

## *Protamine Inhibits Plasma Carboxypeptidase N, the Inactivator of Anaphylatoxins and Kinins*

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Protamine given to neutralize heparin after extracorporeal circulation can trigger a catastrophic reaction in some patients. While searching for a biochemical basis for this reaction, protamine was tested as an inhibitor of human plasma carboxypeptidase N (CPN) or kininase I, the inactivator of anaphylatoxins and kinins. Human plasma and CPN purified from human plasma, (Mr = 280 K) or its isolated active subunit (Mr = 48 K) were the sources of enzyme. The hydrolysis of furylacryloyl (FA)-Ala-Lys was measured in a UV spectrophotometer and that of bradykinin and the synthetic C-terminal octapeptide of anaphylatoxin C3a (C3a8) by high performance liquid chromatography. Protamine inhibited the hydrolysis of FA-Ala-Lys by CPN, (IC<sub>50</sub> = 3.2 × 10<sup>-7</sup> M); added human serum albumin (30 mg/ml) increased the IC<sub>50</sub> to 7 × 10<sup>-6</sup> M. When plasma was the source of CPN, the IC<sub>50</sub> was 2 × 10<sup>-6</sup> M. Protamine more effectively inhibited the hydrolysis of bradykinin and C3a8. The IC<sub>50</sub> for protamine was 5 × 10<sup>-8</sup> M with CPN and bradykinin, 7 × 10<sup>-8</sup> M with CPN and C3a8 and with the 48 K subunit and bradykinin it was 7 × 10<sup>-8</sup> M of protamine. Heparin competes with CPN for protamine, because in high concentration (18 U/ml) it reverses the inhibition by protamine. Protamine did not inhibit angiotensin I converting enzyme (kininase II) or the endopeptidase 24.11 (enkephalinase). Kinetic studies showed the mechanism of protamine inhibition to be partially competitive; about 10-20% of the hydrolysis of bradykinin by CPN was not inhibited by protamine. Thus, by blocking the inactivation of mediators released in shock, protamine inhibition of CPN may be partially responsible for the catastrophic reaction observed to occur in some patients. (Key words: Blood, coagulation: protamine. Enzymes: carboxypeptidase N; kininase I. Peptides: anaphylatoxin; bradykinin, C3a; C4a; C5a.)

PROTAMINE is routinely used to reverse the anticoagulating effect of heparin after extracorporeal circulation.<sup>1</sup> In some patients administration of protamine is followed by pulmonary hypertension and bronchoconstriction with subsequent systemic hypotension.<sup>2-7</sup> This syndrome has been attributed to activation of the complement system<sup>8</sup>

by the heparin-protamine complex resulting in release of anaphylatoxins<sup>2-7</sup> and the synthesis of thromboxane.<sup>7</sup> As an additional consequence of protamine administration, Factor XII is also activated and could, in turn, lead to activation of plasma kallikrein and the release of bradykinin.<sup>9</sup> In some cases after neutralization by protamine, the effect of heparin rebounds; this phenomenon is probably due to the enzymatic metabolism of protamine in blood,<sup>10-13</sup> possibly by carboxypeptidase N (CPN).<sup>11,14</sup>

The human plasma enzyme CPN (EC.3.4.17.3), cleaves the C-terminal lysine or arginine of peptides or proteins.<sup>15-17</sup> Because it inactivates bradykinin by the release of arginine, it was first named kininase I. CPN was also called anaphylatoxin inactivator because it inactivates anaphylatoxins C3a, C4a, and C5a of the complement system by the same mechanism as it inactivates kinins.<sup>18</sup> Indeed, during the purification of active, intact C3a from human plasma, an inhibitor of CPN had to be used, otherwise only the inactive des-arginine derivative could be extracted.<sup>18,19</sup>

Protamine is actually a mixture of simple proteins isolated from fish sperm, which contain a high percentage of arginine.<sup>20</sup> All protamines contain four C-terminal arginine residues, which can be cleaved sequentially by CPN; thus, they are substrates of this enzyme.<sup>21</sup>

Because the catastrophic reaction that sometimes follows the administration of protamine<sup>2-9</sup> can involve the release of hypotensive and bronchoconstrictor peptide substrates of CPN (kinins and anaphylatoxins), it was important to know if protamine inhibits CPN. Such an inhibition would potentiate the actions of anaphylatoxins and kinins and contribute significantly to the development of the reaction. Using both human plasma and homogeneous, purified human CPN, we found that protamine at concentrations lower than would occur in blood after protamine administration *in vivo* is indeed a potent inhibitor of CPN *in vitro*.

### Materials and Methods

Human CPN was purified to homogeneity from outdated plasma using ion-exchange and affinity chromatography.<sup>22</sup> The high molecular weight inactive subunit (Mr = 83,000; 83 K) and low molecular weight active subunit

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Received from the Departments of Anesthesiology and Pharmacology, University of Illinois College of Medicine at Chicago, Chicago, Illinois. Accepted for publication August 30, 1988. Supported by NIH grants HL36473, HL36081, and HL36802.

