Biochimica et Biophysica Acta, 614 (1980) 134—143

BBA 69019

CHARACTERIZATION OF A THIOL PROTEINASE SECRETED BY MALIGNANT HUMAN BREAST TUMOURS

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(Received October 30th, 1979)

Key words: Thiol proteinase; Secretion; (Mammary tumor)

Summary

It has previously been demonstrated (Poole, A.R., Tiltman, K.J., Recklies, A.D. and Stoker, T.A.M. (1978) Nature 273, 545—547) that malignant human breast tumours maintained in organ culture secrete elevated amounts of a thiol proteinase. This enzyme has been shown to possess enzymic properties similar to those of cathepsin B (EC 3.4.22.1) with respect to specificity, affinity and pH optima for synthetic substrates. However, the tumour enzyme is much more stable than human liver cathepsin B to inactivation above neutral pH, and it also has a larger molecular size and a more acidic isoenzyme pattern.

The stability of this enzyme under physiological conditions may allow it to play a role in tumour invasion and metastasis.

Introduction

In the pathology of cancer the two most critical steps are the invasion of the surrounding tissue by tumour cells and the metastatic spread of these cells to distant sites. Both these processes involve at least partial destruction of the connective tissue matrix surrouding the tumour and thus could be due to the action of proteinases released by the tumour tissues. Several proteinases, such as plasminogen activator [1], collagenase [2,3] and lysosomal proteinases [4—6] have been implicated in the destructive action of malignant tumours. Until recently no direct evidence for the active secretion of proteinases from neoplastic tissue was available.

Abbreviations: Bz-Arg-nA, α-*N*-Benzoyl-DL-arginine 2-naphthylamide; Cbz,α-*N*-benzyloxycarbonyl; -4-OMenA, 4-methoxy-β-naphthylamide; thus Cbz-Arg-Arg-4-OMenA, α-*N*-benzyloxycarbonyl-L-arginyl-L-arginine 4-methoxy-β-naphthylamide; Mes, 2-(*N*-morpholino)-ethanesulphonate.

Previous studies [7] have indicated that malignant human breast tumours maintained in organ culture, secrete high levels of a 'cathepsin B-like' enzyme into the culture medium, when compared to the amounts released by non-malignant tumours or normal tissue. This enzyme was described as 'cathepsin B-like' because it is a thiol proteinase and it hydrolyses the synthetic substrate Bz-Arg-nA. However, cathepsin B (EC 3.4.22.1) rapidly loses activity at and above neutral pH [8], hence active cathepsin B would not be expected to accumulate in organ culture medium maintained under physiological conditions (pH 7.4).

Further characterization of this tumour thiol proteinase has shown that although it is catalytically similar to human liver cathepsin B, it has different physical properties and is stable and retains activity at and above neutral pH.

Materials and Methods

Organ culture of tumour specimens. Human mammary adenocarcinoma specimens were obtained at surgery and established in organ culture as previously described [7]. Explants were maintained in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin, streptomycin 100 μg/ml and 5% adult sheep serum. The serum was inactivated at 56°C for 30 min and acid-treated according to Harpel [9] to inactivate α_2-macroglobulin, a major proteinase inhibitor [10]. In some cases serum was omitted from the culture medium. Culture media were replaced every 48 h, and thiol proteinase activity was determined using Bz-Arg-nA as substrate. Media rich in activity were pooled and stored frozen for further analysis. Pooled culture media were concentrated by ultrafiltration using a Millipore PSAC membrane.

Human liver cathepsin B. Cathepsin B was isolated from human liver essentially as described by Barrett [8] with the addition of isoelectric focusing in a Sephadex G-75 gel [11] as a final purification step. The major isoenzyme was used in these studies.

