Regulation of Elastase-Catalyzed Hydrolysis of Insoluble Elastin by Synthetic and Naturally Occurring Hydrophobic Ligands†

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ABSTRACT: Sodium dodecyl sulfate significantly alters the substrate properties of insoluble elastin resulting in a severalfold stimulation of the rate at which this structural protein is hydrolyzed by pancreatic elastase (H. M. Kagan, G. D. Crombie, R. E. Jordan, W. Lewis, and C. Franzblau (1972), *Biochemistry 11*, 3412). The present report describes the hydrophobic and ionic properties of synthetic and naturally occurring ligands which induce similar changes in the susceptibility of elastin to proteolytic attack. Distinct chain-length requirements are in evidence for alkyl sulfate mediated effects with a minimum chain length of eight carbons necessary for sodium dodecyl sulfate-like rate enhancement. A similar minimum hydrophobic requirement is also observed for the induction of circular dichroic (CD) spectral changes in soluble α-elastin by these same compounds. This correlation between enhanced elastolytic activity and the induction of CD spectral changes points to a mechanism of stimulation involving ligand-induced conformational changes in elastin polypeptide chains. In addition to a minimum hydrophobic content, however, there is a clear requirement for an anionic polar group since neutral and nonionic detergents are without effect on the rate of elastin digestion while cationic agents are strongly inhibitory. These results are consistent with a proposed electrostatic attraction between the cationic enzyme and the anionic elastin substrate (A. Gertler (1971), *Eur. J. Biochem. 20*, 541) and suggest that effective stimulating agents bind hydrophobically to elastin to increase the net anionic character of the substrate. Although charge reciprocity is essential for elastolytic activity, the present study also reveals that the normal charge relationships between enzyme and substrate can be effectively reversed such that elastin is readily digested by an anionic derivative of elastase in the presence of a cationic detergent. Predictably, physiological compounds which are anionic and sufficiently hydrophobic also stimulate the rate of elastolytic digestion. These include bile salts and the sodium salts of saturated and unsaturated fatty acids. Like dodecyl sulfate, these compounds readily bind to and form tight complexes with insoluble elastin. Perhaps most interesting is the ability of such ligands to reverse the inhibitory effect of salt on the rate of elastolysis. Thus, at physiological ionic strength, the stimulation afforded by certain of these agents may approach 30-fold. These results may be of potential significance to certain disease states in which the participation of elastolytic enzymes has been implicated.

We have previously reported that the rate of digestion of insoluble elastin by pancreatic elastase can be enhanced by as much as sixfold by the detergent, sodium dodecyl sulfate. It was shown, moreover, that this stimulation results from the formation of an elastin–detergent complex with markedly altered properties as a substrate for elastolysis (Kagan *et al.*, 1972).

These results point toward the potential of elastin ligands to serve as cosubstrates for elastolytic enzymes by positively or negatively influencing the susceptibility of elastin toward enzymatic degradation. This consideration seems all the more important in view of the essential role played by this structural protein in tissues such as blood vessels, ligament, and lung and in view of the avidity of elastic fibers for hydrophobic ligands *in vivo* (Kramsch *et al.*, 1971). Indeed, elastolytic mechanisms have been implicated in cardiovascular disease (Loeven, 1969) and in emphysema (Galdston *et al.*, 1973). We have explored, therefore, the ability of a variety of elastin ligands to influence the proteolytic attack of this protein by pancreatic elastase *in vitro*. The present results describe hydrophobic and ionic properties of elastin ligands which result in stimulation of elastolysis and extend these studies to certain naturally occurring hydrophobic ligands of this connective tissue protein. A preliminary report of these results has been presented (Jordan *et al.*, 1973).

Experimental Section

Materials

Highly purified elastase was obtained from the Whatman Co., Maidstone, Kent (England). The chromophoric elastase substrate, *p*-nitrophenyl *tert*-Boc-L-alaninate, was obtained from Mann Research Laboratories. Maleic anhydride was obtained from Fisher Scientific Co.

Alkyl Sulfates. Alkyl sodium sulfates with six or less carbons (hexyl, pentyl, butyl, ethyl, and methyl) were obtained from Eastman Organic Chemicals. Longer chain compounds (octyl, decyl, dodecyl, and myristyl sodium sulfates) were products of Schwarz/Mann. Sodium dodecyl [^{35}S]sulfate (specific activity of 1.41 Ci/mol at time of purchase) was obtained from New England Nuclear Corp., Boston, Mass.

Fatty Acids. Octanoic and decanoic acids were obtained from Analabs, Inc., lauric acid was obtained from Eastman Organic Chemicals, and [1-^{14}C]lauric acid (2.92 Ci/mol) was purchased from New England Nuclear Corp. Oleic and linoleic acids were acquired as the sodium salts from Sigma Chemical Co.

