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pH Dependence of Salt Activation of Human Leukocyte Elastase[1]

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The effects of pH on salt stimulation of the rates of hydrolysis of three substrates by human leukocyte elastase were studied. The enzyme was most active at pH 10.5, 8.0–8.5, and 9.5 for the hydrolyses of fluorescein isothiocyanate-labeled *S*-carboxymethylated bovine serum albumin (FITC-CM-BSA), succinyl-L-Ala-L-Pro-L-Ala-7-methylcoumaryl-4-amide (Suc-APA-MCA), and succinyl-L-Ala_3-*p*-nitroanilide (Suc-Ala_3-pNA), respectively, in the absence of NaCl. The enzyme was activated by 0.5 M NaCl similarly at all pHs tested for the hydrolysis of Suc-Ala_3-pNA, but more at neutral and alkaline pH values, respectively, for the hydrolyses of FITC-CM-BSA and Suc-APA-MCA. Thus, in the presence of 0.5 M NaCl, the enzyme was most active at pH 8.0 and 10.0 with FITC-CM-BSA and Suc-APA-MCA, respectively. In contrast, the proteolytic activity of porcine pancreatic elastase was somewhat inhibited by 0.5 M NaCl.

Three groups of investigators have reported that the activities of human leukocyte elastase against several substrates, such as azo-casein, elastine, fibrinogen, hemoglobin and *N*-benzoxycarbonyl-L-Ala-2-naphthyl ester, are greatest at pH 8.0 to 8.5 (1-3). Since the pH dependence of purified leukocyte elastase was examined only in media below pH 10 (1, 3), it is unknown how much activity it has above pH 10. The proteolytic activity of a crude extract of human leukocytes was found to be maximal at pH 10.0, showing a broad peak with a shoulder at pH 8.0 (4). It is unknown why the crude extract has high activity at alkaline pH, since the major neutral protease in human leukocytes was recently reported to be elastase (5, 6). To examine the reason for the high proteolytic activity of the crude extract at alkaline pH's, we purified the elastase and reinvestigated its properties.

In this work we found that NaCl has different effects in stimulation of the elastase activity at various pH's, resulting in changes in the pH optimum of the enzyme. The effects of salt on porcine pancreatic elastase activity at various pH's were examined for comparison.

MATERIALS AND METHODS

Materials. All fluorogenic substrates used and Suc-Ala_3-pNA[3] were obtained from the Protein Research Foundation, Minoh, Osaka, Japan. Porcine pancreatic elastase was obtained from Sigma Chemical Company, St. Louis, Missouri. FITC-CM-BSA was prepared as described previously (7).

Purification of human leukocyte elastase. Human leukocytes were prepared from peripheral blood of healthy donors as described by Bakkenist *et al.* (8),

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[3] Abbreviations used: FITC-CM-BSA, fluorescein isothiocyanate-labeled, *S*-carboxymethylated bovine serum albumin; Suc-APA-MCA, succinyl-L-Ala-L-Pro-L-Ala-7-methylcoumaryl-4-amide; Suc-Ala_3-pNA, succinyl-L-Ala_3-*p*-nitroanilide.

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and kept frozen at −20°C until use. Elastase was purified by the method of Bough and Travis (9) with slight modifications. Leukocytes were thawed and suspended in cold 0.34 M sucrose in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA. The cells were homogenized in a Potter-Elvehjem glass homogenizer operated by motor, and the homogenate was centrifuged at 540*g* for 10 min. The elastase activity was extracted from the pellet fraction with 1 M NaCl in 0.1 M sodium acetate buffer (pH 4.0), adsorbed on a column of *S*-carboxymethylated bovine serum albumin-bound Sepharose CL-4B, and eluted with 2 M urea/0.1 M sodium acetate (pH 3.0) after washing the column with 1 M NaCl/0.1 M sodium acetate buffer (pH 4.0). After stepwise elution from a CM-cellulose column with 0.4 M NaCl/0.1 M sodium acetate buffer (pH 5.5), the purified elastase fraction separated into one major and one minor protein band on polyacrylamide gel electrophoresis at pH 4.3, each exhibiting elastase activity with Suc-APA-MCA. The preparation also separated into two protein bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the major and minor bands having molecular weights of 28,000 and 29,000, respectively, like those reported for human elastase (9). The purified preparation of human leukocyte elastase was stored at −80°C in 1 M NaCl/0.4 M Tris-HCl (pH 8.0) until use.