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(Mr = 48,000; 48 K) were isolated by gel filtration after the dissociation of CPN with 3 M guanidine.<sup>22</sup> In other experiments citrated plasma was used as the source of CPN activity. Angiotensin I converting enzyme was solubilized from a membrane fraction of human kidney with trypsin and purified to homogeneity.<sup>23</sup> Neutral endopeptidase 24.11 was extracted from a membrane fraction of human kidney (after the removal of ACE with trypsin) with Triton X-100 and purified to homogeneity as reported.<sup>24</sup>

Bradykinin, des-Arg<sup>9</sup>-bradykinin, Trizma Base, N-( $\alpha$ -rhamnopyranosyloxyhydroxyphosphinyl)-leucyl-tryptophan, (phosphoramidon) and poly-L-lysine (Mr = 14,000) were from Sigma Chemical Co., St. Louis, MO. Trifluoroacetic acid (TFA), sequanal grade, and Triton X-100 were from Pierce Chemical Co., Rockford, IL. Furylacryloyl-L-Ala-L-Lys (FA-Ala-Lys) and 2-(N-morpholino)ethanesulfonic acid (MES) were from Calbiochem, La Jolla, CA and the N-2-hydroxyethylpiperazine (Hepes) was from United States Biochemical Co., Cleveland, OH. Protamine sulfate injection, USP (10 mg/ml) was from Eli Lilly Co., Indianapolis, IN and heparin-NA injectable was from Elkins-Sinn Inc., Cherry Hill, NJ. Acetonitrile (HPLC grade) was from American Burdick and Jackson, Muskegon, MI, the human albumin from Pentex, Kankakee, IL, the FA-Phe-Gly-Gly from Bachem Inc., Torrance, CA, the glutaryl-Ala-Ala-Phe-4-methoxynaphthylamine (Glut-Ala-Ala-Phe-MNA) from Enzyme Systems Products, Livermore, CA and the aminopeptidase M from Boehringer Mannheim, Indianapolis, IN. The C-terminal octapeptide of anaphylatoxin C3a (Ala-Ser-His-Leu-Gly-Leu-Ala-Arg; C3a8) was a gift from Dr. T. Hugli of Scripps Clinic and Research Foundation, La Jolla, CA.

#### ENZYME ASSAYS

Bradykinin or C3a8 (final concentration = 100  $\mu$ M) was incubated for 0–40 min at 37° C in 0.05 M Hepes, pH 7.0 with either 15–150 ng CPN or with its 48 K active subunit (final volume = 100  $\mu$ l). All reactions were performed in duplicate. The enzymatic reaction was stopped by adding 20  $\mu$ l of 5% TFA. In the zero time reaction controls, TFA was added immediately after addition of substrate. When the effects of protamine and heparin were studied, they were added as aqueous solutions replacing water in the 100  $\mu$ l reaction volumes. Protamine, at various concentrations, was preincubated with the enzyme for 15 min on ice before addition of substrate. When heparin (1 U/ml) was used, it was preincubated with the enzyme for 15 min on ice followed by the addition of protamine for an additional 15 min. Reaction products were separated and quantitated by high performance liquid chromatography (HPLC) as described below.

The hydrolysis of FA-Ala-Lys was measured with a

continuous spectrophotometric assay by following the decrease in absorbance at 336 nm in a thermostatted cuvette at 37° C.<sup>25</sup> Briefly, the reaction mixture contained FA-Ala-Lys (500  $\mu$ M), either purified CPN or 20  $\mu$ l of citrated plasma and buffer (50 mM Hepes at pH 7.50 with 250 mM NaCl) in a final volume of 1 ml. Protamine was preincubated with enzyme for 5–10 min at 37° C before starting the reaction with FA-Ala-Lys. When tested, heparin or human albumin was incubated with enzyme for 10 min at 37° C prior to the addition of protamine.

The activity of angiotensin I converting enzyme was measured spectrophotometrically as described for CPN except the substrate was  $5 \times 10^{-4}$  M FA-Phe-Gly-Gly and the buffer was 0.05 M Tris, pH 7.5 with 0.3 M NaCl. The effects of protamine were determined by preincubating converting enzyme with various concentrations of protamine for 15 min at 37° C prior to addition of substrate.

The activity of neutral endopeptidase 24.11 was measured by a two-step spectrofluorometric assay with Glut-Ala-Ala-Phe-MNA as substrate in 0.1 M MES, pH 6.5, with 0.1% Triton X-100.<sup>26</sup> In the second step the fluorogen MNA is released by cleaving Phe-MNA with aminopeptidase M. Fluorescence was measured in a Perkin-Elmer LS-5 spectrofluorometer with excitation at 340 nm and emission at 425 nm. As above, protamine was pre-equilibrated with the enzyme for 15 min at 37° C prior to starting the assay. All experiments were done in duplicate two or more times.

#### HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC analyses were conducted in a Waters automated gradient system consisting of an M-6000A pump, an M-45 pump, a Wisp 710B automatic injector, an 840 Data and Chromatography Control Station and an M-490 Multiwavelength Detector. Peptide products were separated on a Waters  $\mu$ Bondapak C18 reverse phase column (3.9 mm  $\times$  30 cm) and eluted with an increasing linear gradient of acetonitrile/0.05% TFA (solvent B) in water/0.05% TFA (solvent A) at a flow rate of 1 ml/min. The column was allowed to reequilibrate for 8 min at initial conditions between each sample injection. Products of bradykinin hydrolysis were separated with a linear gradient of 25% solvent B to 35% solvent B in 10 min. Products of the hydrolysis of C3a8 were separated with a linear gradient of 20% solvent B to 60% solvent B in 10 min. Peptide products were detected by absorbance at a wavelength of 214 nm.

