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CARBOXYPEPTIDASE IN BLOOD AND OTHER FLUIDS—III

THE ESTERASE ACTIVITY OF THE ENZYME*, †

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Abstract—The carboxypeptidase of human blood plasma hydrolyzes hippuryl-L-lysine (HLL) and bradykinin. In addition the enzyme, called carboxypeptidase N, has an esterase activity as well; it cleaves the ester substrate hippuryl-L-argininic acid (HLAa) even faster than HLL. This esterase activity increases in human serum during pregnancy. Carboxypeptidase N has been purified from human plasma. The best preparation showed a 252-fold-increased peptidase and a 212-fold-concentrated esterase activity. The hydrolysis of HLAs is inhibited by various agents including argininic acid, cadmium, and metal-complexing compounds. The presence of a second kininase in human plasma was observed.

PREVIOUS publications of one of us described the existence of a carboxypeptidase in blood plasma that inactivates bradykinin.[1, 2] The enzyme was called carboxypeptidase N. Plasma contains a similar or identical enzyme, which cleaves HLL, hippuryl-L-arginine, and related substrates.[3, 4] The plasma enzyme is different from swine pancreatic[5] or kidney[6] carboxypeptidases.

The aims of the present studies were to characterize the esterase action of the carboxypeptidase in blood plasma with HLAa [(*o*-benzoylglycyl)-α-L-hydroxyl-δ-guanidino-*n*-valeric acid] substrate and to purify the enzyme further.

METHODS

The enzyme was purified from heparinized blood plasma obtained from healthy donors. Human serum was used in most of the routine assays as the source of enzyme. Swine serum was collected in a slaughterhouse. Swine pancreatic carboxypeptidase B was purified in this laboratory according to Folk *et al.*[5]

HLAa came from different sources. Samples were donated by Dr. J. E. Folk of the National Institutes of Health. Another batch was obtained through the courtesy of Dr. R. O. Studer of Hoffman-La Roche & Co., Basle. The latter batch contained 0·7

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† References 3 and 4 are to parts I and II of this series.

‡ Work (N. Manning) done in the Mellon Institute, Pittsburgh, Pa., during the tenure of a fellowship.

mole benzenesulfonic acid/mole HLAa. Finally HLAa was also synthesized by one of us with some modifications of the method of Wolff *et al.*[7] We obtained an approximately 95 per cent pure product by including a cellulose column chromatography with butanol:H_2O:ethanol (4:1:1) as eluent in the purification process.

The enzymatic hydrolysis of the substrates was determined quantitatively and qualitatively. In peptide mapping,[2, 3] one of the split products of the hydrolysis of HLAa was identical with argininic acid.

The initial rate of enzymatic hydrolysis of the ester bond of HLAa was followed in a Radiometer pH-Stat at 37° in 0·15 M NaCl by adding 10^{-3} N NaOH to maintain the pH constant at 8. The serum used was dialyzed against 2 × 1 l. of saline for 2 hr at 4°. The final dilution of serum in saline was 1:100.

The inactivation of bradykinin was measured on the isolated rat uterus with a special device[8] used to record contractions of smooth muscles.

Samples of the incubation mixture of bradykinin and enzyme were withdrawn for assay every 5 min. The relative specific activity of the enzyme was calculated from the time necessary to inactivate over 50 per cent of a bradykinin solution (3·3 μg/ml):

$$v = \frac{1}{t_{\min} \times \text{enzyme conc. in mg protein}}$$

Most experiments were carried out in a Cary model 15 recording u.v. spectrophotometer at $\lambda = 2540$ Å.[3] If not otherwise indicated, the determination was done at 37° in 0·1 M Tris buffer, pH 7·1. The concentration of substrate was 1×10^{-3} M. In the spectrophotometric studies the final dilution of serum in buffer was 1:80.

When the level of the enzyme was studied in the blood of normal individuals and pregnant women, the microcuvettes of the spectrophotometer contained 5 μl serum and 0·25 μmole HLAa in 0·5 ml buffer, pH 8 (0·05 M Tris).

