from the CA1 zone should result in a $^3H/^{14}C$ ratio that is smaller for the CA1 fraction than that for the dentate fraction; that is, R_H should be less than R_D. This type of result was observed in five experiments, where a decrease in the cytoplasmic proteins for the potentiated section was between 26 and 61 percent. Experiments 16 to 19 give the results for the slices in which the dentate was potentiated through the perforant pathway. Again there was a substantial decrease for the cytoplasmic proteins of the potentiated region of the slice, between 26 and 63 percent. These results are consistent with the concept that enhanced secretion from the locus of potentiation gives rise to a decrease in the amount of labeled proteins remaining in the cytoplasmic fraction.

Our studies indicate that LTP is linked with the metabolic process that leads to protein synthesis in the slice preparation. A coupling between neurophysiological events and the local secretion of newly synthesized proteins is observable 2 hours after tetanizing trains are applied, suggesting that the biochemical changes remain long after the neurophysiological events have been completed. The development of LTP appears to be essential for the protein changes since nonspecific stimulation or stimulation without potentiation does not produce enhanced secretion. In this respect, LTP is similar to the effects of training on the pattern of protein synthesis in vivo (*8*) in the goldfish brain and focuses attention on the proteins that are released into the extracellular fluid.

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References and Notes

1. T. V. P. Bliss and T. Lømo, *J. Physiol. (London)* **232**, 331 (1973).
2. B. E. Alger and T. J. Teyler, *Brain Res.* **110**, 463 (1976).
3. P. A. Schwartzkroin and K. Wester, *ibid.* **89**, 107 (1975).
4. R. M. Douglas and G. V. Goddard, *ibid.* **86**, 205 (1975).
5. H. McIlwain, L. Buchel, J. D. Cheshire, *Biochem. J.* **48**, 12 (1951).
6. M. Browning, T. Dunwiddie, W. Bennett, W. Gispen, G. Lynch, *Science* **203**, 60 (1979).
7. V. E. Shashoua, *Brain Res.* **111**, 347 (1976).
8. ———, *ibid.* **166**, 349 (1979).
9. T. J. Teyler, in *Advances in Psychobiology*, vol. 3, *Neural Models of Behavioral Plasticity*, A. Riesen and R. F. Thompson, Eds. (Wiley, New York, 1976), pp. 301–326.
10. Changes in the synaptic efficacy were expressed as a percentage of the I-O determination before tetanus. Only slices that showed no changes in their population excitatory postsynaptic potential thresholds and significant potentiation (200 percent or more) at the 5- and 25-minute I-O determinations were considered valid for biochemical studies.
11. The crude extracellular fluid fraction was purified by centrifugation at 90,000*g* for 30 minutes at 0°C to yield a supernatant free from particulate matter. This was mixed with an equal volume of brain protein solvent buffer containing 2 percent sodium lauryl sarcosinate, 0.08*M* EDTA, and 5*M* urea in 0.02*M* phosphate buffer, *p*H 10. The mixture was incubated for 2 hours at 37°C to hydrolyze any labeled aminoacyl transfer RNA molecules that might be present and then dialyzed four times against 1 liter of 0.03*M* phosphate, *p*H 7.0, containing 1 m*M* nonradioactive valine, and twice against 1 liter of distilled water. Each dialysis step required 18 hours. The products were then concentrated to 0.1 ml, and 10-μl portions were used for the radioactivity determinations in a liquid scintillation counter. All products, including glycoproteins with molecular weight > 5000, are measured by this procedure.
12. Each experimental slice was homogenized with a control slice in 0.7 ml of 0.32*M* sucrose containing 1 m*M* unlabeled valine, in a Teflon glass homogenizer (0.05-mm gap) at 0°C with 31 strokes at 300 rev/min. The mixture was separated by centrifugation at 90,000*g* at 0°C for 30 minutes into a supernatant cytoplasmic fraction and a sediment containing the mixed membrane-nuclear fraction. The supernatant and sediment were mixed with an equal volume and 3 ml of brain protein solvent, respectively, and purified according to the procedure for the extracellular fluid fraction (*11*) for analysis of ^3H and ^{14}C content.
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Lysosomal Cathepsin B: Correlation with Metastatic Potential