#### EFFECT OF PROTAMINE ON THE BINDING OF CPN TO DEAE CELLULOSE

Four aliquots of CPN (20  $\mu$ g each) were diluted in 1 ml 25 mM Tris-HCl, pH 7.2, with 50 mM NaCl containing: 1) no addition, 2) 300  $\mu$ g protamine, 3) 300  $\mu$ g protamine

and 300 U of heparin, or 4) 300  $\mu\text{g}$  polylysine. The mixtures were incubated at 37° C for 15 min. The individual mixtures were divided in half. To one aliquot was added 40 mg DEAE-cellulose (DE-52) previously equilibrated with the above buffer, while the other aliquot served as a control. The mixtures were then incubated at 4° C for 2 h with rotary mixing followed by centrifugation at 15,000  $\times g$  for 5 min. The supernatants were assayed for CPN activity using FA-Ala-Lys as substrate.

#### REMOVAL OF THE C-TERMINAL ARGININES OF PROTAMINE

Protamine (1 mg) was incubated with 10  $\mu\text{g}$  pure CPN in 1 ml 0.1 M HEPES, pH 7.5, for 18 hours at 37° C, conditions that should result in complete removal of the C-terminal arginines.<sup>21</sup> In order to separate the residual CPN activity from protamine, the sample was filtered in an Amicon Centricon-10 (10,000 molecular weight cutoff) filter, in which the low molecular weight protamine ( $M_r = 4626$ ) passed through the filter and the CPN ( $M_r = 280,000$ ) was retained. No CPN activity could be detected after this treatment. Control protamine samples were carried through the same procedure except distilled water was added instead of CPN. Control and treated protamine samples were then tested for inhibition of FA-Ala-Lys hydrolysis by CPN as described above.

#### Results

The inhibition of CPN by protamine *in vitro* was shown in several different types of assays. In UV spectrophotometric experiments, the hydrolysis of FA-Ala-Lys ( $5 \times 10^{-4}$  M) was inhibited by protamine (fig. 1). When purified CPN was used, the  $IC_{50}$  of protamine was 1.5  $\mu\text{g}/\text{ml}$ . Assuming a molecular weight of 4,626 for protamine,<sup>20</sup> 50% of the enzyme activity was inhibited with  $3.2 \times 10^{-7}$  M protamine. When diluted, citrated human plasma was the source of the enzyme, the inhibition curve was shifted to the right, and one-half the activity of CPN was inhibited by 9.4  $\mu\text{g}/\text{ml}$  ( $2.0 \times 10^{-6}$  M) protamine. Even at high concentrations of protamine, the hydrolysis of FA-Ala-Lys by purified CPN or plasma could not be completely inhibited and about 30–40% of the activity remained. This indicates that protamine inhibits CPN by a mechanism other than that of simple competitive inhibition. Interestingly, the hydrolysis of FA-Ala-Lys by the separated, active 48 K subunit of CPN was not significantly inhibited by protamine; even 1,000  $\mu\text{g}/\text{ml}$  protamine inhibited only 12% of the activity (fig. 1).

The inhibition of the cleavage of C-terminal arginine from a vasoactive peptide substrate of CPN was determined by HPLC. Protamine inhibited the hydrolysis of the nonapeptide bradykinin ( $1 \times 10^{-4}$  M) by CPN at lower concentrations than that of FA-Ala-Lys (fig. 2) with an

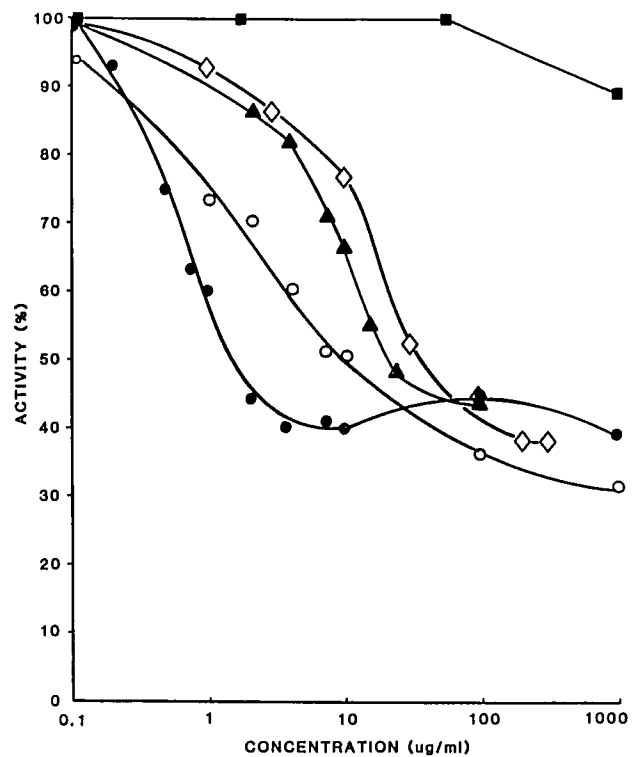


FIG. 1. Inhibition of carboxypeptidase N and the active 48 K subunit by protamine. The effect of various concentrations of protamine (abscissa, plotted on a log scale) on the activity of carboxypeptidase N or 48 K subunit was measured with FA-Ala-Lys substrate. Isolated 48 K active subunit of carboxypeptidase N (■); homogeneous human carboxypeptidase N (●); citrated human plasma (○); carboxypeptidase N pretreated with 1 U/ml heparin (▲). Carboxypeptidase N pretreated with 1 U/ml heparin and assayed in the presence of 30 mg/ml human serum albumin (◇).