The hydrolysis of Ac-L-Phe-L-Arg was measured with a spectrophotometric ninhydrin assay.[3, 9]

In zone electrophoresis, a co-polymer of polyvinyl chloride and polyvinyl acetate, "Pevikon C-870", was the supporting medium in an E-C horizontal, water-cooled apparatus.[10] Barbital buffer[10] was used at pH 8·6, and phosphate (0·05 M) at pH 6·5. In a typical experiment, 1·6 kg Pevikon was poured into a block, 21·5 × 37 × 1 cm. A 1-cm-wide slice was removed from the block; it was mixed with 0·8–1·0 g of plasma globulins and re-applied in the block. The average run took 38–40 hr at approximately 4° with a potential of 200 V and a current of about 50 mA. After the completion of the electrophoresis the Pevikon block was cut into 1-cm-wide segments. The segments were eluted individually in 37 glass columns (1·5 × 36 cm) with 30 ml of 0·15 N NaCl solution. Because of the presence of the barbital buffer, the protein content was measured at $\lambda = 2840$ Å or according to Lowry *et al.*[11]

Since the barbital buffer interferes with the determination of the enzyme, it was removed during overnight dialysis against 1×10^{-4} M $CoCl_2$ solution.

Sephadex gel columns were prepared according to the specifications of the manufacturer.

Mongrel dogs under sodium pentobarbital anesthesia (25 mg/kg) were used in the shock experiments.

RESULTS

Esterase

HLAa is hydrolyzed by normal human plasma or serum faster than HLL[3] at the 1×10^{-3} M concentration. One ml pooled normal human serum cleaves 1·98 μmole HLAa and 0·79 μmole HLL per min[3] under standard conditions. (The hydrolysis of HLL was measured in the presence of 1×10^{-4} M $CoCl_2$).[3] With the technique used for the investigation of enzyme activity in individual blood sera (see Methods), the mean rate of hydrolysis of HLAa was found to be 3·9 times higher than that of HLL. Two samples of sera split HLAa on the average 17 per cent faster than the corresponding heparinized plasma. It was shown previously that HLL is also split somewhat more rapidly in serum than in plasma.[3]

The K_m of HLAa was determined according to Lineweaver and Burk[12] in the pH-Stat (Fig. 1). The points represent the mean of experiments done in duplicate. They

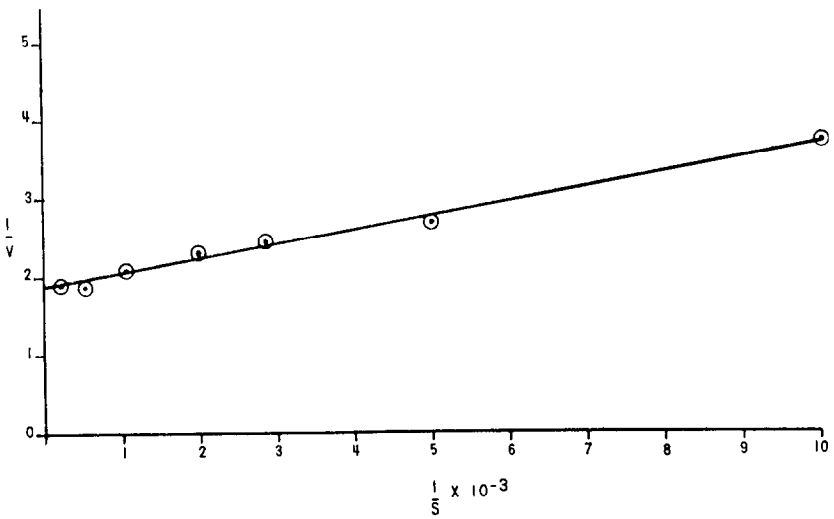

FIG. 1. Determination of the K_m of HLAa in human serum. For details see text.

were carried out twice at the highest substrate concentration and three or four times at the lower concentrations. The K_m was 1×10^{-4} M in the serum.

Figure 2 shows the effect of pH and various buffers on activity. The esterase activity was highest at pH 8·7 in a Tris buffer in the spectrophotometer. The peak of esterase activity is broader in the alkaline range than that of the peptidase activity.[3]

Inhibition

Numerous compounds such as heavy metals and chelating agents inhibited the plasma carboxypeptidase. Table 1 lists the agents and per cent inhibition obtained after 2-hr preincubation. For the sake of comparison the table also summarizes the inhibition of the peptidase activity from a previous publication.[3] Figure 3 shows the curves obtained with two of the most active compounds, $CdSO_4$ and argininic acid. The I_{50} values were 2×10^{-7} M and 4×10^{-5} M respectively.

