Bimane-labelled pepstatin, a fluorescent probe for the subcellular location of cathepsin D

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(Received 26 May 1981/Accepted 3 July 1981)

1. Pepstatinyl-cystamine was synthesized. The disulphide bond was cleaved and the pepstatin-bound thiol group was made to react with monobromobimane. The fluorescent *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol was purified. 2. Human cathepsin D showed tight binding of the bimane-labelled pepstatin at pH 3.5. The titration curves were used to determine the apparent dissociation constant, K_D ; values of approx. 1×10^{-10} M were obtained. 3. Gel-chromatographic experiments showed that, like that of pepstatin, the binding of *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol to cathepsin D was strongly pH-dependent. Binding was seen at pH 5.0, but could not be demonstrated at pH 7.4. 4. Cultured human synovial cells were fixed and incubated with the fluorescent inhibitor at pH 5.0 or pH 7.4. When examined by fluorescence microscopy the cells stained at pH 5.0 showed a punctate perinuclear distribution of bimane fluorescence. By contrast, the cells stained at pH 7.4 showed no fluorescence. 5. The distribution of cathepsin D, determined by indirect immunofluorescence at pH 7.4, closely resembled that of the fluorescent inhibitor seen at pH 5.0. 6. We conclude that *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol is a fluorescent probe selective for the active conformation of cathepsin D.

We have proposed (Knight *et al.*, 1980) a new method for the subcellular location of proteinases that may be active under the conditions existing in living tissues. Briefly, the method employs the enzyme-antibody capture technique developed by Poole *et al.* (1973, 1974) to immobilize and accumulate the proteinase as insoluble immune complexes at the sites of release in the living tissue. These complexes are then treated *in situ* with a tight-binding, or irreversible, active-site-directed inhibitor carrying a 'reporter' group. Since such inhibitors require the native active enzyme structure for efficient interaction (Knight, 1977), the binding of the inhibitor, assuming no non-specific interactions, demonstrates the presence of active enzyme in the immune complex.

In our initial studies (Knight *et al.*, 1980) we analysed the interaction between cathepsin D (EC

3.4.23.5) and dinitrophenyl(Dnp)-labelled derivatives of the tight-binding inhibitor pepstatin [isovaleryl-L-valyl-L-valyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid]. The bi-functional *N*-pepstatinyl-*N'*-Dnp-1,6-diaminohexane was able to bind cathepsin D and fluorescein-labelled antibody to Dnp simultaneously. Because the cathepsin D-pepstatin interaction is stoichiometric (Knight & Barrett, 1976), it is probable that subcellular location of enzyme via the binding of fluorescent anti-Dnp antibody will be a more sensitive method than the direct localization of a fluorescent inhibitor, especially since the commonly employed fluorophores are readily photobleached (Nairn, 1969). This quenching phenomenon can only be overcome by employing multiply-labelled antibodies.

However, Kosower *et al.* (1978) have developed a new class of fluorescent labelling agents, the bromobimanes (Kosower & Pazhenchevsky, 1980). These reagents react with thiol groups to give highly fluorescent products, which are resistant to fading under conditions of continuous irradiation (Kosower *et al.*, 1979, 1980), such as those employed in fluorescence microscopy (Gainer & Kosower,

Abbreviations used: bimanyl, 3,6,7-trimethyl-4-methylene-1,5-diazabicyclo-[3.3.0]octa-3,6-diene-2,8-dione; Dnp, dinitrophenyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate.

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1980). We report below the synthesis and inhibitory properties of a bimane-labelled pepstatin and describe its application to the subcellular location of lysosomal cathepsin D in cultured human synovial cells.

Materials and methods

Biochemical reagents

Pepstatin was from Protein Research Foundation, Osaka, Japan. Benzotriazol-1-yloxytris(dimethyl-amino)phosphonium hexafluorophosphate (BOP) (Castro *et al.*, 1976) was kindly given by Dr. G. Evin, INSERM Unité 36, Paris, France. Cystamine dihydrochloride was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Dithiothreitol and monobromobimane (4-bromomethyl-3,6,7-trimethyl-1,5-diazabicyclo-[3.3.0]octa-3,6-diene-2,8-dione) were from Calbiochem-Behring Corp., Bishops Cleeve, Loughborough, Leics., U.K. Cathepsin D from human liver was kindly given by Dr. A. J. Barrett of this laboratory. All other reagents were obtained from commercial sources and were of the purest grades available. Sephadex LH-20 and G-100 were from Pharmacia, Hounslow, Middx., U.K. AG-50W-X2 resin (H^+ form; 200–400 mesh) was from BioRad Laboratories, Bromley, Kent, U.K. Silica-gel sheets (Merck 5717) for preparative layer chromatography were from BDH Chemicals, Poole, Dorset, U.K.

