Characterization of Leukocyte Enzymes Involved in the Release of Amino Acids in Incubated Blood Cell Lysates*

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The neutral leukocyte proteases involved in the release of free amino acids in incubation mixtures of blood cell lysates and of human serum albumin with leukocyte lysates were characterized by several techniques including 1H nuclear magnetic resonance spectroscopy, electrophoresis, high performance liquid chromatography, gel filtration, amino acid analysis, and NH₂-terminal analysis. The data suggested that the enzymes which contributed significantly to the extensive protein hydrolysis observed were two endopeptidases and three exopeptidases. Identification and analysis of the products obtained in incubation mixtures of human serum albumin with elastase and leucine aminopeptidase were compared with the products obtained in incubation mixtures of the protein with leukocyte lysates.

Classical studies of specific isolated enzyme activities have provided much information about metabolic machinery; however, metabolic maps do not constitute the final picture of metabolism. Understanding the whole demands that individual enzymes are integrated with metabolically related neighbors and with the cellular infrastructure. The construction of more global, and physiologically relevant, models thus requires methodologies with the potential to examine specific enzyme processes in complex, heterogeneous systems. In the preceding study (1) we outlined one such approach, which allowed protease activity to be monitored in incubated blood cell lysates. Similar procedures have been applied now to the characterization of the leukocyte enzymes involved in the proteolysis.

After identifying the resonances observed in the 1H NMR spectrum of incubated blood cell lysates as arising from methyl moieties of free aliphatic amino acids produced as products of putative neutral protease action in leukocytes (1), we investigated the enzyme or enzymes which take part in the hydrolysis. The extensive proteolysis suggested the action of endopeptidases and the fact that considerable amounts of free amino acids were released suggested the involvement of exopeptidases. Another result which needed investigation was the very small number of low molecular weight peptides present after incubations lasting between 15 and 20 h. In the complex mixture of proteins found in hemolysates, it was reasonable to expect that the action of leukocyte proteases would yield a wide gamut of peptides of many different sizes and compositions, but this was not the case. It could be argued that in blood cell lysates erythrocyte proteases would also contribute to the hydrolysis initiated by the leukocyte enzymes, providing thus an explanation for the thorough proteolysis. But since extensive hydrolysis was also observed in preparations containing only leukocyte lysates and large concentrations (180 mg ml⁻¹) of human serum albumin (1), the answer must be found in the properties of the leukocyte enzymes. In the present study we have attempted to establish which leukocyte neutral proteases were the main contributors to the hydrolysis observed in blood cell lysates, and to ascertain whether these enzymes are able to produce the observed proteolysis in the complex mixture of substrates that constitutes a hemolysate.

EXPERIMENTAL PROCEDURES

Materials

Carbonic anhydrase, cytochrome c, human hemoglobin, fatty acid-free human serum albumin, insulin, ovalbumin, elastase (EC 3.4.21.36) type IV, microsomal leucine aminopeptidase (EC 3.4.11.2) type VI-S, cytosolic leucine aminopeptidase (EC 3.4.11.1) type V, and carboxypeptidase A (EC 3.4.12.2) type II were obtained from Sigma. Antipain, chymostatin, elastinol, diithiothreitol, iodoacetamide, leupeptin, phenylmethanesulfonyl fluoride, Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), Nα-tosyl-L-phenylalanine chloromethyl ketone (TPCK), Nα-p-tosyl-L-arginine methyl ester, pepstatin A, phosphoramidon, and bestatin were purchased from Sigma. All other chemicals were analytical reagent-grade.

Methods

Erythrocyte Preparation—Human blood was collected by venipuncture and transferred into tubes containing 3 volumes of physiological saline (0.154 M). Cell suspensions were centrifuged for 10 min at 350 X g, 277 K. The platelet-rich supernatant was decanted, and the cells were resuspended (5 volumes) in physiological saline, and centrifuged under the same conditions. After removing the supernatant, the cells were washed four times with Krebs bicarbonate buffer (2) with antibiotics, pH 7.4, 277 K, 5 volumes, by centrifuging at 3600 X g for 5 min. A final wash was carried out in Krebs buffer constituted in ²H₂O (99.96% ²H; Institute for Nuclear Science and Engineering, Lucas Heights, NSW, Australia) to provide an NMR lock signal for the spectrometer. Blood cell lysates, including theuffy coat, were prepared by twice freezing (78 K) and thawing the samples. Leukocyte-free erythrocyte cell suspensions were obtained by the method of Beutler et al. (3), filtering whole blood through a column of microcrystalline Sigmagel Type 50 and α-cellulose fiber. The filtered cells were washed in Krebs bicarbonate buffer as above. Erythrocyte lysates were made by twice freezing and thawing the cells. White cell lysates were prepared from fresh concentrates supplied by the Blood Bank. Platelets were removed as above, and the buffy coat was collected and washed five times (room temperature, 5 volumes) in

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1 The abbreviations used are: TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; TPCK, Nα-tosyl-L-phenylalanine chloromethyl ketone; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; HPLC, high pressure liquid chromatography; PMN, polymorphonuclear; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
physiological saline with 0.5 mM EDTA and 0.2 mM EGTA. The chelators were removed by twice washing in saline and twice in saline constituted in D₂O. The cells were suspended in a solution of human serum albumin (130 mg ml⁻¹) in saline D₂O and lysed by twice freezing and thawing.

Ultrafiltration—Separation of cells with different molecular weights was carried out by filtration through YM-2, YM-10, YM-30, or XM-50 membranes in an Amicon 8010 (10 ml) ultrafiltration cell.

Nuclear Magnetic Resonance Spectroscopy—For 'H NMR experiments, 0.65-ml samples were dispensed into 5-mm high-precision tubes (Wilmad). Spectra were observed at 400 MHz with a Varian XL-400 spectrometer operated in the Fourier transform mode. Spectral width was 4,800 Hz over 16,384 data points. The 90° pulse was approximately 15 μs long, and the relaxation delay was 3 s. Each spectrum was the average of 320 transients. Chemical shifts were referenced with respect to the resonance of internal dimethyl sulfoxide at 2.5 ppm. Spin-echo spectra were acquired at 310 K with the Hahn pulse sequence (4) with an echo time of 0.060 s. A standard curve was constructed by addition of valine and leucine amino acids to lysates. The integral of the low frequency methyl resonances of the incubated lysates, relative to the integral of the valine and leucine methyl resonances of the reference, was determined and compared with the standards.

Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with the Laemmli modification (5) of the discontinuous buffer system of Ornstein (6) and Davis (7), with a 5% stacking gel and a 12% resolving gel. Samples (5–10 μg of protein) applied in 20 μl of loading buffer (0.05 M Tris, pH 6.8, 2% sodium dodecyl sulfate, 8% glycerol, 0.005% bromphenol blue) were preheated for 5 min at 373 K before electrophoresis. Gels were fixed in 50% methanol, and silver-stained by the method of Wray et al. (8).

Liquid Chromatography—HPLC separations were carried out with an LKB 2150/1/2 instrument (Bromma, Sweden), and CI-10 Milton Roy recording unit (Florida). Samples of 20 μl were injected onto a C-18 reverse-phase column (Lichrosorb, 10 μm, 25 × 0.4-cm, LKB). Separation was achieved by two linear gradients with mobile phases 0.01 M acetic acid, pH 5.2 (A), and acetonitrile made 2% in the same buffer (B). The first step was a 9-min gradient from 1% to 2% B, and the second a 10-min development to 80% B. The flow-rate was 1.0 ml min⁻¹, and the absorbance was monitored at 210 nm with a 5-μl flow-cell and typical absorbance units full scale of 0.16. Amino acid standards and peptides were supplied by Sigma.

Get Filtration Chromatography—Peptides separated by HPLC were lyophilized, resuspended in 0.5 M proponic acid, and loaded onto either a Sephadex G-75 or a Sephadex G-25 Fine (Pharmacia, Uppsala, Sweden) column (88 × 2-cm) equilibrated with 0.8 M proponic acid. The void and total volumes of the column were determined employing Blue Dextran and potassium ferricyanide or tyrosine, respectively. The elution volume of the G-75 column was calibrated with carbonic anhydrase (29 kDa), cytochrome c (12.5 kDa), insulin (5.7 kDa), and tyrosine. The G-25 column was calibrated with insulin (5.7 kDa), reduced insulin B-chain (2.9 kDa), the heptadecapeptide FKLGGDRSR (1.9 kDa), and the nonapeptide FKLGGDGR (1.05 kDa).

Amino Acid Analysis—A Waters Pico-Tag (Ryde, N. S. W.) system was employed to determine the amino acid composition of samples which had been derivatized with phenyl isothiocyanate.

Leukocyte Count—Leukocyte counts were estimated electronically using a Coulter Counter (model ZF, Dunstable, United Kingdom). Three drops of Zaponin "Stromatolysing Agent" were added to 10 ml of the diluted sample (1 in 250 or 1 in 500 with Isoton diluent), and after repeated inversion the sample was counted in triplicate. Duplicate dilutions of each sample were counted, and coincidence correction was not used due to the low counts.

Concentration Measurements—Total concentrations of solutes were determined by measuring the osmolality of solutions with a Wescor vapor osmometer (Logan, Utah). 

NH₂-terminal Residue Analysis—Peptides were derivatized with dansyl chloride by the De Jong et al. (9) adaptation of the method of Tapuh et al. (10). Peptides were then hydrolyzed for 24 h at 383 K in 6.0 M HCl prior to HPLC. The samples were dissolved in 0.01 M acetic acid, pH 5.0, and applied to a reverse-phase C₈ column (μBondapak, 25 × 0.4-cm) which was developed essentially as described by Weiner and Tishbee (11). The elution of dansyl-amino acids was detected by monitoring the absorbance at 330 nm, and identification was made by comparison with dansyl-amino acid standards (Sigma).

RESULTS

An incubation mixture was prepared by adding white cell lysate (3.2 × 10⁶ cells ml⁻¹) to a red cell lysate (hematocrit 0.85), and adjusting the osmolality of the mixture of 350 mOsm kg⁻¹. Chemical species were separated by size by filtering aliquots of the mixture through membranes with molecular weight cutoffs ranging from 2 × 10³ to 300 × 10³ nm. Separations were carried out at 277 K. Under these conditions the amount of hemoglobin that appeared in the filtrates was very small, less than 2% in the fraction separated with the largest membrane pore. The retentate and the ultrafiltrate obtained in each separation were incubated for 20 h at 310 K. The proteolytic activity was determined by measuring in the 1H NMR spin-echo spectrum of each fraction the integral of the methyl resonances of valine and leucine formed, relative to the integral of the internal dimethyl sulfoxide resonance. No free valine or leucine were detected in the incubated ultrafiltered fractions. The relative amount of product formed in the retentate fractions plotted as a function of membrane pore size is shown in Fig. 1. Full activity was observed after remixing the fractions separated by either the 100 × 10³ or 300 × 10³ Mₐ cutoff membrane. Assuming that hemoglobin is the main substrate in the proteolytic reactions in which free valine and leucine are released, the 20–25% decrease in product formed in the retentate fractions obtained by filtration through membranes of Mₐ cutoff larger than 50 × 10³, was interpreted as the result of separating one or several of the active proteases from the retentate mixture containing the substrate hemoglobin.

The effect of divalent cations on the enzymic action of the proteases was investigated by comparing the amounts of product formed in incubations carried out in the presence of different cations. Two stock incubation mixtures were prepared. One contained a solution of human serum albumin (180 mg ml⁻¹) and white cell lysate (9.5 × 10⁶ cells ml⁻¹), and the other was made of a blood cell lysate, without platelets, with 6.5 × 10⁶ white cells ml⁻¹. Before lysis, the cells used in these two preparations had been washed six times in physiological saline containing 0.5 mM EDTA and 0.2 mM EGTA to remove extracellular divalent cations. Both chelators were in turn removed by washing the cell suspensions five times in

FIG. 1. Proteolytic activity of retenates separated through membranes with different pore sizes. Preparations were incubated for 20 h at 310 K. Activities were determined by measuring the integral of the methyl resonances of the valine and leucine formed relative to the integral of an internal dimethyl sulfoxide resonance. The amount of product formed is given relative to the control preparation which was not filtered.
physiological saline. Aliquots of each mixture with 2 mM of the chloride salt of each divalent cation were incubated for 18 h at 310 K. Fig. 2 shows the $^1$H NMR spin-echo spectra of the incubated blood cell lysate used as control in these experiments and of lysates incubated in preparations containing MgCl$_2$ and CaCl$_2$. The resonances corresponding to the methyl moieties of alanine, threonine, valine, and leucine released as products are indicated on the figure. The product concentration in each incubation mixture was normalized by dividing the integral of the corresponding resonance by the integral of an internal dimethyl sulfoxide resonance. Typical product concentrations, obtained in four measurements, of free alanine, threonine, and valine and leucine, relative to that of a control preparation to which no divalent cation was added, are shown in Fig. 3.

