POLYMERS CONTAINING GROUPS OF POTENTIAL BIOLOGICAL ACTIVITY

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STUDIES ON THE REACTIVITY OF POLYMERIC THIOL GROUPS

Introduction

The activity of many enzymes has been shown to depend on the presence of sulphhydryl groups[1–3], not all of which are of equal reactivity nor of equal importance for the proper functioning of the enzyme. Three types of sulphhydryl groups are recognized depending on their reactivity with oxidizing, alkylating and mercaptide-forming reagents. These sulphhydryl groups are classified as freely reactive, sluggishly reactive and " masked ". The " masked " groups become freely reactive when the protein is denatured. It is believed that this is due to the unfolding of the peptide chains, thus making these hitherto inaccessible sulphhydryl groups freely available[4]. Resistance to oxidation of some of the sulphhydryl groups of the enzyme is thought to be due to the distance between sulphhydryl in the native protein, which prevents the formation of a disulphide bond. Barron has reported that for a series of dithiols, the greater the distance between the thiol groups the slower will be the oxidation[5]. The presence of electronegative groups in a molecule was also found to influence the ease of oxidation[5], the effect being a decrease in the degree of dissociation of the —SH bond resulting in a decrease in the rate of oxidation.

High energy irradiation of water containing dissolved oxygen has been shown to produce the oxidizing agents hydroxyl and hydroperoxyl radicals[6]. Since thiols are easily oxidized by mild oxidizing agents, it was not surprising to find that ionizing radiation would rapidly oxidize biologically essential sulphhydryl groups and thus be detrimental to life[6]. However, reversal of inhibition and, further, protection on addition of sulphhydryl compounds such as cysteine and glutathione was shown to take place[7–10]. The detoxification of hydroxyl and hydroperoxyl radicals is most probably accomplished by a rapid reaction of these radicals with the thiol groups, a reaction of relatively low activation energy. Thus, the efficiency of sulphhydryl compounds as protectors against the lethal effects of radiation has been shown to be related to their ease of oxidation[11].

Recently[34], the suggestion was made that damage by irradiation was due to the oxidation of biologically necessary cuprous ion to cupric. Protection

thus operates by shielding the cuprous ion against the oxidizing agents produced during irradiation. This protection is claimed to be due to chelation, and among the effective chelates are dithiols and β-mercaptoamines.

One of the most serious shortcomings of the known protective agents[12] is their relatively short-lived protection. Being mostly low molecular weight compounds they are soon voided from a biological system. It appears reasonable to assume that high molecular weight sulphhydryl-containing polymers would be advantageous in that they would only slowly be metabolized and thus their degree of permanency would be enhanced.

In order to understand more clearly the factors which govern the reactivity of enzymic sulphhydryl groups and to point out useful pathways for improving chemical protection against the effects of lethal radiation, it was thought that addition polymers containing sulphhydryl groups would be useful models to study. Specifically, the extent to which the oxidation of the sulphhydryl group is affected by the restraints imposed by a polymeric backbone in preventing disulphide formation was one of the purposes of these investigations. For comparison, monomeric thiol compounds in a similarly constituted environment were also examined.

Synthetic polymeric thiol compounds

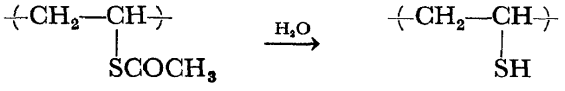
Synthetic polymeric thiol compounds have been prepared *via* both addition and condensation polymerization. Direct synthesis of a free thiol polymer by vinyl polymerization is impractical since the sulphhydryl group adds readily to an unsaturated linkage and, in addition, has a very high chain transfer constant in radical initiated polymerization. Two synthetic approaches are available:

(*a*) the polymerization of a monomer containing the sulphhydryl group which is protected by a subsequently removable " blocking " group;

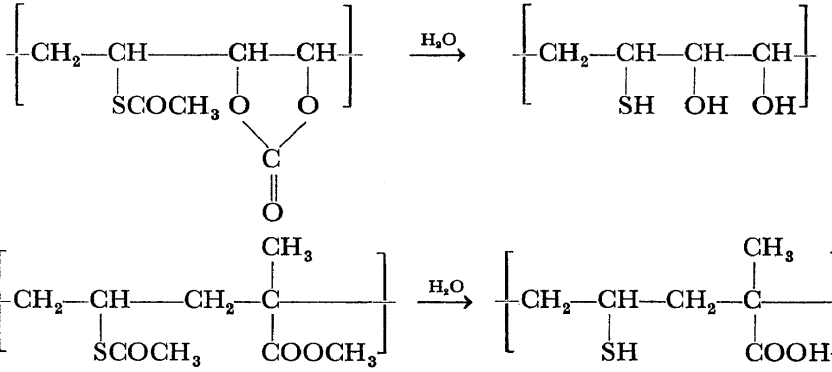
(*b*) the introduction of the thiol group into a previously formed polymer by conversion of some convenient group already on the chain.

The first of these methods is considerably superior for the purposes described in the introduction since it has the advantage of yielding a polymeric material in which the positions, environment and number of thiol residues are predetermined.

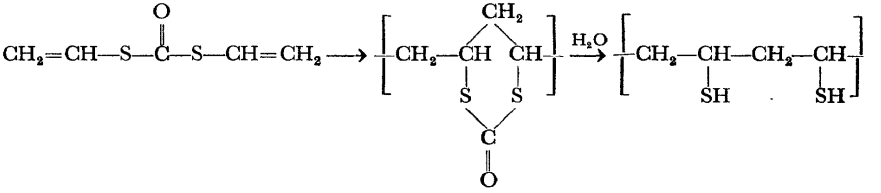
The preparation, polymerization and copolymerization of vinyl thiolacetate has been described[13–15]. Hydrolysis of the homopolymer by both basic and acidic catalysis was reported to give an insoluble poly(vinyl mercaptan)[16]. Under air-free conditions, basic hydrolysis gave a soluble polymer[15].



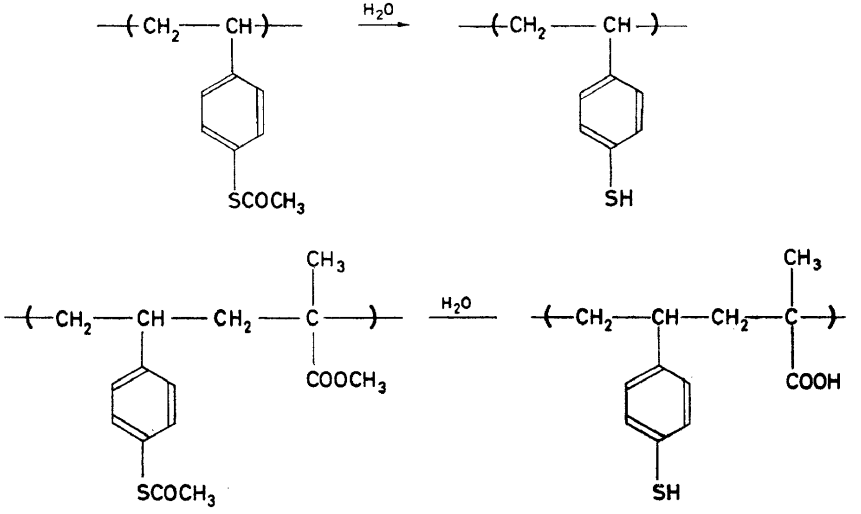
Copolymerization with vinylene carbonate[14] and methyl methacrylate[17], as well as the hydrolysis of these polymers, has been described.



