radioactivity in the body of each pup was then calculated from the radioactivity found in an aliquot of whole body homogenate (prepared in 10 volumes of 0.1*N* HCl). The amount of radioactivity in pups treated for 5 or more days was found equal to that in their saline-treated littermates. In another experiment half of each of several litters were treated with methadone for the first 5 days of life, and then the drug was discontinued. When these mice reached the age of 4 weeks they weighed the same as their untreated littermates. Evidently the initial anorectic effect of methadone, to which tolerance rapidly occurs, is not the cause of the growth retardation seen in the chronically treated young mice.

6. Dr. S. Mulé kindly provided *d*-methadone.
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Pentapeptide (Pepstatin) Inhibition of Brain Acid Proteinase

Abstract. *The pentapeptide pepstatin obtained from culture filtrates of actinomycetes completely inhibited brain acid proteinase (cathepsin D) at exceedingly low concentrations. Among the brain enzymes tested, the effect is specific for acid proteinase because addition of 1000-fold higher concentrations was without effect on neutral proteinase, aminopeptidase, and arylamidases. Pepstatin also inhibits pepsin as tested with hemoglobin or with* N-*acetylphenylalanyl-*L-*diiodotyrosine as substrate. Pepstatin must be regarded as the most powerful agent yet described that inhibits intracellular acidic proteolytic enzyme in brain.*

Cathepsin D or acid proteinase is one of the major intracellular enzymes involved in the breakdown of proteins. This enzyme is of considerable clinical interest because its activity is elevated in a number of degenerative conditions, including muscular dystrophy, denervation atrophy, inflammatory and allergic conditions (*1*), experimental allergic encephalomyelitis, and multiple sclerosis (*2, 3*). Thus agents modifying the activity of intracellular proteolytic enzymes may have great potential in treatment of degenerative conditions. Our recent finding of endogenous factors present in brain extracts influencing protein breakdown (*4*) indicated the need for studying the effect of other naturally occurring inhibitors of acid proteinase. One inhibitor of great promise is a pentapeptide (pepstatin), obtained from the culture filtrates of actinomycetes (*5*), which inhibits pepsin and some intracellular proteinases present in liver and uterus (*6*). We now report the effect of added pepstatin on some typical intracellular proteolytic enzymes extracted from brain and tumor tissue, as compared to the effect of this inhibitor on extracellular enzymes active at acid and neutral *p*H.

Inhibition by pepstatin was studied on representative brain enzymes, some of which had already been purified (*7*). Acid proteinase was purified from an acetone-dried powder prepared from the crude mitochondrial fraction of pig brain, enriched in lysosomes, as follows: the enzyme was extracted with acetone-formate buffer (0.2*M*, 20 percent acetone by volume) and the supernatant was subjected to Sephadex gel filtration and chromatography on diethylaminoethyl (DEAE)-cellulose and DEAE-Sephadex. Acid proteinase exists in isoenzymatic forms, with molecular weights from approximately 27,500 to 48,000 (*4, 7, 8*). One component with a molecular weight of 27,500 giving a single band on sodium dodecyl sulfate–disc gel acrylamide electrophoresis was selected for our study. The purified enzyme can be lyophilized and is stable when stored at 0°C. Neutral proteinases are labile and were prepared (fresh for each series of experiments) by extraction of rat brain with ten volumes of 0.32*M* sucrose; the extracts were then subjected to Sephadex gel filtration and DEAE-cellulose chromatography (*8*). Aminopeptidase showing a marked specificity for leucylglycylglycine (Leu-Gly-Gly) and an arylamidase specific for monoacylated naphthylamides were prepared from the sucrose supernatants by chromatography on DEAE-cellulose and DEAE-Sephadex with elution by a salt gradient different from that used for the other proteolytic enzymes (*9*). Crystalline pepsin and trypsin were obtained from Worthington (Freehold, N.J.). Pepstatin (isovaleryl-L-valyl-L-valyl-L-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-methylheptanoic acid) was a gift from Dr. H. Umezawa (Banyu Pharmaceutical Co., Osaka).