It should be pointed out that 1×10^{-6} M $CdSO_4$ inhibited the enzyme 75 per cent; increasing the concentration of the metal 330-fold to $3{\cdot}3 \times 10^{-4}$ M could not cause complete inhibition of the enzyme. The inhibition by a split product of the substrate

hydrolysis, by argininic acid, becomes more evident after a period of preincubation. For example, 1×10^{-4} M argininic acid inhibited 56 per cent after 60 min of pre-equilibration with the enzyme but only 36 per cent when added simultaneously with the substrate in two experiments. The enzyme was also inhibited by EDTA and its

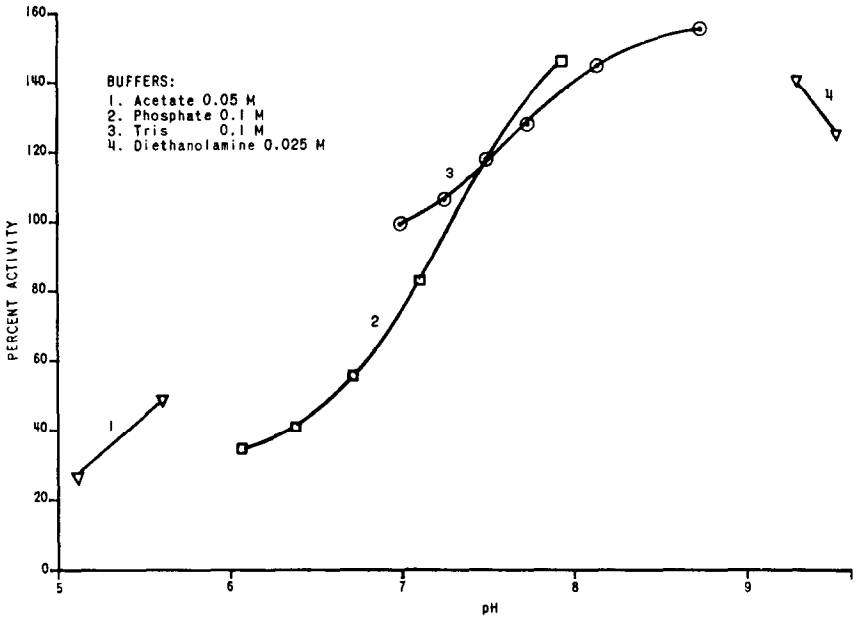

FIG. 2. Esterase activity of the carboxypeptidase in pooled human blood serum at various pH values. Ordinate: per cent activity; 100 = the rate of hydrolysis of HLAa in 0·1 M Tris, pH 7·0. Abscissa: pH.

TABLE 1. INHIBITION OF THE ESTERASE* AND PEPTIDASE† ACTIVITY OF PLASMA CARBOXYPAPTIDASE

Inhibitor	Concentration (M)		Per cent inhibition	
	HLAa	HLL	HLAa	HLL
$CdSO_4$	$2 \cdot 10^{-7}$	$4 \cdot 10^{-6}$	50	50
$HgCl_2$	$1 \cdot 10^{-4}$	$4 \cdot 10^{-5}$	88	50
$CoCl_2$	$3 \cdot 10^{-4}$	$3 \cdot 10^{-4}$	0	a‡
$NiSO_4$	$1 \cdot 10^{-4}$	$1 \cdot 10^{-3}$	43	a‡
$ZnSO_4$	$1 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	30	50
EDTA	$3 \cdot 10^{-3}$	$3 \cdot 10^{-3}$	79	100
2-Mercaptoethanol	$1 \cdot 10^{-2}$	$1 \cdot 10^{-3}$	68	100
ε-Amino-*n*-caproic acid	$5 \cdot 10^{-3}$	$2 \cdot 10^{-3}$	51	50
Argininic acid	$4 \cdot 10^{-5}$		50	

* Substrate: HLAa.
† Substrate: HLL.
‡ a = activates.

complexes such as Mg-EDTA, Ca-EDTA, and Ni-EDTA. Zn-EDTA (3×10^{-3} M) on the other hand enhanced the activity by 10 per cent. The inhibition by EDTA, by its complexes, and by mercaptoethanol[3, 13] is assumed to be caused by binding of a metal cofactor.