Assay of proteolytic activity. The activity was assayed with 0.25 mg FITC-CM-BSA in 0.25 ml 0.4 M Tris-HCl (pH 8.0) at 37°C for 1 h. After adding 0.05 ml 2% bovine serum albumin, the reaction was terminated with 0.2 ml 0.44 M trichloroacetic acid. The mixture was briefly centrifuged, the supernatant fluid (0.1 ml) was mixed with 0.9 ml 0.1 M sodium carbonate buffer (pH 10.0), and its fluorescence intensity at 515 nm, with excitation at 490 nm, was measured.

Assay of amidolytic activity against fluorogenic substrates. Increase of fluorescence at 460 nm with excitation at 380 nm was monitored at 37°C in 1 ml 0.4 M Tris-HCl (pH 8.0) using 125 nmol substrate, 0.3 μg elastase, and 2.5% dimethyl sulfoxide.

Amidolytic activity against the chromogenic substrate Suc-Ala_3-pNA (0.5 mM) was assayed spectrophotometrically by measuring the increase of absorbance at 405 nm at 37°C using 5 μg elastase in 1 ml of the buffer.

For examining pH dependence a mixture of 0.1 M concentrations of Tris, H_3BO_3, and NaH_2PO_4 with or without 0.5 M NaCl was adjusted to the desired pH with 0.3 M NaOH with or without NaCl.

The concentration of human leukocyte elastase was determined spectrophotometrically using a value of $A^{1\%}_{1cm}$ at 280 nm = 9.85 (10).

RESULTS

Of the fluorogenic substrates tested, purified leukocyte elastase hydrolyzed Suc-APA-MCA most rapidly (Table I). The enzyme did not hydrolyze succinyl-L-Ala_2-L-Pro-L-Phe-MCA, a substrate for chymotrypsin-like protease, indicating that it was free from cathepsin G. It showed low activity with three compounds with an arginyl residue as a C-terminal amino acid, which are substrates of trypsin-like proteases. The activity against the three compounds was partly inhibited by elastatinal (0.1 mg/ml), but not by either leupeptin or antipain (each 0.1 mg/ml), indicating that its slight degradation of these three compounds was due to endogenous activity of elastase. Human leukocyte elastase did not hydrolyze three other fluorogenic compounds, Suc-Gly-L-Pro-MCA, Suc-Gly-L-Pro-L-Leu-Gly-L-Pro-MCA, and Suc-L-Arg-L-Pro-L-Phe-L-His-L-Leu_2-L-Val-L-Tyr-MCA.

Elastase measured with FITC-CM-BSA as substrate showed a wide pH range of activity in the absence of NaCl, with an apparent maximum at pH 10.5 (Fig. 1A). But in the presence of 0.5 M NaCl, the enzyme was most active at pH 8.0. In the presence of 0.5 M NaCl the activity was increased over the pH range tested, but the degree of increase varied with the pH; it was increased most in the neutral pH region, and least at pH 9.5. The enzyme showed two pH optima in the presence of 0.5 M NaCl.

The pH dependence of elastase activity with Suc-APA-MCA as substrate is shown

TABLE I

SUBSTRATE SPECIFICITY OF HUMAN LEUKOCYTE ELASTASE

Substrate	Activity (nmol min^{-1} mg^{-1})
Suc-APA-MCA	235
Suc-Ala_2-Pro-Phe-MCA	0
Benzoyl-Arg-MCA	0.116
Glt-Gly-Arg-MCA	0.098
Boc-Val-Pro-Arg-MCA	0.441
Suc-Gly-Pro-MCA	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0
Suc-Arg-Pro-Phe-His-Leu_2-Val-Tyr-MCA	0

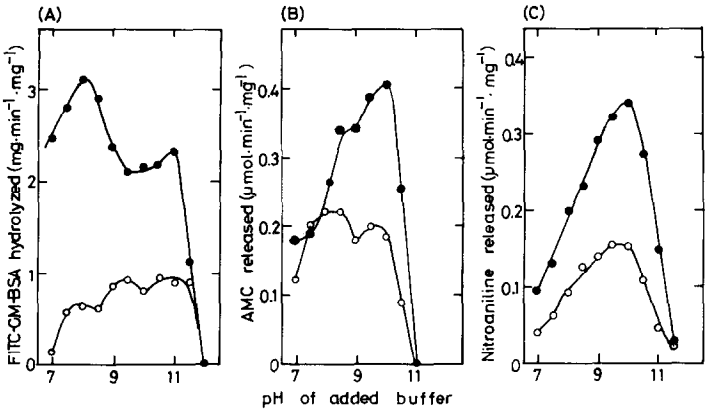

FIG. 1. pH dependence of salt stimulation of human leukocyte elastase. The buffers were prepared as described under Materials and Methods. (A) Proteolytic activity with (●) or without (○) 0.5 M NaCl. (B) Amidolytic activity for Suc-APA-MCA with (●) or without (○) 0.5 M NaCl. (C) Amidolytic activity for Suc-Ala_3-MCA with (●) or without (○) 0.5 M NaCl.

