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CARBOXYPEPTIDASE IN BLOOD AND OTHER FLUIDS—I.
PROPERTIES, DISTRIBUTION, AND PARTIAL PURIFICATION OF THE ENZYME*

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Abstract—A carboxypeptidase was described, which cleaves basic C-terminal amino acids and hydrolyzes HLA, HLL, HLO, and N-acetyl-L-phenylalanyl-L-arginine substrates. The carboxypeptidase exists in the blood sera of mammals, birds, amphibia, and other animals, in lymph, and in human urine and thrombocytes, but not in erythrocytes. No significant amount of carboxypeptidase A-type activity has been seen in human serum. The enzyme is activated by cobalt and to a lesser degree by nickel. It is inhibited by some heavy metals, ε-amino-*n*-caproic acid, benzoyl-L-arginine and chelating agents. The enzyme activity is lower in the plasma of guinea pigs during anaphylactic shock. The serum enzyme is different from pancreatic carboxypeptidase B. The enzyme has been purified from human plasma by means of ammonium sulfate precipitation, and DEAE-cellulose column chromatography followed by DEAE-Sephadex column chromatography. The partially purified enzyme hydrolyzes HLL and inactivates bradykinin. Bradykinin has also been destroyed by urine extract and by lymph. The similarities and differences in cleaving shorter (e.g. HLL) and longer peptide substrates (bradykinin) and the possible existence of several carboxypeptidases in blood are discussed.

ONE of us reported[1, 2] in 1961, that synthetic bradykinin and probably kallidin are destroyed in a human plasma fraction by a carboxypeptidase-type enzyme that cleaves the C-terminal arginine. Others have confirmed this finding.[3, 4] Since the enzyme is different from pancreatic carboxypeptidase A and B, it has been provisionally named carboxypeptidase N.[5] In searching for a more available and simpler substrate than bradykinin with which to extend our studies, we have found that HLA,* HLL, and other peptides are hydrolyzed by carboxypeptidase N or similar carboxypeptidases in blood serum.[6] Part I of this communication deals with the distribution of this activity in the blood and other body fluids of several animal species, with activation and inactivation of the enzyme activity, and with additional evidence for distinguishing the activity and carboxypeptidase B. Part II of the communication will discuss clinical aspects of the work in another journal.

Preliminary reports have been submitted elsewhere.[7, 8]

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Abbreviations used are HLA, hippuryl-L-arginine; HLL, hippuryl-L-lysine; HLO, hippuryl-L-ornithine; HLP, hippuryl-L-phenylalanine.

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MATERIALS AND METHODS

The hydrolysis of the substrates was measured with an ultraviolet spectrophotometric technique, with a photometric ninhydrin determination, or with peptide mapping.

In the Cary model 15 recording u.v. spectrophotometer, the method of Folk *et al.*[9] was used at $\lambda = 2{,}540$ Å. The concentration of HLL, HLA, HLO, and HLP was $1{\cdot}10^{-3}$ M in an 0·1 M Tris buffer of pH 7·4. The temperature was kept at 37°. The source of energy was a deuterium lamp. The optical compartment of the instrument was constantly flushed with a slow stream of nitrogen to avoid the build-up of ozone. The optical density in the reference cells was increased with neutral density filters. The final dilution of the sera samples was 1 : 40 (v/v). The instrument was equipped with a delay switch to reduce the noise, with an extended scale slide wire, and with an automatic cuvet positioner. Activators were preincubated with the enzyme for 105 min at 31° and for 5 min at 37°. If not otherwise indicated, the activity of carboxypeptidase in pooled human serum was determined by the u.v. spectrophotometric technique with HLL substrate and $1{\cdot}10^{-4}$ M $CoCl_2$.

In the inhibition studies, the inhibitors were usually preincubated with the enzyme for 60 min after the 105-min preincubation period with $1{\cdot}10^{-4}$ M $CoCl_2$.

The photometric ninhydrin reaction was based on the method of Moore and Stein.[10] The substrates were HLA, HLP, L-phenylalanyl-L-arginine acetate and N-acetyl-L-phenylalanyl-L-arginine acetate. A sodium diethylbarbiturate buffer[11] was used with pH of 7·4 with HLA and 7·1 with N-acetyl-L-phenylalanyl-L-arginine substrate. The final dilution of serum was 1 : 15 (v/v). Aliquots were taken at regular intervals from a 37° incubation bath, the protein precipitated with picric acid, and the intensity of the color of ninhydrin reaction read at $\lambda = 5{,}700$ Å.