Bile Acids. Cholic and deoxycholic acids were products of

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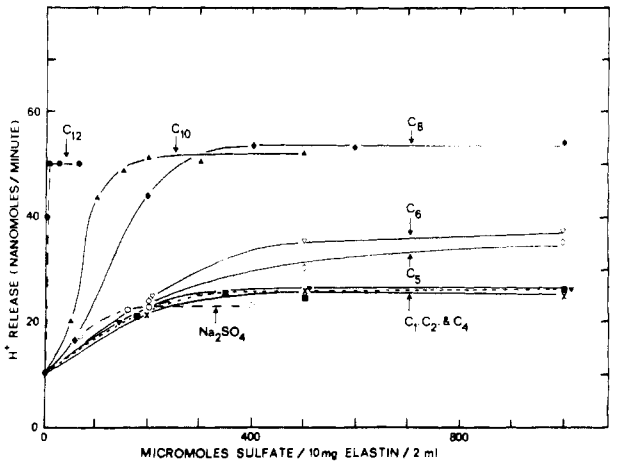

FIGURE 1: The effect of preincubation of insoluble elastin with various alkyl sodium sulfates on the rate of elastase-catalyzed peptide bond hydrolysis. Elastin was pretreated with the given concentrations of alkyl sulfates or sodium sulfate in water prior to resuspension in detergent-free water solution before addition of elastase (10 μg).

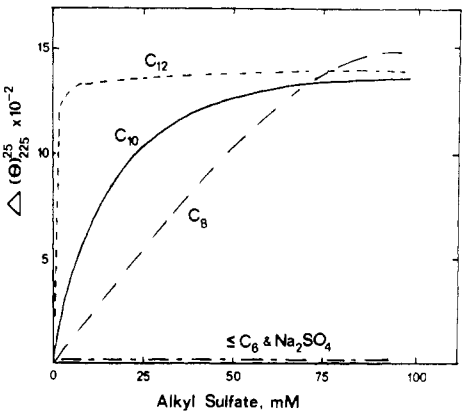

FIGURE 2: Effect of anionic detergents of varying carbon chain length and of sodium sulfate on the CD spectrum of soluble α-elastin. Results are expressed as the change in mean residue ellipticity at 225 nm of α-elastin resulting from the presence of ligands at the given concentrations.

Matheson Coleman and Bell. Taurodeoxycholic and glycocholic acids were obtained from Sigma Chemical Co. [^{3}H]Cholic acid (3.2 Ci/mmol) was a product of the New England Nuclear Corp.

Neutral Detergents. Triton X-100 and Tween-80 were purchased from Schwarz/Mann and Fisher Chemical Co., respectively. Nonidet P-40 was a product of Shell Chemicals. Purified egg-white lysolecithin was a gift of Dr. Martin Carey of Boston University School of Medicine.

Methods

Bovine ligamentum nuchae elastin was prepared according to the procedure of Partridge *et al.* (1955). Solubilized α-elastin was prepared by successive extractions of the ligament elastin powder with 0.25 N oxalic acid, according to Partridge *et al.* (1955).

The enzyme assays using elastin as substrate were performed in a pH-Stat at pH 8.9 titrating hydrogen ion release upon enzymatic hydrolysis of peptide bonds. Assays were done at 37° in water unless otherwise specified.

The effects of alkyl sulfates were initially determined by pretreating an elastin suspension (5 mg/ml) with the desired concentration of detergent for 30 min at 37°. Following centrifugation, the pelletted elastin-detergent complex was resuspended in 2 ml of water by repeated pipetting and this suspension was used directly as the substrate for pH-Stat assay. This pretreatment method for generating elastin complexes was employed in order to avoid the possibility of inhibition of elastase activity due to excess free detergent present in the assay as was previously shown to be the case in studies with sodium dodecyl sulfate (Kagan *et al.*, 1972).

Preincubation of elastin with ligand was also necessary in those cases where the agent being tested was not totally soluble at pH 8.9. In the case of certain longer chain unsaturated fatty acids (oleate and linoleate), elastin was preincubated with solutions of these agents at pH 10.3 and 10.1, respectively, centrifuged, resuspended in water or salt solution, and used as an elastolytic substrate at pH 8.9.

In all other instances, the effects of compounds on the elastolytic rate were determined by adding directly to assay suspensions at the specified concentrations without prior removal of free ligand. Conditions employed in each instance are indicated in the legends to the figures.

Enzyme Inhibition. The effects of the various compounds on the esterolytic activity of elastase were determined by the spectrophotometric method of Visser and Blout (1972). Elastase was preincubated with the test compound at 37° for 30 min in 0.01 M potassium phosphate buffer at pH 6.5. Esterase assays were then performed by removing an aliquot of the preincubated enzyme, diluting it at least 30-fold into the assay cuvet, and following the hydrolysis of *p*-nitrophenyl *tert*-Boc-L-alaninate at 420 nm in a Gilford recording spectrophotometer.

Circular Dichroism (CD). The effects of various ligands on the CD spectra of α-elastin and elastase were determined with a Cary Model 61 circular dichroism spectropolarimeter. Spectra were recorded at 25° in a 1.0-mm cell with a protein concentration of 0.2 mg/ml in dilute phosphate buffer at pH 7. Base lines were recorded before and after each spectral titration.