The following methods of analysis were used. Acid proteinase and pepsin were measured with denatured hemoglobin in 0.05*M* citrate buffer at *p*H 3.2, the reaction was terminated by addition of 6 percent trichloroacetic acid, and the acid soluble fraction was quantified by the Folin procedure (*10*); in addition, pepsin was assayed with a synthetic substrate *N*-acetylphenylalanyldiiodotyrosine (APDT) in 10 m*M* HCl, *p*H 2.0, with a modified ninhydrin procedure (*11*); neutral proteinase was determined with denatured hemoglobin by a standard quantitative ninhydrin procedure (*8*); aminopeptidase was assayed with Leu-Gly-Gly by the ninhydrin procedure of Yemm and Cocking (*12*); arylamidase was assayed with arginyl-β-naphthylamide; and trypsin was assayed with benzoylarginylnaphthylamide by a coupled diazonium procedure (*9*).

Pepstatin at exceedingly low concentrations (in the nanogram range) inhibited purified and crude brain proteinase preparations (Fig. 1A). Crude enzyme was prepared from normal whole brain, and also from a sample of human astrocytoma, by extraction with ten volumes of a hypotonic buffer, 10 m*M* tris-HCl, *p*H 7.6, and was

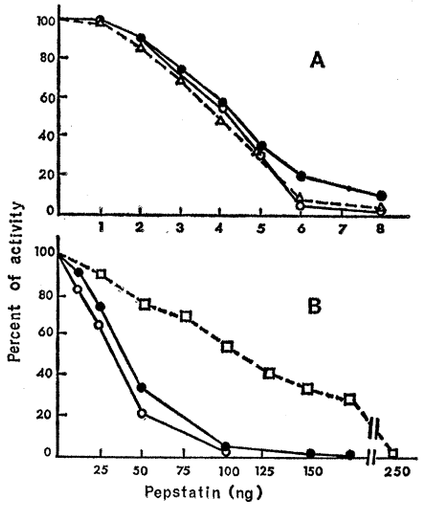

Fig. 1. Inhibition of brain acid proteinase (A) or pepsin (B) with increasing concentrations of pentapeptide (pepstatin). The incubations with hemoglobin as substrate were done as follows: 5.0 mg (●) or 0.6 mg (○) of hemoglobin in 1 ml of 50 m*M* citrate buffer (*p*H 3.2) containing 25 μg of purified porcine acid proteinase or 5 μg of pepsin was incubated for 1 hour at 37°C. When crude enzyme was prepared by hypotonic extraction of rat brain, the mixture was incubated with 100 μg of enzyme protein and 5.0 mg of hemoglobin (△). Inhibition of pepsin by pepstatin was measured with the synthetic substrate *N*-acetylphenylalanyl-L-diiodotyrosine (□), in 1 ml of 10 m*M* HCl (*p*H about 2.0) and incubated for 45 minutes at 37°C. Inhibition is expressed as the percentage of activity remaining after addition of pepstatin relative to that in absence of inhibitor.

Table 1. Pepstatin and inhibition of different proteolytic enzymes. Assay mixtures contained 25 to 100 μg of protein with purified brain enzyme, or 100 to 200 μg with crude enzyme; they were incubated at the appropriate *p*H for 1 hour at 37°C. The abbreviations are APDT, *N*-acetylphenylalanyl-L-diiodotyrosine; BANA, benzoylarginyl-β-naphthylamide; Arg-βNA, arginyl-β-naphthylamide; and Hb, hemoglobin.

Enzymes	Species	Substrate	Pepstatin	
			Amount (μg)	Inhibition (%)
		Brain enzymes		
Acid proteinase (*p*H 3.2)				
Purified	Porcine	Hb	0.01	100
Crude	Rat	Hb	0.01	100
Astrocytoma	Human	Hb	0.01	100
Neutral proteinase (*p*H 7.6)				
Crude	Rat	Hb	1–10	0
Purified	Rat	Hb	1–10	0
Aminopeptidase (*p*H 7.6)				
Purified	Rat	Leu-Gly-Gly	1–10	0
Crude	Rat	Leu-Gly-Gly	1–10	0
Arylamidase (*p*H 7.6)				
Purified	Rat	Arg-βNA	1–10	0
Crude	Rat	Arg-βNA	1–10	0
		Extracellular enzymes		
Pepsin (*p*H 3.2)	Bovine	Hb	0.10	100
Pepsin (*p*H 2.0)	Bovine	ADPT	0.25	100
Trypsin (*p*H 7.6)	Bovine	BANA	1–10	0

centrifuged at 30,000*g* for 30 minutes. The enzyme activity was present in the supernatant.