Synthesis of *N*-pepstatinyl-cystamine

Pepstatin (10 mg, $14.7 \mu\text{mol}$) and cystamine dihydrochloride (33.5 mg, $150 \mu\text{mol}$) were dissolved in dimethyl sulfoxide/dimethylformamide (5:1, v/v; 1.2 ml). Triethylamine (64 μl , $460 \mu\text{mol}$) and BOP (13 mg, $29 \mu\text{mol}$) were added. After the mixture had been stirred at 21°C for 24 h, the solvent was evaporated at 40°C *in vacuo*. The residue was dissolved in ethanol (1 ml), and applied to a column (1 cm $\times$ 60 cm) of Sephadex LH-20 and eluted with ethanol (12 ml/h). Fractions (1 ml) representing the first ninhydrin-positive peak eluted from the column were combined and evaporated to dryness (9.5 mg), the residue was redissolved in aq. 50% (v/v) methanol (1.5 ml) and the solution was applied to a column (1 cm $\times$ 10 cm) of AG-50W-X2 (triethylammonium form). The column was washed with aq. 50% (v/v) methanol (65 ml), and *N*-pepstatinyl-cystamine was eluted with methanol/water/triethylamine (9:9:2, by vol.). The ninhydrin-positive fractions were evaporated to dryness at 40°C *in vacuo* to give a colourless solid. *N*-Pepstatinyl-cystamine (yield: 6 mg, 50%) was homogeneous by t.l.c. on silica gel (R_F 0.6; methanol) and was used without further purification.

Synthesis of *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol

N-Pepstatinyl-cystamine (3 mg, $3.7 \mu\text{mol}$) in methanol (0.5 ml) was added to dithiothreitol (0.56 mg, $3.7 \mu\text{mol}$) in 20 mM-sodium borate buffer, pH 8.0 (0.5 ml). The mixture was stirred for 30 min at 21°C , and monobromobimane (5 mg, $18 \mu\text{mol}$) in acetonitrile (0.2 ml) was added. After the mixture had been left in darkness at 21°C for 48 h, the solvent was evaporated at 40°C *in vacuo*. The residue was dissolved in ethanol (1 ml), and was applied to a column (1 cm $\times$ 60 cm) of Sephadex LH-20 and eluted with ethanol (12 ml/h). A portion (1 μl) of each fraction (1 ml) was taken for the assay of cathepsin D inhibition (Knight & Barrett, 1976). Inhibitory fractions that fluoresced under u.v. light (365 nm) were combined and evaporated to yield a yellow solid (1.5 mg). This material was dissolved in methanol (0.2 ml) and applied to a silica-gel preparative layer chromatography plate, which was developed in methanol. The major yellow and u.v.-fluorescent band was scraped off and eluted with ethyl acetate/ethanol (2:1, v/v; 2 ml). The solvents were removed at 40°C *in vacuo* to leave *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol (0.65 mg, 19%). This material was ninhydrin-negative and homogeneous by t.l.c. (R_F 0.80, methanol; R_F 0.20, butan-1-ol). It was used without further purification. Fluorescence excitation and emission spectra were recorded with a Farrand mark 1 spectrofluorimeter.

Inhibitor titrations with cathepsin D

Cathepsin D activity was measured under the standard assay conditions as described by Barrett (1970), with approx. 1 unit of activity and a 20 min incubation time. One unit of activity is defined as that producing an increase in absorbance (ΔA_{280}) of 1.0 in the assay during 60 min under the specific conditions, a linear response being assumed. Inhibitor titrations were made as described previously (Knight & Barrett, 1976; Knight *et al.*, 1980).