The pH of the various buffers was measured at room temperature, but the pH values in Fig. 5 were not corrected for the difference in temperature between the room and the optical compartment of the spectrophotometer. The buffers used were: Tris, 0·025 M and 0·1 M; phosphate, 0·05 M; imidazole 0·025 M; and acetate 0·1 M.

Peptide mapping was done according to Katz *et al.*[12] Descending paper chromatography was followed by high-voltage paper electrophoresis. The papers were stained with Sakaguchi's reagent on one side and ninhydrin on the other, or only with ninhydrin. The substrate concentration, during the incubation with enzyme preceding the peptide mapping, was 1 or $2{\cdot}10^{-2}$ M[5, 13]. The buffer was Tris, pH 7·4, at 31°.

The destruction of bradykinin *in vitro* was measured on the isolated rat uterus. The contractions of the muscle were registered with a special device.[14]

Swine pancreatic carboxypeptidase B was purified here according to Folk *et al.*[9]

During purification procedures, the approximate protein concentrations were estimated by measuring the optical density of the solutions at 2,780 Å.

Various sources of enzymes were used. Blood was collected from donors in a blood bank and the sera were pooled. Pooled swine, bovine, carp, alligator, and chicken sera were purchased from the Colorado Serum Co. Unanesthetized guinea pig plasma(7) and dog serum(2) were used as individual samples; cat(5) and rabbit plasma(6) were collected from animals in narcosis and not pooled. Turtle plasma,(2) frog plasma,(5) and rat serum(4) were obtained after decapitating the animals; the enzyme levels were determined in the individual samples. Earthworm blood was pooled. Rat lymph(3) and dog lymph(2) were collected via the thoracic duct. Human thrombocytes were obtained by

means of repeated centrifugations and disrupted with sonication. Red blood cells were washed three times in saline and hemolyzed. Pooled urine was collected from several healthy donors.

The effect of anaphylactic shock on the level of enzyme was studied in guinea pigs: 28 animals were divided into three groups; 6 were used as control; 22 were sensitized with an i.v. injection of 5 mg of bovine γ-globulin. Twelve of the 22 animals were shocked three weeks later with a challenging dose of the same protein i.v. All animals were killed by decapitation, and heparinized plasma was collected immediately after death.

RESULTS

Human serum

Human blood serum and heparinized plasma contain a carboxypeptidase-type enzyme that hydrolyzes HLL, HLO, and HLA. The hydrolysis of HLO is very slow but is evident in peptide mapping after 18-hr incubation of the enzyme and substrate. The cleaving of HLL was studied in the u.v. spectrophotometer and qualitatively confirmed in peptide mapping. The splitting of arginine from HLA was followed with all three techniques described in Methods.

Activation and inhibition

It was also observed that metals, especially $CoCl_2$, can activate the enzyme. Figure 1 summarizes the results with $CoCl_2$ and $NiSO_4$. Initially the rate increased steeply with increasing concentrations of $CoCl_2$, but it leveled off at higher values.

At the 1×10^{-3} M concentration of $CoCl_2$, a slight precipitation was sometimes observed, which may explain the fairly wide range of activity at this point (Fig. 1).

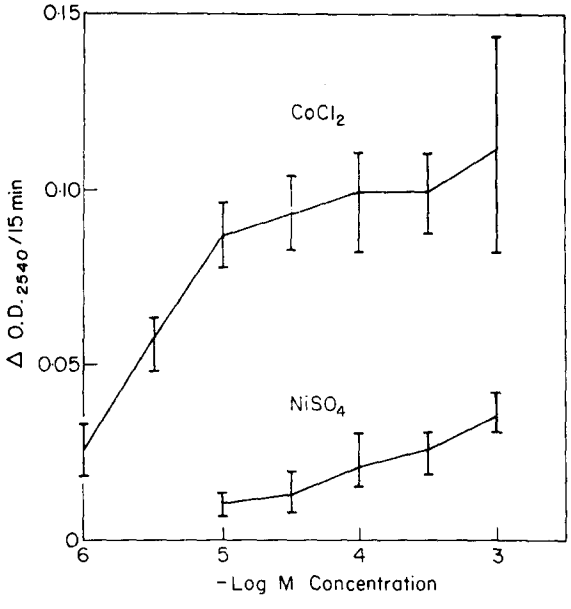

FIG. 1. Activation of the hydrolysis of HLL by human serum carboxypeptidase. Vertical bars show the range in the various samples.

