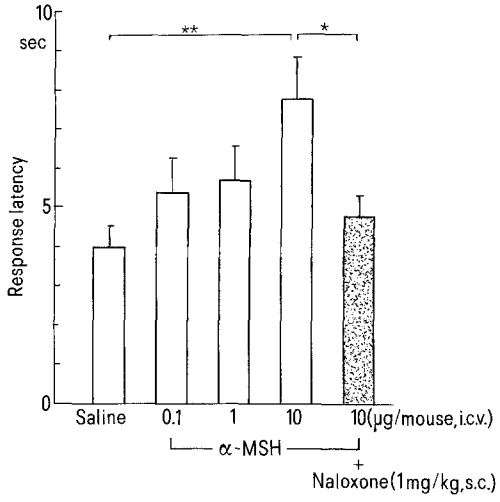
ments were carried out 5 min after i.c.v. injection of MSH. Only the 10 μg dose of α-MSH produced analgesia during 20 min period. Sandman and Kastin report that intraventricular administration of MSH induces hyperalgesia in rats. Our present data contrast strikingly with their findings and may suggest the existence of some species differences in the capacity of rats and mice to activate the opioidergic system in response to MSH. There is an evidence that an analog of $ACTH_{4-9}$ produces significant analgesia, and it is reported that physiologically β-endorphin and ACTH may have effects that are functionally similar in nature[13]. ACTH, α-MSH and β-endorphin coexist within the arcuate nucleus and pituitary cells[14,15]. And it is also reported that α-MSH induces excessive grooming similar to that seen with ACTH and β-endorphin[8] and that the grooming activity correlates well with the intrinsic analgesic activity of these LPH fragments[9]. We also observed that α-MSH elicited grooming in concurrence with analgesic action and these were reversed together by naloxone. These suggest that MSH may have a functional role in the analgesic mechanism similar to those of ACTH and β-endorphin.



The control group received saline i.c.v. and s.c. Each histogram represents the mean ± SE of 12 animals. * $p < 0.05$. ** $p < 0.01$.

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Magnesium enhances human pancreatic elastase digestion of ^{125}I-labeled elastin

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Summary. The effect of some divalent cations, especially Mg^{++}, on elastinolysis by porcine or human pancreatic elastase has been determined using ^{125}Iodine-labeled elastin as substrate. Elastin degradation was significantly increased in the presence of 10^{-3} M Mg^{++}. If elastin was pre-incubated with 0.5 (w/v) Triton, there was a further increase in elastinolysis to 2.6 times the original rate.
Key words. Porcine and human pancreatic elastase; elastin; Triton-Mg^{++}.

Previous attempts to establish the effects of cations and or anions as well as other modifying agents on pancreatic elastase digestion of insoluble elastin have yielded ambiguous results[1-3]. Although it is known that an increase in extracellular Mg^{++} concentrations induces relaxation and vasodilation, and hypomagnesia can potentiate contractile activity, the role of Mg^{++} in the genesis of vascular diseases[4] and the relationship between Mg^{++} levels and atherosclerosis is poorly understood. Even less is known with regard to connective tissue metabolism. Bernier et al.[5] reported that Mg^{++} increased the initial velocity of elastinolysis two-fold at 10^{-2} M concentration. This activation is of great potential interest especially as it may enhance the activity of human pancreatic elastase II which when purified according to Largman et al.[6] or Rabaud et al.[7] exhibits only 30% of the activity of the same amount of porcine pancreatic elastase. In the present study we have explored the effect of Mg^{++} on the elastinolysis of ^{125}I-labeled elastin as well as the role of some other factors in the modulation of the activity of porcine and human pancreatic elastases on insoluble labeled elastin.

Materials and methods. Porcine pancreatic elastase purified by the method of Shotton[8] was obtained from Choay (Paris). Its elastinolytic activity was determined in our laboratory by comparison with known standards of elastase from Elastin Prod-

