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STUDIES ON CHITIN

AND CHITOSAN

by

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ABSTRACT

Title: Studies on chitin and chitosan

By: Beryl D. Gummow

High molecular weight chitosan having a very low residual N-acetyl content has been produced by dissolution and reprecipitation of partially deacetylated chitin, followed by a further alkaline treatment. Attempts to carry out this second deacetylation step in solution using enzymes having amidase activity were unsuccessful, hydrolysis of the polymer chain occurring without any noticeable decrease in the N-acetyl content.

The uptake of Cu(II) ions from solution by chitosan films has been followed by spectrophotometry after treatment of the films with metal complexing reagents. The equilibrium uptake was found to be inversely dependent upon film thickness, indicating that there is a concentration gradient within the films. When Cu(II) ions complexed with a tridentate ligand were used, interaction occurred with all the amine groups.

The ability of solutions of chitosan to induce metachromasy in anionic dyes has been studied. Reversal of metachromasy by addition of neutral electrolyte, urea and ethanol, and by increase in temperature, was found to be similar to that reported for anionic polyelectrolyte/cationic dye systems. The stoichiometry of the reaction between cationic groups on the polymer chain and anionic groups on the dye ions has been shown to be 1:1, and metachromatic titration techniques for determining the degree of N-acetylation of chitosan or measuring its concentration in solution, have been developed. The metachromatic behaviour of a considerable number of dyes has been studied and the results show that the metachromatic shift may be either hypsochromic or bathochromic, and that there is no requirement for the charge on the dye ion to be delocalised. A new theory of metachromasy that does not invoke specific dye/site interaction is proposed and two experiments designed to test it: the dependence of the ease of reversal of metachromasy on the molecular weight of the polyelectrolyte and the stoichiometry of the interaction between chitosan and dibasic dyes. The results obtained, which cannot be explained in terms of current theory, support the new theory proposed here.

Dedication

To my parents,

Eileen and Dennis,

And to my children,

David and Richard.

The ladies of St. Jame's!
They're painted to the eyes,
Their white it stays forever,
Their red it never dies:
But Phyllida, my Phyllida!
Her colour comes and goes;
It trembles to a lily, -
It wavers to a rose.

From

The Ladies of St. Jame's,

By Austin Dobson.

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CHAPTER 1

INTRODUCTION

Chitin, and its deacetylated derivative, chitosan, are important skeletal materials found in a wide range of invertebrates, particularly the Arthropoda. In recent years interest in these high molecular weight, natural polymers has grown rapidly - from biological and structural analogy with cellulose and with the realisation of their abundance as a waste material from seafood processing plants and their potential application in a wide variety of industries.

Chitin and chitosan may be considered as extremes of a continuous series of copolymers consisting of [β -(1→4)-2-acetamido-2-deoxy-D-glucose] and [β -(1→4)-2-amino-2-deoxy-D-glucose] units. Pure chitin would ideally be the fully N-acetylated polysaccharide (Figure 1a) with chitosan the fully deacetylated derivative (Figure 1b). In practice, it is unlikely that chitin can be isolated without some hydrolysis of the N-acetamido groups, whilst chitosan, whether occurring naturally, or produced by hydrolysis of chitin, normally contains residual N-acetyl groups. The name chitosan is generally applied to that range of the copolymers soluble in dilute acid solution.

Lack of characterisation of the degree of N-acetylation of the particular material under discussion has led to considerable difficulty in comparing the results obtained by different workers, since the number and distribution of deactylated units may greatly affect both the physical properties and the biological roles of the polymer. Attempts to produce completely deacetylated chitosan (ideal chitosan) have led to low molecular weight products due to the harsh treatments

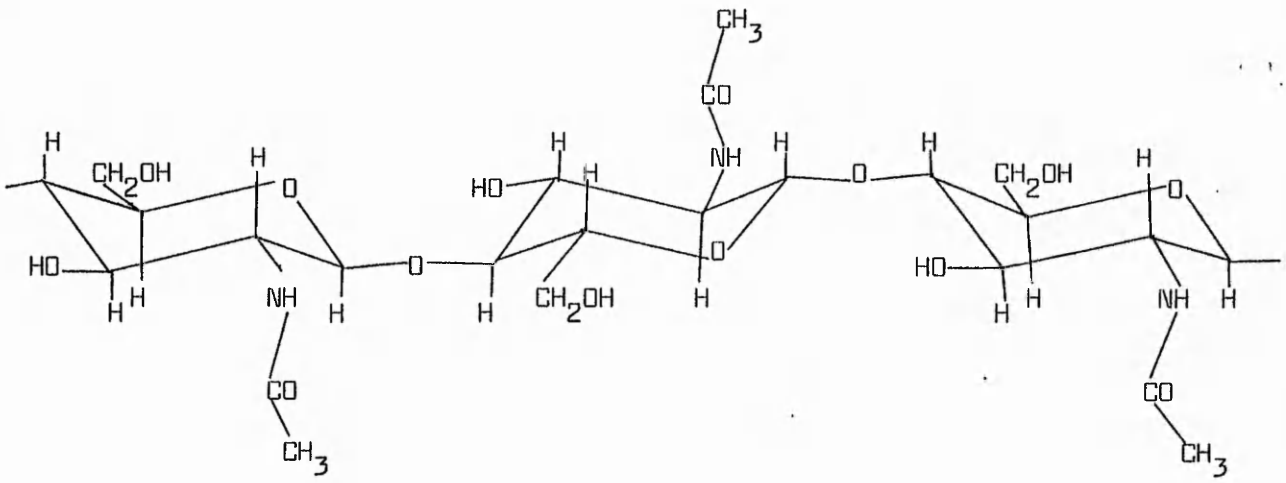


Figure 1a. Idealised structure of chitin.

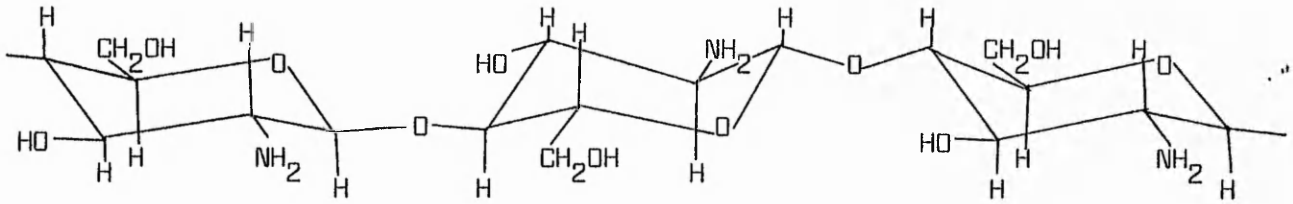


Figure 1b. Idealised structure of chitosan.

required.

The presence of amine groups in chitosan gives the polymer the property of binding a number of metal ions, particularly transition metal ions, very strongly. It is frequently described as chelating with metal ions, but to date no evidence of chelate ring formation has been presented. Residual amine groups may also account for the metal binding properties reported for chitin; the idealised chitin structure (Figure 1a) would not seem likely to permit metal binding.

Finally, a large portion of this thesis is concerned with the use of chitosan as a cationic chromotrope to induce metachromatic shifts in the absorption spectra of a number of anionic dyes. Metachromasy has been observed previously as a change in the absorption spectra of some cationic dyes induced by the presence of anionic polyelectrolytes. The present work represents the first study of a cationic polyelectrolyte/anionic dye system showing metachromatic behaviour, and is the first report of metachromasy induced by chitosan.

CHAPTER 2

REVIEW

2.1 Discovery of chitin

Chitin was described for the first time in 1811, by Braconnot[1] who isolated a product which he called 'fungine' from various mushrooms including Agaricus volvaceus. He stated that 'fungine seems to contain more nitrogen than wood', concluding that it was 'a distinct substance among those identified in plants'. In 1823 Odier[2] established a relationship between insect cuticle and plant tissue, and proposed the name 'chitin', from the Greek χιτών - a tunic or envelope - for the substance that he isolated. Lassaigne[3], in 1843, demonstrated the presence of nitrogen in chitin from the silkworm, Bombyx mori, while Ledderhose[4] showed that chitin is composed of glucosamine and acetic acid. The presence of glucosamine was confirmed by Gilson[5] in 1894.