Abstract. *Although lysosomal enzymes are implicated in the processes of tumor invasion and metastasis, their cellular origin within the tumor is unclear. The activity of the lysosomal proteinase cathepsin B is significantly elevated in a variant of the B16 melanoma with high metastatic potential. The cathepsin B activity is localized to the lysosomes of the tumor cells.*

Metastasis is the result of an intricate sequence of events initiated when the primary tumor cells invade normal tissue, detach, and disseminate to distant sites, and culminated when the neoplastic cells invade and establish secondary tumors. The malignancy of a tumor depends on its ability to invade normal tissue both at the primary and secondary sites of growth. Increases in lysosomal enzyme activities and in lysosomal enzyme release have been correlated with the ability of primary tumors to invade normal tissue as well as with the occurrence of metastatic tumors (*1–3*). Up to 11 times more of the lysosomal proteinase cathepsin B is released from malignant human breast tumors than from normal breast tissue or nonmalignant tumors (*1*). Further evidence linking cathepsin B to tumor malignancy is the positive correlation between serum cathepsin B levels and vaginal clear-cell adenocarcinoma (*4*). Because solid tumors contain heterogeneous cell types (macrophages, lymphocytes, and stromal cells as well as tumor cells), the cellular source of elevated or released enzymes has not been demonstrated definitively. Using the technique of centrifugal elutriation (*5*) to separate the actively growing tumor cells from other cell types dispersed from the solid tumor, we have determined the activities of five lysosomal enzymes in metastatic variants of the mouse B16 melanoma (*6*).

Variants of the B16 melanoma with low ($B16F_1$) and high ($B16F_{10}$) metastatic potential (*6*) were propagated in vivo by subcutaneous injection into syngeneic C57BL/6J male mice (Jackson Laboratory). The $B16F_{10}$ cells when injected intravenously into the tail vein of syngeneic mice consistently produced a greater number of metastatic foci in the lungs than did the $B16F_1$ cells (*7*). The lysosomal enzymes, cathepsin D, β-*N*-acetylglucosaminidase, β-glucuronidase, acid phosphatase, protein, and DNA were assayed fluorometrically as described by Sloane and Bird (*8*). Cathepsin B was assayed fluorometrically (*4*) with carbobenzyloxy-Ala-Arg-Arg-4-methoxy-β-naphthylamine (Enzymes Systems Products, Livermore, California) as substrate (Ala, alanine; Arg, arginine). Enzyme assays were standardized to ensure linearity. Subcutaneous $B16F_1$ and $B16F_{10}$ tumors (0.5 to 1.5 g) were removed, homogenized, and assayed for lysosomal enzyme activities. Cathepsin B activity was threefold higher in homogenates of the $B16F_{10}$ tumor than in those of the $B16F_1$ tumor (Table 1). Cathepsin B activity in these homogenates was inhibited more than 99 percent by the cysteine proteinase inhibitor leupeptin (1 μg/ml). No differences in activity were detected between the $B16F_1$ and $B16F_{10}$ tumors for cathepsin D, β-*N*-acetylglucosaminidase, β-glucuronidase, or acid phosphatase when expressed per gram of wet weight, per milligram of protein, or per microgram of DNA (not illustrated). The threefold elevation in cathepsin B activity in the $B16F_{10}$ tumor with its higher metastatic potential may simply reflect a difference in the macrophage content of the two tumors. Possible changes in the number of macrophages have been suggested as contributing to changes with age in lysosomal

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enzyme activity of Lewis lung carcinoma tumors (*2*). However, determination (latex bead uptake) of the percentage of macrophages in dispersed cells of $B16F_1$ and $B16F_{10}$ tumors revealed no significant difference (*7*).