Since the activation did not increase much above 1×10^{-4} M concentration of cobalt, the metal was kept at this level in most of the routine examinations. On the average, 1×10^{-4} $CoCl_2$ accelerated the enzyme sixfold in 11 samples of pooled normal human serum. $NiSO_4$ had only one third of that effect in four samples. The hydrolysis of HLL in pooled normal serum was about 3·5 times faster (0·79 μmole/min per ml) than that of HLA (0·23 μmole/min per ml).

The activity of the enzyme in serum and in heparinized plasma was slightly different. When blood was taken from 18 individuals and immediately divided into serum and plasma, the mean values in serum were 9% above that in plasma. Various explanations can be offered for this difference, but they are speculative.

Other metals inhibited the enzyme. $HgCl_2$, $ZnSO_4$ or $ZnCl_2$, and $CdSO_4$ were strongly inhibitory (Fig. 2). The I_{50} values were, in the presence of 1×10^4 M of $CoCl_2$, 4×10^{-5}, 2×10^{-5}, and 4×10^{-6} M respectively. ϵ-Amino-*n*-caproic acid and benzoyl-L-arginine also inhibited (Fig. 2). The corresponding I_{50} concentrations were 2×10^{-3} and 1×10^{-4} M. The cleaving of HLA in serum also was blocked by 1×10^{-4} $ZnSO_4$.

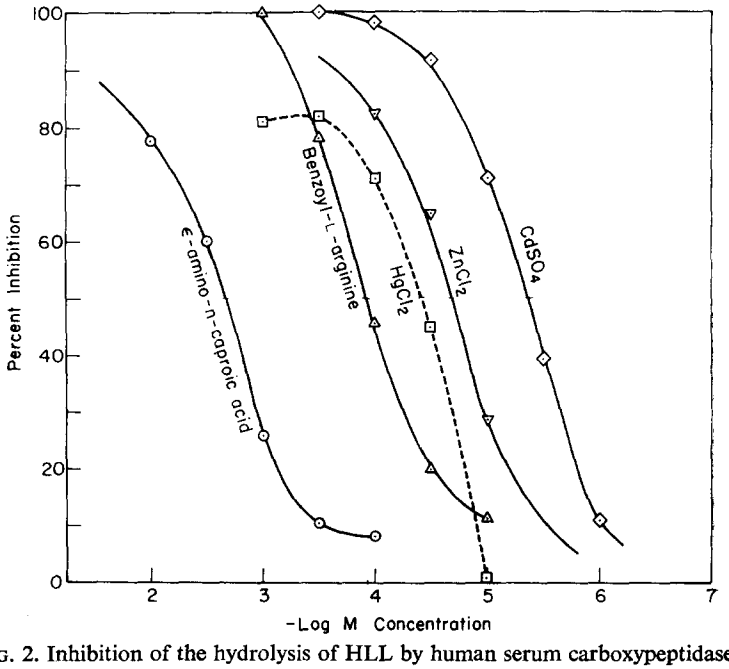

FIG. 2. Inhibition of the hydrolysis of HLL by human serum carboxypeptidase.

The enzyme requires a period of pre-equilibration with the activating metal ions. Figure 3 shows the relationship of activation with length of preincubation period at 31°.

The rate of hydrolysis of HLL increases with increasing serum concentrations. Figure 4 shows the correlation between the volume of the serum used in the 4 ml absorption cells and activity in the spectrophotometer.

In the absence of added activating cobalt ions, chelating agents such as EDTA (3×10^{-3} M), mercaptoethanol (1×10^{-3} M), or 2,3-dimercaptopropanol (1×10^{-4} M) inhibited the enzyme completely.