The “alkaline earths” do not appear to have much effect in the formation of proteolysis products in the incubations of leukocytes with human serum albumin; only the addition of Mg$^{2+}$ produced a 15% increase in activity. In the incubations of blood cell lysates, on the other hand, the presence of Ca$^{2+}$ stimulates the production of free alanine, and the presence of Mg$^{2+}$ stimulates the production of threonine, valine, and leucine. The presence of Fe$^{3+}$, Co$^{2+}$, Ni$^{2+}$, and Cu$^{2+}$ generally had an inhibitory effect. The strongest inhibitions were that of copper in incubation of leukocytes with human serum albumin and that of iron in the whole-blood cell lysate preparation. The presence of Zn$^{2+}$ had opposite effects on either type of preparation; it produced a medium degree of inhibition of proteolysis in the white cell/human serum albumin incubation and a strong stimulation of the proteases in the blood cell lysates. Cd$^{2+}$ and Hg$^{2+}$ showed various degrees of protease inhibition in both types of preparations, with greater inhibition in leukocyte/human serum albumin preparations than in those of blood cell lysates. Sn$^{2+}$ and Pb$^{2+}$ showed a small and a medium degree of proteolysis inhibition, respectively, in leukocyte lysates incubated with human serum albumin.

The specificity of low molecular weight protease inhibitors was employed as an aid to identify the enzymes involved in the release of free amino acids from polypeptides. Preparations of leukocyte lysates (9.5 x 10$^6$ cells ml$^{-1}$) with human serum albumin (130 mg ml$^{-1}$) were incubated with synthetic inhibitors (0.5 mM) for 18 h at 310 K. The effect of the inhibitors was quantified by measuring the amount of valine and leucine released relative to a control sample with no inhibitor present. Typical results obtained in four different measurements are given in Fig. 4, where the inhibitors have been grouped into four categories. The first three correspond to inhibitors of endopeptidases with specificity for thiol, carboxyl, and metallo proteinases, respectively (12). Bestatin, an inhibitor of exopeptidases, forms the fourth category (12). The strongest inhibition was effected by phenylmethanesulfonyl fluoride and by chymosin, which reduced the release of amino acids by more than 50%. The amount of product formed in the presence of either EDTA or bestatin was about 70% of the product formed in the control preparation. Elastinal decreased proteolysis by about 20%.

The decrease in activity observed in the presence of inhibitors specific for endopeptidases and for exopeptidases provided further evidence that the release of free amino acids was catalyzed by several proteases. We concluded in the previous study (1) that the neutral enzymes involved in proteolysis are localized in the white cell membrane and/or were released into the extracellular medium. This conclusion was explored further by measuring the release of amino acids in incubations of human serum albumin with intact and lysed leukocytes. The results, given in Table I, indicate a greater extent of hydrolysis in incubations with lysates than with intact cells.

Pancreatic elastase was incubated with human serum albumin and with hemolysates to obtain information on the products released from these substrates by the endopeptidase. Data on the products formed from the same substrates by exopeptidases were obtained from incubations with kidney microsomal and cytosolic leucine aminopeptidases. Fig. 5 shows the $^1$H NMR spin-echo spectra of incubation mixtures of human serum albumin or hemolysates with added microsomal leucine aminopeptidase, with elastase, and with both enzymes together. In the incubations with human serum albumin, leucine aminopeptidase released almost no products. The proteolytic action of elastase yielded more products, mostly in the form of polypeptides, characterized by broad resonances in the $^1$H NMR spectrum. Narrow spectral lines with low intensity observed at chemical shifts corresponding to free amino acids suggested that small amounts of these molecules were also released as products of elastase. Larger quantities of free amino acids appeared as products in preparations containing both enzymes, but the broader resonances...
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**FIG. 3.** Relative concentration of alanine, threonine, and leucine and valine formed in incubations with different divalent cations. Incubation mixtures were prepared by adding 2 mM of the chloride salt of the divalent cation to a solution of human serum albumin (180 mg ml⁻¹) and leukocyte lysates (9.5 × 10⁶ cells ml⁻¹) (vertical hatching) or to blood cell lysates without platelets (6.5 × 10⁶ cells ml⁻¹) (inclined hatching). Samples were incubated for 18 h at 310 K. The amino acid concentration in the products is given relative to the concentrations in a control sample with no divalent cation. Product concentrations in each incubated sample were measured from the resonances in the ¹H NMR spin-echo spectrum employing the same method as for Fig. 1.

**FIG. 4.** Relative amounts of valine and leucine released in incubations of human serum albumin and leukocyte lysates with low molecular weight protease inhibitors. Preparations containing 130 mg ml⁻¹ of human serum albumin and white cell lysates (9.5 × 10⁶ cells ml⁻¹), with 0.5 mM of inhibitor, were incubated at 310 K for 18 h. The effect of each inhibitor was quantified from the ¹H NMR spin-echo spectrum as for Fig. 1. Results are given relative to a control sample containing no inhibitor. The first group in the figure corresponds to inhibitors of thiol endopeptidases. Pepstatin A is an inhibitor of carboxyl proteinases; and EDTA and phosphoramidon are inhibitors of metallo endopeptidases. Bestatin is an inhibitor of leucyl aminopeptidase and of other exopeptidases.