Chitosan was first produced by Rouget[6] in 1859 when he treated chitin with concentrated potassium hydroxide solution and the 'modified chitin' was named chitosan by Hoppe-Seiler[7] in 1894. Subsequent research concentrated initially on the distribution of chitin in living organisms, and its degradation by bacteria. Many dissimilar soil flora cultures were shown to degrade chitin[8-11], and bacteria capable of utilising chitin were shown to occur throughout the marine environment[12]. Concurrently the chemistry of chitin was also studied. After considerable controversy over the chemical form of nitrogen in chitin Purchase and Braun[13] (1946) described chitin as a polysaccharide of glucosamine, and a structure tailored on the cellulose model for

chitin and chitosan had gained acceptance by 1950. A bibliography of papers and books dealing with the chemistry and biological occurrence of chitosan is given by Muzzarelli[14].

2.2 Morphology of chitin and chitosan

X-ray diffraction studies[15,16] have shown that chitin has a highly ordered crystalline structure occurring in three polymorphic forms: α -, β - and γ -chitin. The tightly compacted α -chitin is the most crystalline form, with anti-parallel chains; the chains are parallel in β -chitin, and in γ -chitin two chains are 'up' to each chain 'down'[17]. α -Chitin is thought to be the most stable form of polymer, since β -chitin assumes this form when reprecipitated from acids[14]. β - and γ -chitin can be converted to α -chitin by treatment with 6N HCl, although there is no interconversion between the three forms on boiling in 5% potassium or sodium hydroxide solutions[18]. Treatment of γ -chitin with lithium thiocyanate can transform it to α -chitin[16].

Rudall[19] suggested that the three forms of chitin were related to diversity of function. α -Chitin is found where extreme hardness is required, as in Arthropod cuticles. The polymer is frequently sclerotised and encrusted with mineral deposits. β -Chitin, which is a crystalline hydrate[20], and γ -chitin are often associated with functions of flexibility and mobility as well as toughness, and may have additional physiological functions. All three forms of chitin have been demonstrated in the squid Loligo[15], whose beak contains α -chitin, whose pen contains β -chitin and whose stomach linings contain γ -chitin, thus relating the polymorphic form to function rather than taxonomic grouping. A review of the present understanding of the macromolecular organisation of chitin, and a comparison with the organisation of cellulose, is given by Blackwell[21].

2.3 Biological occurrence of chitin

2.3.1 Chitin in lower plants

Chitin is present as a structural component in the cell walls of plants metabolising considerable amounts of nitrogen, such as fungi and moulds. Only a few major classes of fungi lack chitin[22,23]. Some lower photosynthesising plants such as the green algae, Chlorophyceae have also been shown to contain chitin[24,25] as have some yeasts[26-28]. Fungal chitin and animal chitin have been shown to have the same identity by physical[29-33], chemical[34] and enzymic[35,36] techniques.

2.3.2 Chitin in animals

Whereas chitin in plants is associated with other polysaccharides, it is associated with proteins, and particularly with collagen, in animals[19,21]. Jeuniaux[37] has described chitin as the major structural polysaccharide of most invertebrates but Chordata, with the exception of the Tunicata, do not contain chitin[38,39]. An extensive review and bibliography of the occurrence of chitin is given by Moore[40]. Other recent reviews of distribution in the animal kingdom are those of Rockstein[41], Hepburn[42] and Jeuniaux[37].

Chitin has been demonstrated in all major classes of the Arthropoda[43], in both exoskeletons and internal tissues, and it is this phylum that has been considered as a source of chitin for industrial use. The Crustacea in particular are a potentially rich source of the polymer[44]. The presence of chitin in marine fauna is estimated to result in several billion tons of sediment per year, largely due to moulted Copepod exoskeletons[45], but no large accumulation of undegraded fossil chitin has been recorded.

The economics of chitin recovery from sea food processing are considered by Murray and Hattis[46], and an evaluation of potential sources of chitin is given by Allan, Fox and Kong[47].

2.4 Enzymic studies on chitin and chitosan

2.4.1 Enzymic synthesis of chitin and chitosan

Muzzarelli[48] has given a pathway of chitin synthesis together with a bibliography of relevant publications. The precursors of chitin include glucose-6-phosphate, N-acetylglucosamine-6-phosphate and uridine diphosphate-N-acetylglucosamine. A yeast chitin synthetase is described by Duran and Cabib[49] whilst Araki and Ito[50] have given an account of chitosan formation in the mould Mucor rouxii by enzymic deacetylation of chitin.

2.4.2 Enzymic degradation of chitin and chitosan

Enzymic degradation of chitin may take place by deacetylation (to chitosan[50]) or by hydrolysis of the $\beta-(1\rightarrow4)$ linkage by chitinase. Chitinases have been shown to occur in bacteria[51], fungi[52], and the digestive glands of animals whose diet includes chitin, - for example several species of mammals and birds[53], the frog Rana temporaria temporaria[54] and the snail Helix peliomphala[55]. In higher plants, a protective role against invasion by fungi has been ascribed to the enzymes with chitinase activity, and these have been shown to be present in sugar maples and oaks[56] and in the papaya tree, Carica papaya[57]. A further important role of chitinase is in Arthropod moult cycles[58]. Otakara et al.[51] described a bacterial chitinase system where chitin was first hydrolysed to chitin-oligosaccharides and then the N,N'-diacetylchitobiose, which was hydrolysed to N-acetylglucosamine by

chitobiase.

Chitosanases have been studied by Fenton et al.[59], Monaghan et al.[60] and Ruiz-Herrera and Ramirez-Leon[61], and have been shown to be widespread in soil flora[59]. Because of the ubiquitous presence of chitinolytic and chitosanolytic micro-organisms, commercial users of chitin and chitosan must also consider the effects of biodegradation[62].

Chitinase (EC 3.2.1.14) is defined[63] as poly[β -(1→4)-2-acetamido-2-deoxy-D-glucoside]glycanohydrolase and produces random hydrolysis of β-(1→4)-2-acetamido-2-deoxy-D-glucoside linkages in chitin and chitodextrin. The problems of purification of chitinase may be highlighted by a qualification to the definition, stating that 'some chitinases also display the activity defined in EC 3.2.1.17' - lysozyme. Lysozymes produce hydrolysis of β-(1→4) linkages between N-acetyl-muramic acid and 2-acetamido-2-deoxy-D-glucose residues in mucopoly-saccharides or mucopeptides, and have been known to be widespread in human and other tissues for many years[64]. The overlap of lysozyme and chitinase activity has led to difficulties in establishing the nature and purity of some chitinolytic extracts[53]. The presence of enzymes with chitinase activity in plants[56,57] and the anti-fungal protection these enzymes are thought to confer, has a corollary in the findings of Araki et al.[65] who showed that lysozyme resistance in Bacillus cereus cell wall peptidogylcans was removed by N-acetyl-ation of glucosamine residues. Cell walls of Rhizopus and Mucor, which contain chitosan as a main component with glycans and chitin, are also particularly resistant to lysozymes[66], but Rhizopus cell walls are decomposed by a chitosanase[67].

The extraction of chitinase for use on a commercial scale as an additive in biological insecticides is described by Smirnoff[68].

2.4.3 Viscometric techniques for assessing enzyme activity

Viscometric methods of assessing enzyme activity have been in use for some time. McLean and Hale[69] studied hyaluronidase activity by viscosity measurements in 1941, and a modified assay was reported in 1947[70]. These methods were based on work by Madinaveitia and Quibell[71], who found that 'half viscosity time' or 'half-life time of the substrate' was inversely proportional to the concentration of the enzyme and independent of the substrate concentration. In 1955, Tracey[72] used a viscometric assay to follow enzymic activity of an extract from Lycopodium on a chitosan acetate substrate. He also determined activity of the same extract on a chitin suspension, by determination of acetylglucosamine produced, and reported positive results in both cases. He described this as chitinase activity.