Materials. Bz-Arg-nA, β-naphthylamine, phenylmethylsulphonyl fluoride, iodoacetic acid, soya bean trypsin inhibitor, aprotinin and proteins used as molecular weight standards were from Sigma (St. Louis, MO, U.S.A.). Cbz-Ala-Arg-4-OMenA, Cbz-Arg-Arg-4-OMenA, Cbz-Gly-Gly-Arg-4-OMenA and 1-methoxy-3-naphthylamine were from Enzyme Systems Products (Indianapolis, U.S.A.); 4-amino-2'3-dimethylazobenzene was from United States Biochemical Corporation (Cleveland, U.S.A.); ampholine from LKB and agarose (Indubiose A45) from l'Industrie Biologique Française were obtained through Fisher Scientific (Montreal). Leupeptin, antipain, pepstatin and elastatinal were obtained through the generosity of the U.S.-Japan Medical Science Program. The *trans*-epoxy-succinate monobenzyl ester was kindly provided by Dr. K. Hanada, Taisho Pharmaceutical Co. Ltd., Japan. Purified human α_2-macroglobulin was a gift from Dr. A.J. Barrett, (Strangeways Laboratory, Cambridge, U.K.) and an antiserum was raised against this preparation in a rabbit.

Enzyme assay. Initial work on the tumour enzyme was carried out using the colorimetric assay described by Barrett [12] using 2 mM Bz-Arg-nA as substrate and an incubation volume of 0.8 ml. It was found however that, as seen with cathepsin B [13], this enzyme hydrolysed the substrates Cbz-Ala-Arg-Arg-

4-OMenA and Cbz-Arg-Arg-4-OMenA more readily. These substrates were therefore used in the characterization of the enzyme.

Studies with thiol reagents, inhibitors and kinetic analyses were carried out with serum-free explant medium and purified human liver cathepsin B. Enzyme dilutions were made in 0.05% (w/v) Brij 35 in distilled water to 50 μl and assays were in 0.05 M sodium Mes/1 mM EDTA/2 mM cysteine (pH 6.0). The incubation volume was 0.2 ml. Cbz-Arg-Arg-4-OMenA and Cbz-Ala-Arg-Arg-4-OMenA were used at a final concentration of 0.5 mM and were added as 1/40 of the assay volume in 5% (v/v) dimethyl formamide. Bz-Arg-nA was used at a final concentration of 2 mM and Cbz-Gly-Gly-Arg-4-OMenA at 0.5 mM. These compounds were dissolved in dimethylsulphoxide and added as above.

For investigation of the effects of thiol reagents and inhibitors, Cbz-Ala-Arg-Arg-4-OMenA was used as the substrate. In studies with the inhibitor iodoacetic acid and phenylmethylsulphonyl fluoride, the inhibitor was incubated with enzyme in 0.05 M sodium Mes/1 mM EDTA in the absence of cysteine for 15 min. Buffer containing cysteine (to give a final concentration fo 2 mM) and substrate was then added and the normal assay procedure followed. The phenylmethylsulphonyl fluoride was dissolved in ethanol which was present at a final concentration of 5% during preincubation. Control experiments demonstrated that this amount of ethanol had no measurable effect on enzyme activity.

Kinetic data were analysed using a non-linear least-squares computer programme derived according to Cleland [14].

Analytical isoelectric focusing. A 2 mm polyacrylamide slab gel containing 5% (w/v) acrylamide/12.5% (w/v) sucrose and equal quantities of pH 4—6 and pH 5—7 ampholine to a final concentration of 5% (v/v) was photopolymerized after addition of 0.3 μg/ml riboflavin. Cathepsin B and human liver homogenate were diluted to give activities similar to that of the tumour medium under investigation. Samples were dialyzed against 1% (w/v) glycine and applied to the gel on pieces of Whatman 3 MM filter paper. Electrophoresis was in an LKB Multiphor apparatus at constant power, reaching a final voltage of 1000 V after 3 h.

The gel was stained for activity using a modification of the method described by Barrett [8]. The gel was overlayed with 0.12 M sodium potassium phosphate (pH 5.5)/0.12 mM EDTA/1 mg/ml cysteine hydrochloride/2 mg/ml Cbz-Ala-Arg-Arg-4-OMenA (initially dissolved at 20 mg/ml in 5% (v/v) dimethyl formamide). After 30 min incubation at 37°C, the gel was immersed in the colour reagent described by Barrett [8], containing double the amount of freshly-diazotized 4 amino-2′,3-dimethylazobenzene, but no Brij 35. After colour had developed the gel was washed with water.

The pH gradient was determined by cutting a 1 cm wide strip of unstained gel in 0.5 cm sections. These were homogenized in 1 ml aliquots of degassed, deionized water and the pH of the resulting solution was measured.