Inhibitor | Product Formed (%)
--- | ---
Control | ![Control Product Formed](image1)
Antipain | ![Antipain Product Formed](image2)
Chymostatin | ![Chymostatin Product Formed](image3)
Elastin | ![Elastin Product Formed](image4)
Dithiothreitol | ![Dithiothreitol Product Formed](image5)
Iodoacetamide | ![Iodoacetamide Product Formed](image6)
Leupeptin | ![Leupeptin Product Formed](image7)
PMSF | ![PMSF Product Formed](image8)
TLCK | ![TLCK Product Formed](image9)
TPCK | ![TPCK Product Formed](image10)
Pepstatin A | ![Pepstatin A Product Formed](image11)
EDTA | ![EDTA Product Formed](image12)
Phosphoramidon | ![Phosphoramidon Product Formed](image13)
Bestatin | ![Bestatin Product Formed](image14)

Also observed at slightly different chemical shifts in the NMR spectrum suggested the presence of polypeptides. Amino acids were released in incubations of hemolysates with either leucine aminopeptidase, elastase, or with both enzymes together. The quantitative results of the incubations with hemolysates are given in Table II. The ¹H NMR spin-echo spectrum of the preparation containing leucyl aminopeptidase showed only spectral lines at the chemical shifts corresponding to amino acids, suggesting that the proteins from which the amino acids had been cleaved remained otherwise largely intact. The spectrum of the hemolysate incubated with elastase showed a number of overlapping resonances with linewidths and chemical shifts corresponding to free amino acids and to residues in polypeptides, indicating a more extensive proteolysis which had resulted in cleavage of protein chains into smaller polypeptides, as well as the release of larger amounts of free amino acids than in the presence of only leucine aminopeptidase. In hemolysates incubated with both enzymes, proteins were thoroughly hydrolyzed with the products almost completely in the form of free amino acids. Perusal of the amount of amino acids released in the control hemolysate incubation (Table II) indicated that erythrocyte proteases did not initiate proteolytic processes in these preparations. On the other hand, the difference between the spectra of preparations with human serum albumin and with hemolysates in each incubation could be ascribed to the action of red cell proteases.

The combined effect of endo- and exopeptidases and inhibitors was investigated by measuring the amount of various products released in incubations with human serum albumin (130 mg ml⁻¹). The results after 20 h at 310 K are given in Table III. The amount of amino acids released was measured from the ¹H NMR spin-echo spectra of the incubated samples as the ratio of the integral of the peaks of the methyl moieties of alanine, threonine, and valine and leucine, to the integral of the internal dimethyl sulfoxide resonance. Chymostatin...
TABLE I

Relative amounts of product formed (+5%) in incubation of human serum albumin (130 mg ml⁻¹) with intact and lysed leukocyte suspensions (40 × 10⁶ cells ml⁻¹) and of red cell lysate (hematocrit 0.85) with intact and lysed buffy coat suspensions (6.5 × 10⁶ cells ml⁻¹)

Buffy coats did not include platelets. Cell suspensions were lysed by twice freezing at 78 K and thawing. Samples were incubated for 15 h at 310 K. The amount of amino acids released was measured as the ratio of the integral of the resonance of the methyl moieties of alanine, threonine, and valine and leucine, to the integral of the internal dimethyl sulfoxide resonance. The values were normalized with respect to the number of cells in each suspension before lysis.

| Sample                  | % Product formed |
|-------------------------|------------------|
|                        | Substrate       | Cells | Treatment | Alanine | Threonine | Valine and leucine |
| Human serum albumin     | Leukocytes       | Intact |           | 17      | 10        | 40                 |
|                         |                  | Lysed  |           | 20      | 27        | 92                 |
| Red cell lysate         | Buffy coat       | Intact |           | 2       | 23        | 82                 |
|                         |                  | Lysed  |           | 9       | 27        | 100                |

Fig. 5. Aliphatic region of the ¹H NMR spin-echo spectra of samples incubated with different enzymes. Preparations contained either 130 mg ml⁻¹ of human serum albumin, or red blood cell lysates (hematocrit 0.85). The enzymes added to the incubation mixtures were microsomal leucine aminopeptidase (0.6 mg), A and D; pancreatic pig elastase (0.4 mg), B and E; and the same quantities of each enzyme added together, C and F. Solutions were adjusted to an osmolality of 350 mOsm kg⁻¹ and were incubated for 20 h at 310 K. Peak assignments of free amino acids are the same as for Fig. 2.

and N°-tosyl-L-arginine methyl ester produced between 74 and 86% inhibition of elastase. The release of free amino acids in preparations containing elastase and leucyl aminopeptidase was reduced to 11–25% in incubations with elastase, and to 41–51% in incubations with bestatin. The presence of both inhibitors together reduced the amount of products formed to 5–12% of the amount released in control preparations.

Several techniques were employed to characterize more completely the size and composition of the proteolysis products obtained in these incubations. Each sample was filtered through a membrane that retained molecular species over 50 kDa. The ultrafiltrates were analyzed in 12% Laemmlí sodium dodecyl sulfate gels. The products, polypeptides, and amino acids, had Mₐ of 10 × 10³ and less. Information about the size of the peptides resulting from proteolysis was obtained by HPLC. The chromatograms of ultrafiltrates obtained by separation through a YM-30 membrane from incubations of human serum albumin with elastase and with elastase and microsomal leucine aminopeptidase are shown in Fig 6 (top); the elution profiles of the products from both incubations are different. The chromatogram of the products of incubations with elastase and microsomal leucine aminopeptidase showed similarities with the chromatogram of incubation products of human serum albumin with white cell lysates (Fig. 5, Ref. 1). The elution profiles of these preparations showed two groups of peaks, one eluting before 8 min and the other between 12 and 17 min. Free amino acids were eluted in the first group


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Hemolysates were prepared by twice freeze-thawing erythrocyte suspensions of 0.85 hematocrit. No enzyme was added to the control sample; the following amounts of enzymes were added to the other preparations: 30 µl carboxypeptidase A (1250 units ml⁻¹), 0.57 mg ml⁻¹ porcine pancreatic elastase (80 units mg⁻¹); 0.86 mg ml⁻¹ microsomal leucine aminopeptidase (9 units mg⁻¹); 0.86 mg ml⁻¹ cytosolic leucine aminopeptidase (90 units mg⁻¹). Samples were incubated for 20 h at 310 K. The amount of amino acids released was measured as the ratio of the integral of the resonance of the methyl moieties of alanine, threonine, and valine and leucine, to the integral of the dimethyl sulfoxide resonance.

Further characterization of the peptides eluted under peaks O₁, O₂, and O₃, are given in Table IV.