Ligand Binding Studies. Direct binding studies of radioactively labeled ligands were carried out as follows: 10 mg of elastin was suspended in 2 ml of 0.05 M sodium borate buffer (pH 8.9) and incubated with the desired concentration of sodium [1-^{14}C]laurate (225 cpm/mg), sodium [^{3}H]cholate (2080 cpm/mg), or sodium dodecyl [^{35}S]sulfate (225 cpm/mg). After incubation for 30 min at 37°, each suspension was quickly centrifuged and an aliquot of the supernatants assayed for radioactivity by liquid scintillation spectrometry to quantitate unbound ligand remaining in solution. Control solutions of each ligand in the absence of elastin were treated identically to determine initial counts/ml of unbound ligand.

Maleylation of Elastase. Elastase was maleylated by the procedure of Butler *et al.* (1969) for chymotrypsinogen as adapted by Gertler (1971b). Elastase (5 mg) was dissolved in 2 ml of H_2O at 2° and brought to pH 9.0 by the addition of NaOH. To this solution, 0.15 ml of a 1 M solution of maleic anhydride in dioxane was added in six aliquots and the pH maintained at 9.0 by the addition of 1 N NaOH on the pH-Stat. After the reaction was complete, the enzyme solution was dialyzed in the cold against several changes of distilled water. These conditions yield an elastase derivative in which all the ϵ-amino groups are maleylated (Gertler, 1971b). The concentration of dialyzed maleylated elastase was determined by optical density readings at 280 nm, as previously described for this derivative (Gertler, 1971b). The esterolytic and elastolytic activities of the maleylated elastase were determined as described above for native elastase.

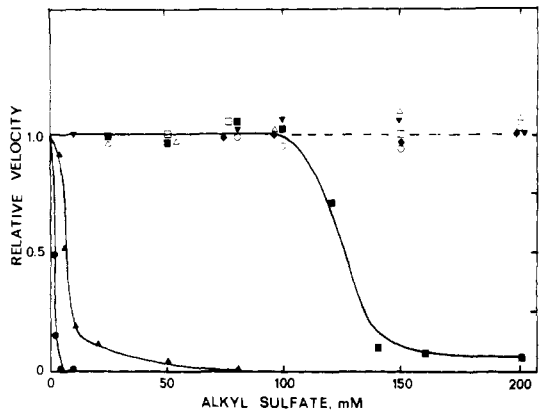

FIGURE 3: Effect of preincubation of elastase in alkyl sodium sulfate solutions on the rate of elastase-catalyzed hydrolysis of *p*-nitrophenyl *tert*-Boc-L-alaninate (see Methods): (●) dodecyl sulfate; (▲) decyl sulfate; (■) octyl sulfate; (○) hexyl sulfate; (♦) pentyl sulfate, (□) butyl sulfate; (▼) methyl sulfate; (△) sodium sulfate.

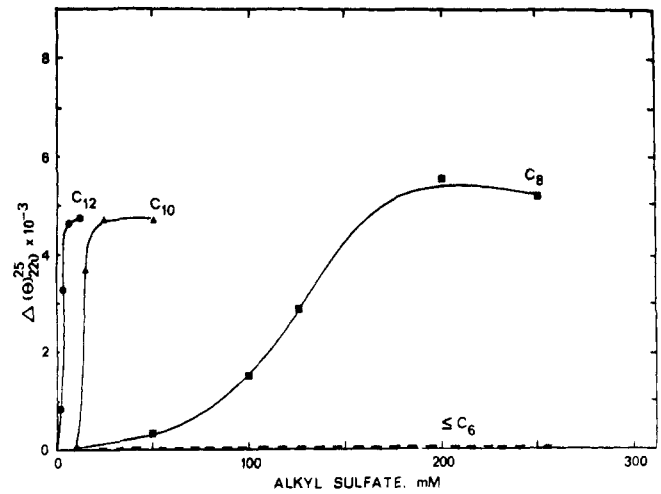

FIGURE 4: Effect of alkyl sulfates of varying carbon chain length on the CD spectrum of elastase. Results are expressed as the change in mean residue ellipticity of elastase at 220 nm in the specified concentrations of the alkyl sulfate solutions. Elastase was present at 0.1 mg/ml in water adjusted to pH 7. The pH of detergent solutions and of enzyme-detergent mixtures was adjusted to pH 7.

Results

Effect of Carbon Chain Length of Detergents. Elastin (10 mg) was suspended in 2-ml solutions of sodium alkyl sulfates of varying chain length and the elastin-detergent pellets isolated by centrifugation were tested for their susceptibility to digestion by elastase (Figure 1). The results are conveniently described both in terms of degree of maximum levels of stimulation and in terms of concentrations of alkyl sulfate required to bring about maximal stimulation. There are two general categories of effect: the rate of elastolysis increases 2.5–3-fold over the control when there are six or less carbon atoms, that is, in the cases of hexyl, pentyl, butyl, ethyl, and methyl sodium sulfates. This degree of stimulation is essentially the same as obtained in the presence of sodium sulfate, alone. Moreover, the concentrations of these shorter alkyl sulfates required to achieve the lower level of stimulation are apparently independent of chain length. The stimulation brought about by Na_2SO_4 or by the short-chain alkyl sulfates is apparently similar in origin and may reflect ionic factors (H. M. Kagan *et al.*, unpublished studies).