Gel filtration. A Sephadex G-75 superfine column (1.5 × 85 cm) was equilibrated with 50 mM sodium acetate (pH 5.5)/200 mM NaCl/1 mM EDTA and eluted with the same buffer at 10 ml/h at 4°C. The column was calibrated with a mixture comprising Blue Dextran 2000 (void volume, V_0)/ovalbumin (M_r 43 000)/chymotrypsinogen (M_r 25 700)/cytochrome *c* (M_r 11 700) (molec-

ular weights were taken from Weber and Osborne [15]). Total bed volume (V_t) was estimated using potassium ferricyanide.

Endopeptidase demonstration. Tumour enzyme partially purified by gel filtration on a Sephadex G-75 was incubated at 4°C with 1 mg/ml α_2-macroglobulin in 50 mM sodium acetate/200 mM NaCl/1 mM EDTA, containing 2 mM cysteine. After 1 h the mixture was chromatographed as described above and the fractions assayed using Cbz-Ala-Arg-Arg-4-OMenA.

Immunological identification of fractions containing α_2-macroglobulin was carried out by double immunodiffusion in 1% agarose containing 20 mM sodium phosphate (pH 7.0)/0.15 M NaCl/0.05% (w/v) NaN_3.

Results

pH stability

Samples of tumour medium and human liver cathepsin B were preincubated at fixed pH at 40°C and the activity remaining was determined at various intervals (Fig. 1). While the liver enzyme lost almost all activity at pH 7.0 in 30 min, in a simple exponential decay, the tumour enzyme was stable at pH 7.0 and lost little activity even at pH 8.0. This enzyme was thus stable under tissue culture conditions, as illustrated by the finding that medium from 48 h of culture gave twice the activity of medium collected after 24 h of culture.

The possibility that carcinomas secrete a stabilizer of cathepsin B was investigated by mixing liver cathepsin B with tumour medium (the amounts of the two components being adjusted to give comparable activities). The combined enzymes were preincubated at pH 5.5 for 30 min then at pH 8.0 as above. After 15 min, only activity corresponding to that from the tumour medium remained. Similarly, preincubation of tumour medium and cathepsin B at physiological pH gave no increase in stable activity. Therefore it must be concluded that if a stabilizer does exist it is not secreted in excess by carcinoma tissue.

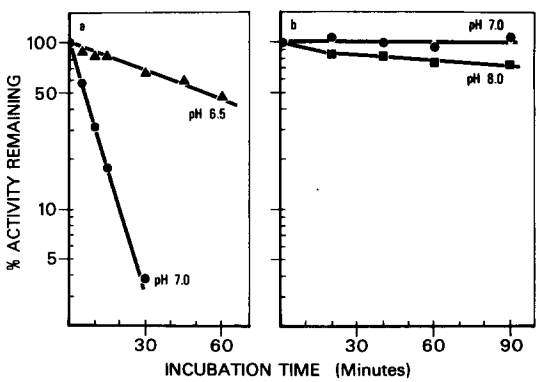

Fig. 1. pH stability of human liver cathepsin B and tumour thiol proteinase. Aliquots (25 µl) of (a) purified human liver cathepsin B, diluted into Dulbecco's modified Eagle's medium containing 5% heat-inactivated acid-treated normal sheep serum and buffered to pH 6.0 with 20 mM sodium Mes or (b) medium from carcinoma explants containing serum as above, were preincubated with 175 µl 0.1 M sodium phosphate, without cysteine at the indicated pH for varying times, after which 0.6 ml of incubation buffer was added and activity assayed with 0.5 mM Cbz-Ala-Arg-Arg-4-OMenA.

Molecular size

A sample of concentrated tumour medium was chromatographed on a calibrated Sephadex G-75 superfine column (Fig. 2). A single symmetrical peak of activity was eluted at a position earlier than that found for liver cathepsin B. The difference in molecular weight between these two species was estimated at 8000. Concentrates of medium from tumours cultured with or without serum and from different patients gave similar profiles.