ucts (Pacific, MO) and found to be 55 U/mg (dry weight). The enzyme gave a single band on polyacrylamide gel electrophoresis by the method of Furthmayr and Tinpl[9]. Human pancreatic elastase (HPE[II]) was purified by the method of Largman et al.[6]. Briefly, 13 human pancreases (600 g), stored at −40 °C were cut into small pieces, homogenized, delipidated by acetone:ether treatment in a Waring blender, dried and then extracted with Tris $CaCl_2$ buffer. After fractional precipitation with saturated $(NH_4)_2SO_4$ the precipitate was dissolved in 25 mM phosphate buffer, pH 6.5, and submitted sequentially to ion exchange chromatography on CM 52 (Whatman, Maidstone, Kent, England) and to gel filtration on G75 Sephadex (Pharmacia, Bois d'Arcy, France). 600 g of wet tissue yielded 300 mg purified elastase. Its activity was determined as described above for porcine elastase and found to be 30 U/mg protein (dry weight). Elastin from bovine ligamentum nuchae was a commercial product from Millipore Corporation (Freehold, NJ).

Elastinolytic activity was determined by a modification[10] of the method of Robert and Robert[11]. Elastin was labeled with ^{125}Iodine which was purchased from C.E.A., Saclay, France as Na solution, according to the protocol of Geokas et al.[12]. Generally, the specific activity of the substrate was about 10^5 cpm/mg. The labeled elastin was washed first with 10 mM EDTA for 30 min, then washed extensively with bi-distilled water and stirred overnight at 20 °C in 10 mM Tris HCl, pH 8.6, with or without addition of Mg^{++} salts or other agents under study, essentially as described by Bernier et al.[5]. In a parallel series of experiments the different salts were added to

Table 1. Amount of protein solubilized by porcine pancreatic elastase digestion of ^{125}I-labeled elastin in the presence of different concentrations of M^{++}

Mg^{++} concentration	ng protein/min/ µg elastase
0	23.9 ± 1.1
10^{-4} M	62.0 ± 2.6
10^{-3} M	74.3 ± 1.7
10^{-2} M	53.8 ± 1.5

Table 2. Change of elastase activity after washing the elastin with Triton 0.5% (w/v) and adding $MgCl_2$ at the start of the assay. PPE, porcine pancreatic elastase; HPE, human pancreatic elastase

Enzyme	cpm/min/µg elastase	
	No addition	$MgCl_2$ + Triton
PPE	91 ± 6.5	237 ± 15
HPE II	18 ± 0.9	48 ± 2.1