Otakara[52,73] has also used viscometric techniques to study the chitinolytic effects of a fungal extract. A preparation of black-koji mould, Aspergillus niger, was used with a substrate of glycol chitin, and shown to cause rapid reduction in the viscosity of substrate solutions. 'Half-life time' was defined as time required to halve the viscosity increment, and was again shown to be inversely proportional to enzyme concentration.

Otakara[74] was able to show that the chitinase hydrolysed the glycosidic linkages of the glycol chitin but was not active with the monosaccharide, or with a deacetylated substrate (glycol chitosan). The extent of deacetylation of the glycol chitin was not stated. Random degradation of glycosidic linkages is assumed and the enzyme described as an endoglucosamidase. Similarity to lysozyme action is also mentioned[73]. Tominaga and Tsujisaka[67] also used change in viscosity of the substrate to study activity of a chitosanase from

a Bacillus strain, and again suggested that the abrupt decrease in viscosity showed that the enzyme hydrolysed the polymer in an 'endo' manner.

More recently, Rinaudo and Milas[75] have followed the kinetics of xanthan hydrolysis by cellulose using a viscometric method.

2.5 Preparation of chitosan

Although chitosan has been found to occur in the cell walls of the fungi Phycomycetes blakesleeanus[76] and Mucor rouxii[50], these have not been used as a source of chitosan. Instead chitosan is normally obtained by alkaline deacetylation of chitin obtained from Crustacea.

2.5.1 Isolation of chitin

Chitin is usually found in close association with other materials, and particularly, in Crustacea, with calcium carbonate and proteins. The severe methods of treatment normally used to isolate the chitin can also cause degradation of the polymer, and it is doubtful whether undegraded samples can be obtained by these methods. Crustacean sources[47] provide the most abundant raw material for chitin production and its preparation generally involves decalcification with acid, followed by deproteination by treatment with hot dilute alkali. In a typical example Hackman[77] prepared chitin from lobster shell by the methods described below.

Lobster shell was cleaned by washing and scraping under running water, then dried at 100°C. The prepared shell (200g) was then digested with 2N hydrochloric acid ($2dm^3$) for 5 hours at room temperature, washed well, dried at 100°C and ground to a fine powder (91g). Extraction of the powder for 48 hours with cold 2N hydrochloric acid

($500cm^3$), with frequent agitation, was followed by centrifugation, washing with water, and extraction at 100^oC with 1M sodium hydroxide ($500cm^3$) for 12 hours, also with frequent agitation.

Muzzarelli[78] has reviewed the methods of various authors, including relatively mild processes using ethylene diamine tetraacetic acid as a decalcifying agent[79] and enzyme deproteination[80]. Karupaswamy[81] reported a method for obtaining a high quality chitin for commercial use from crab, but again conditions were severe (5% boiling NaOH for 24 hours). It was claimed that by choosing an appropriate stage in the moult cycle a product of much higher viscosity could be obtained. With most of these methods however, some deacetylation during the alkaline stage and reduction of molecular weight by acid hydrolysis, are still possible.

2.5.2 Deacetylation of chitin

The cleavage of acetamido groups adjacent to trans-related hydroxyl groups, as is the case with chitin, requires much more vigorous basic conditions than those sufficient for cleavage of cis- related analogues[82]. Thus chitosan is generally prepared by deacetylation of chitin by concentrated alkali and the quality of the deacetylated product will obviously be partly dependent on the quality of the chitin sample used. Wu and Bough[83] have considered the effects of varying the concentration of alkali and the time of treatment on the deacetylation of chitin, and Peniston and Johnson[84] have described a method for preparing chitosan from crab shell that eliminates the acidic demineralisation stage and allows recycling of most of the process chemicals and recovery of protein, sodium acetate and calcium carbonate as by-products. The same authors[85] assessed the probable costs of chitin

and chitosan production from Crustacea processing wastes in the United States.

Madhavan and Ramachandran[86] and Moorjani et al.[87] have tabulated the results of varying conditions of demineralisation on the viscosity of solutions of chitosan samples, and the latter authors also list the effects on the viscosity of the chitosan of bleaching at different stages of processing, and of deacetylating with alkali.

The biosynthesis of chitosan by the action of chitin deacetylase on chitin has been described by Araki and Ito[50]. A method of preparation and purification of this enzyme is given by the authors, but no indication is given as to whether the extent of deacetylation obtained would be sufficient to be useful in a commercial process. However, Iwasaki and Shimahara[88] prepared chitosan which was 50-60% deacetylated by incubating prawn chitin with chitin-deacetylating bacterial strains, and in particular with a strain isolated from soil. No cleavage of the polymer chain was observed. A review of methods of chitosan production is given by Moore[40].

2.6 Structural characteristics of chitosan

2.6.1 Introduction

The distribution and amount of the minor structural component - glucosamine in chitin and N-acetylglucosamine in chitosan - are likely to have a considerable effect on the biological function and physical properties of the polymer. These effects range from resistance to lysozyme to the stability of metal complexes.

As already mentioned (Section 2.4.2) cell walls of Rhizopus and Mucor which contain chitosan as a main component, are particularly resistant to lysozyme attack[66], while lysozyme sensitivity in Bacillus

cereus could be induced by N-acetylation of glucosamine residues in the cell wall peptidoglycans. Similarly, Hirano et al.[89] found that N-acetylated chitosans were hydrolysed more rapidly by chitinase from Streptomyces griseus than was the original chitosan[65].

Distinguishing chitosan from chitin on the basis of the solubility of the former in dilute acid emphasises the importance of the extent of deacetylation in conferring solubility. However the solubility of chitin in organic solvent systems was found to depend inversely on the degree of deacetylation[90]. Sannan et al.[91] studied the relationship between solubility and degree of deacetylation of chitin and found that samples deacetylated to about 50% residual N-acetyl content, under homogeneous conditions, were water soluble. Samples of greater or lesser degree of deacetylation were found to be either gel-forming or insoluble. The distribution of the free amine groups was considered to be important and the water solubility of the approximately 50% deacetylated sample was attributed [92] to homogeneous deacetylation, giving a random distribution of N-acetyl-D-glucosamine and D-glucosamine units along the chain. Chitosan produced by a heterogeneous deacetylation process was considered to have a block copolymer structure, resulting in greater crystallinity and a lack of water solubility.

Hauer[93] studied the metal complexing ability of chitosan, basing the estimation of ion exchange capacity on the amine content of the polymer. Subramanian[94] considered oceanic transport of metals and the roles played by chitin and chitosan through metal complexing, concluding that the stability of chitosan-metal complexes[95] means that the deacetylated polymer forms a 'sink' for metals, while the relatively unstable chitin-metal complex[12] suggests a transport mechanism. Kurita et al.[96] studied the effect of degree of deacetylation on adsorption of metal cations, and found increasing adsorption with

increasing $-NH_2$ content up to about 50% deacetylation. The number of primary amine groups present was also found to be important in immobilising enzymes on chitin and chitosan columns[97]; chitosan columns showed greater activity and did not require an intermediate to aid enzyme immobilisation.

In view of the importance of the extent of deacetylation on the properties of chitin and chitosan, precise characterisation of the samples used by various workers is desirable and necessary but is often not carried out. A number of methods are now available, based on both physical and chemical techniques.

2.6.2 Techniques for determination of the degree of N-acetylation

2.6.2.1 Titration methods

Measurement of the primary amine group content has several advantages over measurement of residual N-acetyl content[98]; it is readily measured by titration, is a direct measure of the functional group of the polymer and is much more sensitive than analysis for nitrogen. Broussignac[99] determined the free amine group content by dissolving chitosan samples in a known excess of acid and titrating the solutions potentiometrically. The curve obtained has two points of inflexion and the difference between these points gives the volume of acid required for neutralisation of the primary amine groups. Similar methods have been used by other authors[91,98]. Hayes and Davies[100] prepared the hydrochloride salt of chitosan and determined the degree of deacetylation by titration with sodium hydroxide and with silver nitrate. Iodide titration following periodate oxidation has also been used, but tended to give low results for the degree of N-acetylation compared with other methods, possibly because of side reactions during

the oxidation stage leading to increased consumption of periodate. Kurita et al.[101] determined the degree of N-acetylation of chitin samples using Elek and Harte's method for estimation of acetyl groups[102]. Samples were hydrolysed with p-toluene sulphonic acid then titrated with sodium thiosulphate, in a relatively complex procedure.