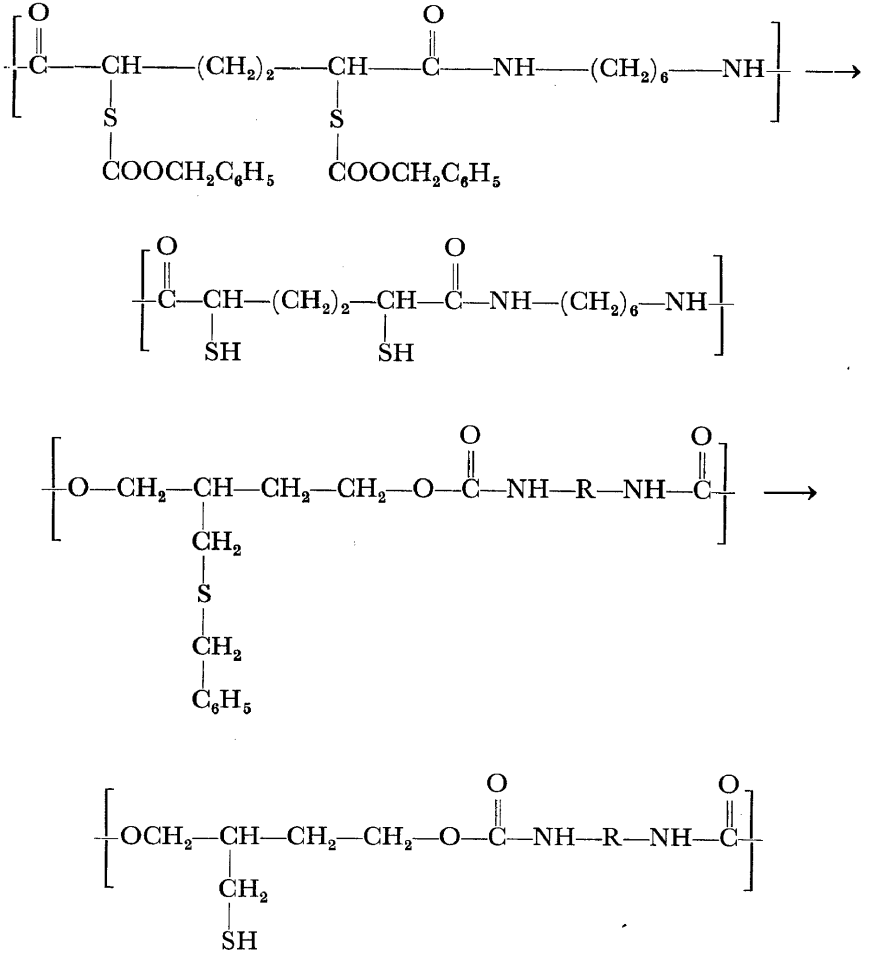
Recently, the preparation of divinyldithiol carbonate and its cyclopolymerization has been described[18]. Basic or acidic hydrolysis afforded an alkali soluble poly(vinyl mercaptan).



p-Vinylphenyl thiolacetate has also been prepared[19]. Saponification of the resultant homopolymer gave a pure homogeneous alkali soluble polymer[19]. Copolymerization with methyl methacrylate gave, upon saponification, another synthetic sulphhydryl-containing polymer.

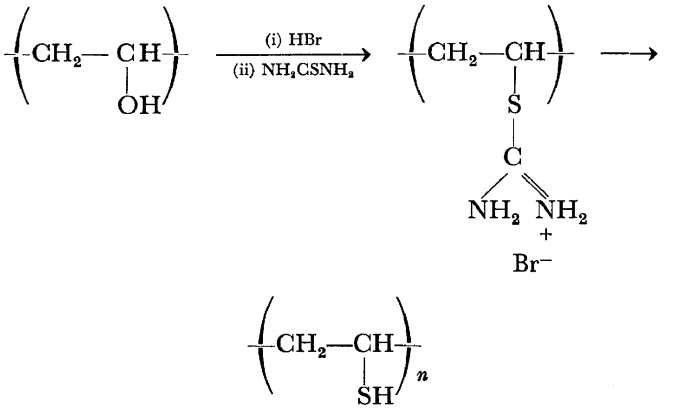


Synthetic methods for the preparation of blocked sulphhydryl monomers and their subsequent polymerization by condensation methods were described recently[20].

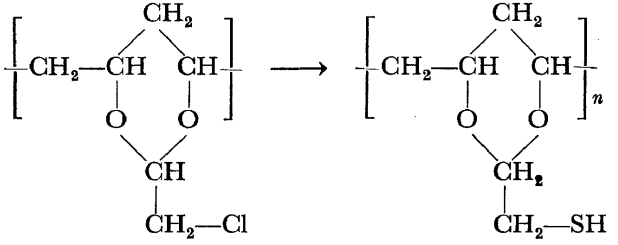


Several examples of the second types of synthetic approach are reported in the literature. The introduction of the chloromethyl group into a cross-linked polystyrene and subsequent treatment with thiourea and hydrolysis gave poly(vinylbenzyl mercaptan)[21] used for ion-exchange purposes. Nitration of polystyrene, hydrogenation, diazotization and treatment with potassium ethyl xanthate followed by hydrolysis gave an insoluble poly(mercaptostyrene)[22] effective as an ion-exchange resin.

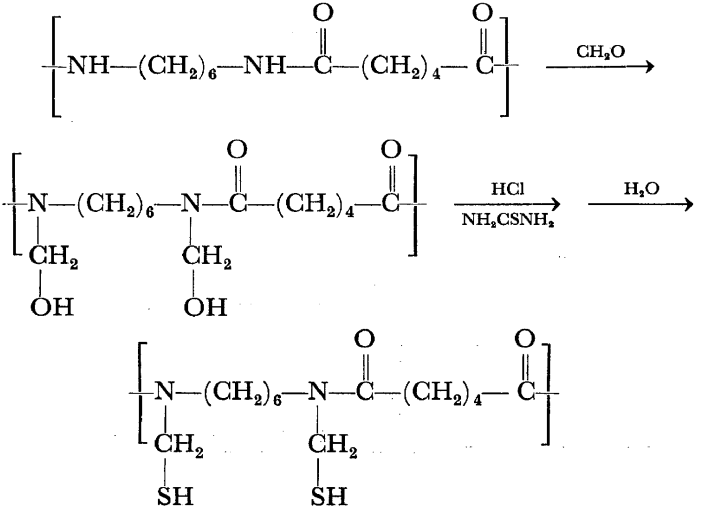
Treatment of poly(vinyl alcohol) with hydrogen bromide and thiourea gave the isothiouronium salt which could be hydrolyzed to an insoluble polymer of vinyl mercaptan[23].



