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The influence of molecular size and pH on the macrocationic inhibition of pepsin by polylysine

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Polylysines of 7–371 lysine residues inhibited pepsin over the pH range 3·6–5·0 in a system using azocoll as substrate. Tetralysine was inactive. An almost 1:1 molar ratio with pepsin gave maximum inhibition for a polylysine containing 59 lysine residues but increase in polylysine molecular size beyond this size was not accompanied by increase in activity on a weight basis although the polylysine:pepsin molar ratio for maximum inhibition decreased and inhibition mechanism varied. Polylysines of 59 and 158 lysine residues which were intermediate in the series were non-competitive inhibitors, whilst polylysines of greater and smaller molecular size were competitive inhibitors, although only the smallest inhibitory polylysine, containing 7 lysine residues, was a pure competitive inhibitor. Polylysine inhibition of pepsin was found to be strongest at pH 5·0 and the pH dependence appeared to be associated with the relative concentrations of the enzyme and inhibitors in ionized form. For each polylysine it was possible to detect a polylysine:pepsin concentration ratio for which inhibition was pH-independent over the range 3·6–5·0.

A role for pepsin in acid peptic disease has continued to sustain suspicion but defy definition. Any control of its protein and peptide destroying activity is unknown, other than by the rather insensitive, albeit effective, method of gastrointestinal pH elevation. However, in the acid secreting environment of the stomach, particularly the hypersecreting stomach, instant alkalinization usually is not readily available to control unwanted peptic activity. Activation of pepsinogen releases pepsin together with pepsin-inhibitory peptides derived from amino acids 1–44 of the pepsinogen. The basic character of certain of these peptides is largely due to lysine and arginine residues. It is believed (Anderson & Harthill 1973; Kumar & Kassell 1977) that these cationic molecules provide interaction sites for the carboxyl anions of pepsin, which are represented in the active site (Tang et al 1973) resulting in macrocationic inhibition of pepsin.

The first systematic studies of the inhibition of peptic activity frequently considered macroanionic inhibitors and related predominantly to secreted luminal pepsin at highly acid pH values at which the mechanism of inhibition was one of substrate protection or depletion (Anderson 1961; Turner et al 1967; Anderson et al 1968). Carbenoxolone also inhibits pepsin in vitro and has been suspected to influence pepsinogen on account of its absorption (Roberts & Taylor 1973).

There appears to be no reason to suppose that any pathogenic pepsin proteolysis may not also be initiated before the enzyme is secreted into the gastric lumen and the enquiry therefore shifts to include inhibition at cellular or near-cellular level raising questions such as specificity and pH possibly up to 5 but certainly higher than those normally encountered in luminal content. Below pH 6 at which destruction of pepsin begins, increase in pH will increase ionization of pepsin carboxyl groups which in turn will promote interaction with cationic centres in suitable molecules. Hence the methods which have been used (Anderson & Harthill 1973; Harboe et al 1974; Kumar & Kassell 1977) to demonstrate inhibitory activity of such substances adopt a pH of around 5, rather than a pH of around 2 which is suitable for haemoglobin digestion. In the latter type of system ionization of pepsin carboxyls will tend to be depressed and opportunity for cation interaction correspondingly decreased.

Based on such considerations a search for the factors which determine peptide inhibition of pepsin and for peptides or similar substances more readily available and hopefully with greater activity appears reasonable. One approach to the study of the factors involved is that of considering the relevant properties of the synthetic poly-α-amino acid, polylysine, which is readily available in different degrees of polymerization, behaves as a macrocation and has been shown to influence biological activities in systems where these activities depend on the presence of one or more macroanions (Donaruma 1975). Polylysines of varying molecular size have therefore been chosen as a means of examining certain of

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the characteristics of macrocationic inhibition of pepsin in the upper pH ranges of its activity.