2.6.2.2 Spectrophotometric methods

Domszy[103] has recently developed a spectrophotometric method of determining the degree of deacetylation of chitin and chitosan samples using salicylaldehyde. Schiff's base formation between the free amine groups of the solid chitosan and salicylaldehyde is allowed to proceed to equilibrium, when the concentration of salicylaldehyde remaining in solution in the methanol reaction medium is determined by measurement at 255nm. Use of the reaction between salicylaldehyde and chitosan has been extended[103] to the determination of free amine group content under homogeneous conditions. Excess salicylaldehyde is added to a solution of chitosan in methanol/aqueous acetic acid, and the concentration of N-salicylidene chitosan formed determined by measurement of the absorbance at 410nm.

A number of workers have explored the use of infrared spectrophotometry to determine the extent of N-acetylation. Sannan et al.[104] and Muzzarelli et al.[105] used the absorbance of the amide II band at 1550cm^{-1}, correcting for sample thickness by reference to the C-H peak at 2878cm^{-1}, whilst Miya et al.[106] described a modification which gave better results for samples of greater than 90% deacetylation by utilizing the amide I band at 1655cm^{-1}. Moore and Roberts[107] had previously described the use of the amide I band, but referred absorbances to the hydroxyl band at 3450cm^{-1}. They pointed out the

advantage given by this method in enabling estimation of the degree of N-acylation, as the value of A_{3450} remains virtually constant with increasing chain length of the N-acyl group, while considerable variation is found with A_{2878}. Furthermore they showed that, at low N-acetyl group contents, the amide II band frequently shifts from $1595cm^{-1}$ to $1550cm^{-1}$ with increase in N-acetyl group content. This may account for the poor results obtained using the amide II band at high degrees of deacetylation.

2.6.2.3 Gas chromatographic methods

Radhakrishnamurthy et al.[108] measured the release of acetic acid from N-acetyl groups in mucopolysaccharides by hydrolysis with hydrochloric acid and chromatography of aliquots of the treated solution. The amount of acetic acid evolved, and hence the number of N-acetyl groups in the original sample, was determined from the calibration curve, using propionic acid as internal standard. Holan et al.[109] used this method in studies on yeast cell walls, and proposed the technique for determining the content of chitin in the cell walls of yeasts, and hence of quantitative differences in cell wall fractions of different yeast species. However, this presupposes that the native chitin is completely N-acetylated.

Muzzarelli et al.[105] also used a gas chromatographic technique with chitin and chitosan, but in this case the method relied on differences in the retention time of methanol when eluted through columns of chitin or chitosan, the retention time increasing with increasing degree of N-acetylation.

2.6.2.4 Mass spectroscopy and nuclear magnetic resonance spectroscopy

The mass spectra of chitin, chitosan and chitosan hydrochloride have

been determined over a range of temperatures[100] and appeared to provide a promising method of characterising samples. Use of the mass spectral peaks attributed to $-COCH_3$ and $-NHCOCH_3$ was proposed for determination of the degree of deacetylation and evaluation of the $-NH_2/-NHCOCH_3$ ratio was postulated. Pyrolysis mass spectra of chitosans of different degrees of N-acetylation were later prepared[110] and gave a correlation with the amine group content of the samples. The identification of the N-acetyl group of amino sugars of complex carbohydrates has been achieved by GLC-mass spectroscopy by Banoub and Michon[111]. The method depends on the acylation of the alditols (formed by reduction of the monosaccharides obtained by acid hydrolysis) with trideuterioacetic anhydride in pyridine at room temperature. Using this method, it was shown that hydrolysis of chitin with 6M hydrochloric acid was accompanied by de-N-acetylation, and that de-N-acetylation of oligosaccharides could be avoided by using 2M trifluoroacetic acid, or 90% acetic acid followed by 0.25M sulphuric acid.

Nuclear magnetic resonance studies of chitin, chitosan and chitosan derivatives have also been carried out[112,113]. Gagnaire[112] studied solutions of chitin in lithium thiocyanate and lithium chloride/dimethylacetamide media. The ^{13}C NMR spectra of N-acetyl-D-glucosamine and chitobiose were assigned and then used to assign the peaks in the ^{13}C NMR spectrum of chitin. The dissolution of chitin in formic acid was subsequently studied and shown to occur with the formation of formyl chitin, and the ^{13}C NMR spectra of diformyl- and diacetylchitin were also worked out.

2.7 Adsorption of metal ions by chitosan

Muzzarelli[114] was the first to describe the interaction of metal ions

with chitin and chitosan as taking place by chelation. Hauer[93] has also studied the chelating ability of chitosan, attributing the property of metal ion adsorption to the $-NH_2$ group. Yaku and Koshijima[115] used a water-soluble glucosamine oligomer to prepare a D-glucosamine/Cu(II) complex as a model of the chitosan/Cu(II) complex and concluded that one mole of cupric ion was coordinated with four moles of D-glucosamine. This result conflicts with that of Blair and Ho[116] who estimated that in chitosan film two moles of D-glucosamine were complexed with one mole of cupric ion, and with that of Muzzarelli et al.[117] who found that at pH 4.0 and pH 5.0 1 or 2 N atoms per cupric ion were involved, while at higher pH the coordination number increased and OH groups were involved.

The exact mechanism of metal ion uptake has not yet been determined - Muzzarelli[118] speculated that ion-exchange, sorption and chelation may all be involved, and Yoshinari and Subramanian[119] suggest that all three processes are important to varying degrees for different metal ions.

Chitosan has great affinity for first row transition metals, while remaining relatively inert towards alkali metal and alkali earth ions[93]. Collection rates depend on several factors[120], the most important of which are polymer grain size, temperature, speed and mode of stirring, the presence of other ions suitable for collection, oxidation number of the ion and pH. The presence of large amounts of ammonium sulphate in test solutions allowed transition metals present at trace levels to be collected, while the presence of anions other than sulphate may also exert important effects - chloride may depress the extent of collection, as may thiocyanate, possibly because it is a complexing agent, whilst acetate alters the surface of polymer

grains because chitosan acetate is soluble. The effect of chemical modification of chitosan on uranyl ion uptake rates has also been studied[121], and a decrease in adsorption rate was found with increasing N-substitution of the chitosan.

In comparison with a synthetic polymer, chitosan showed consistently higher uptakes of metal ions than poly(p-aminostyrene)[122]. Although chitosan and poly(p-aminostyrene) are both polyamine polymers, the greater basicity of the aliphatic primary amine group compared with the aromatic amine group may account for the greater effectiveness of chitosan.

Uptake of transition metal ions is not affected by the presence of alkali metals or alkali earth ions[118]; high capacity and binding rate for many ions are combined with good filtering properties allowing high flow rates and a buffering capacity for hydrogen ions. In addition the free amine groups present scope for chemical modification[123]. Taken with the fact that chitosan is a renewable resource, the polymer seems to offer excellent scope for applications involving metal ion recovery. Nair and Madhavan[124] examined the metal binding properties of chitosan from different sources and concluded that the origin of the chitosan did not greatly affect uptake. Absorbance was found to be virtually complete within one hour, and the pH of the solution did not influence the rate of uptake or the quantity of metal ion absorbed.

