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The Interaction of Papain with Polycations

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Abstract—An investigation has been made of the interactions of the enzyme papain with the polycations protamine, polybrene, poly(L-lysine), spermine, spermidine and the neutral polymer polyvinylpyrrolidone (PVP). At low concentrations, each behaves as an inhibitor of the enzyme. As their concentrations increased above a certain level, the activity of the systems increased, and their inhibition of the enzyme appeared to be less pronounced. When acting by themselves in the presence of the substrate haemoglobin, each of the polycations was a weak proteolytic catalyst with a ranking of catalytic effectiveness of protamine > polybrene > poly(L-lysine) > polyvinyl-pyrrolidone > spermidine > spermine. This effect could explain the anomalous inhibition of papain by these polycations. The interaction of papain with dansyl protamine (DNSP) and the extent of complex formation were studied using a fluorescence polarization technique and the results showed that there was a strong interaction occurred. The strength of binding was assessed by determination of the critical electrolyte concentration (0·2 M, $NaNO_3$). The stoichiometry of the DNSP-papain complex was found to be 63 base moles of DNSP to one mole of papain.

Pepsin has been shown to be inhibited by low concentrations of several polycations (Herriot 1962). In each case, as the concentration of the polycation was increased above a critical value, the activity of the system increased (Lawton & Mekras 1985a). To investigate if polycations are general inhibitors for proteases, we have examined several enzymes and herein describe our work with papain, the effect of inhibitors on which has received little attention. Stockwell & Smith (1957) and Smith & Parker (1958) investigated the effect of pH variations on the inhibition caused by carbobenzoyl-L-glutamate and found that the inhibition decreased as the pH increased. Arnon & Shapira (1967) isolated anti-papain antibodies and found that these inhibited the papain catalysed hydrolysis of casein by a maximum of 85%.

Since papain contains a sulphydryl group at its active site, it is readily inhibited by heavy metal ions (Hwang & Ivy 1951) and must be activated and maintained in an active state (Mitchell et al 1970) by the use of a suitable additive such as sodium cyanide, which we used in the present work. The substrate chosen for the enzyme was denatured haemoglobin (Anson 1938) and the work was carried out at pH 7 and 25°C using several polycations and the neutral polymer polyvinyl-pyrrolidone as potential inhibitors. In one case, using protamine labelled with a dansyl group, we were able to show that a definite complex is formed between the enzyme and the polycation using the technique of fluorescence depolarization (Lawton & Mekras 1985b). By a further application of that technique it was possible to estimate the 'Critical Electrolyte Concentration' (Scott 1973) for the polycation-enzyme complex. Equation (1) represents the interaction of the polycation (PCX_{nm}) with the papain

$$PCX_{nm} + nPM_m = PC - Pn + nmMx \qquad (1)$$

Here X^- and M^+ are the counterions that are present because of the ionic nature of the materials.

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Materials and Methods

Materials

Papain: No. P4762 (Papainase, E.C.3.4.22.2) from papaya latex; type IV had been twice crystallized and the lyophilized powder was obtained from the Sigma Chemical Company and stored at 0–5°C in a desiccator. Haemoglobin which had been prepared from washed, lysed and dialysed bovine erythrocytes, was obtained from Sigma (type 1) and stored at 0–5°C in a desiccator. Sigma also supplied protamine sulphate (Grade X) which had been obtained from salmon. We used the material in extensive gel electrophoresis experiments and found it to be homogeneous. Poly(L-lysine hydrobromide), mol. wt 2500 Daltons; and polybrene (1,5, dimethyl 1,5-diazaundecamethylene polymethobromide) were both of synthetic origin and were obtained from Sigma, as were spermine tetrachloride, spermidine trichloride, polyvinylpyrrolidone and Folin Ciocalteu's phenol reagent. The other chemicals were of analytical purity. Concentrations of each polycation have been expressed as 'base mol^{-1}' where one base mole is the mass of a polycation associated with one positive charge. Details are given in Table 1.

Preparation of denatured haemoglobin substrate

A solution containing 8·0 mL of 1·0 M sodium hydroxide, 72 mL triple distilled water, 36 g urea and 10 mL of 22% w/v haemoglobin was kept at 25°C for 60–75 min to denature the haemoglobin. It was then mixed with a solution containing 10 mL of 1·0 M potassium dihydrogen phosphate and 4 g of urea. The final pH of the buffer after addition of urea was 7·2. Then, 5–6 drops of toluene was added to 100 mL of the haemoglobin solution as a preservative. The haemoglobin solution was stored at 5°C and it was stable for 12 weeks. It was found that the haemoglobin in any given batch gave consistent results, but there was a small variation between batches. To ensure that the experimental results were not distorted by this variation, all the experiments with a given polycation were carried out with the same batch of denatured haemoglobin.