Subcutaneous $B16F_1$ and $B16F_{10}$ tumors (1 to 3 g) were removed, and the cells were dispersed and separated into two fractions by centrifugal elutriation. The β fraction contained tumor cells that incorporate [^{3}H]thymidine and the α fraction contained macrophages, stromal cells, and tumor cells that do not incorporate [^{3}H]thymidine (*7*). When the two fractions were injected subcutaneously into syngeneic mice, only the β fraction cells produced tumors (*7*). Cathepsin B activity was again threefold higher in the dispersed cells of the $B16F_{10}$ tumors than in those of the $B16F_1$ tumors (Table 1). No significant difference in cathepsin B activity was found between the α fractions of the $B16F_1$ and $B16F_{10}$ tumors (Table 1). Most (90 percent) of the cathepsin B activity was found in the β fractions of the two tumors. Comparison of the β fractions of the $B16F_1$ and $B16F_{10}$ tumors revealed a fourfold higher activity of cathepsin B in the $B16F_{10}$ fraction (Table 1). The activity of β-*N*-acetylglucosaminidase, a marker enzyme for lysosomes (*9*), was twice as great in the β fraction of the $B16F_{10}$ tumor (1441 ± 99 n*M* 4-methylumbelliferone per microgram of DNA per minute) as it was in the $B16F_1$ tumor (641 ± 296 n*M*/μg-min). The content of the two enzymes in the $B16F_{10}$ and $B16F_1$ tumor cell lysosomes may differ, or, alternatively, the concentration of an endogenous inhibitor to cathepsin B may differ (*10*).

In contrast to the findings with cathepsin B, the activities of β-*N*-acetylglucosaminidase, β-glucuronidase, and acid phosphatase were evenly distributed between the α and β fractions of both $B16F_1$ and $B16F_{10}$ tumors. Cathepsin D activity was concentrated in the α fractions, perhaps reflecting the presence of residual bodies in the macrophages of the α fractions (*7, 11*).

This is definitive proof that the elevated activity of a lysosomal enzyme, the proteinase cathepsin B, originates from the tumor cells. Many studies have suggested that lysosomal proteinases and glycosidases may have elevated activities in tumors or that they may be released from tumors (*1–4, 12*) and also that activity of these lysosomal enzymes may be correlated with the invasive properties and onset of metastasis (*2*), but none of these studies identified the cellular source of the lysosomal enzymes.

It has been suggested that cathepsin B in human breast tumors might not be lysosomal in origin since its secretion differs from that of cathepsin D (*1*). Therefore, to determine the subcellular localization of cathepsin B in the $B16F_1$ and $B16F_{10}$ tumors, we fractionated 1 to 3 g of subcutaneous tumors by differential centrifugation (Fig. 1). The majority of lysosomes sedimented in the L fraction (*13, 14*), with larger autophagic and heterophagic vacuoles or residual bodies sedimenting in the M fraction (*13, 14*). In both the $B16F_1$ and $B16F_{10}$ tumors, the pattern of cathepsin B activity paralleled that of β-*N*-acetylglucosaminidase, β-glucuronidase, and cathepsin D.

Cathepsin B is a cysteine proteinase with significant activity at neutral *p*H (*1, 15*). Since cathepsin B can degrade pericellular protein (*16*), it is possible that cathepsin B release from tumor cells (*1, 4*) may contribute to detachment of the cells from the primary tumor and hence to metastasis. Two of the major constituents of the extracellular matrix, collagen and proteoglycan, can be degraded by cathepsin B (*15*), as can the major constituents of the venule wall, actin and myosin (*10*); thus cathepsin B may facilitate both invasion and extravasation. Ca-

Table 1. Comparison of cathepsin B activity between low ($B16F_1$) and high ($B16F_{10}$) metastatic melanoma variants. Cell suspensions from primary tumors were obtained by a modification of our procedure (*19, 20*). Cathepsin B activity is expressed as means ± standard error of 4-methoxy-β-naphthylamine formed per minute on the basis of wet weight, protein, or DNA content. A two-tailed Student's *t*-test was used; $N = 3$ in each case.