Pattern on isoelectric focusing

Samples of concentrated tumour medium were compared with pure cathepsin B and homogenates of human liver by analytical isoelectric focusing in a polyacrylamide slab gel (Fig. 3). On staining the gel for activity, using the synthetic substrate Cbz-Ala-Arg-Arg-4-OMenA, the isoenzyme pattern due to the tumour medium clearly showed three species, all more acidic than the predominant activity found for human liver homogenate and the major cathepsin B isozyme. Similar patterns were obtained with tumour media from different patients. Human liver does however seem to contain a minor activity with a p*I* similar to that of the middle isoenzyme in the tumour medium.

Characterization of the tumour enzymes as a thiol endopeptidase

Endopeptidase demonstration. The enzyme was demonstrated as a true endopeptidase, rather than a naphthylamidase, by its ability to bind to α_2-macroglobulin. This high molecular weight proteinase inhibitor has been shown to bind all endopeptidases tested while exopeptidases do not bind [10,16]. Although proteinases bound to α_2-macroglobulin are inactive with protein substrates, the complexes usually retain activity with small molecular weight substrates [17]. When the tumour proteinase was incubated with pure α_2-macroglobulin and the mixture chromatographed on a Sephadex G-75 superfine most of the activity, tested with Cbz-Ala-Arg-Arg-4-OMenA, eluted in the void volume, as would be expected for a complex with this large inhibitor. α_2-Macroglobulin was identified at the void volume by double immunodiffusion analyses of the column fractions against a specific antiserum to α_2-macroglobulin. Control experiments demonstrated that the α_2-macroglobulin preparation itself had no effect on the substrate.

Thiol requirement. Sulphydryl reagents were required for enzyme activity. Of the reagents tested, at a thiol concentration of 2 mM, cysteine permitted the highest proteinase activity. Activities in the presence of dithiothreitol, reduced glutathione and 2-mercaptoethanol were 89, 56 and 36%, respectively, of that obtained with cysteine. In the absence of thiol, only 5% of this maximum value was seen.

Inhibitors. The susceptibility of the tumour proteinase to inhibitors also served to demonstrate its thiol dependence. Assay without cysteine in the presence of 0.1 mM *p*-chloromercuribenzoate abolished the small amount of activity detected without thiol reagent. Preincubation with 1 mM iodoacetate inactivated the enzyme irreversibly. The microbial inhibitors leupeptin and antipain gave 84 and 63% inhibition respectively, when present at 1 μg/ml. These compounds are very effective inhibitors of cathepsin B but are not specific for thiol proteinases [18]. A derivative of the E64 thiol proteinase

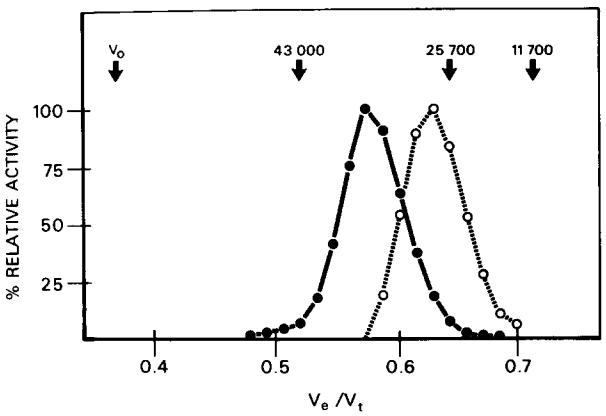

Fig. 2. Molecular size estimation of tumour thiol proteinase. Medium from carcinoma explants (5 ml) was concentrated to 0.25 ml by ultrafiltration, mixed with 0.5 ml 1 mg/ml bovine serum albumin then chromatographed on a Sephadex G-75 superfine calibrated column. Fractions were assayed for activity with Cbz-Ala-Arg-Arg-4-OMena, ●——●. Purified liver cathepsin B (0.4 mg) was chromatographed separately on the same column, ○- - - - -○. Arrows show elution positions of the calibration standards.

inhibitor, *trans*-epoxysuccinate monobenzyl ester, which is thought to be a specific thiol proteinase inhibitor [19,20] gave 45% inhibition at 4 μM (1 μg/ml). Inhibitors of carboxyl proteinases (pepstatin, 1 μg/ml) and serine proteinases (1 mM phenylmethylsulphonyl fluoride, 1 μg/ml elastatinal; 0.1 mg/ml soya bean trypsin inhibitor, and 0.1 mg/ml aprotinin) did not inhibit the enzyme. The omission of EDTA from the assay did not cause any increase in activity.