MATERIALS AND METHODS

Pepsins. Two pepsins were used: (a) twice crystallized pig pepsin (Sigma); and (b) pig pepsin prepared from pepsinogen which had been extracted according to Ryle (1970). The latter pepsin was prepared by activation of 75 mg pepsinogen in 8 ml water at 14 °C brought to pH 2·0 by rapid addition of a chloroacetic acid - HCl mixture maintained at pH 2·0 and 14 °C for exactly 20 min at which time 4M sodium acetate buffer was added to give pH 4·4 (Rajagopalan et al 1966). The activation mixture was then applied promptly to a column of SP-Sephadex C-25 and eluted with 0·001 M acetic acid (Trujillo & Schlamowitz 1969) before dialysis. Homogeneity of both pepsins was determined by chromatography on hydroxylapatite after Rajagopalan (1966). Fresh solutions of pepsin in 0·025 M sodium acetate buffer were prepared daily for the inhibition experiments.

Azocoll substrate. Calbiochem Ltd., London. Azocoll as supplied is a granular solid, subject to granule size variation. However, whilst between batch variation could amount to 25% in the rate of pepsin digestion (at pepsin concentration of 0·05 mg $(3\ ml)^{-1}$) large ($\geqslant 76\mu m$) and small ($<76\mu m$) granules separated from within a batch showed only non-significant differences in activity; and in any case reasonably uniform distribution of granule sizes in use could be ensured by inversion of the container before removal of the sample.

Poly-L-lysine hydrobromides. These poly-α-amino acids, here referred to as polylysine, were obtained from Miles-Yeda, Rehovoth, Israel. They had been prepared from 6-*N*-benzyloxycarbonyl-L-lysine and had stated molecular weights (sedimentation) 1250, 4800, 12300, 33000, 43870 and 77000. Corresponding degrees of polymerization were 7, 23, 59, 158, 210, 371, and they are referred to as lys_7, etc. Lysine in each polylysine hydrobromide was determined (Locarte Mk IV Amino acid analyser) following hydrolysis with 6 M HCl and the results were in good agreement with the stated degrees of polymerization. Dialysed polylysines behaved in the inhibition experiments in the same manner as undialysed polylsines which were used without further purification.

Buffer solutions were 0·025 M sodium acetate at various pH values.

Azocoll method for peptic activity. Following Kay (1975) this was conducted at 39 °C. Except when variations in substrate concentration or in pH were studied, 15 mg azocoll was weighed into 25 ml conical flasks and suspended in 2 ml acetate buffer at pH 5. The pepsin solution (usually 0·05 mg twice crystallized pepsin or an equivalent amount of chromatographically purified pepsin as judged by the present method) was added and the reaction allowed to proceed for 20 min in a shaking $(100\ min^{-1})$ water bath. The reaction was stopped by removing residual azocoll by filtration and the absorbance of released dye, which does not participate in the enzyme reaction but which is a measure of digestion of the azocoll substrate, read at 520 nm in 1 cm path length microcells. Peptic activity was expressed as filtrate absorbance (adjusted for blanks and controls) relative to 20 min proteolysis.

Pepsin inhibition. When polylysine inhibitor was included in the enzyme system the polylysine dissolved in 1 ml buffer was mixed with 1 ml pepsin solution and the enzyme-inhibitor interaction allowed to proceed at 20 °C usually for 0·5 h (pre-incubation) when 1 ml of the mixture was added to the azocoll dispersion (total digest volume 3 ml) and the digestion allowed to proceed as described above. Inhibition, i, was calculated as the difference between uninhibited and inhibited absorbances expressed as a fraction of the uninhibited.

RESULTS

Azocoll method. A plot of absorbance units $(20\ min)^{-1}$ against pepsin concentration (0·01–0·08 mg $(3\ ml)^{-1}$ crystallized pepsin) was linear whilst at each pepsin concentration used to construct that plot, absorbance was linearly related to time of digestion, up to 20 min. Coefficient of variation was 7% in uninhibited and $\leqslant 11\%$ in inhibited reactions.