Metastatic melanoma variant	Amount of 4-methoxy-β-naphthylamine formed per minute		
	μ*M*/g wet weight	μ*M*/mg protein	n*M*/μg DNA
	Whole tumor		
$B16F_1$	607 ± 9	3.8 ± 0.2	226 ± 42
$B16F_{10}$	1596 ± 164*	11.8 ± 2.8†	664 ± 54*
	Dispersed tumor cells		
$B16F_1$		2.1 ± 0.4	84 ± 9
$B16F_{10}$		5.1 ± 1.1†	269 ± 28‡
	Elutriated α fraction		
$B16F_1$		0.8 ± 0.2	16 ± 5
$B16F_{10}$		1.7 ± 0.9	21 ± 10
	Elutriated β fraction		
$B16F_1$		2.6 ± 1.4	66 ± 37
$B16F_{10}$		9.3 ± 0.6§	260 ± 21§

*$P < .005$. †$P < .05$. ‡$P < .025$. §$P < .01$.

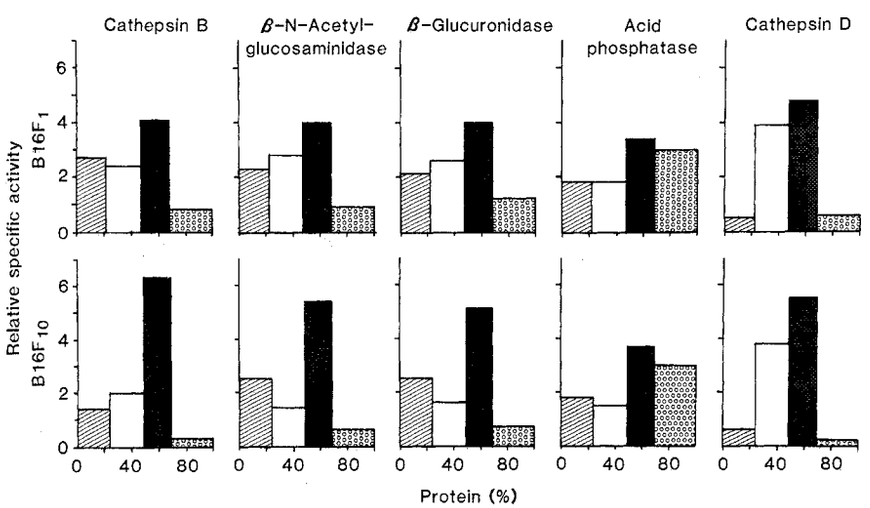

Fig. 1. Subcellular distribution of lysosomal enzymes in $B16F_1$ and $B16F_{10}$ melanoma variants. A $B16F_1$ tumor (upper graphs) and a $B16F_{10}$ tumor (lower graphs) were homogenized as described in Table 1 and subjected to differential centrifugation in a Beckman J2-21 centrifuge. Four fractions were separated as described in (*8*): (crosshatched bar) N, or nuclear fraction at 800*g* for 10 minutes; (white bar) M, or heavy mitochondrial fraction at 10,000*g* for 5 minutes; (black bar) L, or light mitochondrial fraction at 23,000*g* for 15 minutes; and (stippled bar) S, or supernatant. These four fractions were assayed fluorometrically for five lysosomal enzymes; the activity of the enzyme in each fraction is expressed as the relative specific activity versus the percentage of protein in each fraction (*13*). A relative specific activity greater than 1 indicates that the enzyme is purified in that fraction.

thepsin B also transforms a procollagenase found in bone to its active form (*17*). Liotta *et al.* (*18*) demonstrated that the activity of a collagenase specific for basement membrane collagen is twice as great in $B16F_{10}$ melanoma cells as it is in $B16F_1$ cells. Since maximal collagenase activity in vitro required trypsin treatment, the elevated cathepsin B activity in $B16F_{10}$ cells (Table 1) might activate the basement membrane collagenase in vivo, thus correlating it with the higher metastatic potential of $B16F_{10}$.

Cathepsin B activity, previously shown to be associated with human breast carcinoma (*1*) and vaginal adenocarcinoma (*4*), is a specific property of the tumor cell. In the mouse model, increased cathepsin B activity is correlated with increased metastatic behavior. These results warrant an investigation of lysosomal cathepsin B activity in human tumors with high metastatic potential. Cathepsin B may be a locus for therapeutic intervention in the metastatic cascade.

