
<i>Inadequate dialysis, adequate PN</i>	197 (30)	6	(99)
<i>Adequate dialysis, inadequate PN</i>	105 (28)	3	(99)
Transplant			
	43 (4)	16	(97)
	76 (33)	16	(107)
<i>Transplant (renal failure)</i>	87 (14)	14	(97)

* Mean \pm SEM (97), interquartile ranges (98), mean \pm SD (75, 98, 99, 107, 109)

† First report which measured carbamylated hemoglobin. The assay, however, could not detect carbamyl groups in normal samples.

‡ Protein nutrition

carbamyated hemoglobin in renal disease (63, 75, 98, 99, 107-109). Kwan and coworkers (75, 99, 107, 109) have published most extensively in this area and have not only developed a new method for measurement of CHb (see Section 1.3), but have provided additional evidence to support the use of CHb as a uremic indicator. They found that CHb in healthy laboratory personnel ($37 \mu\text{g CVal/g Hb}$, $n = 30$) and unselected hospital inpatients with normal renal function ($38 \mu\text{g CVal/g Hb}$, $n = 33$), were not significantly different from insulin-dependent diabetic patients ($38 \mu\text{g CVal/g Hb}$, $n = 29$) (107). There were significant differences ($p < 0.001$), between the control groups and patients with renal disease including CRF ($n = 43$), CAPD ($n = 18$), HD ($n = 27$) and TX ($n = 16$). However, CHb concentrations in CAPD patients were no different from CHb concentrations in HD patients. Furthermore, there were no sex or age distinctions, nor was there a difference between the pre- and post-dialysis CHb concentrations. In addition, pre-dialysis urea concentrations, as compared to creatinine concentrations, were significantly correlated to CHb concentrations in all groups.

In another study they looked at the relationship between UKM derived indices (TAC, G, PCR) and CHb in 20 twice-weekly hemodialyzed patients (99). Again there was a significant correlation between urea and CHb concentrations ($r = 0.69$), but there was a slightly stronger correlation between TAC and CHb concentrations. CHb was also weakly correlated with the urea generation rate ($r = 0.53$), and PCR ($r = 0.52$). Using the UKM parameters they classified their patients into four categories (adequate/inadequate dialysis combined with adequate/inadequate protein nutrition), and then calculated the mean CHb concentration for each of these groups. Adequately dialyzed patients had a CHb concentration of $142 (\pm 29) \mu\text{g CVal/gHb}$, and underdialyzed patients had a CHb concentration of $197 (\pm 30) \mu\text{g CVal/gHb}$.

Carbamyated hemoglobin has also been found to be able to differentiate acute from chronic renal impairment (98). An assessment was made on 43 patients who had a serum creatinine concentration of greater than 500 mmol/L .

and a provisional diagnosis of acute renal failure. Standard biochemical and hematological tests were similar in all patients, but differences in CHb concentrations were seen. Patients were later divided into groups based on their subsequent diagnosis. Patients with acute renal failure (ARF) had a lower concentration of CHb compared to patients with acute on CRF (AonCRF). Also, the AonCRF group had a lower CHb concentration compared to the CRF group. Within the ARF group, patients presenting with symptoms longer than 10 days had significantly higher CHb concentrations than did patients with symptoms of less than 10 days. Overall, patients with reversible renal failure (ARF and AonARF) had a CHb concentration of $< 190 \mu\text{g VH/g Hb}$. The sensitivity of this cutoff was 100% with a positive predictive value of 62%. Furthermore, there was a higher positive predictive value when the ratio of CHb to creatinine was used (to correct for renal function). A cutoff of 0.2 gave a sensitivity of 100% and a positive predictive value of 80%. Therefore, based on the initial measurement of CHb, patients with ARF could be rapidly diagnosed and treated sooner.

The latest study by Stim *et al.* with CRF and ARF patients (24) corroborates many of the points discussed above. This study also determined that factors such as phosphate and bicarbonate do not affect carbamylation. They state that the significant predictors for carbamylation are predialysis urea and the urea reduction ratio, but their data is a bit weak to support this conclusion.

1.5.2.2 Plasma proteins

Relatively little work has been done on the carbamylation of plasma proteins in renal failure. The earliest report (110) showed that there was significantly more carbamylated plasma protein in patients with renal failure as compared to normal subjects. A few other studies have reported the same findings and quantitated the number of carbamyl groups on plasma proteins from normal individuals as compared to uremic patients (Table 1.4). In one study, carbamylation of both amino and sulfhydryl groups of plasma proteins was measured (32). Only lysine carbamylation was found. However, erythrocytes

TABLE 1.4

Carbamylated Protein Values in Normal Individuals and Patients with Renal Failure

Protein	Normal ($\mu\text{g HC/g protein}$)*	Uremic ($\mu\text{g HC/g protein}$)	Ref.
Total protein			
Carbamyl groups	ND**	1247 (284)	(17)
Homocitrulline†	~ 208	~ 473	(32)
Homocitrulline‡	219 (164 - 329)	737 (55 - 903)	(78)
Albumin			
Carbamyl groups	ND	938 (378)	(36)

For comparison purposes carbamyl groups were assumed to be homocitrulline residues.
 Conversion factors used were 69,000 g/mol for HSA and 189 g/mol for homocitrulline.
 Values in parentheses are SD's or ranges.

* $\mu\text{g homocitrulline/g protein}$

** Not detected

† Estimated from a graph.

‡ Calculated from molar ratio's.

were found to contain carbamylated sulfhydryl groups also in addition to homocitrulline residues.

Kraus and Kraus have done the majority of studies concerning carbamylated proteins and amino acids in patients on CAPD and HD (48, 57, 82, 111, 112). Carbamylated amino acids were found in the plasma of uremic patients, but not in normal individuals (57, 82, 112). Non-denaturing polyacrylamide electrophoresis has shown differences in the separation of albumin and gamma globulin from CAPD and HD patients as compared to normal individuals (111). *In vitro* carbamylated plasma protein exhibited a similar pattern of heterogeneity. They also report that after one year of treatment, the CAPD group showed less carbamylation as compared to the HD group.

The most recent study looked at carbamylated leukocyte proteins in patients on CAPD compared to normal individuals (48). The study used a specific fluorescent labelled antibody to homocitrulline (carbamyl lysine) for detection; quantitation of homocitrulline was not done. They found fluorescence in the cytoplasm and on the cell surface of polymorphonuclear neutrophils (PMN), and monocytes in CAPD patients. Fluorescence in PMN cells from normal individuals was associated only with the perinuclear membrane. Monocytes from these individuals were unlabelled. Furthermore, exposure of normal PMN's with cyanate resulted in an inhibition of the release of superoxide. Therefore, carbamylation of PMN's could be one reason why they have altered functions in patients with renal disease.

Other studies have been concerned with the acidic drug binding defect of albumin seen in patients with renal failure (17, 36, 78, 81, 113). *In vitro* studies showed a strong correlation between the amount of free drug and the degree of albumin carbamylation (17, 78). In addition, they have shown that plasma proteins isolated from uremic patients contain more carbamyl groups and bind less drug (salicylate) than do plasma proteins isolated from normal individuals. Furthermore, a more recent study (113) has shown that the binding defect for

small acidic molecules with HSA is due to not only structural modifications of HSA (carbamylation), but also to competitive inhibition with other physiological ligands.

It has also been reported that the acidic dye bromocresol green (BCG), used to assay for albumin, underestimates albumin in plasma from uremic patients, and this underestimation could be correlated with the degree of albumin carbamylation (36). The difference in albumin content in pooled uremic sera was approximately 10% less when measured by the BCG assay as compared to an electrophoretic method.

In another drug study, the plasma protein alpha₁-acid glycoprotein (AAG), which binds basic drugs more avidly than albumin, has also been found to be carbamylated in patients with renal failure (37). *In vitro* carbamylation of AAG or normal serum protein, causes decreased binding of the basic drug penbutolol. However, in uremic patients there is no difference in free penbutolol as compared to normals. The overall affinity constant for the binding of penbutolol to AAG was decreased, but the counter balance to this was an increased amount of AAG.

1.5.2.3 Lipoproteins

Abnormalities in Renal Insufficiency

Chronic renal failure is accompanied by changes in lipid metabolism (114). The abnormalities are primarily in triglyceride-rich lipoproteins and are reflected in the apolipoprotein component rather than the lipid profile. Hypertriglyceridemia can develop in advanced renal failure, but cholesterol levels are usually in the normal range.

Apolipoprotein concentrations change as follows: reduced levels of apoA-I and apoA-II; normal or slightly reduced levels of apoE; normal or slightly increased levels of apoB and apoC-I; increased levels of apoC-II; and significantly increased levels of apoC-III. The abnormalities of the lipoproteins cause impaired catabolism such as lipolysis and receptor mediated uptake. The increased residence time of lipoproteins (VLDL and LDL), albeit not concentration, may be an atherogenic potential, cardiovascular and

glomerulovascular. Alteration in lipoproteins also includes modifications such as acetaldehyde, formaldehyde, glucose, and cyanate derivatization which have been shown to alter the metabolism of LDL *in vitro* and *in vivo* (49). The pathophysiology and clinical significance of these alterations is complex and remains to be clarified.

Decreased Clearance of LDL in Uremic Patients

There is strong evidence that LDL is an important risk factor for the development of atherosclerosis (114). The defects in either LDL particles or the LDL apoB/E receptors may cause alteration in its metabolism (115). Studies have shown that *in vitro* carbamylated LDL (116) or LDL isolated from uremic patients does not bind as well to apoB/E receptors on human fibroblasts and subsequently its clearance is reduced (117). Hörkko *et al.* have found that in rabbits, *in vitro* carbamylated apoB causes reduced binding to LDL apoB/E receptors (49). However, this reduced binding was dependent upon the extent of carbamylation. When LDL was carbamylated (9 - 20% of free amino groups derivatized), reduced uptake was seen, but when modification was greater (>20%), there was accelerated clearance of LDL. Only heavily carbamylated LDL was recognized by scavenger receptors. They also isolated LDL from uremic individuals and found there to be reduced binding to LDL apoB/E receptors in rabbits. No estimation on the amount of carbamylation on uremic LDL particles was done. However, due to the short half-life of LDL in plasma, the relative amount of carbamylation is unlikely to be extensive enough to be taken up by the scavenger pathway.

Studies in man have shown that *in vitro* carbamylated LDL (6%) is cleared more slowly than normal, whereas the clearance of 18% carbamylated LDL is accelerated (118). The clearance of LDL from patients on hemodialysis and continuous ambulatory peritoneal dialysis has been compared (115). LDL clearance in hemodialysis patients was found to be decreased slightly and markedly decreased in CAPD patients.

Low LDL clearance might also be due to alterations in the LDL receptor.

In a study on the impaired binding of LDL to LDL apoB/E receptors in uremic guinea pigs, it was shown that the decreased binding was due to a reduced number of receptors rather than to altered receptor affinity (119). A supporting study (115) showed that LDL apoB/E mRNA expression was lower than normal in lymphocytes isolated from uremic patients. Another possibility that has not been explored is receptor modification, for example, by carbamylation.

Immunogenicity of Carbamylated LDL

Steinbrecher *et al.* (50) carried out a study to see if small chemical substituents on lysine residues of homologous proteins could render the protein immunogenic. They found that guinea pig LDL or albumin when carbamylated, acetylated, ethylated or methylated was immunogenic in guinea pigs. Furthermore, the antibodies generated against modified LDL were exclusively immunogenic towards the derivatized lysine on any protein or the free form (e.g. homocitrulline for carbamylation). Antibodies generated against other modified proteins reacted primarily towards that protein and not towards any other protein with that derivative or the free form.

This finding was used for the generation of a region-specific monoclonal antibody against glycated proteins (120) with *in vitro* glycated LDL (murine and human combined). The murine monoclonal antibody reacted only towards glycated proteins and not to nonderivatized proteins.

Then in 1991 Kraus *et al.* (63) using *in vitro* carbamylated guinea pig LDL immunized guinea pigs and obtained polyclonal antisera to homocitrulline. These antisera were able to detect homocitrulline in the immunogen as well as in other carbamylated blood proteins in guinea pigs and in humans.

1.5.2.4 Other carbamylated compounds

Kraus *et al.* (57, 82) has found alterations in tyrosine and phenylalanine in plasma from patients with renal failure. A longitudinal study was done to investigate the usefulness of measuring N-carbamyl tyrosine for evaluation of patients with end-stage renal disease on CAPD (57). Renal patients were found to have approximately one N-carbamyl tyrosine (70.1 $\mu\text{mol/L}$ N-C-Tyr) for every

tyrosine (77.2 $\mu\text{mol/L}$ Tyr) molecule. Since diet influences the Tyr values, the ratio of N-C-Tyr/Tyr or carbamylation index (CI) was used for the evaluation. They found that a reduction in the number of dialysis exchanges were related to increases in CI and urea. In contrast, when the dialysis exchanges were added, or if there was supplemental hemodialysis, the CI decreased followed by a decrease in urea. Also, episodes of peritonitis increased the CI with little effect on Tyr or urea concentration. This indicated that there was a difference in the phenomena that these parameters measured. They therefore suggested its use as another evaluation tool for CAPD patients.

1.6 Research Proposal

The determination of carbamylated proteins in patients with renal failure has been shown to be significant as discussed above. However, the complete utility of measuring carbamylated proteins has not been investigated adequately. Based on the present state of knowledge, the benefits of measuring carbamylated hemoglobin, albumin and others is still unclear. Furthermore, the investigation of the pathological significance of carbamylated proteins has only been touched upon.

Carbamylation research in renal failure is relatively new and the lack of good methodologies limit the advancement of this area. The research that has been done so far has concentrated on carbamylated hemoglobin. By analogy to glycosylated proteins in diabetes, there is also a potential utility of both carbamylated long-lived proteins (hemoglobin), and short-lived proteins (albumin) as indicators in renal failure.

The principal focus of this study is to examine if the measurement of short-lived proteins can be of benefit for patients with renal failure. As a first assessment, the difference between carbamylated total protein values in normal individuals and uremic patients will be determined. These values will then be correlated to the carbamylated hemoglobin concentrations from the same

samples.

An enzyme-linked immunoassay for carbamylated human serum albumin, the major serum protein, will also be developed. This type of assay will allow it to be used routinely in a clinical laboratory where it can be of most value for patient management. Polyclonal antibodies will be produced in rabbits against *in vitro* prepared CHSA.

A six-month longitudinal study of hemodialyzed patients will examine the clinical utility of measuring carbamylated proteins. This will be done by comparing carbamylated hemoglobin and carbamylated total protein with currently used uremic indices (pre-dialysis urea and Kt/V).

In addition, this study will look at albumin carbamylation in terms of time dependency and its purification from plasma. Also, the diacetyl monoxime assay for carbamylated proteins and the valine hydantoin HPLC assay for carbamylated hemoglobin will be evaluated.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals were purchased from various companies and are listed in Table 2.1.

All water used in preparation of solutions was Type II water or better. Distilled water was passed through either a mixed bed ion-exchanger, or a second distillation apparatus and/or a mixed bed ion-exchanger. Type I water was produced using the Milli-Q water system.

2.1.2 Enzymes and Proteins

All enzymes and proteins were purchased from Sigma Chemical Company (St. Louis, MO) (see Table 2.2).

2.1.2.1 Antibodies

See Table 2.3.

2.1.3 Chromatography Resins

Chromatography resins used included Affi-Gel Blue Gel (50-100 mesh), Affi-Gel Protein A Agarose, Affi-Gel 15 Gel, and Bio-Gel® P-6 (200-400), purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON).

CM-cellulose [C-4031], and Sephadex G-25 [G-25-150] were purchased from Sigma Chemical Co. (St. Louis, MO).

2.1.4 Test Kits

Albumin (BCP), total hemoglobin, protein assay kits (Bio-Rad Protein Assay: Kit I *bovine gamma globulin standard*, Kit II *bovine serum albumin standard*; and DC Protein Assay), and urea (rate), were purchased from Sigma Diagnostics Canada (Mississauga, ON).

2.1.5 Supplies

See Table 2.4.

TABLE 2.1

List of Chemicals

Acetic acid, glacial, <i>Assured ACS</i> ⁴
Acetone, <i>Assured ACS</i> ⁴
Acetonitrile, <i>OmniSolv</i> ⁴
Acrylamide, 99.9% ⁵
2-Aminobenzoic acid, 98%+ ¹
2-Amino-5-bromobenzoic acid, 97% ¹
2-Amino-5-chlorobenzoic acid, 90% ¹
4-Aminodiphenylamine, 98% (semidine) [A-7657] ¹²
2-Amino-5-iodobenzoic acid, 97% ¹
Ammonium persulfate ³
Biolyte® 6/8 Ampholyte, 40% ⁵
Biolyte® 4/6 Ampholyte, 40% ⁵
Biolyte® 3/10 Ampholyte, 40% ⁵
Bis (N,N'-Methylene- <i>bis</i> -acrylamide) ⁵
Cadmium chloride, ACS ³
N-Carbamyl-DL-aspartic acid [C-4250] ¹²
N-Carbamyl-DL-norvaline [C-5375] ¹²
N-Carbamyl-DL-valine [C-6250] ¹²
L-Citrulline (L-2-amino-5-ureidovaleic acid) ¹⁰
Coomassie Brilliant Blue R 250 ¹¹
Crocein Scarlet ⁵
Diacetyl monoxime, <i>AnalaR</i> ⁴
Dicyclohexylammonium salt [U-7050] ¹²
Diethanolamine, <i>AnalaR biochemical</i> ⁴
Diethyl ether, <i>Assured ACS</i> ⁴
N,N-Dimethylaniline, 99% ¹
Ethanol, 95% ⁸
Ethanol, anhydrous ⁶
Ethylenediaminetetraacetic acid tetrasodium salt (EDTA), >99% [E-5391] ¹²
Ferric chloride (lump) ⁸
Glycine <i>Assured</i> ⁴
Glycerol, approx. 99% [G-7757] ¹²
L-Homocitrulline ¹⁰
Hydrochloric acid, <i>AnalaR</i> ⁴
Lauryl sulfate sodium salt ¹⁴
Methanol, <i>OmniSolv</i> ⁴
Methanol ⁸
MOPS (3-[N-morpholine]propanesulfonic acid) [M-1254] ¹²
<i>p</i> -Nitrophenylphosphate, picrylsulfonic acid hydrate, 97% (TNBS) ¹
N-phenyl- <i>p</i> -phenylenediamine·HCl ¹²
Polyacrylamide solution, 30% [37.5:1] ⁵
Potassium phosphate monobasic ⁸

Table 2.1 List of Chemicals - continued

Potassium chloride, *Assured ACS*⁴
 Potassium [¹⁴C]cyanate (52 mCi/mmol)²
 Riboflavin-5'-phosphate, sodium salt dihydrate⁵
 Sodium acetate, anhydrous, *Assured ACS*⁴
 Sodium azide⁸
 Sodium borate (tetra-crystal)⁸
 Sodium carbonate (anhydrous), *ACS*⁴
 Sodium chloride, *Assured ACS*⁴
 Sodium citrate, *ACS*⁴
 Sodium cyanate, 96%¹
 Sodium hydrogen carbonate, *AnalaR*⁸
di-Sodium hydrogen orthophosphate heptahydrate, *Assured ACS*⁴
 Sodium hydroxide, 10N, *Assured*⁴
 Sulphuric acid, *Assured ACS*⁴
 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate system [T-8540]¹²
 N,N,N',N'-Tetramethylethylenediamine (TEMED)⁵
 Thiosemicarbazide⁷
 Trichloroacetic acid solution, 12%¹³
 Tris (hydroxymethyl)methylamine, *Assured ACS*⁴
 Tween-20, *EIA purity*⁵
 Uracil (2,4-dihydropyrimidone; crystalline) [U-0750]¹²
 Urea [U-5128]¹²
 L-Valine⁹

¹Aldrich Chemical Company, Inc. (Milwaukee, WI)²Amersham International plc (Buckinghamshire, England)³Anachemia Chemicals Ltd. (Toronto, ON)⁴BDH Inc. (Toronto, ON)⁵Bio-Rad Laboratories Ltd. (Mississauga, ON)⁶Commercial Alcohols Inc. (Brampton, ON)⁷Eastman Organic Chemicals (Rochester, NY)⁸Fisher Scientific Company (Fair Lawn, NJ)⁹Fluka Chemical Corp. (Ronkonkoma, NY)¹⁰ICN Biomedicals Inc. (Aurora, OH)¹¹Merck (Darmstadt, Germany)¹²Sigma Chemical Company (St. Louis, MO)¹³Sigma Diagnostics Canada (Mississauga, ON)¹⁴United States Biochemical Corporation (Cleveland, OH)

TABLE 2.2

List of Enzymes and Proteins

Albumin *bovine, initial fractionation by heat shock, Fraction V, approx. 98-99%* [A-6793]
 Albumin *human, approx. 99%* [A-8763]
 Albumin *human, Fraction V, 96-99%* [A-1653]
 Carbonic anhydrase I *IEF marker from human erythrocytes, pl 6.6* [C-5653]
 Carbonic anhydrase II *IEF marker from bovine erythrocytes, pl 5.4* [C-3666]
 Carbonic anhydrase II *IEF marker from bovine erythrocytes, pl 5.9* [C-6403]
 Fibrinogen *Fraction I Type III: from human plasma* [F-4129]
 Glucose oxidase *IEF marker from Aspergillus niger, pl 4.2* [G-7146]
 Hemoglobin *human* [H-7379]
 IEF mix, *pl 3.6 - 6.6* [I-8012]
 β -Lactoglobulin A *IEF marker from bovine milk, pl 5.1* [L-5137]
 Lectin from lens *culinaris* [L-9267]
 Lipoprotein, *low density from human plasma* [L-5402]
 Myoglobin *IEF marker from horse heart, pl 6.8, 7.2* [M-9267]
 MW-SDS-200 Kit, *for molecular weight range 30,000-200,000* [SDS-6H]
 Trypsin inhibitor *IEF marker from Soybean, pl 4.6* [T-1021]
 Urease *Type III: from Jack Beans* [U-1500]
 Urease *attached to 4% corss-linked beaded agarose* [U-7878]

Table 2.3

List of Antibodies

Anti-human albumin, *developed in rabbit IgG fraction of antiserum* [A-0433]²
 Anti-rabbit IgG (whole molecule) *peroxidase conjugated adsorbed with human serum protein* [A-4914]²
 Anti-rabbit IgG (whole molecule) *alkaline phosphatase conjugate affinity isolated antibody* [A-8025]²
 Sheep anti-human albumin *alkaline phosphatase*¹

¹Biodesign International (Kennebunk, ME)²Sigma Chemical Company (St. Louis, MO)

TABLE 2.4
List of Key Supplies

Concentrators	
Centricon-30	
Microcon-30	
	Amicon Canada Ltd. (Beverly, MA)
Dialysis Tubing	
Spectra/Por® 1 (MWCO 6,000-8,000)	
Spectrum® (Houston, TX)	
Filters	
Acrodisc LC13 PVDF 0.2µm syringe filters, minispikes outlet (4450)	
Gelman Sciences (Ann Arbor, MI)	
Millipore membranes Type HVLP 0.45µm	
Millipore Corporation (Milford MA)	
Gel Support Film (IEF)	
Gel support for acrylamide, 125 x 65 mm	
Bio-Rad Laboratories Inc. (Mississauga, ON)	
HPLC Column	
Reverse phase APEX ODS II 5µ	
Jones Chromatography Ltd. (Lakewood, CO)	
HPLC Vials	
Vial, clear screw top (1.5 mL)	
Chromatographic Specialities, Inc. (Brockville, ON)	
Ion-exchange Cartridge	
HN Ultrapure (mixed bed dl)	
Barnstead Thermolyne Corporation (Dubuque, IA)	
Microtitration Strip Wells	
Costar EIA/RIA 8 well strip (2580)	
Costar Corporation (Cambridge, MA)	
Coming ELISA wells 1x8 strips (24101-8)	
Coming Inc. (Coming, NY)	

2.1.6 Instrumentation

See Table 2.5.

2.1.7 Human Sera

Blood samples used in this study were obtained from either the Windsor Western Hospital Laboratory or the Hotel Dieu-Grace Hospitals Laboratory (Grace site), Windsor, Ontario. All blood used was leftover blood obtained from routine phlebotomies. EDTA whole blood was stored at 4°C for up to 2 weeks. The red cells were separated from the plasma by centrifugation, washed 3 times with phosphate buffered saline and resuspended in an equal volume of PBS. Both the plasma and red blood cells were frozen at -20°C until required.

Outpatients with normal urea and glucose concentrations were considered as normal subjects.

2.1.8 Renal Patients

Patients used in this study were selected from the Renal Dialysis Unit at the Hotel Dieu-Grace Hospitals (Grace site). Approval for the study was given by both the University of Windsor Ethics Committee and the Salvation Army Grace Hospital (now Hotel Dieu-Grace Hospitals), Windsor, Ontario. Consent forms were also obtained from the patients in order to obtain diagnostic, demographic and laboratory data from their hospital charts. All blood used was leftover blood obtained from routine phlebotomies. The documentation used in this study is contained in the APPENDIX.

All patients who were referred to the Renal Unit at the Salvation Army Grace Hospital were potential subjects for this study. However, only the patients who gave consent for the study were considered (82 patients). These included 50 patients on hemodialysis, 9 CAPD patients, 9 transplant patients, and 2 predialysis patients. The 12 patients unaccounted for died before completion of the study.

The initial longitudinal study was to be for 1 year, but due to a number of reasons, the study was shortened to 6 months. Only blood that was leftover

TABLE 2.5
List of Instruments

Balances

- Electronic balance (Model ER-60A)
A&D Company Ltd. (Toyko, Japan)
- Mettler P1000
Mettler Instrument Corp. (Hightstown, NJ)

Centrifuges

- Eppendorf centrifuge 5415C
Eppendorf Inc. (Hamburg, Germany)
- International clinical centrifuge (Model 3781M-9)
International Equipment Co. (Boston, MA)
- Sorvall superspeed RC2-B automated refrigerated centrifuge
Sorvall (Newtown, CN)

Cuvettes

- quartz glass (1cm x 1mL)
- special optical glass (1cm x 3.5mL; 1cm x 1.5mL)
Hellma (Canada) Ltd. (Concord, ON)

Densitometer

- Imaging Densitometer (Model GS-670)
Bio-Rad Laboratories Inc. (Mississauga, ON)

Dri-bath

- Thermolyne Dri-Bath (Model DB28125)
Barnstead|Thermolyne Corporation (Dubuque, IA)

Electrophoresis Systems

- Model 111Mini IEF Cell (with casting tray)
Bio-Rad Laboratories Inc. (Mississauga, ON)
- SE 250 Mighty Small II
Hofer Scientific Instruments (San Francisco, CA)

HPLC System

- SPD-10A uv-vis detector
- LC-10AD solvent delivery module (2)
- SCL-10A system controller
- SIL-10A autoinjector
- C-R4A Chromatopac (data processor and printer)
Shimadzu Corporation (Kyoto, Japan)

Microplate Reader

- Micro Reader III
Hyperion Inc. (Miami, FL)
-

Table 2.5 List of Instruments - continued

pH Meter

Coming pH meter 340
Coming Inc. (Coming, NY)

Pipetting Systems

Eppendorf Repeater® pipette
Brinkman Instruments Inc. (Wesbury, NY)
Gilson pipetman (Models P-20, P-100 and P-1000)
Mandel Scientific Co. Ltd. (Guelph, ON)
Nichiryo digital micropipette (Model 5000DG)
Nichiryo Co., Ltd. (Toyko, Japan)

Power Supply

Bio-Rad (Model 500) power supply
Bio-Rad Laboratories Ltd. (Mississauga, ON)

Shakers

Lab-line orbit shaker (Model 3520)
Labline Instruments, Inc. (Melrose Park, IL)
Thermolyne Type 16700 mixer
Barnstead|Thermolyne Corporation (Dubuque, IA)
Wallac Delfia plate shake (Model 1296-002), microtitration plate shaker
Wallac Oy (Turku, Finland)

Spectrophotometers

Hitachi F-3100 Fluorescence Spectrophotometer
Hitachi (Toyko, Japan)
Response® UV-VIS Spectrophotometer
Ciba-Coming Diagnostics Corp. (Toronto, ON)
Shimadzu UV-169 uv-visible recording spectrophotometer
Shimadzu Corporation (Kyoto, Japan)

Stirrer/Hotplate

Coming stirrer/hotplate
Coming Inc. (Coming, NY)

Water Bath

10-L water bath
Fisher Scientific (Pittsburgh, PA)

Water Heater

Water heater (Model ED 66537)
Haake (Berlin, Germany)

Water System

Milli-Q water system
Millipore Corporation (Milford, MA)

from routine tests was used for this study and unfortunately not all these samples were obtainable. Also, one month of samples was lost during a freezer breakdown. Therefore, no individual patient had samples available for 12 consecutive months. In the end only 7 patients were used in the study.

2.2 Methods

2.2.1 Automated and Test Kit Assays

Protein, total hemoglobin, albumin and urea (rate) assays were performed according to the instructions given in the test kits. Table 2.6 lists the test methodologies used for each kit. Test kits were purchased from Sigma Diagnostics Canada (Mississauga, ON). Human serum albumin (99%) was the standard used in both protein assays.

Automated assays were performed on the Kodak Ektachem 700 or 700XR Analyzer. Table 2.7 lists the test methodologies for the analytes.

2.2.2 Preparation of Carbamylated Proteins

Human serum albumin (99%), fibrinogen and hemoglobin were carbamylated by incubation with 100 mM sodium cyanate in 0.1 M phosphate buffer, pH 7.4 at R.T.¹. The incubation time varied depending on the extent of carbamylation required. Unreacted cyanate was removed from the protein by gel filtration (Bio-Gel P-6G).

The extent of carbamylation was estimated with the trinitrobenzene-sulfonic acid assay (see Section 2.2.4).

2.2.3 Measurement of Cyanate

The method used was adapted from Guilloton and Karst (9).

Solutions

20 mM 2-Aminobenzoic acid. 2-Aminobenzoic acid (137 mg) was

¹ The designation R.T. will be used throughout this dissertation to represent room temperature.

TABLE 2.6
Test Kit Methodologies

Test	Methodology	Absorption Maximum
Albumin	Albumin + Bromcresol purple ⇨ Blue-purple color complex	600 nm
Hemoglobin	① Hemoglobin + Alkaline potassium ferricyanide ⇨ Methemoglobin	540 nm
	② Methemoglobin + Potassium cyanide ⇨ Cyanmethemoglobin	
Protein (Bradford)	Protein + Coomassie Brilliant Blue G-250 + H ⁺ ⇨ Blue color complex	595 nm
Protein (Lowry)	① Protein + Alkaline copper tartrate (alkaline) ⇨ Copper-protein complex	750 nm
	② Copper-protein complex + Folin-Ciocalteu reagent ⇨ Folin-Ciocalteu reagent (reduced)	
Urea	① Urea + urease ⇨ (NH ₄) ₂ CO ₃	340 nm
	② NH ₄ ⁺ + α-Ketoglutaric acid + NADH + glutamate dehydrogenase + ADP + H ⁺ ⇨ NAD ⁺ + Glutamic acid	

TABLE 2.7
Kodak Ektachem Test Methodologies

Test	Methodology	Maximum Absorption
Albumin	Albumin + Bromocresol green (BCG) ⇨ BCG-Albumin complex	630 nm
Creatinine	Creatine + H ₂ O + Creatine amidohydrolase ⇨ Creatine	670 nm
	Creatine + H ₂ O + Creatine amidinohydrolase ⇨ Sarcosine + Urea	
	Sarcosine + O ₂ + H ₂ O + Sarcosine oxidase ⇨ Glycine + HCO + H ₂ O ₂ H ₂ O ₂ + Leuko dye + Peroxidase ⇨ Colored dye	
Total Protein	Protein + Copper tartrate + LiOH ⇨ Colored complex	540 nm
Urea	Urea + H ₂ O + Urease ⇨ 2 NH ₃ + CO ₂	670 nm
	NH ₃ + pH indicator dye ⇨ Change in absorbance spectrum of dye	

Method details can be found in: Spayd RW, Bruschi B, Burdick BA, Dappen GM, Eikenberry JN, Esders TW, Figueras J, Goodhue CT, LaRossa DD, Nelson RW, Raud RN, Wu T-W. Multilayer film elements for clinical analysis: Applications to representative chemical determinations. *Clin Chem* 1978; 24: 1343-50.

dissolved in 50 mL hot water. Note that other derivatives of this compound required the presence of a small amount of alcohol to get them into solution.

Procedure

Sample or standard (0.5 mL) was mixed with 0.5 mL 20 mM 2-aminobenzoic acid and incubated for 10 min at 40°C. If the solution contained protein, 2.5 mL 12% TCA were added and then centrifuged for 1 min. The supernatant (1 mL), not done if TCA step was omitted, was removed and 1 mL 12 N HCl added. The mixture was boiled for 1 min and then cooled to R.T.. Absorbances were read at 310 nm against a blank containing no cyanate. Unknown cyanate concentrations were determined from the standard curve (0.005 - 1 μ M).

Modifications

Several other derivatives of 2-aminobenzoic acid were investigated to find out if the assay could be made more sensitive. The reactants were: 20 mM 2-amino-5-chlorobenzoic acid; 20 mM 2-amino-5-bromobenzoic acid; and 20 mM 2-amino-5-iodobenzoic acid. Preliminary results showed that bromo and iodo derivatives gave no peak between 280 nm to 400 nm. The chloro derivative produced a bathochromic shift (378 nm) and a hyperchromic effect.