The double reciprocal plot, 1/v versus 1/S was linear for both pepsins giving for twice-crystallized pepsin and chromatographically purified pepsin respectively $K_m = 12{\cdot}20$ mg $(3\ ml)^{-1}$; $V = 0{\cdot}061$ absorbance units min^{-1}; $K_m = 6{\cdot}79$ mg $(3\ ml)^{-1}$; $V = 0{\cdot}050$ absorbance units min^{-1}. K_m and K_m app, V (V_{max}), v, S, I, K_i have their usual meanings.

Preincubation of enzyme and inhibitor. The two smallest polylysines (lys_7, lys_{23}) gave maximum inhibition only after enzyme-inhibitor pre-incubation at 20 °C for 0·5 h; all other polylysines interacted instantaneously, although a pre-incubation period was included for all.

Inhibition and molecular size of polylysine. Tetralysine (0·001–1 mg $(3\ ml)^{-1}$; preincubation periods up to 1 h; pH 3·6, 5·0) did not inhibit pepsin. All other polylysines did so with maximum inhibition

at pH 5·0; the various relationships are given in Table 1.

Order of addition. Enzyme-inhibitor pre-incubation, followed by addition of substrate, in every instance resulted in several-fold greater inhibition than substrate-inhibitor pre-incubation followed by addition of enzyme. On the other hand, addition of inhibitor to an enzyme-substrate mixture resulted in no inhibition.

Table 1. Comparative pepsin inhibitory data for a series of polylysines

Polylysine	i_{max}, %	I for i_{max} mg (3 ml)$^{-1}$	Polylysine : pepsin molar ratio for i_{max}	No. of $-NH^+_3$ in polylysine per mole pepsin for i_{max}
lys_7	76	0·30	220	1760
lys_{23}	87	0·15	23	552
lys_{59}	90	0·02	1·10	66
lys_{158}	98	0·02	0·42	67
lys_{210}	94	0·02	0·31	65
lys_{371}	100	0·02	0·18	67

i_{max}, maximum inhibition; I, inhibitor concentration; column 5 indicates *total* possible ionized polylysine. ε-amino groups available to one mole pepsin at i_{max}.

Reversibility of enzyme-inhibitor interaction. When conditions favoured insoluble enzyme-inhibitor complex formation, dissolution of the complex could be brought about by addition of sodium chloride, the macroanion heparin, excess of the inhibitor, polylysine, or by increase in temperature. When such a pepsin-polylysine complex was caused to dissolve by the addition of sufficient heparin the inhibition normally caused by the polylysine was markedly reduced or even eliminated, indicating that the stronger macroanion heparin had replaced pepsin, thus forming heparin-polylysine and confirming the existence of a dissociable link between pepsin and polylysine in the pepsin-polylysine complex.

The plot of velocity of enzyme reaction versus enzyme concentration (Ackerman & Potter 1949), frequently used to indicate reversibility, gave linear regressions passing through the origin for all polylysines except lys_{59} and lys_{158}, which double reciprocal plots showed to be non-competitive inhibitors. However, the straight line intercepts on the concentration axis for these polylysines in the Ackerman & Potter plots were small enough to allow the chemical evidence for reversibility adduced above to stand.

Effect of pH. Within the range pH 3·6–5·0 rate of digestion of azocoll was linearly related to enzyme concentration with a maximum at pH 4. Beyond pH 5·0 the integrity of pepsin is prejudiced.

Except for lys_7, the polylysine of lowest mol. wt, a common pattern of inhibition as a function of pH, consistently emerged for all polylysines. Thus, above a certain 'critical' inhibitor concentration, inhibition increased with increasing pH, whilst below this critical inhibitor concentration inhibition decreased with increasing pH. It follows (Fig. 1) that at this critical inhibitor concentration (or concentration range) inhibition was independent of pH.