In contrast to the shorter chain length effects, a higher maximal level of stimulation of about five times that of the control is brought about by pretreatment of elastin with the sodium salts of octyl, decyl, and dodecyl sulfates. In this case, moreover, there is a marked chain length dependency of the concentration of alkyl sulfate needed to bring about this fivefold level of stimulation.

Circular dichroic spectral studies with soluble α-elastin reveal that these same ligands can also be classified into two categories on the basis of their ability to induce a conformational change in α-elastin. In this series of experiments, complete CD spectra were recorded in each case between 250 and 190 nm. For the sake of clarity, however, the titrations of conformational changes induced are plotted as the mean residue ellipticity at 225 nm *vs.* the concentration of ligand (Figure 2). As shown, neither hexyl, pentyl, butyl, ethyl, or methyl sodium sulfate nor sodium sulfate, itself, induces a measurable change in conformation at concentrations as high as 100 mM. However, octyl, decyl, dodecyl, and myristyl sodium sulfates do alter the CD spectrum of the elastin solution. In each case, the final perturbed spectrum is essentially the same as that previously reported for sodium dodecyl sulfate treated α-elastin in that the initial spectrum which reflects an apparently random conformation of α-elastin was altered to one which is similar to the CD spectra of proteins containing α-helical structure (Kagan *et al.*, 1972).

The CD titrations further indicate that the relative effectiveness of the octyl, decyl, and dodecyl compounds in altering conformation closely resembles their relative effectiveness at stimulating the elastolytic rate, as judged by the concentration dependencies in each instance.

It has been previously demonstrated that although sodium dodecyl sulfate stimulates elastolysis, it readily inactivates this activity of the enzyme if elastase and detergent are first preincubated together in the absence of elastin (Kagan *et al.*, 1972). The sensitivity of elastase to the series of alkyl sulfates was similarly determined by preincubation of elastase with the designated concentrations of test compound at 37° for 30 min followed by assay of aliquots of control and alkyl sulfate pretreated enzyme for esterase activity (Figure 3) using *tert*-Boc-L-alaninate *p*-nitrophenyl ester as substrate (Visser and Blout, 1972).

As shown, the 8-, 10-, and 12-carbon alkyl sulfates inactivate the esterolytic activity of elastase, while no significant effect on the catalytic rate was observed with any of the other compounds at the concentrations employed (Figure 3).

The relative concentration dependencies of the 8-, 10-, and 12-carbon alkyl sulfates differ in the case of esterase inhibition as compared to their effect on stimulation of elastolysis (see Figures 1 and 3). The interaction of these detergents with elastase was also quantitated by CD measurements of conformational changes which they induced in the enzyme. The titration curves so obtained indicate concentration dependencies for the 8-, 10-, and 12-carbon detergents similar to those shown in the case of esterase inhibition studies (Figure 4).

Therefore, it appears that the stimulation of elastolysis reflects the interaction of these detergents with elastin while the inhibition of esterase activity reflects the interaction of these agents with elastase, inactivating the enzyme by the induction of conformational changes. The differing concentration dependencies for the 8-, 10-, and 12-carbon detergents which result in changes in the CD spectrum of α-elastin and stimulation of elastolysis, on one hand (see Figures 1 and 2), and result in changes in the CD spectrum and esterase activity of elastase (see Figures 3 and 4), on the other hand, point toward differing

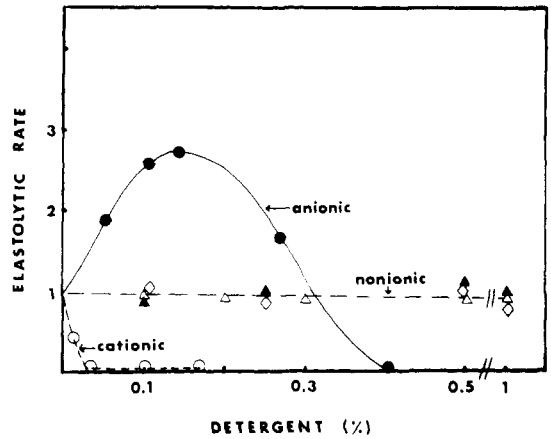

FIGURE 5: The effects of detergents of different charge on the rate of elastase-catalyzed digestion of insoluble elastin at pH 8.9 in water. Anionic detergents are represented in the figure by sodium dodecyl sulfate (●). The nonionic detergents are Tween-80 (▲), Triton X-100 (◇), and Nonidet P-40 (△). The cationic detergent depicted is dodecyltrimethylammonium bromide (○).

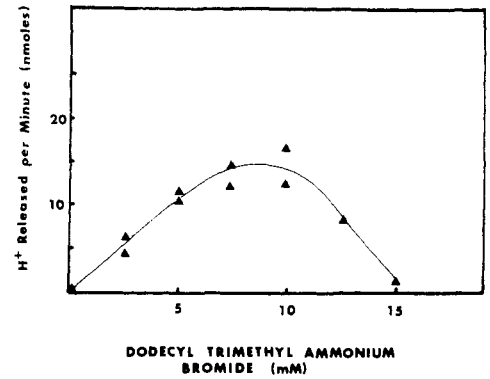

FIGURE 6: The digestion of insoluble elastin (10 mg) by maleylated elastase (12.5 μg) in various concentrations of a cationic detergent. Digestions were carried out in water at pH 8.9 and 37° with dodecyltrimethylammonium bromide present during the assay.

specificities of the binding sites for these detergents in elastin and elastase.