in Fig. 1B. The enzyme was most active at pH 8.0 to 8.5 in the absence of NaCl as reported by others (1–3). However, in the absence of 0.5 M NaCl, it showed a second peak of activity at pH 9.5, like the proteolytic activity. Addition of 0.5 M NaCl stimulated the amidolytic activity considerably. However, NaCl stimulated the activity in the alkaline pH range most, resulting in one main peak of activity with an optimum at pH 10.0. The amidolytic activity of human leukocyte elastase against Suc-Ala_3-pNA showed one pH optimum at pH 9.5 to 10.0 in the absence of NaCl (Fig. 1C). This activity was also stimulated by 0.5 M NaCl, but since the extent of stimulation of activity by NaCl was almost constant over the pH range tested, the activity of elastase against Suc-Ala_3-pNA in the presence of 0.5 M NaCl was also maximum at pH 10.0. These results indicate that the pH for maximum activation of human leukocyte elastase by NaCl varies with the substrate.

The pH dependence of the hydrolyses of FITC-CM-BSA and Suc-APA-MCA by a human leukocyte homogenate are shown in Fig. 2. The amidolytic activity of the homogenate showed a single, broad peak with an optimum at pH 9.5 in the absence of NaCl. Addition of 0.5 M NaCl stimulated the activity almost equally at all pH values. The peak of amidolytic activity of the homogenate seemed to correspond to the second, low peak of pure elastase on the alkaline side shown in Fig. 1B, while the major peak of pure elastase at pH 8.0 to 8.5 was not seen with the homogenate. In the presence of 0.5 M NaCl, the proteolytic activity of the leukocyte homogenate showed two pH optima like the activity of pure elastase. The proteolytic activity of the homogenate at pH 8.0 without NaCl was 1/12th of that in the presence of 0.5 M NaCl (data not shown in Fig. 2). Since cathepsin G, another protease in leukocytes, has a pH optimum of 7.5 with hemoglobin (2), 7 to 7.7 with benzoyl-Tyr ethyl ester (11), 7.5

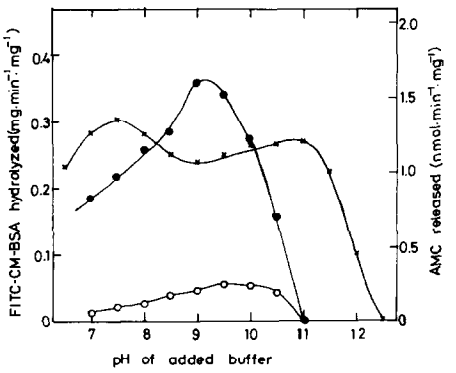

FIG. 2. pH dependence of protease activities in a human leukocyte homogenate. Proteolytic activity (×); amidolytic activity with (●) or without (○) 0.5 M NaCl.

with casein (12), and 7.5 to 7.8 with azocasein (13), the results in Figs. 1 and 2 indicate that the hydrolysis of FITC-CM-BSA was mainly due to elastase in the leukocyte homogenate under the present assay conditions.

The proteolytic and amidolytic activities in a leukocyte homogenate that had been frozen for several months were measured at pH 8.0 (Table II). Hydrolysis of Suc-APA-MCA by the homogenate was stimulated 65-fold and that of FITC-CM-BSA 9-fold by 0.5 M NaCl. The slight differences in specific activities for hydrolysis of FITC-CM-BSA and Suc-APA-MCA by the fresh and stored homogenates at pH 8.0 in the presence of 0.5 M NaCl can be explained by differences in the ion compositions of the buffers used for the assay. Since the amidolytic activity of a freshly prepared leukocyte homogenate was stimulated 9-fold by 0.5 M NaCl, the results in Table II suggest a loss of the activity during storage of leukocyte homogenate and its assay in the absence of NaCl.