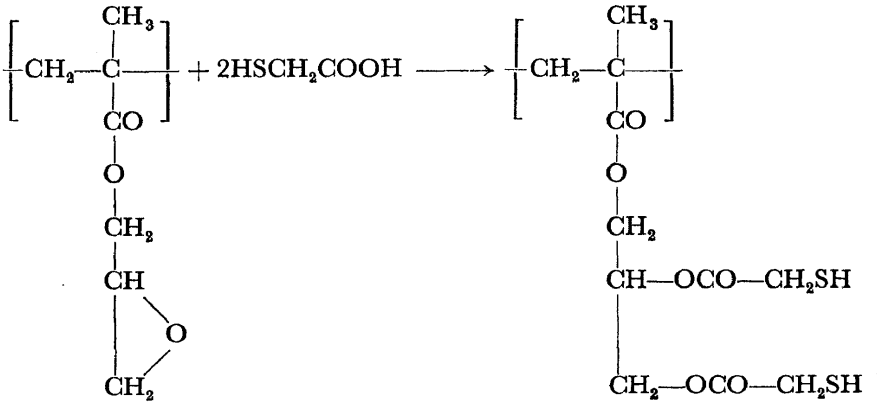
Treatment of poly(vinyl alcohol) with chloroacetaldehyde gave a cyclic acetal with a replaceable chloride. Reaction with thiourea followed by saponification gave a polythiol[24].



Poly(hexamethylene adipamide) (nylon 66) was used as a backbone on which to graft the mercaptomethyl fragment by treatment with formaldehyde and ammonia. Subsequent treatment with thiourea and hydrogen chloride gave the isothiouronium salt from which a soluble polythiol could be obtained[25].

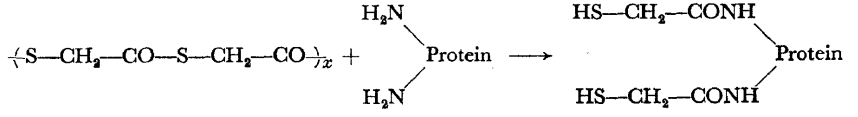


Soluble graft copolymers containing pendant mercaptan groups were obtained by treatment of poly(glycidyl methacrylate) with thioglycolic acid[26].

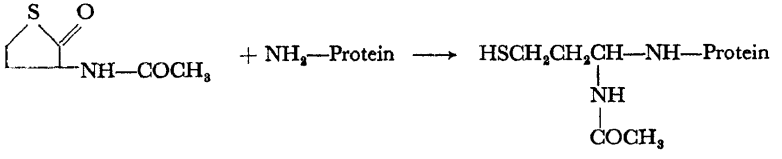


Poly(*p*-lithiostyrene) was obtained from iodinated polystyrene and lithium[27]. By treatment with sulphur and acidification, an alkali soluble poly(*p*-mercaptostyrene) was obtained containing 62 mole per cent mercaptostyrene.

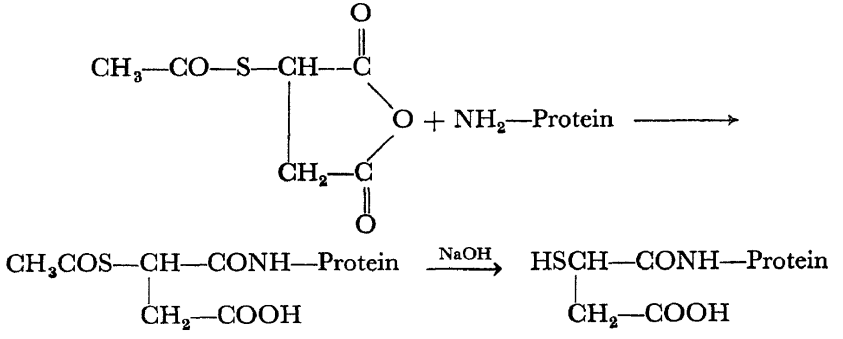
Several successful attempts have been made at introducing thiol residues *de nova* into proteins. Using thioglycolides, a highly thiolated casein and ovalbumin was prepared[28].



An alternate method used *N*-acetylhomocysteine thiolactone[29].

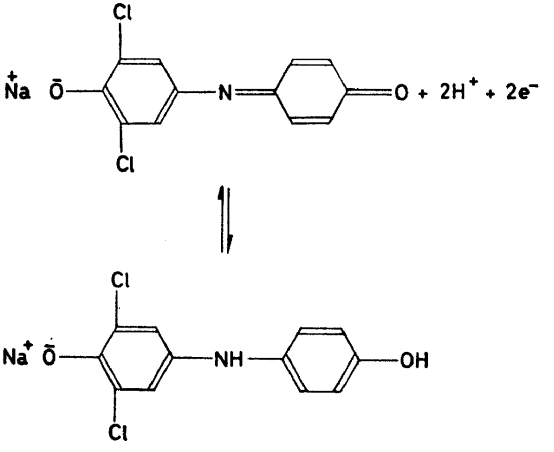


Still another method used *S*-acetylmercaptosuccinic anhydride[32].



Oxidation reactivity

In order to determine relative rates of oxidation of the polymers and monomeric compound described below, the spectrophotometric procedure of Basford and Huennekens[31] was modified for use in pH 10 aqueous buffer solution and in organic solvents. In this system, a redox dye, which has an absorption maximum in the visible region of the spectrum, is allowed to come in contact with an easily oxidized molecule, *e.g.*, a mercaptan, and undergoes reduction to its leuco form. The rate of reduction of the dye, which depends on the rate of oxidation of the other molecular species, is determined by following spectrophotometrically the rate of disappearance of the peak in the visible. In this way, the rate of oxidation of the other species is automatically known. The dye, 2,6-dichlorobenzeneoneindophenol, in neutral or basic solution, possesses an absorption maximum at 600 to 605 mμ and in dimethyl formamide at 643 mμ.



Thus, colorimetric methods are available for the study of monomeric and polymeric sulphhydryl compounds which dissolve in water or in dimethyl formamide.