Effect of Ionic Charge of Detergents on the Rate of Elastolysis. Studies have indicated that the initial stage in the elastolysis of insoluble elastin involves electrostatic attraction between enzyme and substrate (Hall and Czerkowski, 1961; Gertler, 1971a). This suggests a role for the anionic moiety of the detergent in the alkyl sulfate mediated stimulation of elastolysis. In fact, our earlier studies have shown that sodium dodecyl sulfate enhances the binding of elastase to elastin under certain conditions (Kagan *et al.*, 1972). In order to more clearly define the contribution of the polar group in this stimulation, we have examined the ability of anionic, neutral, and cationic detergents to influence the rate of elastolysis.

The cationic detergent, cetyltrimethylammonium bromide, was shown by Hall and Czerkowski (1961) to inhibit the rate of elastolysis. Similarly, dodecyltrimethylammonium bromide, a cationic analog of sodium dodecyl sulfate, totally inhibits digestion of elastin at concentrations much lower than those at which sodium dodecyl sulfate stimulates the reaction (Figure 5). Moreover, this inhibition does not result from inactivation of elastase since the activity of the enzyme against the ester substrate is not affected by concentrations of this cationic detergent which totally inhibit elastolysis (*e.g.*, 0.5 mM). These results are consistent with the generation of a cationic detergent–elastin complex which has decreased electrostatic affinity for elastase, in accordance with the proposed charge relationship between the normally positively charged enzyme and slightly negatively charged substrate (Hall and Czerkowski, 1961; Gertler, 1971a).

The effects of neutral detergents on the rate of elastolysis are also shown in Figure 5. Of the neutral detergents tested, Tween-80, Triton X-100, and Nonidet P-40 were all without effect even when present in the assay at a concentration of 1%. Egg-white lysolecithin, which contains both anionic and cationic groups with a net charge of zero at the pH of assay, was also without effect.

Gertler (1971b) has shown that the electrostatic relationship between enzyme and substrate can be similarly disturbed by maleylation of the ε-amino groups of elastase lysine residues. This enzyme derivative is inactive in elastolysis whereas the esterolytic activity is essentially unchanged from that of native elastase (Gertler, 1971b). Since maleylation alters elastase from a cationic protein (pI = 9.6) to an anionic protein at the pH of assay, it was of interest to determine whether the enzyme–substrate charge relationships could be reversed to yield a productive enzyme–substrate complex in which the enzyme is negatively charged by maleylation, and the substrate is positively charged by complex formation with the cationic detergent. Indeed, this anionic enzyme derivative efficiently digests elastin in the presence of the cationic detergent. Thus, at the optimum concentration range of dodecyltrimethylammonium bromide, elastolysis by maleylated elastase proceeds at a rate approximating that obtained when native elastase is assayed in the absence of detergent (Figure 6).

Studies with Naturally Occurring Compounds. The combined properties of hydrophobicity and negative charge shown essential for the detergent-induced stimulation of elastolysis characterize certain classes of compounds which occur physiologically, including fatty acids and bile salts. Not surprisingly, at least a fivefold rate enhancement of elastolysis results when such compounds are introduced into the elastolytic assay under the appropriate conditions (Table I). It was necessary to vary the method used for exposure of elastin to these compounds due to the differing solubility characteristics of these agents in aqueous systems (detailed in the legend of Table I). In each case, conditions were employed to ensure that each agent would be totally dissolved before mixing with insoluble elastin or directly adding the agent to the assay mixtures. As shown, the sodium salts of saturated fatty acids (decanoate and laurate), unsaturated fatty acids (2-dodecenoate, palmitoleate, oleate, and linoleate) as well as bile acids (cholate, deoxycholate, glycocholate, and taurodeoxycholate) all induce a similar stimulation in the elastolytic rate. The sodium salt of octanoic acid did not affect the elastolytic rate at concentrations as high as 100 mM present in the assay system. Therefore, like the anionic detergents, a minimum number of carbon atoms is required for fatty acid salts to stimulate elastolysis.

These results indicate that the dual requirements for hydrophobicity and anionic character may be satisfied by compounds which differ considerably with regard to their chemical composition. Thus, hydrophobic groups may include linear saturated and linear unsaturated carbon chains. Further, the number and position of unsaturated double bonds may vary. Similarly, cyclic saturated carbon rings provide the appropriate hydrophobicity for stimulation of elastolysis. The requirement for negative charge may clearly be satisfied by organically bound sulfate ions or carboxylate groups. Presumably, these flexible requirements will extend to a variety of other compounds which are sufficiently hydrophobic and negatively charged.

TABLE I: Stimulation of Elastolysis by Synthetic and Naturally Occurring Compounds.