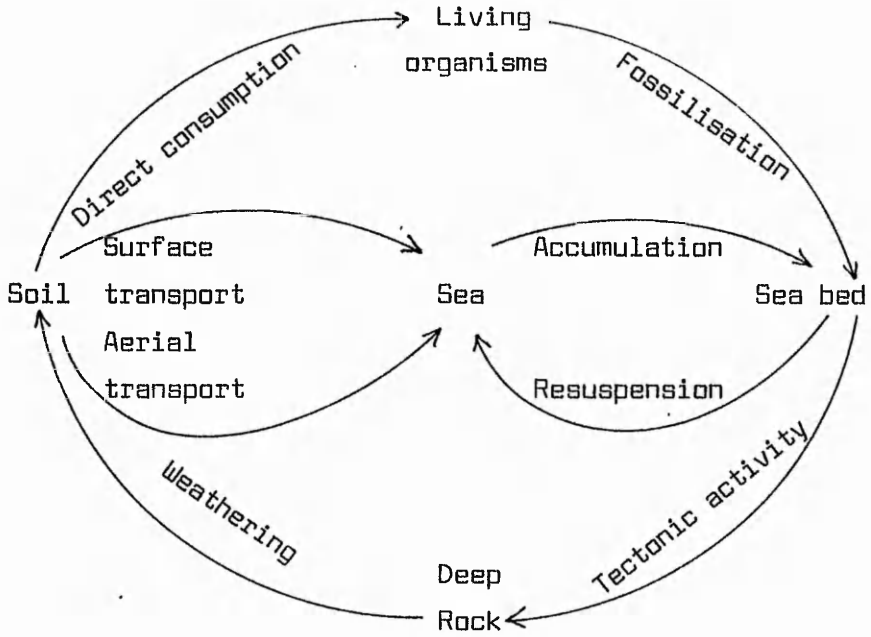
One major drawback of chitosan as a metal ion absorbent is its solubility in dilute acidic media with the exception of sulphuric acid, which forms insoluble chitosan salts at ambient temperature. Pretreatment, or conditioning, of chitosan with sulphate media has been shown to enhance transition metal ion capacity under some conditions[125,126], and short term cycling of chitosan columns with dilute acid (<3%) was

felt to have little degradative effect[93] and to be a possible means of avoiding the problem of solubility in acidic media; an alternative solution is the partial cross-linking of chitosan with glutaraldehyde[123].

Potential industrial applications of the metal ion binding capabilities of chitosan are numerous and range from treatment of electroplating wastes to improving the quality of wool dyeing effluents[123]. Chitosan from prawn waste[127] and chitin, chitosan and shellfish powders[128] have been studied for treatment of wastes and have been found to be very promising. The use of chitin, chitosan and chitosan-glucan complexes from fungal sources has also been explored[129,130], and inactivated material from Streptomyces griseus cultures was found to be particularly promising. Such material is available in large amounts as a waste product of fermentation plants producing Streptomycin for the pharmaceutical industry.

The extensive occurrence of chitin, and particularly its presence in large amounts in zooplankton exoskeletons gives cause for consideration of its role in the cycling of metals in the biosystem. Biodegradation of chitin-metal complexes has been studied by Subramanian[94], who considered that the decomposition of these complexes may be the source of localised metalliferous sediments in the marine environment. Concentrations of cadmium, copper, cobalt, iron, magnesium, manganese, nickel, lead, strontium and zinc in surface zooplankton specimens was found to be lower than in zooplankton samples collected at depths of 100 metres or more, presumably because food-dependent moulting rates were lower at depth, giving longer for accumulation of metal ions[131]. When the estimated annual biosynthesis of chitin by zooplankton alone is considered - greater than one billion tons[132] - the scale of metal ion uptake in the exoskeletons of marine Crustacea can be seen to be

considerable. A diagrammatic representation of a generalised biogeochemical cycle of the elements is given below[94].



2.8 Dyeing of chitin and chitosan

Very few studies on the dyeing of chitin and chitosan have been carried out. Giles et al.[133,134] studied the adsorption of sulphonated azo dyes, and also other organic solutes, on chitin obtained from the from the carapaces of Nephrops norvegicus, the Norwegian lobster, and compared the results with those of similar studies carried out on cellulose. The chitin used was not thoroughly characterised, but estimated to be about 13% deacetylated. Affinity for the dyes rose with increasing number of benzene nuclei - increasing planar area of the anion - and fell with increasing degree of sulphonation of the dyes, steric effects preventing interaction at cationic sites of the second and subsequent sulphonate groups, which remain dissolved in the water and tend to desorb the anion from the substrate. The

adsorption of sulphonated dyes can be regarded as an ion-exchange process at the acetamido groups, and the reaction of a dye with the cationic site in a sulphuric acid medium may be represented as given below[133].

$$R\text{-}\overset{+}{N}H_2COCH_3SO_4^{2-}{}_{/2} + NaDye \rightleftharpoons R\text{-}\overset{+}{N}H_2COCH_3Dye^- + \tfrac{1}{2}Na_2SO_4$$

Chitin was found to exert weaker van der Waals' forces than cellulose, probably because of the less regular molecular chain contour. Hydrogen bonding may also take place, and as with ion-exchange the interaction occurs at the acetamido groups, as the glucoside hydroxyl groups appear to be protected by solvated water molecules.

McKay, Blair and Gardner[135-137] have also studied the adsorption of dyes on chitin. Adsorption isotherms were plotted for a direct, a mordant, an acid and a premetallised acid dyestuff, and film mass transfer coefficients and intraparticle diffusion processes were considered in a series of studies which examined the effects of various parameters on dye adsorption, including temperature, particle size, agitation, solution pH and initial dye concentration. An earlier study by Mataga and Koizumi[138] found that the adsorption and fluorescence spectra of solutions of chitosan hydrochloride and glucosamine hydrochloride in combination with eosin and erythrosin (C.I. 45380 and 45430) showed modification of the adsorption and fluorescence spectra when the polymer but not the monomer was present. In contrast a closely related dye, uranine (C.I. 45350) did not show the same kind of modification. This paper will be considered more fully in the following section.

Applications involving the use of chitosan in dyeing have been suggested to facilitate the colouration of glass fabrics[139], polyester-cotton fibres[140], and plastic fabrics[141]. Printing of polyester-cotton textiles with a solution of chitosan in 1% acetic acid followed by drying and dyeing to give intensified colours on the printed sections has been suggested[140], as has the use of chitosan solutions containing pigments. A novel application has also been put forward by Knorr[142], who proposes chitin and chitosan as potential carriers of food colouring materials.

2.9 Metachromasy

2.9.1 Introduction

The phenomenon of metachromasy, or metachromasia, has been known since the middle of the 19th century. It was first observed as a visible change in the colour of certain dyes used as histological stains when these were applied to particular tissues, and has since been studied with increasing interest both histologically and in solution. Although many workers have devoted considerable effort to determining the physical basis of metachromasy, the underlying cause of the change in the wavelength of maximum adsorption has still to be described satisfactorily. Even the definition of a metachromatic shift in wavelength varies from one group of workers to another.

For the purposes of this thesis, the following definition will be adopted:

> Metachromasy is a characteristic reversible colour change that any dye may undergo by virtue of a change in its environment not involving chemical reaction of the dye[143].

The following review of work on metachromasy up to the present will

concentrate on studies carried out on dyes in solution, and will describe work carried out to vary the environment of the dyes studied and the theories presented as a result of such work, as background to the results to be described in Section 3.

2.9.2 Metachromasy in tissues and solid substrates

One of the few reviews of metachromasy, and by far the most detailed, is that written by Schubert and Hamerman in 1956[144], and the earliest references to metachromatic dye/tissue interactions are taken from their paper. In 1875, Cornil[145], Heschl[146] and Jurgens[147] appear to have noticed simultaneously that several triphenylmethane dyes stained amyloid a colour characteristically different from the colour of the dyes in dilute solution. In 1879, Ehrlich[148] found that the same dyes stained both granulated cells and plasma cells of connective tissue in a remarkable manner and Schubert and Hamerman[144] attribute the first use of the description 'metachromatic' to Ehrlich. Since that time metachromasy has been used to study tissue sections, and has been particularly associated with observations carried out on tissues containing high molecular weight polysaccharides containing sulphate ester groups - for example mast cells, cartilage and mucilages. Michaelis and Granick[149] noted that nucleic acids stained the 'normal' colour of the dye, and that the colour of metachromatically staining tissues was more variable according to conditions. Michaelis[150] applied ideas already current in 1947 on the importance of 'polymerisation' of dyes in the formation of the metachromatic colour in solution to his studies of agar, and of tissues containing nucleic acids, and Kelly[151] pointed out that the thiazine dyes - for example Toluidine Blue, Azure A and Methylene Blue, were useful histological stains. Methylene Blue was particularly useful instrumentally,

although visually its sensitivity was limited. The problems of purity mentioned by Kelly were also underlined by Sylven, who thought that the use of metachromatic staining as an indication of the presence of specific chemical groups in histological sections was very limited[152]. By contrast Szirmai and Balazs[153] thought that the quantitative use of metachromasy in the investigation of tissue constituents was potentially useful.