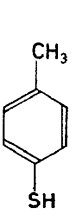
For a comparison of oxidizabilities, Basford and Huennekens[31] demonstrated the validity of comparing initial rates of oxidation. This was expressed in micromoles of indophenol reduced per ml per min and was calculated from the change in $A_{600m\mu}$ over the first $\frac{1}{2}$ minute. In the work described below, the initial velocity, V_0, was calculated from the value of the optical density at 20 seconds. The reduced initial velocity, V_0', was determined by converting V_0 to a ratio of thiol to dye of 1 : 1.

Reaction velocities of thiols at pH 10

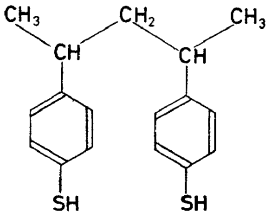
Table 1 lists thiol oxidizabilities in pH 10 buffered solution[32]. Several interesting comparisons can be made. Compound (VII), which is the repeating unit of the hydrolyzed homopolymer (VIII), is almost 7 times as fast as *p*-thiocresol(I).

Table 1. Thiol oxidation rates in pH 10 borate buffer solution

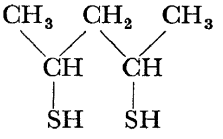
Compound	*Observed oxidation rates* (μm ml^{-1}min^{-1}—SH^{-1}) $\times 10^3$	*Ref.*	*Relative oxidizability*
(I) *p*-Thiocresol	1·17	33	1·00
(II) 2,2-Dimethyl-4-(*p*-mercaptophenyl)valeric acid	1·04	17	0·89
(III) Hydrolyzed copolymer of vinyl thiolacetate and methyl methacrylate	1·21	17	1·04
(IV) 2,2-Dimethyl-4-mercaptovaleric acid	2·16	17	1·85
(V) Hydrolyzed copolymer of *p*-vinylphenyl thiolacetate and methyl methacrylate	3·29	33	2·81
(VI) Thioglycolic acid	4·26	33	3·64
(VII) 2,4-Di(*p*-mercaptophenyl)-pentane	7·51	33	6·43
(VIII) Hydrolyzed homopolymer of *p*-vinylphenyl thiolacetate	8·88	33	7·58
(IX) 2,4-Pentanedithiol	23·0	15	19·7



(I)



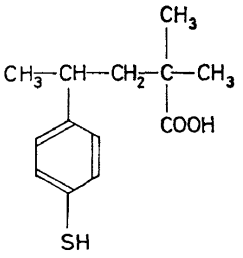
(VII)



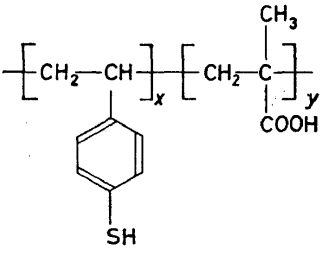
(IX)

Since the rates were determined at 10^{-8} molar in —SH, it is reasonable to argue that a thiol group of (VII) will have a nearer neighbouring thiol group than will a thiol group in compound (I). 2,4-Pentanedithiol, compound (IX), oxidizes almost 20 times as fast as *p*-thiocresol and about 3 times as fast as (VII). This seems to indicate, as shown before[5], that oxidation is increased as ease of disulphide formation increases.

An interesting comparison is between the hydrolyzed copolymer (V) and its model compound (II).



(II)



(V)

The copolymer (V) oxidizes about 3 times as fast as its model compound (II). This can be understood by the observation that in the copolymer, *p*-mercaptostyrene entered the polymer faster than methyl methacrylate indicating that there are undoubtedly some blocks of *p*-mercaptostyrene. Since this sequence is known to enhance oxidizability (VII and VIII) this would explain the increased rate. It is interesting to note that compound (II) is about as fast as the simple aromatic mercaptan, *p*-thiocresol, indicating that the carboxyl group of (II) has very little effect on the rate.

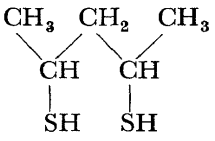
Reaction velocities of thiols in dimethyl formamide

Table 2 lists thiol oxidizabilities in dimethyl formamide (DMF). The relative rates indicate that oxidizabilities increase as the distance between

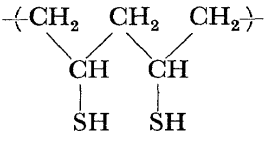
Table 2. Thiol oxidation rates in DMF[15]

Compound	*Observed oxidation rate* (μm ml^{-1}min^{-1}—SH^{-1}) $\times 10^5$	*Relative oxidizability*
(X) β-Mercaptoethanol	1·7	1
(XI) 2,5-Hexanedithiol	3·9	2·3
(XII) 2,4-Pentanedithiol	9·7	5·7
(XIII) Polyvinylmercaptan	91·4	53·8

the thiols decreases. Compound (XIII), polyvinylmercaptan is more than 50 times as fast as the monothiol, β-mercaptoethanol, compound (X), and almost 10 times as fast as its model, compound (XII).



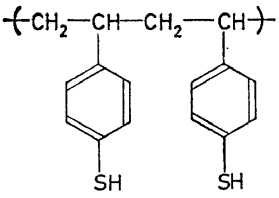
(XII)



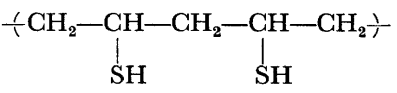
(XIII)

This is assumed to be primarily a statistical effect. In the polymer, a single thiol group has two nearest neighbours while in compound (XII), a single mercaptan has only one nearest neighbour. Since compound (XII = IX) has been compared to both polyvinylmercaptan, compound (XIII) *Table 2*, and polymercaptostyrene, compound (VIII), *Table 1*, it can be

seen that polyvinylmercaptan is oxidized almost 25 times as fast as poly-*p*-mercaptostyrene.



Poly-*p*-mercaptostyrene
1·0



Polyvinylmercaptan
24·5

Thus it seems reasonable to assume, that the presence of phenyl rings in poly-*p*-mercaptostyrene increases the steric requirements of the phenyl thiol group to a degree sufficient to inhibit intramolecular disulphide formation compared to polyvinylmercaptan.

POLYMERS WITH IMIDAZOLE SIDE CHAINS

Introduction

In recent years much research activity has centred about the investigation of the mechanism by which enzymes catalyse chemical reactions. The proteolytic enzymes, which cleave ester or amide linkages, have been the subject of intensive investigation, since these enzymes are simple proteins and do not require the presence of a prosthetic group or a co-enzyme for their activity[35]. The reactivity must be an integral part of the protein structure itself. Research has involved a study of the enzymes themselves, both structurally and kinetically, and the study of simple model systems, which eliminate the complexities of the protein structure, most of which is thought not to be involved directly in the catalytic process. It has been suggested that much of the structural features of proteins was derived by evolutionary processes, rather than by the steric or electronic requirements of the active sites of these proteins[36]. In fact, in certain cases such as papain, a degradation fragment containing only seventy-six out of the original one hundred and eighty-five amino-acid residues in the protein retained the complete activity of the intact enzyme[37].