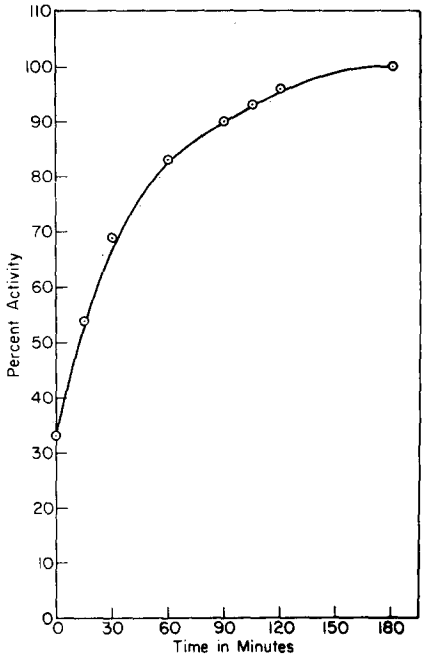

FIG. 3. Effect of the length of preincubation with 1×10^{-4} M $CoCl_2$ on activity. Ordinate: per cent activity; 100 = the activity of pooled normal human serum preincubated with the metal for 3 hr.

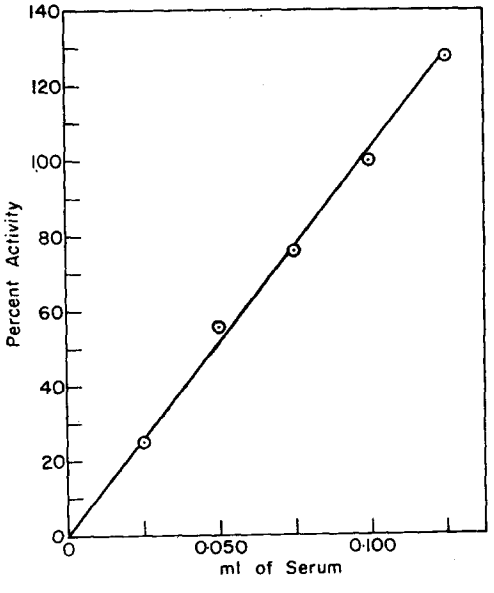

FIG. 4. Effect of concentration of serum on the rate of hydrolysis of HLL. Ordinate: per cent activity; 100 = the activity of 0·1 ml of pooled normal serum per 4-ml volume in the absorption cells. Abscissa: ml serum diluted to 4 ml.

The K_m of HLL in the presence of 1×10^{-4} M $CoCl_2$ was $1{\cdot}4 \times 10^{-3}$ M. The determination was made in pooled human sera according to Lineweaver and Burk.[15]

It was recently indicated that 1-butanol accelerates the hydrolysis of HLA by carboxypeptidase B.[16] In our experiments, the hydrolysis of HLL by the serum enzyme was enhanced by 39% in the presence of 0·3 M 1-butanol and 18% by 0·1 M 1-butanol. HLA gave very similar results.

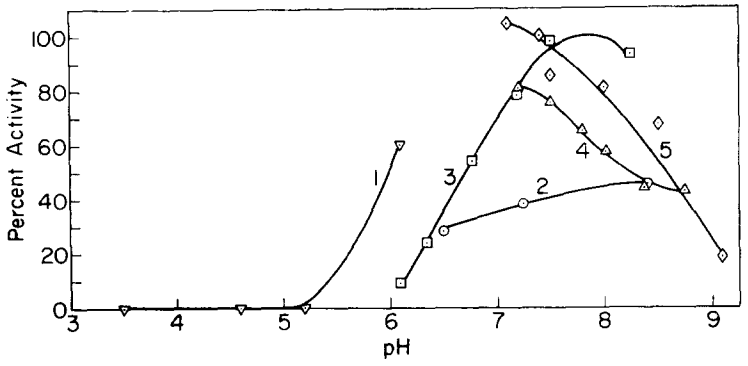

FIG. 5. Activity of the carboxypeptidase of pooled human serum at various pH values. Ordinate: per cent activity; 100 = the rate of hydrolysis of HLL in presence of 1×10^{-4} M $CoCl_2$ and in 0·1 M Tris (pH 7·4). Abscissa: pH. Buffers: 1, acetate 0·1 M; 2, imidazole 0·025 M; 3, phosphate 0·05 M; 4, Tris 0·025 M; 5, Tris 0·1 M.