Between 25 to 50 μg of enzyme protein was incubated at different substrate concentrations of hemoglobin (Fig. 1A). Under these conditons only 4 ng of pepstatin was required to obtain a 50 percent inhibition, with complete loss of activity on addition of about 10 ng. In the case of crude enzyme obtained from hypotonic extracts of whole rat brain, or human astrocytoma tumor, the pattern of inhibition was similar (Fig. 1A) (Table 1). In contrast, no inhibition was observed on addition of 100- to 1000-fold higher concentrations of pepstatin to other brain enzymes, such as neutral proteinase, aminopeptidase, and arylamidase (Table 1).

As observed by others, pepstatin was shown to inhibit pepsin on incubation of 2 to 5 μg of enzyme with hemoglobin at concentrations similar to those used for acid proteinase. Activity was inhibited 50 percent by 35 ng of pepstatin, and 100 percent by 100 ng (Fig. 1B). There have been no reports of inhibition of pepsin by pepstatin when assayed with synthetic rather than protein substrates. Geratz (*13*) observed that some amino acid analogs such as β-phenyl pyruvate were effective only with hemoglobin as the substrate, and not with synthetic substrates. To determine if the same limitations apply to pepstatin, pepsin was assayed with the substrate APDT at 0.2 to 0.8 m*M*. Although peptic activity was considerably lower, requiring more enzyme, it was blocked completely by pepstatin at a ratio of enzyme to inhibitor similar to that observed for hemoglobin as substrate (Fig. 1B and Table 1).

The amino acid sequence of pepsin and cathepsin D and the nature of their catalytic sites are not known. These enzymes show no requirement for added cofactors or metal ions and do not appear to contain a free -SH grouping at the active center (*7*). Pepsin is differentiated from cerebral acid proteinase, however, by its lower *p*H optimum and ability to cleave smaller peptide substrates. Both enzymes show a preference for peptide bonds adjacent to aromatic (hydrophobic) residues such as phenylalanyl (Phe) and tyrosinyl (Tyr) when tested with the B chain of insulin and synthetic polypeptide substrates (*7*). The hydroxy side chains of pepstatin may be a factor in the affinity of this material for the intermediates of the catalytic reaction. Study of the kinetics did not provide additional information of the inhibitory mechanism. Inhibition at different substrate concentrations did not conform to a purely competitive inhibition when plotted by the method of Dixon and Webb (*14*), but indicated a mixed type inhibition; as such it appears to be similar to inhibition of pepsin by pepstatin reported in other studies (*6*).

On the basis of a molecular weight for pepsin of 35,000, the ratio of enzyme to inhibitor was approximately 1:1; in the case of brain acid proteinase the higher ratio indicates the presence of protein impurities in our enzyme preparations. The availability of a specific inhibitor of acid proteinase may be useful for establishing the purity of cathepsin D preparations.

Among synthetic materials reported to inhibit acid proteinase is diazoacetylnorleucine methyl ester and azoacetyl-*N*-(2,4-dinitrophenyl) ethylenediamine; but these, which are regarded as nonspecific, act rather slowly, and require the presence of heavy metals such as Cu^{2+} (*7*, *16*). The full range of enzymes against which pepstatin can act as an inhibitor is still unknown. Of the known intracellular enzymes present in a variety of tissues, pepstatin appears to be specific only for cathepsin D and not other proteinases active at low *p*H such as cathepsin B and C. In blood it is reported to inhibit the conversion of angiotensin-angiotensinogen by renin measured at *p*H 7.4 both in vitro and in the intact animal (*17*). Of the extracellular enzymes studied, pepstatin is active only against pepsin and shows no activity against typical serine hydrolases such as trypsin and chymotrypsin.

Our finding of a potent inhibitor of acid proteinase of brain may hold promise for treatment of degenerative conditions involving the labilization or release of lysosomal enzymes. Demyelination, for example, represents breakdown of myelin by proteolytic and lipolytic enzymes, although the mechanisms responsible for their release and their mode of action are unknown (*2*). The availability of a material of low molecular weight and low toxicity which could possibly penetrate the brain may affect the induction and duration of myelin degeneration. This material may also modify other symptoms consequent upon release of acid proteinases. In addition, pepstatin can be useful for investigating alterations of protein turnover and the role of breakdown.