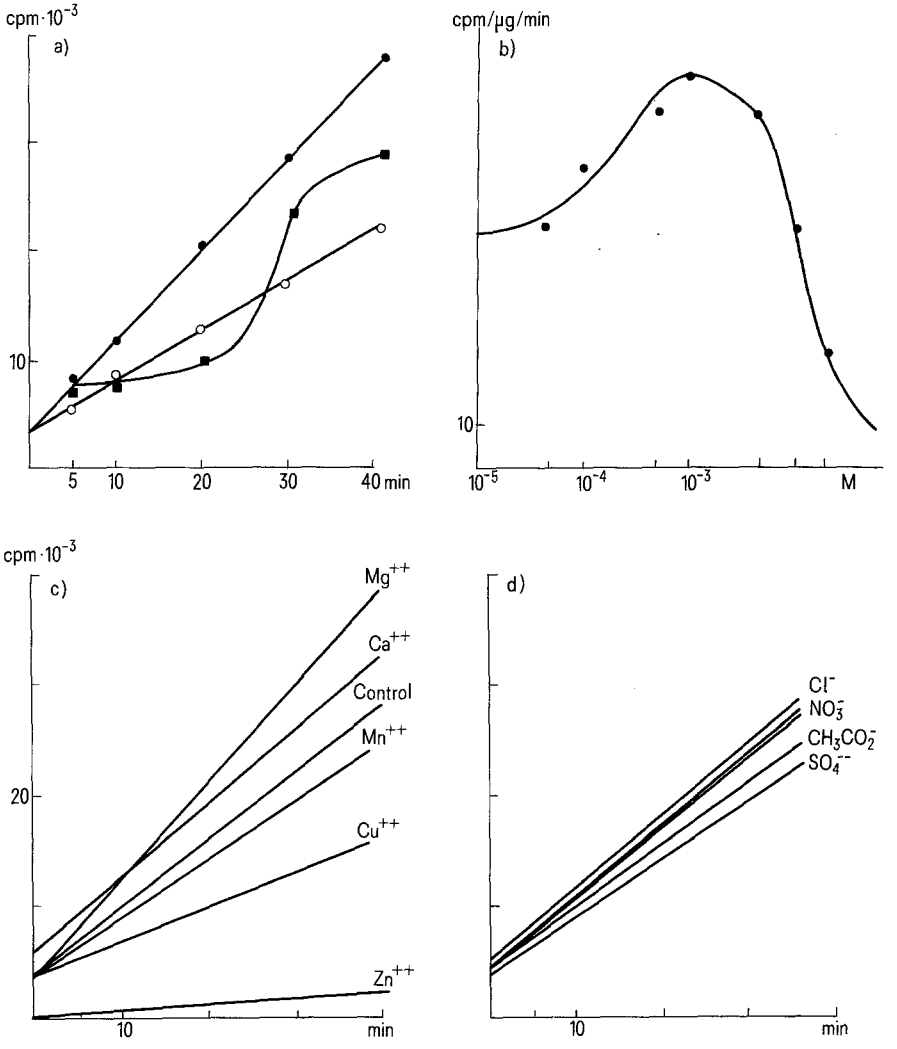

Figure 1. *a* Kinetic measurements of elastinolysis. ■–■, with Mg^{++} in the washing buffer; ●–●, with Mg^{++} in the reaction buffer. ○–○ without Mg^{++}. *b* Change of porcine pancreatic elastase activity in the presence of Mg^{++} concentrations 10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2} and 2×10^{-2} M. *c* Change of porcine pancreatic elastase activity with different cations at 10^{-3} M concentration. The cations are added at the start of the reaction. *d* Kinetic measurements in the presence of various Mg^{++} salts at 10^{-3} M concentration. Each point is the mean of 3 observations.

the buffer at the beginning of the reaction. Assays were carried out as follows: 5 mg/ml of ^{125}I-labeled elastin was suspended in Tris HCl buffer with or without adding a cation and was equilibrated at 37 °C in a Dubnoff-type incubator. The elastase, 0, 5, 10 µg in 2.5 ml of the same buffer, was then added and allowed to react for 0, 5, 10, 30 or 40 min. In each case the reaction was stopped by filtration through Whatman paper No. 40 and the radioactivity of 1 ml of the filtrate was measured in an automatic gamma spectrometer (GS 500 ICN). Protein concentration of the filtrates was determined in each aliquot by the procedure of Lowry et al.[13] using bovine serum albumin as a standard.

Results. Kinetic measurements of elastinolysis with and without Mg^{++} were carried out after 17 h pre-incubation of the cation with elastin and eliminating the excess by washing or with the cation added to the buffer at the beginning of the assay. As shown in figure 1a the activity of pancreatic elastase was significantly increased in the presence of Mg^{++}. This increase was much more pronounced when the cation was added to the buffer at the start of the assay. The sigmoid-like curve obtained when elastin was pre-incubated with Mg^{++} suggests that the cation may have been adsorbed on the fiber. The difference in the slope of the two parts of the kinetic curve (fig. 1a) supports this hypothesis. When Mg^{++} was added to the buffer under our experimental conditions the optimum concentration of Mg^{++} was approximately 10^{-3} M (fig. 1b). The same concentration of Mg^{++} also showed maximum solubilization of protein (table 1).

Comparison of the effect of Mg^{++} with that of other divalent cations, some of which had been previously studied by Bernier et al.[5] is shown in figure 1c. Since these authors have reported maximum effects between 10^{-2} M and 10^{-3} M for all cations and 10^{-3} M was found optimum for the enhancement of elastinolysis by Mg^{++}, kinetic measurements were carried out at this concentration. Only Mg^{++} and Ca^{++} increased elastinolysis, all other cations tested, i.e. Mn^{++}, Cu^{++} and most markedly Zn^{++} decreased elastinolytic activity. As shown in figure 1d no significant effect could be discerned with various anions.