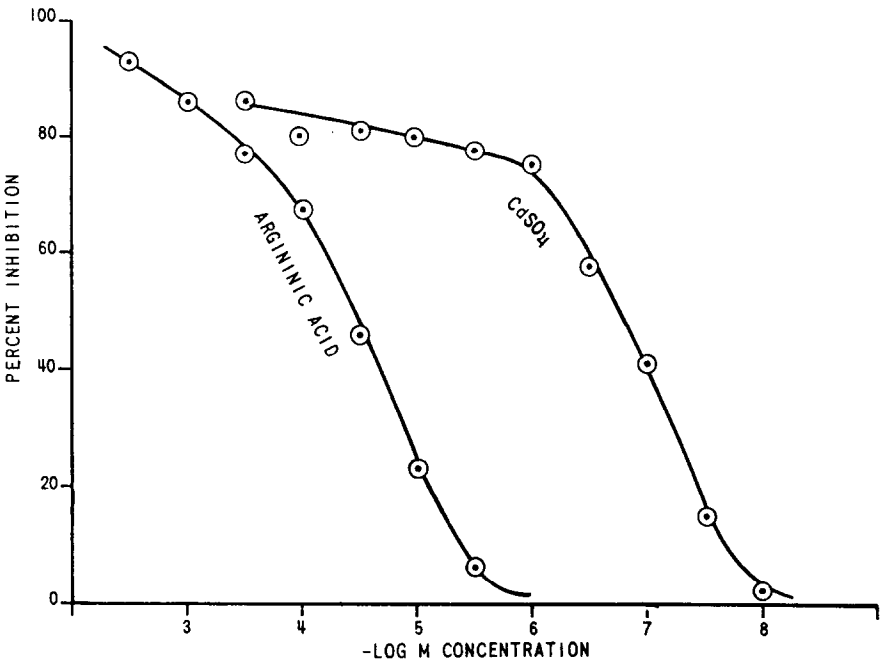

FIG. 3. Inhibition of the esterase activity of human plasma carboxypeptidase by argininic acid and by $CdSO_4$.

Swine serum

Swine serum hydrolyzed HLAa at the rate of 5·2 μmole/min/ml serum. Since it is known that the esterase activity of swine pancreatic carboxypeptidase B[14] is enhanced by Cd^{2+} and the enzyme is not inhibited by EDTA,[5, 7] it was of interest to test these two compounds on swine serum carboxypeptidase. EDTA (3×10^{-3} M) inhibited 90 per cent of the ester cleavage, $CdSO_4$ 76 per cent at 10^{-5} M and 71 per cent at 10^{-6} M concentration. Thus swine serum carboxypeptidase is inhibited by EDTA and $CdSO_4$ just as the human enzyme is. This behavior toward inhibitors suggests additional differences between the swine serum carboxypeptidase and swine pancreatic carboxypeptidase B.[3]

Other substrates

Our previous studies[3] indicated that human plasma carboxypeptidase cleaves hippuryl-L-arginine and hippuryl-L-ornithine although at a lower rate than HLL. The substrate of carboxypeptidase A, hippuryl-L-phenylalanine, on the other hand, was not hydrolyzed.

In the present studies no carboxypeptidase A-type esterase activity was seen in the human serum either, since hippuryl-DL-β-phenyllactic acid,[15, 16] an ester substrate of carboxypeptidase A, was not split by human blood serum as assayed by the pH-Stat procedure.

Biocytin (ε-*N*-biotinyl-L-lysine) was not hydrolyzed by carboxypeptidase N. Peptide mapping failed to reveal the cleavage of the bond between the carboxyl group of biotin and ε-amino group of lysine.

Purification

The enzyme in 280 ml of plasma was precipitated between 30 and 55 per cent saturation with ammonium sulfate. Subsequently it was dialyzed at 4° against 1×10^{-4} M $CoCl_2$ solution. The active proteins were adsorbed on a DEAE-Sephadex A-50 column that had been pre-equilibrated with 0·02 M phosphate buffer, pH 6·8. The proteins were eluted with stepwise-increased concentrations of NaCl in 0·02 M phosphate buffer. The concentrations of NaCl were 0·075, 0·15, 0·2, and 0·3 M.