Thus, if the catalytic process of the enzyme can be reduced to the interaction of a limited number of functional groups, there should exist model systems which would approximate to the active site of the enzymes. Comparison of the model systems with the enzymes would then lead to a knowledge, although indirect, of the enzymatic catalytic processes.

Initial information, however, must come from a study of the enzymes and their interaction with the substrate, whether it be protein, peptide or simple ester, such as *p*-nitrophenyl acetate (NPA). Thus it has been found that enzymes, of which the most studied, α-chymotrypsin (ChTr), will be used as example, react with phosphorylating agents, such as the nerve

gas di-isopropyl phosphofluoridate (DFP), to give a stable, covalently bound, completely inactive phosphorylated enzyme[38]. The reaction is stoicheiometric, one mole of enzyme reacting with one mole of DFP, indicating that there is most probably only one active site per molecule of enzyme. The enzyme–DFP compound is sufficiently stable to survive degradation techniques, as is not usually the case with intermediates from the reaction of the enzyme with esters or amides. The evidence is clear-cut that the group which becomes phosphorylated is the hydroxy group of a single serine residue in the protein. Upon degradation of the protein, serine phosphoric acid is found[39]. Using similar techniques with radio-active phosphorous, the immediate sequence of amino-acids about the reactive serine was determined, and was found to be [40]

—asp—ser—gly–

Surprisingly, this same sequence has been found for a number of enzymes[41]. The reason for this is not immediately evident. It may be that the small glycine residue is necessary for steric reasons, and the aspartic acid may conceivably be involved in the catalytic process, but possibly the enzymes involved had a common biological origin, and developed differently with different specificities by independent mutations[36].

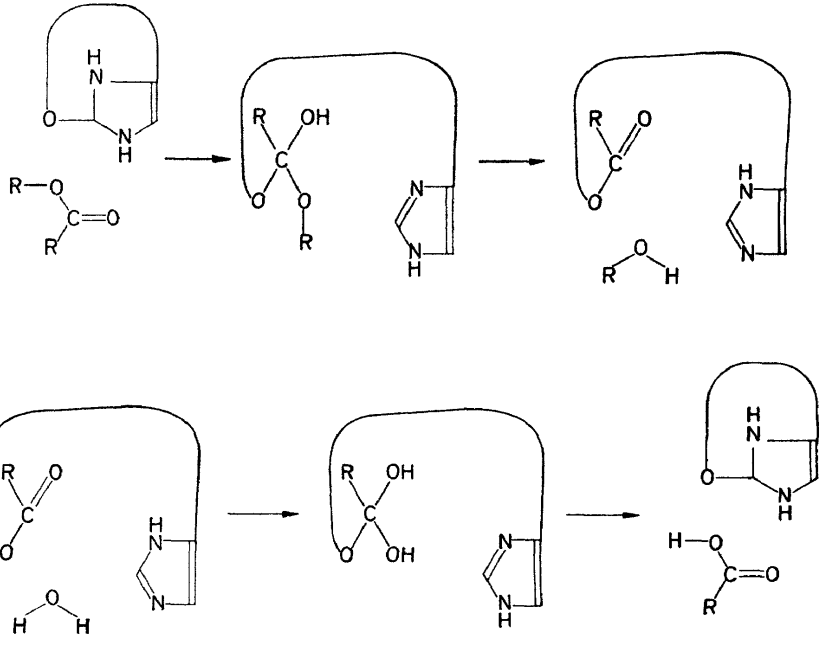
Much evidence has accumulated which indicates that the imidazole ring of a histidine residue is also involved in the catalytic process. It is assumed that the imidazole ring, in order to participate in the interaction, of whatever nature it may be, with the comparatively small ester or amide function, must be in the vicinity of the serine hydroxyl group. Since histidine is not found immediately adjacent to serine, juxtaposition must occur by proper folding of the helical peptide chain or positioning of an adjacent chain of the same molecule to bring the groups in close proximity. The tertiary structure of enzymes is necessary for their catalytic activity, suggesting that groups on different parts of the molecule interact to provide the catalysis.

In photo-oxidation experiments with ChTr, loss of activity of the enzyme is concurrent with oxidation of one imidazole ring of the two histidine residues present in the protein[42]. In addition, the rate of loss of enzyme activity on photo-oxidation is approximately equal to the sum of the rates of oxidation of a histidine residue and a methionine residue[43]. In ChTr, one histidine reacts readily with 2,4-dinitrofluorobenzene while the other reacts sluggishly under mild conditions. In ChTr inactivated by DFP, both residues are reactive, suggesting that some interaction between serine and imidazole is destroyed on phosphorylation, allowing the previously sluggish imidazole to react[44].

In the catalysis of hydrolysis of NPA by ChTr, the reaction was found to occur in three steps: (i), rapid adsorption of the substrate on the enzyme; (ii), acylation of the enzyme with concomitant liberation of *p*-nitrophenol; (iii), liberation of acetate and reactivation of the enzyme. The pH dependence of the third step was found to be similar to that previously found for the over-all rate of ChTr-catalysed reactions and in agreement with the results obtained from other substrates[45], that is, the rate was found to be dependent upon a group with a pK of about 6·5; it was suggested that this

involves the imidazole group of a histidine residue (pK 6·5). The second step was suggested to involve the acetylation of the hydroxy group of a serine residue[46]. At pH 5·0, the reaction of NPA with ChTr results in the formation of a stable monoacyl derivative, which is identical with the intermediate in the hydrolysis at higher pH's[47]. Thus the acylation and deacylation steps can be separately studied. Spectral studies of the acetylation reaction did not detect any measurable change at 245 mμ, the characteristic absorption of acetylimidazole. It was also found that deacetylation did not occur if the acetylated enzyme was denatured in 8 M urea[47], suggesting that two groups are required for catalytic activity; one to which the acetyl group is bound (*e.g.*, serine hydroxyl) and the other not immediately adjacent on the chain to the first, which is necessary for the deacylation reaction (*e.g.*, an imidazole group of an adjacent helix)[41].