Chromatography of cathepsin D-*N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol complexes on Sephadex G-100

Gel-filtration experiments were made at pH 5.0 and pH 7.4 with a column (1.5 cm $\times$ 26 cm, 46 ml) of Sephadex G-100 eluted with the appropriate buffer at 4°C . The buffering components were 50 mM-sodium acetate/acetic acid at pH 5.0 and 50 mM- $\text{NaH}_2\text{PO}_4/\text{NaOH}$ at pH 7.4. Both buffers contained 0.1 M-NaCl and 0.1% Brij 35. Cathepsin D (50 units, 1.37 nmol) and *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol (0.32 μg , 0.34 nmol) were mixed in

elution buffer (1ml) and left at 21°C for 10min before being applied to the column. The flow rate was 15ml/h and 0.8ml fractions were collected. A portion (0.1ml) of each fraction was taken for assay of cathepsin D activity. A further portion (0.7ml) was taken for the measurement of bimeane fluorescence. The elution characteristics of the column were determined with standards of known molecular weight as described by Knight *et al.* (1980).

Materials for cell culture

Plastic Petri dishes and flasks were from Sterilin, Teddington, Middx., U.K. Dulbecco's modified Eagle's medium and foetal calf serum were from GIBCO, Uxbridge, Middx., U.K. Collagenase type V and bovine trypsin type I were from Sigma Chemical Co., Poole, Dorset, U.K.

Cell culture

Human synovial cells were prepared from sterile biopsy tissue obtained from the Department of Orthopaedic Surgery, New Addenbrooke's Hospital, Cambridge, U.K. Minced synovium (0.5g) was placed in Dulbecco's modified Eagle's medium (10ml) containing collagenase type V (1mg/ml) at 37°C in an atmosphere of CO₂/air (1:19). After incubation overnight tissue fragments were removed. Clumps of cells were centrifuged at 350g for 5min, resuspended in fresh medium (1ml) and centrifuged again. This procedure was repeated twice, and the final cell pellet was resuspended in medium (2ml) containing bovine trypsin type I (0.25mg/ml) and 1mM-EDTA (sodium salt). After incubation for 30min at 37°C, the trypsin was inactivated by adding an equal volume of medium containing 20% (v/v) heat-inactivated foetal calf serum. The cell suspension (approx. 1×10^5 cells/ml) was plated on acid-etched glass coverslips (2.2cm $\times$ 2.2cm) at a density of 1×10^5 cells/cm² and cultured at 37°C in CO₂/air (1:19) in medium containing 10% (v/v) foetal calf serum. Confluent cultures were obtained in 7–10 days.

Staining with N-pepstatinyl-S-bimanyl-2-aminoethanethiol

Confluent cultures of human synovial cells were rinsed three times in serum-free Dulbecco's modified Eagle's medium. The cultures were divided into two groups. Fixation and staining procedures were made with the first group at pH5.0 with 50mM-acetate buffer and with the second group at pH7.4 with 50mM-phosphate buffer. Both buffers contained 0.1M-NaCl and 0.1% Brij 35. The cells were fixed for 3min at 21°C in paraformaldehyde (4%, w/v) and then washed for 30min in several changes of buffer at the same pH value.

After rinsing the coverslips were immersed in *N*-pepstatinyl-S-bimanyl-2-aminoethanethiol (1μM) at either pH5.0 or 7.4 and incubated in a moist atmosphere for 30min at 21°C. The cells were then briefly rinsed in the corresponding buffer and mounted in glycerol.

Fluorescence microscopy

The cells were examined in a Leitz Ortholux fluorescence microscope equipped with a 200W mercury vapour lamp. Bimeane fluorescence was excited between 360 and 400nm by using a Leitz BG12 and a Balzers K1 filter combination. Optimal bimeane fluorescence was observed with the use of a Leitz Ploem 2 emission filter complex with a 460nm bandpass filter. All observations were recorded on Kodak Tri-X, or on Ektachrome 400 film that was uprated to ASA 1600.

Immunohistochemical subcellular location

Human synovial cells were fixed with paraformaldehyde at pH7.4 in the phosphate-buffered saline as described above. After being washed three times with the same buffer the cells were delipidated in acetone at -20°C for 5min in order to enhance the permeability of the lysosomal membranes for the antibodies. After extensive washing at pH7.4, one group of cells was incubated for 30min at 21°C with univalent fragments (Fab) of a partially purified sheep immunoglobulin G (0.5mg/ml) directed against human cathepsin D. Another group of cells was incubated under the same conditions with normal sheep Fab fragment. The cells in both groups were washed again in buffer for 30min and then immersed for 30min at 21°C in buffer containing a fluorescein-labelled pig Fab fragment (0.2mg/ml) directed against sheep Fab fragment. After a final wash with buffer, the cells were mounted in glycerol and examined in the Leitz fluorescence microscope with fluorescein optics.