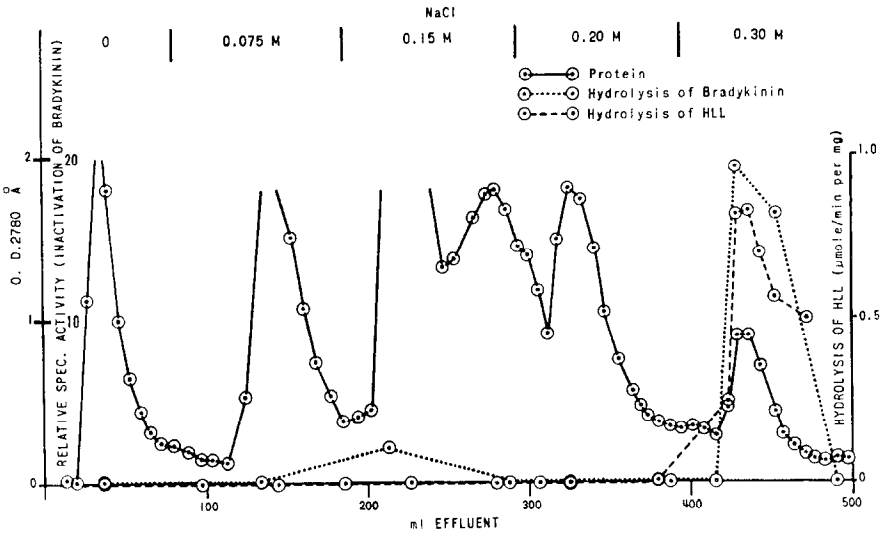

FIG. 4. Purification of the enzyme on DEAE-Sephadex A-50 column. One gram protein was purified by means of stepwise elution with NaCl. The volume of the effluent is shown on the abscissa. The ordinates show the concentration of protein, the bradykininase activity per mg protein, and the rate of hydrolysis of HLL. The protein peak eluted with 0·3 M NaCl had peptidase, esterase (not shown), and bradykininase activities.

The active fraction appeared in 0·3 M NaCl (Fig. 4). The enzyme was then rechromatographed directly on a second DEAE-Sephadex column and eluted with NaCl, but the salt concentrations were changed in smaller increments (Fig. 5). This second chromatography offered an additional purification (2–2·5-fold) over the first column (Table 2), as measured with HLL. The three activities, peptidase, esterase, and kininase, emerged as a single peak after the second column chromatography as well.

The final product gave a 252-fold purification of peptidase and 212-fold purification of esterase activity of the native plasma (Table 2). The bradykininase activity of globulins precipitated with ammonium sulfate increased after column chromatography 67-fold (step 2 in Table 2). This was measured on the isolated rat uterus after preincubation of the enzyme with 1×10^{-3} M $CoCl_2$ at 4° for 1 hr. The data indicate that the three activities of the purified enzyme increased fairly proportionally. (This statement, however, applies only to carboxypeptidase N, since another bradykininase present in human blood plasma was not purified by this procedure.)

The 0·15 M NaCl eluate of the first DEAE-Sephadex column contains an enzyme that inactivates bradykinin but cleaves HLAa only very slowly. The characterizations