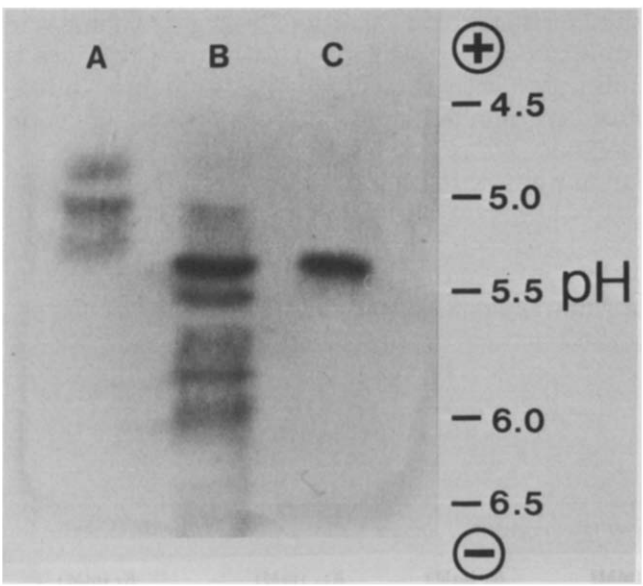

Fig. 3. Analytical isoelectric focusing. (A) tumour thiol proteinase, (B) human liver homogenate, (C) purified human liver cathepsin B. The gel was stained for activity using Cbz-Ala-Arg-Arg-4-OMenA as substrate.

TABLE I

RELATIVE ACTIVITIES OF TUMOUR THIOL PROTEINASE AND CATHEPSIN B WITH SYNTHETIC SUBSTRATES

Activities are all relative to that obtained with 2 mM Bz-Arg-na. Cbz-Gly-Gly-Arg-4-OmenA was assayed with 50 mM sodium phosphate/1 mM EDTA/2 mM cysteine, pH 5.5 for 16 h, and the other substrates were assayed with 50 mM sodium Mes/1 mM EDTA/2 mM cysteine, pH 6.0, for 2 h.

Substrate (0.5 mM)	Tumour thiol proteinase	Human liver cathepsin B
Cbz-Arg-Arg-4-OMenA	21.1	20.5
Cbz-Ala-Arg-Arg-4-OMenA	20.3	20.7
Cbz-Gly-Gly-Arg-4-OMenA	0.12	0.10

Substrate properties

The tumour enzyme was routinely assayed using the synthetic substrate Bz-Arg-nA. The pH optimum with this compound is pH 5.5, which is the same as that for purified human liver cathepsin B. Significantly more activity was found in the region pH 6.5—7, which probably reflects the increased stability of the tumour enzyme.

As cathepsin B has been shown to have a high affinity for substrates containing two adjacent arginine residues [13], the action of the tumour enzyme on two of these substrates was investigated (Table I). The rates of hydrolysis of Cbz-Arg-Arg-4-OMenA and Cbz-Ala-Arg-Arg-4-OMenA at 0.5 mM (which was the concentration giving maximal activity) were approx. 20-fold higher than compared with Bz-Arg-nA at 2 mM (solubility limit). The increased activities seen with these substrates were similar in magnitude to those found with liver cathepsin B under the same conditions.

The action of the tumour thiol proteinase on Cbz-Gly-Gly-Arg-4-OMenA was also studied. Trypsin [21], urokinase and plasminogen activator [22] have a high affinity for synthetic substrates with the Gly-Gly-Arg-sequence, unlike cathepsin B [13]. The tumour enzyme resembled cathepsin B in its poor activity on this substrate (Table I).

A kinetic analysis of the tumour enzyme using the more specific substrates

TABLE II

KINETIC CONSTANTS FOR TUMOUR THIOL PROTEINASE AND CATHEPSIN B

Each enzyme was assayed at pH 6.0 through the substrate range 0.045 mM—2.7 mM. The data were fitted to the equation,

$$V = \frac{VS}{K_m + S + S^2/K_i}$$

Kinetic constants ± S.E.