Figure 5 shows the activity of the enzyme at various hydrogen ion concentrations. As with carboxypeptidase B,[17] the activity below neutrality depends very much on the choice of buffer used. For example, phosphate buffer gives sharply decreasing rates between pH 6 and 7; in acetate buffer the rates are much higher at pH 6. The behavior of the enzyme between pH 6 and 7 in phosphate buffer resembles the curves showing destruction of bradykinin below pH 7 in this medium.[18]

Other substrates

HLP,[19] the substrate of carboxypeptidase A, gave negative or negligible results with all three methods used. L-Phenylalanyl-L-arginine, which represents the C-terminal end of bradykinin, was not attacked significantly by the enzyme. When the free amino group of phenylalanine residue of this dipeptide was acetylated (N-acetyl-L-phenylalanyl-L-arginine) the rate of hydrolysis became substantial. This was shown quantitatively with ninhydrin assay where the rate was approximately half that of HLA and qualitatively in peptide mapping in one experiment. The difference in the hydrolysis of a short peptide substrate with and without protective groups on the N-terminal end is in good agreement with the carboxypeptidase character of the enzyme investigated.

Erythrocytes

Hemolyzed human red blood cells showed no carboxypeptidase activity with HLA substrate, either in the ninhydrin assay or in peptide mapping.

Thrombocytes

The two different preparations of platelets split HLL very slowly; after 18 hr incubation, partial hydrolysis was seen in the combined paper chromatography and electrophoresis.

Urine

Fresh urine was collected from healthy subjects. To 1 l. of urine, solid ammonium sulfate was added to 30% saturation, and the precipitate was removed in the centrifuge. The supernatant was then brought to 55% saturation and the precipitate separated in the centrifuge, dissolved in water, and dialyzed against 0·9% saline and 1×10^{-4} M $CoCl_2$ overnight in the cold room. The final volume was 17 ml. A 1 : 20 (v/v) dilution of the solution destroyed bradykinin under standard conditions[5] within a half hour. The experiments were repeated three times.

Peptide mapping showed that during the 18-hr incubation period both HLL and HLA were hydrolyzed by the urine extract. Peptide mapping with bradykinin substrate demonstrated two main spots which were similar to those obtained with carboxypeptidase[5] from other sources. Those spots have been identified previously as arginine$_9$ and the octapeptide derivative of bradykinin. In addition, there were indications of further breakdown of the peptide by the urinary enzymes.

Human plasma fractions

Human plasma fractions IV-1, IV-4, and IV-6-3 contain a carboxypeptidase that cleaves HLA and HLL. The rate of hydrolysis of HLL by IV-1 was 0·034 μmole/min per mg.

Lymph

Lymph has been reported by others[20] to inactivate bradykinin. We can confirm this finding. In our experiments rat and dog lymph diluted 1 : 20 (v/v) destroyed more than half of 3·4 μg/ml of bradykinin within half an hour. In a single orientation experiment the activity of bradykinin was preserved in the presence of 1,10-phenanthroline (1×10^{-3} M).[5, 21]

No hydrolysis of HLL was observed in rat lymph in absence of cobalt ions. In the presence of 1×10^{-4} M $CoCl_2$, rat lymph hydrolyzed HLL very slowly; thus the activity was substantially lower than in rat serum. The rate in the one sample of dog lymph tested was very similar. In exploratory studies, lymph of both animals split HLA, when determined with the photometric techniques in presence of $1 \cdot 10^{-4}$ M $CoCl_2$ (rat 0·04, dog 0·06 μmole/min per ml).

Animal sera

Carboxypeptidase occurs in the sera or plasma of many animals including mammals, birds, amphibia, fish, etc. (Table 1). Alligator showed only traces of activity with HLL. This is in contrast to the relatively high rate of destruction of bradykinin in alligator sera.[6] Although guinea pig blood has been described as very potent in inactivating bradykinin,[20] other species hydrolyzed HLL faster than the guinea pig. The hydrolysis of HLA by dog, swine, rabbit, and guinea pig sera was also shown previously in peptide mapping.[2]

TABLE 1. HYDROLYSIS OF HIPPURYL-L-LYSINE BY BLOOD SERA OR PLASMA IN PRESENCE OF 1×10^{-4} M $CoCl_2$

	Activity (μmole/min per ml)
Human	0·79
Swine	1·01
Ox	0·26
Newborn calf	0·43
Rabbit	0·28
Guinea pig	0·70
Rat	1·02
Cat	1·04
Chicken	0·45
Alligator	trace*
Frog	0·28
Turtle	0·87
Carp	0·56
Earthworm	trace*

* Activity shown with peptide mapping only.