2.2.4 Determination of Free Amino Groups (TNBS Assay)

This assay is an indirect method to detect free amino groups (71). The extent of carbamylation is inversely proportional to the number of reactive amino groups.

Solutions

0.1 M Borate buffer. NaB_4 (3.81 g) was dissolved in 100 mL of water and the pH adjusted to 9.3.

0.03 M TNBS. TNBS (0.22 g) was dissolved in 25 mL borate buffer. This solution must be made fresh each day.

Procedure

To a 50- μ L sample or control (1 mg/mL HSA), 950 μ L borate buffer and 25 μ L TNBS were added. The solution is mixed well and incubated for 30 min at

R.T.. The absorbance is read at 420 nm.

The loss of free amino groups was expressed as a percentage and was calculated as follows:

$$\% \text{ loss} = [1 - (\text{sample absorbance}/\text{standard absorbance})] \times 100\%$$

2.2.5 Electrophoresis

2.2.5.1 Isoelectric-focusing

The procedure for isoelectric focusing (IEF) used was similar to that outlined in the instruction manual for the Model 111 Mini IEF Cell. The only modification was in the catalyst solution. The ammonium persulfate volume was increased to 40 μL versus 15 μL and TEMED to 5 μL versus 3 μL . This was necessary in order for polymerization to occur within 1 h. Staining and destaining was done by all methods, but the most frequently used method was Method B which incorporated the fixing step with the staining step.

Solutions

Monomer-ampholyte solution. Monomer concentrate (2 mL), H_2O (5.5 mL), 25% glycerol (2 mL) and 4/6 ampholyte, 40% (0.5 mL) were mixed and degassed for 5 min under vacuum.

Catalyst solution. Ammonium persulfate, 10% (w/v) (40 μL) was prepared fresh and combined with 50 μL riboflavin-5'-phosphate, 0.1% (w/v), and 5 μL TEMED (neat).

Stain B. Crocein scarlet (0.5 g) was dissolved in 270 mL ethanol. To this was added 100 mL of acetic acid, 0.4 g Coomassie Brilliant Blue (R-250), 5 g CuSO_4 and diluted to 1 L with H_2O .

First destaining solution. CuSO_4 (0.5 mg) was dissolved in 120 mL of ethanol. To this solution was added 70 mL of acetic acid and diluted to 1 L with H_2O .

Second destaining solution. Ethanol (250 mL) was mixed with 70 mL of acetic acid and diluted to 1 L with H_2O .

Procedure

Gels (125 x 65 x 0.4 mm) were cast using two polyacrylamide gel supports adhered to glass plates placed on the casting tray. Immediately before use, the catalyst solution was added to the monomer-ampholyte solution and swirled gently. A pipette was used to gently dispense the polyacrylamide solution between the casting tray and gel support/glass plate. The gels were irradiated for 1 h using a fluorescent desk lamp. Following polymerization the samples (desalted, 2-5 μ L) were applied to the gel using the sample applicator and allowed to diffuse for 5 min. The gel (sample side down) was then placed onto graphite electrodes (moistened with H₂O) of the Mini IEF Cell and run for 90 min under constant voltage in a stepped fashion (100 V, 15 min; 200 V, 15 min; and 450 V, 60 min). Gels were then stained with stain B for about 1 h, destained twice with the first destaining solution (15 min periods), and then destained with the second destaining solution (about 1 h). The total time for staining and destaining was kept under 4 h since the gel would detach from the gel support if left longer. The gel was then left on the bench to dry overnight.

IEF markers were run on each gel, the combination depending on the IEF range of the gel. The markers included: trypsin inhibitor, *pI* 4.6; β -lactoglobulin, *pI* 5.1; carbonic anhydrase II, *pI* 5.4, *pI* 5.9; carbonic anhydrase I, *pI* 6.6; myoglobin, *pI* 6.8, 7.2; lectin from lens culinaris, *pI* 7.8, 8.0. Premixed IEF markers (3.6 - 6.6) were also used. The relative *pI* of the unknown proteins was calculated from a standard curve constructed with these markers (Figure 2.1).

2.2.5.2 SDS polyacrylamide gel electrophoresis

The polyacrylamide gels made were discontinuous with a 10% resolving gel and a 4% stacking gel. The solutions and the procedure followed was as described in the instruction manual for the SE 250 Mighty Small II. After destaining the gels were scanned using Bio-Rad's Imaging Densitometer. Gels were also stored for a short time in a ziplock bag containing a some water.

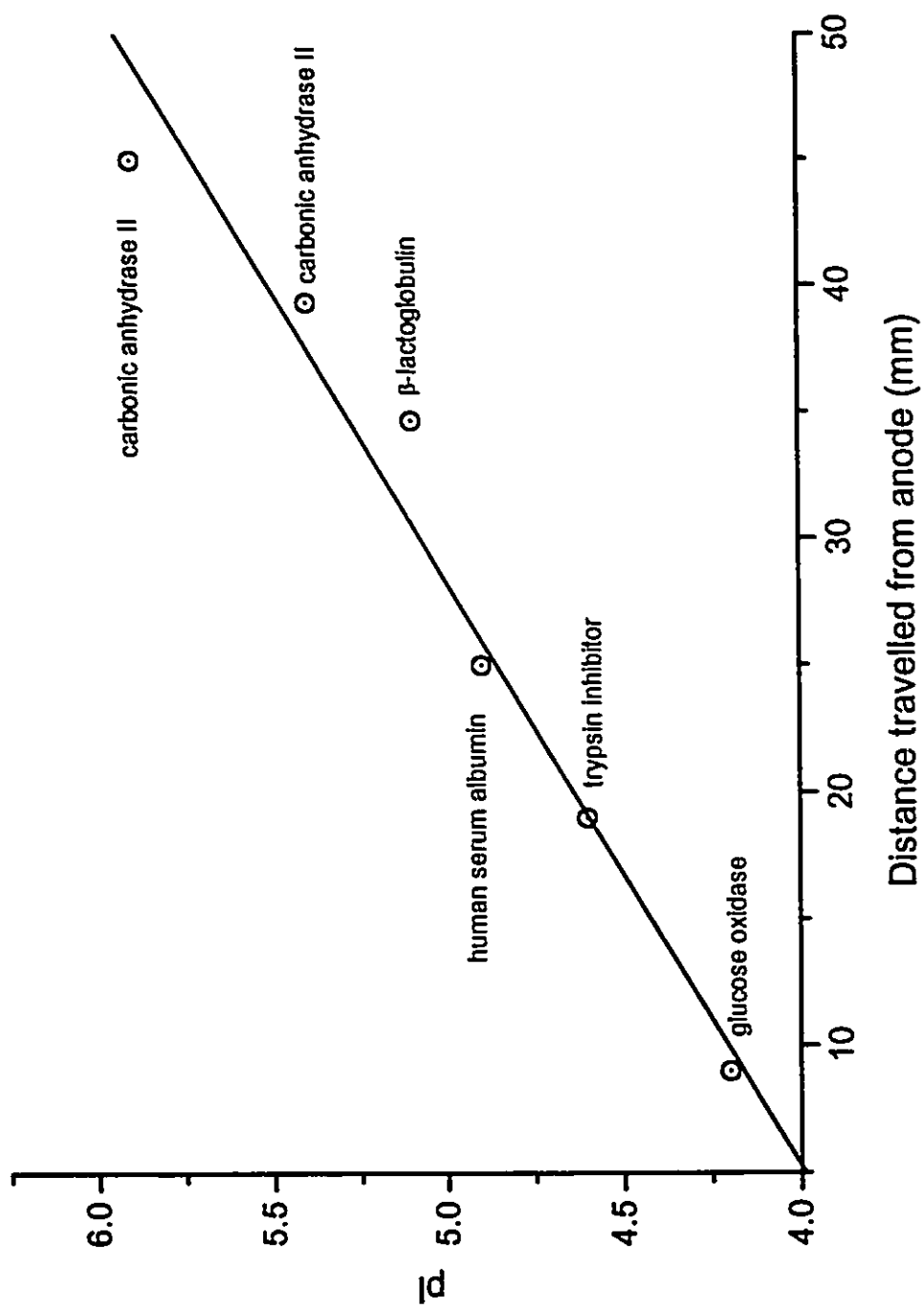
Solutions

Separating gel (10%T 2.7%C). Polyacrylamide solution (30%T 2.7%C)

FIGURE 2.1**IEF Standard Curve***Legend*

IEF markers were run on a Bio-Lyte 4/6 gel (125 x 65 x 0.4 mm) in a Model 111 Mini IEF Cell (100 V for 15 min, 200 V for 15 min and 450 V for 1 h). The relative distances travelled of each marker were plotted against the pI of the marker to obtain a standard curve. This is an example of a typical standard curve ($y = 0.043x + 3.77$, $r = 0.98$).

FIGURE 2.1



(3.33 mL), 2.5 mL resolving gel buffer (1.5 M Tris-Cl, pH 8.8), 0.1 mL 10% SDS, and 4 mL H₂O were mixed well and degassed for 5 min under vacuum. Immediately before use 50 µL ammonium persulfate (10%) and 5 µL TEMED (neat) were added to the solution.

Stacking gel (4%T 2.7%C). Polyacrylamide solution (30%T 2.7%C) (0.67 mL), 1.35 mL stacking gel buffer (0.5 M Tris-Cl, pH 6.8), 50 µL 10% SDS, and 3 mL H₂O were mixed well and degassed for 5 min under vacuum. Immediately before use 25 µL ammonium persulfate (10%) and 2 µL TEMED (neat) were added.

Treatment buffer. Stacking gel buffer (0.5 M Tris-Cl, pH 6.8) (2.5 mL), 4 mL SDS (10%), 2 mL glycerol, 1 mL 2-mercaptoethanol, and a few crystals of bromphenol blue (tracking dye) were mixed and diluted to 10 mL with H₂O.

Stain. Coomassie Brilliant Blue R-250 (0.125 g) was dissolved in 200 mL methanol. To this solution was added 35 mL acetic acid and then diluted to 500 mL with H₂O.

Destaining solution I. Methanol (400 mL) and 70 mL acetic acid were mixed and diluted to 1 L with H₂O.

Destaining solution II. Methanol (50 mL) and 70 mL acetic acid were mixed and diluted to 1 L with H₂O.

Procedure

Gels (8 x 10 x 0.075 cm) were cast using the SE 250 Mighty Small II gel apparatus. The stacking gel was poured in first and allowed to polymerize (1 h) before the resolving gel solution was added. Samples were prepared by mixing equal volumes of protein solution with treatment buffer. This mixture was boiled for a few minutes and then cooled before application to the gel. Gels were run at constant current (20 mA/gel) for about 1 h (tracking dye at bottom of gel). The gels were stained with shaking for 2-6 h, destained with destaining solution I for 1-4 h, and then left in destaining solution II overnight. Gentle heating was also used to speed up the destaining procedure.

Protein markers (MW-SDS-200) were run on each gel and consisted of:

carbonic anhydrase (29 kDa); ovalbumin (45 kDa); BSA (66 kDa); phosphorylase B (97.4 kDa); β -galactosidase (116 kDa); and myosin (205 kDa).

2.2.6 Purification of Albumin From Plasma

Affi-Gel Blue affinity gel was used to selectively bind albumin. Several methods were employed during the progress of this research in order to determine which one would yield the best albumin preparation (121-124). The differences between the methods were with the buffers strengths and pH's and with the type of eluting agent used.

The predominant method used was a slightly modified procedure of Bio-Rad's Bulletin 1107. Briefly, 5 mL of Affi-Gel Blue gel was washed with equilibrating buffer (0.02 M phosphate buffer, pH 7.4) and 1 mL of equilibrated sample applied. The column was then washed with equilibrating buffer until protein was no longer detected. This was done by taking 8 μ L of effluent and mixing it with 2 μ L of Bio-Rad protein dye reagent. The resulting color was compared to the buffer blank. The bound albumin was eluted with 1.4 M NaCl until no protein was detected as before. The column was regenerated with 10 mL of 2 M guanidine-HCl and then reequilibrated with 10 mL of equilibrating buffer.

2.2.7 Measurement of Carbamylation

Two methodologies for the measurement of carbamylation were performed. The original assay was developed by Hunninghake and Grisolia (76) and then modified by Wybenga *et al.* (77) in order to reduce the causticity of the assay.

In both cases it was necessary to remove any contaminating urea or urea-like compounds from the carbamylated protein. This was done by several methods.

2.2.7.1 Urea removal

Alcohol Precipitation

Plasma or serum proteins were precipitated with alcohol (95% ethanol or methanol) in a 1:10 ratio according to the method of Erill *et al.* (17) using 10-mL

borosilicate test tubes. The mixture was centrifuged in a clinical centrifuge and decanted. The precipitate was washed twice with alcohol and resuspended in water. The precipitate was too compact if the precipitation was done in Eppendorf tubes and then spun in a microfuge.

Gel Filtration

Sephadex G-25 and Bio-Gel P-6 were both used for gel exclusion chromatography. Small samples (<100 μ L) were desalted on microcolumns containing 1 mL of hydrated gel. The microcolumn was placed in a 1.5-mL Eppendorf tube and spun briefly in a microfuge. This type of desalting was sufficient for use for IEF analysis and the removal of low concentrations of cyanate. However, it did not allow for complete removal of urea from plasma or serum samples. Sephadex was the preferred matrix due to its better flow rate over Bio-Gel P-6.

Complete salt or urea removal was obtained by using a 1 x 10 cm column packed with approximately 8.5 mL of Sephadex G-25. Using this column, a 0.5-mL mixture of 100 mM NaOCN and 40 mg/mL HSA could be separated completely (see Figure 2.2). Gel filtered plasma samples showed no detectable amounts of urea or creatinine.

Urease-Agarose Treatment

A 500- μ L sample of undiluted plasma or urea standard (50 mM in PBS) was placed in a 1.5-mL Eppendorf tube and to it was added a 50 μ L suspension of urease-agarose (50 mg/mL). The mixture was incubated for 1 h at R.T. on an orbit shaker. Figure 2.3 illustrates the loss of urea over time.

2.2.7.2 Hunninghake and Grisolia assay (76)

Solutions

3% Diacetylmonoxime (DAM). DAM (3.0 g) dissolved in 100 mL water.

0.2% Semidine (4-aminodiphenylamine). Semidine (200 mg) dissolved in 10 mL 95% ethanol and made up to 100 mL with water, filtered and stored for up to 6 weeks at 4°C.

0.001 M H_2SO_4 - $FeCl_3$. This solution was prepared by adding 1 mL of

FIGURE 2.2**Cyanate Removal by Gel Filtration***Legend*

A 0.5-mL mixture of albumin (40 mg/mL) and NaOCN (100 mM) was separated using a 1 x 10 cm column filled with 8.5 mL of Sepadex G-25. The flowrate by gravity was 1.15 mL/min and fractions were collected at 1-min intervals using 0.1 M phosphate buffer, pH 7.4.

FIGURE 2.2

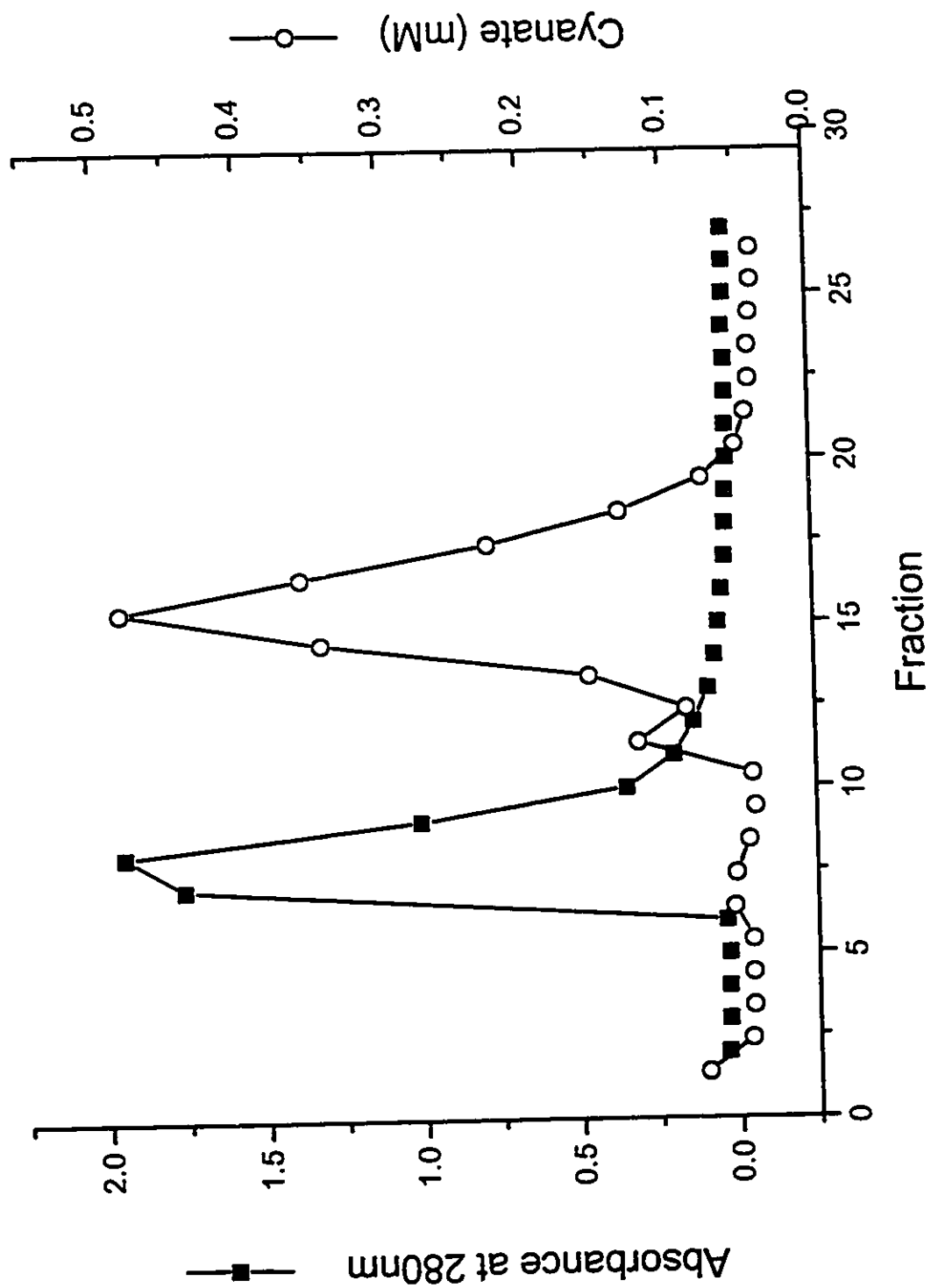
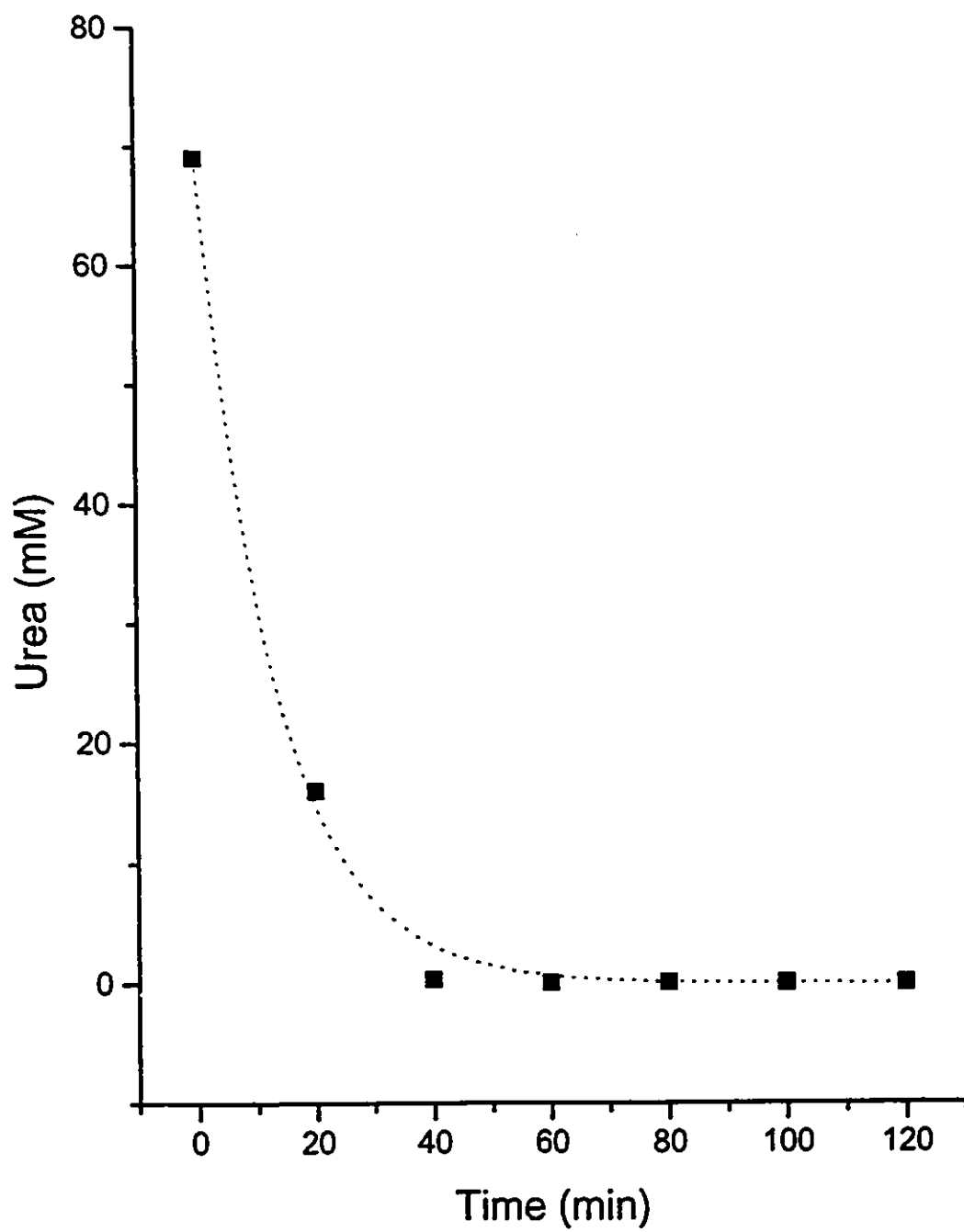


FIGURE 2.3**Hydrolysis of Urea by Urease-agarose Beads***Legend*

Rate of hydrolysis of 50 mM of urea with urease-agarose beads (5 mg/mL).

Duplicate determinations were done. The exponential decay equation describing the curve is $y = 69.14e^{-(x-x_0)/12.92}$.

FIGURE 2.3



0.1 M FeCl_3 (0.13 g FeCl_3 in 10 mL water) to 99 mL H_2SO_4 .

Procedure

Into a 10-mL borosilicate test tube the following was added in order: 1 mL sample or standard, 0.5 mL DAM, 0.5 mL H_2SO_4 , and 0.5 mL semidine. The mixture was vortexed and heated for 10 min at 90°C. The mixture was then allowed to cool for 5-10 min (temperature of solution should be between 30° to 60°C). Then, 1 mL H_2SO_4 - FeCl_3 was added and mixed thoroughly. The mixture was cooled to R.T. and then absorbances read within 1 h at 525 nm. Positive samples were a purplish color.

2.2.7.3 Wybenga et al. Assay (77)

Solutions

Urea nitrogen reagent. The following was combined in order into 100 mL of water contained in a 1 L beaker: 44 ml sulfuric acid and 66 mL 85% phosphoric acid. The mixture was cooled to R.T.. CdCl_2 (2.0 g) and thiosemicarbazide (60 mg) were added while stirring and the mixture diluted to 1 L. The solution was stored in an amber bottle at 4°C for up to 4 months.

Diacetylmonoxime (DAM). DAM (2.0 g) was dissolved in 100 mL water with slight heating. The solution was stored at 4°C.

Procedure

To 0.5 mL of sample, standard or control 2.5 mL urea nitrogen reagent was added. Samples were heated at 100°C for 5 min. Then 0.5 mL DAM was added and samples were heated at 100°C for a further 20 min. After cooling to R.T. the samples were read absorbance at 525 nm. Positive samples were a pinkish color.

2.2.8 Measurement of Carbamylated Hemoglobin

2.2.8.1 Preparation of valine hydantoin standard

Valine hydantoin (VH) standard was prepared by incubating carbamyl valine (13.5 mg) with 11.6 M HCl (5 mL) for 8 h in a dri-bath set at 120°C and then left overnight at R.T.. The solution was rotoevaporated until only a few drops of yellow liquid remained. The tube was then left under vacuum overnight

to remove the last traces of liquid. The resulting residue was slightly yellowish and had a melting point of 145°C which was consistent with reports in the literature (125). The residue was redissolved in approximately 3 mL of water and filtered through a 2- μ m PVDF filter syringe. The filtrate was then left to recrystallize overnight on a watch glass. The crystallized preparation was white.

2.2.8.2 Preparation of valine hydantoin from hemoglobin

The preparation of valine hydantoin was based on the method by Manning *et al.* (74). A 0.5-mL aliquot of red blood cells was placed into a heavy walled 15-mL screw top test tube. To this was added 5 mL of 2%(w/v) HCl in acetone (4°C). The mixture was vortexed, centrifuged for 3 min at 3000 x g and the supernatant discarded. The pellet was washed 3 times with 5 mL of acetone to remove all the heme. The off-white pellet was then washed with 5 mL of diethyl ether, centrifuged and heated in warm water (*ca.* 45°C) until dry. The pellet was then suspended in 0.5 mL of 50% glacial acetic acid and 0.5 mL of concentrated HCl. The tube was capped with a glass marble and the mixture heated for 1 h at 100°C in a dri-bath. The globin is subsequently hydrolyzed. Carbamyl valine residues are released which then spontaneously cyclize to form valine hydantoins (Figure 2.4). The hydrolyzate is cooled immediately in an ice bath. To this were added 0.8 mL of 10 N NaOH and 0.5 mL of saturated NaCl and the resulting suspension mixed well. The pH at this point should be between 4-5 for optimal extraction. Then 100 μ L of the internal standard, 100 mg/mL carbamyl norvaline, was added. The valine hydantoin and carbamyl norvaline are then extracted by adding 5 mL of ethyl acetate and vortexing for 1 min. The ethyl acetate supernatant (4 mL) was transferred to a clean 10-mL borosilicate tube and evaporated to dryness under a stream of air in a dri-bath (70°C). The residue was then placed over NaOH pellets in a vacuum dessicator overnight to remove the last traces of acid.

2.2.8.3 HPLC analysis of valine hydantoin

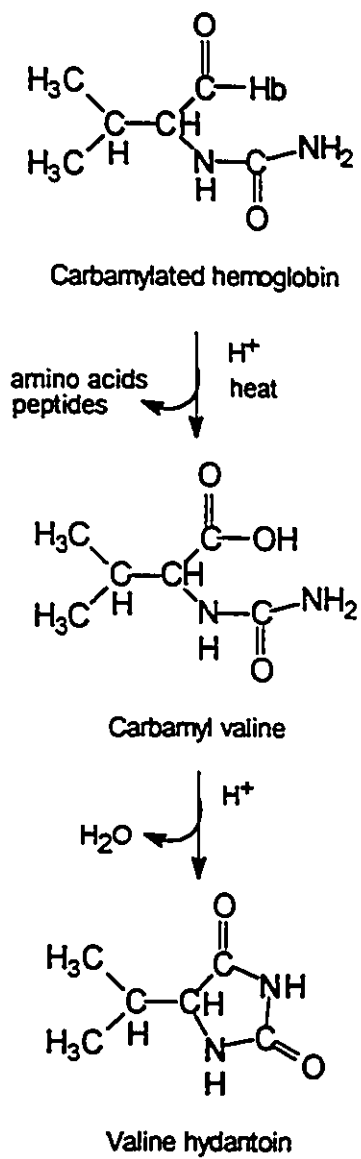
Chromatography

The method of Kwan *et al.* (75) was adapted for the measurement of

FIGURE 2.4**Formation of Valine Hydantoin***Legend*

Spontaneous cyclization of carbamyl valine to valine hydantoin under acid conditions (see Section 2.2.8.2).

FIGURE 2.4



valine hydantoin. The mobile phase (6% acetonitrile) was prepared by first adjusting approximately 900 mL of Type I water to pH 4.0 with 0.5 M acetic acid (HPLC), adding 60 mL HPLC grade acetonitrile and diluting to a final volume of 1 L. The prepared extract (see above) was resuspended with 0.5 mL of the mobile phase, filtered through a 2- μ m PVDF filter syringe, and 50 μ L were injected onto an Apex II reversed-phase column. The column was heated by inserting the column into an insulated distillation tube (wrapped with cotton batting and then covered with aluminum foil), and passing warm water (45°C) through it. The absorbance was read at 210 nm. Peak integration was performed by the Chromatopac. Each run included two standards, 50 μ L of valine hydantoin (10 μ g/mL) and 50 μ L of carbamyl norvaline (10 μ g/mL).

Calculation of Results

Carbamylated hemoglobin, expressed as μ g valine hydantoin/g hemoglobin (μ g VH/g Hb) was calculated according to the following formula:

$$\text{CHb} = (\text{A} \times 0.5 \mu\text{g/B} \times \text{B/C} \times 20)/\text{g Hb}$$

where: A = peak height of VH in the sample
 B = peak height of the 50 μ L (0.5 μ g) CNVal (external standard)
 C = peak height of CNVal in sample (internal standard)

The ratio 0.5 μ g/B converts the peak height of the sample VH to μ g VH, since the absorbance of VH is the same as CNVal at 210 nm (see Figure 3.4). The ratio of B/C is a correction factor for loss during extraction and the value 20 adjusts for the volume injected (50 μ L) compared to the total volume (1 mL) prepared from 0.5 mL of washed red blood cells. The amount of Hb in the sample is calculated by multiplying the concentration of Hb (g/L) in the sample by the sample volume processed (0.5 mL).

2.2.9 Polyclonal Antibody Production

Polyclonal antibodies were made by Lampire Biological Laboratories (Pipersville, PA). Rabbits (SPF) were immunized with *in vitro* prepared carbamylated albumin (containing 74% of the lysine residues carbamylated).

The antigen, emulsified in 1-mL Freund's complete suspension [killed *Mycobacterium tuberculosis* (H37RATCC25177), 0.85 mL mineral oil, 0.15 mL mannide monooleate; emulsified with PBS, pH 7.4], was injected subcutaneously (0.5 mL) and intradermally (0.5 mL). Booster injections were made on days 7, 14, 28, and then monthly with antigen emulsified in Freund's incomplete suspension [*Mycobacterium tuberculosis* (H37RATCC25177), 0.85 mL paraffin oil, 0.15 mL mannide monooleate; emulsified with 0.85% NaCl]. The blood obtained was centrifuged and the serum collected, and then frozen at -20°C.

2.2.10 Preparation of Antibody

Antibody Purification

Rabbit serum was processed through Affi-Gel protein A columns and specific affinity columns. The resulting IgG's were used to develop anti-CHSA immunoassays. All columns procedures were run at R.T. unless otherwise stated.

Protein A

Affi-Gel protein A columns were equilibrated with 10 bed volumes of binding buffer (10 mM phosphate buffer, 0.15 M NaCl, pH 8.2). Rabbit serum, heat inactivated (56°C, 10 min) and pH adjusted to 8.2 with NaOH (~ 1 M), was applied to an equilibrated column. The volume of serum to gel was 1:1. The column was washed with binding buffer until no protein was detected (spot test, see Section 2.2.6). Immunoglobulins were eluted with 0.1 M sodium citrate, pH 3.0, in 1-mL aliquots into 1.5-mL Eppendorf tubes containing 120 µL 3 M Tris-Cl, pH 8.0 (final pH ~ 7.5). Approximately three column volumes of binding buffer were required to elute all the IgG. Aliquots containing the largest amount of IgG were combined and dialyzed against PBS overnight at 4°C. Final IgG concentration was determined using the Lowry assay with bovine IgG as standard. The column was stripped with 1.5 M sodium thiocyanate and then reequilibrated with 10 column volumes of binding buffer containing 0.05% sodium azide and stored at 4°C.

Affi-gel 15 (N-hydroxy-succinamide ester derivatized to agarose gel)

A suspension of Affi-Gel 15 (usually 2 mL) was poured into a glass fritted Buchner funnel and washed with several volumes of water (4°C). The moist cake was transferred to a 5-mL glass sample vial. A protein solution (HSA, CHSA or CHb) was prepared by equilibration with coupling buffer by dialysis or gel filtration. The coupling buffers used were 0.1 M MOPS, pH 7.5 for HSA and CHSA, and 0.1 M MOPS, 0.3 M NaCl, pH 7.5 for CHb. The equilibrated protein solution was then added to the gel to give a final concentration of 18, 20.8, and 3 mg protein/mL gel for HSA, CHSA and CHb, respectively. The suspension was incubated on a rotator overnight at 4°C. Following the incubation, the slurry was poured into 10-mL Econo column and washed with one or more of the following solutions: 1 M NaCl; 0.1 M glycine-HCl, 0.2 M HCl, pH 2.5; and 0.1M glycine-HCl, pH 7.5 containing 10% (v/v) dioxane. The column was then reequilibrated with the appropriate coupling buffer.