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References and Notes

1. A. R. Poole, K. J. Tiltman, A. D. Recklies, T. A. M. Stoker, *Nature (London)* **273**, 545 (1978); A. D. Recklies, K. J. Tiltman, T. A. M. Stoker, A. R. Poole, *Cancer Res.* **40**, 550 (1980).
2. L. Dobrossy, Z. P. Pavelic, M. Vaughan, N. Porter, R. J. Bernacki, *Cancer Res.* **40**, 3281 (1980).
3. A. Lage, J. W. Diaz, F. Hernandez, *Neoplasma* **26**, 57 (1979).
4. R. J. Pietras, C. M. Szego, C. E. Mangan, B. J. Seeler, M. M. Burtnett, M. Orevi, *Obstet. Gynecol.* **52**, 321 (1978).
5. R. J. Sanderson and K. E. Bird, *Methods Cell Biol.* **15**, 1 (1977); M. L. Meistrich, D. J. Grdina, R. E. Meyn, B. Barlogie, *Cancer Res.* **37**, 4291 (1977).
6. I. J. Fidler, *Nature (London)* **242**, 148 (1973); *Methods Cancer Res.* **25**, 399 (1978); *Cancer Res.* **38**, 2651 (1978).
7. B. F. Sloane, K. V. Honn, J. G. Sadler, W. A. Turner, J. J. Kimpson, J. D. Taylor, in preparation.
8. B. F. Sloane and J. W. C. Bird, *Am. J. Physiol.* **232**, E423 (1977).
9. T. J. Peters, M. Müller, C. de Duve, *J. Exp. Med.* **136**, 117 (1972).
10. W. N. Schwartz and J. W. C. Bird, *Biochem. J.* **167**, 811 (1977); J. W. C. Bird, J. H. Carter, R. E. Triemer, R. M. Brooks, A. M. Spanier, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 20 (1980).
11. W. Th. Daems, E. Wisse, P. Brederoo, in *Lysosomes: A Laboratory Handbook*, J. T. Dingle, Ed. (North-Holland, Amsterdam, 1972), p. 150.
12. B. Sylven, R. Ottoson, L. Revesz, *Br. J. Cancer* **13**, 551 (1959); B. Sylven, *Eur. J. Cancer* **4**, 463 (1968); H. B. Bosman and G. Z. Pike, *Life Sci.* **9**, 1433 (1970).
13. C. de Duve, B. C. Pressman, R. Gianetto, R. Wattiaux, F. Appelmans, *Biochem. J.* **60**, 604 (1955).
14. F. Appelmans, R. Wattiaux, C. de Duve, *ibid.* **59**, 438 (1955).
15. M. C. Burleigh, A. J. Barrett, G. S. Lazarus, *ibid.* **137**, 387 (1974).
16. B. Sylven, *Eur. J. Cancer* **4**, 559 (1968).
17. Y. Eeckhout and G. Vaes, *Biochem. J.* **166**, 21 (1977).
18. L. A. Liotta, S. Abe, P. Gehron Robey, G. R. Martin, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 2268 (1979); L. A. Liotta, K. Tryggvason, S. Garbisa, I. Hart, C. M. Foltz, S. Shafia, *Nature (London)* **284**, 61 (1980).
19. K. V. Honn, J. R. Dunn, L. R. Morgan, M. Bienkowski, L. J. Marnett, *Biochem. Biophys. Res. Commun.* **87**, 795 (1979).
20. Subcutaneous tumors were removed, diced, and placed in sterile Eagle's minimal essential medium (MEM) buffered with sodium bicarbonate (15 m*M*) and Hepes (25 m*M*). The MEM used for tumor cell dispersion contained collagenase type III (Worthington, 1 mg/ml; 174 U/mg), deoxyribonuclease I (Sigma, 50 μg/ml), soybean trypsin inhibitor (Worthington, 100 μg/ml), and fatty acid–free human serum albumin (Sigma, 10 mg/ml). Cells were dispersed (for 30 minutes and then for 60 minutes at 37°C) under air in a Dubnoff metabolic shaker (90 oscillations per minute). Supernatants were collected through cheesecloth and centrifuged (100*g* for 10 minutes); the pellets were resuspended in MEM and fractionated by centrifugal elutriation (*5*) into an α and a β fraction. Elutriation was accomplished with a Beckman JE6 elutriator rotor fitted with a Sanderson separation cell and operated (1300 rev/min) in a Beckman J2-21 centrifuge at 25°C. The separation medium (MEM) was pumped through the system with a Cole Palmer Master Flex pump (model 7014). The pump control was modified with a ten-turn potentiometer [W. D. Grant and M. Morrison, *Anal. Biochem.* **98**, 112 (1979)]. Monodispersed (> 99 percent) cells were introduced into the chamber and washed (3.3 ml/min) for 15 minutes to remove cellular debris and red blood cells. Fifty-milliliter fractions were then elutriated at flow rates of 3.7, 7.8, 9.5, 11.8, 30.0, and 43.5 ml/min. Each fraction was examined microscopically for composition. The first three fractions were designated α and the last three fractions were designated β. The viability (trypan blue dye exclusion) of β fraction cells was generally > 97 percent, with homogeneity > 99 percent. Whole tumors, dispersed cells, α fractions and β fractions were homogenized in 250 m*M* sucrose with 5 m*M* EDTA, *p*H 7.3, at 4°C with two 5-second bursts of a Tekmar homogenizer at full speed.
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Selective Protection of Methionine Enkephalin Released from Brain Slices by Enkephalinase Inhibition