Carboxypeptidase in anaphylactic shock

Since bradykinin[22] and proteolytic[23] enzymes (as well as other substances) have been implicated in anaphylactic shock, we investigated the level of the enzyme in the heparinized plasma of shocked animals. Table 2 shows the results.

All animals in the third group, with one exception, were in severe shock when killed by decapitation. The animal which was not shocked had the highest enzyme level in that group. The guinea pigs in the other groups did not receive antigen and were killed in the same way. The guinea pigs which were only sensitized and those which were both sensitized and shocked showed lower values than the controls with HLL substrate. The difference in enzyme activity between the first and third group is statistically significant ($P < 0{\cdot}01$).

TABLE 2. PLASMA CARBOXYPEPTIDASE LEVELS IN SENSITIZED AND SHOCKED GUINEA PIGS

	No. of animals	HLL hydrolyzed (μmole/min per ml)	± S.E.
Control	6	0·74	0·06
Sensitized	10	0·54	0·06
Sensitized and shocked	12	0·50	0·02

Differences between the pancreatic and the serum enzyme

Folk and Gladner[24] stated that swine pancreatic carboxypeptidase B hydrolyzes HLA much faster than HLL at the 1×10^{-3} M concentration level. We wanted to determine whether the difference between the human serum carboxypeptidase and the swine pancreatic carboxypeptidase B was caused by the difference in species or in site of origin of the enzymes. Therefore, comparison was made of the hydrolysis

ratios of two substrates, using human serum, swine serum, and swine pancreatic carboxypeptidase B as enzyme sources.

All three enzyme preparations contained $CoCl_2$ (1×10^{-4} M). Table 3 shows the results. Human and swine serum hydrolyzed HLL faster than HLA, while carboxypeptidase B gave opposite results. The serum enzymes were much more accelerated by cobalt than the pancreatic enzyme.

TABLE 3. COMPARISON OF THE ACTIVITY OF SERUM AND PANCREATIC CARBOXYPEPTIDASE

Source of enzyme	Human serum	Swine serum	Swine pancreatic carboxypeptidase B
Ratio of hydrolysis HLL/HLA	3·5	2·4	0·33
Per cent acceleration of HLL hydrolysis by 1×10^{-4} M $CoCl_2$	550	383	49

Thus the characteristics of swine serum enzyme are closer to the human serum carboxypeptidase than to the swine pancreatic carboxypeptidase B. These characteristics, taken with the information gained in clinical material, also indicate that the serum enzyme is different from the pancreatic carboxypeptidase B and possibly does not originate from the pancreas.

Purification

Heparinized plasma was collected from healthy individuals. About 200 ml plasma was brought to 30% saturation with solid ammonium sulfate at 4°. After stirring for half an hour, the precipitate was separated in the centrifuge at 14,000 *g* in 30 min. Ammonium sulfate concentration in the supernatant was increased to 55% saturation, the precipitate was collected in the centrifuge, and the supernatant discarded. The precipitate obtained was redissolved in a few milliliters of water and dialyzed overnight in the cold against two or more changes of 5 liters of 1×10^{-4} M $CoCl_2$. Some solids present in the dialysis sacks were removed in the centrifuge at 10,000 *g* in 10 min.

The ammonium sulfate precipitation was similar to the procedure applied by Folk *et al.*[9] The subsequent steps of purification, however, were different.

DEAE-cellulose (35 *g*) was washed in 0·2 M phosphate buffer of pH 6·7. The material was packed into a column (1·5 × 29 cm) under air pressure with 0·01 M phosphate buffer, pH 6·7. Roughly 280 mg of serum proteins from the ammonium sulfate precipitate was taken up in the buffer and adsorbed on the column at 4°. Nonadsorbing impurities were washed off the column with 100 ml of phosphate buffer. The proteins were eluted stepwise with NaCl, with increased concentrations (up to 0·2 M) in 0·01 M phosphate buffer of pH 6·7.[25] The rate of flow was about 90 ml/hr. The enzyme activity was eluted with 0·15 M NaCl. Table 4 and Fig. 6 show the results. (Table 4 suggests the relative degree of purification since, after column chromatography, contents of a number of tubes were pooled before additional steps were taken.) The tubes corresponding to each of the peaks of the 2,780 Å absorbing material were combined, lyophilized, dissolved in water, and dialyzed against several changes of 1×10^{-4} M $CoCl_2$ in the cold room overnight.