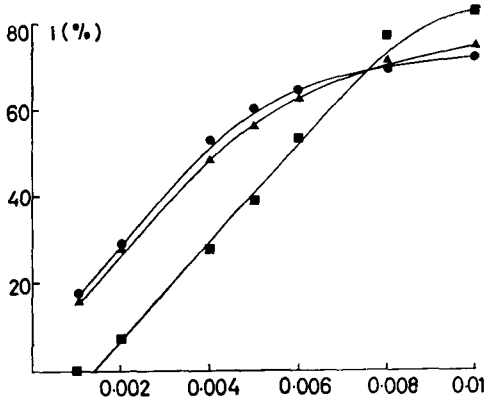

FIG. 1. Polylysine (lys_{210}) inhibition, i%, of pepsin at several pH values: ●, pH 3·6; ▲, pH 4·0; ■, pH 5·0. Abscissa: I mg (3ml)$^{-1}$.

Another general feature observed was that at pH $<5·0$ maximum inhibition was reached at lower inhibitor concentrations than those required for maximum inhibition at pH 5·0 where the inhibition maxima were higher than for lower pH values. Thus it appeared that, for identical concentrations, more inhibitor was available to effect more inhibition at pH 5·0 than at lower pH. Fig. 1 depicts the results for lys_{210} which, allowing for minor quantitative variations, was representative for the other polylysines (except lys_7).

Relevant data are in Table 2. lys_{59} which effects

Table 2. Critical polylysine concentrations at which inhibition was pH-independent.

Poly-lysine	concn mg (3 ml)$^{-1}$	polylysine: pepsin molar ratio at crit. concn	No of $-NH_3^+$ mol^{-1} pepsin at crit. concn	inhib., % at crit. concn
lys_{23}	0·45	62	1488	55
lys_{59}	0·14	0·78	47	78
lys_{158}	0·01	0·21	33	64
lys_{210}	0·008	0·12	25	68

the highest pH-independent inhibition has the inhibitor : enzyme ratio nearest unity and also gives highest inhibition at pH <5·0.

For lys_7, the polylysine of lowest molecular size, the pH dependence of inhibition was such that inhibition was higher at pH 4·6 and 5·0 than at pH 3·6 and 4·0, particularly at the higher concentration levels.

Inhibition mechanism. Double reciprocal plots indicated that the polylysines of intermediate size, lys_{59} and lys_{158} were non-competitive inhibitors whilst those of larger and smaller size, lys_7, lys_{23}, lys_{210}, lys_{371} were competitive inhibitors of pepsin at pH 5·0 (Fig. 2 a–f). At pH 3·6 corresponding plots for lys_{59} and lys_{210}, representing non-competitive and competitive inhibition respectively, allowed similar conclusions. Confirmatory plotting, 1/v versus I, v versus v/S, S/v versus S all gave results supporting the conclusions from the double reciprocal plots. Deviation from linearity at some low substrate - high inhibitor concentrations, could be detected but usually was not included in the double reciprocal plots. In replots of the slope of the double reciprocal plot against inhibitor concentration, only lys_7 gave a completely straight line. It appears therefore that in the system used, lys_7 is a pure competitive inhibitor of pepsin, lys_{23}, lys_{210} and lys_{371} are partial competitive inhibitors, whilst lys_{59} and lys_{158} are