Results

Synthesis of N-pepstatinyl-S-bimanyl-2-aminoethanethiol

Monobromobimane is a non-fluorescent compound that forms highly fluorescent products when the bromine atom is displaced by a suitable nucleophile, such as a thiol group (Kosower *et al.*, 1979). In order to label pepstatin with the bimeane fluorophore it was necessary to synthesize a derivative carrying a free thiol group. We therefore coupled the disulphide cystamine to the free carboxy group of pepstatin, cleaved the disulphide bond with 1 mol-equiv. of dithiothreitol (Cleland, 1964) and made the liberated thiol group react with mono-

bromobimane. *N*-Pepstatinyl-*S*-bimanyl-2-aminoethanethiol (Fig. 1) in aqueous solution had a fluorescence excitation maximum at 380 nm, with an emission maximum at 460 nm.

Inhibition of cathepsin D by N-pepstatinyl-S-bimanyl-2-aminoethanethiol

N-Pepstatinyl-*S*-bimanyl-2-aminoethanethiol behaved like pepstatin and bound tightly and stoichiometrically to human cathepsin D at pH 3.5. The shape of the titration curve (Fig. 2) enabled the calculation of an apparent dissociation constant, K_D , from the deviation from stoichiometric inhibition in the region of the equivalence point (Green & Work, 1953; Knight & Barrett, 1976). Two independent experiments gave K_D values of 1.4×10^{-10} M and 0.9×10^{-10} M. Since the precise concentration of the inhibitor was not known, the cathepsin D concentration used in the assays was defined by titration with pepstatin (Knight & Barrett, 1976). The concentration of *N*-pepstatinyl-*S*-bimanyl-2-amino-

Fig. 1. Structure of *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol

Fig. 2. Inhibition of cathepsin D by *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol

Standard assay conditions were used as described by Barrett (1970). The assays were started by the addition of buffered haemoglobin to the mixture of cathepsin D and inhibitor at 45°C. After 20 min the trichloroacetic acid-soluble products of digestion of haemoglobin were measured by their A_{280} .

ethanethiol determined from the equivalence points was equal to the apparent weight concentration, indicating the absence of non-inhibitory impurities.

Effect of pH on the stability of the enzyme-inhibitor complex

We have shown previously (Knight & Barrett, 1976; Knight *et al.*, 1980) that radiochemically labelled derivatives of pepstatin remained bound to cathepsin D during gel-permeation chromatography on Sephadex G-100 at pH 5.0. *N*-Pepstatinyl-*S*-bimanyl-2-aminoethanethiol behaved similarly, and all the detectable fluorescence eluted from the column was found in fractions that also contained enzyme (Fig. 3a). However, at pH 7.4 the fractions containing cathepsin D were not fluorescent (Fig. 3b). These results were very similar to those obtained with pepstatin and pepstatinyl-[³H]glycine (Knight & Barrett, 1976). Thus at pH values above 5.0 the binding of pepstatin derivatives to cathepsin D becomes progressively weaker as the catalytic activity of the enzyme falls essentially to zero (Barrett, 1977). We have used this phenomenon to distinguish between active-site-directed and non-specific binding of the fluorescent inhibitor.

Subcellular location of cathepsin D in human synovial cells

When briefly fixed human synovial cells were incubated with *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol (1 μM) at pH 5.0, the fluorescent inhibitor was distributed primarily in granules located in the perinuclear regions of most cells (Plate 1a), although

EXPLANATION OF PLATE 1

Localization of cathepsin D in human synovial cells in culture

Details of the cell culture and staining methods are given in the Materials and methods section. (a) Cells stained with *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol at pH 5.0 showed a predominantly granular distribution of fluorescence around the nuclei (N), although some diffuse reticular staining was also seen. The arrows indicate typical regions with punctate and diffuse staining. (b) No fluorescence was detected around the nuclei of cells stained with *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol at pH 7.4. (c) Cells treated at pH 7.4 with monospecific sheep Fab fragment directed against human cathepsin D, followed by a fluorescein-labelled pig Fab fragment directed against sheep Fab fragment. This indirect immunohistochemical method revealed numerous fluorescently stained granules around the nuclei (cf. a). (d) When normal sheep Fab fragment was used in the immunohistochemical procedure the cells (arrows) were unstained.