The active component in the 0·15 M NaCl peak hydrolyzed HLL, HLA, acetyl-L-phenylalanyl-L-arginine, and bradykinin, but not HLP. In addition, bradykinin was inactivated by a serum component which hydrolyzed HLL only very slightly. That fraction was collected after starting to elute with 0·08 M NaCl, and it was several times less active than the main fraction. In both active fractions, the inactivation of bradykinin was inhibited by 1×10^{-3} M 1,10-phenanthroline.

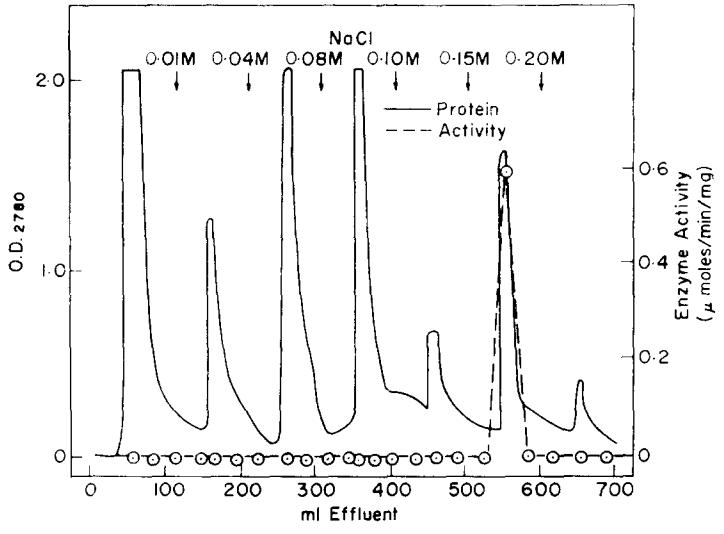

FIG. 6. Purification of the enzyme on DEAE-cellulose column. The activity was measured with HLL substrate.

TABLE 4. PURIFICATION OF THE CARBOXYPEPTIDASE OF HUMAN PLASMA

	Relative specific activity with HLL	Yield (%)
Plasma	1	100
Ammonium sulfate precipitation	3·4	58
DEAE-cellulose column chromatography	31	31
DEAE-Sephadex column chromatography	80	18

Additional purification was obtained in exploratory studies with a column of DEAE-Sephadex G-50 medium gel prepared according to the suggestions of the manufacturer (Pharmacia). The gel was treated overnight in 0·02 M phosphate buffer, pH 6·8, which contained 0·2 M NaCl. It was subsequently washed free of NaCl with the buffer. The gel was poured into a column (1 × 19 cm). Approximately 20 mg of protein containing the enzyme from the active fraction was added to the column and eluted stepwise with increasing concentrations of NaCl (Fig. 7). The rate of flow was 10 to 18 ml/hr. The enzyme was collected between 80 and 110 ml of effluent. The activity was concentrated by freeze drying; the dry material was redissolved and dialyzed against 1×10^{-4} M $CoCl_2$. The active sample hydrolyzed HLL,

HLA, and bradykinin. Here also the destruction of bradykinin was prevented by preincubating the active enzyme with 1,10-phenanthroline.

DISCUSSION

These experiments have shown that in various animals, blood sera and some other fluids contain a carboxypeptidase which, like carboxypeptidase B, cleaves basic C-terminal amino acids. Our conclusions are that serum carboxypeptidase and pancreatic carboxypeptidase B are not identical and that some experimental evidence suggests the existence of several carboxypeptidases in blood. This latter consideration leads to the assumption that bradykinin may be the substrate of more than one carboxypeptidase.