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References and Notes

1. I. M. Weinstock and A. A. Iodice, in *Lysosomes in Biology and Pathology*, J. T. Dingle and H. B. Fell, Eds. (North-Holland, Amsterdam, 1969), vol. 2, p. 450.
2. J. F. Hallpike and C. W. M. Adams, *Histochemistry* **1**, 559 (1969); P. J. Riekkinen, J. Clausen, H. J. Frey, T. Fog, U. K. Rinne, *Acta Neurol. Scand.* **46**, 349 (1970); E. R. Einstein, in *Handbook of Neurochemistry*, A. Lajtha, Ed. (Plenum, New York, 1972), vol. 7, p. 107.
3. M. F. Kerekes, T. Feszt, A. Kovacs, *Experientia* **21**, 42 (1965); J. Wood and N. King, *Proc. Amer. Soc. Neurochem.* **2**, 118c

(1971); O. P. Kuzulina, Ya. T. Terletska, L. P. Syrovatska, *Ukr. Biokhim. Zh.* **44**, 145 (1972).
4. N. Marks, B. D'Monte, A. Lajtha, *Texas Rep. Biol. Med.*, in press.
5. H. Morishima, T. Takita, T. Aoyagi, T. Takeuchi, H. Umezawa, *J. Antibiot. Tokyo Ser. A* **23**, 263 (1970).
6. H. Ikezawa, T. Aoyagi, T. Takeuchi, H. Umezawa, *ibid.* **24**, 488 (1971); J. F. Woessner, *Biochem. Biophys. Res. Commun.* **47**, 965 (1972); A. J. Barrett and J. T. Dingle, *Biochem. J.* **127**, 439 (1972).
7. N. Marks and A. Lajtha, in *Handbook of Neurochemistry*, A. Lajtha, Ed. (Plenum, New York, 1971), vol. 5A, p. 49.
8. ———, *Biochem. J.* **89**, 74 (1965); N. Marks, B. D'Monte, A. Lajtha, in preparation.
9. N. Marks, *Int. Rev. Neurobiol.* **11**, 57 (1968); ———, R. K. Datta, A. Lajtha, *J. Biol. Chem.* **243**, 2882 (1968).
10. S. Serra, A. Grynbaum, A. Lajtha, N. Marks, *Brain Res.* **44**, 579 (1972).
11. J. Tang, *Methods Enzymol.* **19**, 406 (1970).
12. N. Marks and A. Lajtha, *ibid.*, p. 343.
13. J. D. Geratz, *Arch. Biochem. Biophys.* **111**, 134 (1965).
14. M. Dixon and E. C. Webb, *Enzymes* (Academic Press, New York, ed. 2, 1964).
15. R. Vogel, I. Trautschold, E. Werle, *Natural Proteinase Inhibitors* (Academic Press, New York, 1968).
16. H. Keilova, K. Bhaha, B. Keil, *Eur. J. Biochem.* **4**, 442 (1968); *Coll. Czech. Chem. Commun.* **33**, 131 (1968).
17. F. Gross, J. Lazar, H. Orth, *Science* **175**, 656 (1972); R. P. Miller, C. J. Poper, C. W. Wilson, E. De Vito, *Biochem. Pharmacol.* **21**, 2941 (1972).
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Gas Chromatographic Assay of Epoxide Hydrase Activity with 3-Methylcholanthrene-11,12-Oxide

Abstract. *Epoxide hydrase has been measured in rat tissue with 3-methylcholanthrene-11,12-oxide as substrate; diol formation was assayed by gas chromatographic separation of the trimethylsilylated derivative of* trans-*11,12-dihydro-11,-12-dihydroxy-3-methylcholanthrene from the corresponding derivative of the 11 (or 12)-hydroxy-3-methylcholanthrene on 3 percent OV-17, which is formed from the 11,12-oxide during the derivatization. The polycyclic hydrocarbons were extracted initially from the incubation mixture with ethyl acetate. The assay is simple, inexpensive, and sensitive.*

Chemical carcinogens of the polycyclic hydrocarbon type are thought to exert their effect after modification to metabolically active derivatives (*1*). In this regard, Boyland proposed that epoxides may be formed from aromatic ring structures as reactive intermediates in the transition to diols, phenols, and conjugates of glutathione (*2*). Since that time, epoxides have been reported as intermediates in the metabolism of polycyclic hydrocarbons (*3*). The K-region oxides are highly reactive substances and produce malignant transformations of cells in vitro (*4*).

Not only is the rate of synthesis of the K-region oxides of the polycyclic hydrocarbons important, but consideration must also be given to their rate of metabolism. Thus, it has been shown that these reactive substances may be converted to *trans*-dihydrodiol derivatives by a microsomal epoxide hydrase (*5*). The hydrase has been partially purified from the microsomes of guinea pig liver and may actually represent a heterogeneous class of enzymes (*6*). Consequently, the intracellular quantity of the reactive epoxide derivative will be a function of the levels of epoxide synthetase and hydrase enzymes.