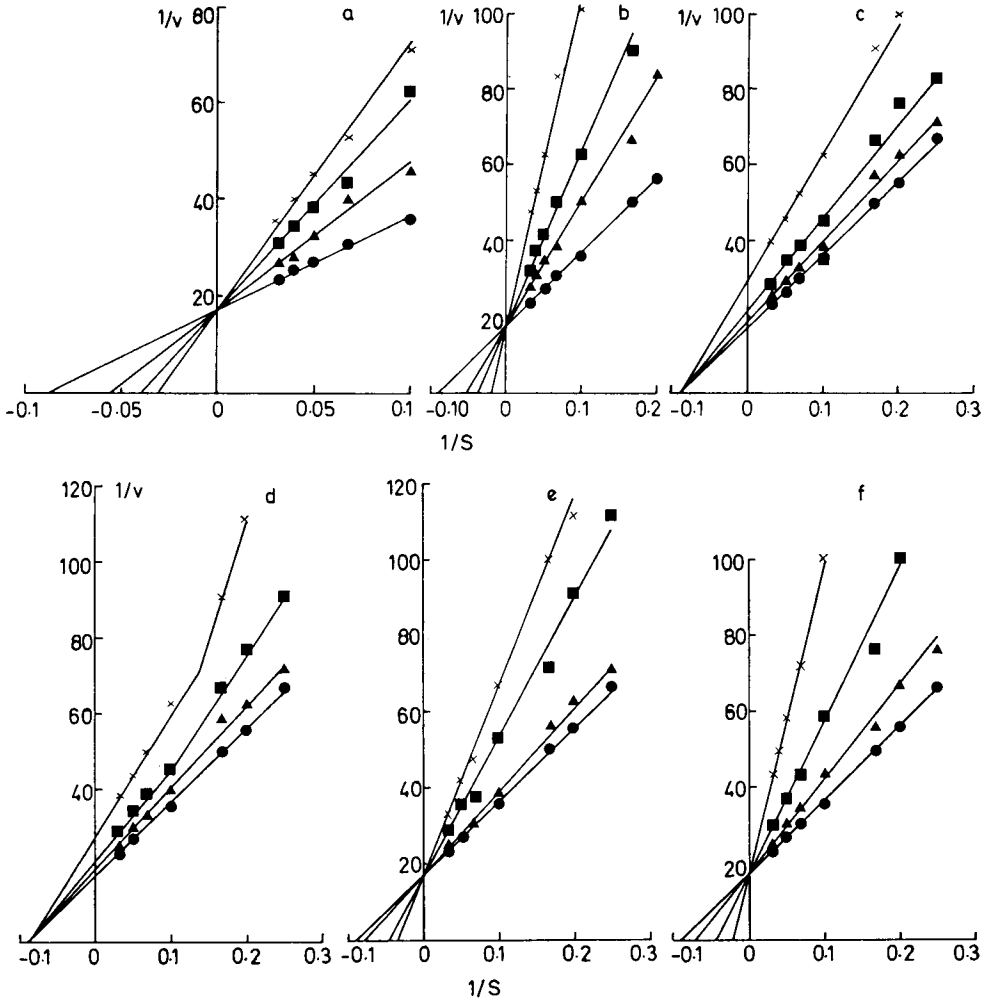

FIG. 2. Double reciprocal plots for polylysine inhibition of pepsin. a. lys_7 concentrations, mg (3 ml)$^{-1}$: ●, 0; ▲, 0·02; ■, 0·05; ×, 0·10. b. lys_{23} concentrations, mg (3 ml)$^{-1}$: ●, 0; ▲, 0·02; ■, 0·04; ×, 0·06. c. lys_{59} concentrations, mg (3 ml)$^{-1}$: ●, 0; ▲, 0·002; ■, 0·004; ×, 0·006. d. lys_{158} concentrations, mg (3 ml)$^{-1}$: ●, 0; ▲, 0·002; ■, 0·004; ×, 0·006. e. lys_{210} concentrations, mg (3 ml)$^{-1}$: ●, 0; ▲, 0·002; ■, 0·004; ×, 0·006. f. lys_{371} concentrations, mg (3 ml)$^{-1}$: ●, 0: ▲, 0·002; ■, 0·004; ×, 0·006. v, absorbance units min^{-1}; S, mg (3 ml)$^{-1}$.

partial non-competitive inhibitors. Deviations from linearity in the plots at low substrate - high inhibitor concentrations suggested substrate - inhibitor interaction and this interpretation was supported by hyperbolic I versus i/(1−i) plots for all polylysines except lys_7.

The direct linear plot (Eisenthal & Cornish-Bowden 1974; Porter & Trager 1977) gave computer-calculated values for K_m and V (obtained using the same data but omitting the deviant low substrate - high inhibitor concentrations) which were constant for K_m, variable for V, for lys_{59}, lys_{158} indicating non-competitive inhibition; for lys_7, lys_{23}, lys_{210} and lys_{371} competitive inhibition was indicated (V, constant; K increased with inhibitor concentration). Data are in Table 3.