Table 1. Percentage inhibition of polycations with papain activated with sodium cyanide using denatured haemoglobin (0·5 mg mL^{-1}) as the substare at 25°C and pH 7·0, compared with that of pepsin* at pH 2·1.

Type of polycations	Concentration of polycation at max. inhibition of papain (10 mol L^{-1})	Base mol of polycation	% inhibition of papain by polycation pH 7·0	% inhibition of pepsin by polycation pH 2·1
Poly(L-lysine)	32×10^{-5}	197	46	27
Spermine	4×10^{-5}	87	27	28
Protamine sulphate	4×10^{-5}	220	22	48
Spermidine	4×10^{-5}	85	22	32
Polybrene	4×10^{-5}	187	20	38
Polyvinylpyrolidone (neutral polymer)	20×10^{-5}	109	11·5	—

*(Lawton & Mekras 1985a)

Activation of papain by sodium cyanide (NaCN)

Papain contains traces of heavy metal ions which might otherwise bind to the essential thiol group of the enzyme and to obtain its maximal hydrolytic activity, the enzyme must be activated (Finkle et al 1958). This is achieved with reducing agents which can also act as chelators. Sodium cyanide was used to remove inhibitors in the haemoglobin solution as well as to activate the enzyme by the elimination of traces of heavy metal ions. This was done as follows: to 0·5 mL papain solution in phosphate buffer, 5 drops of 2·0 M sodium cyanide was added. After 3 min at 25°C, 9·25 mL of triple distilled water was added. Dilution if needed was with a solution of 5 drops of 2·0 M sodium cyanide in 10 mL of water.

Assay of papain activated with sodium cyanide using denatured haemoglobin as a substrate at pH 7·0 and 25°C

Most of the work was with denatured haemoglobin as a substrate since it gave reproducible results. Activated papain (0·5 mL of $4{\cdot}28 \times 10^{-6}$ M) in phosphate buffer, was added from an automatic pipette, to a series of tubes to which was added a known volume of phosphate buffer (0·01 M, pH 7·0) to 5·0 mL after different amounts of haemoglobin had been added. The tubes were incubated at 25°C for 20 min and the reaction terminated with trichloroacetic acid (TCA) (5·0 mL 10%). The precipitate was centrifuged at 8000 rev min^{-1} for 30 min. Supernatant from each tube (1·25 mL) was transferred to clean, dried tubes and NaOH (2·5 mL of 0·5 M) and Folin & Ciocalteu's reagent (0·75 mL, 1:3 dilution) was added. The mixture was shaken well, left for 10 min for the blue colour to develop, and the relative absorbance recorded spectrophotometrically at 420 nm against a blank of the reaction medium without the haemoglobin or the enzyme. A plot of relative absorbance against substrate concentration was then constructed.

The effect of protamine, polybrene, poly(L-lysine), spermine and spermidine on the reaction of papain with denatured haemoglobin was investigated at pH 7·0 and 25°C. All the solutions were pre-equilibrated at 25°C for 30 min before mixing. The polycation (10^{-3} base mol L^{-1} in phosphate buffer) was added in chosen amounts to the activated enzyme ($4{\cdot}38 \times 10^{-6}$ M in phosphate buffer 0·01 M, pH 7·0), followed by a measured volume of the phosphate buffer, and well-mixed by shaking. The mixture was incubated for 5 min at 25°C and amounts of haemoglobin solution (2·2%) were added to 5 mL. The mixture was incubated for 20 min at 25°C before the reaction was terminated with TCA (5 mL 10%) and the mixture cooled in ice before centrifuging at 4°C. The extent of the hydrolysis of the haemoglobin was determined by the method of Anson (1938) using freshly prepared Folin & Ciocalteu's reagent (diluted 1:3 with water) and sodium hydroxide (0·5 M). The absorbances of the solutions were measured at 420 nm. The reagent reacted to a small extent with the polycation and a blank experiment was done in each case to allow the results to be corrected for that effect.