To gain some insight into the role of cations in the elastinolytic process, Mg^{++} was eliminated from the reaction mixture. This was done by adding EDTA during the pre-incubation with elastin or at the start of the assay, or by extensive dialysis of the elastase solution. When elastin was incubated overnight with 10^{-2} M EDTA, then extensively washed with the reaction buffer to eliminate any trace of EDTA; there was no significant deviation from the kinetic parameters of standard elastinolysis controls (fig. 2). On the other hand addition of EDTA (10^{-2} M final concentration) at the start of the assay resulted in a diminution of enzyme activity until a plateau was reached. These results indicate that elastase exhibits basic activity in the absence of added cations and that cations intervene only as activating factors. Similarly when elastase was extensively dialyzed against distilled water (fig. 3) enzyme activity decreased. Subsequent addition of Mg^{++} increased the activity but did not restore it completely, suggesting that other cations may be involved separately or synergistically. To further modulate the enzymatic activity, elastin was subjected to a washing period either in sodium dedecyl sulfate (SDS) or in Triton, or these substances were added to the reaction buffer. When SDS (0.01, 0.05, 0.1 and 0.5% w/v) was added or incompletely removed from the batch, the elastinolytic activity was completely abolished as previously reported by Kagan et al.[14]. If, after 0.5% SDS had been used overnight in the washing process for elastin, the substrate was carefully equilibrated with its buffer the activity of pancreatic elastase was not affected. In contrast, Triton treatment increased elastinolysis. This effect may be ascribed to swelling of elastin in Triton, facilitating enzyme-substrate interaction, rather than modulation of the enzyme activity. If Triton was added at the start of the assay the elastinolytic process was slightly decreased. However, if Triton was added to the washing buffer (0.5% w/v) and then completely removed before the assay, elastin degradation was enhanced by approximately 20% (fig. 4).

Discussion. The combined effect of washing elastin with Triton and adding $MgCl_2$ to the reaction buffer was an increase of 150% in the measured elastinolysis as shown by comparison of the values obtained in figures 1a and 4. Such an enhancement of the elastinolytic activity may be of special interest as it affects human pancreatic II which is much less potent than porcine pancreatic elastase. Table 2 lists the values determined for both human and porcine pancreatic elastase with and without Mg^{++} Triton treatment. In both cases elastinolytic activity was 2.6 times greater when the elastin had been exposed to Triton and $MgCl_2$ was present. Triton may exert its effect in several ways. As it causes swelling of the macromolecular elastin it may enable release of entrapped anions of iodine as well as

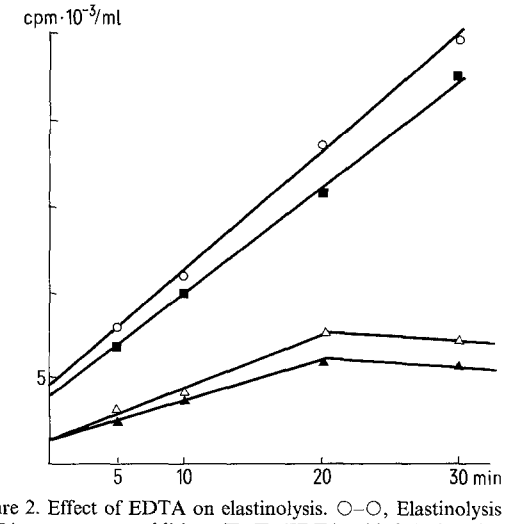

Figure 2. Effect of EDTA on elastinolysis. ○–○, Elastinolysis without EDTA exposure or addition; ■–■, EDTA added during the washing process only; △–△, EDTA added at the start of the assay without pre-incubation; ▲–▲, EDTA added at the start of the assay following pre-incubation during the washing process. Each point is the mean of duplicate observations.

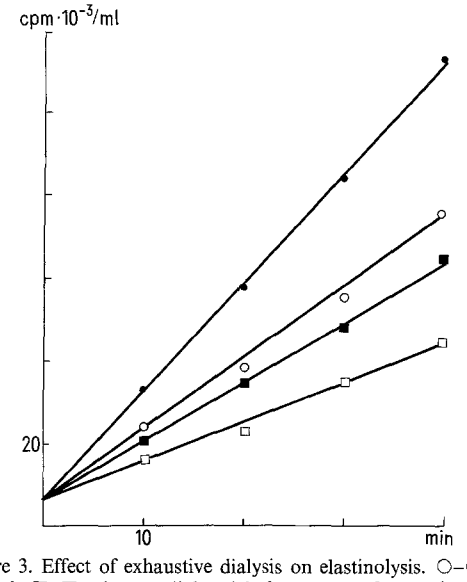

Figure 3. Effect of exhaustive dialysis on elastinolysis. ○–○, Elastase control; □–□, elastase dialyzed before assay; ●–●, elastase control assayed in the presence of 10^{-3} M $MgCl_2$; ■–■, dialyzed elastase assayed in the presence of 10^{-3} M $MgCl_2$. Each point is the mean of duplicate observations.