It seemed likely that only the amidolytic activity of elastase against Suc-APA-MCA was unstable in the absence of NaCl, because the leukocyte homogenate was prepared in a low-salt buffer (0.34 M sucrose/1 mM EDTA/50 mM Tris-HCl, pH 7.4). To confirm this idea, we examined the effect

TABLE II

PROTEOLYTIC AND AMIDOLYTIC ACTIVITIES OF HUMAN LEUKOCYTE HOMOGENATE AFTER STORAGE AT −20°C

Substrate	Enzyme	Activity		Stimulation
		− NaCl	+ NaCl	
		$\mu g\ min^{-1}\ mg^{-1}$		
FITC-CM-BSA	Fresh[a]	23	280	12.2
	Stored	40	355	8.9
		$nmol\ min^{-1}\ mg^{-1}$		
Suc-APA-MCA	Fresh[a]	0.13	1.15	8.8
	Stored	0.03	1.94	64.7

Note. Human leukocyte homogenate (5.56 mg protein/ml of 0.34 M sucrose, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4) was kept frozen for several months at −20°C.

[a] Taken from Fig. 2.

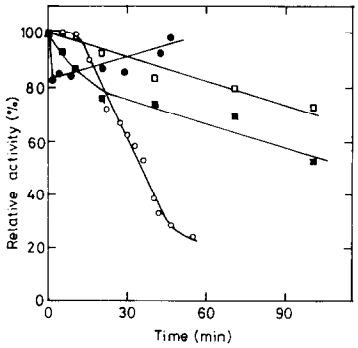

FIG. 3. Changes of proteolytic and amidolytic activities of human leukocyte elastase on dilution with low-salt buffer. Elastase (1 mg/ml in 1 M NaCl) was diluted 30 times with 0.4 M Tris-HCl (pH 8.0) and incubated at 37°C. The activities of aliquots (0.01 ml) taken at the indicated times were measured in 0.4 M Tris-HCl (pH 8.0) with or without 1 M NaCl. Amidase with (●) or without (○) NaCl; protease with (■) or without (□) NaCl.

of dilution with low-salt buffer on pure elastase activity (Fig. 3). Elastase diluted with 0.4 M Tris-HCl (pH 8.0) rapidly lost its amidolytic activity when assayed without NaCl. However, when the elastase was assayed with NaCl, the activity of the diluted enzyme was maintained at nearly the original level even after 60 min. On incubations of the elastase at 37, 20, and 0°C after its dilution, the amidolytic activity decreased similarly (data not shown). The activities of elastase immediately after dilution were 50.8 and 117 nmol min^{-1} mg^{-1} in the absence and presence of 1 M NaCl, respectively. Addition of NaCl increased the amidolytic activity of elastase about two- and fivefold at 0 and 60 min after dilution, respectively. Results indicate that the extent of stimulation of the amidolytic activity of elastase by NaCl depended on the storage period in low-salt medium.

The elastase activity was almost constant at between 20 and 55°C in 0.4 M Tris-HCl (pH 8.0), as reported for the proteolytic activity (14), while it was maximum at 40°C in 1 M NaCl (Fig. 4). These results indicate that, at lower temperatures, human leukocyte elastase becomes more active in the presence of 1 M NaCl than in its absence.

There are no reports of studies on the effect of NaCl at various pH's on porcine

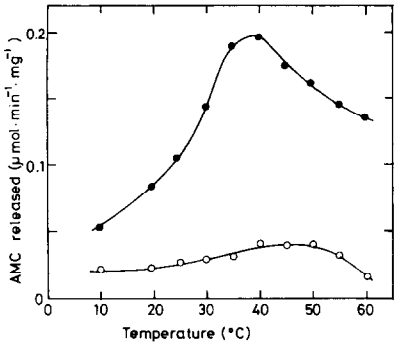

FIG. 4. Effect of temperature on salt stimulation of human leukocyte elastase. Elastase was diluted the same as for Fig. 3, and amidolytic activity with (●) or without (○) 1 M NaCl was measured at the indicated temperatures.

pancreatic elastase. Therefore, we examined whether its pH-activity curve was also affected by salt. As shown in Fig. 5, the proteolytic activity of the elastase against FITC-CM-BSA showed a typical bell-shaped pH curve with maximal activity at pH 9.0 both in the presence and absence of 0.5 M NaCl, but NaCl reduced the activity. This result is consistent with a report that salt inhibited the elastolytic activity of pancreatic elastase (15), although the proteolytic and esterolytic activities of the enzyme were not affected by various concentrations of salt (16). NaCl inhibited the amidolytic activities of the enzyme almost equally at all pH's tested. These observations show that the curious pH dependence of the hydrolysis of FITC-CM-BSA and Suc-APA-MCA by human leukocyte elastase is due to inherent properties of the enzyme.