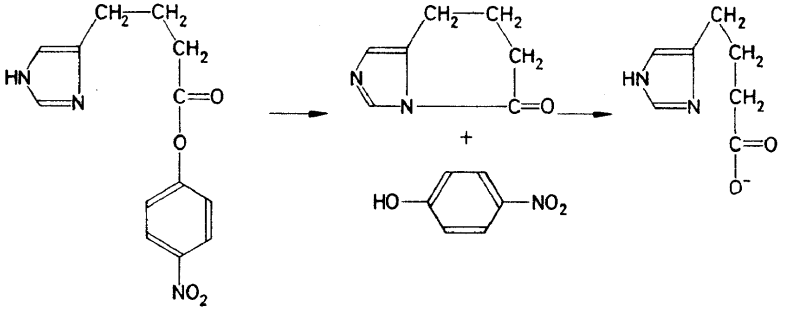
More recently, the actual mechanism of the catalytic reaction has been studied by a number of investigators. Bender and co-workers[48, 49] studied the reaction of *o*-nitrophenyl cinnamate with ChTr. Spectrophotometric studies indicated the formation of a cinnamoyl-enzyme intermediate in which the acyl group is attached to the hydroxyl group of serine. Results from pH and deuterium oxide isotope studies led these workers to postulate as the mechanism of catalysis either general basic catalysis by imidazole in both the acylation and deacylation steps, or nucleophilic catalysis by imidazole accompanied by general acid catalysis by a group with a pK of 12·5 or higher. A mechanism proposed by Bender which is consistent with both the pH dependence and the isotope effects involves the formation of a tetrahedral addition compound of imidazole and the serine hydroxyl group[48].



Studies of the effects of pH changes and of various inhibitors on the enzyme acetylcholinesterase have been carried out and conclusions have been arrived at which seem to be consistent with the results for other enzyme systems[50]. It was again found that both an acid and a basic group are involved in the enzyme-catalysed hydrolysis reaction. The basic group was found to have a pK of 6·5 and the acid group a pK of 9·3, in general agreement with the values obtained for ChTr. The basic group is probably imidazole and the acid group could be an ω-amino or a phenolic group. From the pH effects it was concluded that the imidazole group interacts with the carbonyl group of the substrate in the Michaelis complex, but not in the acyl-enzyme[51]. From the inhibition studies, a model of the active site was able to be constructed. In this model, which, in the case of cholinesterase, contains an anionic site but which is probably very similar for other enzymes, the imidazole group of histidine, the hydroxyl group of serine and the acid group are spatially arranged in proximity to each other, and the fit of the substrate at these three sites on the enzyme is readily seen[52].

Further evidence for the participation of histidine in the active site comes from model experiments in non-enzymic systems, keeping in mind the limitations of model systems compared to enzyme systems. Imidazole and its derivatives are efficient catalysts in the hydrolysis of certain esters[53]. However, comparison of the imidazole-catalysed hydrolysis of NPA, with the ChTr-catalysed hydrolysis, taking into account the formation of the Michaelis complex which is absent with imidazole, reveals that ChTr is far more efficient in both the acylation and deacylation steps[54]. In addition, imidazole does not cleave simple alkyl esters or amides whereas ChTr does. Thus, imidazole itself as a nucleophilic catalyst is not a suitable model for enzyme catalysis.

A significant achievement in the preparation of a model system was the hydrolysis of p-nitrophenyl-γ-(4-imidazolyl) butyrate[55]. The hydrolysis occurs by intramolecular nucleophilic participation by the imidazole ring, and it was found that the rate constant and pH dependency for the liberation

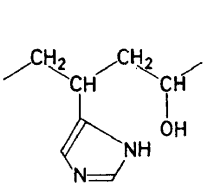


of p-nitrophenol from this ester are almost identical to those for the liberation of p-nitrophenol from the ChTr–NPA complex. This means that if NPA were bound to a protein so that the ester group had the same steric relationship to an imidazole ring of an histidine residue as in the model, the pH dependency and rate for the acylation step would be the same as that for NPA with ChTr, and no other groups would be necessary. However,

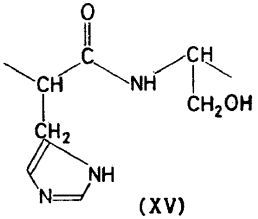
this intramolecular assistance is absent in the corresponding methyl ester, indicating the lack of some essential feature as an enzyme model.

Discussion

With this extensive background in the literature concerning the mechanism of enzyme action, we proposed to prepare polymeric materials, synthesized by addition polymerization and copolymerization of vinyl monomers, with pendant imidazole groups, along with other functional groups as required by the known facts concerning the mechanism, such as hydroxyl or carboxyl groups. The imidazole groups would be provided by the monomer 4(or 5)-vinyl imidazole. Since these polymers would be expected to have some tertiary structure in solution, it was expected that by random coiling an imidazole ring would be brought into the proper steric relationship with the other groups to provide greater reactivity than would be expected from a similar polymeric chain containing imidazole groups alone, or from simple imidazole compounds. The structure of a repeat unit of a hydrolysed copolymer of 4-vinyl imidazole and vinyl acetate (XIV), for instance, would approximate a peptide with neighbouring histidine and serine (XV), such as prepared by Katchalski, *et al.*[56] and would be a model in accord with early proposals of the enzyme mechanism, involving acyl transfer from serine to imidazole[57]. Of course,



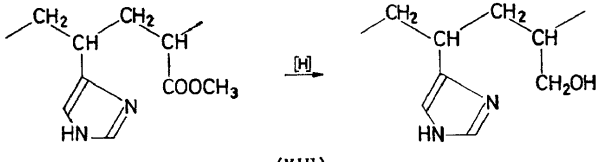
(XIV)



(XV)

this sequence is not found in chymotrypsin, but a histidine residue (one of the three in the protein) has been found adjacent to the phosphorylated serine in the enzyme phosphoglucomutase[58].

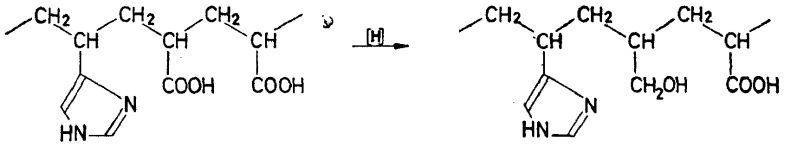
Since a copolymer with vinyl acetate would give a less reactive secondary alcohol[59] compared to the primary alcohol of serine, copolymerization with a monomer such as methyl acrylate (XVI) followed by reduction would give a primary alcohol in the polymer. In addition, copolymerization



(XVI)

with vinylene carbonate would provide an hydroxyl group, after hydrolysis, on a carbon atom closer to the carbon bearing the imidazole ring. Since an acidic group is probably also necessary for the enzymic catalysis, this

third site could be provided, for example, by a terpolymerization, or by a partial reduction of a copolymer with acrylic acid which had neighbouring carboxyl groups (XVII). Also, monomers other than 4-vinyl imidazole can be considered, such as benzimidazole derivatives with the vinyl group



(XVII)

on the benzene ring[60]. These are only a few examples of the many interesting possibilities which exist by which different groups and different stereochemical situations may be attained on a polymeric chain with a hydrocarbon backbone by various combinations of copolymerization, hydrolysis, reduction, *etc.* Thus, our initial hopes were that these polymers would function as models, although crude ones, of the active sites of the enzymes in question, and that they would show enhanced catalytic activity in the hydrolysis of esters, specifically *p*-nitrophenyl acetate.