For CHSA- or CHb-affinity columns, IgG (in 0.1 M MOPS, pH 7.5 or 0.1 M MOPS, 0.3 M NaCl, pH 7.5 for CHSA and CHb, respectively) was applied to the affinity column and washed with buffer until no protein was detected. The column was then washed with 1 M NaCl (in starting buffer) until no protein was again detected. The bound IgG was eluted (1-mL aliquots) using 0.1 M glycine-HCl, pH 2.5 containing 10% (v/v) dioxane into 1.5-mL Eppendorf tubes containing 140 μ L 3 M Tris-Cl, pH 8.0. Aliquots containing the majority of the protein were combined and dialyzed against PBS overnight at 4°C, or applied to a Centricon-30 concentrator for buffer exchange. Final IgG concentration was determined using the Lowry assay with bovine IgG as standard. The column was reequilibrated with starting buffer containing 0.05% sodium azide and stored at 4°C.

The HSA-affinity column was run with 0.1 M MOPS, pH 7.5. The IgG applied to the column was also in this buffer. Aliquots (1-mL) were collected until no protein was detected. The aliquots containing the majority of the protein were combined and processed as described above. The column was stripped of

bound IgG using several bed volumes of 0.1 M sodium citrate, pH 2.5, and 1 M NH_4OH . The column was then reequilibrated with the starting buffer containing 0.05% sodium azide and stored at 4°C.

Antibody Specificity

Immuno-electrophoresis and Ouchterlony studies were done by Dr. K. Kithier, Department of Pathology, Wayne State University, Detroit, MI.

2.2.11 Enzyme-Linked Immunosorbant Assay

2.2.11.1 Competitive assay

Solutions

Phosphate buffered saline (PBS). NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), KH_2PO_4 (0.24 g) were dissolved in 800 mL water. The pH was adjusted to 7.4 with NaOH and the solution diluted to 1 L.

Phosphate buffered saline containing Tween (PBST). Tween-20 (0.05%) in PBS.

Blocking buffer. BSA (3%) in PBS.

Substrate buffer. Diethanolamine (0.53 g) and MgCl_2 (0.05 g) were dissolved in 400 mL water. The pH was adjusted to 9.0 and the solution diluted to 500 mL.

Antibody dilutions. Antibodies were diluted with PBST.

Substrate solution. 1 mg/mL *p*-NPP was dissolved in substrate buffer.

Procedure

This assay system was first optimized in terms of the amount of antigen coated to wells, blocking time, 1° and 2° antibody concentration, and incubation time.

Microtitre plated wells were coated with 50 μL of carbamylated albumin (2 $\mu\text{g}/\text{mL}$ in PBS). The plate was covered with parafilm and incubated overnight at 4°C. Wells were washed three times with PBS (300 $\mu\text{L}/\text{well}$), and then blocked with 250 $\mu\text{L}/\text{well}$ of blocking buffer. After 2 h at R.T. the wells were washed three times with PBST. A mixture (100- μL) containing 50 μL sample or standard (dilutions of 74% carbamylated HSA) and 50 μL of a limiting amount of antibody

was incubated for 2 h on a shaker at R.T.. After washing wells three times with PBST, 100 μ L of 1:10,000 alkaline phosphatase conjugated goat anti-rabbit IgG were added. The plate was incubated for 1 h on a shaker at R.T.. The wells were washed again three times with PBST and then once with substrate buffer. Substrate solution (100 μ L) was added to each well and incubated for a convenient time (usually 20 min). The reaction was stopped by adding 100 μ L of 2 M NaOH.

The well absorbances were read at 405 nm using the Micro Reader III. Data was calculated as B/B_0 in which B represents the amount of binding of antibody in the presence of a competitor, and B_0 in the absence of any competitor.

2.2.11.2 Sandwich assay

Solutions

The solutions used in the sandwich assay were as described in the competitive assay (see above) except for the addition of anti-human albumin conjugated to alkaline phosphatase (anti-HSA-AP).

Procedure

Microtitre plated wells were coated with 50 μ L of anti-HSA (HSA-affinity purified, 2 μ g/mL in PBS). The plate was covered with parafilm and incubated overnight at 4°C. Wells were washed three times with PBS (300 μ L/well), and then blocked with 250 μ L/well of blocking buffer. After 2 h at R.T. the wells were washed three times with blocking buffer. Sample (100 μ L) or standard (100 μ L, dilutions of 74% carbamylated HSA), diluted in PBST, were added to wells and incubated for 2 h on a shaker at R.T.. After washing wells twice with PBST 100 μ L of 1:5000 anti-HSA-AP was added to each well. The wells were incubated for 1 h on a shaker at R.T.. The wells were washed twice with PBST and twice with substrate buffer. Substrate solution (100 μ L) was added to each well and incubated for a convenient time (usually 20 min). The reaction was stopped by adding 100 μ L of 2 M NaOH. The well absorbances were read at 405 nm using the Micro Reader III.

CHAPTER 3

RESULTS

3.1 Diacetyl Monoxime Assay

3.1.1 Evaluation

The diacetyl monoxime (DAM) reaction was found to be limiting when applied to the measurement of carbamyl groups on proteins. The Hunninghake and Grisolia procedure (76) had a high blank averaging 0.230, when read against water, and reacted with noncarbamylated protein. When commercial human serum albumin (HSA) preparations were tested, they gave an absorbance above the blank (see below). This absorbance could be due to the endogenous carbamyl groups present, since the HSA preparation is prepared from human plasma, but it could also mean that other parts of the protein react with this reagent, or a combination of both.

Carbamylated hemoglobin could also be detected by this assay. Interference from heme was very small at the wavelength used. One mg each of Hb and CHb were tested with resulting absorbances of 0.0333 and 0.2933, respectively.

Other noncarbamylated proteins were then tested to see if they also reacted with the diacetyl monoxime reagent. Chicken egg ovalbumin (4 mg) and wheat acid phosphatase (4 mg) both gave an absorbance above the blank (0.263 and 0.252, respectively). Since acid phosphatase is a plant protein it would not be expected to have carbamyl groups on it. Therefore, the absorbance must be due to the noncarbamyl groups on the protein.

In the modification of this assay by Wybenga *et al.* (77), there is no reaction with the commercial HSA preparation, nor is it able to detect low levels of carbamylated protein. In this assay system, plasma protein carbamylation could not be detected due to its limited protein capacity. When amounts of protein equivalent to those in the Hunninghake and Grisolia method for detection

of carbamylated plasma proteins are used, precipitation occurs. This is most likely a result of the lower acid concentration (0.6 M) in the reagent. The Hunninghake and Grisolia reagent, which contains 4.5 M H₂SO₄, initially precipitates the protein, but then upon heating and the addition of more H₂SO₄ (final 9 M), resolubilizes it. The high amount of acid, therefore, causes some hydrolysis resulting in solubilization of the protein.

3.1.1.1 Urea removal

Various methods were examined to remove urea from plasma samples. Urea would interfere with the detection of carbamylated protein. The methods tried included alcohol precipitation, Microcon ultrafiltrators, gel exclusion chromatography (Bio-Gel P-6DG and G-25 Sephadex), and urease treatment. The alcohol precipitation technique (17) was found to be inappropriate because it was unable to precipitate all the protein particularly carbamylated proteins. In addition, the precipitate was usually very sticky and difficult to get back into solution. Microcon ultrafiltrators were not useable either because of their limited protein capacity. The protein content in a 0.5-mL plasma sample (maximum volume for the Microcon) is about 35 mg and causes the membrane to become clogged which therefore prevents filtration. Gel exclusion chromatography and urease treatment were both satisfactory techniques.

G-25 Sephadex was better because of its higher rate of filtration compared to Bio-Gel P-6DG. There was no significant difference in absorption using the DAM assay between G-25 filtered and urease treated samples. However, only 'old' plasma samples were used which may have reduced the influence of potential interfering compounds found in the plasma (*e.g.*, citrulline, drugs). Therefore, the filtration technique is preferred to ensure the removal of all contaminating small molecules.

Incubation of urease-agarose with carbamylated HSA (CHSA) standard overnight did not alter the absorbance (DAM) of the plasma samples. Therefore, under these conditions urease does not hydrolyze carbamyl groups from carbamylated proteins.

3.1.2 Standard Curves

The DAM assay reacted linearly with the amount of CHSA, but not with the amount of HSA (Figure 3.1). The assay was linear with CHSA up to highest concentration measured (2 mg/mL). The reaction was linear up to 5 mg/mL and then there was a gradual levelling out of the reaction with increasing amounts of HSA. The difference between the absorbance of CHSA and HSA, at the same concentration, will be the absorbance due to the carbamyl groups alone. The correction factor will be different depending on what concentration of HSA is being measured, up to 5 mg/mL. Furthermore, it is not known whether this correction could be directly applied to total plasma protein.

The average protein concentration in the patient samples analyzed was 5 mg/mL which gave an absorbance range of 0.5 to 0.8. This concentration of protein corresponds to a background HSA absorbance of 0.32 (including the blank absorbance of 0.25). This means about half of the patient sample absorbance is due to the blank and nonspecific protein absorbance.

The within-assay imprecision for hemodialyzed patient samples ($n = 6$) was found to be 8.9%.

3.1.2.1 Homocitrulline

The standard curve relating CHSA absorbance to homocitrulline absorbance (Figure 3.2) could not be used because of the absorptivity differences between the carbamyl lysine groups on HSA and free carbamyl lysine (homocitrulline). The absorbance of approximately equal amounts of carbamyl lysine (HSA) and homocitrulline (free) differed by almost 10-fold in favour of the free homocitrulline. That is, the absorbance from free homocitrulline is about 10-fold greater than the absorbance of carbamyl lysine residues on HSA. The high acid and heat conditions used to detect carbamyl groups causes some protein hydrolysis. The result is partial release of homocitrulline residues which is probably what is detected in the reaction. This would explain the low absorbance readings from what should be a highly carbamylated protein. An alternative assay to detect carbamyl groups involves conditions that are less harsh

FIGURE 3.1**Standard Curves For the DAM Assay***Legend*

Standard curves showing the absorbance at 525 nm plotted against HSA (A) and CHSA (B) concentrations (see Section 2.2.7.2). The line of best fit for Figure A is given by $y = 0.26x + 0.033$ ($r = 0.99$). Each point is the mean of duplicate determinations.

FIGURE 3.1

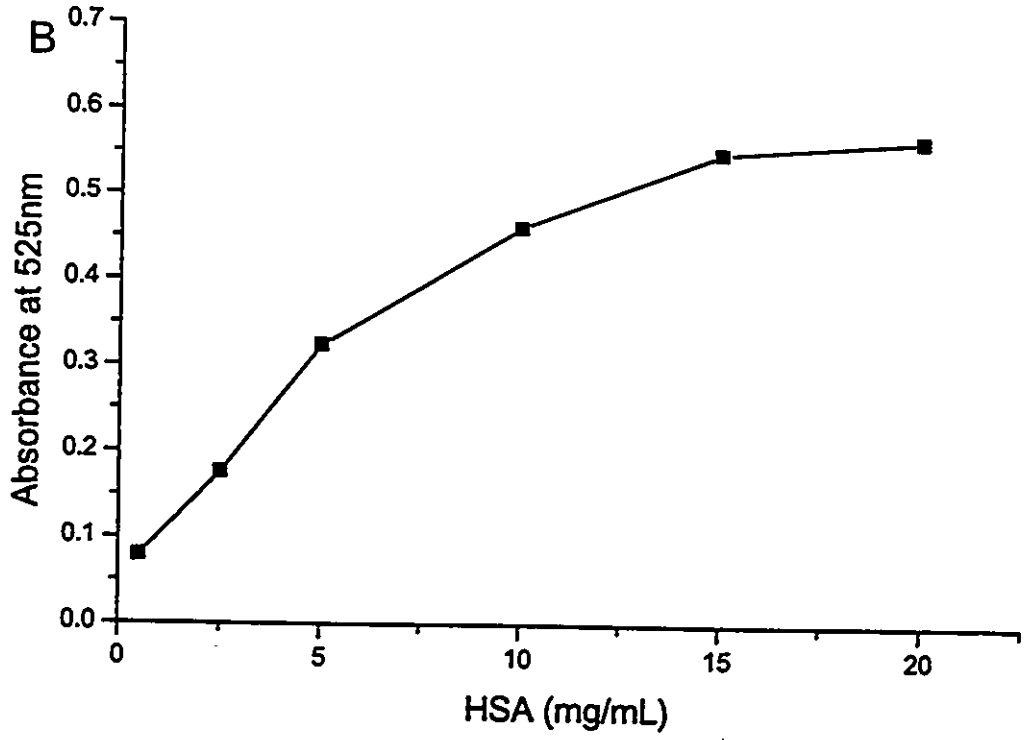
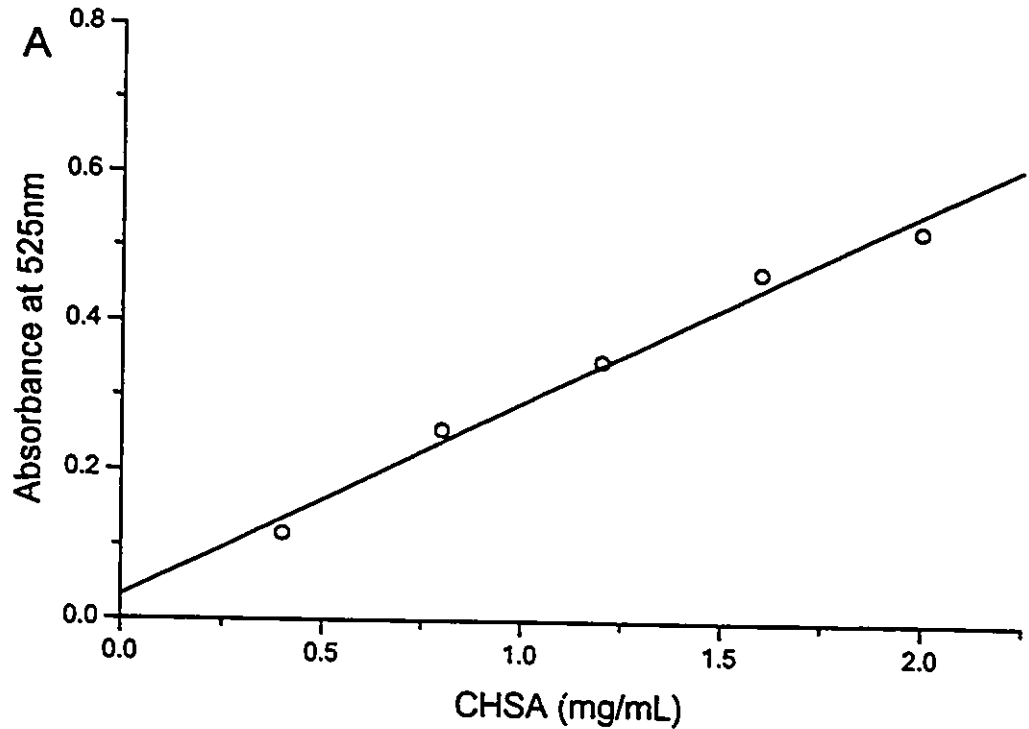
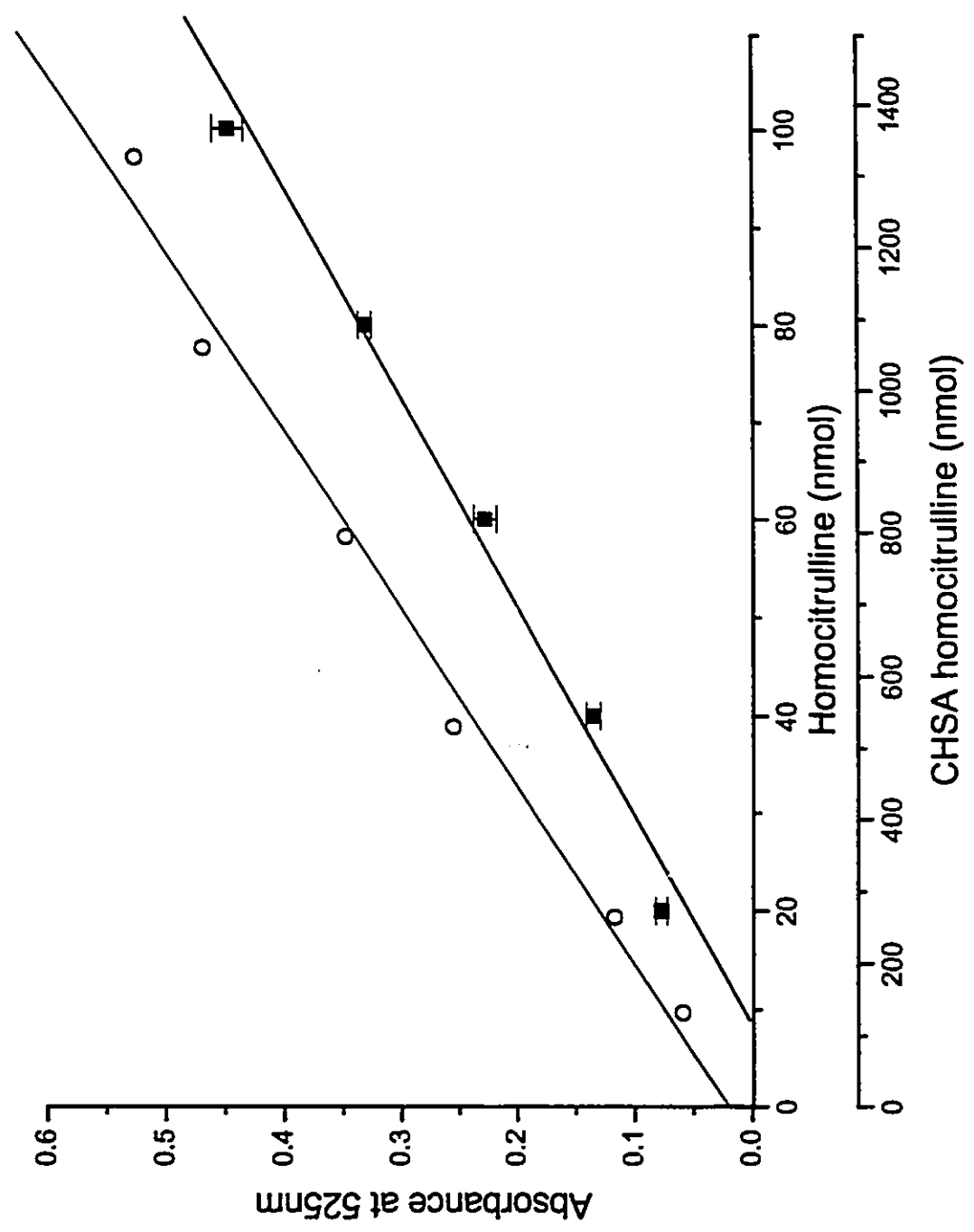


FIGURE 3.2

CHSA and Homocitrulline Standard Curves*Legend*

Comparison between standard curves using CHSA (O) and homocitrulline (■) to estimate carbamyl group content. The regression lines were $y = 4 \times 10^{-4}x + 0.02$ ($r = 0.994$) and $y = 0.0047x - 0.037$ ($r = 0.993$), for CHSA and homocitrulline, respectively. CHSA determinations were done only once and homocitrulline values are a mean of six determinations. See section 2.2.7.2 for assay details.

FIGURE 3.2



(Wybenga *et al.* assay, see Section 2.2.7.3), but using this method no carbamylation is detected on CHSA. The reaction mixture in this case is very turbid compared to a transparent solution in the previous case, indicating protein denaturation but not hydrolysis.

3.1.2.2 [¹⁴C]Cyanate standard curve

Studies were done to estimate the extent of carbamylation on HSA by incorporation of [¹⁴C]cyanate and relation to absorbance in the DAM assay according to Erill *et al.* (17). After incubation of [¹⁴C]cyanate with HSA several techniques were used to remove the unreacted [¹⁴C]cyanate. Precipitation with acetone or ethanol (-20°C) followed by centrifugation and washing, or ethanol precipitation and filtration through nitrocellulose, did not effectively remove unreacted [¹⁴C]cyanate. The control sample (0 min) still had a substantial amount of radioactivity in it. In addition, when samples were analyzed 6 days later they were found to have anywhere from 22-82% decrease in radioactivity. This decrease is probably due to unreacted [¹⁴C]cyanate decomposing to [¹⁴C]CO₂.

Sephadex G-25 gel filtration gave the best separation of [¹⁴C]cyanate-HSA from [¹⁴C]cyanate, though the radioactivity did not reach baseline between peaks. However, there was a linear relationship between radioactivity and incubation time (0 - 200 min). Experiments were not continued because it was decided that this form of standard curve would not give an accurate estimation of carbamylation.

There is an inherent amount of carbamyl groups on HSA, in addition to the [¹⁴C]cyanate that is added. This means that the total absorbance (525 nm) is not only due to the added [¹⁴C]cyanate that can be quantitated, but also the unknown amount of native carbamylation that cannot be quantitated. The DAM assay has its limitations as well (see Section 3.1), in particular its nonspecific absorbance. Also, if carbamylation of total plasma protein was being analyzed, the correct standard curve should be made with total plasma protein and not just HSA.

3.1.4 Low Density Lipoprotein

Several papers have looked at a binding defect of LDL in uremia, and have indirectly related it to carbamylation (see Section 1.5.2.3). Therefore a crude experiment was done to see if there were any carbamyl groups on commercial LDL. LDL (0.38 mg protein) was found to have an increase in absorbance of 0.05 using the DAM assay. This absorbance was doubled when twice the amount of LDL was tested. However, since the Hunninghake and Grisolia's version of the DAM assay contains $\text{FeCl}_3\text{-H}_2\text{SO}_4$, and cholesterol (present in LDL) also reacts with this mixture to give a colored complex, there could have been some interference (126). Therefore, LDL (0.57 mg) was delipidated according to the procedure of Harake (72) and retested. It was found that delipidated LDL still exhibited an absorbance over that of the blank, but less than that of nondelipidated LDL (33% lower). In addition, cholesterol alone gave a strong absorbance under these assay conditions. Therefore, the the DAM reagent reacted with LDL protein alone. However, it was not determined whether the absorbance was from the specific reaction with carbamyl groups, or from some nonspecific protein reaction (see Section 3.1.2).

3.2 HPLC Analysis of Carbamylated Hemoglobin

3.2.1 Method Evaluation

In setting up the HPLC procedure, a few complications arose. Samples and/or standards often gave only one large peak, and samples and/or standards may have worked one time were not repeatable. In order to discern what these complicating factors were, a number of experiments were done. From these experiments a few interesting method points were noted. Many of the experiments were found unimportant because, in the end, the chromatography problem had to do with a serious malfunction of the autoinjector.

3.2.1.1 Sample preparation, stability and recovery

Sample Preparation

Several variations for sample preparation were tried (74, 75, 97). The sample preparation method that was decided upon was similar to Manning's (74), except the red blood cells were isolated first before globin purification (see Section 2.2.8.2). This preparation gave a clear sample with no precipitates as compared to the other methods in which heme and undissolvable material were often present. When this method was compared to Kwan *et al.*'s method (75), no difference was found between the CHb values. Figure 3.3 shows a typical chromatogram of a uremic patient's sample using the modified method.

Stability

A freezer breakdown prompted the study of the effect of freeze/thaw cycles on the stability of carbamylated hemoglobin to be evaluated. The concentration of carbamylated hemoglobin was found not to be affected by two freeze-thaw cycles. In addition the prepared sample was found to be stable for at least 3 weeks (no significant change in peak heights or areas).

Recovery

The extraction recovery for the internal standard carbamyl norvaline was 57% (SD = 4.2%, $n = 31$). Since only 80% of the ethyl acetate layer is removed for evaporation, the total mean recovery was actually 68%. This is a very similar to the recovery found in the procedure of Kwan *et al.* (64%).

3.2.1.2 Absorption spectra and linearity

The absorption spectra at pH ~2.7 showed the maximum absorbance for CNVal to be 210 nm and for CVal 200 nm. This is the pH when the mobile phase contains 0.1% acetic acid, though the procedure says it should be 'about pH 4' (75). At pH 4 (Figure 3.4), the maximum absorbance for CNVal and VH remains at 210 nm and CVal's peak shifts to below 200 nm.

Figures 3.5A and B show that peak area or peak height versus sample volume injected was linear for VH, CNVal and CVal. In all cases, the y-intercept was close to zero except for the peak height line of CVal. However, the line was

FIGURE 3.3**HPLC Chromatogram of Carbamylated Hemoglobin From a Uremic Patient***Legend*

The valine hydantoin peak (7.33 min) is representative of the carbamylated valine residues on the patient's hemoglobin and carbamyl norvaline (8.77 min) is the internal standard. The samples were run on a reversed-phase column at 45°C (see Sections 2.2.8.2 and 2.2.8.3). The chromatographic conditions were:

Mobile phase - 6% acetonitrile (pH adjusted to 4.0)

Mode - isocratic

Pump speed - 1.5 mL/min

Detection wavelength - 210 nm

FIGURE 3.3

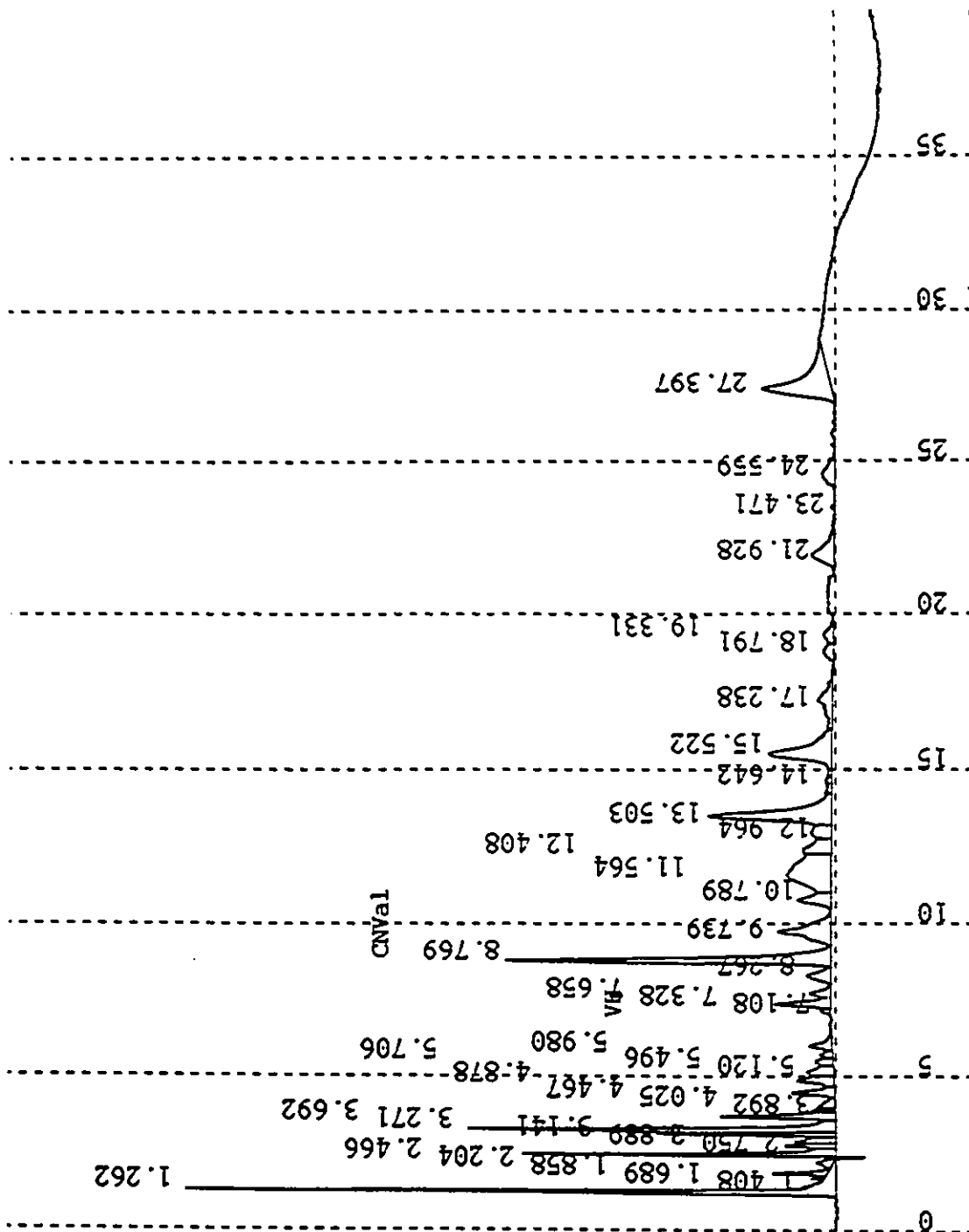


FIGURE 3.4**Absorption Spectra For Carbamyl Norvaline, Carbamyl Valine, and Valine Hydantion***Legend*

Absorption spectra for CNVal (—), CVal (---) and VH (—). The concentration was 0.2 mg/mL in 6% acetonitrile, pH 4.

FIGURE 3.4

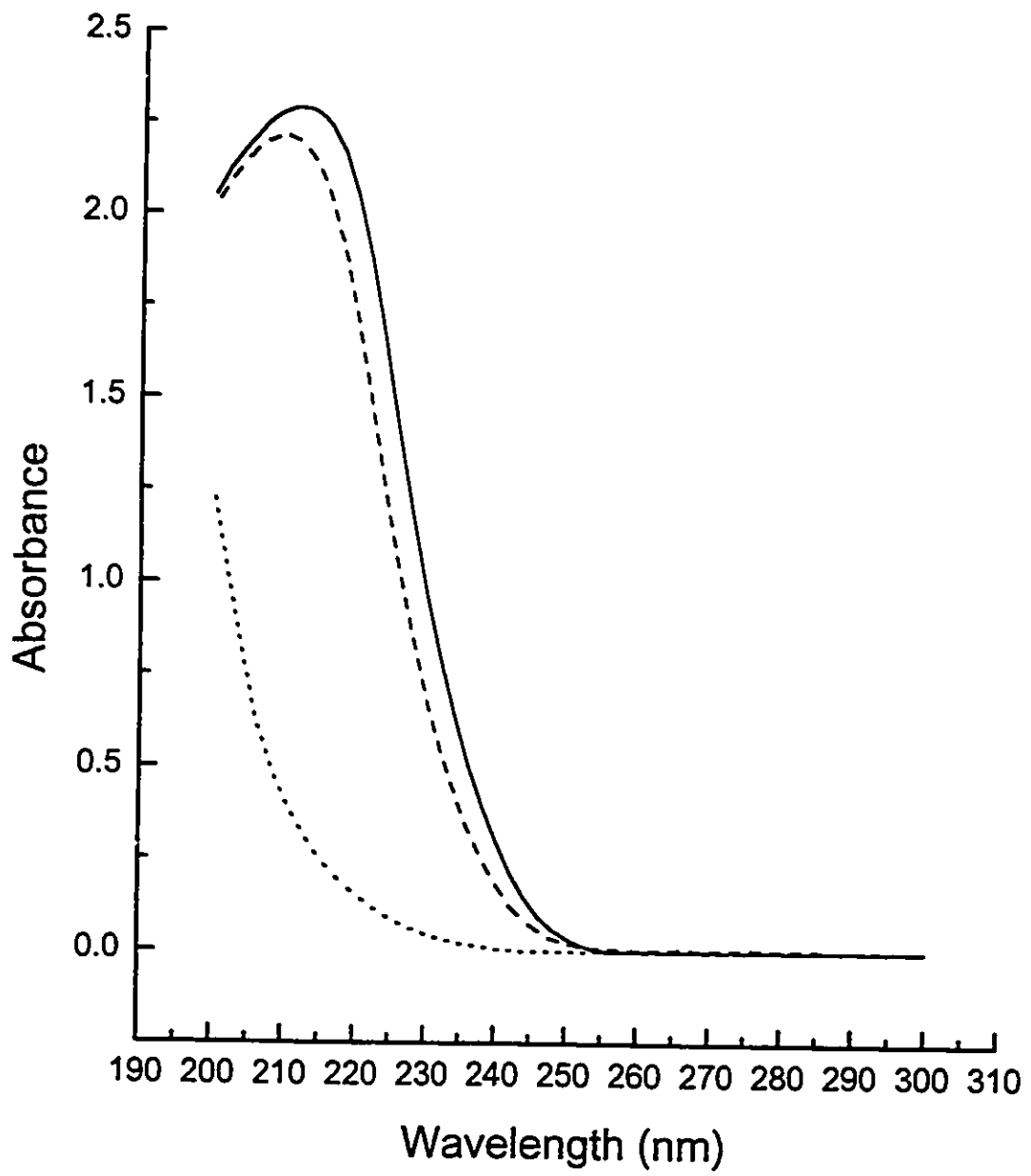


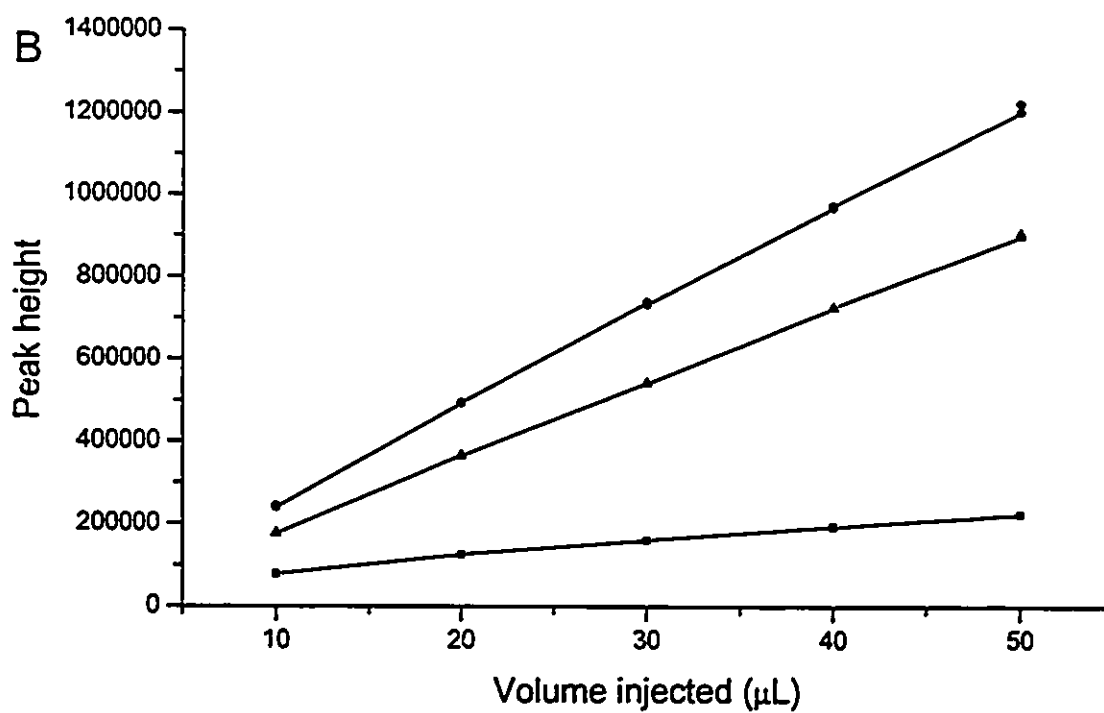
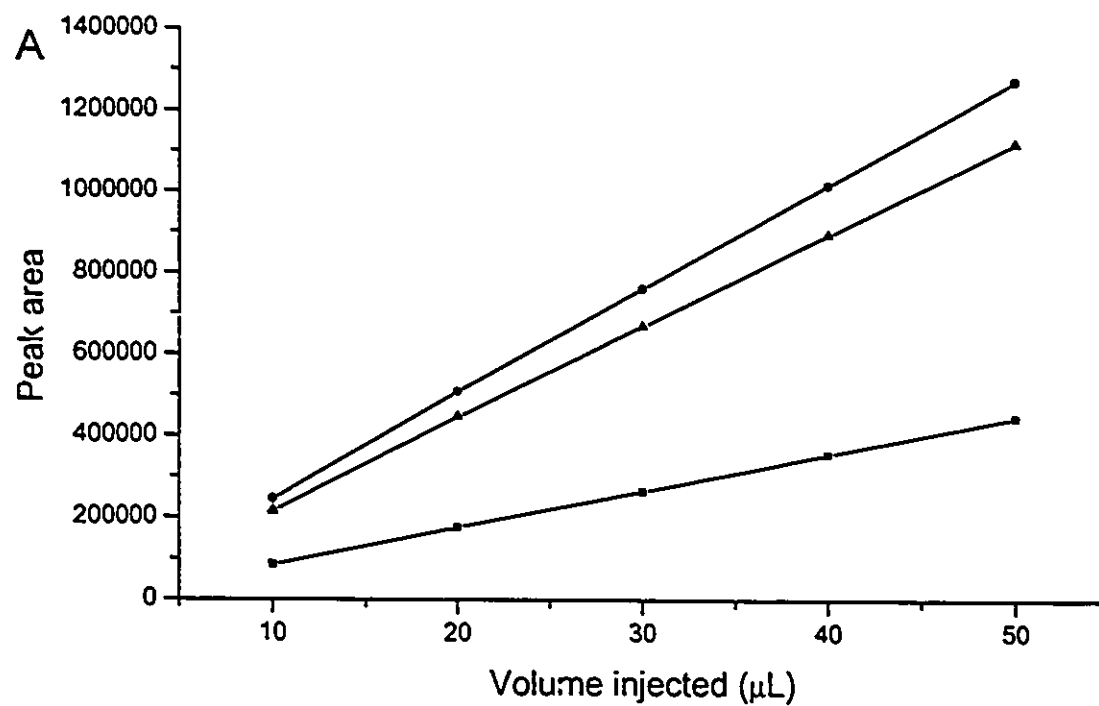
FIGURE 3.5

Linearity of Valine Hydantoin, Carbamyl Norvaline, and Carbamyl Valine*Legend*

Linearity of VH (●), CNVal (▲) and CVal (■) with respect to peak area (A) and peak height (B). Sample concentration was 100 µg/mL (6% acetonitrile, pH 4). Each measurement was done in duplicate. The regression equations were:

	A (peak area)	B (peak height)
VH	$y = 2565472x - 6092$	$y = 242578x + 575$
CNVal	$y = 2260189x - 6724$	$y = 181665x + 8.5$
CVal	$y = 899930x - 3159$	$y = 36297x + 4951$

FIGURE 3.5



found to pass through the origin when a 10-fold lower concentration of CVal was used (data not shown).