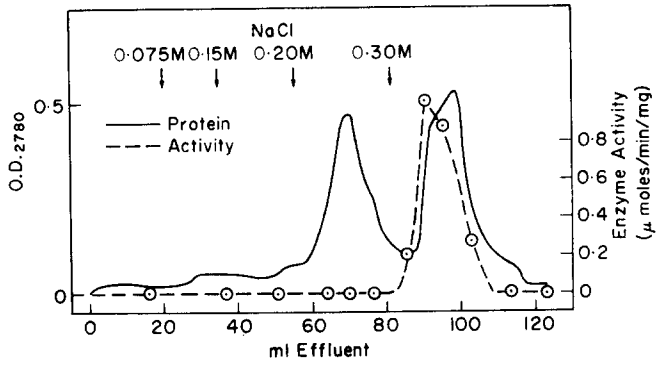

FIG. 7. Purification of the enzyme on DEAE-Sephadex column.

Carboxypeptidase B had been found originally in zymogen form in swine[26] and bovine pancreas,[11] but carboxypeptidases were thought to be absent from the blood.[27] A catheptic carboxypeptidase B, active at acid pH, has been described recently in tissues.[28] Another carboxypeptidase B may exist in brain.[29] Crude pancreatic kallikrein preparations contain carboxypeptidase B as an impurity.[13] After closing the experimental part of this work, we noticed a communication mentioning the excretion of carboxypeptidase B in rat urine.[30]

There are numerous differences between pancreatic carboxypeptidase B and blood enzymatic activity with HLL and HLA. Table 3 summarizes two of these dissimilarities: substrate specificity and metal activation. There are other points of contrast. For example, bovine pancreatic carboxypeptidase B hydrolyzes some of the substrates of carboxypeptidase A,[11] but the blood enzyme is inactive or only very slightly active in this respect. We have found in clinical subjects two cases of pancreatitis,[31] but with normal enzyme level, whereas a number of patients with cirrhosis of the liver had an abnormally low value. This we may take as an additional indication that the human enzyme does not originate in the pancreas. Furthermore, swine pancreatic carboxypeptidase B is unaffected by EDTA,[9] but the serum enzyme is inhibited by this chelating agent. Carboxypeptidase N with bradykinin substrate behaves similarly in this respect.[5] In addition, the hydrolysis of both HLL and bradykinin in serum is inhibited by metal-sequestering thiols.[3, 32, 33] Carboxypeptidase B contains zinc in the active center which can be replaced by various other metals to form active or

inactive complexes.[9, 24] In contrast, serum carboxypeptidase in our experiments was strongly inhibited by $ZnSO_4$ even in the presence of $CoCl_2$.

Some properties of the enzyme in blood plasma that inactivates bradykinin (carboxypeptidase N) differentiate it from carboxypeptidase B.[5] However, it is not yet certain that bradykinin and HLL are hydrolyzed in sera by a single enzyme. Similar carboxypeptidases with differing specificities toward the two substrates could be present. Some differences in the sera activity toward the substrates, as well as the similarities reported here, have been noted. For example, butanol accelerates the splitting of HLL but not the inactivation of bradykinin.[5] $CoCl_2$ accelerates the breakdown of HLL much more than that of bradykinin. N-acetyl-L-phenylalanyl-L-arginine has not yet been used in a sufficient number of experiments to solve the question of one vs. several carboxypeptidases.

A major point of disagreement was found, nevertheless, during the purification procedures. In addition to the active fraction shown in Fig. 6, bradykinin was inactivated by plasma proteins in another fraction which had low activity with HLL.

We should like to point out, however, that the behavior of even highly purified carboxypeptidase B depends very much on the structure of the substrate used. For instance, the metal sensitivity of the enzyme is determined by selecting ester or peptide-type substrates.[24] It is also known that the inhibition of carboxypeptidase B with short peptide substrates varies.[34]

Another reason for dissimilarity might be the difference of more than 300-fold in concentration between bradykinin and the other peptide substrates used in our experiments. Since experiments with inhibitors both *in vitro* and *in vivo* indicated that the destruction of bradykinin is due to a carboxypeptidase,[2, 5, 6, 32, 33] it is possible that bradykinin might be the substrate of several carboxypeptidases with affinity for basic C-terminal amino acids. Thus the situation may resemble that of leucyl-aminopeptidase in blood. This peptidase is probably not a single enzyme but a group of related aminopeptidases.[35]

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