$IC_{50} = 0.25 \mu\text{g}/\text{ml}$  ( $5 \times 10^{-8}$  M). In contrast to the results obtained with FA-Ala-Lys, protamine also inhibited the hydrolysis of bradykinin by the active 48 K subunit of CPN with an  $IC_{50}$  of 0.30  $\mu\text{g}/\text{ml}$  or  $6 \times 10^{-8}$  M, only slightly higher than that obtained with the tetrameric CPN (fig. 2).

Because anaphylatoxins are thought to be involved in the catastrophic reaction to protamine administration and anaphylatoxins are inactivated by removal of the C-terminal arginine by CPN, we investigated the inhibition of the hydrolysis of the synthetic C-terminal octapeptide of anaphylatoxin C3a (C3a8) by CPN. Protamine inhibited the cleavage of C3a8 by CPN as established in HPLC (fig. 3). The  $IC_{50}$  value of protamine (0.33  $\mu\text{g}/\text{ml}$ ;  $7 \times 10^{-8}$  M) was similar to that obtained with bradykinin.

To see whether the inhibition of CPN by protamine was due to a specific interaction with the enzyme or only to a nonspecific positive charge effect due to the guanidino group of arginine, polylysine, another highly charged basic molecule, was tested as inhibitor. Polylysine did not

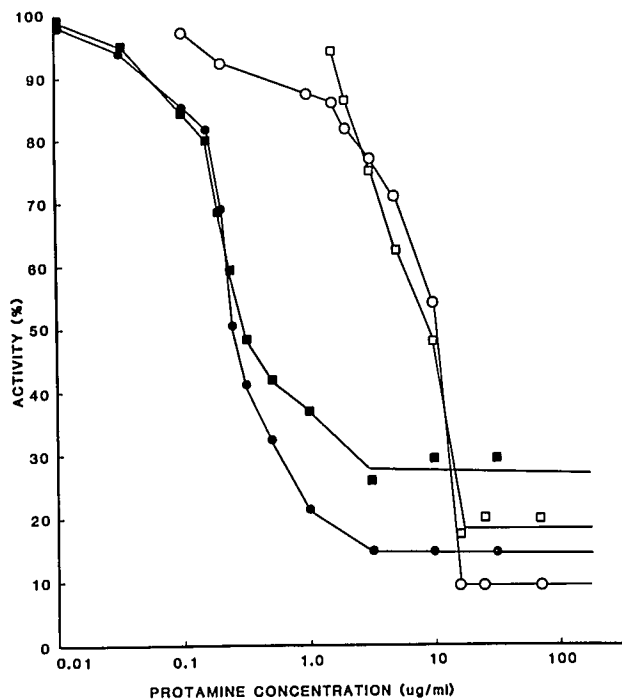


FIG. 2. Effect of heparin on protamine inhibition of the hydrolysis of bradykinin by carboxypeptidase N and by the 48 K subunit. Carboxypeptidase N (●) or the isolated 48 K subunit (■) was preincubated with various concentrations of protamine (abscissa, plotted on a log scale), and the hydrolysis of 100  $\mu$ M bradykinin measured by HPLC. Heparin (1 U/ml) was preincubated with carboxypeptidase N (○) or the 48 K subunit (□) for 15 min prior to the addition of protamine.

inhibit the hydrolysis of FA-Ala-Lys by CPN at concentrations up to 100  $\mu$ g/ml.

#### EFFECT OF HEPARIN

Because heparin binds to protamine, we wanted to determine whether heparin could reverse the inhibition of CPN by protamine. Heparin itself had no effect on the hydrolysis of FA-Ala-Lys (at up to 10 U/ml concentration) or bradykinin (at 1 U/ml) by CPN (not shown). However, heparin did reverse protamine inhibition of FA-Ala-Lys hydrolysis by CPN in a dose-dependent manner. Heparin at 1 U/ml shifted the protamine inhibition curve to the right, increasing the  $IC_{50}$  from 1.5  $\mu$ g/ml to 20.4  $\mu$ g/ml (fig. 1). When the concentration of heparin was increased at a constant protamine concentration of 10  $\mu$ g/ml, the inhibition of CPN was reduced and at 18 U/ml heparin CPN became fully active (fig. 4). Heparin had a similar effect on protamine inhibition of bradykinin hydrolysis by CPN. Heparin at a concentration of 1 U/ml increased the  $IC_{50}$  from 0.25  $\mu$ g/ml to 10.6  $\mu$ g/ml for CPN and raised the  $IC_{50}$  from 0.3  $\mu$ g/ml to 8.9  $\mu$ g/ml with the 48 K subunit (fig. 2).

#### IMPORTANCE OF C-TERMINAL ARGININE

To determine the importance of the C-terminal arginine residues of protamine for inhibition of CPN, protamine (1 mg) was incubated for 18 h at 37° C with 10  $\mu$ g CPN. Under these conditions all C-terminal arginines of protamine should be cleaved.<sup>21</sup> After incubation CPN was separated and retained by ultrafiltration on a membrane, which allows only molecules smaller than 10,000 daltons (e.g., protamine) to pass. Control samples of protamine were treated in the same way except that distilled water was used instead of CPN. In the control, untreated protamine (10  $\mu$ g/ml) inhibited the hydrolysis of FA-Ala-Lys by 59%, while protamine preincubated with CPN was less effective, inhibiting only 42%. This indicates that, although the C-terminal arginines of protamine play a role in the inhibition of CPN, other components of the protamine molecule also contribute to the inhibition.