The effects of the polycations, at pH 7·0 were similarly investigated. Absorbance values were reproducible to $\pm 0{\cdot}002$ absorbance units.

Preparation of DNSP by cyclohepta-amylose-dansyl chloride (CDC)

The compound CDC. A sample of 20 mg of CDC, prepared by the method Kinoshita et al (1974), was added little by little to an ice cold solution of protamine sulphate 20 mg in 2 mL of 0·1 M phosphate buffer. The mixture was magnetically stirred at 0° for 50 min then centrifuged twice at 3000 rev min^{-1} retaining the supernatant, for fractionation on Sephadex Gel G25.

Fractionation of DNSP by gel filtration

A column (0·9 × 30 cm) was packed with Sephadex G25 to a bed height of 27 cm. The above supernatant was loaded onto it and then eluted to remove residual dansyl chloride, dansyl amide, dansyl sulphonic acid and β-cyclodextrin. The flow rate was maintained at approximately 0·2 mL min^{-1}. The column was eluted with distilled water and the elute collected in 1·5 mL fractions. The absorbance of each fraction was measured spectrophotometrically at 334 nm against water. An elution profile was then constructed and two major peaks were obtained on the graph (Mekras 1985). The first peak was due to DNSP (high molecular weight), the second peak to residual dansyl sulphonate (low molecular weight).

The concentration of dansyl group was calculated using $A_{334} = 3400$ mol^{-1} cm^{-1} as given by Kinoshita et al (1974). Finally the protein concentration of DNSP was carried out using the method of Lowry et al (1951).

The degree of labelling was given by

$$\frac{\text{Mol of bound DNS group}}{\text{base mol of polycation}}.$$

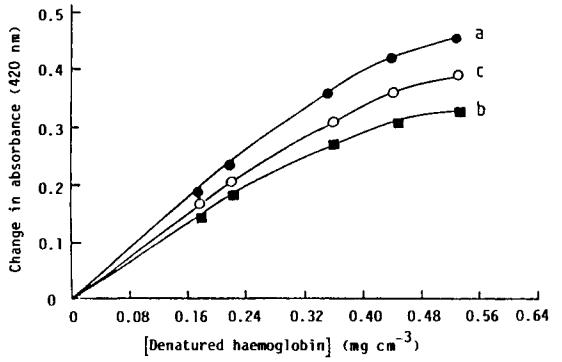

FIG. 1. The effect of protamine sulphate on the hydrolysis of denatured haemoglobin (2·2%) by papain ($4{\cdot}28 \times 10^{-6}$ M) activated with NaCN and pH 7·0 and temperature 25°C. Protamine sulphate concentrations: (base mol L^{-1}): a, (—●—) none; b, (—■—) 4×10^{-5}; c, (—○—) 20×10^{-5}.

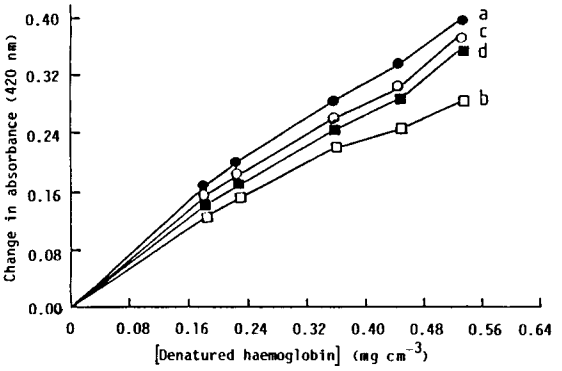

FIG. 3. The effect of spermine on the hydrolysis of denatured haemoglobin (2·2%) by papain ($4{\cdot}28 \times 10^{-6}$ M) activated by NaCN at pH 7·0 and temperature 25°C. Spermine concentrations: (base mol L^{-1}); a, (—●—) none; b, (—○—) 4×10^{-5}; c, (—□—) 10×10^{-5}; d, (—■—) 20×10^{-5}.

Fluorescence polarization measurements

The method of Lawton & Mekras (1985b) was used. The polarization of the fluorescence was determined using an instrument similar to that described by Teichberg & Shinitzky (1973).