More recent workers have used diffuse reflectance spectrophotometry to examine metachromatic colour changes in dyes adsorbed onto solid substrates[154,155]. Polyanionic celluloses prepared under different conditions were used to study the effects of the nature of the anionic group and the intersite distance on the metachromasy exhibited by Methylene Blue and good agreement was found with studies on metachromatic dyes in aqueous solution of polyanions (considered in detail in the following sections).

Metachromatic dyes are still finding wide application in histological and cytological studies - for example in the examination of the role of polyamines in the expression of the differentiated phenotype of chondrocytes in culture[156], and in the identification of different blood leukocytes in normal and leukaemic patients[157].

2.9.3 Metachromasy in solution in the absence of polymer

2.9.3.1 Effect of increasing dye concentration

In a long paper dealing with the effects of environment and aggregation on the absorption spectra of dyes, Sheppard discussed the behaviour of dyes in aqueous solutions[158]. He noted the difference in spectral characteristics evident between solutions of the same dye in organic

solvents and in water and made a series of observations that were to be much quoted by later workers. These observations referred back to work on cyanine dyes carried out by Sheppard in 1906 and 1907 and included the findings that:

i) in organic solution, eg. alcohol, the absorption obeyed Beer's Law for all spectral bands;

ii) in water, Beer's Law was not obeyed, but the ratio of band intensities (extinctions) depended on the concentration; with increasing concentration the shorter wave bands gained in strength at the expense of the main wave band - this behaviour was reversible;

iii) on raising the temperature, the aqueous solution showed spectral characteristics more like those found in organic solution - this behaviour was also reversible.

Sheppard concluded that the spectral changes in water were due to a reversible molecular aggregation, whereas in organic solvents the dye was molecularly dispersed. The main bands in the dye spectra, which were also the absorption bands at longest wavelength, were termed the α bands by Sheppard, who called the subsidiary bands β and γ bands, the γ band occurring at the shortest wavelength.

From a consideration of the structures of the dyes that he studied, and from electrochemical measurements made on dye solutions of different concentration, Sheppard concluded that the most likely dye aggregate was a dimer, and that the planar dyes exhibiting dimerisation must be coordinated with a water molecule.

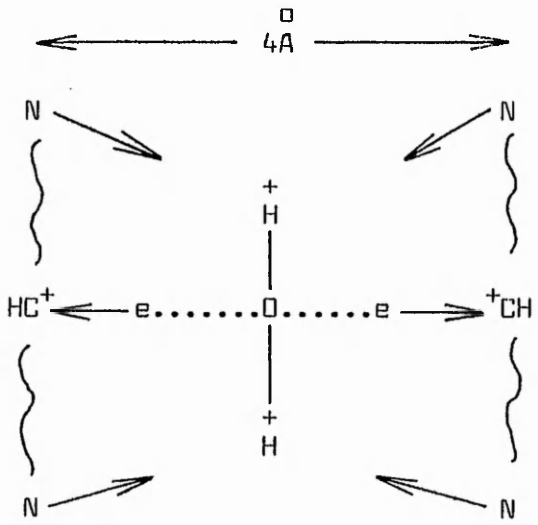


Figure 2. Suggested structure of dimer[158].

Dimer formation was thought to account for the spectral deviations observed in aqueous solution. The β and γ bands, however, were not assigned specifically to dimer or higher aggregates; rather, dimer formation enhanced the transition probability of bands produced in the molecule by coupling of the electronic transition with vibrations.

Schubert and Hamerman's review of 1956[144] collected together work on basic dyes in solution and summarised the findings and the problems. They emphasised the fact that dyes showing metachromatic behaviour do not conform to Beer's Law and brought together some of the theories put forward to account for spectral shifts. In particular they described the work of several authors who assigned the α band (also called the M band) to monomeric dye, and the β (or D) band to dimeric dye, and the γ (or μ) band to higher aggregates (with wavelength $\alpha > \beta > \gamma$) and concluded that the deviations from Beer's Law in aqueous solutions could be best interpreted by the assumption that dyes can exist in solution as monomers, dimers and higher polymers, all in

equilibrium, and that changes in dye concentration change the proportions of these molecular species in the equilibrium. Polymerisation of dyes was thought to affect loosely constrained π electrons with a consequent effect on the spectrum of the dye[144].

A series of papers by McKay and Hillson examined the behaviour of dyes in water and in solvents of different dielectric constant[159-162]. They used the terms M band (α band) and H band (lower wavelength band assigned to aggregated dye), and also described a shorter wavelength band (the L band) that appeared near the solubility limit of two of the dyes studied. From a study of the effect of increasing dye concentration in aqueous solution ('dye-induced' metachromasy) it was found that the M bands of the dye spectra diminished while the H bands increased in intensity as dye concentration was increased. The dyes studied were nearly all cationic dyes with the ionic charge delocalised over the whole chromophore. Three exceptions were noted: 9-methyl-3,3'-di(3-sulphobutyl)thiacarbocyanine iodide - an anionic dye by virtue of the sulphonate groups, but with a positive charge on the chromophore system - showed metachromatic behaviour; Solway Ultra Blue B (C.I. 62055, an anthraquinone dye) and Janus Yellow G (C.I. 26060, an azo dye) did not show metachromatic behaviour over the concentration range 10^{-6} to 10^{-3}M. In these last two dyes the ionic charge is not part of the chromophoric system[159].

The effect of increasing dye concentration was also studied in a series of solvents and solvent mixtures of differing dielectric constant, where it was found that the lower the dielectric constant, the greater the metachromasy induced[160]. This finding was taken as further support for the view that the metachromatic behaviour of dyes

should be attributed to interaction between dye and ions and counter-ions advanced in an earlier paper[159]. However, this view was largely withdrawn when solvents of high dielectric constant, including water, were considered[162]. Spectral data for two metachromatic dyes dissolved in water (1,1'-diethyl-2,2'-carbocyanine bromide and Methylene Blue) showed marked metachromasy, which was not shown by dye solutions prepared in the other solvents examined, with the exception of glycerol, in which metachromasy was much less marked than in water. The tendency of water molecules, and to a lesser extent polyhydric alcohols, to form hydrogen bonds confers a high degree of structural order, and the metachromasy of dyes in water was attributed largely to this property.

The L band of Hillson and McKay[159] was termed the H band by Padday[143], who reviewed studies of dye concentration metachromasia and examined the ways in which the absorption spectra of metachromatic dyes have been analysed by various authors to provide quantitative confirmation of the aggregation theory of metachromasy. Sculthorpe[163] reiterated that metachromatic colour change is only shown by dyes in which the ionic charge is an integral part of the chromophore system, and that dyes showing concentration metachromasia all failed to obey Beer's Law.

2.9.3.2 Effect of addition of electrolytes

Both Sheppard[158] and Michaelis[150] noted that metachromatic changes could be induced in dilute dye spectra by addition of neutral salts, when colloidal particles of dye which remained in relatively stable solutions showed a γ adsorption band. Michaelis and Granick[149] stated that the degree of this effect was dependent upon the conc-

entration of the salt while McKay and Hillson[159] found that addition of salt was relatively less effective than addition of further dye. The effect was also dependent upon the valency of the cation - barium and calcium chlorides produced changes similar in magnitude to those produced by sodium chloride at half the molar concentration. The concentration of dye at which the salts were added also affected the results. In apparent ignorance of this previous work Wollin and Jaques claimed[164], as late as 1973, to have made the first observation of metachromasy induced by an electrolyte - in this case 3M NaCl.