#### EFFECT OF ALBUMIN

As albumin was reported to form a complex with protamine,<sup>27</sup> we investigated whether physiologic concentrations of albumin could abolish the inhibition of CPN by protamine. Albumin at a concentration of 30 mg/ml (the approximate concentration in normal human plasma) decreased the inhibition of CPN by protamine (10  $\mu$ g/ml) from 60% to 28% and increased the  $IC_{50}$  from 1.5  $\mu$ g/ml to 33.5  $\mu$ g/ml. These values did not change significantly in presence of heparin (fig. 1). Albumin alone did not affect the hydrolysis of FA-Ala-Lys by CPN.

Because the cleavage of the short FA-Ala-Lys in the spectrophotometric experiments could not be inhibited completely by protamine, as 30–40% of the activity remained, we also calculated the concentration of prot-

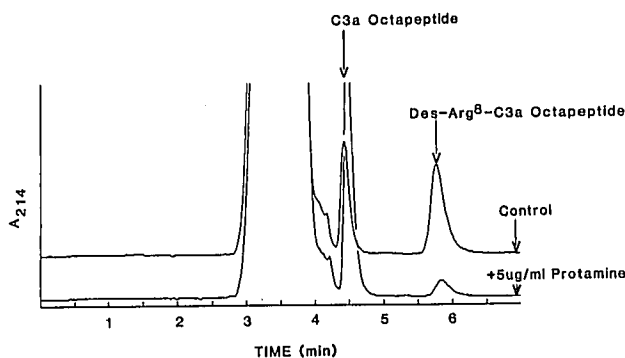


FIG. 3. Hydrolysis of the C-terminal octapeptide of anaphylatoxin C3a (C3a8) by carboxypeptidase N and its inhibition by protamine. C3a8 (100  $\mu$ M) was incubated with 15 ng carboxypeptidase N in the presence (bottom tracing) or absence (upper tracing) of 5  $\mu$ g/ml protamine for 40 min at 37° C and the products separated by HPLC as described. Carboxypeptidase N cleaved Arg.<sup>8</sup> Abscissa: time in minutes for elution. Ordinate: UV absorbance at 214 nm.

amine which reduced to half the inhibitable activity. This was 0.6  $\mu\text{g}/\text{ml}$ , the value shifted to 2.2  $\mu\text{g}/\text{ml}$  in diluted plasma, to 12.6  $\mu\text{g}/\text{ml}$  in presence of albumin, or to 7.3  $\mu\text{g}/\text{ml}$  with added heparin (1 U/ml). These values are lower than the  $\text{IC}_{50}$  values given above.

#### EFFECT OF PROTAMINE ON OTHER PEPTIDASES

To determine whether the effect of protamine on carboxypeptidase N is specific, we tested it as an inhibitor of other purified peptidases. Protamine did not inhibit the angiotensin I converting enzyme (kininase II) or the neutral endopeptidase 24.11 (enkephalinase) or aminopeptidase M at concentrations of up to 100  $\mu\text{g}/\text{ml}$ .

#### BINDING OF PROTAMINE TO CPN

The finding that protamine did not inhibit the hydrolysis of FA-Ala-Lys by the 48 K subunit of CPN indicates that the inhibition of CPN by protamine is not due to a simple interaction with the active center of CPN. Because protamine is a highly basic molecule with a net positive charge, if it binds to CPN, it should make the net charge of the complex with CPN more positive. Protamine slightly altered the electrophoretic mobility of CPN in nondenaturing polyacrylamide gel electrophoresis, suggesting the formation of a stable CPN-protamine complex (not shown). This complex formation was also shown in experiments in which we studied the effect of protamine on the binding of CPN to the anion exchange resin DEAE-cellulose. At pH 7.2, CPN has a net negative charge and when incubated at pH 7.2 for 2 h at 4° C with DEAE-cellulose, 97% of the activity was bound to the positively charged ion exchange resin (table 1). When 20  $\mu\text{g}$  CPN was preincubated with 300  $\mu\text{g}$  protamine and then added to the DEAE-cellulose, only 32% of the activity bound, indicating that protamine complexed CPN and altered its charge, thereby preventing its interaction with the ion exchange resin. This action of protamine on CPN appears to be specific. Polylysine ( $M_r = 14,000$ ), another highly charged basic molecule, was incubated with CPN under the same conditions. However, it failed to block the binding of CPN to DEAE-cellulose (table 1). Heparin reversed the binding of protamine to CPN as 95% of the activity was bound to the resin (table 1) when 300 units of heparin were added to 300  $\mu\text{g}$  protamine in the preincubation mixture.

#### INHIBITION KINETICS

The kinetics of inhibition of CPN by protamine were studied with the bradykinin substrate. CPN, preincubated with varying concentrations of protamine, was subsequently incubated with bradykinin (20 to 200  $\mu\text{M}$ ). The hydrolysis of bradykinin was measured by HPLC and the