Very little research has been done on the reactivity of polymers as enzyme model systems. Merrifield and Woolley[61] compared the reactivity of histidine and several poly(L-histidines) which were capped at the carboxyl end by L-glutamic acid or L-seryl-L-glutamic acid as catalysts in the hydrolysis of ethyl *p*-nitrophenyl carbonate. The turnover numbers (moles of substrate per milligram catalyst per minute) found were as follows: L-histidine, 7·7 ; chymotrypsin, 4·1 ; poly(L-histidine), 70 ; (poly-L-histidyl)-L-glutamic acid, 80; (poly-L-histidyl)-L-seryl-L-glutamic acid, 40. The authors concluded from their data that one or more histidines in the polymers was activated. While these results are interesting, it has been pointed out[62] that the catalytic activity should be compared per mole of un-ionized imidazole, not per weight of catalyst, since the pK of free histidine is higher than the pK of imidazole in the polymers[63]. Thus, at the same pH, there would be more un-ionized imidazole in the polymer than in free histidine. Katchalski *et al.* [56], in work which parallels our own, prepared poly(L-histidine) and copolymers of L-histidine with L-serine, L-aspartic acid, L-lysine and L-glutamic acid and studied their catalysis of the hydrolysis of NPA Their results will be discussed later.

Results

The synthesis of the monomer 4(or 5)-vinyl imidazole (XVIII) will be discussed in a forthcoming paper. 4-Vinyl imidazole was found to be a very reactive monomer using free radical initiation, readily forming homo- and copolymers. Of the co-monomers tested to date, all were found to be less reactive than (XVIII), by comparison of the monomer feeds and copolymer compositions. Generally the polymers were water soluble and exhibited association at high concentrations, as shown by gel effects. This paper will be concerned mainly with some of the results obtained with vinyl acetate as co-monomer. Vinyl acetate was a far less reactive monomer than 4-vinylimidazole in the copolymerization. For instance, with

a 5 mole per cent vinylimidazole monomer feed, the copolymer had a 75 mole per cent imidazole content. Difficulties were encountered in the attempted hydrolysis of the acetate groups using basic catalysts. Basic treatment led to either polymer degradation or to intractable materials. It was finally found that the acetate groups could be hydrolysed by dilute acid treatment. That some hydrolysis had occurred in a copolymer on mere dissolution in water was confirmed by two facts. The infra-red spectrum of the polymer after solution in water and precipitation from dioxane showed a decrease in the extinction coefficient of the ester carbonyl stretching frequency at 1725 cm^{-1} compared to that of the polymer before being dissolved in water. Also, elemental analysis of the polymers obtained from two successive precipitations from water into dioxan showed a decrease in the percentage of carbon, as would be expected if acetate groups had been replaced by hydroxyl groups. We assume that this hydrolysis occurred with nucleophilic participation of neighbouring imidazole groups on the polymer chain.

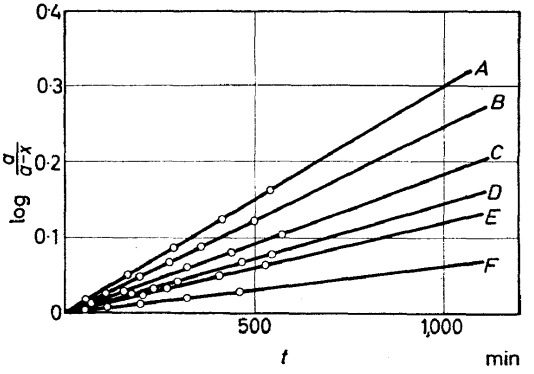


Figure 1. Typical first-order plots of the appearance of *p*-nitrophenol (400 mμ) in the hydrolysis of *p*-nitrophenol acetate catalysed by imidazole-containing polymers; 25°C, *tris*-(hydroxymethyl)-aminomethane-HCl buffer, pH 7·0, 0·05M, 28·5% (v/v) ethanol-water; [imidazole] = 10^{-4}M

A: imidazole
B: poly(4-vinylimidazole)
C: hydrolysed copolymer of 4-vinylimidazole and vinyl acetate (60 mole % 4-vinylimidazole)
D: hydrolysed copolymer of 4-vinylimidazole and vinyl acetate (44 mole % imidazole)
E: copolymer of 4-vinylimidazole and methyl acrylate (29 mole % imidazole)
F: blank (TRIS-HCl buffer, 0·05M, pH 7·0, 28·5% (v/v) ethanol-water)

Kinetics of the hydrolysis of *p*-nitrophenyl acetate catalysed by a number of polymers were studied[64]. The polymers used were poly-4-vinylimidazole, hydrolysed copolymers of vinyl acetate and 4-vinylimidazole, and for comparison, copolymers of methyl acrylate and vinylimidazole. The results were compared with the catalytic effect of imidazole itself. Reactions were followed by observing the appearance of *p*-nitrophenol at 400 mμ. Ordinary phosphate buffers could not be used, since the polymers were insoluble at pH's ranging from 6 to 8, even at buffer concentrations of 0·0025 M. The buffer system required was a *tris*-(hydroxymethyl)-aminomethane-hydrochloric acid buffer of pH 7·0 in an ethanol–water mixture, even though *tris*-(hydroxymethyl)-aminomethane itself appreciably catalyses the hydrolysis of NPA[65]. Concentrations of substrate and catalyst were

chosen to give a conveniently slow reaction time, with a half-life of 1000 minutes for imidazole itself. First-order kinetics were followed in all cases for at least 25 per cent of the reaction. Representative first-order plots of the appearance of *p*-nitrophenol are shown in *Figure 1*. The catalytic rate constants k_2' were calculated from the equation[53]:

$$k_2' = \frac{k_1 - k_{obs}}{c}$$

where k_1 is the observed first-order rate constant with added catalyst, k_{obs} the observed first-order rate constant of the buffer system in the absence of imidazole compounds, and c is the total molar concentration of imidazole groups. For the buffer system used, k_{obs}, was found to be $1{\cdot}43 \times 10^{-4}$ min^{-1}.

The most efficient catalyst found was imidazole, and the best polymeric catalyst was the homopolymer of 4-vinyl imidazole. The catalytic rate constant k_2' for imidazole in our system was found to be 5·42 l.mole^{-1} min^{-1} and for the homopolymer of 4-vinyl imidazole the value was 3·90 l.mole^{-1} min^{-1}. The value for one of the hydrolysed copolymers with vinyl acetate, with 60 mole per cent imidazole was 3·25 l.mole^{-1} min^{-1}. The pertinent data are summarized in *Table 3*, with the catalysts arranged in order of decreasing efficiency. The reaction was first-order in imidazole concentration, as shown by the linearity of the plot of observed first-order rate constant *v.* concentration of imidazole groups (*Figure 2*) obtained from a single copolymer. The value of the catalytic rate constant obtained from the slope is in good agreement with that obtained as the average values of the individual points.