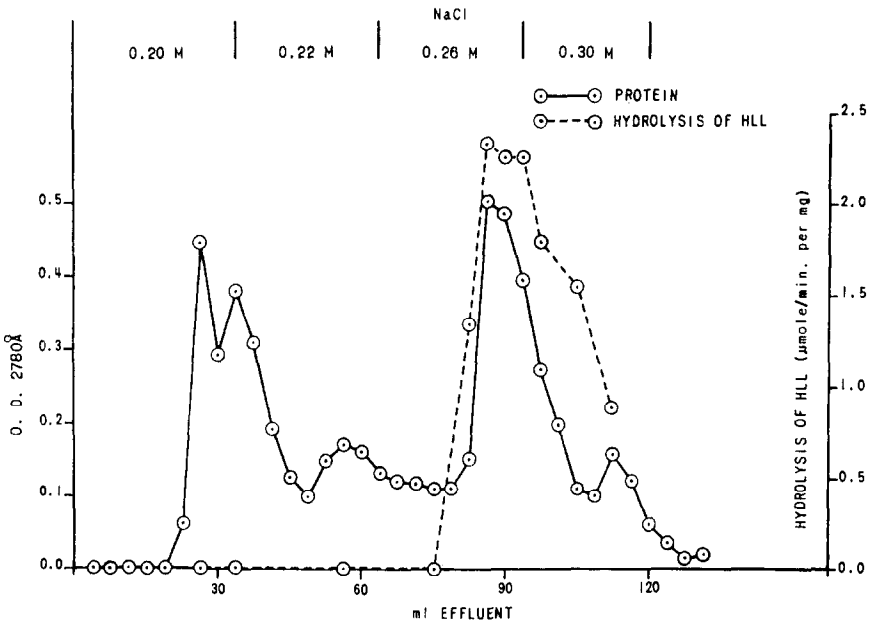

FIG. 5. Purification of the enzyme on a second DEAE-Sephadex column; 22 mg protein was purified by means of stepwise elution with NaCl. The ordinates show the concentration of protein and the rate of hydrolysis of HLL. A single protein peak had peptidase, esterase, and bradykininase activities.

of this second bradykininase of human blood plasma are not included in this communication.

Other techniques of purification not shown in Table 2 involved the use of CM-cellulose. Chromatography of the ammonium sulfate precipitate on CM-cellulose

TABLE 2. PURIFICATION OF THE CARBOXYPEPTIDASE OF HUMAN PLASMA

Steps	Relative specific activity			HLL* (% yield)
	Peptidase*	Esterase†	Kininase‡	
1. Plasma	1·0	1·0		100
2. Ammonium sulf. ppt (30–55%)	2·9	2·8	1·0	88
3. DEAE-Sephadex 1	110			35
4. DEAE-Sephadex 2	252	212	67	14

Substrates:
* Hippuryl-L-lysine.
† Hippuryl-L-argininic acid.
‡ Bradykinin.

The hydrolysis of hippuryl-L-lysine and hippuryl-L-argininic acid was calculated as μmole substrate hydrolyzed in 1 min by 1 mg enzyme protein. For the calculation of the rate of hydrolysis of bradykinin see Methods.

column in 0·05 M acetate buffer, pH 5, gave about a 10-fold purification but only an 11 per cent recovery. The active fraction containing the carboxypeptidase was not absorbed on CM-cellulose at pH 5.

Zone electrophoresis

To establish whether or not a single enzyme in blood plasma would have all three activities (esterase, peptidase, and bradykininase), we tried to separate them in zone electrophoresis. Human plasma globulins precipitated with ammonium sulfate gave a single activity peak with HLL, HLAa, and bradykinin in Pevikon zone electrophoresis at pH 8·6. The enzyme was concentrated in sections 18–20 (Fig. 6), giving