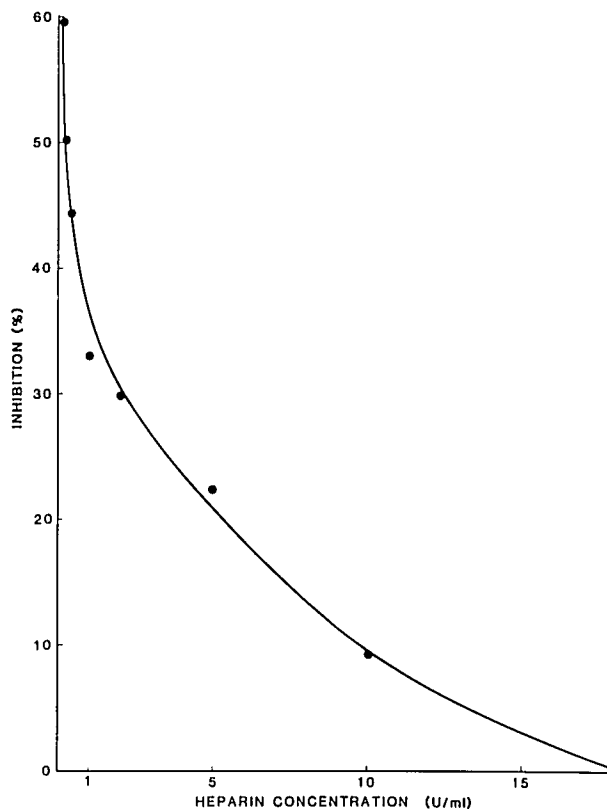


FIG. 4. Effect of heparin on the inhibition of carboxypeptidase N by protamine. Carboxypeptidase N was preincubated with various concentrations of heparin for 10 min at 37° C and then with 10  $\mu\text{g}/\text{ml}$  protamine for an additional 5 min. The activity was then assayed with FA-Ala-Lys substrate. Abscissa: concentration of heparin.

data were plotted according to Lineweaver-Burk and fit to the best straight line by linear regression. As shown in figure 5, the lines intersected on the y-axis, indicating that protamine is a competitive inhibitor of CPN.<sup>28</sup> However, protamine is not a simple competitive inhibitor of CPN as a replot of figure 5 (slope *vs.* protamine concen-

TABLE 1. Effect of Protamine on the Binding of Carboxypeptidase N to DEAE-Cellulose

Carboxypeptidase N Pretreatment*	Carboxypeptidase N Bound (%)
300 $\mu\text{g}$ protamine	32
300 $\mu\text{g}$ protamine + 300 U heparin	95
300 $\mu\text{g}$ polylysine	94
No addition	97

\* Carboxypeptidase N (20  $\mu\text{g}$ ) was preincubated for 15 min at 37° C with the agents in a final volume of 1 ml in 25 mM Tris-HCl buffer, pH 7.2, and 50 mM NaCl. One-half of each sample was rotated with 40 mg DEAE-cellulose for 2 h at 4° C and the other half served as control. Mixtures were centrifuged and the supernatant and the control sample assayed for activity with FA-Ala-Lys. Results shown are the average of two separate experiments.

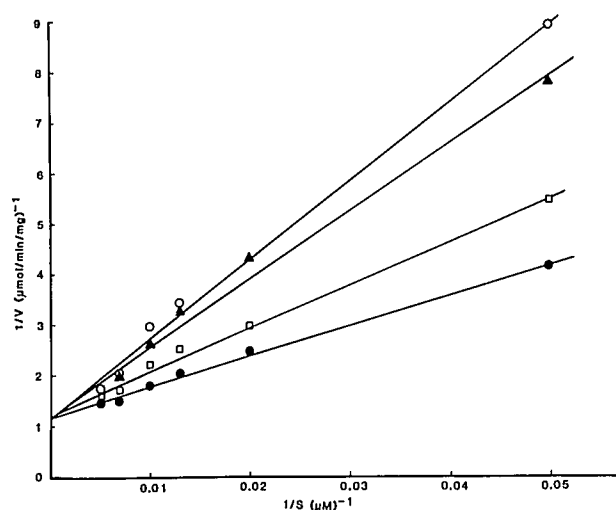


FIG. 5. Lineweaver-Burk plot of the protamine inhibition of bradykinin hydrolysis by carboxypeptidase N. The rate of hydrolysis of bradykinin (20–200  $\mu\text{M}$ ) by carboxypeptidase N was measured at protamine concentrations of: 0.4  $\mu\text{g}/\text{ml}$  (●), 0.6  $\mu\text{g}/\text{ml}$  (□), 0.8  $\mu\text{g}/\text{ml}$  (▲), and 1.0  $\mu\text{g}/\text{ml}$  (○). Abscissa: reciprocal of substrate concentration. Ordinate: reciprocal of reaction rate.

tration) gave a hyperbolic curve (not shown) instead of a straight line. This indicates that protamine is a partially competitive inhibitor of CPN, meaning the enzyme-inhibitor complex still has residual activity.<sup>28</sup>

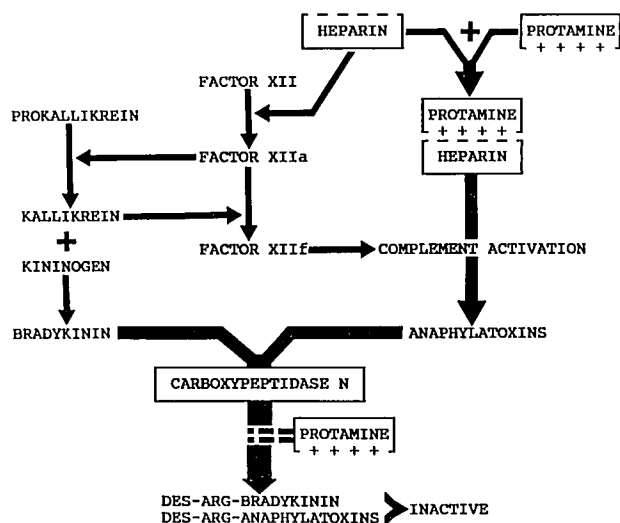


FIG. 6. Scheme showing the events that may follow the neutralization of heparin with protamine. Heparin may activate the complement system directly, when complexed with protamine, or indirectly by activating Factor XII first. In addition, activated Factor XIIa can convert prokallikrein to kallikrein and kallikrein releases bradykinin from plasma kininogen substrate. The activation of the complement system results in the liberation of anaphylatoxins. Protamine can inhibit carboxypeptidase N, the enzyme that inactivates kinins and anaphylatoxins by the release of C-terminal arginine.