Table 3. Kinetic data for polylsyine inhibition of pepsin.

Poly lysine	Concn in digest mg (3 ml)$^{-1}$	$K_{m\,app}$ mg (3 ml)$^{-1}$	V absorbance units min^{-1}
lys_7	0·02	19·40	0·063
	0·05	22·00	0·055
	0·1	31·83	0·057
lys_{23}	0·02	16·75	0·055
	0·04	24·41	0·053
	0·06	50·94	0·057
lys_{59}	0·002	12·56	0·057
	0·004	12·44	0·049
	0·006	11·52	0·035
lys_{158}	0·002	14·07	0·065
	0·004	14·24	0·049
	0·006	14·35	0·039
lys_{210}	0·002	13·38	0·059
	0·004	20·46	0·059
	0·006	27·20	0·057
lys_{371}	0·002	14·60	0·057
	0·004	24·00	0·063
	0·006	52·55	0·061

This latter method of dealing with the results (for which a computer programme was kindly supplied by Dr. Cornish-Bowden) removes many of the difficulties related to inherent errors and their distribution, and not least the difficulties which accompany attempts to ensure the most accurate determination of K_m and V from the normal data plots. Ki for the various polylysines (molar) were lys_7–lys_{371} respectively: $1·2 \times 10^{-5}$; $0·14 \times 10^{-5}$; $0·1 \times 10^{-6}$; $0·6 \times 10^{-7}$; $0·19 \times 10^{-7}$; $0·12 \times 10^{-7}$. *Chromatographically purified pepsin.* Polylysines lys_{158} and lys_{210} selected as representative non-competitive and competitive inhibitors respectively were also studied using chromatographically purified pepsin prepared from pepsinogen. Non-competitive double reciprocal plots (K_m = 7·13 mg (3 ml)$^{-1}$; V = 0·044, 0·038, 0·025 absorbance units min^{-1}) were obtained for lys_{158}, as for crystallized pepsin of commercial origin, but lys_{210} (K_m = 7·15 mg (3 ml)$^{-1}$; V = 0·044, 0·041, 0·034 absorbance units min^{-1}) which was (partially) competitive using crystallized pepsin also inhibited chromatographically purified pepsin non-competitively.

DISCUSSION

Control by enzyme inhibition at cellular or near-cellular level where unwanted proteolytic activity could be ulcerogenic, so far appears not to have received attention, although secretion inhibiting effects are well known. The polylysines are similar in structure and activity to the highly basic pepsinogen activation peptides which may be involved in such control and in the present work these synthetic poly-α-amino acids are used to demonstrate the variation in inhibitory properties which can attend the relatively simple change involved in increasing the chain length of these macroationic materials.

Basic amino acids such as lysine and arginine do not inhibit pepsin at least in reasonable concentrations (unpublished results from this laboratory) and in the present study the 4-amino acid tetralysine showed no inhibitory activity which in the series studied appeared in a polylysine containing 7 lysine residues. Although lys_5 and lys_6 were not examined the differences between the activity of lys_7 and that of other polylysines examined, particularly its several-fold lesser activity (Table 1) than lys_{23} and its different pH dependence, suggested that significant inhibitory activity appeared with 7 lysine residues. One naturally occurring inhibitory peptide containing lysine has 16 amino acids (Anderson & Harthill 1973) and inhibitory activity can probably not be expected if that is reduced below 12 (Dunn et al 1976; Kumar & Kassell 1977) whilst still containing the important -lys- lys- sequence. The inhibition of pepsin by polylysines is an example of macrocationic enzyme modification and many of the variables likely to govern the underlying interaction with the macroanionic pepsin have been identified although our understanding of their operation in vivo as the basis of an enzyme control system is certainly incomplete.