Katchalski and co-workers[56] in working with poly (α-amino acids) found

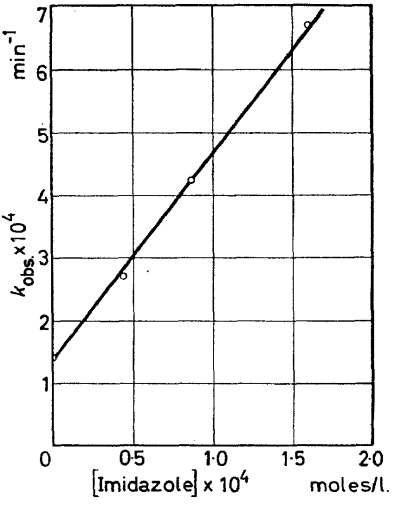


Figure 2. Plot of k_{obs} *v.* imidazole concentration for a hydrolysed copolymer of 4-vinyl imidazole and vinyl acetate (60 mole % vinyl imidazole); slope = 3·28 l. mole^{-1} min^{-1}

Table 3. Hydrolysis of *p*-nitrophenyl acetate catalysed by imidazole derivatives, 25°C, pH 7·0 *Tris*-HCl buffer, 0·05M; solvent: 28·5% (v/v) ethanol–H_2O. [NPA] = $0{\cdot}9 \times 10^{-4}$M

Catalyst	*Imidazole groups* (mole %)	[*Imidazole*] $\times 10^4$	$k_{obs} \times 10^4$ (min^{-1})	k'_2 ($l.mole^{-1}min^{-1}$)
Imidazole	100	1·00	6·85	5·42
Poly 4-vinylimidazole	100	1·10	5·69	3·90
Copolymer-4-vinylimidazole, hydrolysed vinyl acetate	60	0·86	4·25	3·28
Copolymer-4-vinylimidazole, hydrolysed vinyl acetate	44	1·11	3·34	1·72
Copolymer-4-vinylimidazole, methyl acrylate	30	1·14	2·19	0·67
Blank	0	0	1·43	0

that all histidine-containing polymers had less than 1/1000 of the activity of chymotrypsin, assuming one active histidine in the enzyme of molecular weight 24,000. The values of the catalytic rate constants were also significantly lower than that found for imidazole, which in their system was 35·3 $l.mole^{-1}$ min^{-1}. The polymers tested all had activities similar to that of histidine, with values of k_2 ranging from 3 to 10 $l.mole^{-1}$ min^{-1}. The authors concluded that all the histidine residues in the polymers tested were equivalent, and that no other assumptions were justified.

Comparison of *Table 3* with the values quoted shows that our results for vinyl polymers are in qualitative agreement with those Katchalski found for polyamides. A plot was made of the variation of the catalytic rate constant with the mole per cent of imidazole groups in the polymers, and is shown in

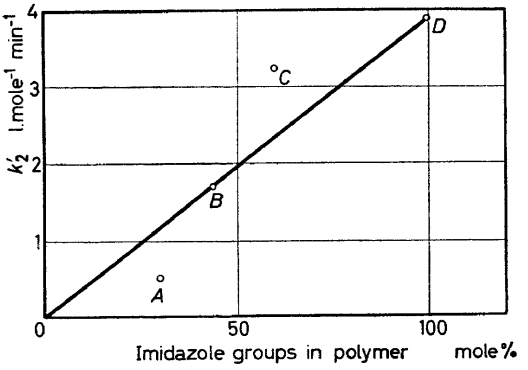


Figure 3. Dependence of k'_2 on mole % of imidazole groups in polymer
A: copolymer with methyl acrylate
B: copolymer with vinylacetate, hydrolysed
C: copolymer with vinyl acetate, hydrolysed
D: poly(4-vinylimidazole)

Figure 3. This dependence is seen to be roughly linear. We can only conclude that in the limited number of polymers tested, the catalytic activity results only from that provided by the reactivity of imidazole groups diluted by an inert polymer chain, and that no enhanced reactivity is caused by the presence of other groups in the polymers. It is hoped that more significant results will be obtained with some of the less crude model polymers mentioned above which are currently being investigated.

Summary

In order to understand more fully the factors which govern the reactivity of protein-sulphhydryl groups and to point out useful pathways for improving chemical protection against the effects of lethal radiation, relative rates of oxidation of a series of polymers, copolymers and low molecular weight analogues were determined. A spectrophotometric method based on the reduction of the redox dye, sodium 2,6-dichlorobenzeneoneindophenol, was used for the rate determinations in both aqueous and nonaqueous systems. The polymers studied in aqueous solution were: the saponified homopolymer of *p*-vinylphenyl thiolacetate, a saponified copolymer of *p*-vinylphenyl thiolacetate and methyl methacrylate and a saponified copolymer of vinyl thiolacetate and methyl methacrylate. The saponified homopolymer of vinyl thiolacetate was studied in dimethyl formamide solution.

Among the low molecular weight analogues studied in aqueous solution were: *p*-thiocresol, 2,4-di(*p*-mercaptophenyl)pentane, 2,2-dimethyl-4-(*p*-mercaptophenyl)valeric acid and 2,2-dimethyl-4-mercaptovaleric acid. In dimethyl formamide solution, the following were studied: β-mercaptoethanol, 2-4,pentanedithiol and 2,5-hexanedithiol. It was found that the saponified homopolymer of *p*-vinylphenyl thiolacetate and its model 2,4-di(*p*-mercaptophenyl)pentane oxidized more than six times faster than the monomeric compound of *p*-thiocresol. The saponified homopolymer of vinyl thiolacetate oxidized about ten times faster than its model 2,4-pentanedithiol and about fifty times faster than β-mercaptoethanol. Comparative rates show that poly(vinyl mercaptan) oxidizes about twenty-five times faster than poly(*p*-mercaptostyrene). These results, as well as other results, are explained in terms of facile intramolecular disulphide formation.

A brief review of the literature concerning the active site of enzymes, such as α-chymotrypsin is presented. The active site is generally agreed to involve the hydroxyl group of a single serine residue, the imidazole group of a histidine residue, and an acidic site in the enzyme. The preparation of model polymeric systems containing imidazole groups, hydroxyl groups and carboxyl groups is described. The imidazole groups are provided by the monomer 4(or 5)-vinyl imidazole, or benzimidazoles with vinyl groups on the benzene ring. The results obtained from a kinetic study of the hydrolysis of *p*-nitrophenyl acetate catalyzed by a number of addition polymers containing imidazole and other functional groups is presented. All of the polymers tested were less efficient catalysts than imidazole. It is concluded that the reactivity of the limited number of polymers tested is due solely to the presence of imidazole groups, with no enhancement provided by the other functional groups present.

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