Abstract. *Methionine enkephalin release was evoked by depolarization of slices from rat striatum with potassium. In the presence of 0.1 μM thiorphan [(N(R,S)-3-mercapto-2-benzylpropionyl)glycine], a potent inhibitor of enkephalin dipeptidyl carboxypeptidase (enkephalinase), the recovery of the pentapeptide in the incubation medium was increased by about 100 percent. A similar effect was observed with the dipeptide phenylalanylalanine, a selective although less potent enkephalinase inhibitor. Inhibition of other known enkephalin-hydrolyzing enzymes—aminopeptidase by 0.1 mM puromycin or angiotensin-converting enzyme by 1 μM captopril—did not significantly enhance the recovery of released methionine enkephalin. These data indicate that enkephalinase is critically involved in the inactivation of the endogenous opioid peptide released from striatal neurons.*

Whether specific inactivation mechanisms similar to those found in the well-known neurotransmitter synapses are responsible for turning off the signals conveyed by neuropeptides has not been established. Regarding the enkephalins Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu (Tyr, tyrosine; Gly, glycine; Phe, phenylalanine; Met, methionine; Leu, leucine), at least three well-characterized peptidases are candidates for this role in the central nervous system: an aminopeptidase (*1*), a peptidyl dipeptide hydrolase, angiotensin-converting enzyme (ACE) (*2*), and the recently characterized enkephalin dipeptidyl carboxypeptidase, enkephalinase (*3–8*). All three enzymes cleave the enkephalins into biologically inactive peptides—the first by releasing the NH_2-terminal Tyr residue, the others by hydrolyzing the Gly^3-Phe^4 amide bond. Enkephalinase may be more strategically located than the others, that is, in the vicinity of putative enkephalinergic synapses (*7, 9, 10*).

One approach to this kind of study consists in ascertaining whether selective inhibitors of the various enzymes protect the endogenous enkephalins released from their neuronal stores. Brain slices constitute a preparation in which the morphological organization is preserved and on which release of endogenous enkephalins by depolarizing agents has been demonstrated (*11, 12*). Inhibition of enkephalinase by the dipeptide Phe-Ala (Ala, alanine) or by thiorphan (*13*) enhances recovery of Met-enkephalin when its release from rat striatal slices is evoked by K^+, whereas selective inhibition of the two other peptidases does not provide such protection.

Selective inhibition of the Tyr-releasing enzyme activity can be obtained with puromycin, a compound that does not significantly affect either enkephalinase or ACE (*7*). Captopril (SQ14,225), a potent ACE inhibitor with an inhibition

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