Studies of peptide (Anderson & Harthill 1973; Kumar & Kassell 1977) and polylysine (Anderson 1970) inhibition of pepsin have usually been based

on the use of milk-clotting systems for enzyme activity determination but these systems are understandably complex, and end point determination involves a subjective observation; furthermore, variability of the dried milk substrate depending on its source and manner of drying make comparison of results difficult (unpublished observations from this laboratory). Many of the problems of the milk clotting tests were avoided in the present work by using the chromogenic substrate azocoll, used by Kay (1975) for pepsin, which has been found suitable for studying polylysine inhibition, the only difficulty being the laborious necessity to weigh out the insoluble substrate into every flask. The linear relationships which necessarily underly quantitative interpretations of the enzyme activity and inhibition results were easily shown. Azocoll is a conjugate of collagen and a red dye, benzidine naphthol-3,6-disulphonic acid which is released quantitatively into solution at a rate which is linearly dependent on peptic activity over the range pH 3·0–5·0.

Whilst the globular macroanionic pepsin molecule behaves as a weak polyacid at pH 5·0 having a uniquely large number of ionizable carboxyl groups, the linear polylysines behave as macrocations of varying flexibility, the regularly spaced ϵ-amino groups being ionizable below pH 10. In the present work all ϵ-amino groups are considered available for interaction with anions. Thus pepsin-polylysine interaction at pH 5·0 is likely to be very largely electrostatic in nature although hydrogen, and to a lesser extent hydrophobic, bonding could conceivably occur to a degree which is determined by the particular polylysine involved. Despite the common interaction mechanism for all polylysines the differences in activity between the different polylysines suggest that detailed aspects of the interactions vary. In a more general way Elbein (1974) reviewing such interactions stressed that each system requires detailed interpretation despite common underlying interaction type. Frequently, electrostatic interactions are easily reversible, for example by increased ionic strength in the system and evidence for reversibility in the present study is to be found not only in the velocity versus enzyme concentration plot but also in the loss of inhibitory activity when the pepsin-polylysine complex formation is reversed by increase in macroanion or macrocation concentration. Results of the order of addition experiments also supported the conclusion of reversibility.

True competitive inhibition suggesting interaction of inhibitor with the enzyme active site was demonstrated only for the inhibitory polylysine of smallest size, lys_7, which required for its maximum inhibition 10 times the number of moles per mole of pepsin required by the next largest polylysine lys_{23}. The shorter the polylysine chain the more influential will be the terminal carboxyl which when ionized will be repelled by the anionic active site of pepsin. For three other polylysines (lys_{23}, lys_{210}, lys_{371}) the partial nature (indicated by additional plots) of their competitive inhibition may have been contributed to by inhibitor-substrate interaction at low substrate concentrations in addition to directive influences relative to the enzyme active site. Two intermediate polylysines in the series (lys_{59}, lys_{158}) showed non-competitive character suggesting interaction with the enzyme but not at the active site. Since the polylysines differ chemically from each other only in number of lysine residues which are linearly arranged, the chains presumably adopting random coil configuration, it may be that the different mechanism of lys_{59}, lys_{158} arises from peculiarity of shape relative to that of pepsin. Table 1 shows that lys_{59} and lys_{158}, particularly lys_{59}, most nearly approximated to molar 1 : 1 interaction ratio with pepsin. Moreover for these two polylysines there was a hint of non-reversibility in the velocity versus enzyme concentration plot, findings which could suggest tight binding of these two polylysines to the enzyme. Although calculation shows that lys_{59}, lys_{158}, lys_{210}, lys_{371} had equivalent numbers of $-NH_3^+$ groups available for interaction with each mole of pepsin it does not prove that all did so or with the same pepsin carboxyls: for lys_{59}, lys_{158} because of similarity in size to pepsin, a larger number of $-NH_3^+$ groups may have been involved than for the other two polylysines. Differences in polylysine chain length are clearly associated with differences in inhibitory characteristics and these could be due to the presence of particular properties in certain members rather than to a simple progression of properties with increasing size.