intervene during the reaction by facilitating enzyme-substrate interaction as the lysis proceeds. The mechanism by which SDS could intervene in elastinolysis is ambiguous. In the first place, it depends upon the nature of the enzyme, since SDS enhances elastinolysis only with macrophage elastase. Then, a main point is to distinguish between SDS action on the substrate (before the reaction) and on the enzyme (during the reaction). Nevertheless, some investigators have suggested that the conformation of elastin could be modified by SDS and so the substrate would be more accessible to degradation[15]. Others[14,15] suggest that SDS could increase the binding of the enzyme to the substrate. In our hands, addition of SDS during the reaction inactivates the enzyme. If following its preincubation with elastin, SDS was removed completely by thorough washing no effect could be discerned.

The reported observations emphasize the role of Mg^{++} salts in the elastinolysis mechanism. A direct effect on the enzymatic reaction is indicated by the greater increase of elastinolysis when Mg^{++} was incorporated in the reaction buffer rather than applied to the substrate as part of the washing process[5]. As pointed out by several authors[4,8] the biological effects of Mg^{++} are due to its ability to form weak chelates. Enzymes activated by Mg^{++} demonstrate a bell-shaped p Mg^{++} dependency similar to that found in this study. As regards the effects of anions it has been reported[3] that iodine may be more effective than chloride in enhancing elastinolysis; however, our findings indicate that anions do not affect the elastinolysis process. A major problem in biological studies is the lack of sufficiently large amounts of material for adequate tests. The 2.6-fold enhancement of elastinolysis may therefore be of great importance. Furthermore, if destruction of elastin in the arterial wall is the first event in the etiology of atherogenesis, the dependence of the elastinolysis rate on Mg^{++} concentration which is highest under physiological conditions will have a significant effect.

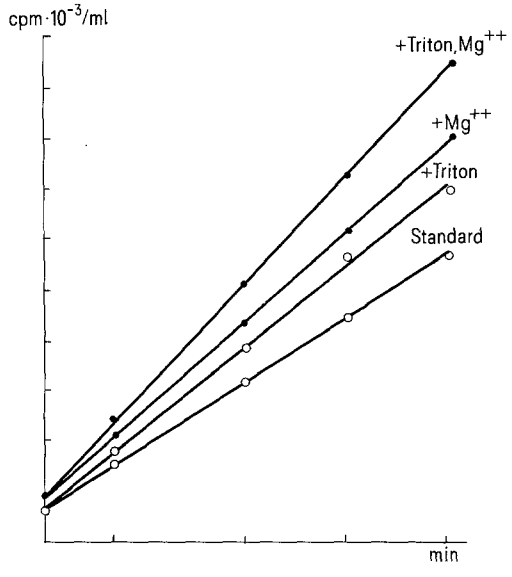

Figure 4. Effect of Triton on elastinolysis with and without added Mg^{++}. O–O, Elastinolysis as previously described with and without pre-incubation with 0.5% (w/v) Triton during the washing of elastin; ●–●, elastinolysis in the presence of 10^{-3} M $MgCl_2$ added at the start of the assay and with and without Triton pre-incubation. Each point is the mean of five experiments.

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Role of phospholipids in the binding activity of vasoactive intestinal peptide receptors[1]

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Summary. Phospholipase digestion of rat intestinal epithelial cell membranes was performed in order to study the influence of membrane phospholipids on the binding activity of VIP receptors. Phospholipases A_2 and C strongly ($ED_{50} \simeq 4 \times 10^{-2}$ and 4×10^{-1} µg/ml, respectively) and rapidly reduced ^{125}I-VIP binding to membranes whereas phospholipase D was ineffective. This suggests an important role of both hydrophobic and hydrophilic groups of phospholipids on VIP receptor binding activity.
Key words. Intestinal epithelium, rat; VIP receptors; phospholipids.

The binding of vasoactive intestinal peptide (VIP) to specific receptors on the exterior surface of target cells initiates cellular responses[2]. The functional properties of VIP receptors[2] as well as their protein nature and molecular size[3,4] have been characterized in the intestinal epithelium. Phospholipids, a major component of membrane lipids, interact with membrane proteins, in particular hormone receptors, and often modulate their functional activity[5]. The influence of phospholipids on