Substrate	Tumour thiol proteinase		Cathepsin B	
	K_m (mM)	K_i (mM)	K_m (mM)	K_i (mM)
Cbz-Ala-Arg-Arg-4-OMenA	0.38 ± 0.03	8.6 ± 1.5	0.21 ± 0.01	6.0 ± 0.7
Cbz-Arg-Arg-4-OMenA	0.19 ± 0.01	9.9 ± 1.9	0.105 ± 0.003	6.1 ± 0.3

Cbz-Ala-Arg-Arg-4-OMenA and Cbz-Arg-Arg-4-OMenA revealed in both cases a fall in activity at high substrate concentrations, indicative of substrate inhibition. This has been reported for cathepsin B [13]. The K_m values for both substrates were found to be slightly higher for the tumour enzyme than for cathepsin B (Table II). However, this could be due to the protein present in the tumour medium acting as a competitive substrate. An example of such an effect is illustrated by the finding that the apparent K_m for Cbz-Arg-Arg-4-OMenA increases almost 3-fold when purified liver cathepsin B is assayed in the presence of 0.25 mg/ml bovine serum albumin.

Discussion

The above results clearly demonstrate that the proteinase secreted in increased amounts by malignant breast tumours [7] is thiol dependent. Of the several tissue thiol proteinases now known, most can be clearly differentiated from the tumour enzyme. Cathepsins L [23] and N (collagenolytic cathepsin) [24] show no activity with the synthetic substate Bz-Arg-nA. Cathepsin H hydrolyses Bz-Arg-nA and leucine naphthylamide which illustrates respectively the endo- and exopeptidase character for this proteinase [25]. Tumour medium was found to contain a low level of activity with leucine naphthylamide but this was not cysteine dependent (unpublished data), and hence is not due to cathepsin H. Neutral thiol proteinases have been found in tissue undergoing inflammatory reactions [26] but their properties, particularly molecular weights, are quite different to that of the tumour thiol proteinase. Furthermore, explants from inflamed human synovia did not release activity comparable to the tumour thiol proteinase (Recklies, A.D. and Poole, A.R., unpublished data).

Pietras et al. [27,28] have described an activity in the serum of women with various neoplastic diseases which hydrolyses Cbz-Ala-Arg-Arg-4-OMenA. The release of 4-methoxynaphthylamine was followed fluorometrically and was found to be inhibited by leupeptin. It seems likely that the thiol proteinase secreted by tumours may accumulate in the blood in vivo. In its enzymic characteristics the similarity of the tumour enzyme to cathepsin B is striking in that the substrate specificities and kinetic constants for Cbz-Ala-Arg-Arg-4-OMenA and Cbz-Arg-Arg-4-OMenA show little difference. In their stabilities above neutral pH and their molecular sizes, however, these enzymes are quite different. Several explanations of these differences are possible. The tumour proteinase may be a separate enzyme species or it could be a complex between cathepsin B and an additional macromolecule that is acting as a stabilizing agent. This latter possibility is rendered unlikely by the lack of the ability of tumour medium to stabilize cathepsin B although it could be argued that excess stabilizer may not be present in this medium. It is also possible that the tumour thiol proteinase is a precursor of cathepsin B which before processing is stable at neutral pH. Studies on the secretion of the tumour proteinase indicate however, that very little enzyme is released from non-viable tissue and secretion halts rapidly after blockage of protein synthesis with cycloheximide [29], suggesting that the enzyme is synthesized for export. Also, homogenates of normal breast tissue and of malignant tumour tissue contain comparable amounts of

cathepsin B activity [7] which like liver cathepsin B is unstable at physiological pH, but only the latter tissue secretes the stable thiol proteinase in culture. Thus, it is unlikely that the stable enzyme is a precursor of lysosomal cathepsin B. The tumour enzyme is also different from cathepsin B in its pattern on isoelectric focusing. However, in homogenates of human liver an activity with a p*I* similar to that of the middle tumour isoenzyme is present. It is possible that human liver contains trace amounts of an activity similar to that secreted by carcinomas but negligible cathepsin B-like activity remains in this tissue after pH inactivation.