Although these lines were linear, they were not superimposable. This would be expected for CVal because of its much lower absorbance compared to VH and CNVal at 210 nm (Figure 3.4). The lines for VH and CNVal should have been very close because their absorbances at 210 nm are the same. The reason for this discrepancy is unclear, since only one peak is detected when the VH standard is run alone. However, it could indicate impurity in the VH preparation since the only analysis done on it was a melting point determination.

Also, the retention time was consistent for different volumes of valine hydantoin and carbamyl norvaline, but steadily increased with sample volume for carbamyl valine (Figure 3.6). These results indicate that carbamyl valine is not the best of available standards to use, even though it is the carbamylated amino acid on hemoglobin.

3.2.1.3 Imprecision and detection limit

The within-run coefficient of variation for the standards was 1.7% for both VH and CNVal. The between-run CV's for the standards VH and CNVal were 2.7% and 3.6%, respectively.

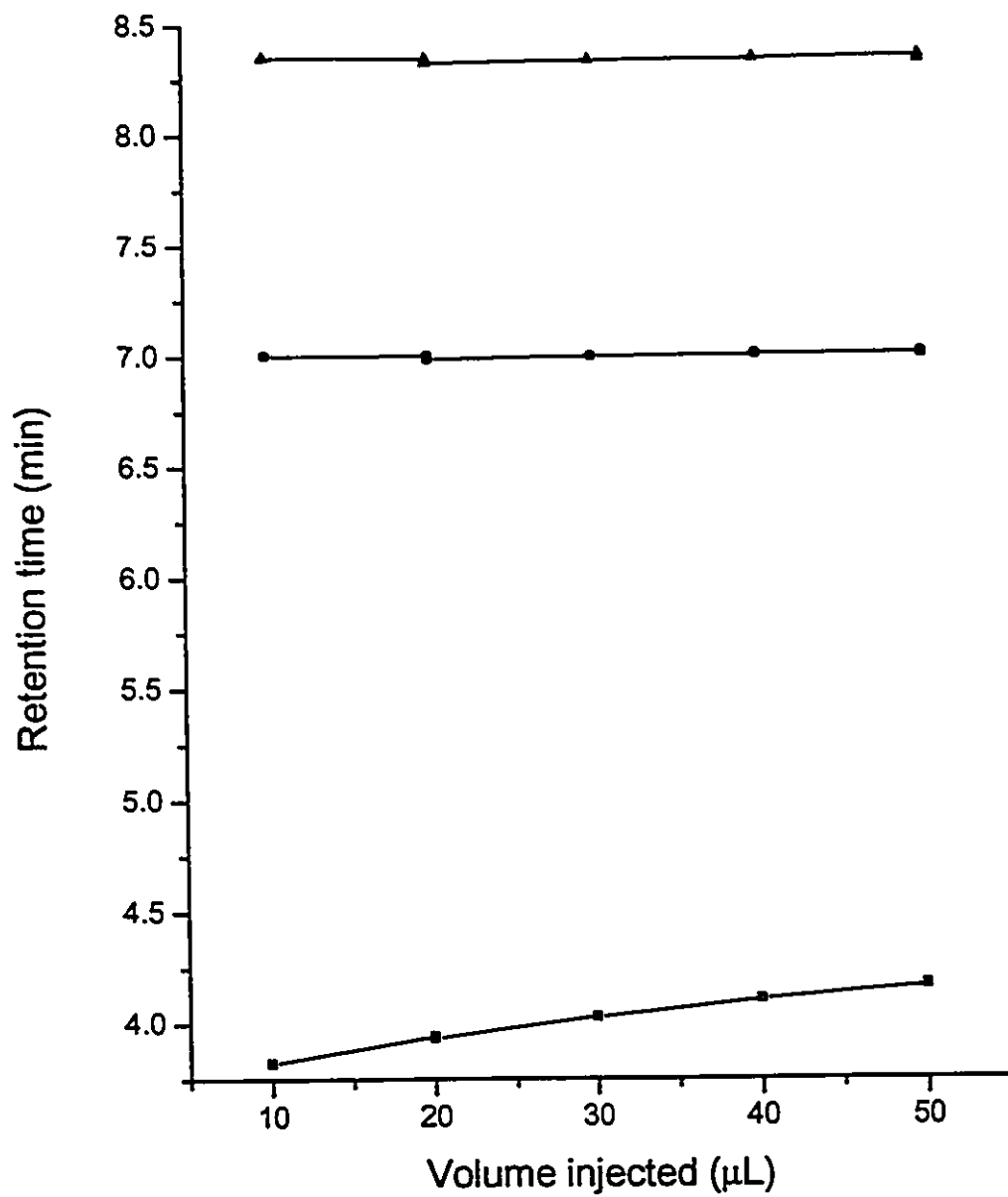
The variation between samples was calculated from three patient samples prepared in duplicate and run in the same batch. The within-run CV, calculated using Tonks' procedure (method 2) (127), was 10.2 % for VH and 2.5% for CNVal.

The detection limit for carbamylated hemoglobin is dependent on the baseline variation of the chromatograph. If the assumption is made that the variation in the standard (0.5 μg CNVal) reflects this variation, then the minimum detection limit, defined as three times the standard deviation for CNVal, is 0.025 μg VH.

FIGURE 3.6**Effect of Injection Volume on Retention Time***Legend*

The retention times of VH (●), CNVal (▲), and CVal (■) expressed in relation to volume injected. Sample concentration was 100 µg/mL (6% acetonitrile, pH 4). Each measurement was done in duplicate.

FIGURE 3.6



3.3 Albumin Carbamylation

3.3.1 Time Dependent *In Vitro* Analysis

Human serum albumin was incubated with sodium cyanate as described in Methods (see Section 2.2.2) and aliquots (100 μ L) of this incubate were removed at various timed intervals up to 76 h. The excess cyanate was immediately removed by gel filtration (microcolumn procedure, see Section 2.2.7.1). The samples were then tested for albumin concentration, loss of free amino groups, presence of carbamyl groups and change in isoelectric point.

3.3.1.1 Indirect measurement of carbamylation

The TNBS assay detects free amino groups, and also reacts with cyanate. Figure 3.7 shows the loss of free amino groups with time of incubation indicating that carbamylation of the free amino groups was taking place. Since there was an excess amount of cyanate present in the reaction mixture, the hyperbolic curve indicated that the carbamylation reaction approached a maximum. Human serum albumin has a total of 60 free amino groups of which 59 are from lysine residues and 1 from the NH_2 -terminal amino group (aspartate). Assuming that native albumin contained no carbamyl groups (128), then at maximal derivatization the number of amino groups carbamylated would equal 46 (equations 1 and 2).

$$\frac{\text{absorbance of native albumin}}{60 \text{ free amino groups}} = \frac{\text{absorbance of carbamylated albumin}}{\text{free amino groups remaining}} \quad (1)$$

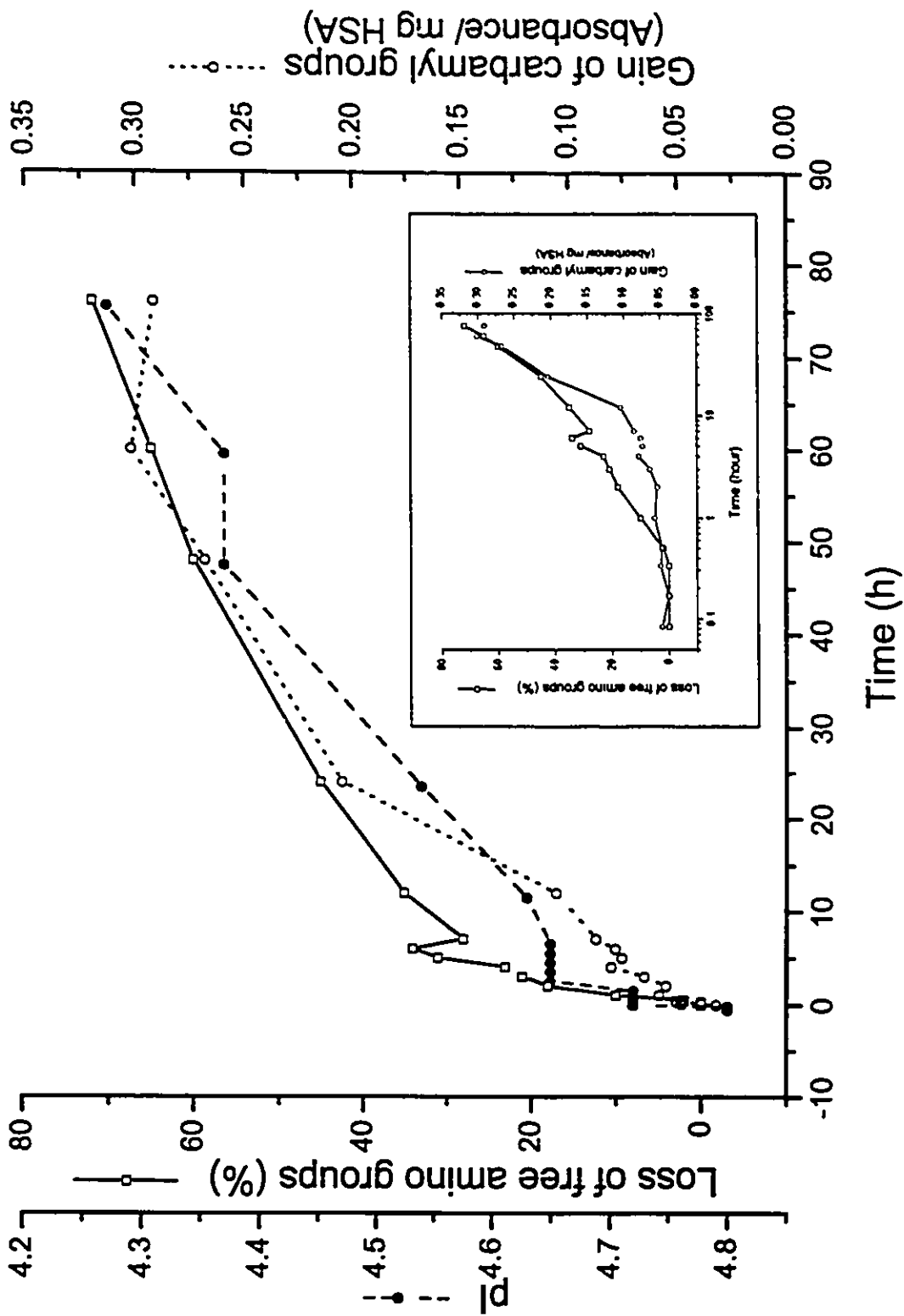
$$60 - \text{free amino groups remaining} = \text{number of carbamyl groups on albumin} \quad (2)$$

Indirectly, one could say that 14 (60 - 46) amino groups were not available for carbamylation. These amino groups may be unavailable for a number of reasons. Native albumin probably contains some carbamyl groups just as there are glucose units on albumin. Furthermore, other compounds may be attached to amino groups (e.g., glucose and acetyl groups). Also, cyanate may not be

FIGURE 3.7**Time-dependent Carbamylation of Human Serum Albumin***Legend*

Time-dependent carbamylation (100 mM NaOCN) of human serum albumin (40 mg/mL) as measured by change in pI (●), loss of free amino groups (□) and gain of carbamyl groups (○). The reaction was stopped at timed intervals by removing excess NaOCN (P-6G microcolumn, see Section 2.2.2). The inset shows the time log relationship between the loss of free amino groups and the gain of carbamyl groups. Measurements were done in duplicate.

FIGURE 3.7



able to access all potential sites.

3.3.1.2 Direct measurement of carbamylation

Figure 3.7 also shows the increase in carbamyl groups with time of incubation. The number of carbamyl groups was expressed in relative terms as absorbance/mg HSA since there was no standard available for comparison. A change in absorbance for this reaction occurred slightly sooner than in the TNBS assay.

3.3.1.3 Effect of carbamylation on the isoelectric point

The isoelectric point of carbamylated albumin changed with increasing incubation time (Figure 3.7 and Figure 3.8). However, this technique was not as sensitive to changes in carbamyl content as compared to the TNBS assay or the DAM assay. Part of the reason was due to the rather broad bands produced on the gel which probably indicates microheterogeneity within each band. It has been shown that electrofocusing of nondefatted albumin, the type used in this study, gives two zones (with and without fatty acids) (129). In addition, these authors noted that their albumin bands were broad and diffuse in gels containing urea (hence carbamylation) compared to those without urea. Also, the technique used to measure the slight change in pI was done by enlarging a photocopy of the gel and measuring the distance each band travelled from the cathode. When pI comparisons are made approximately every 5 h then significant changes can be noted.

Generally speaking, all three curves in Figure 3.7, representing three different parameters for measuring carbamylation, show a similar time-dependent course. The inset in Figure 3.7 shows the relationship of the TNBS assay to the DAM assay on a time log scale.

3.3.2 Other Characteristics of Carbamylated Albumin

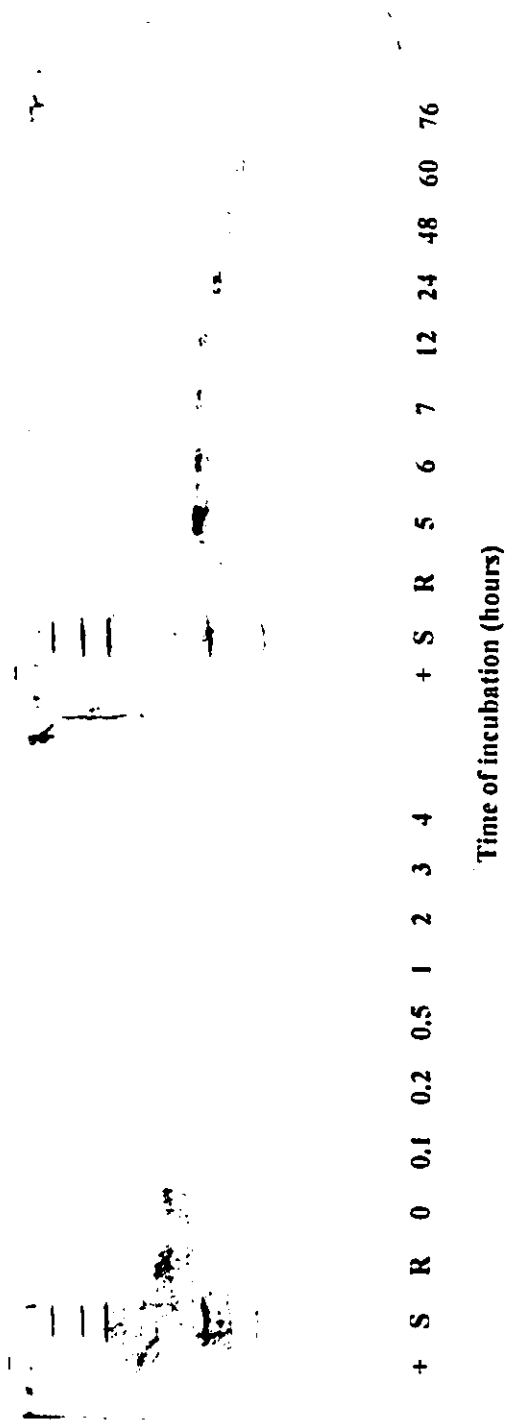
Absorption Spectrum

The absorption spectrum of 5.3 mg/mL of carbamylated albumin is the same as the absorption spectrum of 5.3 mg/mL of native albumin (data not shown).

FIGURE 3.8**Isoelectric Focusing of HSA Carbamylation Time Course Samples***Legend*

The samples were focused on a Bio-Lyte 4/6 gel (125 x 65 x 0.4 mm) in a Mini IEF Cell (100 V for 15 min, 200 V for 15 min and 450 V for 1 h) and stained with Coomassie Blue (see Section 2.2.5.1). Standard pI markers (lane S) were glucose oxidase (4.2), trypsin inhibitor (4.6), β -lactoglobulin (5.1), and carbonic anhydrase (5.4,5.9). Lane R contains native albumin (4.8). The remaining lanes represent the different incubates.

FIGURE 3.8



Fluorescent Spectrum

The fluorescent emission spectra were taken on the same solutions used in the absorption spectra study and excited at 280 nm. The emission spectrum was found to be shifted to the right slightly compared to a solution of native albumin. The peak at 327 nm (peak A) shifted to 331.2 nm, and the peak at 614.8 nm (peak B) shifted to 623.6 nm. In addition to the shift there was also a difference in the amount of light emitted. Peak A was increased by 77% and peak B by 48%. Amino groups are known auxochromes and carbamylation adds amino groups so this could explain the differences seen between CHSA and HSA.

Albumin Assay

Carbamylated albumin has been found to interfere with albumin measurement when detected using the bromocresol green (BCG) dye. However, there is no interference if albumin is measured using the bromocresol purple (BCP) dye (36). The BCP albumin assay could not be used to monitor albumin concentrations less than approximately 6 mg/mL without significant increases in the CV ($n = 10$; CV = 25.5% at 2 mg/mL, CV = 14.3% at 4 mg/mL, and CV = 9.2% at 6 mg/mL).

3.3.3 Albumin Purification Using Affi-Gel Blue

Affi-Gel Blue is known to bind albumin, and has been used to purify albumin from serum (20, 122-124, 130). This approach was investigated to see if it could be used to remove both carbamylated and noncarbamylated albumin from patient serum. Several purification strategies were investigated.

Initially, a method was chosen which described a very simple procedure for isolating albumin from serum (124). It was based on the method described in Bio-Rad's Bulletin 1107. A 1.5-mL column was used and to it 150 μ L serum was applied. In this procedure, the volume (2 mL) of equilibrating buffer (0.02 M phosphate buffer, pH 7.1) was found to be inadequate to wash out nonspecific protein binding and urea contamination. Also, the volume (1.5 mL) of eluting buffer (0.02 M phosphate buffer, 1.5 M NaCl, pH 7.1) allowed only ~50% of the

albumin to be desorbed. This method was then modified to ensure adequate washing and elution.

A uremic serum sample was first treated with urease (see Section 2.2.6) and then applied to an Affi-Gel Blue column. The column was washed until the absorbance at 280 nm was constant, then albumin was eluted with the salt containing buffer until the absorbance was again constant. Figure 3.9 shows the elution profile for this column. Protein is eluted in two major peaks, one with the wash buffer and the other with the eluting buffer. Similarly, carbamylated proteins were detected in both protein fractions. Albumin concentration could not be quantitated because of the limited sensitivity of the albumin assay (BCP). In order to find out which proteins were contained in each fraction, SDS-PAGE was done on several peak fractions (Figure 3.10). The electrophoresis showed that the first peak (lanes 2-5) contained many proteins, but almost no albumin. However, the second peak not only contained albumin, but several other proteins as well (lanes 6-9). If the column is eluted further, a pure albumin fraction can be obtained, but it is not positive for carbamyl groups. When native albumin ($n = 5$) was used instead of plasma in this procedure approximately 17% was not bound, and 74% was eluted after 10 mL of buffer.

The binding capacity of carbamylated albumin and noncarbamylated to Affi-Gel Blue was investigated next. This time the equilibrating buffer was 0.05 M Tris-HCl/0.05 M NaCl and the eluting buffer was 0.05 M Tris-HCl/2.5 M NaCl (20). Figure 3.11 shows that no carbamylated albumin bound to Affi-Gel Blue, whereas almost all the native albumin was bound.

In another method, an albumin rich sample was first prepared by ammonium sulfate fractionation of plasma (123). SDS-PAGE revealed that the ammonium sulfate precipitation was not effective in removing albumin from the other proteins. In fact, the supernatant of the fractionation contained mostly albumin, whereas the precipitate contained a mix of albumin and all the other proteins. Affi-Gel Blue chromatography of the precipitate showed as before that many proteins bind, but it also showed that if additional eluting buffer was added

FIGURE 3.9**Affi-Gel Blue Chromatography of a Uremic Serum Sample***Legend*

The 16 cm x 1 cm column was equilibrated with 0.02 M phosphate buffer, pH 7.1 at a flow rate of 1 mL/min. Urea was removed from the serum sample by urease and 2 mL applied to the column (see Section 2.2.7.1). The column was washed with equilibrating buffer until the absorbance at 280 nm remained constant. The bound protein was eluted with 0.02 M phosphate buffer containing 1.5 M NaCl, pH 7.1. Sample fractions were tested for total protein (Lowry assay, see Table 2.6) and carbamylated protein (Hunninghake and Grisolia assay, see Section 2.2.7.2). Numbers associated with data points indicate fractions that were subjected to SDS-PAGE (see Figure 3.10).

FIGURE 3.9

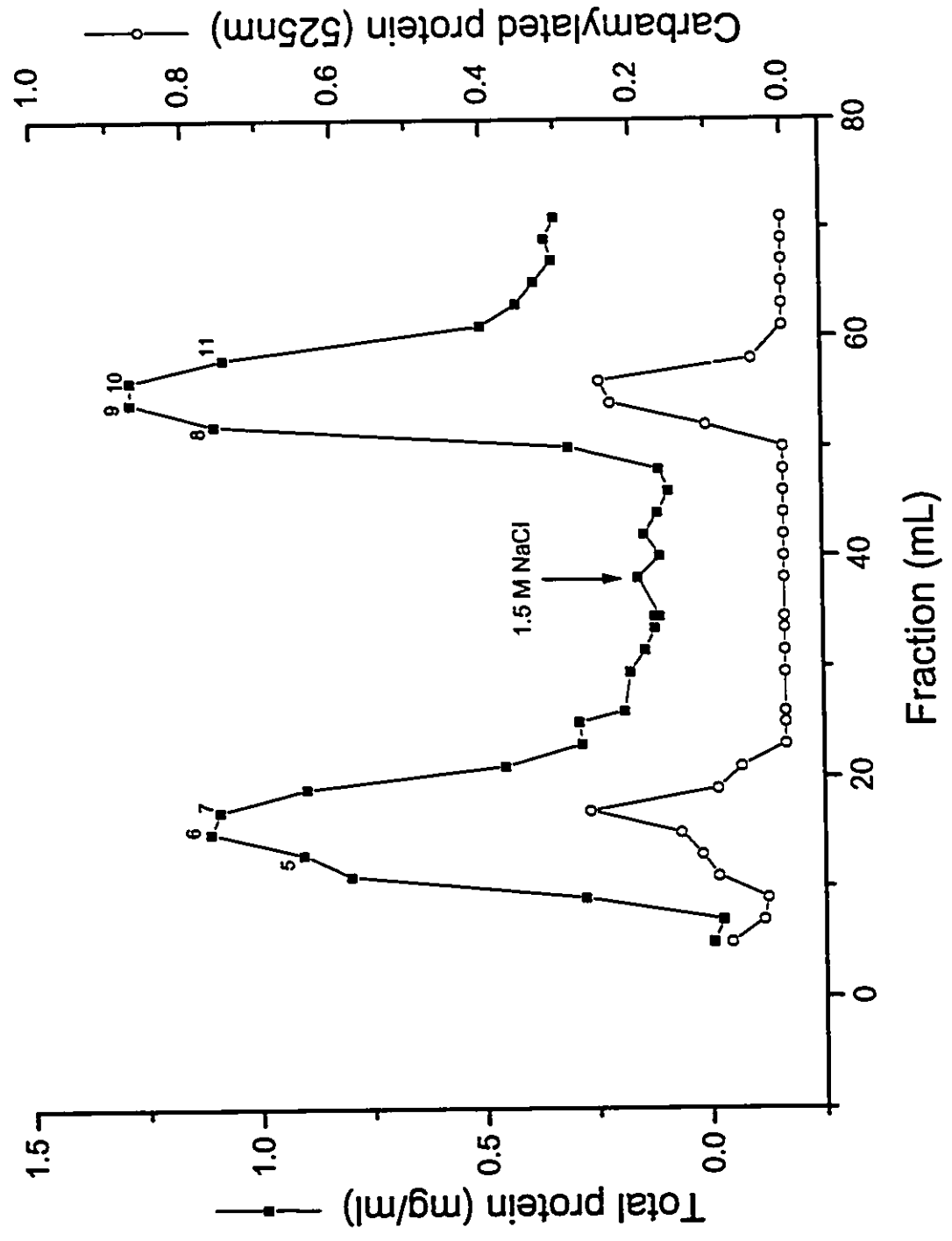


FIGURE 3.10**SDS-PAGE of Selected Fractions From the Affi-Gel Blue Chromatography of Uremic Serum***Legend*

Samples were applied to a 10% SDS gel and run at constant current (20 mA/gel) for about 1 h. The gel was stained with Coomassie Brilliant Blue for 4 h, destained with destaining solution I for 1 h, and then left in destaining solution II overnight (see Section 2.2.5.2). Lane 1, is native HSA (5 µg); Lanes 2-5, are elution fractions 11, 10, 9 and 8, respectively; Lanes 6-8, are wash fractions 7, 6 and 5, respectively; and Lane 9, is the serum sample diluted 50-fold.

FIGURE 3.10

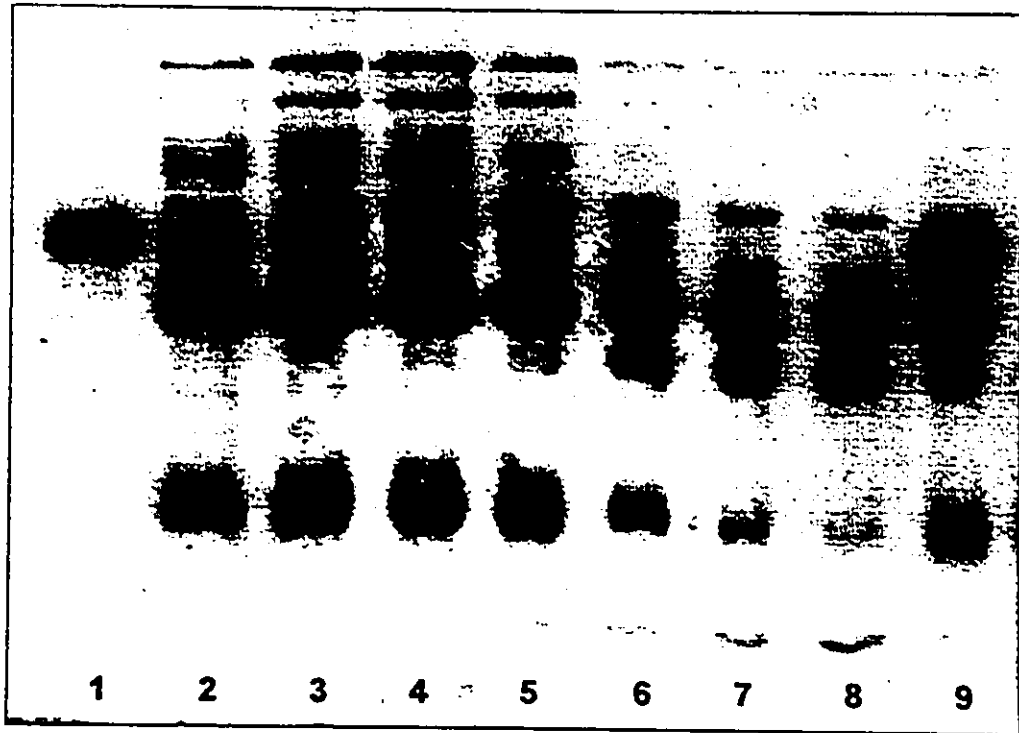
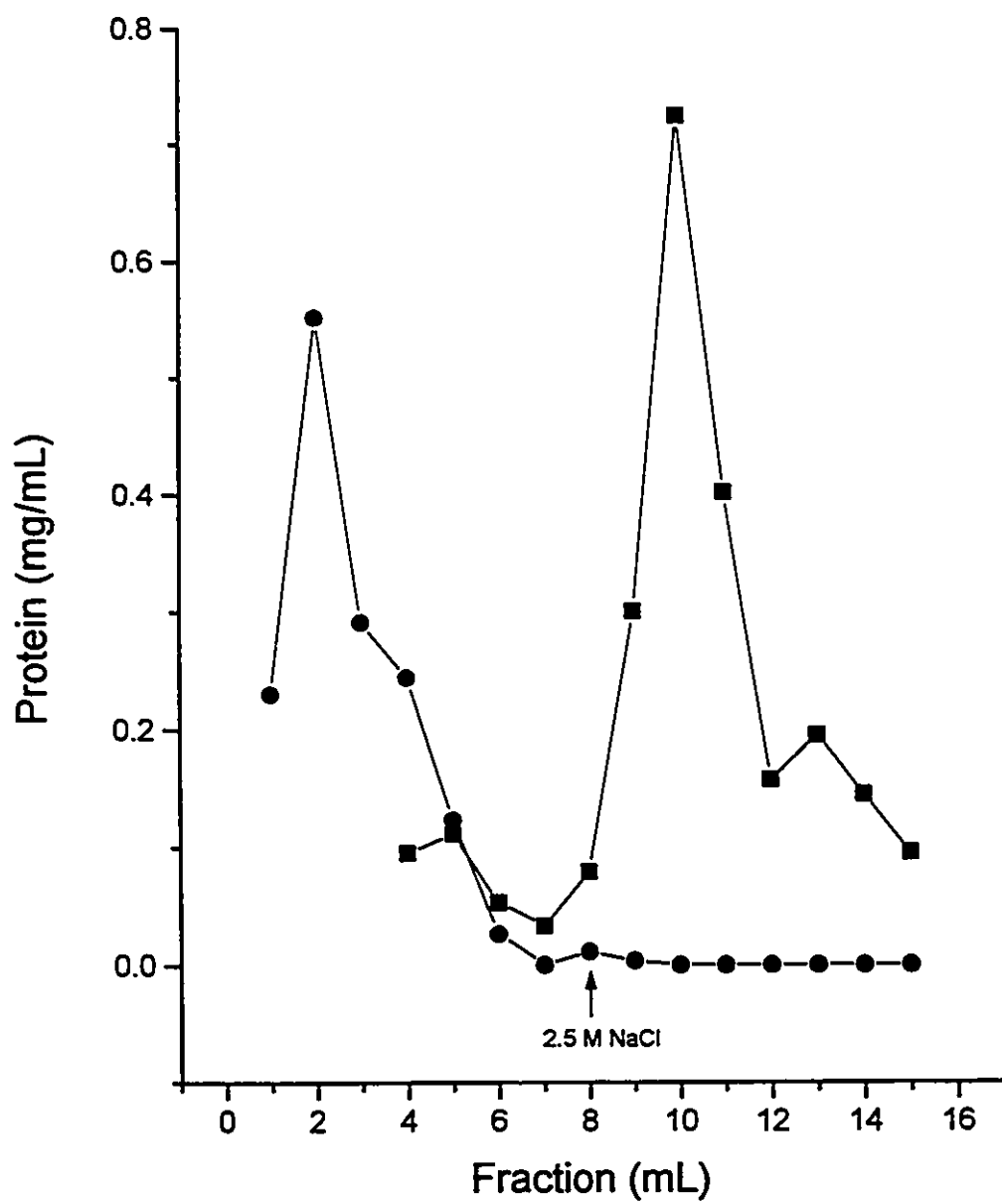


FIGURE 3.11**Binding of Carbamylated Albumin and Native Albumin to Affi-Gel Blue***Legend*

CHSA (●) (400 μ L x 5 mg/mL) and HSA (■) (400 μ L x 14 mg/mL) were applied to individual columns (2 cm x 1 cm) (see Section 2.2.6). The wash buffer was 0.05 M Tris-HCl, pH 8.0 and the elution buffer was 0.05 M Tris-HCl, 2.5 M NaCl, pH 8.0. Aliquots were taken in 1-mL volumes and analyzed for protein (Bradford method, see Table 2.6).