With chromatographically purified pig pepsin freshly prepared from pepsinogen and maintained in solution without drying to ensure stability, lys_{158} was non-competitive, as it was for crystalline pepsin, but lys_{210} was also non-competitive although it was partially competitive for crystalline pepsin. An explanation of the difference observed would be of interest but cannot at present be offered. A finding of this type, not entirely unexpected, might be repeated were all the known pepsins of the human and the pig to be examined for inhibition by polylysines. However, the more relevant pepsin to use

of all those which have been identified is not at present known and identifying it is for future work although certain human pepsins have been associated with ulcer disease (Taylor 1970). These pepsins have not so far been prepared in adequate quantity for this type of study.

The size of the polylysine molecule was found to influence activation of ferrodoxin NADP reductase (Schneeman & Krogmann 1975) although reasons were not adduced. The significance of deductions from kinetic studies regarding mechanism and other qualitative aspects underlying the chemical reactions involved has recently been stressed by Cleland (1977).

The effects of pH on polylysine inhibition of pepsin at constant ionic strength indicate that the underlying interaction between enzyme and inhibitor depends on the degree of ionization in both polyionic molecules. Increased ionization brought about by change in pH results in an increase in ionic content of the system thus tending to decrease the amount of enzyme-inhibitor interaction. Clearly (Fig. 1) there is an inhibitor concentration or small concentration range ('critical' concentration) at which inhibition is constant at all pH values between 3·6 and 5·0 indicating either absence of excess of any ionic species at equivalence or non-effectiveness of any slight excess at points within this range. At inhibitor levels above and below the 'critical' concentration interaction (and hence inhibition) increases and decreases respectively with pH as in Table 4 which gives results for lys_{210} (Anderson et al 1978).

This may be explained as follows. As inhibitor concentration is lowered to sub-'critical' levels, pepsin concentration which has remained constant may assume a relative excess over the cationic inhibitor and increasing pH will by promoting ionization of the pepsin carboxyl groups and depressing ionization of the lysine amino groups effectively exaggerate this anionic excess sufficiently to decrease the electrostatic pepsin-polylysine interaction and therefore also the inhibition. Conversely, raising inhibitor concentration to higher than 'critical' level will create a relative cation excess over the anionic pepsin which has remained constant. This cationic excess will be exaggerated at lower pH values, and the decrease in pepsin-polylysine interaction will result in decreased inhibition with decreasing pH. This trend is clearly seen in Table 4, data for lys_{210}, which is representative of the other polylysines, except lys_7. The other polylysines differed from lys_{210} only in 'critical' concentration which was respectively: 0·45 mg (3 ml)$^{-1}$ (lys_{23}); 0·14 mg (3 ml)$^{-1}$ (lys_{59}); 0·01 mg (3 ml)$^{-1}$ (lys_{158}). Thus the ionic contribution resulting from the effect of pH on ionization of the macroions influences the effect of overall inhibitor concentration in determining the inhibition. The degree of electrostatic binding will play a large part in determining the tightness of enzyme-inhibitor binding and the solubility of the complex so formed.

Although maximum inhibition and the inhibitor concentration required to cause it varied with pH for the pure competitive inhibitor lys_7, there was no 'critical' concentration for this low molecular weight polylysine which probably lacks the length of chain to engage in configurational changes or to contribute to the pH effect just described. Thus it tends to be excluded from participation in the more complicated inhibitory mechanisms deduced for the polylysines of higher molecular weight each of which showed a partial type of inhibition in distinction to lys_7 which exhibited pure competitive inhibition.

Table 4. Effect of concentration and pH on polylysine (lys_{210}) inhibition of pepsin.

I	Inhibition, % pH			
mg (3 ml)$^{-1}$	3·6	4·0	4·6	5·0
0·002	29	28	18	7
0·004	52	48	39	29
0·005	60	56	49	39
0·006	62	61	57	53
0·0076*	68	68	68	68
0·008	70	71	74	80

I = inhibitor concentration; *, 0·0076 determined graphically = critical concentration.

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