2.9.3.3 Reversal of metachromasy in the absence of polymer

On raising the temperature of an aqueous solution of a metachromatic dye, the spectrum of the dye becomes similar to that of the same dye dissolved in an organic solvent - that is, the α band increases in intensity, and the β and γ bands decrease in intensity or disappear. This effect is wholly reversible[158]. Padday[143] felt that the effect of raising the temperature of a solution of dye was to hinder the formation of dye aggregates and to favour the presence of monomer, and was able to base thermodynamic calculations on the temperature dependence of the equilibrium between monomer and aggregated dye.

The metachromasy of aqueous solutions of Methylene Blue was shown to be reduced by the addition of large quantities of sucrose[162]. The increase in intensity of the M band (α band) was accompanied by a slight bathochromic shift, which was attributed to the increase in the refractive index of the solution containing sucrose over the aqueous solution.

2.9.4 Metachromasy in the presence of natural anionic polymers

2.9.4.1 Introduction

The study of the metachromasy shown by some basic dyes with several natural polyanions followed naturally from the work of histologists as an attempt to explain and quantify the phenomenon. In spite of many years of investigation the mechanism of metachromasy has yet to be explained to the complete satisfaction of all workers; a wide range of natural and synthetic polyelectrolytes have now been investigated, and several applications of metachromasy in solution, and of the related characteristics exhibited by fluorescent metachromatic dyes have been found. Virtually all work has concentrated on cationic (basic) dyes, while the polymers or other chromotropes inducing the metachromasy are almost entirely anionic. Indeed, Schubert and Hamerman[144] expressed their regret at the absence of polycation - anionic dye metachromasy and reflected on the potential that such a system would have as a research technique.

2.9.4.2 The influence of the polyanion

In 1956, Schubert and Hamerman[144] stated that 'all known chromotropes are anionic, or negatively charged, in aqueous solution, and in addition, are of high molecular weight, or if of low molecular weight are capable of associating into aggregates of high molecular weight'. The list of examples that they gave included among animal products - heparin, chondroitin sulphate, hyaluronate and nucleates, and among plant products - agar and alginate. An extensive summary of work on isolated systems of metachromatic dye and chromotrope in solution is given and assessed. Among the work mentioned is that of Lison[165], and of Bank

and Bungenberg de Jong[166]. Lison [165] found no evidence for a stoichiometric relationship between dye and chromotrope and proposed that the dye existed in solution in two tautomeric forms, the equilibrium between the two being displaced sharply in favour of the metachromatic form. Bank and Bungenberg de Jong[166] were able to show that the charge density of the chromotrope was important in inducing metachromatic colour and also found that the character of the anoinic group determined the extent of chromotropic action, in the order sulphate > phosphate > carboxylate. This work was largely carried out by visual comparison against a set of arbitrary standards in which agar was the chromotrope. Spectrophotometric studies were carried out on agar and nucleic acids by Michaelis and Granick who found that metachromasy could be induced by agar, but not with nucleic acid under the conditions of their experiments (pH 4.6, 3% solution of nucleic acid). They inferred from their results that a salt-like compound was formed from nucleic acid and basic dyestuffs in stoichiometric amounts, whereas with agar a reversible polymerisation of dye molecules took place, the dye aggregate subsequently being adsorbed onto the agar. Levine and Schubert[167] studied solutions of hyaluronate, chondroitin sulphate, heparin and alginate with Methylene Blue and Crystal Violet. All of the polysaccharides behaved as chromotropes for the dyes and induced metachromatic behaviour. However, as the concentration of the chromotrope was increased, metachromasy began to disappear from the solutions. The results of dialysis experiments led the authors to believe that dye binding was not suppressed by increasing chromotrope concentration. Comparison with similar studies carried out on detergents[167] suggested that micelle formation by the chromotropes could be involved in metachromasy.

The interactions of Toluidine Blue with a range of natural polymers and derivatives were studied by Walton and Ricketts[168] with the aim of improving the histological use of the dye. The polysaccharide samples used varied in the extent of their polymerisation, in the number and nature of the attached acidic groups and in the nature of their component saccharide units. In particular a series of dextran derivatives were prepared having differing extents of polymerisation, from which dextran sulphates were prepared. It was found that, over a threshold of four glucose units, metachromatic activity was not dependent on degree of polymerisation of the dextran sulphates, but was linearly dependent upon degree of sulphation. When the metachromatic activities of carboxymethyl dextrans were compared with a heparin standard, the activity was found to be several hundred times less than the heparin at pH 2.04. At pH 7.3 the activity of the carboxylated sample had doubled but was still at least one hundred times less than that of heparin at the same pH. The stoichiometry of interaction with the dextran sulphates was also estimated and found to give good agreement with a model assuming one dye molecule per sulphate group[168]. The metachromatic activity of carboxylic groups was also considered by Szirmai and Balazs[153], who used the sodium or potassium salts of various naturally occurring polysaccharides, and found that at pH 6.7 there was a 1:1 stoichiometry between anionic sites per disaccharide unit and the number of molecules of dye bound per disaccharide unit. The relative participation of carboxyl and sulphate groups was further examined by varying the pH of the solutions. At pH 3 binding by carboxyl groups was suppressed while sulphate ester groups remained active. Unlike Walton and Ricketts, who measured the unbound dye remaining in solution after the metachromatic complex had been extracted

by organic solvent[168], Szirmai and Balazs[153] measured the spectra of solutions containing free and bound dye. These workers also found that at the point at which all anionic sites were saturated with dye, the polysaccharide/dye complex precipitated and could be removed from solution by centrifugation. Their data suggested that the supernatant solution showed either the spectrum of the free dye, with the α band reduced in intensity to some degree, or the spectrum of the metachromatic complex, but not any of the transitional spectra recorded for the same solutions prior to centrifugation. The dye in this case was Azure A. By contrast Pal and Schubert[169], who also removed the metachromatic complex formed from Methylene Blue and chondroitin sulphate from solution by centrifugation, found that the supernatant solution showed the orthochromatic spectrum with a single peak at 665nm. Subtraction of this spectrum from that of the solution before centrifugation gave a difference spectrum assigned to the metachromatic complex, and having a single peak at 570nm. Direct analysis of the sediments showed them to contain polyanion/dye complex at a polymer:dye equivalent sites ratio (P/D) of 1:1, even though the original solutions had P/D values of up to 20:1. The authors suggested that in the presence of excess polyelectrolyte the dye cations were not uniformly distributed, but saturated some polymer molecules preferentially, leaving the rest almost free of dye cations.

Similar findings were reported by the same authors[170] on using a different method of extraction of the metachromatic complex. Solutions of Methylene Blue and Crystal Violet were allowed to interact with chondroitin sulphate to form the metachromatic complex, which was then adsorbed by one of the three insoluble calcium salts which had been shown to adsorb neither free dye nor chromotrope. The salts were

calcium hydrogen phosphate ($CaHPO_4$), calcium carbonate ($CaCO_3$) and calcium oxalate (CaC_2O_4).

The metachromasy shown by another dye, Acridine Orange, was examined in relation to samples of native and denatured nucleic acids, polyadenylic acid, polyuridylic acid and heparin, and again stoichiometric complexing was observed[171,172]. The techniques developed were later applied in the classification of sulphated plant polysaccharides on the basis of their ability to act as chromotropes[173]. The properties of Acridine Orange and Methylene Blue were later compared on the basis of quantitative spectral measurements and found to show marked differences in their sensitivity to chromotrope average intersite distance[174]. The spectral parameters used were total band intensity (oscillator strength) and intensity weighted average frequency of the band (centre of gravity), and were considered to give separate measures of the two aspects of metachromasy shown by these dyes - hypochromism (reduction of intensity of the main absorbance band) and frequency shift (in this case, and generally, hypsochromic - to shorter wavelengths). The measurements were carried out on dye/chromotrope complexes in solution at a P/D ratio of 1:1. Whereas the change in centre of gravity for Methylene Blue showed a marked dependence on the intersite distance of the acidic sites on the polymer, Acridine Orange showed only minimal dependence. The dyes also varied in the extent to which their metachromasy was affected by the strength of the acidic sites.