FIGURE 3.11



to the column pure albumin fraction could be obtained.

3.3.4 Purification of Noncarbamylated HSA

Commercial HSA, since it is purified from human plasma, contains several derivatives of HSA due to post-translational modifications. An attempt was made to purify noncarbamylated HSA from this commercial preparation so that it could be used as a standard or control.

The same system used by Day *et al.* (130) for the separation of glycosylated HSA from nonglycosylated HSA was tried. The assumption was made that glycosylated albumin and carbamylated albumin are somewhat similar because they both remove positive charges from HSA. HSA (99%), in 0.01 M sodium acetate, pH 4.65, was applied to a carboxymethylcellulose column and washed with 10 bed volumes of starting buffer. The column was eluted with a 150-mL gradient of 0.01 to 0.5 M sodium acetate, pH 4.65. Only one peak was eluted. The reason might be because in the original method, the HSA was purified from fresh human serum by Affi-Gel Blue and gel chromatography, a milder process compared to the commercial preparation.

To look at the separation system more efficiently, an FPLC cation-exchange column (Mono SHR 5/5) was tried. Sodium acetate buffer, pH 4.65 was also used, but different gradients were made. In all cases, only one peak came off. The peaks were usually broad and seemed to be heterogeneous because of their roughness.

3.4 Immunoassays for Carbamylated Albumin

3.4.1 Specificity of Antiserum

3.4.1.1 Ouchterlony testing (immunodiffusion)

The HSA affinity purified antibody preparation (anti-CHSA) reacted with HSA, CHSA, normal plasma, and uremic plasma. A reaction of identity was formed between CHSA and HSA, HSA and normal plasma, and normal plasma with uremic plasma. In addition, a reaction of partial identity was noticed

between the CHSA and HSA wells by evidence of a faint spur pointing toward the HSA well. This suggested that the antibody solution probably contained another antibody that was specific to CHSA and not to HSA, that is, an antibody specific toward carbamyl groups on HSA.

3.4.1.2 Immuno-electrophoresis

Electrophoresis of HSA, CHSA, normal plasma, and uremic plasma was followed by immunodiffusion with anti-HSA. Only single arcs appeared in the albumin region with anti-CHSA. Carbamylated HSA, however, ran more anodically than HSA (decreased positive charges), but still showed only a single precipitan arc.

Electrophoresis of normal plasma, CHSA and HSA, followed by immunodiffusion with goat-anti-human serum showed a characteristic arc pattern for normal plasma. There was a strong single arc corresponding to albumin for HSA, plus an additional three thin, faint arcs indicating lack of purity in the preparation. The CHSA antigen showed a strong precipitan arc with this antibody in a position anodic to the albumin region.

These results show that there are similar epitopes on CHSA and HSA that allow for recognition with both anti-CHSA and anti-human plasma.

3.4.2 Antibody Purification

Initial purification strategies included nitrocellulose affinity purification using carbamylated fibrinogen and carbamylated hemoglobin, and a combination of ammonium sulfate (AmS) precipitation of IgG and HSA-affinity purification. Antibodies eluted from the nitrocellulose matrix bound equally to CHSA and HSA. The AmS/HSA-affinity preparation, reacted more with CHSA compared to HSA. When this preparation was passed through the HSA-affinity column two more times, the difference between reactivity with CHSA and HSA increased several fold.

A pure IgG preparation was made by passing whole serum through a protein A column. This preparation was then passed through either a CHSA-affinity column or a HSA-affinity column, removing 96.5% and 77% of the applied

IgG, respectively. The IgG preparations, total IgG, HSA-affinity purified IgG and CHSA-affinity purified IgG were tested for their specificity towards CHSA. Figure 3.12 shows that total IgG had the highest titre compared to the affinity purified preparations. However, HSA-affinity purified IgG gave the widest linear detection range.

Antibody specificity was tested using homocitrulline, HSA, and pooled plasma. All antibody preparations were diluted to give the same absorbance for maximal binding (B_0). No competition was seen with homocitrulline (0.31 - 20 mg/mL) and any antibody preparation (data not shown). Figure 3.13A shows that HSA (0.64 - 20.5 mg/mL) reacted completely with the total IgG preparation, but only slightly with the affinity purified preparations. There was less cross-reactivity seen with the HSA-affinity purified preparation than with the CHSA-affinity purified preparation. Similarly, plasma diluted 2 to 4000-fold, reacted completely with the total IgG preparation (Figure 3.13B). All competition with plasma was removed after a 50-fold dilution when the affinity purified antibody preparations were used.

Based on the data from these experiments, the HSA-affinity purified antibody preparation was selected as the antibody for the carbamylated albumin enzyme-linked immunosorbant assay (ELISA). The antibody was always diluted to a titre which did not react with HSA (40 mg/mL) and was limiting with respect to the antigen coated wells. The titre of the HSA-affinity purified antibody varied during the course of the evaluation because of the different preparations used and their instability. The loss of titre in one preparation after two weeks was reduced 50-fold. Precipitate in the antibody solution was observed with time which may explain the loss of titre. Freezing also affected the stability of the antibody.

There was no difference in titre between the three HSA-affinity purified preparations from the three immunized rabbits. All three dilution curves were superimposable (data not shown). The evaluation was done using equal IgG concentrations that were serially diluted and added to CHSA coated wells.

FIGURE 3.12**Antibody Dilution Curves For Total IgG, HSA- , and CHSA-affinity Purified Antibody***Legend*

Antibody dilution curves for total IgG (■), HSA-affinity purified IgG (○), and CHSA-affinity purified IgG (▲). Serial dilutions of antibody (0.7 mg/mL) were incubated in microtitre wells coated with CHSA. The amount of antibody was detected by using the secondary antibody goat-anti-rabbit-IgG-AP and *p*-NPP.

FIGURE 3.12

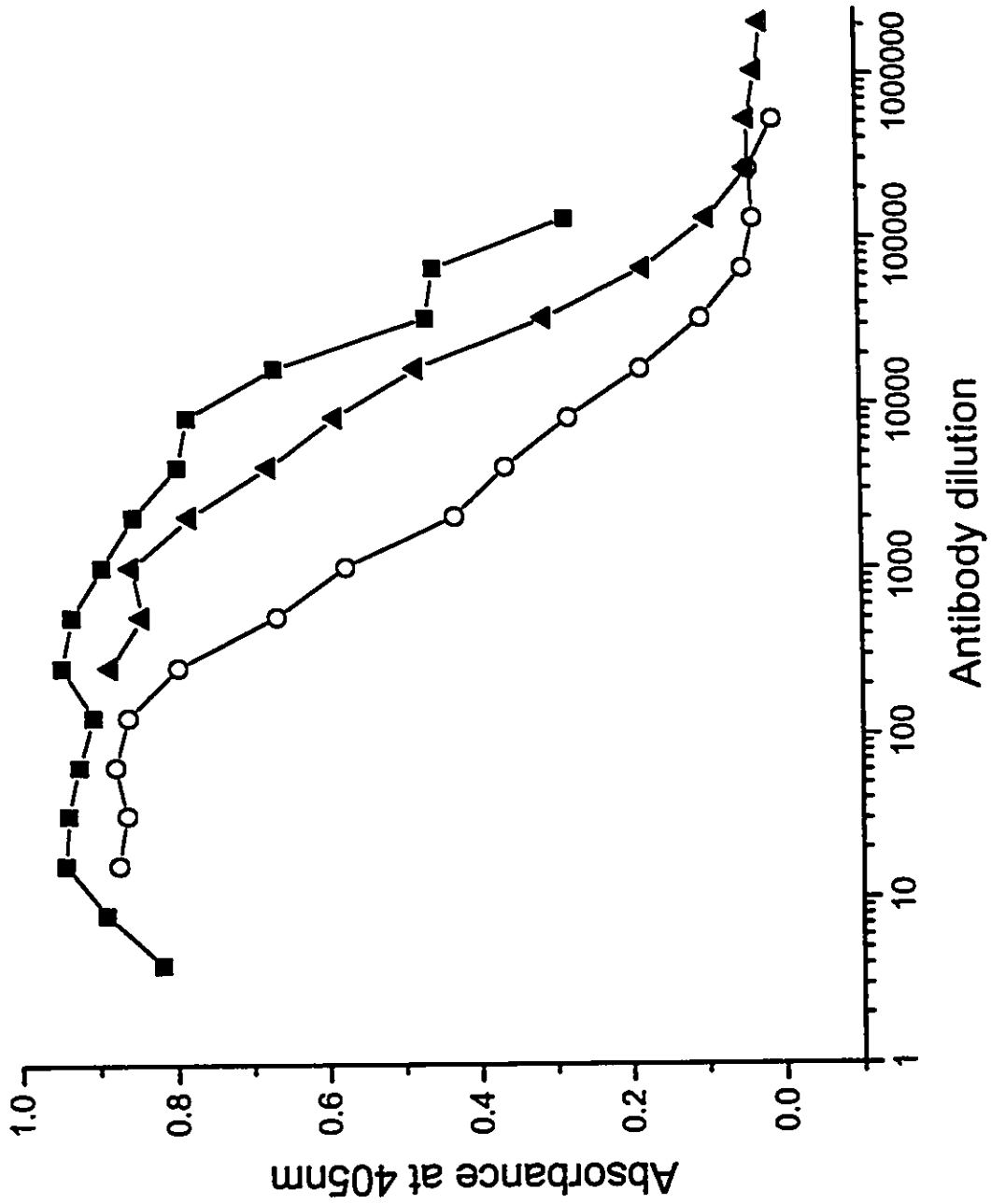
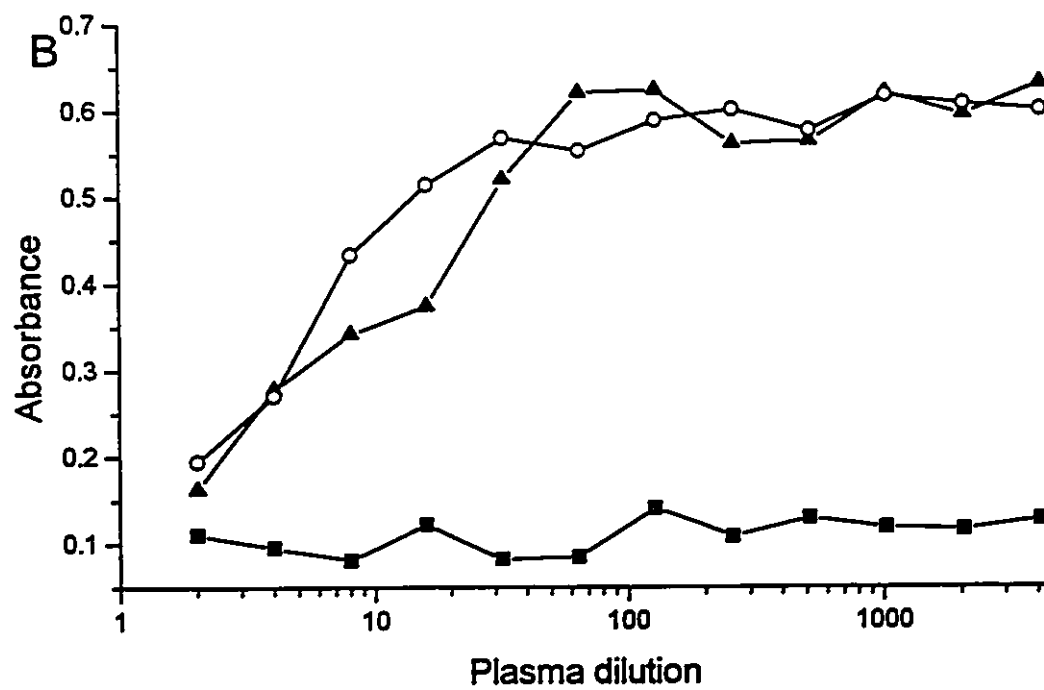
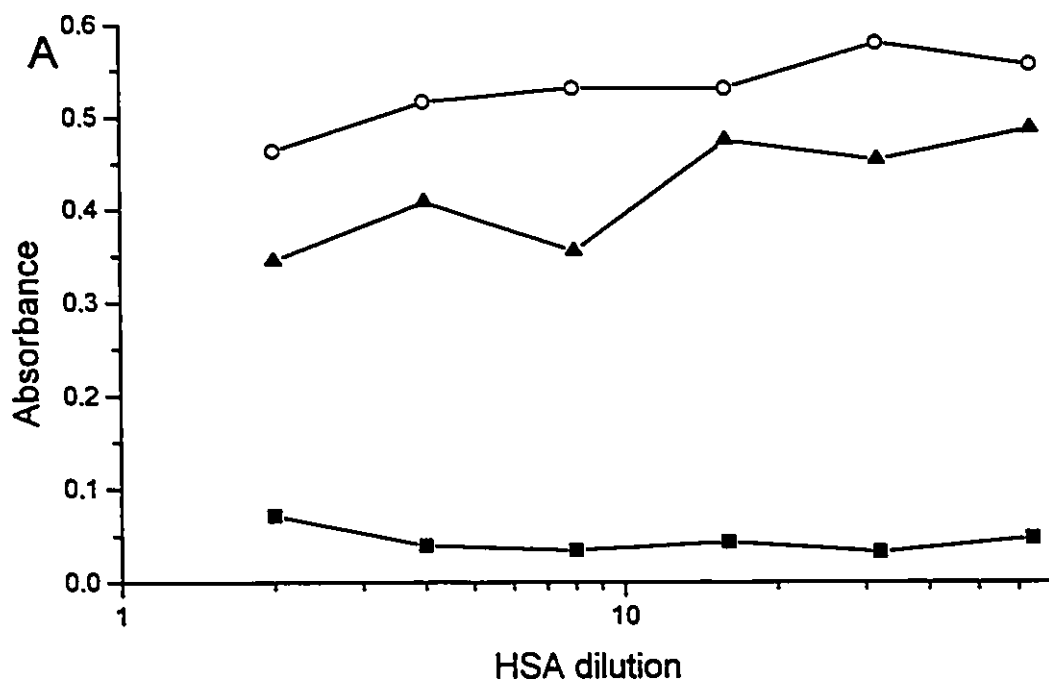


FIGURE 3.13**Competition of Anti-CHSA's Against HSA and Plasma***Legend*

Competition of total IgG (■), HSA-affinity purified IgG (○), and CHSA-affinity purified IgG (▲) against HSA (initial concentration 41 mg/mL) (A) and plasma (B). A limiting amount of antibody, calculated from Figure 3.12, was mixed with an equal amount of diluted competitor and preincubated for 1.5 h before addition to CHSA coated wells. The amount of antibody was detected by using the secondary antibody goat-anti-rabbit-IgG-AP and the substrate *p*-NPP (see Section 2.2.11.1).

FIGURE 3.13



Antibody was also purified on an Hb-affinity column. The results using this preparation will be discussed separately.

3.4.3 Competitive Assay

3.4.3.1 Linearity, detection limit, and imprecision

Figure 3.14 shows a representative calibration curve for the assay plotted as a dose-response curve (A) and its log/logit transformation (B). The minimum detection limit of the assay, calculated as $B_0 - 3(NSB)$, is 25 pmol of carbamyl groups. The maximum amount of carbamyl groups that can be detected is $3(NSB)$ or 100 nmol. The within-assay CV's for 33.1 nmol and 0.52 nmol carbamyl groups were 7.5% and 1.2%, respectively. The within-assay CV calculated using Tonks' procedure (method 2) (127) between replicates of plasma samples, normal ($n = 6$) and uremic ($n = 8$), was 7.8%.

The immunoassay's response to albumin carbamylated to different degrees was tested next. Figure 3.15A illustrates the change in absorbance associated with the change in albumin carbamylation. A minimum level of approximately 6 carbamyl groups must be present on HSA for the ELISA to be able to detect a change from native HSA. The change in absorbance per carbamyl group is 0.007 (Figure 3.15B). However, since the difference between replicate sample absorbance measurements can range from 0.001 - 0.04 (mean = 0.02), a change in one carbamyl group cannot easily be detected by this assay. A minimum difference of about 3 carbamyl groups is probably needed.

Antibody dilution curves were then done using wells coated with the various CHSA preparations (Figure 3.16). There was a significant difference in response with slightly carbamylated HSA preparations, but preparations of 45% carbamylation and above responded almost the same.

3.4.3.2 Specificity studies

HSA-affinity Purified Antibody

Figure 3.17 shows the specificity of AmS/HSA-affinity purified antibody. Carbamylated and noncarbamylated preparations of HSA, fibrinogen and hemoglobin were tested. All antigens showed some response, but only at low

FIGURE 3.14**Standard Curves For the Competitive ELISA of Carbamylated Albumin***Legend*

Dose-response curve (A). Log/logit transformation of A (B). Each point is a mean (\pm SD) of 3 measurements. The concentration of CHSA was converted to nmol of carbamyl residues using a MW of HSA of 69 kDa and a ratio of 44 carbamyl groups to 1 HSA molecule (see Section 2.2.11.1).

FIGURE 3.14

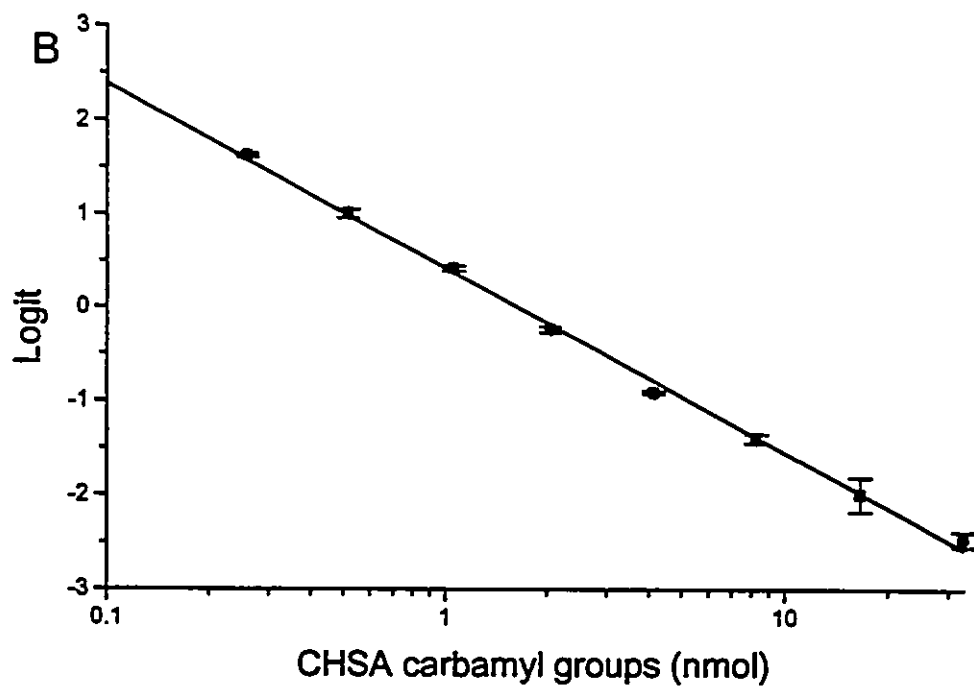
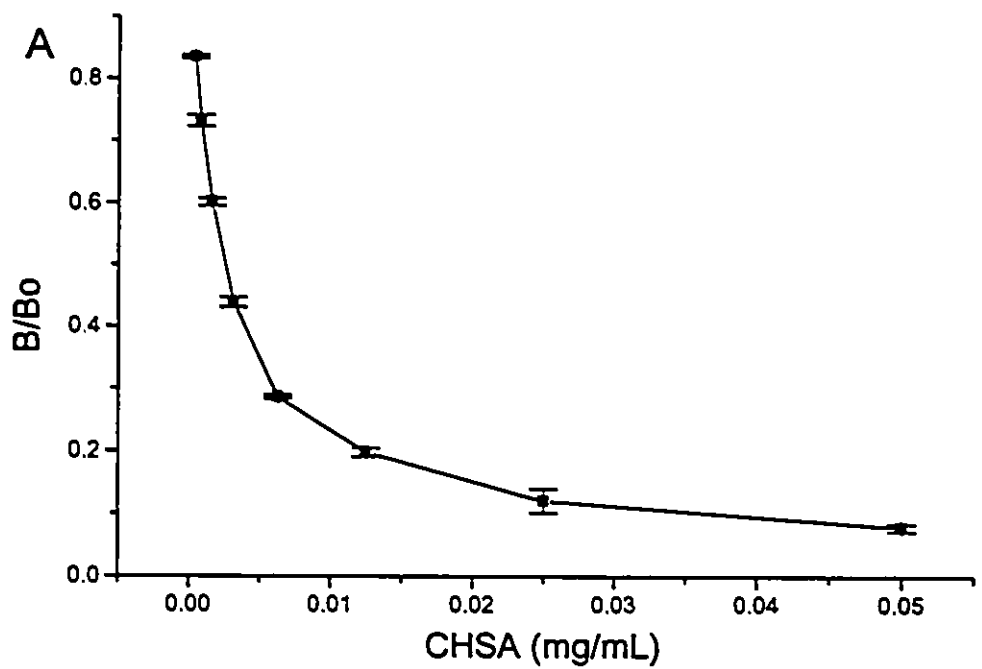


FIGURE 3.15**Detection of Albumin Carbamylated to Various Degrees***Legend*

(A) HSA was carbamylated for various lengths of time (see Section 3.3.1) and the extent of carbamylation detected by the TNBS assay (see Section 2.2.4) and the CHSA ELISA (see Section 2.2.11).

(B) Relationship between percent carbamylated HSA and the absorbance from the CHSA ELISA. The best fit line was $y = -0.0072x + 0.51$ ($r = -0.97$).

FIGURE 3.15

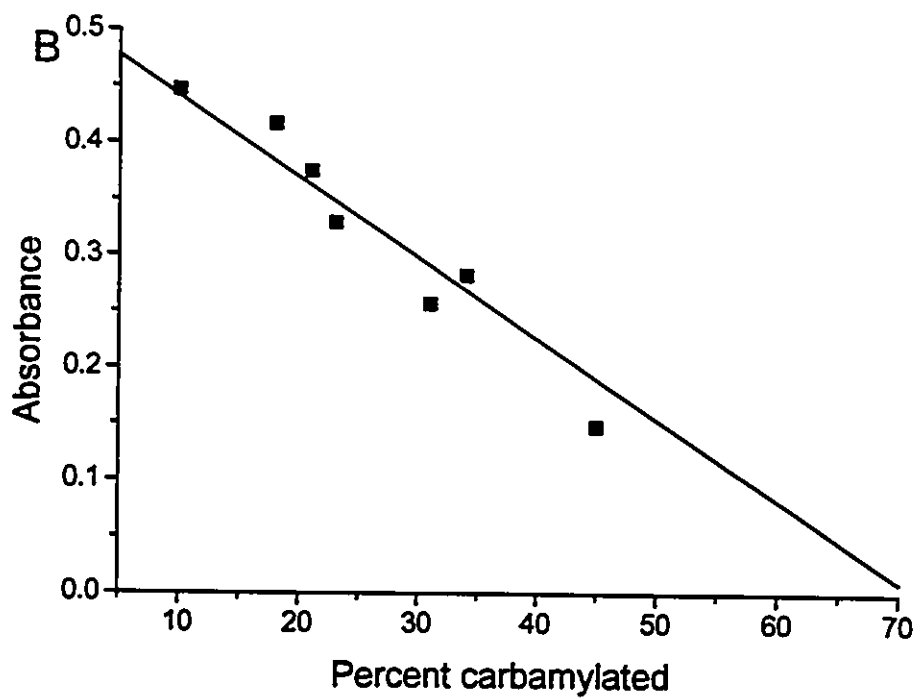
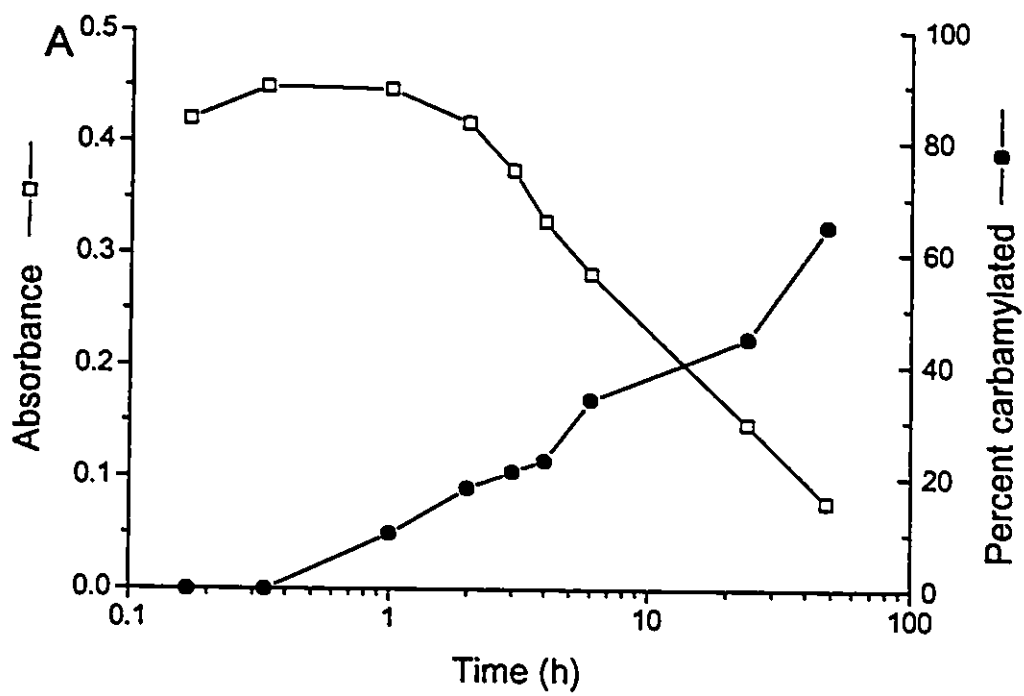


FIGURE 3.16**Antibody Dilution Curves With Various Preparations of CHSA***Legend*

HSA was carbamylated for various lengths of time (see Section 3.3.1) and the extent of carbamylation detected by the TNBS assay (see Section 2.2.4). The legend expresses the preparations in terms of the percent of free amino groups carbamylated (60 free amino groups on HSA). Samples were not corrected for NSB.

FIGURE 3.16

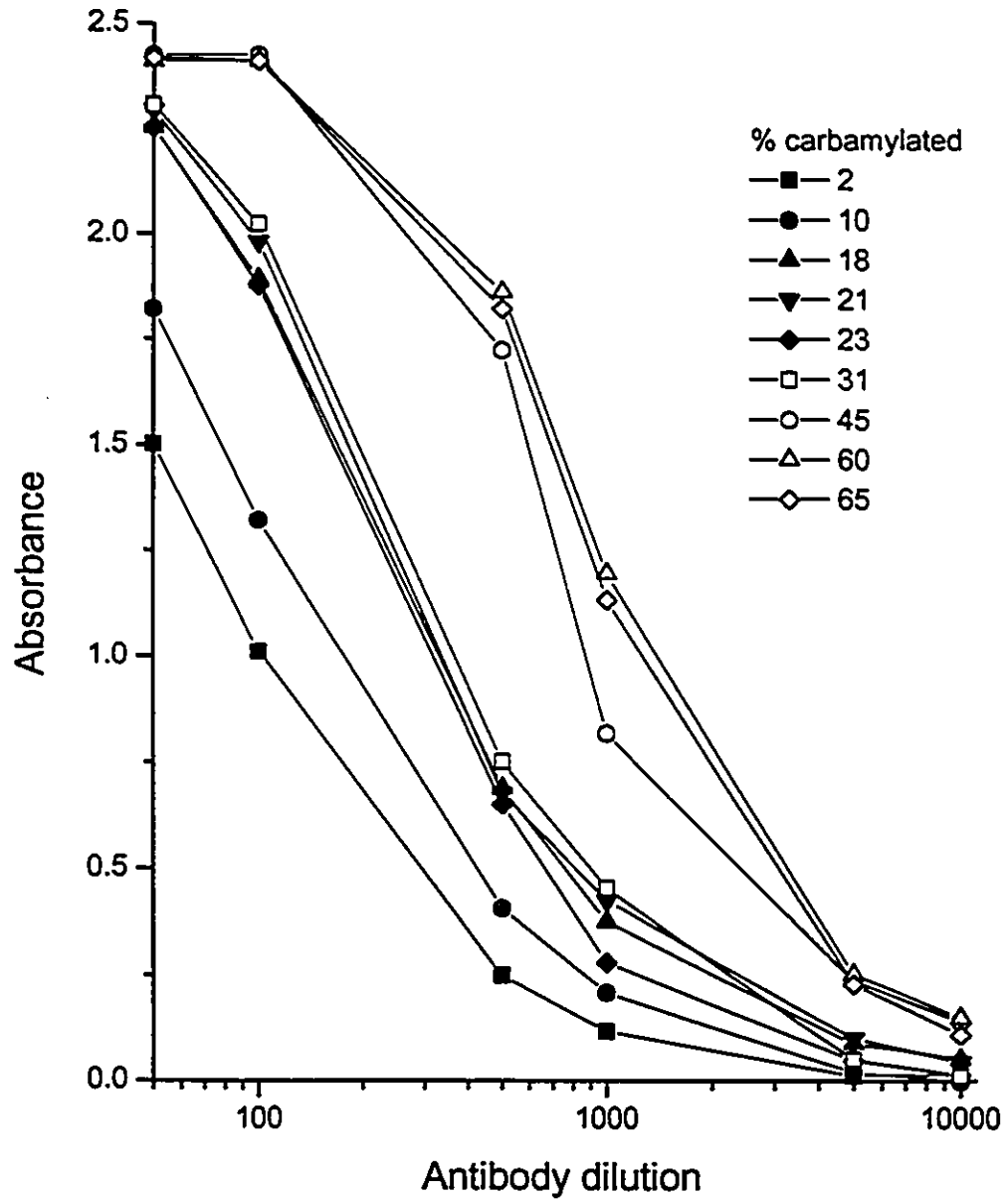
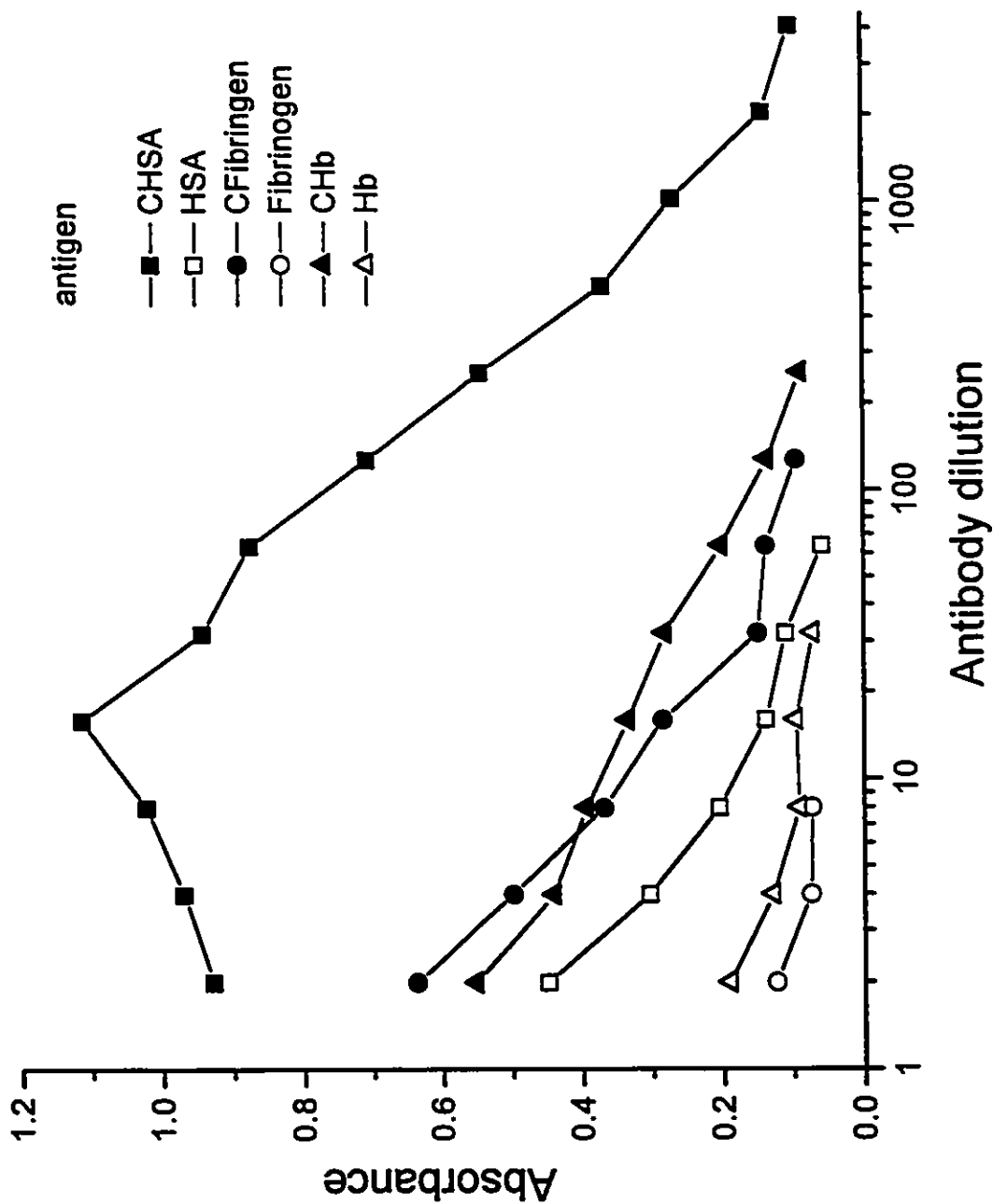


FIGURE 3.17**Specificity of the HSA-affinity Purified Antibody Preparation***Legend*

Carbamylated and noncarbamylated HSA, fibrinogen and hemoglobin were coated on microtitre wells and incubated with serially diluted antibody. The amount of bound antibody was detected by using the secondary antibody goat-anti-rabbit-IgG-AP and the substrate *p*-NPP.