Agent	Concn Required for Maximum Effect (mM)	Method[b]	Rate[a] (nmol of H^+ Released min^{-1} 10 μg of Elastase^{-1})
Control			10
Alkyl sulfates			
Sodium tetradecyl sulfate	3.0	1	71
Sodium dodecyl sulfate	7.5	2	50
Sodium decyl sulfate	150	2	51
Sodium octyl sulfate	400	2	52
Saturated fatty acids			
Sodium laurate	7.5	3	51
Sodium decanoate	25	3	48
Unsaturated fatty acids			
Sodium 2-dodecenoate	60	2	50
Sodium palmitoleate	8.0	2	50
Sodium oleate	6.0	4	50
Sodium linoleate	2.5	5	55
Bile salts			
Sodium deoxycholate	15	3	67
Sodium cholate	15	3	61
Sodium taurodeoxycholate	20	3	58
Sodium glycocholate	20	3	60

[a] Titrimetric assay of peptide bond hydrolysis; 10 mg of elastin was suspended in a total volume of 2 ml of water at pH 8.9 and 37°. [b] (1) Sodium tetradecyl sulfate present during the assay in 0.15 M NaCl; (2) elastin was preincubated with ligand at the given concentration in water, centrifuged, and resuspended in water before addition of elastase; (3) ligands present during the assay in water at the given concentrations; (4) elastin was preincubated with oleate at pH 10.3, pelleted from suspension, and resuspended in water at pH 8.9 before addition of elastase; (5) elastin was preincubated with linoleate at pH 10.1 in 0.15 M NaCl, pelleted from solution, and resuspended in 0.15 M NaCl before addition of elastase at pH 8.9. Control elastolytic rate at this ionic strength is approximately 2 nmol of H^+ released/min.

Direct Binding Studies. The binding to elastin of agents which stimulate elastolysis was studied by measurement of the formation of pelletable complexes of isotopically labeled sodium dodecyl sulfate, sodium laurate, or sodium deoxycholate with insoluble elastin, as described (see Methods). Other studies indicate that there is an ionic component to the stimulation which can be satisfied by inorganic salts (see Figure 1). Therefore, binding of the hydrophobic ligands was studied at a low ionic strength of 0.018 and at a higher ionic strength of 0.15, the latter chosen to simulate the physiological condition. As shown in Figure 7, the binding affinity of elastin differs for cholate, laurate, and dodecyl sulfate especially at the lower ionic strength. The maximal binding levels of laurate and dodecyl sulfate become similar at the higher ionic strength, in contrast to their behavior at a μ of 0.018. Interestingly, the binding curves for sodium deoxycholate are essentially identical at the high and low ionic strengths employed. The maximum binding level of this ligand is significantly lower than those for laurate at a μ of 0.018 and sodium dodecyl sulfate at either ionic

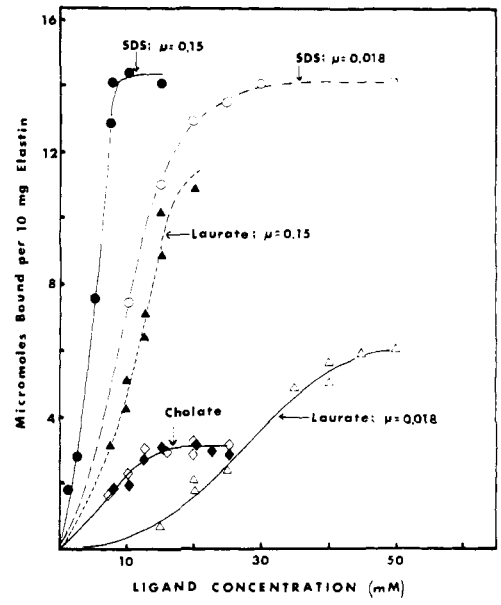

FIGURE 7: The effect of ionic strength on the binding of sodium dodecyl sulfate, sodium laurate, and sodium cholate to insoluble elastin at pH 8.9. Results are expressed as the micromoles of ligand bound to 10 mg of elastin in 0.05 M sodium borate buffer (μ = 0.018) and in borate buffer adjusted to an ionic strength of 0.15 with NaCl.

strength. These apparently differing binding modes of linear alkyl sulfates and fatty acids, on the one hand, and the deoxycholate salt, on the other, are consistent with the salt-dependent behavior of such compounds in solution. Thus, increasing ionic strength favors the tendency of such linear, ionic detergents and fatty acids to associate in micellar aggregates while bile salts do not exhibit such salting-out behavior at either ionic strength used in this study (Carey and Small, 1969). These results suggest, therefore, that the linear compounds may "salt out" as aggregates on the elastin surface.