## Discussion

The results suggest a biochemical basis for the catastrophic reaction that can occur when protamine is given to neutralize heparin after extracorporeal circulation (fig. 6). The patients in whom such a reaction occurs have shown increased capillary permeability, interstitial fluid and leukocyte accumulation, fever, and other signs.<sup>2–7</sup> It has been suggested that the initial reaction triggering these events is the activation of the complement system by the protamine-heparin complex (fig. 6). This is followed by the subsequent liberation of anaphylatoxins C3a, C4a, and C5a. Anaphylatoxin C3a, a 77 amino acid peptide, is spasmogenic to smooth muscle, releases histamine, and increases vascular permeability. Anaphylatoxin C5a, in addition to these properties, interacts with receptors on leukocytes to promote chemotaxis and the release of lysosomal enzymes and superoxide.<sup>19</sup>

A second factor that may contribute to this syndrome is the nonapeptide bradykinin. Activation of Factor XII in blood<sup>9</sup> (e.g., by heparin) leads to the activation of plasma kallikrein with the subsequent liberation of kinins from kininogen (fig. 6). Kinins also cause hypotension, bronchoconstriction, and pain, and increase capillary permeability.<sup>17</sup>

A third contributing factor is thromboxane.<sup>7</sup> The blood level of thromboxane greatly increased in two patients who developed pulmonary hypertension following protamine after open heart surgery but did not change in nonresponders. These authors suggested that thromboxane may be the major mediator of pulmonary hypertension and bronchoconstriction after protamine administration.

Both kinins and anaphylatoxins have a C-terminal arginine.<sup>15,19</sup> Most, although not all, of the activities of these peptides depend on this amino acid; thus a carboxypeptidase, which removes this residue, controls the activity of these peptides. Human and animal plasma contain such a soluble CPN. Its concentration in human plasma is 30–40  $\mu\text{g}/\text{ml}$  or about  $10^{-7}$  M<sup>17</sup>. Although a genetically determined lower level of this enzyme was observed in one family,<sup>29,30</sup> a complete absence of CPN has not been detected and is not considered compatible with life. The enzyme originates from the liver.<sup>17</sup> A membrane-bound carboxypeptidase is present in many organs (placenta, kidney, lung) and cells (fibroblasts, endothelial, epithelial cells).<sup>31,32</sup> This enzyme (carboxypeptidase M) cleaves the same peptides as does CPN and is inhibited similarly, but it is a distinctly different enzyme protein.<sup>31,33</sup>

The substrates of CPN range from small peptides such as kinins (which were chronologically the first to be used, hence the name kininase I<sup>17</sup>), and enkephalin hexapeptides (Arg<sup>6</sup>- or Lys<sup>6</sup>-enkephalins),<sup>31</sup> to anaphylatoxins (Mr = 9,000)<sup>18,19</sup> and protamine (Mr = 4626).<sup>15,21</sup> It cleaves

even higher molecular weight proteins, for example, creatine kinase ( $M_r = 80,000$ ).<sup>34</sup>

Human plasma carboxypeptidase N is a complex molecule (fig. 7). The naturally occurring form in human plasma has a molecular weight of 280,000 daltons. It is composed of two low molecular weight subunits (48 K) containing the active center and two high molecular weight glycoprotein subunits (83 K), which keep the lower molecular weight active subunits in the circulation and stabilize them at body temperature.<sup>22</sup> The two high and two low molecular weight units are held together by non-covalent forces.

We report here that CPN is inhibited *in vitro* by protamine at a concentration 1/30th to 1/100th of that can be expected in blood after its administration to neutralize heparin. This was shown with purified homogeneous enzyme (280 K) and a short synthetic substrate as well as with bradykinin and/or the anaphylatoxin derivative C3a8. When plasma was a source of the enzyme, the inhibition curve was shifted to the right, to higher concentrations of protamine. This might have been due to the binding of protamine by albumin<sup>27</sup> because albumin itself did not affect the activity of CPN. Heparin and CPN may compete for protamine because the addition of heparin (1 U/ml) shifted the inhibition curve to the right and high concentrations (18 U/ml) abolished the inhibition of 10  $\mu\text{g/ml}$  protamine, and reactivated the enzyme.