Further characterization of the peptides eluted in the HPLC peaks was carried out by gel filtration. The elution profiles of peak O₁, and of peaks O₁ and O₂ are shown in Fig. 6 (bottom). Separation through a Sephadex G-25 column resolved peak O₁ into several main components of molecular masses 5.3, 4.9, 3.7, and 2.05 kDa. The size of the peptide eluted about fraction 54, 3.7 kDa, is similar to the size of the largest peptide fragment obtained in incubations with white cell lysates (Fig. 5, Ref. 1). The separation of peaks O₁ and O₂, resulted in peptides of molecular masses 5.9, 5.1, 4.6, 4.1, and 3.5 kDa. The Mₛ of the peptide eluting about fraction number 60, 3.5 \times 10⁶, is similar to the molecular weight of one of the peptides obtained in the separation of peak O₁, a product of the incubation of human serum albumin with white cell lysates. A final characterization of these peptides was made by NH₂-terminal analysis of each polypeptide. The results of the analyses of peptides produced in incubations of human serum albumin with white cell lysates, with elastase, and with elastase and leucine aminopeptidase are shown in Table V. They indicated that peptides obtained in fractions 49–52 and 55–62 in incubations with white cell lysates have the same NH₂-terminal residue as peptides obtained in fractions 48–60 in incubations with elastase, and in fractions 55–64 in incubations with elastase and leucine aminopeptidase, respectively.

DISCUSSION

The methyl resonances observed in the ¹H NMR spectra of incubated blood cell lysates and in preparations of intact lysed leukocytes with human serum albumin were identified as arising from products of leukocyte neutral proteases (1). The extensive hydrolysis observed and the variety of amino acids released suggested that several enzymes take part in the proteolysis. The data obtained for retentates separated from blood cell lysates (Fig. 1) indicated that proteases of at least two different sizes participated in the release of amino acids in the incubated samples; one size appeared to be in the range 20–40 kDa and the other over 100 kDa. Endopeptidases in erythrocytes and leukocytes usually have a size range between 25 and 65 kDa (13), whereas exopeptidases are much more complex proteins consisting of several subunits with sizes

| Sample | Enzyme(s) | Alanine (arbitrary units) | Threonine (arbitrary units) | Valine and leucine (arbitrary units) |
|--------|-----------|--------------------------|----------------------------|-----------------------------------|
| Hemolysate Control | 11 | 29 | 130 |
| Carboxypeptidase A | 4 | 8 | 38 |
| Microsomal leucine aminopeptidase | 62 | 62 | 314 |
| Elastase Control | 11 | 7 | 73 |
| Chymostatin | 11 | 32 | 146 |
| TAME | 32 | 3 | 33 |
| Elastase and microsomal leucine aminopeptidase | 30 | 28 | 139 |
| Elastase and bestatin | 6 | 4 | 39 |
| TAME | 58 | 58 | 276 |
| Elastase and cytosolic leucine aminopeptidase | 70 | 68 | 328 |
| Elastinal | 17 | 9 | 72 |
| Bestatin | 6 | 4 | 39 |
| TAME | 58 | 58 | 276 |

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**Table II**

Amount of amino acids released in incubations of hemolysates with endo- and exopeptidases

| Sample | Product formed (arbitrary units) |
|--------|---------------------------------|
| Hemolysate Control | 130 |
| Carboxypeptidase A | 21 |
| Microsomal leucine aminopeptidase | 38 |
| Elastase Control | 139 |
| Chymostatin | 39 |
| TAME | 276 |
| Elastase and microsomal leucine aminopeptidase | 328 |
| Elastinal | 72 |
| Bestatin | 39 |
| TAME | 276 |
| Elastase and cytosolic leucine aminopeptidase | 314 |
| Elastinal | 73 |
| Bestatin | 146 |
| Elastase and bestatin | 33 |

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**Table III**

Amount of amino acids released in incubations of human serum albumin (130 mg ml⁻¹) with endo- and exopeptidases, and with inhibitors

No enzyme or inhibitor was added to the control sample. Enzyme amounts and inhibitor concentrations were the same as for Table II; inhibitor concentrations were 0.5 mM. Samples were incubated for 20 h at 310 K. Relative amounts of amino acids were measured as for Table II. In the spectra of these incubations there was a substantial overlap between resonances arising from amino acids and from peptide residues; consequently the errors in determining the content of free amino acids were as high as 40%. TAME, N°-p-tosyl-L-arginine methyl ester.

| Sample | Enzyme(s) | Inhibitor(s) | Alanine (arbitrary units) | Threonine (arbitrary units) | Valine and leucine (arbitrary units) |
|--------|-----------|-------------|--------------------------|----------------------------|-----------------------------------|
| Elastase Control | 11 | 29 | 130 |
| Chymostatin | 4 | 8 | 38 |
| TAME | 58 | 58 | 276 |
| Elastase and microsomal leucine aminopeptidase | 70 | 68 | 328 |
| Elastinal | 17 | 9 | 72 |
| Bestatin | 6 | 4 | 39 |
| TAME | 58 | 58 | 276 |
| Elastase and cytosolic leucine aminopeptidase | 62 | 62 | 314 |
| Elastinal | 11 | 7 | 73 |
| Bestatin | 32 | 32 | 146 |
| Elastase and bestatin | 4 | 3 | 33 |

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of peaks (Fig. 5, Ref. 1). In the second group the two major peaks, O₁ and O₂, corresponding to peptides produced in the incubation, appeared at elution times close to those of the two major peaks, O₁ and O₂, observed in the incubation of human serum albumin with elastase and leucine aminopeptidase. The amino acid compositions of the peptides eluted under peaks O₁, O₂, and O₃, are given in Table IV.
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Fig. 6. Top: high performance liquid chromatography of ultrafiltrates separated from human serum albumin solutions incubated with pig pancreatic elastase or pig pancreatic elastase and microsomal leucine aminopeptidase. The filtrates were obtained by separation through an Amicon YM-30 membrane. Incubation conditions were the same as for Fig. 5. The amino acid compositions of the peaks labeled O₁, O₂, and O₃ are given in Table IV. Bottom, elution profiles of peaks O₁, O₂, and O₃ obtained by gel chromatography. The Sephadex G-75 (fine) column was equilibrated with 0.8 M propionic acid. The void and total volumes were determined by running Blue Dextran and tyrosine, respectively. The column was calibrated with the following standards: nonapeptide (1.05 kDa); heptadecapeptide (1.9 kDa); reduced insulin B-chain (2.9 kDa); and insulin (5.7 kDa). Left, elution profile of peak O₃; right, elution profile of peaks O₁ and O₂.