Titration of DNSP with papain

DNSP (4 mL, $2{\cdot}5 \times 10^{-6}$ base mol L^{-1}) was placed in a fluorimeter cell and the polarization and the total fluorescence intensity were recorded. To that amount of DNSP, amounts (μL) of papain ($4{\cdot}23 \times 10^{-6}$ M in water) were added and the readings again recorded. To a complex having a composition identical to that giving maximum polarization, known amounts of sodium nitrate were added and the decrease in polarization was from the breakdown of the complex recorded. All the results are means of triplicate determinations. The time-dependence of DNSP-papain complex formation was overcome by mixing the solutions vigorously and then allowing them to stand for 15 min before any measurements were taken (Mekras 1982).

Results

To prevent the time of contact between the enzyme and the polycation affecting the rate of hydrolysis, the polycation and the enzyme were pre-mixed and incubated for 30 min before the haemoglobin was added (Mekras 1985).

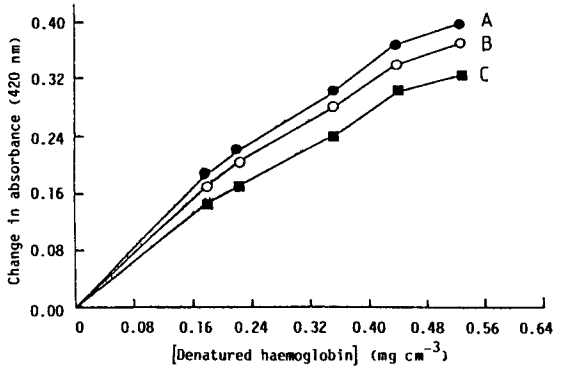

FIG. 2. The effect of polyvinylpyrrolidone (PVP) on the hydrolysis of denatured haemoglobin (2·2%) by papain ($4{\cdot}28 \times 10^{-6}$ M) activated with NaCN at pH 7·0 and temperature 25°C. PVP concentrations: (base mol L^{-1}): A, (—●—) none; B, (—○—) 4×10^{-5}; C, (—■—) 20×10^{-5}.

Effect of polybrene, poly(L-lysine) and protamine on the hydrolysis of haemoglobin by papain, at pH 7·0 and 25°C

Fig. 1 shows the spectrophotometric results of the hydrolyses of denatured haemoglobin by the enzyme, papain ($4{\cdot}28 \times 10^{-6}$ M) in the presence of polybrene.

The reaction in its absence is also shown. All determinations were in duplicate and the work was repeated several times. Corrections for the Folin & Ciocalteu's reagent were made in each case to allow for the small reaction of the phenol in the reagent with the polycation. Errors were of ca $\pm 0{\cdot}002$ absorbance units.

For polybrene, poly (L-lysine) and protamine sulphate there was inhibition of the activity of papain on denatured haemoglobin that was most marked at a low poly-ion concentration. With each of these compounds the concentration of haemoglobin was from 0 to 0·64 mg mL^{-1} and the rate of hydrolysis was proportional to the change of absorbance at 420 nm (see Fig. 1 shown for protamine sulphate; the other compounds gave similar responses).

The effect of a PVP (a neutral polymer) on the hydrolysis of denatured haemoglobin by activated papain is shown in Fig. 2. Unlike the other polycations PVP at low concentrations (line B), causes the papain to behave more actively. As the concentration of PVP increases (line C), there is a noticeable inhibiton of papain. In the case of spermine, Fig. 3, the papain is inhibited at all concentrations, although the maximum inhibition occurs at the lowest concentration and thereafter as the concentration of spermine is increased, the papain is reactivated relative to line b, but is still inhibited relative to line a, the control.

Spermidine gives a similar graph to spermine but is less effective at 10 and 20×10^{-5} mol L^{-1}.

Table 1 presents the percentage inhibition of the polycations with papain. The calculations were made by taking the hydrolysis of denatured haemoglobin (0·2 mg mL^{-1}) by the enzyme papain alone as 100%. Fig. 4 shows that the study of the catalytic properties of protamine, polybrene, poly(L-lysine), polyvinylpyrrolydone, spermine, and spermidine relative to papain using denatured haemoglobin as the substrate. The reaction was carried out in sodium acetate