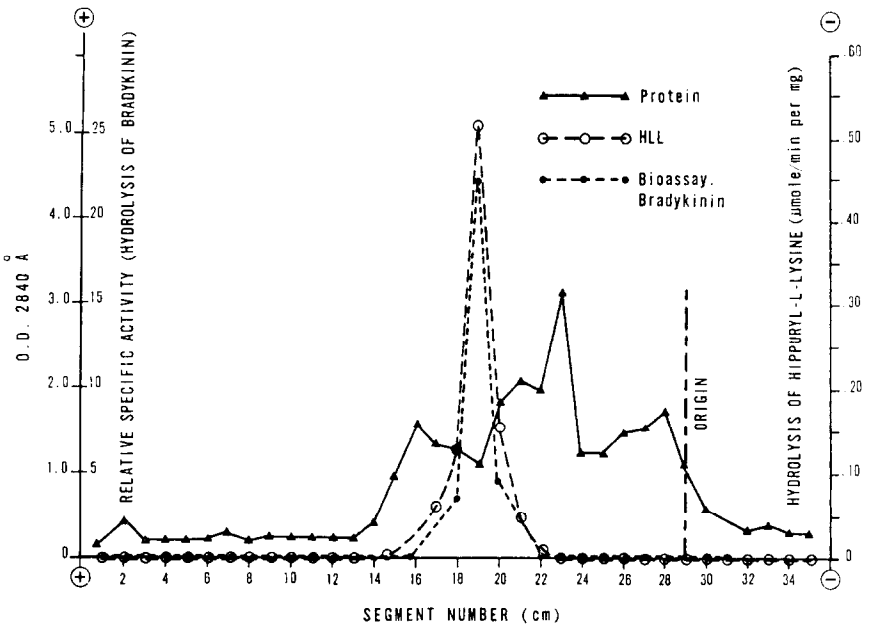

FIG. 6. Purification of human plasma carboxypeptidase by zone electrophoresis on "Pevikon C-870." Segments 18–20 contained the peptidase (HLL), esterase (HLAa, not shown), and bradykininase activities. The source of enzyme was 0·8 g of plasma globulins precipitated with ammonium sulfate. The ordinates show the concentration of protein, the bradykinin activity per mg protein, and the rate of hydrolysis of HLL. The abscissa indicates the segment number.

15–20-fold purification with a 30–45 per cent yield. Owing to the acid sensitivity of the enzyme, the active fraction was rerun in the electrophoresis at a pH not lower than 6·5. The esterase, peptidase, and bradykininase activities migrated again as a single unit. The experiments do not contradict the working hypothesis that the plasma enzyme (carboxypeptidase N) can split the three types of substrates. Although the electrophoresis did not indicate it, human plasma contains a second enzyme that inactivates bradykinin, as suggested above. The subject will be discussed elsewhere.

Level in blood

The hydrolysis of HLAa was measured in the blood serum of 52 healthy individuals of both sexes. The mean initial rate of hydrolysis at 37° in a pH 8, 0·05 M Tris buffer was 3·01 μmole/min per ml serum at a 5×10^{-4} M concentration of substrate. The range of activity of the enzyme was about the same in the blood samples collected from male and female donors.

As observed with HLL[4] or bradykinin,[17] the rate of hydrolysis of HLAa also increased during pregnancy to 4·45 ± 0·048 S.E. μmole/min per ml (Table 3). The difference between the normal group and pregnant women is significant at the $P < 0·01$ level. The seven women in the first trimester had lower values (3·71 μmole/min per ml) than 17 subjects in the second (4·26), or 30 subjects in the third trimester (4·74), indicating a rise in enzyme level during the progress of pregnancy.

TABLE 3. HYDROLYSIS OF HLAA IN HUMAN BLOOD SERA

Donors	Hydrolysis				Inhibition by $1·10^{-6}$ M $CdSO_4$		
	No. of samples	(μmole/min per ml)	± S.E.	P	No. of samples	Percent inhibition	Range
Normal subjects	52	3·01	0·028		12	62	51–72
Pregnant women (total)	54	4·45	0·048	<0·01	16	64	52–75
First trimester	7	3·71	0·46	<0·5			
Second trimester	17	4·26	0·25	<0·01			
Third trimester	30	4·74	0·013	<0·01			

The activity of the enzyme in serum of pregnant women in second and third trimesters was significantly higher than normal.

The inhibition of carboxypeptidase by 1×10^{-6} M $CdSO_4$ was studied in individual samples of blood sera under the conditions of the screening process. In 12 normal, healthy donors, 1×10^{-6} M $CdSO_4$ inhibited 62 per cent on the average (range 51–72), and in 16 pregnant women 64 per cent (range 52–75). Thus $CdSO_4$ inhibited the enzyme to the same extent both in normal sera and sera of pregnant women.

Enzyme in shock

The esterase activity was measured in heparinized dog plasma before and during endotoxin shock. Mongrel dogs received 2 mg Difco *Escherichia coli* endotoxin/kg.[18] The mean rate of hydrolysis of HLAa in 9 untreated dogs was 5·2 μmole/min per ml plasma. In 8 treated dogs the activity of the enzyme decreased by 12 per cent 15 min after the injection of endotoxin, and 19 per cent after 2 hr.