Fig. 3. Chromatography on Sephadex G-100 of cathepsin D/*N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol mixtures at (a) pH 5.0 and (b) pH 7.4

A mixture of cathepsin D (50 units) and *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol (0.32 μ g) was applied to a column (1.5 cm $\times$ 26 cm, 46 ml) of Sephadex G-100 at 4°C and eluted at 15 ml/h with (a) 50 mM-sodium acetate/acetic acid, pH 5.0, or (b) 50 mM-sodium phosphate buffer, pH 7.4. Both buffers contained 0.1 M-NaCl and 0.1% Brij 35. A portion (0.1 ml) of each fraction (0.8 ml) was taken for assay of cathepsin D activity (●), and the remainder was taken for the measurement of bimane fluorescence (○). The arrows show the elution volumes of the following markers: 1, Blue Dextran 2000; 2, bovine serum albumin (mol.wt. 66500); 3, cytochrome *c* (mol.wt. 11700).

some diffuse staining was also seen. By marked contrast, cells incubated with the inhibitor at pH 7.4 showed no bimane fluorescence (Plate 1b), although the treated cells were clearly visible under phase-contrast optics.

The distribution of lysosomal cathepsin D in human synovial cells was also determined at pH 7.4 by indirect immunofluorescence staining (Poole *et al.*, 1974). The enzyme was located with a univalent specific antibody raised in sheep, counterstained with a fluorescein-labelled pig antibody to sheep Fab fragment. The granular distribution of fluorescein fluorescence (Plate 1c) closely resembled that seen with the pepstatinyl-bimane at pH 5.0 (Plate 1a). No staining was seen in cells treated with normal sheep Fab fragment (Plate 1d).

We concluded from these results that at pH 5.0 *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol was a selective fluorescent probe for the active conformation of the lysosomal cathepsin D.

Discussion

Fluorescent substrates and inhibitors of proteinases have been used primarily in studies of active-site specificity and polarity (Fruton, 1980). The present study has demonstrated the novel application of a fluorescent proteinase inhibitor to subcellular location of an enzyme. The suitability of *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol as a location reagent for cathepsin D results from two important characteristics. It is a tight-binding

inhibitor specific for cathepsin D in the active conformation and it contains the photostable bimane fluorophore. The binding characteristics were very similar to those of other pepstatin derivatives (Knight & Barrett, 1976; Knight *et al.*, 1980), although somewhat lower values of the apparent dissociation constant, K_D , at pH 3.5 were obtained. At pH 7.4 binding of the inhibitor to the free enzyme or to the enzyme in lysosomes could not be demonstrated.

Monobromobimane was chosen as the labelling reagent because reaction with thiols gives highly fluorescent products that resist photobleaching (Kosower *et al.*, 1979; Gainer & Kosower, 1980). In preliminary experiments (I. T. W. Matthews, W. Hornebeck & C. G. Knight, unpublished work) we had prepared fluorescent inhibitors by reaction of *N*-pepstatinyl-1,2-diaminoethane with dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride or Lissamine Rhodamine B sulphonyl chloride. The fluorescence of the dansyl derivative was lost during the purification in daylight, and the Rhodamine derivative faded rapidly in the fluorescence microscope. By contrast, the bimane-labelled pepstatin could be isolated readily without decomposition, and cells stained with this reagent at pH 5.0 could be examined in the microscope without an appreciable decrease in fluorescence intensity.

A notable feature of the location studies was the absence of non-specific staining. This was particularly apparent in the cells treated with *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol at pH 7.4 (Plate 1*b*), but even at pH 5.0 the distribution of bimane fluorescence among the cells was uneven (Plate 1*a*), closely resembling in this respect the cells stained by the indirect immunofluorescent method (Plate 1*c*). Nor was the extent and pattern of staining changed when the pepstatinyl-bimane concentration was increased to 100 μ M. It appears, therefore, that the only factor controlling the extent of fluorescent inhibitor binding at pH 5.0 is the intracellular content of cathepsin D.