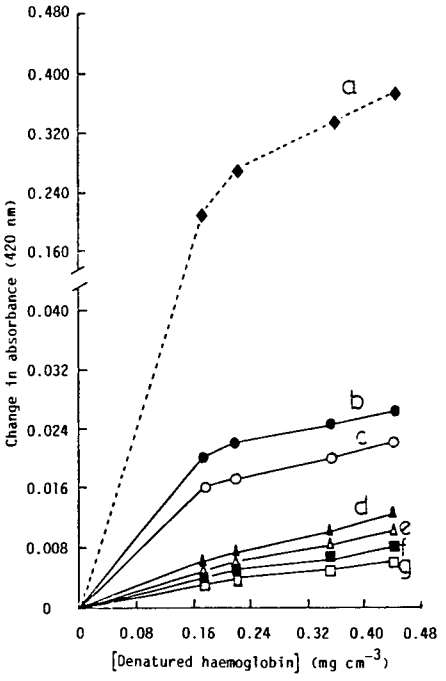

FIG. 4. A study of the catalytic properties of protamine sulphate, polybrene, poly (L-lysine), polyvinylpyrrolidone, spermine, and spermidine relative to papain ($4{\cdot}28 \times 10^{-6}$ M) using denatured haemoglobin as the substrate in sodium acetate buffer (0·1M, pH 5·2) at 25°C. Concentrations: (base mol L^{-1}): a, (- - ◆ - -) papain; b, (—●—) protamine sulphate (20×10^{-5}); c, (—○—) polybrene (20×10^{-5}); d, (—▲—) P-L-L (20×10^{-5}); e, (—△—) PVP (20×10^{-5}); f, (—■—) spermidine (20×10^{-5}); g, (—□—) spermine (20×10^{-5}).

buffer (0·01 M) at pH 5·2 and 25°C. The results shown graphically are those after subtraction of appropriate blanks. The order of catalytic effectiveness is: Protamine > polybrene > poly(L-lysine) > polyvinylpyrrolidone > spermidine > spermine. In another experiment at pH 7·0, the order of catalytic effectiveness was: Polybrene > protamine > poly(L-lysine) (Mekras 1985).

Study of the complex formation of activated papain using dansyl protamine in aqueous solution at 27°C, and pH 6·5

Fig. 5 shows the results of fluorescence titrations of dansyl protamine (DNSP) $2{\cdot}5 \times 10^{-6}$ (b. mol L^{-1}) with papain ($4{\cdot}23 \times 10^{-6}$ M) at pH 6·5 and 27°C. The interaction was followed by measuring the polarization of the fluorescence of

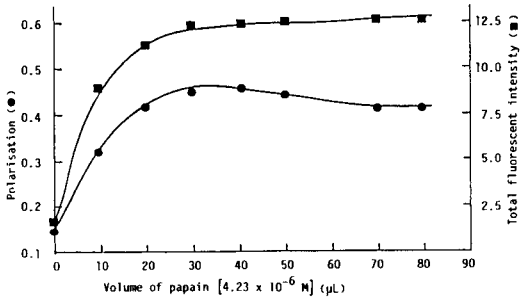

FIG. 5. Changes in the polarization (—●—) and in the total fluorescence intensity (—■—) accompanying the addition of papain $4{\cdot}23 \times 10^{-6}$ M to DNSP $2{\cdot}5 \times 10^{-6}$ base mol L^{-1} in aqueous solutions at 27°C.

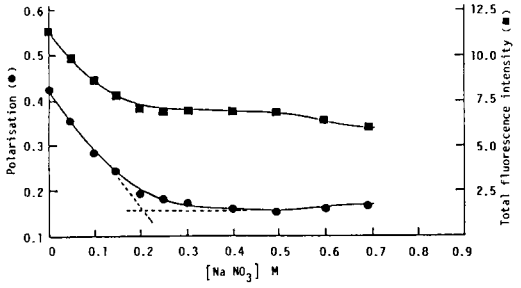

FIG. 6. Changes in the polarisation (—●—) and in the total fluorescence intensity (—■—) on addition of sodium nitrate to papain—DNSP complex. (DNSP = $2{\cdot}5 \times 10^{-6}$ base mol L^{-1}), in aqueous solution at 27°C.

the dansyl group at various DNSP : papain ratios. Changes in the polarization were interpreted as evidence that complex formation has taken place. The intersection of the two straight-line portions of the upper trace gives a direct estimate of the stoichiometry of DNSP base mol mol^{-1} of enzyme (63:1). Both polarization and total fluorescence intensity changes appear to behave similarly, that is, there is an initial increase and at the point of complex formation at levels off as more enzyme is added. The results were interpreted in the light of Perrin's equation as used, Wahl & Lami (1967):

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_o}-\frac{1}{3}\right)\left(1+\frac{RT}{\eta V_o}\right) \qquad (2)$$

Where P is the polarization, P_o is the limiting or intrinsic polarization, R the universal gas constant, T the absolute temperature, η the viscosity and V_o the partial specific volume. The equation implies that if T, and η are constant then P will increase if V_o increases as a consequence of complex formation.