The binding data at physiological ionic strength were compared to the corresponding effects of the agents on the elastolytic rates obtained in 0.15 M NaCl (Figure 8). It should be noted that the control rate of elastolysis in the complete absence of added hydrophobic ligand is markedly inhibited by the salt present (*i.e., ca.* 2 μmol of NaOH/min *vs. ca.* 10 μmol of

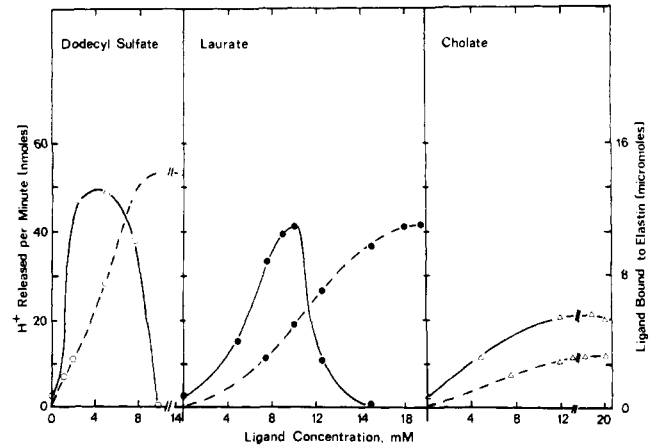

FIGURE 8: Comparison of binding curves (- - -) of hydrophobic elastin ligands with effects of these agents on the rate of elastolysis (—). Both binding and elastolytic rate determinations were carried out at pH 8.9 at 37° at an ionic strength of 0.15.

NaOH/min in water). In each case, however, the added elastin ligand can reverse this inhibition at least to the level of activity seen in water or, in the cases of sodium dodecyl sulfate and sodium laurate, even cause a stimulated rate which is 2–2.5 times the rate obtained in water (*i.e.*, 40–50 μmol/min) yielding a total stimulation of approximately 10-fold, 20-fold, or 25-fold that of the salt-inhibited rate, in the cases of deoxycholate, laurate, and dodecyl sulfate, respectively.

There is general agreement between the concentration ranges over which binding of ligand and stimulation of activity occur (Figure 8). The inhibitory effects on elastolysis noted in the cases of sodium dodecyl sulfate and sodium laurate and absent in the case of the bile salt are readily explicable by the differing effects of these agents on the intrinsic catalytic ability of elastase. As noted, sodium dodecyl sulfate readily inactivates the esterase activity (see Figure 3). Sodium laurate similarly inhibits this catalytic function to the extent of 70% inhibition at 10 mM laurate. However, the bile salt does not measurably affect esterase activity at the concentrations employed in the experiment of Figure 8.

Discussion

The present data extend the studies previously reported on the effect of sodium dodecyl sulfate on the elastolysis of insoluble elastin (Kagan *et al.*, 1972). Thus, octyl, decyl, and tetradecyl sodium sulfates similarly stimulate elastolysis by interacting with elastin and altering its susceptibility to elastase digestion. Elastin–detergent interaction is revealed both by direct binding studies and by the observation that the 8-, 10-, and 12-carbon compounds alter the conformation of α-elastin with approximately the same relative concentration dependencies as observed in elastolytic stimulation. These effects cannot be attributed to an increase in the catalytic ability of elastase since these same compounds inhibit enzyme activity as measured by the esterase assay or by the elastolytic assay if elastase is preexposed to sodium dodecyl sulfate (Kagan *et al.*, 1971). Similarity between the CD and enzyme data suggests that conformational alterations induced by these alkyl sulfates and by fatty acids in elastin may underlie the enhancement of elastolysis to a significant degree, possibly by exposing otherwise unavailable peptide bonds to enzymatic attack. However, a more direct measure of conformational effects in the insoluble substrate is obviously desirable to establish this unequivocally.

The observation that a minimal detergent chain length of 8 carbons is required to achieve the highest level of stimulation and a change in conformation suggests that a minimal number of multiple, apolar contacts must form between elastin and the detergent to produce effective binding and stimulation of elastolysis. A similar conclusion has been suggested in the case of bovine serum albumin which also shows a similar dependency upon the carbon chain length of alkyl sulfate detergents which bind to this serum protein (Steinhardt and Reynolds, 1969).

It is of interest that the eight-carbon alkyl sulfate stimulates elastolysis, albeit at a relatively high concentration, while a minimum chain length of ten carbons is necessary with the saturated fatty acids to achieve stimulation. This likely reflects the fact that the oxygen atom bridging the carbon and sulfur atoms of alkyl sulfates does not contribute significantly to the polarity of the detergent while it also permits all carbons to function as methylene groups to take part in hydrophobic interactions. The carboxyl group of the fatty acids, however, while counting in the enumeration of chain length, is polar at the pH of assay due to the oxyanions. Nevertheless, the requirement for minimal hydrophobic binding energy pertains in both classes of compounds.

As we have previously suggested (Kagan *et al.*, 1972) at least part of the mechanism of stimulation by these ligands is likely due to the augmentation of the net anionic character of the substrate which they impose, consistent with the proposed electrostatic interaction between the normally anionic elastin and cationic elastase (Gertler, 1971a; Hall and Czerkowski, 1961). In fact, elastin-bound sodium dodecyl sulfate results in greater adsorption of elastase to elastin as a pelletable complex under certain conditions as well as an increase in the proteolytic rate (Kagan *et al.*, 1972). However, the relationship between pelletable adsorptivity of enzyme and hydrolytic enzymatic activity directed toward elastin is not clear at present and is the subject of further study. This proposed role of the anionic moiety of stimulating agents is supported by the present data which indicate that a complex of elastin with a cationic detergent is not a functional substrate for elastase, consistent with the previous demonstration that a cationic detergent inhibits the formation of the sedimentable enzyme–elastin complex (Hall and Czerkowski, 1961).