The subcellular distribution of cathepsin D revealed by *N*-pepstatinyl-*S*-bimanyl-2-aminoethanethiol (Plate 1*a*) was very similar to that seen by the immunohistochemical method (Plate 1*c*). Both techniques showed that in human synovial cells cathepsin D resides primarily within a perinuclear array of granules, although a pattern of somewhat more diffusely stained material was often seen in the cytoplasm (Plate 1*a*). Clearly, most of the enzyme is in secondary lysosomes (Poole *et al.*, 1972; Decker & Wildenthal, 1980), but our observations imply that cathepsin D can also be found elsewhere in the cell. This apparent dual subcellular location of the enzyme may be explained by the studies conducted by Erickson & Blobel (1979). They demonstrated that cathepsin D was synthesized and glycosylated

within the rough endoplasmic reticulum, and it seems possible that our diffuse fluorescence may represent newly synthesized enzyme *en route* to the lysosomal vacuolar apparatus.

Our present observations demonstrate a great potential for developing labelled active-site-directed inhibitors for the location of specific enzymes within the cell. Unlike the immunohistochemical approach, which requires extensive enzyme purification and antibody production before location studies can begin, the pepstatin probes are specific for the aspartate proteinases regardless of species (Barrett, 1977). An additional advantage of such reagents is that they allow the distribution of potential enzyme activity to be assessed. With the current development of techniques for the introduction of low-molecular-weight probes into living cells (cf. Barack *et al.*, 1980) it may be possible to use specific probes to reveal where a particular enzyme is physiologically functional within the cell.

I. T. W. M. thanks the Nuffield Foundation for financial support. R. S. D. is an Established Investigator of the American Heart Association.

References

- Barack, L. S., Yocum, R. R., Nothnagel, E. A. & Webb, W. W. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 980–984
- Barrett, A. J. (1970) *Biochem. J.* **177**, 601–607
- Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 209–248, North-Holland, Amsterdam
- Castro, B., Dormoy, J. R., Dourtoglou, B., Evin, G., Selve, C. & Ziegler, J. C. (1976) *Synthesis* **751**
- Cleland, W. W. (1964) *Biochemistry* **3**, 480–482
- Decker, R. S. & Wildenthal, K. (1980) in *Degradative Processes in Heart and Skeletal Muscle* (Wildenthal, K., ed.), pp. 389–418, North-Holland, Amsterdam
- Erickson, A. H. & Blobel, G. (1979) *J. Biol. Chem.* **254**, 11771–11774
- Fruton, J. S. (1980) *Mol. Cell. Biochem.* **32**, 105–114
- Gainer, H. & Kosower, N. S. (1980) *Histochemistry* **68**, 309–315
- Green, N. M. & Work, E. (1953) *Biochem. J.* **54**, 347–352
- Knight, C. G. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 583–636, North-Holland, Amsterdam
- Knight, C. G. & Barrett, A. J. (1976) *Biochem. J.* **155**, 117–125
- Knight, C. G., Hornebeck, W., Matthews, I. T. W., Hembry, R. M. & Dingle, J. T. (1980) *Biochem. J.* **191**, 835–843
- Kosower, E. M. & Pazhenchevsky, B. (1980) *J. Am. Chem. Soc.* **102**, 4983–4993
- Kosower, E. M., Pazhenchevsky, B. & Hershkowitz, E. (1978) *J. Am. Chem. Soc.* **100**, 6516–6518
- Kosower, N. S., Kosower, E. M., Newton, G. L. & Ranney, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3382–3386

- Kosower, N. S., Newton, G. L., Kosower, E. M. & Ranney, H. M. (1980) *Biochim. Biophys. Acta* **622**, 201–209
- Nairn, R. C. (1969) in *Fluorescent Protein Tracing* (Nairn, R. C., ed.), pp. 61–94, Livingstone, Edinburgh and London
- Poole, A. R., Dingle J. T. & Barrett, A. J. (1972) *J. Histochem. Cytochem.* **20**, 261–265
- Poole, A. R., Hembry, R. M. & Dingle, J. T. (1973) *Calcif. Tissue Res.* **12**, 313–321
- Poole, A. R., Hembry, R. M. & Dingle, J. T. (1974) *J. Cell Sci.* **14**, 139–161