(no PTH), in the group treated with PTH, and in 2 PTH-treated groups incubated with minocycline in a final concentration of 6 or 20 μg/ml, were found to contain 7 μg, < 1 μg, 3.5 μg and 7.5 μg of hydroxyproline, respectively (the hydroxyproline values represent the mean of duplicate analyses of a pool of 5 bones per group). This pattern of change indicated that collagen loss, in this bone resorbing system, paralleled mineral dissolution as expected; that is, PTH hormone-stimulated bone collagen breakdown was partially inhibited by the lower dose (6 μg/ml) and completely inhibited by the higher dose (20 μg/ml) of minocycline.

PTH stimulates the production of hydrolytic[11,12] and collagenolytic[4,13] enzymes in bone in organ culture, and the hormonal effect on lysozomal enzymes appears to precede the release of calcium[11]. Recently, using an organ culture system, the inhibition of collagenase activity was shown to suppress bone resorption[14]. Thus, the ability of tetracyclines to inhibit bone resorption observed in the current study probably reflects its newly identified anti-collagenolytic enzyme property[2]. Preliminary studies in our laboratory indicate that tetracyclines can also suppress PGE_2 and endotoxin-stimulated bone resorption in this culture system, which indicates a general ability of this class of antibiotics to inhibit enhanced resorption, not only to inhibit resorption stimulated by PTH. A proposed mechanism for this effect has been described by us previously[2] and involves the ability of tetracyclines to chelate cations[10] since collagenase and some other collagenolytic neutral proteases are dependent on metals (calcium, zinc) to maintain their normal hydrolytic activity[15-18]. In support of this proposed mechanism, minocycline has been found to directly inhibit leukocyte collagenase in vitro, an effect that was completely reversed by adding extra calcium to the incubation mixture[2].

We are currently attempting to determine (1) the mechanisms by which tetracyclines inhibit bone resorption, using histological and biochemical techniques (the latter to monitor alterations in collagen degradation), and (2) whether tetracyclines can reduce pathologically excessive bone resorption in vivo. Our studies to date suggest that this newly identified property of these drugs, independent of their antibacterial function, could be therapeutically useful in the treatment of diseases characterized by excessive connective tissue breakdown including pathologically enhanced bone resorption.

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Molecular mechanism for the production of multiple forms of MM creatine kinase

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Summary. Incubation of human, canine or rabbit MM creatine kinase with carboxypeptidase-N or B resulted in the production of 2 additional enzyme forms with increased anodal migration on polyacrylamide gels. The C-terminal amino acid of tissue MM creatine kinase from all 3 species was shown to be lysine, a specific substrate for carboxypeptidase-N and B.
Key words. Creatine kinase, MM; myocardium; isoenzymes.

Cytosolic creatine kinase [EC 2.7.3.2] is a dimeric isoenzyme exhibiting 3 forms: MM, MB, and BB creatine kinase. The enzyme catalyzes the reversible transfer of a phosphate group from ATP to creatine. The release of creatine kinase into the plasma is used as a diagnostic marker for myocardial infarction and to assess the extent of myocardial damage or infarct size[1]. Several groups have shown that tissue MM creatine kinase exists as a single form which upon release into plasma is converted into 2 additional isoforms[2-6]. Preliminary studies in our laboratory demonstrated that the plasma factor responsible for conversion is heat-labile, non-dialyzable, and temperature-dependent[7]. In vitro conversion of tissue MM creatine kinase (MM_3) to MM_2 and MM_1 was not inhibited by non-specific protease inhibitors but was completely inhibited by guanidinoethylmercaptosuccinic acid, a specific inhibitor of carboxypeptidase-N and B[8]. To determine the molecular mechanism for production of MM creatine kinase subtypes tissue MM creatine kinase purified from human and canine myocardium, as well as rabbit skeletal muscle, was subjected to proteolysis by carboxypeptidase-N and B and the carboxy-terminal amino acid was determined for the tissue form of each MM creatine kinase.