Further characterization of the tumour enzyme and the resolution of these points will require a means of producing much larger quantities of the proteinase. Due to the small amounts of material available so far, techniques for studying the proteinase must depend on monitoring the enzyme activity. This severely limits the properties amenable to characterization.

The stability of the tumour thiol proteinase would favour its accumulation at extracellular sites. This together with the enhanced secretion of the thiol proteinase from malignant tissues would be compatible with a role for this enzyme in the expression of malignancy.

Acknowledgements

The authors thank Dr. W. Duguid, Chief Pathologist, Montreal General Hospital for supplying surgical specimens and Chantal Barbeau for expert technical assistance. We are also grateful to Dr. K. Hanada for the gift of the E64 derivative. This work was supported by the National Cancer Institute of Canada and the Shriners of North America.

References

1 Reich, E. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B. and Shaw, E., eds.), pp. 333–353, Cold Spring Harbor Press, Cold Spring Harbor
2 Dabbous, M.K., Roberts, A.N. and Brinkley, S.B. (1977) Cancer Res. 37, 3537–3544
3 Liotta, L.A., Ave, S., Robey, P.G. and Martin, G.R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2268–2272
4 Sylvén, B. (1968) Eur. J. Cancer 4, 463–474
5 Weiss, L. (1978) Int. J. Cancer 22, 196–203
6 Poole, A.R. (1978) in Lysosomes in Biology and Pathology (Dingle, J.T., ed.), Vol. 3, pp. 303–337, Elsevier/North-Holland, Amsterdam
7 Poole, A.R., Tiltman, K.J., Recklies, A.D. and Stoker, T.A.M. (1978) Nature 273, 545–547
8 Barrett, A.J. (1973) Biochem. J. 131, 809–822
9 Harpel, P.C. (1976) Methods Enzymol. 45, 639–652
10 Starkey, P.M. and Barrett, A.J. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A.J., ed.), pp. 663–696
11 Radola, B.J. (1974) Biochim. Biophys. Acta 386, 181–195
12 Barrett, A.J. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A.J., ed.), pp. 181–208
13 McDonald, J.K. and Ellis, D. (1976) Life Sci. 17, 1269–1276
14 Cleland, W.W. (1967) Adv. Enzymol. 29, 1–32
15 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
16 Barrett, A.J. and Starkey, P.M. (1973) Biochem. J. 133, 709–724
17 Haverback, B.J., Dyce, B., Bundy, H.F., Wirtschafter, S.K. and Edmondson, H.A. (1962) J. Clin. Invest. 41, 972–980
18 Umezawa, H. and Aoyagi, T. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A.J., ed.), pp. 637–662, Elsevier/North-Holland, Amsterdam
19 Hanada, K., Tarnai, M., Morimoto, S., Adachi, T., Ohmura, S., Sawada, J. and Tanaka, I. (1978) Agric. Biol. Chem. 42, 537–541

20 Inaba, T., Hirayama, Y. and Fujinaga, N. (1979) Agric. Biol. Chem. 43, 655—656
21 Nachlas, M.M., Plapinger, R.E. and Seligman, A.M. (1964) Arch. Biochem. Biophys. 108, 2566—274
22 Zimmerman, M., Quigley, J.P., Ashe, B., Dorn, C., Goldfarb, R. and Troll, W. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 750—753
23 Kirschke, H., Langner, J., Wiederanders, B., Ansorge, S. and Bohley, P. (1977) Eur. J. Biochem. 74, 293—301
24 Evans, P. and Etherington, D.J. (1978) Eur. J. Biochem. 83, 87—97
25 Kirschke, H. (1977) Acta Biol. Med. Ger. 36, 1547—1548
26 Hayashi, H. (1975) Int. Rev. Cytol. 40, 101—151
27 Pietras, R.J., Szego, C.M., Mangan, C.E., Seeler, B.J., Burtnett, M.M. and Orevi, M. (1978) Obstet. Gynecol. 52, 321—327
28 Pietras, R.J., Szego, C.M., Mangan, C.E., Seeler, B.J. and Burtnett, M.M. (1979) Gynecol. Oncol. 7, 1—17
29 Recklies, A.D., Tiltman, K.J., Stoker, T.A.M. and Poole, A.R. (1980) Cancer Res. 40, 550—556