DISCUSSION

The rates of hydrolyses of Suc-APA-MCA (amidase) and FITC-CM-BSA (peptidase) by human leukocyte homogenate were stimulated 9- and 12-fold by 0.5 M NaCl at pH 8.0, as shown in Fig. 2. It is possible that this stimulation was due to solubilization of proteases in the homogenate by salt solution, as reported by Pryce-Jones *et al.* (4). However, salt also stimulated the rates of hydrolyses of both FITC-CM-BSA, as presented here, and of azo-casein (3) by purified human leukocyte elastase. Thus, since salt activated the protease(s) in human leukocytes directly, part of the stimulation of the peptidase in a human leukocyte homogenate by salt was due to direct activation of elastase. The extents of salt stimulation of the protease(s) in a human leukocyte homogenate at pH 8.0 were almost equal with the different substrates used.

When the peptidase and amidase activities were assayed in the presence of 0.5 M

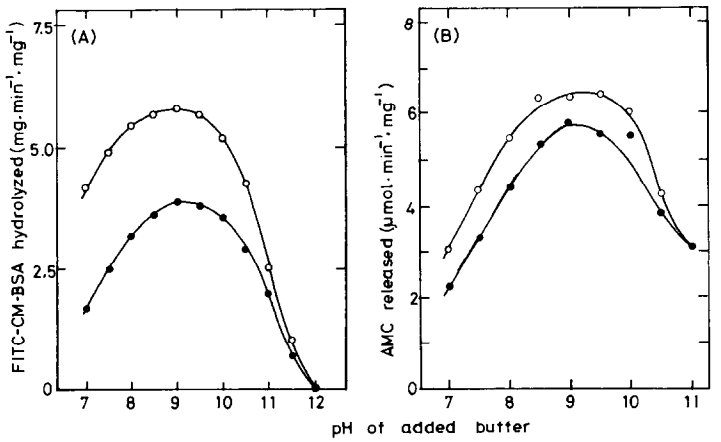

FIG. 5. Effect of NaCl on the proteolytic and amidolytic activities of porcine pancreatic elastase. (A) Proteolytic activity with (●) or without (○) 0.5 M NaCl. (B) Amidolytic activity for Suc-APA-MCA with (●) or without (○) 0.5 M NaCl.

NaCl at pH 8.0 during purification of proteases from human leukocytes, the total activities recovered in the three subcellular fractions (the pellets at 540*g* and 8000*g*, and the supernatant at 8000*g*) were 183% (peptidase) and 130% (amidase) of those of the homogenate. These results indicate that the peptidase and amidase activities in human leukocytes were both increased during the fractionation (data not shown). One possible reason for this increase during fractionation was the release from inhibition by some inhibitor present in leukocytes, as reported by Janoff and Blondin (17). The total activities recovered in the three fractions in the absence of NaCl were 212% (peptidase) and 425% (amidase) of those in the homogenate (data not shown). Ratios of recoveries in the presence/absence of 0.5 M NaCl were 0.86 and 0.31 for the peptidase and the amidase, respectively. This observation suggests that the inhibitor, if present, is not dissociated from the proteases by 0.5 M NaCl.

The following findings showed that hydrolysis of FITC-CM-BSA by a human leukocyte homogenate was due mainly to the action of elastase, although it had a second pH optimum in the alkaline pH region in addition to that at pH 7.5 in the presence of 0.5 M NaCl. First, we tried to purify an alkaline protease from human leukocytes using the nonspecific substrate for assay of activity, but consistently obtained only elastase and cathepsin G. Extensively purified human leukocyte elastase was active for hydrolysis of FITC-CM-BSA at pH 10.5 in the absence of NaCl, and showed a second peak of activity in the alkaline pH region in the presence of 0.5 M NaCl (Fig. 1). The pH-activity curve of hydrolysis of FITC-CM-BSA by the homogenate was very similar to that by pure elastase. Second, the extent of salt stimulation of the hydrolysis of FITC-CM-BSA by the human leukocyte homogenate was very similar to that of the elastase. Third, elastase is the main neutral protease in human leukocytes (5, 6).