Methods. Purification of creatine kinase isoenzymes. The MM isoenzymes from human and canine myocardium were purified as previously described[9]. Rabbit skeletal muscle MM creatine kinase was obtained from Sigma Chemical Co., St. Louis, MO 63178. *Creatine kinase activity assay.* Creatine kinase activity was assayed spectrophotometrically by the coupled enzyme system of Rosalki[10] using the Gemeni Miniature Centrifugal Analyzer (Electronucleonics, Inc., Fairfield, NJ 07006). Activity was expressed as international units per liter (IU/L). *Protein determination.* Protein concentrations were determined by the procedure of Lowry et al.[11]. *Polyacrylamide gel electrophoresis.* Non-denaturing polyacrylamide gel electrophoresis was done in a 7.5% acrylamide separating gel overlaid with a 3.5% stacking gel. The electrode buffer was 25 mM TRIS-HCl, 192 mM glycine pH 8.3. Samples containing 2 IU were applied in 20% glycerol and electrophoresed overnight at 4°C at 80 V. Migration of creatine kinase was detected by incubating the gel with a cellulose acetate overlay which had been previously saturated with NADPH-generating medium. The gel was then photographed with UV illumination[12]. *Production of MM creatine kinase isoforms by carboxypeptidase B and N digestion.* Carboxypeptidase B (Sigma Chemical Co.) was added to purified human, canine and rabbit tissue MM creatine kinase at a concentration of 1 µg of carboxypeptidase to 100 µg of MM creatine kinase in 25 mM TRIS-HCl (pH 7.3) and incubated at 37°C. Aliquots were removed over a period of 1 h and analyzed for creatine kinase activity by gel electrophoresis as described above. Human plasma carboxypeptidase-N (a generous gift of Dr Thomas H. Plummer Jr, Division of Laboratories and Research, New York State Department of Public Health, Albany, New York) was added to tissue MM creatine kinase in a concentration of 4 IU/l and aliquots removed and analyzed as above. *Carboxy-terminal amino acid determination.* Carboxypeptidase-B was added to purified human, canine or rabbit MM creatine kinase at an enzyme/substrate ratio of 1:125 in 100 mM N-ethylmorpholine acetate (pH 6.0). Aliquots were removed at 0, 5, 10, 15, 20, 30, 45, 60, and 120 min and the reaction terminated by the addition of 200 mM sodium citrate (pH 2.2). The samples were analyzed on a LKB automated amino acid analyzer.

Results and discussion. Incubation of human, canine or rabbit MM creatine kinase purified from tissue with carboxypeptidase-B resulted in the production of 2 additional creatine kinase isoforms (fig. 1) with increasing anodal mobility (identical results were observed with carboxypeptidase-N and with plasma). Rabbit skeletal muscle MM creatine kinase has an electrophoretic mobility different from that of canine and human myocardial tissue MM creatine kinase, but was nevertheless converted into 2 additional isoforms. Amino acid analysis of

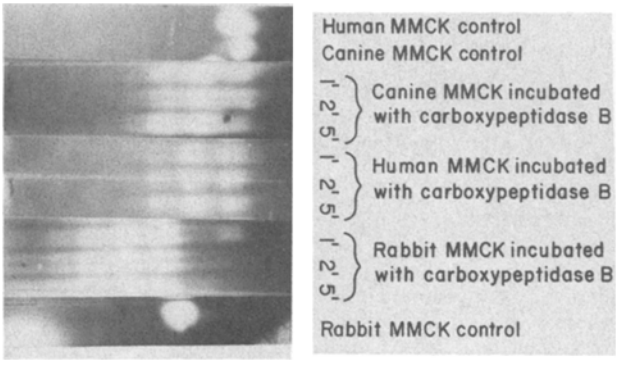

Figure 1. Effect of incubation of tissue MM creatine kinase with carboxypeptidase-B. Incubation of human, canine and rabbit tissue MM creatine kinase (MMCK) with carboxypeptidase-B for 1 to 5 min resulted in the production of 2 additional creatine kinase isoforms. The cathode is at the top.