Although the hydration of styrene-oxide has been employed as the basis for the hydrase assay in previous work (*7*), this reaction might not reflect an accurate assay of the formation of *trans*-dihydrodiol derivatives of polycyclic hydrocarbons. Accordingly, we have developed a relatively simple and sensitive gas chromatographic method in which the formation of the *trans*-11,12-dihydrodiol of 3-methylcholanthrene (3MC) is measured as a trimethylsilyl derivative. We have reported the gas chromatographic characteristics and the mass spectral patterns of the trimethylsilylated derivatives of the 11,-12-oxide and of the *cis*- and *trans*-11,12-dihydrodiols (*8*). We now report the details of the assay system.

Gas chromatography was conducted on an instrument (Nuclear-Chicago model 5000) containing a U-tube glass column (4 mm by 1.8 m) which was packed with Gas Chrom Q (100 to 120 mesh) coated with 3 percent OV-17 (Applied Science). The injection temperature was 335°C; the detector temperature was 255°C; the carrier gas was N_2, flowing at 60 ml/min. The chromatogram was programmed from 220° to 280°C at 3° per minute. The 11,12-oxide and *trans*-dihydrodiol derivatives were prepared by the procedure of Sims (*9*). Microsomes were prepared from livers of adult male Charles River rats after homogenization of the tissue in 0.25*M* sucrose (1 g of tissue per 5 ml of sucrose). The homogenate was centrifuged at 9,000*g* for 15 minutes at 0°C, and the supernatant was recentrifuged at 100,000*g* for 1 hour at 0°C. The

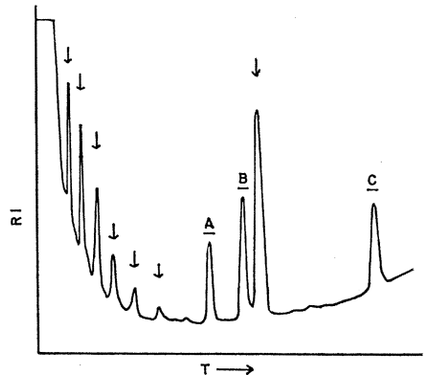

Fig. 1. Gas chromatogram of participants in the epoxide hydrase assay. The chromatogram was programmed from 220° to 280°C at 3° per minute. *A*, Silylated *trans*-diol; *B*, C_{32} standard [dotriacontane (Applied Science)]; *C*, silylated phenol (from the oxide). Rat liver microsomes (2.3 mg of protein) were first incubated in 0.1*M* sodium phosphate buffer, *p*H 8, in a total volume of 0.4 ml for 2 minutes at 37°C. The 11,12-oxide (50 μg in 0.05 ml of dimethyl sulfoxide) was then added, and the incubation mixture was shaken at 37°C for an additional 10 minutes. This amount of dimethyl sulfoxide had no effect on the enzyme activity. Boiled microsomes served as controls in this and all further experiments. After the incubation, the tubes were plunged into an ice bath to halt the reaction, and 2.0 ml of ethyl acetate was added. The polycyclic hydrocarbons were extracted with shaking for 10 minutes and the suspension was then centrifuged at 600*g* for 5 minutes. The organic solvent layer (top) was withdrawn, and the aqueous layer was reextracted with an additional 1.0 ml of ethyl acetate. The ethyl acetate extracts were combined, a 2.5-ml portion was removed, and the organic solvent was evaporated under nitrogen. The residue was washed with approximately 1 ml of anhydrous ethyl ether and the latter was also evaporated off under nitrogen. The remaining residue was trimethylsilylated under the following conditions: To each tube was added 20 μl of Tri-Syl (Pierce) and, after the resultant stoppered mixture was heated at 60°C for 30 minutes, 10 μl of *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (Pierce) was introduced. The solution was brought to a final volume of 70 μl by the addition of acetonitrile, and the derivatives were stored at 5°C for no longer than 12 hours. At this juncture thin-layer chromatography of the silylated derivative in a benzene-ethanol system (19 : 1) showed no underivatized diol present; then 5-μl portions were injected into the gas chromatograph for analysis. The ordinate is given as relative intensity (*RI*) and abscissa as increasing temperature (*T*). The derivatives are stable under these conditions.