The effect of salt ($NaNO_3$) on the complex of DNSP and papain in aqueous solution at 27°C

When the spectrofluorometric experiments were repeated with several salts a decision was made to use sodium nitrate because of its high solubility in water. Sodium nitrate in aqueous solution (5 or 10 M) was added in μL quantities to a solution of the complex, followed by vigorous shaking. The system was allowed to equilibrate (15 min) before measurements were made. The effect is shown in Fig. 6. The changes are interpreted as the addition of salt dissociates the complex. The point at which the complex is completely dissociated is difficult to determine. An estimation of the critical electrolyte concentration (CEC) was made by taking the concentration of salt at which there was no further reduction in polarization. This was 0·2 M for the complex with a stoichiometric ratio of 63:1 (Fig. 6). The stoichiometry is given as base mol of DNSP mol^{-1} of papain. The CEC when compared with the values obtained for other enzymes (Lawton & Mekras 1985b), shows the complex of DNSP with pepsin to have a value of 0·52 M with a stoichiometric ratio 10:1 indicating that the complex of pepsin-DNSP is stronger than that of papain-DNSP.

Discussion

Effect of polycations on the activity of papain

The results show that papain is inhibited by low concentrations of the polycations: protamine, polybrene, spermine, spermidine, poly(L-lysine) and also by the neutral polymer. In each case, as the concentration of the polycation, or PVP, was increased, the activity of the system reversed and increased towards that of the pure enzyme. The simplest explanation of the results is that each polycation (and also PVP) can behave as a proteolytic catalyst under the conditions of the experiment when present in an uncomplexed state. That this is so, is shown clearly in Fig. 4. As might be expected, the enzyme is a much better catalyst than the polymers. Since no precipitate was observed in any of our solutions, we consider that the sequence of events following the addition of a polycation to papain is probably that a polycation—papain complex is formed, and that any excess polycation will behave as a proteolytic catalyst. This is supported by Fig. 5 where the results show that a complex is formed between papain and dansyl-protamine at an enzyme to polycation ratio of 1 mol enzyme to 63 base mol protamine. Since the enzyme was present in the catalysis experiments at $4{\cdot}28 \times 10^{-7}$ M the amount of the polycation required to form the complex in our experiments would correspond to $2{\cdot}69 \times 10^{-5}$ base mol L^{-1}. Thus maximum inhibition should occur at about that level, possibly slightly above it due to the need to have a slight excess of polycation to ensure that all the enzyme molecules were complexed. These ideas are supported by the results shown in Fig. 1.

Thus it appears that papain behaves in a similar manner to pepsin (Lawton & Mekras 1985a, c) in the presence of polycations. The effect of PVP on papain is also similar to its effect on pepsin (Mekras, 1985).

Fig. 4 shows that at pH 5·2 the order of catalytic effectiveness of the free polycations is: protamine > polybrene > poly(L-lysine) > PVP > spermidine > spermine. While the best catalyst is protamine, Table 1 shows that the best inhibitor of papain is poly(L-lysine).

Catalysis experiments have also been made at pH 7·0 (Mekras 1985) and showed that the catalytic activity was less at pH 7·0 than at pH 5·2.

Fluorescence polarization studies

In addition to showing that a complex was formed between pepsin and dansyl protamine Fig. 5, these studies also allowed us to determine that a concentration of 0·2 M of sodium nitrate was needed to dissociate the complex (Fig. 6). This represents the CEC (Scott 1973) and shows that a strong complex is formed.

It has been suggested to us that the polycations may act by masking the susceptible sites on the haemoglobin, rather than by binding to the enzyme. Although there is no positive evidence for that type of interaction, we accept that it would explain our results.

In conclusion, it may be stated that protamine, polybrene, poly(L-lysine), spermidine, spermine and polyvinylpyrrolidone all act as inhibitors of papain at low concentrations. Each of the polymers acts as a proteolytic catalyst for the substrate haemoglobin when used by itself. Thus, as the concentration of the given polymers is increased in a papain-polymer-haemoglobin system, the initial inhibition is reversed.

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