FIGURE 3.17



antibody titres. The carbamylated antigens were all more reactive than their noncarbamylated counterparts. These results indicate that this antibody is able to detect carbamyl groups on proteins other than the carbamylated protein it was made against, namely CHSA.

A titration curve was done to look at the competition of unbound CHSA with bound CHSA (wells were coated with 2×10^{-5} mg of CHSA). Serially diluted CHSA (0.01 mg), with an equal volume of antibody (final volume 100 μ L), was added to the wells. The amount of free CHSA that was needed to remove all the antibody, so none was available for bound CHSA, was 0.005 mg. This is an amount 250-fold greater than the amount that could be maximally bound. This result shows the antibody's greater avidity for bound versus free CHSA. Furthermore, this also shows that the assay's sensitivity is reduced since the relationship is not one-to-one (bound-to-free).

CHb-affinity Purified Antibody

CHb-affinity purified antibody was tested for its reactivity with carbamylated and noncarbamylated hemoglobin and albumin (Figure 3.18). There was no dilution of antibody that was sufficient to exclude detection of noncarbamylated HSA or Hb, but not their carbamylated counterparts.

3.4.3.3 Patient studies

Plasma samples from normal individuals and uremic patients were tested to determine if a difference in the degree of carbamylation between these groups could be detected. Plasma titration curves were done for a normal and uremic sample of approximately equal albumin concentrations (Figure 3.19). Differences between these two samples could only be seen at low dilutions (<10 fold). The initial study was encouraging. It showed a significant difference between normal ($n = 6$) and uremic ($n = 7$) subjects ($p = 0.0014$). The plasma from the uremic patients showed a higher competition, or more carbamyl groups, than did the plasma from normal individuals. The amount of carbamylation was expressed as absorbance \times albumin(mg) in order to normalize the values with respect to albumin concentration. If the absorbance (ELISA) values were

FIGURE 3.18**Specificity of the CHb-affinity Purified Antibody Preparation***Legend*

Carbamylated and noncarbamylated HSA and hemoglobin were coated on microtitre wells and incubated with serially diluted antibody. The amount of bound antibody was detected by using the secondary antibody goat-anti-rabbit-IgG-AP and the substrate *p*-NPP.

FIGURE 3.18

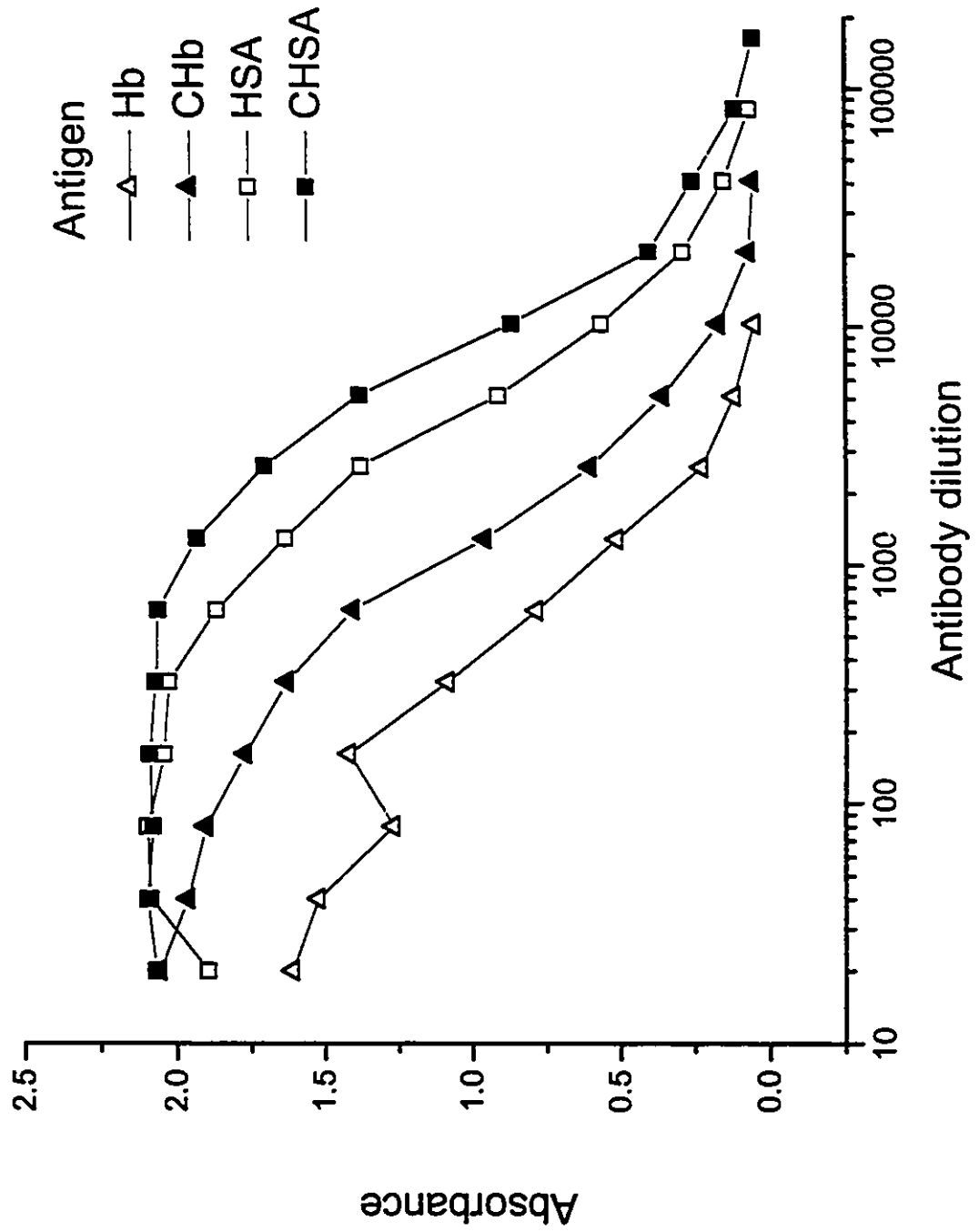
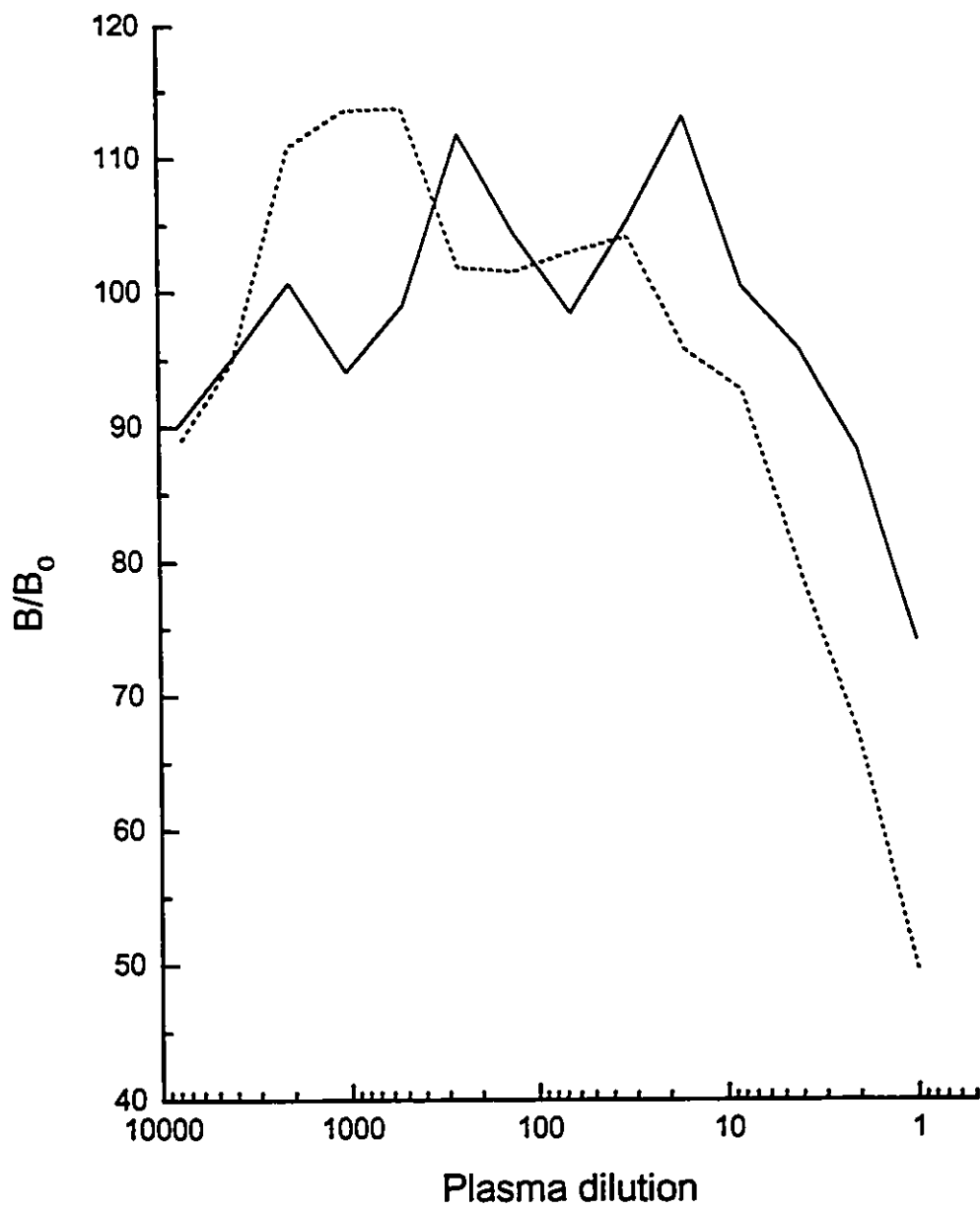


FIGURE 3.19**Plasma Titration Curves***Legend*

Plasma from a uremic patient (-----) and a normal individual (——) were diluted with an equal amount of diluted antibody and preincubated for 1 h before adding to CHSA coated wells (see Section 2.2.11.1).

FIGURE 3.19



multiplied by total protein instead of albumin no significance was seen ($p = 0.2$). These samples were also significantly different when assayed by the DAM assay ($p = 0.024$). However, the association between the immunoassay and DAM assay was unclear (Figure 3.20), probably due to the small sample size. If the two outliers (■*) were removed (no basis to do this though), then the correlation was fair ($y = -3.96x + 0.77$, $r = -0.66$, $p = 0.029$).

Repeat experiments were done using the same antibody preparation as well as newly prepared antibody with more normal and uremic subjects. There was no consistency, that is, there was either no difference between the groups or a difference that showed the normal group as having more carbamyl groups than the uremic group.

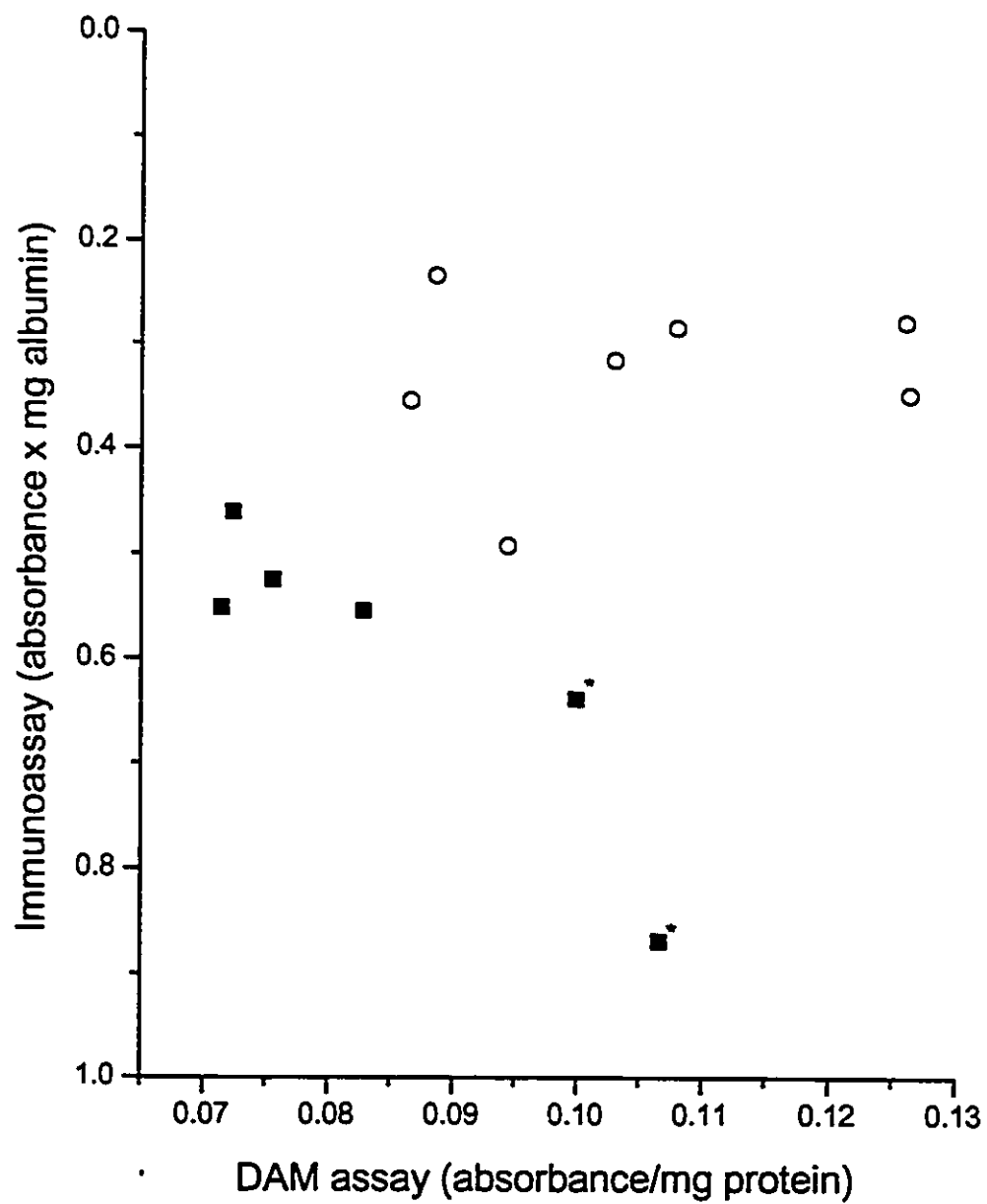
In one study, which tested the same samples as in the preliminary study using a fresh antibody preparation, different results were obtained. That is, there was no difference between the normal and uremic groups, but more interesting was the way the values changed. All values were higher than in the first analysis though the uremic group changed by twice as much as the normal group did. The CHSA standard gave the same B/B_0 as in the first study, but the HSA standard had a much lower B/B_0 . Therefore, this result seems to show a change in the specificity of the antibody preparation. It is unlikely the effect is due to a change in the carbamylated proteins because they have been shown to be stable.

Since the plasma samples were used neat without dilution, it was necessary to determine if there were potential nonprotein interferents. Therefore, to test this samples were dialyzed or passed through Affi-Gel Blue columns. There was no difference between dialyzed and non-dialyzed pooled uremic plasma. The Affi-Gel Blue preparations (lyophilized and reconstituted to the applied sample volume), were found to produce a smoother dilution curve. However, there were no differences between the normal and uremic plasma sample preparations (data not shown). In fact, they paralleled the dilution curve of the HSA standard. Furthermore, a spiking experiment showed very little

FIGURE 3.20**Comparison Between the CHSA Immunoassay and the DAM Assay***Legend*

Plasma from normal (■) and uremic subjects (○) were tested for the presence of carbamyl groups with the competitive ELISA (see Section 2.2.11.1) using HSA-affinity purified antibody, and the DAM assay (see Section 2.2.7.2). Each point was an average of duplicate measurements. Data points with an asterix denote outliers.

FIGURE 3.20



matrix interference (data not shown).

There was also no significant difference among the three HSA-affinity antibody preparations from the three immunized rabbits and patient plasma.

Patient studies with CHb-purified antibody were not possible because of the high cross-reactivity with native HSA. The absorbance was lower for native HSA than the absorbance for patient samples (equivalent HSA concentration).

Therefore, in order to gain more specificity for the assay a sandwich type immunoassay was attempted.

3.4.4 Sandwich Assay

3.4.4.1 Standard curve

HSA-affinity Purified Antibody

The linear range of this assay was found to be from 0.05 - 5 μg CHSA, or 32 - 3,200 pmol of carbamylated residues (Figure 3.21A). The minimum detection limit of the assay was determined by multiplying the NSB value ($n = 12$) by 2, and was found to be 1 pmol. The within-run imprecision, estimated by triplicate analysis was 0.8% for 5 μg CHSA and 11.3% for 0.05 μg CHSA. No between-run imprecision studies were done.

3.4.4.2 Cross-reactivity studies

HSA-affinity Purified Antibody

Figure 3.21B shows response of noncarbamylated HSA in this assay as compared to carbamylated HSA. The figure clearly shows that noncarbamylated HSA is detected by this assay, but a much higher concentration is required than for carbamylated HSA. When noncarbamylated HSA is diluted to 5 μg there is no longer any reaction. This indicates that the noncarbamylated HSA still has some carbamyl residues on it, or the antibody preparation has a few antibodies that are directed against the noncarbamylated sites on HSA. Whatever the case, this interference can be excluded if the samples that are being measured contain less than 5 μg of HSA.

CHb-affinity Purified Antibody

Cross-reactivity studies (Figure 3.22) in a sandwich ELISA showed that

FIGURE 3.21**CHSA Sandwich Immunoassay***Legend*

(A) Standard curve for the CHSA sandwich immunoassay. The wells were coated with anti-CHSA (HSA-affinity purified), the secondary antibody was goat-anti-HSA-AP and the substrate was *p*-NPP (see Section 2.2.11.2). Serial dilutions of *in vitro* CHSA (74%) were made in triplicate. The concentration of CHSA was converted to nmol of carbamyl residues using a MW of HSA of 69 kDa and a ratio of 44 carbamyl groups to 1 HSA molecule. The curve was linearized by taking the log of both sides.

(B) Comparison of CHSA (■) and HSA (○) reactivity.

FIGURE 3.21

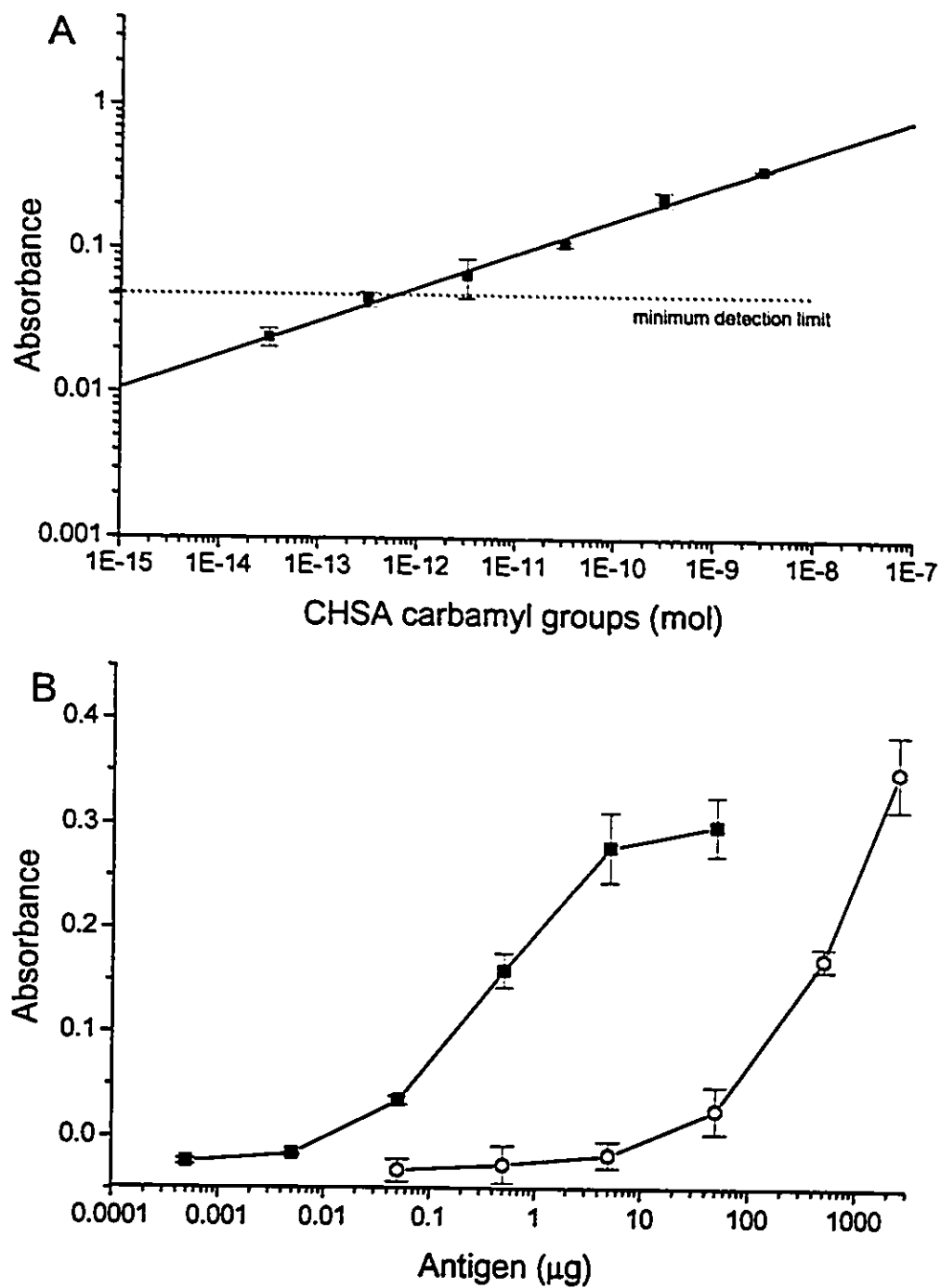
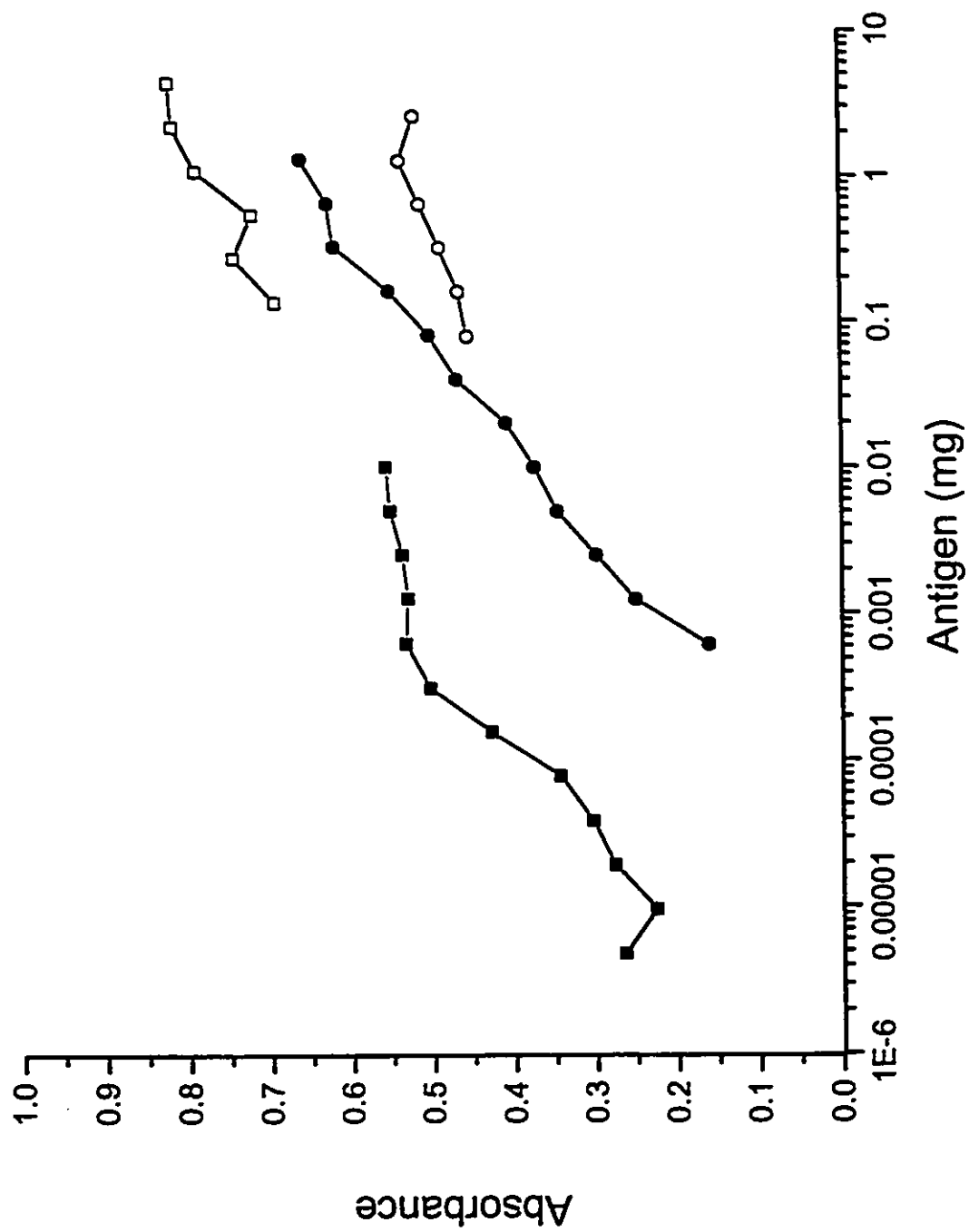


FIGURE 3.22**Cross-reactivity of CHb-affinity Purified Antibody in a Sandwich ELISA***Legend*

Serial dilutions of Hb (○), CHb (●), HSA (□) and CHSA (■) were incubated in HSA-affinity purified antibody coated microtitre wells for 1 h. After washing to remove unbound antigen, sheep anti-human albumin alkaline phosphatase was added. Following another incubation of 1 h, wells were washed again and substrate *p*-NPP added. Absorbances were not corrected for NSB (mean = 0.23).

FIGURE 3.22



this CHb-affinity purified antibody reacted not only to CHb, but also to a slightly lesser degree with noncarbamylated hemoglobin. This antibody also detected both carbamylated and noncarbamylated albumin. In addition, three uremic and three normal plasma samples (serially diluted up to 64-fold), were tested in this sandwich assay. No differences could be detected between them (data not shown).

3.4.4.3 Patient studies

There was no significant difference between serial dilutions of uremic and normal plasma samples (previously frozen and unfiltered) (Figure 3.23). Absorbance values decreased with increasing dilutions. The CV's were high (mean CV's = 40% and 18% for normal and uremic dilutions, respectively), and the absorbances lower than expected when compared to HSA or CHSA standards in buffer (Figure 3.21B). The absorbance of HSA (20 mg/mL) was about 4.5-fold greater than for normal plasma (HSA concentration ~ 20 mg/mL).

The above results seemed to indicate a matrix interference problem and therefore a spiking experiment was done to see if this was the case. Pooled normal plasma was filtered, serially diluted, and spiked with a limiting amount of CHSA (500 ng). Filtering increased sample absorbances and also lowered CV's (21%). However, no additive effect was seen in the spiked samples compared to the nonspiked samples (Figure 3.24B). In fact, the absorbance values were all very close to CHSA (500 ng) in buffer.

There was also no difference between pooled and filtered normal and uremic samples using HSA- or CHSA-affinity purified antibody.

Assays were also tested using PBS and Tris buffered saline (TBS) buffers. It was found that samples (pooled and filtered, normal and uremic) assayed in PBS buffer gave higher absorbance readings than samples assayed in TBS buffer. However, there was no significant difference between normal and uremic samples using either buffer.

FIGURE 3.23

Sandwich Immunoassay For CHSA in a Normal and a Uremic Plasma Sample

Legend

Sandwich immunoassay for CHSA in a normal (■) and a uremic plasma sample (●). Each dilution was done in triplicate. See Section 2.2.11.2 for procedure.