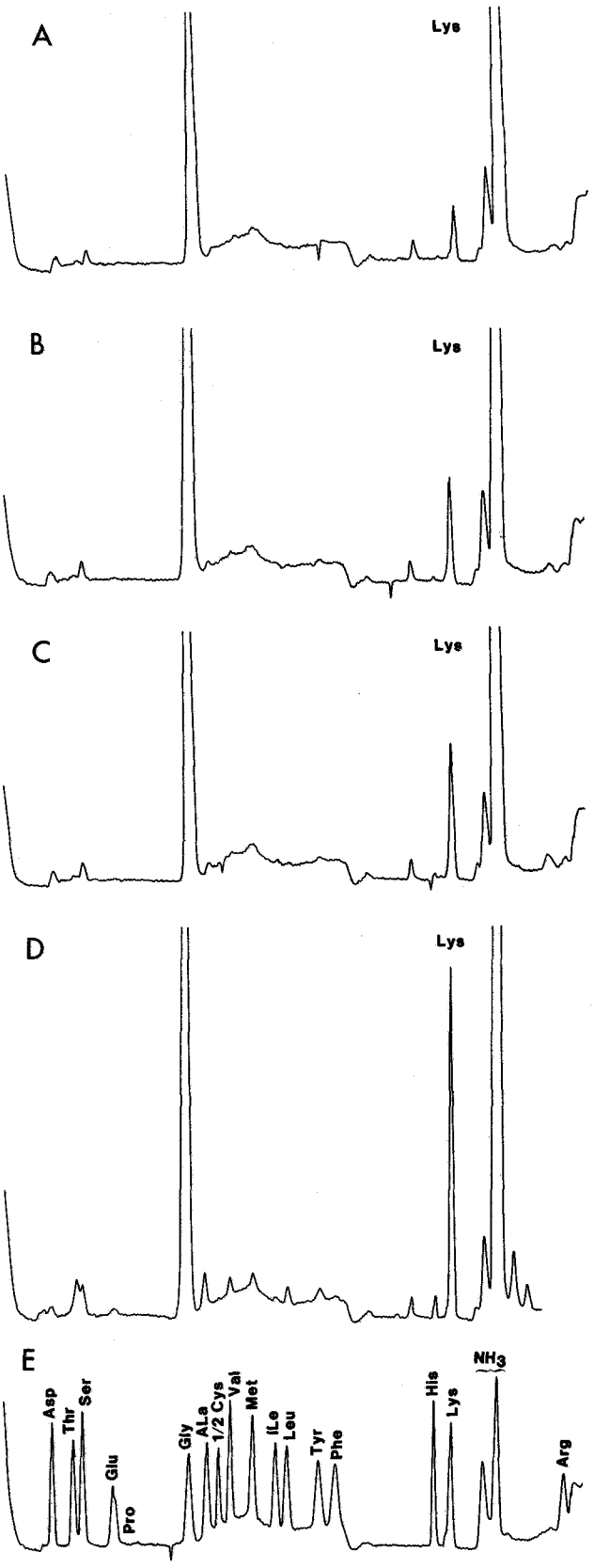

Figure 2. Amino acid analysis of rabbit MM creatine kinase following hydrolysis by carboxypeptidase-B. Shown are the elution profiles of rabbit MM creatine kinase following 0 (A), 10 (B), 30 (C) and 120 min (D) digestion with carboxypeptidase-B. Panel D is the elution profile of an amino acid standard mixture.

the carboxypeptidase-B proteolysis product of human, canine, and rabbit tissue MM creatine kinase showed lysine to be the C-terminal amino acid. Identical results were obtained with human and canine tissue MM creatine kinase. These results confirm that lysine is the C-terminal amino acid of rabbit MM creatine kinase[13] as well as the C-terminal amino acid for both human and canine myocardial MM creatine kinases. We conclude that multiple subforms of MM creatine kinase are produced in plasma by hydrolysis of the C-terminal lysine from 1 polypeptide chain of tissue MM creatine kinase (MM_3) to produce MM_2. Hydrolysis of the C-terminal lysine from the second polypeptide chain of MM_2 produces MM_1. It is most probable that carboxypeptidase-N is the responsible agent since it is found in plasma[14] while carboxypeptidase-B is found primarily in the pancreas[15]. Since the detection of MM_3 indicates recent release into plasma it can be used as a marker for recent myocardial injury or vessel patency following thrombolytic therapy.

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Exocytosis of secretory granules – a probable mechanism for the release of neuromodulators in invertebrate neuropiles

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Summary. Presynaptic terminals typically contain secretory granules, usually 100–200 nm in diameter, in addition to the smaller synaptic vesicles. Evidence is presented that granule exocytosis is a widespread phenomenon in invertebrate neuropiles. Such secretory release is apparently associated with morphologically unspecialized regions of the plasmalemma, rather than synaptic thickenings.
Key words. Invertebrate neuropile; exocytosis; synapses; neuromodulators.

It has long been known[1,2] that presynaptic terminals whether in vertebrates or invertebrates, in central or peripheral nervous systems, typically contain dense-cored granules, usually 100–200 nm in diameter, in addition to the smaller synaptic vesicles. Neurosecretory endings within neurohemal organs are identical in ultrastructure[3–5]. Secretory release from both types of terminals is thought to occur by exocytosis – i.e., following fusion of the bounding membranes of the secretory inclusions with the plasmalemma. However, whereas discharge from presynaptic terminals is generally assumed to involve synaptic vesicles[6,7]; release within neurohemal organs such as the corpus cardiacum of insects and the neurohypophysis of vertebrates involves the larger granules (ref. 8, 9 for review). Work in this laboratory has previously shown that a process of granule exocytosis apparently identical in most respects to that occurring within neurosecretory fibers is a feature of synaptic terminals in annelids[10,11] and we now provide evidence that this form of secretory activity, which was virtually unknown with respect to central nervous systems until recently, is a widespread phenomenon in invertebrate neuropiles (see also Roubos et al.[12]).

Cerebral ganglia of *Dendrocoelum lacteum* (Platyhelminthes), *Lumbricus terrestris* and other earthworms (Annelida) and *Helix aspersa* (Mollusca) were prepared for electron microscopy following fixation in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.2, sometimes following 2.5% glutaraldehyde in the same buffer. In addition, some specimens were stimulated by incubation for various periods of time in K^+-rich (50 mM) Ringer solution or 1 mM 4-aminopyridine (4AP) in Ringer solution[13]. Some were processed using the tannic acid-glutaraldehyde-OsO_4 (TAGO) method[14,15] using 1% tannic acid and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 1 h, followed by 1% OsO_4 in the same buffer.

The neuropiles we have examined are richly endowed with secretory granules (fig. 1A). These inclusions show great variety in size, form, electron density of the core, etc., and can be used as a basis for classifying the axon varicosities and synaptic boutons in which they are contained. Synaptic vesicles show less variation and, unlike the granules, they are usually concentrated adjacent to synaptic thickenings.

Sites of granule exocytosis within the neuropiles have been recognized by the incidence of 'omega profiles' together with the presence of electron dense material within the indentations (fig. 1B, C). They have been encountered in each of the ganglia examined (and also within the cerebral ganglion of the arthropod *Balanus hameri* – A.S. Clare, personal communication).