Elastase and Leucine Aminopeptidase

Elastase, cathepsin G, and PMN-chymotrypsin are proteases able to digest a wide range of proteins, whereas the action of the other endopeptidases is directed to specific proteins like casein, fibrin, proteoglycans, collagen, gelatin, and elastin. It was shown in the previous study (1) that the leukocyte proteases involved in the release of free aliphatic amino acids in incubations of blood cell lysates also hydrolyzed other proteins like concanavalin A, cytochrome c, hemoglobin, human serum albumin, and lysozyme. Thus, it is unlikely that any of the more specific white cell neutral endopeptidases contribute significantly to the release of free amino acids in incubations with red blood cell lysates or with human serum albumin. In the group of endopeptidases with broad specificity, leukocyte elastase digests elastin and proteins generally, and it hydro-

over 150 kDa (14). The principal neutral endopeptidases found in polymorphonuclear leukocytes are neutrophil elastase, cathepsin G, polymorphonuclear (PMN) chymotrypsin, and neutrophil proteinases 3 and 4, located in the azurophil granules (15-17), neutrophil gelatinase and neutrophil collagenase, located in the specific granules (15), and neutrophil proteinase 5, located in the cell membrane (18). In monocytes the main neutral endopeptidase is macrophage elastase (19). Neutral exopeptidases found in leukocytes include leucine aminopeptidase (20), soluble arginine aminopeptidase (21), microsomal alanine aminopeptidase (22), and cytoplasmic proline dipeptidase (23).

Leukocyte neutral endopeptidases can be grouped into two types according to the range of substrates that they hydrolyze.
and hydrolyzes a number of proteins including hemoglobin, by incubating different enzymes with human serum albumin or with red blood cell lysates for 20 h at 310 K. The ultrafiltrates were analyzed by HPLC, and the isolated peaks were lyophilized prior to gas-phase hydrolysis in 6 M HCl at 383 K for 24 h. No correction was made for the participation of serine proteases in the hydrolysis. The compositions of peptides in peaks 0, 0, and 0 are given in parentheses. The values given in parentheses are the rounded figures.

### Table IV

| Amino acid | Elastase | Microsomal leucine aminopeptidase and elastase |
|------------|----------|---------------------------------------------|
|            | Peak 0   | Peak 0                                      | Peak 0                                      |
| Asx        | 2.1 (2)  | 2.7 (3)                                     | 3.4 (3)                                    |
| Glx        | 2.7 (3)  | 3.8 (4)                                     | 6.1 (6)                                    |
| Ser        | 0.7 (1)  | 3.1 (3)                                     | 2.2 (2)                                    |
| Gly        | 0.8 (1)  | 9.6 (10)                                    | 2.6 (3)                                    |
| His        | 0.3 (0)  | 0.0 (0)                                     | 0.8 (1)                                    |
| Arg        | 0.6 (1)  | 2.6 (3)                                     | 1.5 (1)                                    |
| Thr        | 2.6 (5)  | 1.2 (2)                                     | 2.1 (2)                                    |
| Ala        | 2.1 (2)  | 0.9 (1)                                     | 5.3 (5)                                    |
| Pro        | 1.0 (1)  | 4.0 (4)                                     | 3.6 (4)                                    |
| Tyr        | <0.2 (0) | 0.0 (0)                                     | <0.2 (0)                                   |
| Val        | 1.2 (1)  | 3.6 (4)                                     | 3.2 (3)                                    |
| Met        | 0.0 (0)  | 0.0 (0)                                     | 0.0 (0)                                    |
| Cys(OH)    | 0.9 (1)  | 1.8 (2)                                     | 0.8 (1)                                    |
| Ile        | 2.4 (2)  | 0.0 (0)                                     | 2.9 (3)                                    |
| Leu        | 0.6 (1)  | 5.7 (6)                                     | 0.5 (0)                                    |
| Phe        | 0.3 (0)  | 2.1 (2)                                     | 1.4 (1)                                    |
| Lys        | 1.4 (1)  | 1.6 (2)                                     | 6.2 (6)                                    |

### Table V

NH₂-terminal residues of peptides obtained by gel filtration from incubated samples of human serum albumin

Samples contained 130 mg ml⁻¹ of human serum albumin as substrate. Enzymes or enzyme mixtures were added as leukocyte lysates (9.5 × 10⁶ cells ml⁻¹), elastase (0.57 mg ml⁻¹, 80 units mg⁻¹), or elastase and microsomal leucyl aminopeptidase (0.86 mg ml⁻¹, 9 units mg⁻¹). Preparations were incubated for 20 h at 310 K. The larger peptides were separated from other incubation products by HPLC. The corresponding elution peaks were further resolved by gel chromatography on a Sephadex G-25 fine column. NH₂-terminal analysis was carried out on the different fractions obtained by gel chromatography.

| Fraction no. | M₄ | Leukocyte lysate | Elastase | Microsomal leucine aminopeptidase and elastase |
|--------------|----|-----------------|----------|---------------------------------------------|
| 28-32        | 5900 | S               |          | G                                           |
| 31-37        | 5300 |                 |          |                                             |
| 33-41        | 5100 |                 |          |                                             |
| 38-47        | 4900 | C               |          |                                             |
| 42-47        | 4600 |                 |          | K                                           |
| 48-64        | 4100 |                 |          |                                             |
| 48-60        | 3700 | A               |          |                                             |
| 49-53        | 3700 | A               |          |                                             |
| 55-62        | 3600 | S               |          |                                             |
| 55-64        | 3500 |                 |          |                                             |
| 61-75        | 2950 | S               |          |                                             |
| 82-87        | 2050 |                 |          |                                             |

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The compositions of peptides in peaks 0, 0, and 0 are given as the molar ratios of residues normalized to either cysteine content, measured as cysteic acid, or relative to Asx. Samples were prepared by incubating different enzymes with human serum albumin or with red blood cell lysates for 20 h at 310 K, and were filtered through Amicon YM-30 membranes. The ultrafiltrates were analyzed by HPLC, and the isolated peaks were lyophilized prior to gas-phase hydrolysis in 6 M HCl at 383 K for 24 h. No correction was made for the participation of serine proteases in the hydrolysis. The compositions of peptides in peaks 0, 0, and 0 are given in parentheses. The values given in parentheses are the rounded figures.