FIGURE 3.23

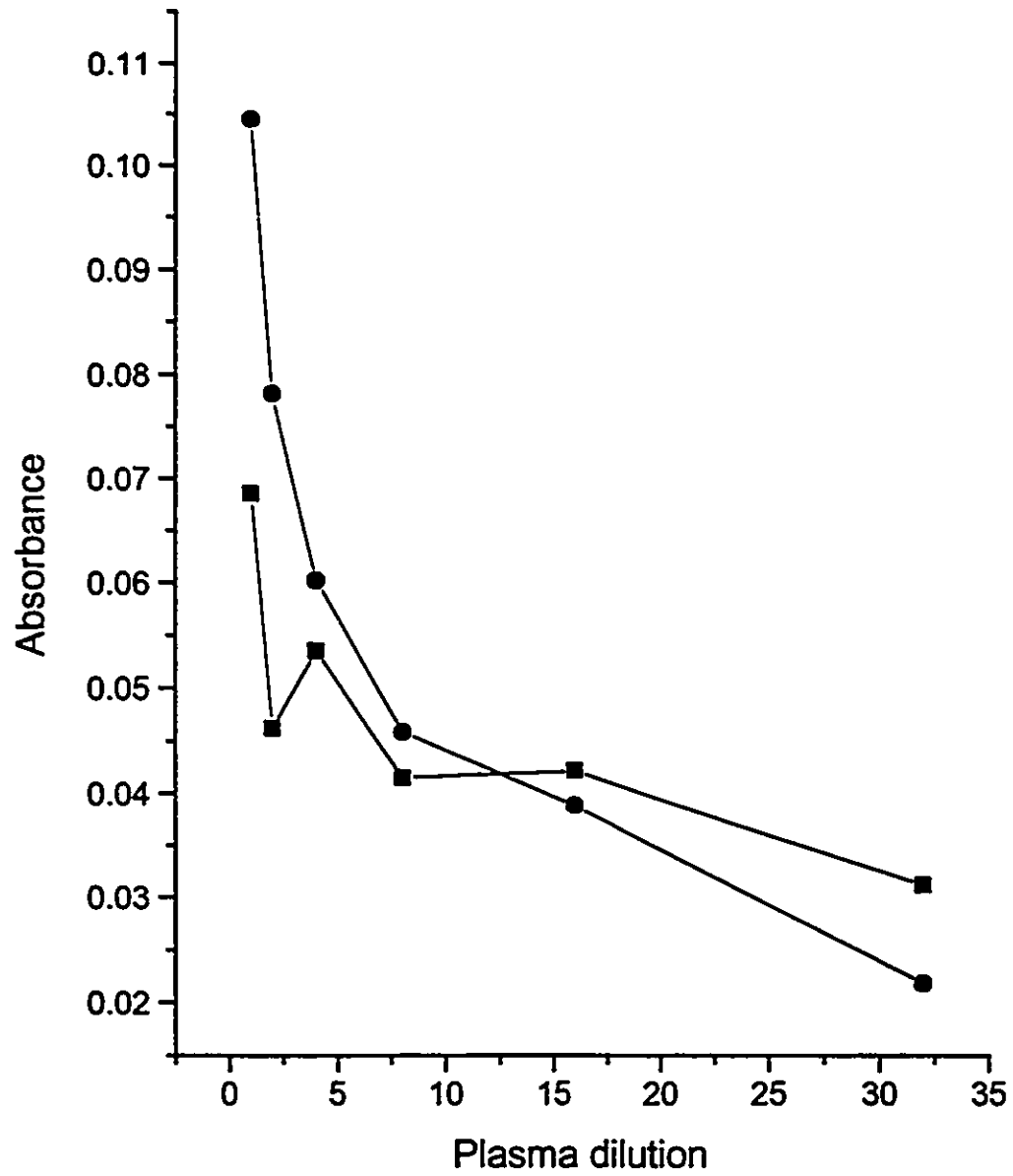
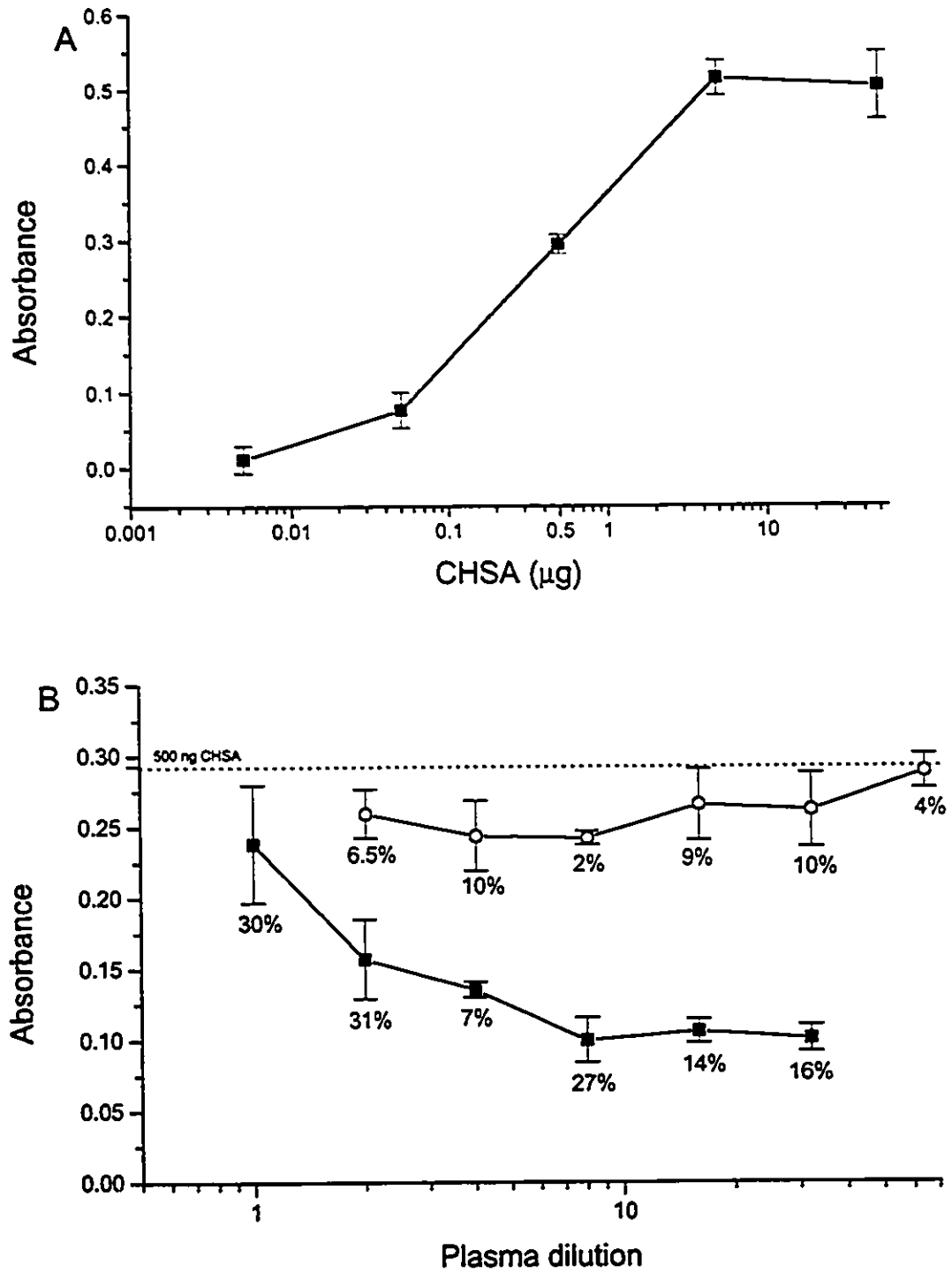


FIGURE 3.24**Plasma Matrix Interference in the CHSA Sandwich Immunoassay***Legend*

(A) CHSA standard curve for this assay. Error bars represent the SD of triplicate measurements.

(B) A pooled normal plasma sample (○) serially diluted and spiked with 500 ng of CHSA compared to a non-spiked sample (■). Values below each error bar (SD) are the corresponding CV's for each dilution. Assay conditions are given in the legend for Figure 3.21. Error bars represent the SD of triplicate measurements.

FIGURE 3.24



3.5 Carbamylated Proteins in Hemodialyzed Patients

3.5.1 Relationship of CHb with CTP

Figure 3.25 shows the relationship between carbamylated hemoglobin and carbamylated total protein in normal ($n = 9$) and uremic patients ($n = 13$). The correlation of carbamylated hemoglobin with carbamylated total protein is highly significant ($p < 0.0001$) with a correlation of 0.87 (SD = 0.01). However, if associations are done separately on the normal and uremic group, the correlations are much different. The normal group shows no association between CHb and CTP values ($r = 0.07$, $p = 0.85$), but the uremic group shows a fair association ($r = 0.61$, $p = 0.026$).

There was also a significant difference found between the normal and uremic groups for both carbamylated hemoglobin ($p < 0.0001$) and carbamylated total protein ($p < 0.0001$). The mean value for carbamylated hemoglobin in normal individuals and uremic patients was found to be $53 (\pm 20)$ $\mu\text{g VH/g Hb}$ and $157 (\pm 40)$ $\mu\text{g VH/g Hb}$, respectively. The mean value for carbamylated total protein in normal individuals and uremic patients was found to be $0.08 (\pm 0.009)$ A/mg protein and $0.117 (\pm 0.011)$ A/mg protein, respectively.

The relationship between carbamylated hemoglobin and carbamylated total protein also shows that the y-intercept crosses at 0.069 (absorbance/mg protein). This is a very high y-intercept considering that the highest y-value is only about twice that value. This may suggest that carbamylated total protein can be detected sooner than carbamylated hemoglobin.

3.5.2 Longitudinal Study: Correlation With Uremic Markers

The results of the longitudinal study comparing urea (pre- and post-dialysis), KtV, carbamylated protein and carbamylated hemoglobin in 7 patients are shown in Figure 3.26. There do not appear to be any clear relationships, or discernable patterns between any sets of curves that are consistent amongst all patients.

Because of the very different half-lives of carbamylated protein and

FIGURE 3.25

Correlation Between Carbamylated Plasma Protein and Carbamylated Hemoglobin in Normal and Uremic Subjects*Legend*

Relationship between carbamylated hemoglobin and carbamylated total protein in 9 normal (O) and 13 uremic (■) patients. The regression line was $y = 2.9E^{-4}x + 0.069$ ($r = 0.87$, $SD = 0.01$, $p < 0.0001$). Duplicate determinations were done for each sample. The methods used for the determination of CTP and CHb can be found in Sections 2.2.7.2 and 2.2.8.3, respectively.

FIGURE 3.25

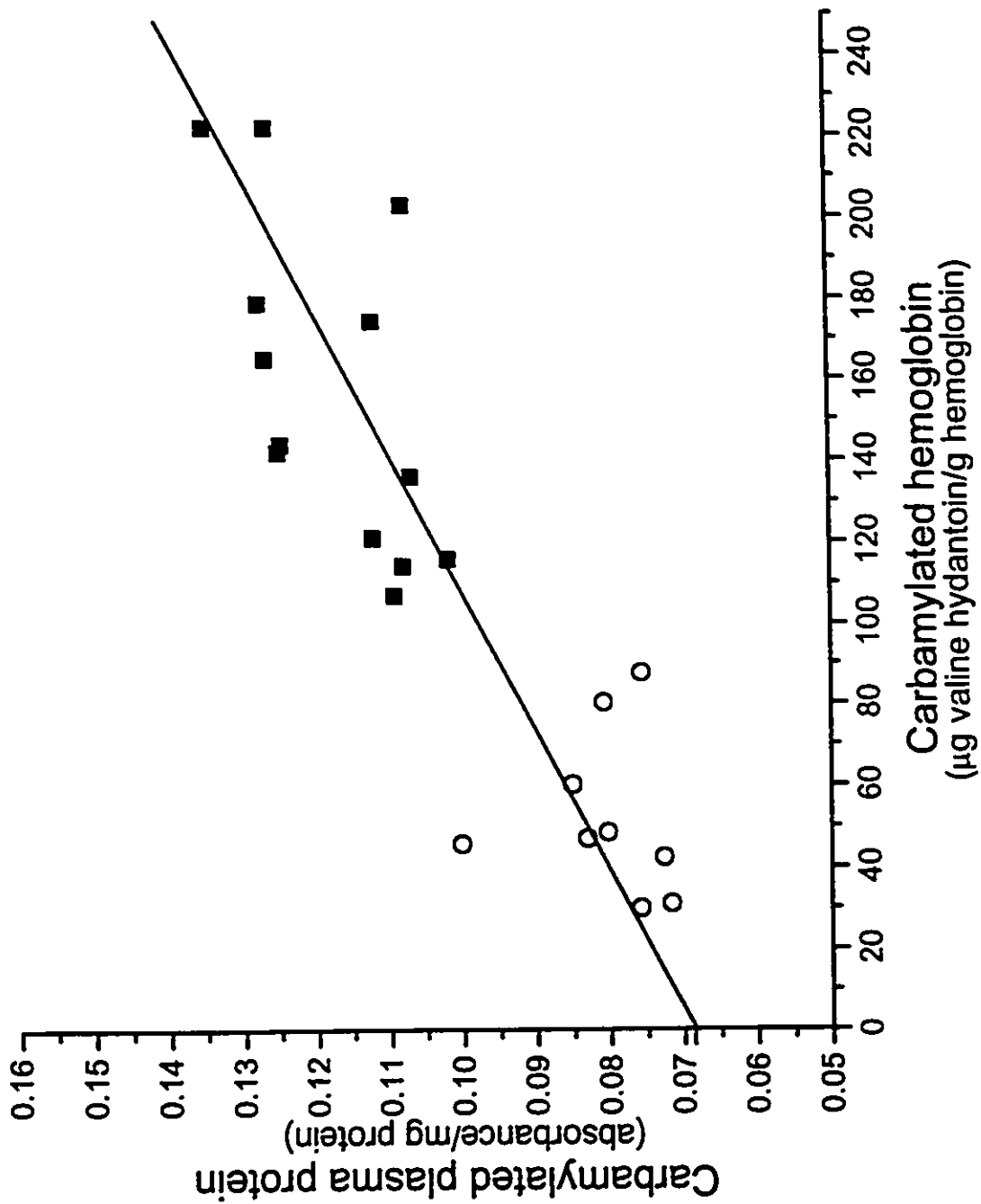


FIGURE 3.26**Serial Measurements of Uremic Markers in Hemodialyzed Patients***Legend*

A six-month longitudinal study of traditional dialysis markers, urea and Kt/V , with time-averaged urea markers, carbamylated hemoglobin and carbamylated protein, in seven thrice weekly hemodialyzed (HD) patients (#3, #33, #40 and #56). The methods used for the measurement of urea, CHb and CTP can be found in Table 2.7, Section 2.2.8.3 and Section 2.2.7.2, respectively. Kt/V was calculated using a pre-programmed calculator (Fresenius-Brent, Toronto, ON) incorporating the variable volume single-pool model developed by Gotch and Sargent (91).

FIGURE 3.26

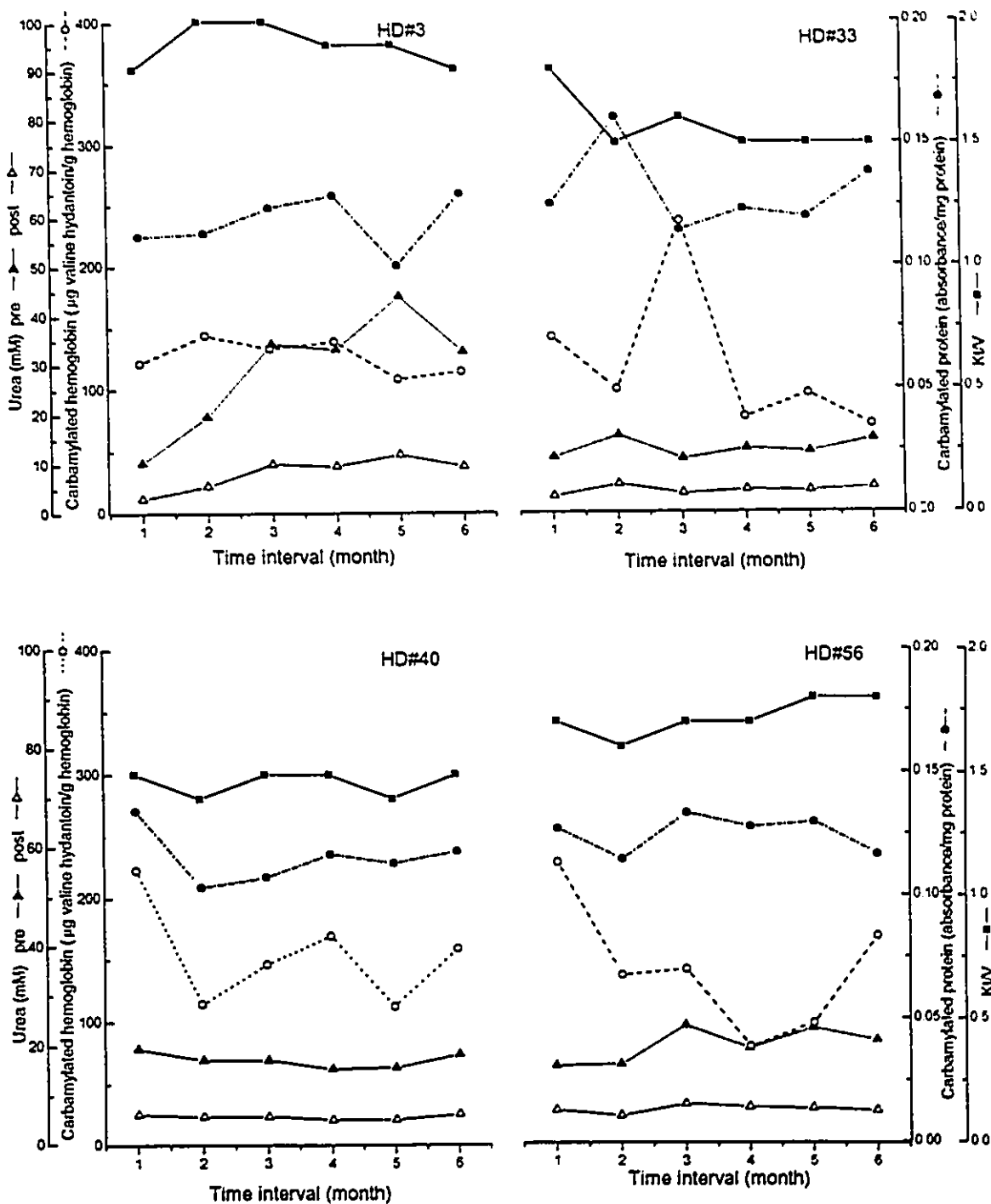
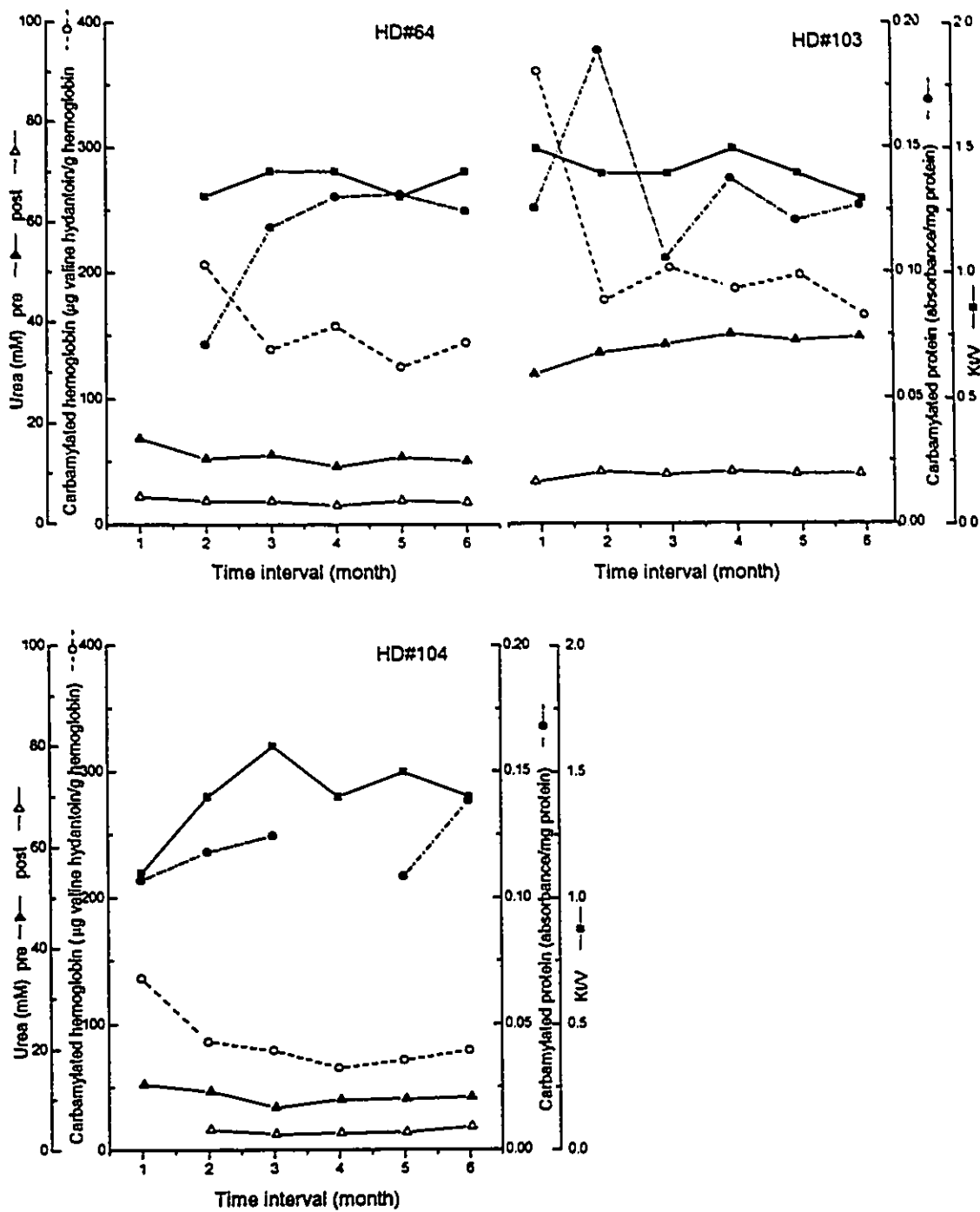


FIGURE 3.26 continued**Serial Measurements of Uremic Markers in Hemodialyzed Patients***Legend*

A six-month longitudinal study of traditional dialysis markers, urea and Kt/V, with time-averaged urea markers, carbamylated hemoglobin and carbamylated protein, in seven thrice weekly hemodialyzed (HD) patients (#64, #103, and #104). The methods used for the measurement of urea, CHb and CTP can be found in Table 2.7, Section 2.2.8.3 and Section 2.2.7.2, respectively. Kt/V was calculated using a pre-programmed calculator (Fresenius-Brent, Toronto, ON) incorporating the variable volume single-pool model developed by Gotch and Sargent (91).

FIGURE 3.26 continued



carbamyated hemoglobin, correlations performed at single times are of little value in assessing the relative clinical utility of the two assays. It was therefore considered that a complementary assessment could be obtained from data on the relative changes of urea, Kt/V, carbamyated albumin and carbamyated hemoglobin, and by application of these in the analysis of a longitudinal study of urea control. Figure 3.27 show the relative % changes in the uremic marker values over time. These figures show more clearly how much one uremic marker has changed relative to any other since the measurement scales are the same. Kt/V values vary the least (6.3%) over this time period whereas CHb has an average fluctuation of nearly 5 times more (30.1%). Pre-dialysis urea and CTP values have similar relative fluctuations (18.3% and 14.9%, respectively), in between Kt/V and carbamyated hemoglobin values. This may indicate that CHb is more sensitive in measuring urea fluctuations than either pre-dialysis urea, CTP or the dialysis dose, Kt/V.

The results of the correlation studies (Table 3.1) do not seem to reveal much more in terms of relationships than did the visual observations of the initial data. A look at the correlation coefficients reveals that there was no correlation in most cases between any two tests. Furthermore, the correlations that did exist between any two tests were mixed, positive and negative, and in very few cases was the correlation significant ($p < 0.05$). The reason for these apparent ambiguities could be due to the nature of the tests themselves, in that they do not measure the same thing at the same time, or the lack of a larger sample size (frequency of sampling and number of patients). There is no reference standard to make the comparisons.

Table 3.2 shows the correlations between pre-dialysis urea and CHb, and pre-dialysis urea and CTP on the data sets ($n = 7$) for each time period ($n = 6$). There was a much higher association between pre-dialysis urea and CHb ($r = 0.97, 0.86, 0.71, 0.52, 0.51, 0.40$) than with pre-dialysis urea and CTP ($r = 0.48, 0.77, -0.11, 0.66, 0.26, 0.08$).

Furthermore, a multiple regression analysis was done to statistically

FIGURE 3.27**Serial Measurements of Uremic Markers in Hemodialyzed Patients
Expressed as Relative % Change***Legend*

Measurement of four uremic markers used to follow seven thrice weekly hemodialyzed patients (#3, #33, #40, #56) over a six-month period. The uremic marker values were calculated as percent change between consecutive months thus allowing for direct comparison between uremic markers.

FIGURE 3.27

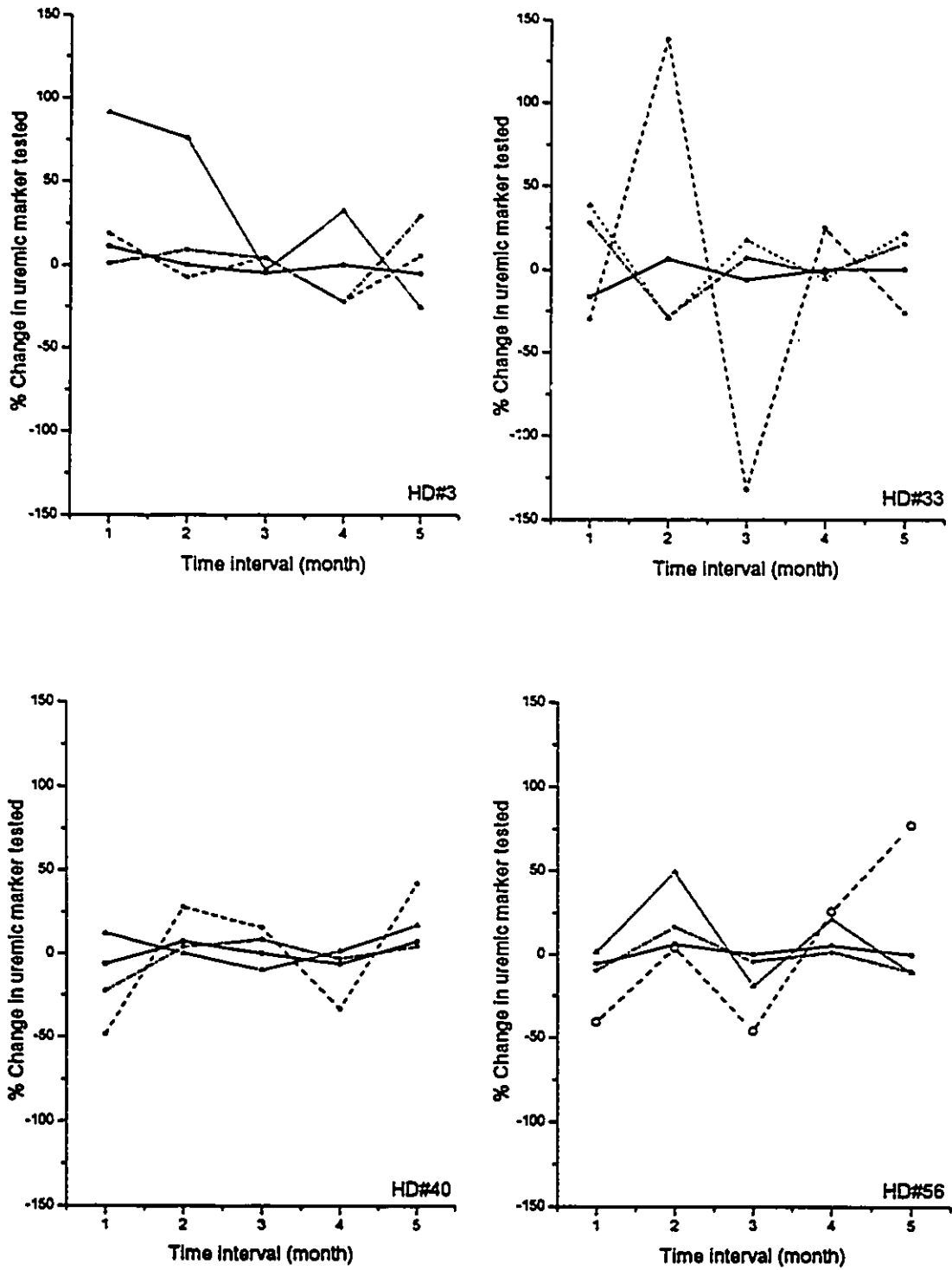


FIGURE 3.27 continued**Serial Measurements of Uremic Markers in Hemodialyzed Patients
Expressed as Relative % Change***Legend*

Measurement of four uremic markers used to follow seven thrice weekly hemodialyzed patients (#64, #103, and #104) over a six-month period. The uremic marker values were calculated as percent change between consecutive months thus allowing for direct comparison between uremic markers.

FIGURE 3.27 continued

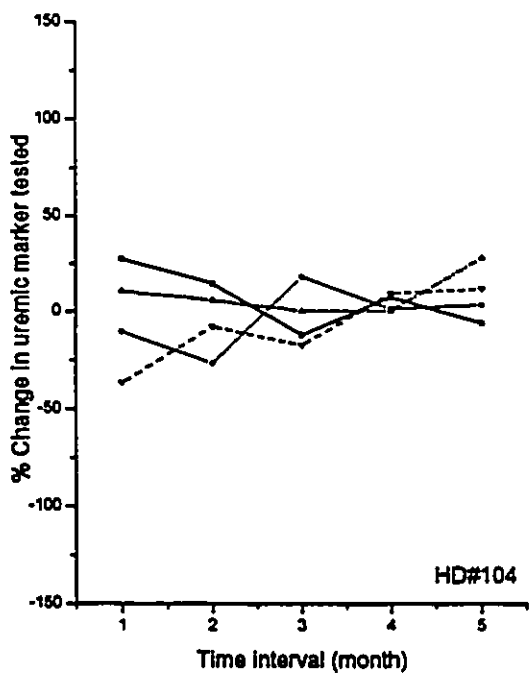
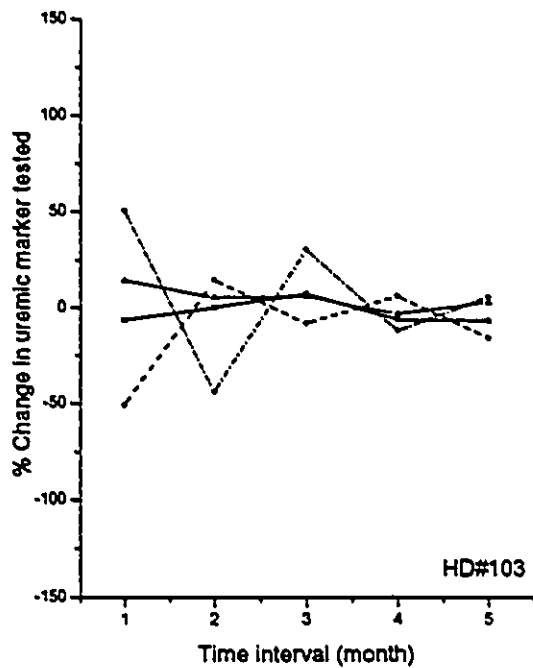
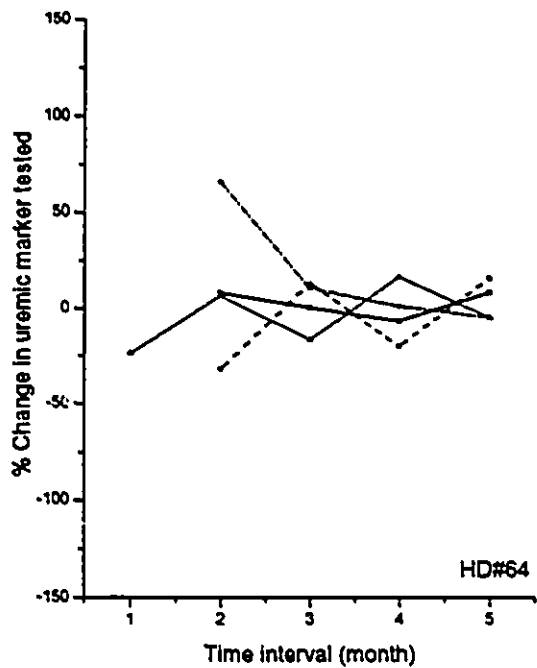


TABLE 3.1
Comparison of Kt/V, Pre-dialysis urea, Carbamylated Hemoglobin and Carbamylated Protein in Hemodialyzed Patients

HD#	Kt/V vs pre-dialysis urea		Kt/V vs CTP		Kt/V vs CHb		CTP vs CHb	
	$y =$	r	$y =$	r	$y =$	r	$y =$	r
3	$y = 6.44x + 33.0$	0.86 (0.06)	$y = -0.95x + 4.5$	-0.34 (0.57)	$y = 0.91x - 0.3$	0.39 (0.52)	$y = 0.46x - 2.2$	0.55 (0.33)
33	$y = -2.62x - 0.1$	-0.87 (0.05)	$y = -2.06x - 2.8$	-0.85 (0.07)	$y = 7.01x + 17.7$	0.62 (0.26)	$y = -3.40x + 8.0$	-0.73 (0.16)
40	$y = 0.15x + 4.0$	0.10 (0.88)	$y = 1.21x - 2.1$	0.68 (0.21)	$y = 5.50x - 0.4$	0.96 (0.01)	$y = 2.68x + 5.6$	0.83 (0.09)
56	$y = 3.80x + 4.0$	0.68 (0.20)	$y = 1.81x - 3.4$	0.81 (0.09)	$y = 5.17x - 22.0$	0.83 (0.17)	$y = 1.96x - 16.4$	0.64 (0.36)
64	$y = -0.71x + 1.4$	-0.35 (0.65)	$y = 2.05x + 13.7$	0.45 (0.55)	$y = 0.42x - 7.0$	0.12* (0.88)	$y = -0.52x + 3.2$	-0.71 (0.29)
103	$y = 0.10x + 5.0$	0.10 (0.87)	$y = 0.02x + 5.9$	0.00 (1.0)	$y = 1.35x - 7.4$	0.33 (0.59)	$y = -0.61x - 7.4$	-0.88 (0.05)
104	$y = -0.80x + 1.6$	-0.08 (0.13)	$y = -0.07x + 9.1$	-0.10 (0.87)	$y = -0.64x - 4.6$	-0.52 (0.37)	$y = 0.47x - 12.5$	0.27 (0.66)

The line of best fit was calculated using the % change values (Figure 3.27).
The numbers in parentheses are the p values significant at $\alpha = 0.05$.