The amidase activity of human leukocyte homogenate was stimulated 9-fold (Fig. 2) and 65-fold (Table II) by 0.5 M NaCl at pH 8.0. When pure elastase stocked in 1 M NaCl was diluted with NaCl-free buffer, the diluted enzyme in the absence of NaCl lost its activity within 60 min (Fig. 3). However, the diluted enzyme in the presence of 0.5 M NaCl showed the same activity as the stocked enzyme even after 60 min. It has been shown that the overall rate constant for reversible denaturation, k_f, and renaturation, k_b, of some pancreatic proteins depend on ionic strength (18). It seems that this mechanism is concerned in the loss of amidolytic activity of human leukocyte elastase on dilution in the absence of 0.5 M NaCl and the complete recovery of activity in the presence of salt. However, the diluted elastase had slightly lower peptidase activity in the presence of 0.5 M NaCl than in its absence (Fig. 3). The pH dependence of the stimulatory effects of NaCl on amidolysis and proteolysis of human leukocyte elastase was different (Fig. 1). It remained unclear whether amidolysis and proteolysis by human leukocyte elastase were catalyzed by slightly different mechanisms, or whether salt had varying effects on the relative accessibility of the different groups on the synthetic substrate.

The hydrolyses of FITC-CM-BSA and Suc-APA-MCA by porcine pancreatic elastase were slightly inhibited by 0.5 M NaCl. Our results are consistent with those obtained for the hydrolyses of elastin (15) and ester substrate (19) by the enzyme. The inhibitions of both activities by NaCl were similar at all pH's tested. The hydrolysis of Suc-Ala_3-pNA by human leukocyte elastase was stimulated constantly at all pH's tested by 0.5 M NaCl. Although NaCl acted reversely on human leukocyte elastase and porcine pancreatic elastase, the constancy of salt stimulation at various pHs for hydrolysis of Suc-Ala_3-pNA by human leukocyte elastase was similar to those of salt-inhibition of porcine pancreatic elastase. Thus, salt affects the activities of mammalian proteases for their substrates in various ways.

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REFERENCES

1. OHLSSON, K., AND OLSSON, I. (1974) *Eur. J. Biochem.* **42,** 519–527.
2. SCHMIDT, W., AND HAVEMANN, K. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355,** 1077–1082.
3. STARKEY, P. M., AND BARRETT, A. J. (1976) *Biochem. J.* **155,** 265–271.
4. PRYCE-JONES, R. H., SAKLATVALA, J., AND WOOD, G. C. (1974) *Clin. Sci. Mol. Med.* **47,** 403–414.
5. HAVEMANN, K., AND JANOFF, A. (1978) Neutral Proteases of Human Polymorphonuclear Leukocytes, Urban & Schwarzenberg, Baltimore/Munich.
6. DELSHAMMAR, M., AND OHLSSON, K. (1976) *Eur. J. Biochem.* **69,** 125–131.
7. OSHIMA, G., YAMADA, M., AND SUGIMURA, T. (1979) *Biochem. Biophys. Res. Commun.* **90,** 158–163.
8. BAKKENIST, A. R. J., WEVER, R., VULSMA, T., PLAT, H., AND VAN GELDER, B. F. (1978) *Biochim. Biophys. Acta* **524,** 45–54.
9. BOUGH, R., AND TRAVIS, J. (1976) *Biochemistry* **15,** 836–841.
10. BARRETT, A. J., AND MCDONALD, J. K. (1980) Mammalian Proteases, Vol. 1, pp. 165–176, Academic Press, New York.
11. GERBER, A. C., CARSON, J. H., AND HARDORN, B. (1974) *Biochim. Biophys. Acta* **364,** 103–112.
12. RINDLER-LUDWIG, R., AND BRAUNSTEINER, H. (1975) *Biochim. Biophys. Acta* **379,** 606–617.
13. STARKEY, P. M., AND BARRETT, A. J. (1976) *Biochem. J.* **155,** 273–278.
14. STARKEY, P. M., AND BARRETT, A. J. (1976) *Biochem. J.* **155,** 255–263.
15. JANOFF, A., AND SCHERER, J. (1968) *J. Exp. Med.* **128,** 1137–1155.
16. GERTLER, A. (1971) *Eur. J. Biochem.* **20,** 541–546.
17. JANOFF, A., AND BLONDIN, J. (1971) *Lab. Invest.* **25,** 565–571.
18. POHL, F. M. (1969) *FEBS Lett.* **3,** 60–64.
19. HARTLEY, B. S., AND SHOTTON, D. M. (1971) *in* The Enzymes (Boyer, P. D., ed.), 3rd ed., Vol. 3, pp. 323–373, Academic Press, New York.