Leukocyte elastase has been shown to have enhanced activity at ionic strengths up to 1.0 M NaCl. Its activity is stimulated by MgCl₂, it is not affected by CaCl₂, and is inhibited by ZnSO₄ (27). The activity of cathepsin G is not greatly affected by ionic strength, is mildly enhanced by MgCl₂, is slightly inhibited by CaCl₂, and is unaffected by ZnSO₄ (28). The enzymatic activity of PMN-chymotrypsin is dependent on ionic strength. In incubations with cations it was observed that Ca²⁺ ions produced a small activation of the enzyme, and Cu²⁺ and Zn²⁺ strongly inhibited it (16). The properties of leukocyte elastase and of PMN-chymotrypsin, but not those of cathepsin G, agree with the observed dependence on ionic strength (1) and with the data for incubations with human serum albumin in the presence of different divalent cations given in Fig. 3.

The low molecular weight inhibitors antipain and leupeptin did not have any effect on the amount of product formed (Fig. 4). Since they inhibit plasmin, trypsin, and cathepsin B (22), which are not found in leukocytes, these results were in agreement with the general conclusion that the proteases involved in the release of amino acids observed in the ¹H NMR spectrum of incubated blood cells are located in leukocytes. Similarly, the very small inhibitor observed in incubations with peptatin, a specific inhibitor of some acid proteases (29), is consistent with the conclusion that the enzymes are neutral proteases. The strong inhibition observed in the presence of phenylmethanesulfonyl fluoride (Fig. 4) indicated the participation of serine proteases in the hydrolysis. The lack of inhibition in the incubation mixtures with the haloalkyl ketones of lysine (TLCK), and of phenylalanine (TPCK) helped to elucidate which endopeptidases have a significant role in the proteolysis. Cathepsin G is inhibited by both TLCK and TPCK (26). Elastase is inhibited by TLCK but not by TPCK (27), and PMN-chymotrypsin is inhibited by TPCK but not TLCK (16). Consequently, the data suggested that there was no significant contribution by cathepsin G, and the results were consistent with the presence of both neutrophil elastase and PMN-chymotrypsin. The proteolytic action of a chymotrypsin-like enzyme was also supported by the strong effect observed in the presence of the inhibitor chymostatin. The loss of activity measured in the presence of EDTA did not correspond to the catalytic properties of either protease. However, a strong inhibition of PMN-chymotrypsin has been reported when Ca²⁺ and EDTA are both present in the incubation medium (16). Although EDTA inhibits many metalloenzymes, and a collagenolytic metalloproteinase is found in the specific granules of human polymorphonuclear leukocytes (19), it is unlikely that this enzyme is a major contributor to the release of amino acids observed in this work because the presence of phosphoramidon, a metalloendopeptidase inhibitor (28), did not affect the amount of products formed. The small degree of inhibition by elastatin indicated the presence of leukocyte elastase. Finally, the lack of inhibition observed in incubations with iodoacetamide and with dithiothreitol was consistent with the proteolytic activity of PMN-chymotrypsin (16).

Leucine aminopeptidase is activated by Mg²⁺ (30), but Ca²⁺ and Mg²⁺ have little effect on the activity of the other common exopeptidases present in white cells. Thus, the increase in product formation observed in preparations incubated with 2 mM Mg²⁺ (Figs. 2 and 3) supported the conclusion that leucine
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aminopeptidase was one of the enzymes catalyzing the release of free amino acids. Soluble arginyl aminopeptidase and proline dipeptidase are inhibited by Cu²⁺, Zn²⁺, Cd²⁺, and Hg²⁺ (31, 32). Since proteolysis was reduced in incubations with these ions, the data in Fig. 3 are consistent with the presence of both enzymes. Moreover, soluble arginine aminopeptidase was suggested by the inhibition measured in incubations with Co²⁺, which inhibits this enzyme even at very low concentrations (33). Thus, the data from incubations with divalent cations provided evidence for the involvements of the four common leukocyte exopeptidases.

EDTA inhibits soluble arginine aminopeptidase, microsomal alanine aminopeptidase, and proline dipeptidase (31-33) but does not inhibit native (Zn-containing) leucine aminopeptidase (25). The native enzyme is activated and stabilized by Mg²⁺ (2 mM), and the Mg²⁺-activated leucine aminopeptidase is inhibited by EDTA. The incubations with EDTA were carried out with preparations in which leucine aminopeptidase was in the Mg²⁺-activated form. Bestatin does not affect proline dipeptidase (32) but is a potent inhibitor of the other three aminopeptidases (30-33). Hence, the data obtained from incubation mixtures with low molecular weight inhibitors supported the presence of three exopeptidases which would preferentially release NH₂-terminal valine, threonine, and arginine residues, but which are also capable of cleaving NH₂-terminal glycine, threonine, alanine, and tyrosine residues.

Information on the products released by the action of endopeptidases was obtained from data of incubation mixtures of porcine pancreatic elastase and/or porcine kidney leucine aminopeptidases with either human serum albumin or hemolysate. Considering that there are important catalytic differences between the porcine and the human enzymes, it was necessary in the first place to establish whether this modeling of the reactions taking place in blood cell lysates would yield results of interest. The 1H NMR spin-echo spectra measured from incubation mixtures of hemolysates with microsomal leucine aminopeptidase, with elastase, and with both enzymes together are shown in Fig. 5. The spectrum of the sample containing the hemolysate and both enzymes (Fig. 5, F) is qualitatively very similar to the spectrum measured in blood cell lysates (Fig. 2) containing hemolysate and leukocyte lysate. Since it was shown that erythrocyte prostates were not able to initiate the reactions observed in hemolysates containing leukocytes, the results of the incubation of hemolysate with elastase and microsomal leucine aminopeptidase demonstrated that the presence of these enzymes was sufficient to initiate proteolysis in human cells.