DISCUSSION

Carboxypeptidases that cleave the bond of C-terminal, basic amino acids of peptides have been found in the pancreas,[5] blood plasma,[1–3] urine,[3] lymph,[3] and kidney cortex,[6, 19] and in catheptic spleen preparations.[20] Our interest in this group of enzymes stems from the observation that some of these carboxypeptidases are very potent inactivators *in vitro* and *in vivo*[21–24] of bradykinin and kallidin. The current experiments showed that the carboxypeptidase in blood plasma has an esterase activity as well. The evidence presented indicates that the same enzyme in human blood plasma hydrolyzes HLL, HLAa, and bradykinin. The advantages in using the ester substrate HLAa in preference to HLL are numerous. Depending on the conditions of the assay, the ester is hydrolyzed 2·5–3·9 times faster than HLL. (It was shown previously that HLL is cleaved 3·5 times faster in human plasma than is hippuryl-L-arginine. It follows that the ester substrate is hydrolyzed by the carboxypeptidase at least 8·8 times faster than the corresponding peptide.) Since more diluted serum or

plasma can be used as source of enzyme, the protein background interferes less in the u.v. range. The enzyme does not require preincubation with $CoCl_2$ as measurement of the peptidase activity with HLL does.[3] In the microcuvettes, 0·25 μmole substrate and 5 μl serum are sufficient for one determination, which takes about 5 min with the extended-scale slide wire of the spectrophotometer. Although most of the present experiments were done in a Cary 15 automatic recording u.v. spectrophotometer, less sophisticated equipment can be used for measuring the amount of acid liberated when the ester bond is split. If the carboxypeptidase is indeed identical with carboxypeptidase N,[2] which inactivates bradykinin in human blood plasma, a chemical assay method would be available for measuring the "kininase" in various conditions.

Serum of pregnant women has 48 per cent higher than normal esterase activity, whereas the peptidase activity assayed with HLL increased 39 per cent during pregnancy.[4]

During purification of the enzyme the peptidase, esterase, and kininase activities increased fairly proportionally. Human blood plasma, however, contains a second kininase;[3] the identification of this substance is in progress. Although Pevikon electrophoresis at two pH values could not separate esterase, peptidase, and bradykininase activities, the second kininase could be separated during DEAE-Sephadex chromatography. The existence of this second kininase was first observed after DEAE-cellulose chromatography of plasma globulins.[3] Although it had a relatively low activity, the absolute values in plasma are quite high when, instead of the ammonium sulfate precipitate, another source of plasma globulins is used as the starting material in the purification procedure. One of the products of hydrolysis of the substrate, argininic acid, is an effective inhibitor of the enzyme after a period of preincubation. Argininic acid and arginine inhibit pancreatic carboxypeptidase B as well.[25] Arginine, ε-amino-*n*-caproic acid and a number of structurally related compounds block the hydrolysis of bradykinin by carboxypeptidase N.[24] Although the plasma carboxypeptidase is a metalloenzyme, as suggested by its inhibition by chelating agents, no metal ions were found which increased the activity significantly. Pancreatic carboxypeptidase B requires Zn^{2+} cofactor with hippuryl-L-arginine and cadmium with HLAa.[14] Plasma carboxypeptidase is activated by $CoCl_2$ or by $NiSO_4$ when HLL is the substrate.[3] Possibly the esterase activity of the plasma enzyme depends on the presence of a metal ion other than cobalt.

Among inhibitors, $CdSO_4$ is the most effective. The enzyme in the blood sera of 28 individuals was inhibited by 10^{-6} M $CdSO_4$ to a similar extent (mean, 63 per cent). The lack of complete inhibition of the esterase activity of serum at a relatively high $CdSO_4$ concentration suggests the presence of a Cd^{2+}-resistant factor in blood. This hypothetical factor could be responsible for 10–15 per cent of the activity found.

Inhibition studies also indicated that the characteristics of carboxypeptidase of swine serum resemble those of human serum, but not swine pancreatic carboxypeptidase B. The hydrolysis of HLAa in the swine and human serum is inhibited by cadmium and EDTA, whereas the esterase activity of swine carboxypeptidase B is activated by cadmium;[14] EDTA does not inhibit pancreatic carboxypeptidase B.[5] These facts, added to the differences found in the hydrolysis of HLL by plasma or pancreatic carboxypeptidases,[3] confirm the conclusion that the two enzymes are not identical.

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