However, the distribution of the complementary charges in the enzyme–substrate pair does not seem to be of great significance in order to generate a catalytically functional interaction. This is clear from the observation that maleylation of elastase, resulting in an enzyme derivative which is anionic at assay pH and, therefore, unable to digest elastin (Gertler, 1971b), will, in fact, hydrolyze elastin if this substrate is rendered cationic by the addition of a cationic detergent. The implications of this result are far reaching. Thus, elastolysis can be controlled not only quantitatively but qualitatively, as well, by elastin ligands since in this case the totally nonproductive enzyme–substrate pair is made to operate efficiently by an elastin ligand which provides an electrostatic charge reciprocal to that of the enzyme on the substrate. In addition, this result indicates that while lysyl side chains of the enzyme may be important to facilitate enzyme–substrate interaction, these ε-amino groups do not precisely orient the enzyme on the substrate so that the active site is in the proper stereochemical relationship to the susceptible peptide bond. Hence, the negative charge of the maleyl group can be pictured to be close to the site of the original cationic amino group since the acylating carboxyl function and the ionized carboxyl function of the acylating agent are cis to each other. However, the four carbons of the maleyl substituent provide molecular bulk at these enzyme sites as does the cationic detergent on the elastin surface. Therefore, it seems likely that reciprocal charge–charge interaction between enzyme and substrate is essential for elastolysis to proceed, but not for the purpose of precise stereochemical alignment of the active site and hydrolyzable peptide bonds on elastin. The role of this charge reciprocity may well be to overcome diffusional limitations between the insoluble substrate and the soluble enzyme. This concept seems to apply to other instances involving heterogeneous catalysis, but usually in consideration of immobilized enzymes and soluble substrates (Goldstein, 1972; Goldman *et al.*, 1971). Immobilized enzyme particles are surrounded by an unstirred layer of solvent in aqueous suspension in which a concentration gradient of soluble substrate is established (Nernst, 1904; Goldman *et al.*, 1971). In the present instance, such a diffusional barrier in the substrate against enzyme mobility seems likely not only in view of the insoluble character of elastin, but also in consideration of its extremely hydrophobic and highly cross-linked nature. It is not difficult to picture an envelope of water molecules organized with limited translational freedom around the intricate network of hydrophobic polypeptide chains. The detergents which stimulate elastolysis, therefore, could interact hydropho-

bically with the protein side chains while enhancing the net ionic character of the substrate to reduce this diffusional limitation by enhancing water mobility and to provide an attractive ionic force for the oppositely charged enzyme. If such effects were coupled with even subtle conformational effects induced in the substrate by hydrophobic ligands, the degree of enzymatic susceptibility could well be considerable.

In summary, the present study demonstrates that the elastolytic process is readily modifiable by a variety of small molecules which can bind to elastin. Therefore, it does not seem unreasonable to propose that ligands with the appropriate chemical characteristics could exert an effect on the metabolism of elastin fibers *in vivo*. For example, fraying of elastic fibers has been observed in lipid-associated aortic plaques. Whereas the majority of lipids which interact with elastin and which are implicated in the pathology of aortic tissues are electrostatically neutral (*i.e.*, cholesterol, cholesterol esters, phospholipids, and triglycerides (Kramsch *et al.*, 1971)), the presence of free fatty acids has also been demonstrated in proximity to elastic fibers in the fatty streak (Chobanian and Manzur, 1972). The cumulative influence on even small amounts of free fatty acids might be considerable over an extended length of time. The possibility of fatty acids playing a regulating role in elastolysis seems even more important in light of the present observation that these ligands can reverse the marked inhibition of elastolysis which occurs at physiological ionic strengths.

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Stimulation of Alkaline Phosphatase by Analogs of Inorganic Pyrophosphate†

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ABSTRACT: Hydrolysis of low concentrations of 4-nitrophenyl phosphate by nonspecific alkaline phosphatases from *Escherichia coli* and from bovine intestine is stimulated by low concentrations of methylene diphosphonate or imidodiphosphate, which are analogs of inorganic pyrophosphate (PP_i). Under similar conditions PP_i, P_i, inorganic phosphite, and EDTA have little or no effect. The observed stimulations are inconsistent with the "flip-flop" mechanism; the results favor a model involving cooperativity between the two subunits of the enzyme, but are also consistent with a model involving two isomeric forms of the enzyme, in which the PP_i analogs shift the equilibrium toward the form which has a higher affinity for substrate.

We have recently reported that inorganic pyrophosphate (PP_i) and its analogs in which the bridge oxygen atom is replaced by either an imido or methylene group competitively inhibit the hydrolysis of 4-nitrophenyl phosphate by nonspecific alkaline phosphatases of *Escherichia coli* and bovine intestine (Kelly *et al.*, 1973). We observed in the course of this investigation that under some conditions the PP_i analogs (but not PP_i itself) had a small but reproducible stimulatory effect upon these enzymes. In this communication we present these observations and their interpretation with respect to the mechanism of action of these enzymes.

Materials and Methods

The source of all materials, including enzymes (both of

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