TABLE 3.1 continued
Comparison of KtV, Pre-dialysis urea, Carbamylated Hemoglobin and Carbamylated Protein in Hemodialyzed Patients

HD#	Pre-dialysis urea vs CTP		Pre-dialysis urea vs CHb	
	r		r	
3	-0.38 (0.52)	$y = -0.14x + 9.2$	0.09 (0.87)	$y = 0.03x - 1.2$
33	0.99 (0.00)	$y = 0.79x - 2.6$	-0.77 (0.12)	$y = -2.88x + 19.2$
40	-0.48 (0.41)	$y = -0.56x + 0.4$	-0.05 (0.93)	$y = -0.21x + 1.5$
56	0.89 (0.04)	$y = 0.35x - 4.2$	0.10 (0.87)	$y = 0.19x + 2.3$
64	0.20 (0.80)	$y = 0.44x + 17.9$	-0.81 (0.19)	$y = -1.35x - 6.2$
103	0.61 (0.27)	$y = 3.52x - 10.8$	-0.76 (0.14)	$y = -3.0x + 3.2$
104	-0.05 (0.94)	$y = -0.03x + 8.6$	0.16 (0.80)	$y = 0.19x - 7.8$

The line of best fit was calculated using the % change values (Figure 3.27).
 The numbers in parentheses are the p values significant at $\alpha = 0.05$.

TABLE 3.2

Linear Regression Equations For Pre-dialysis Urea Versus Carbamylated Hemoglobin and Carbamylated Protein

Time Interval (Month)	Urea vs CHb			Urea vs CTP				
	Regression equation	r	p	n	Regression equation	r	p	n
1	$y = 12.3x - 1.2$	0.98	<0.01	6	$y = 6.9 \times 10^{-3}x + 0.110$	0.48	0.34	6
2	$y = 3.2x + 66$	0.86	0.03	6	$y = 3.7 \times 10^{-3}x + 0.065$	0.77	0.04	7
3	$y = 2.5x + 83.5$	0.71	0.11	6	$y = -1.0 \times 10^{-4}x + 0.120$	-0.11	0.82	7
4	$y = 2.4x + 75.7$	0.52	0.23	7	$y = 4.0 \times 10^{-4}x + 0.119$	0.66	0.16	6
5	$y = 1.6x + 80.0$	0.51	0.24	7	$y = -3.2 \times 10^{-4}x + 0.125$	-0.38	0.40	7
6	$y = 0.72x + 114.3$	0.21	0.66	7	$y = 5.0 \times 10^{-5}x + 0.126$	0.07	0.88	7

Correlations were done for the six time intervals (month).

answer the question as to whether there is a relationship between any two tests within a patient. The overall correlation coefficient was obtained by removing the variation due to individual subjects (131). No significant correlation was found with any combination of tests. This is probably a reflection of the variation in the correlations of the individual patients, *i.e.*, regression to the mean.

The data can also be looked at in terms of between subject correlation. That is, it can answer the question whether on average do patients with high (or low) values for test A also tend to have high (or low) values for test B.

The weighted correlation coefficients were determined between various tests using the subject means. The significance of the correlation was based on the number of subjects ($n = 7$) and not the number of data pairs ($n = 40$) because of the repeated observations per subject (132). This significant correlation value was obtained from a critical value table (133), and at $\alpha = 0.05$ was 0.6786. Using this value, the correlations that were significant were between CHb versus CTP ($r = 0.707$) and CHb versus pre-dialysis urea ($r = 0.671$). The correlation between CTP versus pre-dialysis urea was almost significant ($r = 0.534$), and there was a very poor correlation between Kt/V versus pre-dialysis urea ($r = 0.278$). The other comparisons done were between Kt/V versus CTP or CHb ($r = -0.327$ and -0.387 , respectively).

Laboratory tests for hemoglobin, albumin, urea and creatinine are listed in Table 3.3 for the seven hemodialyzed patients used in this study. All patients have low hemoglobin concentrations and low hematocrits for all times except for patient #56 at time intervals 4,5 and 6 that are normal. All albumin values were within the reference interval. Pre-dialysis urea values are above the reference interval in all patients. Post-dialysis urea values are all lower than pre-dialysis urea values and in patients #3, #40, #64 and #104 the values are within the reference range. Creatinine concentrations are all well above the reference interval before dialysis and decrease after dialysis, but remain above the reference interval.

Other laboratory tests looked at included potassium, sodium, chloride,

TABLE 3.3
Biochemical Values in Hemodialyzed Patients Used in This Study

Test Time interval (Month)	Patient (HD#)						
	3	33	40	56	64	103	104
Age/sex	24F	66M	82F	66F	91M	68M	69M
Hemoglobin							
(120-180 g/L)							
1	86	109	77	93	101	110	108
2	97	109	89	100	96	110	102
3	90	98	89	116	96	115	108
4	102	100	89	128	88	113	114
5	116	93	91	140	92	105	110
6	114	90	84	126	98	104	111
Albumin							
(30-50 g/L)							
1	35	39	36	37	39	35	38
2	42	41	39	38	37	37	34
3	43	37	39	37	38	37	34
4	41	39	NA	39	35	36	35
5	42	37	35	40	38	36	35
6	39	36	36	38	38	35	34
Urea							
(1.8-7.1 mmol/L)							
1	10.2	11.2	19.3	15.6	16.7	29.5	12.9
2	19.5	15.5	17	15.8	12.7	33.7	11.5
3	34.3	10.9	17	23.6	13.5	35.4	8.4
4	33.2	12.8	15.3	19.1	11.2	37.4	9.9
5	43.9	12.1	15.5	23.2	13	36.1	10
6	32.6	14.7	18.1	20.7	12.3	36.9	10.3
Creatinine							
(62-150 µmol/L)							
1	650	528	695	626	769	677	617
2	716	618	786	636	791	755	580
3	734	527	756	787	754	851	605
4	831	535	707	782	682	828	595
5	1035	556	769	885	777	876	630
6	935	585	783	733	830	843	656

Note: All values are pre-dialysis.
 Values in parentheses refer to the normal range of the analyte.

carbon dioxide, glucose, calcium (total and ionized), phosphate, alkaline phosphatase, γ -glutamyl transferase, and aspartate aminotransferase. Phosphate concentrations were above the reference interval in patients (time intervals are in parentheses) #3 (3-5), #33 (1,2), #40 (2-6), #64 (1-6), and #104 (1-6). Carbon dioxide concentrations were below the reference interval in all patients for almost all time intervals. Potassium in patients #3 (3), #40 (1,2), and #64 (6) were elevated. Glucose was elevated only in the two diabetic patients #40 and #103. The enzyme test showed alkaline phosphatase to be elevated in patients #3 and #104 for all time periods. γ -Glutamyl transferase was also elevated in #3 for all time periods. The other laboratory tests were within the reference range.

CHAPTER 4 DISCUSSION

4.1 Protein Carbamylation

The underlying mechanism for carbamylation is generally regarded to be between isocyanate and an uncharged amino group (Figure 1.1). However, this does not explain why lysine residues ($pK_a \sim 10$) are carbamylated and why NH_2 -terminal amino groups may or may not be carbamylated (4). When HSA was carbamylated (see Section 3.3.1), there was a time-dependent increase in its anodicity. Increases in anodicity have been found with many carbamylated proteins and is correlated with an increase in homocitrulline carbamyl residues (39, 55, 134). These data clearly show that positively charged groups (lysine) are carbamylated.

In nonenzymatic protein glycation, a similar contradiction has been described (20). Nonenzymatic glycation involves a Schiff base formation between glucose and a free amino group of a protein followed by an Amadori rearrangement (135). The pK_a of the amino group was found to have little importance on whether or not it would be glycated. This peculiarity was explained by considering the three-dimensional structure of the protein and the environment of the lysine residue (20). Consideration was given to the fact that the Amadori rearrangement is enhanced by acid-base catalysis, and at physiological pH, the most likely proton donors are the basic amino acids. It was found that all the glycated lysine residues in albumin and hemoglobin were located in a sequence of basic amino acids. This observation therefore validated the requirement for local acid-base catalysis. This explanation could also be used for the carbamylation reaction since it too involves an acid-base rearrangement step.

4.2 Measurement of Carbamylated Proteins

Clinical research and application of carbamylated proteins in renal failure has been limited because of inadequate detection methods. An efficient and robust assay to measure carbamylated proteins is needed in order to establish its true merits as a marker for renal failure. An overview of the methods available to detect carbamyl groups were presented in the INTRODUCTION (see Section 1.3). No one method incorporated the attributes necessary for use in a clinical laboratory, particularly those of performance, ease, and speed. Therefore, one of the intents of the present study was to develop a method that would incorporate these attributes, specifically an immunoassay. Furthermore, during the course of this study several of the published methods for measuring carbamyl groups were evaluated. Particular aspects of these methods will now be discussed.

4.2.1 Diacetyl Monoxime Assay

The diacetyl monoxime assay is not a very good method for detecting carbamyl groups on proteins. There are several reasons for this. The assay method itself is particular, cumbersome, and uses caustic reagents. Most importantly though, is the difference in molar absorptivity for different carbamylated compounds (76). This is especially limiting in a protein which can be carbamylated at different sites and is only partially hydrolyzed. This point is well illustrated in Figure 3.2 which shows that the theoretical number of carbamyl groups is several-fold greater from that estimated using a homocitrulline standard curve. This reflects both the lack of an appropriate standard and the limited amount of hydrolysis that occurs.

Carbamylated Protein Standard

There are a number of problems associated with obtaining an appropriate standard. In the DAM assay, the absorbance comes from carbamyl groups which may be present on lysines (homocitrulline), on NH_2 -terminal amino acids, on sulfhydryl groups, free or part of a peptide fragment. The absorptivities of

these species are all different. Furthermore, there is an inherent nonspecific absorbance (not due to carbamyl groups). Therefore, a standard must contain a known number of carbamyl groups and determining this is not simple. Erill *et al.* (17) did this by constructing a standard curve made with [¹⁴C]carbamylated plasma protein which related absorbance to μmol of [¹⁴C]cyanate (dpm). However, it did not include the nonradioactive carbamyl groups normally present on plasma proteins. Consequently, carbamyl groups on plasma protein from normal individuals were not detected by this method. Also, this procedure used protein precipitation as a means of removing nonprotein interferents (*e.g.*, urea). However, precipitation or coagulation of protein is counteracted by carbamylation (10), and therefore carbamylated proteins could be selectively left in solution. Bachman *et al.* (78) used a homocitrulline standard curve, but homocitrulline was first isolated from the carbamylated protein (see Section 1.3).

One way of getting around the 'native' amount of carbamyl groups would be to start with noncarbamylated HSA. The technique to do this was borrowed from the approach used in the separation of glycated HSA from nonglycated HSA and involved cation-exchange chromatography (20, 130). Since CHSA involves the loss of positive charges as does glycated HSA, it seemed reasonable that CHSA could be separated from noncarbamylated HSA this way also. However, using this procedure and modifications of it, no separation was achieved. No further steps were taken to pursue this separation because of the time that would be needed to do it. Also, it became less clear if this separation was really necessary in order to fulfill the basic purpose of the study. The necessity was to have a preparation of CHSA that could be used to make comparisons against. The absolute quantitation of carbamyl groups was not the key purpose, although that would be optimal.

Recombinant HSA was contemplated, but it was found to involve a synthesis step which used urea and was therefore a source of potential carbamylation bias.

HPLC analysis of hydrolyzed carbamylated protein to determine carbamyl

content is somewhat limiting because it can only detect one type of carbamylated amino acid at a time. Most often it is the ϵ -carbamyl lysine (homocitrulline). However, homocitrulline is not stable under the high acid and temperature conditions used in the hydrolysis procedure (73). A portion of homocitrulline residues converts to lysine residues, the amount is specific for a particular protein, and probably depends on where the carbamyl group is in the protein, and also the hydrolysis conditions. More studies would have to be done to ascertain which factors are involved.

An accurate quantitation of protein carbamylation could probably be done by proteolytic digestion followed by fast atom bombardment mass spectroscopy (FAB-MS) analysis (30). Using this technique as a reference method, other techniques could be compared to it.

Units For Expression of Carbamylation - Protein Half-life

Carbamylated protein (CTP) values were expressed as a function of protein concentration based on the assumption that carbamylation is dependent on both protein concentration and urea concentration. Though this is the accepted practice, it may not be necessary because the assumption is based largely on *in vitro* experiments, without regard to the half-life of proteins. *In vivo*, the half-life of proteins is dependent on their concentration *i.e.*, plasma proteins at a lower concentration will remain in circulation longer (136). A decrease in albumin of about one-third is equivalent to a 50% decrease in catabolism (137).

A study on glycated albumin showed that albumin or total protein correction of fructosamine measurements was misleading (137). Lower albumin concentrations were associated with a greater extent of glycation (longer half-life). Similarly, higher albumin concentrations showed a lesser extent of glycation (shorter half-life). Therefore, if the fructosamine concentration is corrected for protein (albumin or total protein), then in cases of lower albumin concentration the value could be falsely elevated (138). In cases of higher albumin concentration the value could be falsely lowered.

This evaluation could also be applied to the carbamylation reaction

because it is also a nonenzymatic reaction with amino groups on plasma proteins. Furthermore, it might also apply to carbamylated hemoglobin because the hematocrit in patients with renal failure is lower (increased red cell fragility and reduced erythropoetin synthesis).

4.2.2 Affi-Gel Blue Chromatography

Purification of albumin from serum using Affi-Gel Blue chromatography was tried exhaustively using several strategies. It was impossible to obtain pure albumin from Affi-Gel Blue chromatography of plasma or serum. Albumin was eluted off the gel, but so were several other proteins (Figure 3.10). Furthermore, carbamylated albumin did not bind well to Affi-Gel Blue (Figure 3.11). This nonspecificity of Affi-Gel Blue has been noted previously (139).

It is still not known how albumin binds to the acidic dye Cibacron Blue F-3-GA (Affi-Gel Blue). It could involve ionic, hydrophobic, aromatic or sterically active binding site interactions (Bio-Rad Bulletin 1107). Since carbamylation reduces the number of negative charges on the protein, and CHSA is not bound well, the binding of HSA to this dye must involve ionic interactions. Furthermore, it is known that binding of small acidic molecules to albumin in patients with chronic renal failure is reduced. For example, a study by Dengler *et al.* (113) has shown that *in vitro* carbamylated and uremic HSA have reduced warfarin (Site I) and indole/diazepam (Site II) binding sites. It could be that these sites are important in the binding of Cibacron Blue F-3-GA.

4.2.3 Antibodies to Carbamyl Groups on Proteins

Polyclonal antibodies were made to CHSA as opposed to monoclonal antibodies for a number of reasons. It was found that polyclonal antibodies could be made specifically against carbamylated albumin and fibrinogen that did not react with other carbamylated proteins or noncarbamylated proteins (50). Also, polyclonal antibody assays have been developed for glycated HSA (140), which is also a nonenzymatic derivative of HSA. Furthermore, since the purpose of the study was really to see if carbamylated HSA measurements could be used diagnostically in renal failure, polyclonal antibody production seemed the fastest

way to start.

Polyclonal antibodies were made against HSA that had 44/60 residues carbamylated. The antiserum was processed through several types of affinity columns with ligands of HSA, CHSA or CHb. The resulting antibody preparations, including the total IgG preparation, all recognized CHSA, but varied in their cross-reactivity characteristics.

Characterization of the HSA-affinity purified antibody showed no reaction with other carbamylated proteins (hemoglobin and fibrinogen), carbamylated amino acids (homocitrulline and carbamyl aspartate), noncarbamylated albumin and other serum proteins. These cross-reactivity studies showed that this antibody was directed against not only the derivatized lysine residue, but to a larger domain on the carbamylated albumin molecule. These specificity results are similar to those found by Steinbrecher *et al.* (50) for antibodies directed against carbamylated guinea pig albumin and fibrinogen. Also, antibodies to isocyanates such as toluene diisocyanate are not directed solely against the hapten, but the combined determinant of hapten and protein (141).

The HSA-affinity purified antibody preparation was used in a competitive ELISA and sandwich ELISA. Both immunoassays could detect carbamyl groups on the CHSA standard in the picomol range (25 and 1 pmol, respectively).

However, there was an inability among the four antibody preparations to differentiate between carbamylated proteins in uremics and normal subjects. This was primarily due to the antibody's recognition domain. The antibodies were not specific to homocitrulline alone. The antibodies were also made against HSA that had a molar ratio of homocitrulline:albumin of 44, compared with *in vivo* ratios of about 0.08 for normal individuals and 0.27 for uremic patients (78). Furthermore, in nonenzymatic glycation (20), derivatization occurs only on a select number of amino acid residues (mainly Lys-199, Lys-281, Lys-439 and Lys-525). Similarly, this is likely the case for nonenzymatic carbamylation, but the specific residues could be different. What this suggests is that the anti-CHSA polyclonal antibody preparations probably have antibodies directed against many

carbamylated lysine residues - therefore diluting out the ones that are specific to *in vivo* carbamylation.

Furthermore, when HSA carbamylated to different degrees was tested in the competitive ELISA, it was found that a molar ratio of approximately six carbamyl residues per HSA molecule was needed before a change above NSB was detected. This ratio is greater than 20-fold the maximum ratio found in uremic patients. Therefore, it would be unlikely for this antibody preparation to be able to detect carbamylated HSA in normal or uremic individuals. The small amount of reactivity seen between the antibody preparations and plasma samples must consequently be with noncarbamylated epitopes on HSA. Moreover, this identifies the reason no difference in absorbance was found between normal and uremic serum. It also explains why reactivity with the CHSA standard was invariably found.

The presence of autoantibodies to carbamylated proteins, which would interfere in an immunoassay, is also a possibility. Precedence can be found in two studies where autoantibodies have been found to carbamylated proteins (50, 141, 142).

A sandwich immunoassay was designed to see if the immunoassay's specificity could be increased. The primary antibody coated to the well surface was the antibody specific to carbamyl groups (HSA- or CHb-affinity purified), and the secondary antibody was a polyclonal antibody to HSA. However, due to a significant plasma matrix problem, owing to the use of undiluted samples, the sandwich assay design was not investigated further.

Antibodies to carbamyl groups can be made as demonstrated in this study and others (48, 50, 63). Antibodies made against carbamylated LDL have been shown to detect homocitrulline residues specifically and to differentiate normal subjects from uremic patients (48, 63). However, anti-homocitrulline antibodies have not yet been used directly to assay uremic plasma samples. It is unknown why not. No antibody preparation has been made yet that can detect carbamyl groups on a specific protein. To do this would require either immunization with a

carbamylated protein purified from serum (or one that has a similar extent of carbamylation), or production of monoclonal antibodies.

4.2.4 Alternative Methods

An immunoassay for example, using monoclonal antibodies against carbamylated LDL, would have some very appealing features from both a clinical and technical viewpoint. This type of test could be automated and carried out by the average clinical laboratory. This would put the test within reach of physicians and should favour more clinical trials.

Several microorganisms have been found to contain *N*-carbamoyl-D-amino acid amidohydrolase and *N*-hydantoinase (143, 144). Their function is in pyrimidine degradation. They have been investigated for the synthesis of D-amino acids that are now being employed for a variety of commercial applications. *N*-Carbamoyl-D-amino acid amidohydrolase has been shown to produce not only D-amino acids from *N*-carbamyl-D-amino acids, but also L- and D-amino acids from *N*-carbamyl-DL-amino acids. The products of this reaction also include ammonia and carbon dioxide. The enzymes isolated so far have optimal pH's between 7 and 9. There could be a possibility that these enzymes could also hydrolyze carbamyl groups off proteins. Potentially, any carbamyl group could be hydrolyzed from any protein at physiological pH. Also, since one of the hydrolysis products is ammonium ion, activity could easily be monitored by any ammonium assay.

4.3 Carbamylated Proteins in Hemodialyzed Patients

4.3.1 Correlations Between Carbamylated Hemoglobin and Carbamylated Protein

4.3.1.1 Cross-sectional study

The correlation between carbamylated hemoglobin and carbamylated total protein concentrations in normal individuals and uremic subjects (Figure 3.25) showed a high correlation ($r = 0.87$). Furthermore, the y-intercept showed a bias

in favour of carbamylated total protein. Similarly, in glycation studies the bias favours glycated albumin (124) or fructosamine (145) versus glycated hemoglobin, *ie.* glycated proteins are short-term indicators of glycemic control compared to glycated hemoglobin (146).

Since assay methodology could also influence this bias, a general idea for how much carbamylation occurs on albumin versus hemoglobin was obtained by interpolating data from two representative studies (75, 78). The amount of carbamylation was assessed by comparing homocitrulline residues for albumin (see Table 1.4) and carbamyl valine residues for hemoglobin (see Table 1.3). This allowed for direct comparison on a mole-to-mole basis between carbamyl groups on albumin and hemoglobin. This comparison showed that there was 5-fold more carbamyl groups on albumin [0.08 (0.06 - 0.12)] as compared with hemoglobin [0.016 (± 0.005)] in normal individuals. Similarly, there were also more carbamyl groups on albumin [0.27 (0.20 - 0.33)] than on hemoglobin [0.066 (± 0.035)] in patients with renal failure (4-fold). Since homocitrulline residues on CHb were not included, the total number of carbamyl groups may be slightly more for CHb (63, 64).

These results are compatible with the metabolism of urea. Urea, produced in the liver, is released into the plasma and in this medium can dissociate to form cyanate. Therefore, the potential for carbamylation is greatest for plasma proteins where the concentration of urea and cyanate are the highest. Urea and cyanate pass freely through membranes where carbamylation of intracellular proteins can occur. However, the degree to which they are carbamylated is lower due to the lower intracellular concentration of urea and cyanate.

Half-lives of Carbamylated Proteins

The half-lives of carbamylated proteins (CTP) and hemoglobin (CHb) are assumed to be similar to the half-lives of plasma proteins and hemoglobin in general (21 days and 60 days, respectively). Detecting a relationship between the CTP and CHb values when measurements are taken only every 30 days is

therefore difficult (Figure 3.27). If time points had been available weekly, the possibility of detecting a trend may have increased. Also, since the patients studied were in good control already (dialysis regime and diet), very little variation or significant difference in any individual test was seen. Untreated patients or patients on continuous ambulatory peritoneal dialysis may have shown more suggestive results.

Carbamylated protein concentrations most likely vary more rapidly in response to changing blood urea control than do carbamylated hemoglobin concentrations (as expected from the relative half-lives of the two analytes). Whether this renders carbamylated protein clinically more useful will depend on the frequency of testing.

4.3.1.2 Longitudinal study

Parameters of dialysis dose (Kt/V), pre- and post-dialysis urea, CHb and CTP for all seven patients were measured and plotted with respect to the time-interval (1 month). Visual observation of these parameters did not show any strong associations or trends (Figures 3.26).

Pre-dialysis Urea Versus CHb and CTP

Although the correlations between pre-dialysis urea and CHb (Table 3.2) is high for most time-periods, they do not agree. This discrepancy is probably due to the small sample size ($n = 7$). The variation in correlations (month-to-month), between pre-dialysis urea concentrations and CHb concentrations probably reflects the large fluctuations in urea generated by dialysis. The large range in correlation values (0.21 - 0.98), seems to suggest that there is no consistent relationship between pre-dialysis urea concentration and CHb. Studies with larger sample sizes have shown inconsistent relationships as well. Kwan *et al.* (75) showed a correlation of 0.69 ($n = 20$) in HD patients for pre-dialysis urea versus CHb. Smith *et al.* (97) using a similar method showed a correlation of only 0.44 ($n = 31$), also in HD patients. However, he found higher correlations in all other groups studied (control $r = 0.55$, $n = 20$; chronic renal failure $r = 0.75$, $n = 40$; continuous ambulatory peritoneal dialysis $r = 0.55$, $n = 24$;

transplant rejection $r = 0.87$, $n = 14$). Another study (32) reported a correlation of 0.84 in a combined group of normal and uremic subjects. However, the normal group in this study biases the correlation; if it is removed the correlation drops notably.

The correlations between pre-dialysis urea and CTP among the six time periods was very poor (range: -0.11 to 0.77). This variation could also be due to the lack of an adequate sample size, but may also demonstrate the dissimilarity between the two measurements. Also, samples were only available at monthly intervals and since the half-life of plasma protein is on average on 21 days, it would have been better if samples could have been obtained more frequently.

A patient on stable hemodialysis has fluctuations in urea that reflect nutritional status and urea removal by dialysis. Time-averaged urea concentration (TAC) is a slightly better measure of urea concentration as compared to a single urea measurement, but it was unavailable for this longitudinal study. A recent study (109) has reported that CHb concentrations in patients ($n = 8$) with renal transplants, reflects the urea level 10 to 25 days earlier. These researchers obtained this range by choosing the highest correlation coefficient calculated between TAC duration and CHb concentrations. This range is surprising because it is not related to the half-life of hemoglobin. In comparison, measurement of glycated hemoglobin reflects the glucose concentrations over the past 8 to 12 weeks. This range was selected by taking the time it took for a diabetic patient's GHb to return to normal after starting treatment. If the CHb data is reinterpreted in a similar manner, it can also show that CHb reflects urea levels between 10 to 12 weeks earlier.

However, time-averaged urea concentration (TAC), is not always calculated the same way. It is unclear what the appropriate way is. Over what time-frame should the measurements be made, and how many should be taken? Is it appropriate to use just pre-dialysis urea values, or post-dialysis values as well? These points need to be considered when TAC measurements are to be made for patients on dialysis.

Kt/V Versus CHb and CTP

The correlations between Kt/V versus CTP and CTP among the patients (Table 3.2) exhibited a wide range of values. No conclusion could be reached from these values. However, about half of the correlations were negative and this is interesting because it indicates that the dialysis dose is inversely correlated to the time-averaged urea concentration. In other words, as the dialysis dose decreases, carbamylated proteins are increased which is a reflection of an increased urea load. When data from another study (99) was interpolated, a negative correlation was also found between Kt/V and CHb ($r = -0.52$). This correlation was also significant ($p = 0.018$), probably due to a larger sample size used ($n = 20$).

Interpretation of CHb and CTP Values in Hemodialyzed Patients

As demonstrated in this longitudinal study, there is no clear relationship between pre-dialysis urea or Kt/V with CTP or CHb measurements. Because hemodialysis patients are maintained on a fairly stable dialysis regime, the utility of measuring carbamylated proteins may be limited. However, they may be of value if concentrations are above a pre-determined cutoff value or a pattern emerges over time. Kwan (99) has reported that CHb concentrations $<175 \mu\text{g CVal/g Hb}$ indicated adequate dialysis. This result was based on comparison to urea kinetic modelling measurements which did not necessarily reflect the patients health.

Interpretation of carbamylated hemoglobin concentration though could be complicated by the anemia in uremia, the shortened life-span of erythrocytes, and transfusions that dilute the erythrocyte mass and the CHb concentration. However, measurement of CTP would not be accompanied by these same problems. The plasma protein content is relatively stable in patients with end-stage renal disease, although lower than normal (nutritional effect).

Nonetheless, measurement of carbamylated hemoglobin may reflect a therapeutic time interval which is most appropriate to the clinical management of the uremic patient in an outpatient setting. The majority of patients are reviewed

at four month intervals. The carbamylated protein concentration, an index of integrated urea levels over the preceding one to three weeks, may therefore reflect dialysis adequacy over too short a time-interval to be of much practical use. However, in cases of acute renal failure the estimation of urea over a shorter time range would be more appropriate (98).

Moreover, major changes in therapy usually require monitoring an interval of not less than a month to allow stabilization on the new regime.

Hypothetically, if a hemodialyzed patient were to have CTP measurements made weekly, then an adjustment could be made on the dialysis dose weekly.

At present, only two other reports in the literature that have done longitudinal studies on carbamylation in renal failure (57, 105) (see Section 1.5.2). Both studies were not able to draw any significant conclusions from their data. Again, the reasons were similar to this present study *i.e.*, lack of more frequent time points, and comparison to a one-point urea measurement (pre-dialysis) instead of TAC or Kt/V.

4.4 Future Studies

The evaluation of a patient on dialysis is really based on clinical acumen. No straightforward interpretation of any singular test associated with dialysis can be made. What is enough dialysis and how can it be measured? The optimal time and quality of dialysis still needs to be established (90). Clinical outcome studies have interpreted successful dialysis in a vague manner (92). That is, the successfully dialysed patient has no overt medical illness, does not require hospitalization and remains alive.

Measurement of CTP as opposed to CHb, may provide the necessary tool to look at the adequacy of dialysis. Carbamylated total protein levels most likely portray a relatively recent history of change in urea levels, be it from deterioration of renal function, diet or dialysis regime. The speculation is, could CTP indicate

something more? If CTP was measured weekly, could changes be detected sooner and more often? What effect would the detection of these more frequent changes have on the treatment of the patient? What would these changes necessarily reflect? More studies are needed to predict their ultimate role in defining adequacy of dialysis.

A plethora of questions remain unanswered in this area. Do other factors affect the rate and extent of carbamylation? Would this be a reflection of the underlying metabolic stability of the patient? How can the real potential of carbamylated proteins be assessed in hemodialyzed patients?

Valid outcome studies are needed to judge the clinical significance of carbamylated proteins. Longitudinal studies need to be done to show how carbamylated protein values change over the progression of all types of renal diseases. The extent of this change must then be related back to other clinical parameters. Consideration must be made for how much of an increase is acceptable and if the cutoff level will depend on the individual patient.

Final Thoughts

Over the last 50 years the quiescent role of carbamylation (via urea) in renal failure has appeared sporadically in the literature. The idea existed, but the substantiation for it has only arisen over the last few years. The presence of carbamylated amino acids, membranes, lipoproteins, plasma proteins, hemoglobin and lens proteins in patients with renal failure signifies the potential and far-reaching pathophysiological consequences of urea elevation. Carbamylated proteins have significance in renal failure, and further research will establish its utility.

APPENDIX

Patient Consent Form and Approval Letters

RESEARCH STUDY

Title: Carbamylated proteins in patients with renal disease
Investigators: University of Windsor
Department of Chemistry and Biochemistry (253-4232 ext.3519)
Cynthia Balion
Dr. R.J. Thibert
The Salvation Army Grace Hospital (255-2370)
Dr. T.F. Draisey

Recently, a new type of marker for the uremic state of patients on dialysis has been demonstrated. This marker is the product of urea and protein, and is called carbamylated protein. Normally urea is excreted by the kidney, but when the kidney function is impaired, urea excretion is limited and it accumulated in the blood. The level of urea in the blood is an indicator of renal function. However, urea levels fluctuate with dietary and metabolic influences, whereas carbamylated proteins do not because of their longer lifetime in the blood. It our hope that this new marker will be more effective in the management of patients with renal disease.

In order to accomplish this study it is necessary to test blood from patients who have renal disease for these proteins. In addition, it is necessary to obtain other information such as the type of renal disease, duration of the disease, age, sex, and other laboratory data. All this information will then be analyzed to determine if this new test will be of benefit to patients with renal disease.

We are asking all patients who attend the Renal Unit at The Salvation Army Grace Hospital to consider participating in the study. All it would require from you is your written consent to allow us to use your leftover blood samples and access you hospital record strictly for the purposes outlined above. If you have any questions or concerns about participating in this study please feel free to contact any of the above investigators.

The results of the research findings will be available upon request.

We greatly appreciate you help. Thank you.

This study has been approved by the University of Windsor Ethics Committee and the Salvation Army Grace Hospital Ethics Committee. Specific questions addressing the conduct of the study can be made to Karen Marrero, Office of Research Services, University of Windsor (253-4232 ext. 3916).

CONSENT FORM

Subject number: _____

I give permission for my hospital records to be accessed to obtain information pertaining to my disease state for the above stated research proposes. In addition, my leftover blood samples drawn for routine analysis may be used in this study.

I understand that all information will remain confidential throughout the study and only group data will be used in any publication resulting from this study.

I also understand that I may withdraw from this study at anytime if I so wish.

Signature: _____ Date: _____

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VITA AUCTORIS
CYNTHIA M. BALION

Education:

Doctor of Philosophy
Clinical Chemistry
University of Windsor, Windsor, ON
February 1996

Dissertation: Development and evaluation of methods for the determination of carbamylated proteins in hemodialyzed patients

Masters of Science
Pathology
University of Saskatchewan, Saskatoon, SK
June 1990

Thesis: Regulation of carnosine metabolism: Carnosinase in rat tissues and human sera

Bachelor of Science (Honors)
Biochemistry
University of Saskatchewan, Saskatoon, SK
April 1986

Employment history:

September 1989 - August 1990
Research Assistant, Department of Medicine, University of Saskatchewan, Saskatoon SK.

September 1986 - June 1987
Research Assistant, Department of Biochemistry, University of Saskatchewan, Saskatoon SK.

May 1986 - August 1986
Student Research Assistant, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon SK.

May 1985 - August 1985
Insect Technologist, Agriculture Canada Research Station, Saskatoon SK.

Presentations:

Balion CM, Draisey TF, Caines PS, Thibert RJ. Development of an enzyme-linked immunoassay for carbamylated plasma albumin. 39th Annual Conference of the CSCC-CAMB, Whistler, BC, April 23-27, 1995.

Balion CM, Caines PS, Draisey TF, Thibert RJ. Carbamylated hemoglobin and carbamylated plasma proteins in normal subjects and hemodialyzed patients. 47th Annual Meeting, American Association for Clinical Chemistry, Anaheim, CA, July 16-20, 1995.

Balion CM, Thibert RJ. Determination of oxalate by luminol chemiluminescence. 46th Annual Meeting, American Association for Clinical Chemistry, New Orleans, LA, July 17-21, 1994.

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Balion C. Carbamylated hemoglobin and carbamylated plasma protein in uremic patients. *American Association for Clinical Chemistry, Michigan Section Spring Seminar*, Frankenmuth, MI, May 20, 1994.

Balion CM, Caines PSM, Draisey TF, Thibert RJ. A time-study analysis to evaluate albumin carbamylation *in vitro*. 37th Annual Conference, Canadian Society of Clinical Chemists, Banff, AB, April 18-22, 1993.

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Publications:

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