Although protamine is cleaved by CPN, the inhibition did not depend entirely on protamine being a competitive substrate owing to its scissile arginines. After the release of the C-terminal arginines, the inhibition of CPN by protamine decreased but was not abolished. Thus, besides C-terminal arginines, other structural elements of the protamine molecule contribute to the inhibition. Protamine inhibited the hydrolysis of the longer physiologic substrates of CPN better than that of the short protected dipeptide. Kinetic studies with bradykinin indicate that protamine inhibition is partially competitive, *i.e.*, the enzyme-inhibitor complex still has some residual activity. These data, together with the evidence showing that protamine forms a complex with CPN, indicate that protamine binds to the enzyme at a site or sites close to the active center, resulting in partial blockage of the active site. With the isolated 48 K active subunit, protamine blocks less of the active site than in the intact enzyme so it remains active with the small FA-Ala-Lys substrate. Possibly protamine not only binds to the active site containing 48 K subunit, but also to the inactive 83 K subunit of the intact enzyme, and that makes it a more efficient inhibitor of the cleavage of short peptides by changing the conformation of this complex protein. Thus, it is possible that with the short protected dipeptide substrate protamine acts as an allosteric modifier of the enzyme *via* the high molecular weight subunit.

PROPOSED SUBUNIT STRUCTURE OF PLASMA CARBOXYPEPTIDASE N

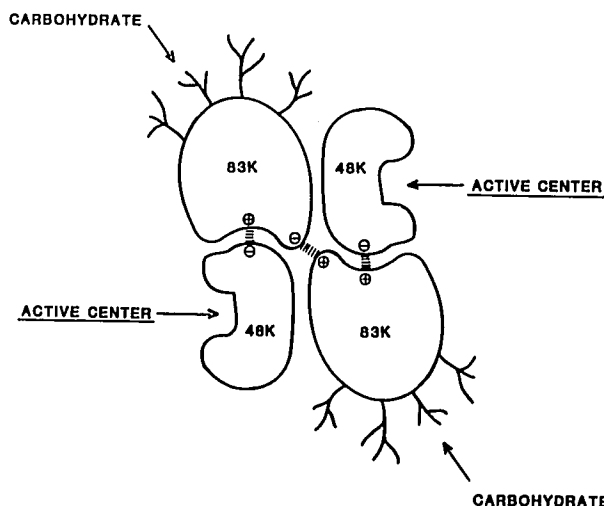


FIG. 7. A model of the tetrameric subunit structure of human plasma carboxypeptidase N. The branched chains represent carbohydrate components; the +/- represent noncovalent interactions (ionic interactions, hydrogen bonds, van Der Waals forces, hydrophobic interactions).

Protamine also inhibited the hydrolysis of the C-terminal octapeptide of anaphylatoxin C3a. Both bradykinin and C3a8 were used in the HPLC assay at a concentration of  $10^{-4}$  M. This is much higher than the concentration of free bradykinin or anaphylatoxin C3a in blood *in vivo* ( $10^{-11}$  M bradykinin and  $10^{-6}$  M C3a after complement activation<sup>2,17</sup>). Because enzyme inhibitors are more effective at lower substrate concentrations, protamine, even in the presence of albumin, may be a more potent inhibitor of the hydrolysis of endogenous substrates by CPN after extracorporeal circulation than reported here.

The importance of inhibition of plasma carboxypeptidase N was shown in experiments done in guinea pigs.<sup>35</sup> These animals died after activation of the complement cascade, which released anaphylatoxins, provided they were pretreated with a potent inhibitor of CPN. Death was due to acute respiratory distress involving intense bronchoconstriction and vasoconstriction, caused by the inhibition of the inactivation of anaphylatoxins.

Most of the bradykinin is inactivated during a single passage through the lung by kininase II or angiotensin I converting enzyme,<sup>17</sup> which is excluded from the circulation during cardiopulmonary bypass. The decreased ability of CPN to inactivate kinins after protamine administration might be especially detrimental in patients who are treated with an inhibitor of the angiotensin I converting enzyme (*e.g.*, captopril or enalapril). These inhibitors block both the conversion of angiotensin I and the inactivation of bradykinin by this enzyme<sup>36</sup> on the pulmonary and peripheral arterial endothelial cells.

The fact that a majority of patients treated with protamine do not have a severe reaction indicates that other factors must be involved in predisposing the patients to this kind of reaction. Protamine is generally given in doses of 100–600 mg, which would yield a final concentration in plasma of about 30–200  $\mu\text{g}/\text{ml}$  (for an average 70 kg patient with 3 l of plasma). Because heparin binds protamine and can reverse the inhibition of carboxypeptidase N, it could be expected that only when protamine is in excess would a problem develop. The free protamine levels that would actually be obtained would thus depend on many factors such as the time passed since the last heparin dose, the rate of heparin clearance, and the rate of protamine infusion. Even if maximally effective concentrations of free protamine were reached, 10–20% of the cleavage of endogenous peptides (*e.g.*, bradykinin) by CPN would not be inhibited by protamine, as suggested by figure 2. Studies of a family with a genetic deficiency of carboxypeptidase N revealed two members with only about 20% of normal enzyme level. This level of activity may be sufficient to protect from the effects of mediators such as kinins and anaphylatoxins, although one person in this family did have recurrent bouts of angioedema associated with this deficiency.<sup>30</sup> However, it has been shown that CPN levels decrease to 20% of normal during cardiopulmonary bypass.<sup>37</sup> Thus, patients with lower levels of CPN due to genetic factors,<sup>29,30</sup> or disease (*e.g.*, cirrhosis of the liver<sup>17</sup>), or with decreased inactivation of bradykinin during treatment with angiotensin converting enzyme inhibitor may be especially sensitive to the effects of protamine inhibition of CPN after cardiopulmonary bypass.

In conclusion, protamine by inhibiting CPN can block the inactivation of kinins and anaphylatoxins and thereby potentiate two of the three factors implicated in the catastrophic reaction to protamine administration after extracorporeal circulation.<sup>7,9</sup>

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