The amino acid analyses of each of the major peptide peaks (O₁, O₂, and O₃) obtained in the chromatograms of both incubations are given in Table IV. Comparison of the compositions of the peptides in peaks O₁ and O₃ obtained in incubations with leukocyte lysates (Table II, Ref. 1), with the composition of the peptides in peaks O₁ and O₂ obtained in incubations with elastase and leucine aminopeptidase (Table IV), revealed a remarkable similarity; only the content of glycine, threonine, alanine, isoleucine, and leucine was significantly different. A final characterization of these peptides was made by determining the first residue in each peptide by NH₂-terminal analysis (Table V).

The most important result was that in the incubations with elastase and leucine aminopeptidase only five different peptides were identified with molecular masses between 3.5 and 5.9 kDa, one of which had the same size and NH₂-terminal residue as a peptide obtained in incubation with leukocyte lysates. These comparisons suggested that, in all probability, the free amino acids and the limited number of peptides detected in incubations of leukocytes with human serum albumin and with hemolysates resulted from an extensive proteolysis by more than one endopeptidase and one exopeptidase; the endopeptidases were most probably neutrophil elastase and PMN-chymotrypsin, and the exopeptidases were most probably leucine aminopeptidase, soluble arginyl aminopeptidase, and microsomal alanine aminopeptidase. These conclusions are consistent with the data presented and with the interpretation of results that has been followed. However, it should be made clear that considering the com-
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Complexity of the systems studied, and the qualitative nature of some of the data presented, the interpretation given is probably not unique, and the participation of other enzymes in the proteolysis has not been ruled out.

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REFERENCES

1. Mendz, G. L., McCall, M. N., and Kuchel, P. W. (1989) J. Biol. Chem. 264, 2100–2107
2. Krebs, H. A., and Henseleit, K. (1932) Z. Physiol. Chem. 210, 33–37
3. Beutler, E., Kuhl, W., Matsumoto, D., and Pangolis, G. (1976) J. Exp. Med. 143, 975–980
4. Hahn, E. L. (1950) Phys. Rev. 80, 580–594
5. Laemmli, U. K. (1970) Nature 227, 680–685
6. Ornstein, L. (1964) Ann. N. Y. Acad. Sci. 121, 321–349
7. Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427
8. Wray, W., Bouliakas, T., Wray, V. P., and Hancock, R. (1981) Anal. Biochem. 118, 197–203
9. De Jong, C., Hughes, G. J., van Wieringen, E., and Wilson, K. J. (1982) J. Chromatogr. 241, 345–359
10. Tapuh, T., Schmidt, D. E., Linder, W., and Karger, B. L. (1981) Anal. Biochem. 115, 123–129
11. Weiner, S., and Tishbee, A. (1981) J. Chromatogr. 213, 501–506
12. Umezawa, H., and Aoyagi, T. (1983) in Proteinase Inhibitors (Katunuma, N., Umezawa, H., and Holzer, H., eds) pp. 3–15, Springer Verlag, Berlin.
13. Barrett, A. J., and McDonald, J. K. (1980) in Mammalian Proteases, Vol. 1, Academic Press, London
14. McDonald, J. K., and Barrett, A. J. (1986) in Mammalian Proteases, Vol. 2, Academic Press, London
15. Baggiolini, M., Bretz, V., and Dewald, D. (1978) in Neutral Proteases of Human Polymorphonuclear Leukocytes (Havemann, K., and Janoff, A., eds) pp. 3–17, Urban & Schwarzenberg, Baltimore
16. Schmidt, W., and Havemann, K. (1978) in Neutral Proteases of Human Polymorphonuclear Leukocytes (Havemann, K., and Janoff, A., eds) pp. 150–162, Urban & Schwarzenberg, Baltimore
17. Ohlson, K., and Olsson, I. (1973) Eur. J. Biochem. 36, 473–481
18. Coblyn, J. S., Austen, K. F., and Wintroub, B. U. (1979) J. Clin. Invest. 63, 998–1005
19. Banda, M. J., and Zerb, Z. (1979) Fed. Proc. Fed. Am. Soc. Exp. Biol. 38, 1339–1342
20. Nagaoka, I., and Yamashita, T. (1980) Biochim. Biophys. Acta 598, 169–172
21. Söderling, E., Knuttila, M., and Mäkinen, K. K. (1977) FEBS Lett. 76, 219–225
22. Smith, G. P., MacGregor, R. R. and Peters, T. J. (1983) Biochim. Biophys. Acta 728, 222–227
23. McDonald, J. K., and Schwahe, C. (1977) in Proteases in Mammalian Cells and Tissues (Barrett, A. J., ed) pp. 311–391, Elsevier Scientific Publishing Co., Amsterdam
24. Janoff, A., and Feinstein, G. (1978) in Neutral Proteases of Human Polymorphonuclear Leukocytes (Havemann, K., and Janoff, A., eds) pp. 102–113, Urban & Schwarzenberg, Baltimore
25. Barrett, A. J. (1978) in Neutral Proteases of Human Polymorphonuclear Leukocytes (Havemann, K., and Janoff, A., eds) pp. 238–239, Urban & Schwarzenberg, Baltimore
26. Rinder-Ludwig, R., Bretz, V., and Baggiolini, M. (1978) in Neutral Proteases of Human Polymorphonuclear Leukocytes (Havemann, K., and Janoff, A., eds) pp. 138–149, Urban & Schwarzenberg, Baltimore
27. Starkey, P. M., and Barrett, A. J. (1976) Biochem. J. 155, 265–271
28. Starkey, P. M., and Barrett, A. J. (1976) Biochem. J. 155, 273–278
29. Aoyagi, T., and Umezawa, H. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D. B., and Shaw, E., eds) pp. 429–455, Cold Spring Harbor Conferences, Colorado
30. McDonald, J. K., and Barrett, A. J. (1986) in Mammalian Proteases, Vol. 2, pp. 23–37, Academic Press, London
31. McDonald, J. K., and Barrett, A. J. (1986) in Mammalian Proteases, Vol. 2, pp. 48–55, Academic Press, London
32. McDonald, J. K., and Barrett, A. J. (1986) in Mammalian Proteases, Vol. 2, pp. 261–268, Academic Press, London
33. McDonald, J. K., and Barrett, A. J. (1986) in Mammalian Proteases, Vol. 2, pp. 59–71, Academic Press, London