An example of a dye showing metachromatic activity with a bathochromic shift (shift to longer wavelength) was cited by Bean, Shepherd, Kay and Walwick[175], who examined the spectra produced by 4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine (DBTC) with several different polyionic macromolecules, and reported spectra showing seven

different complex band maxima. Their notation appears to differ considerably from that of previous authors, but examination of the spectra shown in the text suggests that different polyionic macromolecules induce metachromatic behaviour to differing extents. Variations in pH were found not to affect the stoichiometry of the complex reactions, even when it was expected that the ionization of carboxyl groups would vary considerably.

The interaction of Methylene Blue with polyanions has been studied by the technique of pulse radiolysis[176]. Hydrated electrons (e^-_{aq}) are generated by pulse radiolysis of the solutions, and the disappearance of e^-_{aq} can be followed as a function of time. Whereas free Methylene Blue reacted extremely rapidly with the hydrated electrons, the rate of reaction dropped to the rate of reaction for heparin when the Methylene Blue was in solution in the presence of the polymer. Pulse radiolysis measurements were compared with spectral measurements for the same solutions at the same temperature, and on the basis of these comparisons it was deduced that aggregation rather than ion-binding was largely responsible for metachromasy. Increase in temperature led to liberation of free monomeric dye and hindered formation of the complex, the amount of dye released at any given temperature being dependent on the nature of the polyanion.

2.9.4.3 Reversal of metachromasy shown by natural organic polyanions

Many authors have studied the reversible nature of metachromasy in solution in attempts to quantify the 'strength' of the chromotrope, and to investigate the mechanism of the metachromatic interaction. Studies have fallen into four broad groups, two of which are similar to those examined for dye systems in the absence of polymer - the

effects of temperature, additional cations, solvent system, and additional polymer.

a) The effect of temperature

Many authors have implicitly recognised the importance of temperature by reporting studies carried out at fixed temperature. Only two direct references to this effect were found however; when Moore, Phillips and Power[176] noted that the nature of the polyanion affected the amount of the free dye released into the system on raising the temperature, and when the reversal of metachromasy by raising the temperature was shown to be completely reversible[177].

b) The effect of additional cations

In Section 2.9.3.2 the effect of additional electrolyte on the aggregation of metachromatic dyes in solution, in the absence of polymer, was described. In contrast to the promotion of aggregation found under these conditions, the metachromasy induced by polyanions is suppressed by the addition of electrolytes. Levine and Schubert[167] reported dialysis experiments showing that potassium chloride (0.0005M) reduced Methylene Blue - chondroitin sulphate metachromasy considerably and that 0.01M KCl suppressed the concentrating effect of dialysis completely. From these results, they inferred that the dye-binding process was disrupted, with consequent disruption of metachromasy. Bank and Bungenberg de Jong[166] had also shown that the addition of neutral salts reversed the production of metachromatic colour by chromotropes and that the effect was more pronounced the higher the charge on the cation of the salt. Bradley and Felsenfeld[172] reported the sensitivity of Acridine Orange - deoxyribonucleic acid metachromasy

to the addition of salts and Davies et al.[178] described the dissociation of dye-polymer complexes at high ionic strength. Pulse radiolysis studies have also been used to follow the change in the ratio of complexed dye to free dye that takes place when sodium chloride is added to heparin-Methylene Blue solutions[177]. When 0.05M NaCl was introduced to a solution of P/D ratio 100:1 the rate of disappearance of e^-_{aq} increased to a rate approaching that for dye alone, and equalled that for dye alone with 0.1M NaCl. Davies et al.[178] continued the pulse radiolysis investigation by measuring the relative binding strengths for different types of anionic site for polyanionic glycosaminoglycans including de-N-sulphated heparin, chondroitin-4-sulphate, chondroitin-6-sulphate, sulphated hyaluronic acid (all potassium salts), sodium alginate and sodium dextran sulphate. The concentration of potassium chloride required to achieve complete release of Methylene Blue from the polyanions varied with the nature of the binding sites present and to a lesser degree with P/D ratio, and was termed the limiting salt concentration, by analogy with the critical electrolyte concentration. The effect of adding potassium chloride to polyanion-cetylpyridinium chloride (CPC) solutions was also measured by pulse radiolysis, and it was shown that CPC was released from the complex; binding affinities for CPC were not, however, found to be the same as those for Methylene Blue, although the order was similar. Binding affinity of the anionic sites (P/D = 1:1) was found to increase in the order:

$$CO_2^- < CO_2^- + O.SO_3^- < CO_2^- + O.SO_3^- + NH.SO_3^- < O.SO_3^-$$

Similar limiting salt concentrations were found for complexes with P/D ratios of 10:1 and 100:1, suggesting that the strength of interaction

was more closely associated with the nature of the anionic site than with the concentration of the counter ion. Cetyl pyridinium chloride was also used by Pal and Biswas[179] who were able to show that CPC displaced dye ions almost stoichiometrically from complexes with heparin or chondroitin sulphate. The dyes used were Methylene Blue and Crystal Violet, and the metachromatic complexes were removed from solution by extraction with carbon tetrachloride, after which the unbound dye remaining in solution was measured spectrophotometrically. It was emphasised that this result contrasts with the non-stoichiometric finds for simple electrolytes.

c) The effects of different solvents

Bank and Bungenberg de Jong[166] found that the addition of alcohol to metachromatic solutions reversed metachromasy (reported by Schubert and Hamerman[144]). They also found that the extent of reversal was greater the longer the chain length of the alcohol. Later, the reversal of metachromasy by ethanol was used by Pal and Schubert[180] to produce a scale of stabilities of twelve metachromatic compounds from different combinations of dyes and polyanions. Urea was also used by these workers to reverse metachromasy, and equivalent results were obtained. The measure used was the concentration of ethanol (or urea) required to increase the α absorbance band to half of the value at which all metachromatic activity was destroyed. It became clear that both dyes and chromotropes differed in their metachromatic strength. For any particular chromotrope, the order of metachromatic strength of the dyes was Toluidine Blue > Acridine Orange > Methylene Blue > Crystal Violet. When the strongest dyes and chromotropes were combined, complete destruction of metachromasia was not achieved even

at the highest ethanol concentration used (60% by volume or about 10M). Above such ethanol concentrations precipitation of chromotropes was sometimes observed. The extent of reversal of metachromasy determined the colour of the precipitate - if reversal was complete before precipitation began, the precipitate was colourless, otherwise the precipitate showed the metachromatic colour. Pal[181] extended the study of the effects of ethanol to other alcohols - methanol, propan-2-ol and 2-methylpropan-2-ol, and found that the suppression of metachromasy for the Acridine Orange/λ-carageenan system correlated with increasing hydrophobicity of the alcohols. Dioxane (10-50% v/v) was also used as a solvent in a comparison of the metachromatic and conductimetric effects of the addition of organic solvents to Methylene Blue or Crystal Violet complexes with potassium chondroitin sulphate or heparin[182]. Crystal Violet/chondroitin sulphate complexes were found to be more sensitive to organic solvents than Methylene Blue/chondroitin sulphate complexes when judged spectrophotometrically, but conductimetric measurements were interpreted as showing, for example, that 75% bound dye was still present when the spectrum indicated that metachromasy had been reduced to 16% (8M urea). Pulse radiolysis studies also showed that the addition of organic solvent can suppress metachromasy - the rate of decay of e^-_{aq} increased when methanol was introduced to the system Methylene Blue/heparin[177].

d) Addition of excess polymer

The importance of the ratio of chromotrope concentration to dye concentration, and in particular of equivalent anionic site concentration to dye concentration has been reviewed by Schubert and Hamerman[144], who describe the changes observed in the spectrum on adding increasing

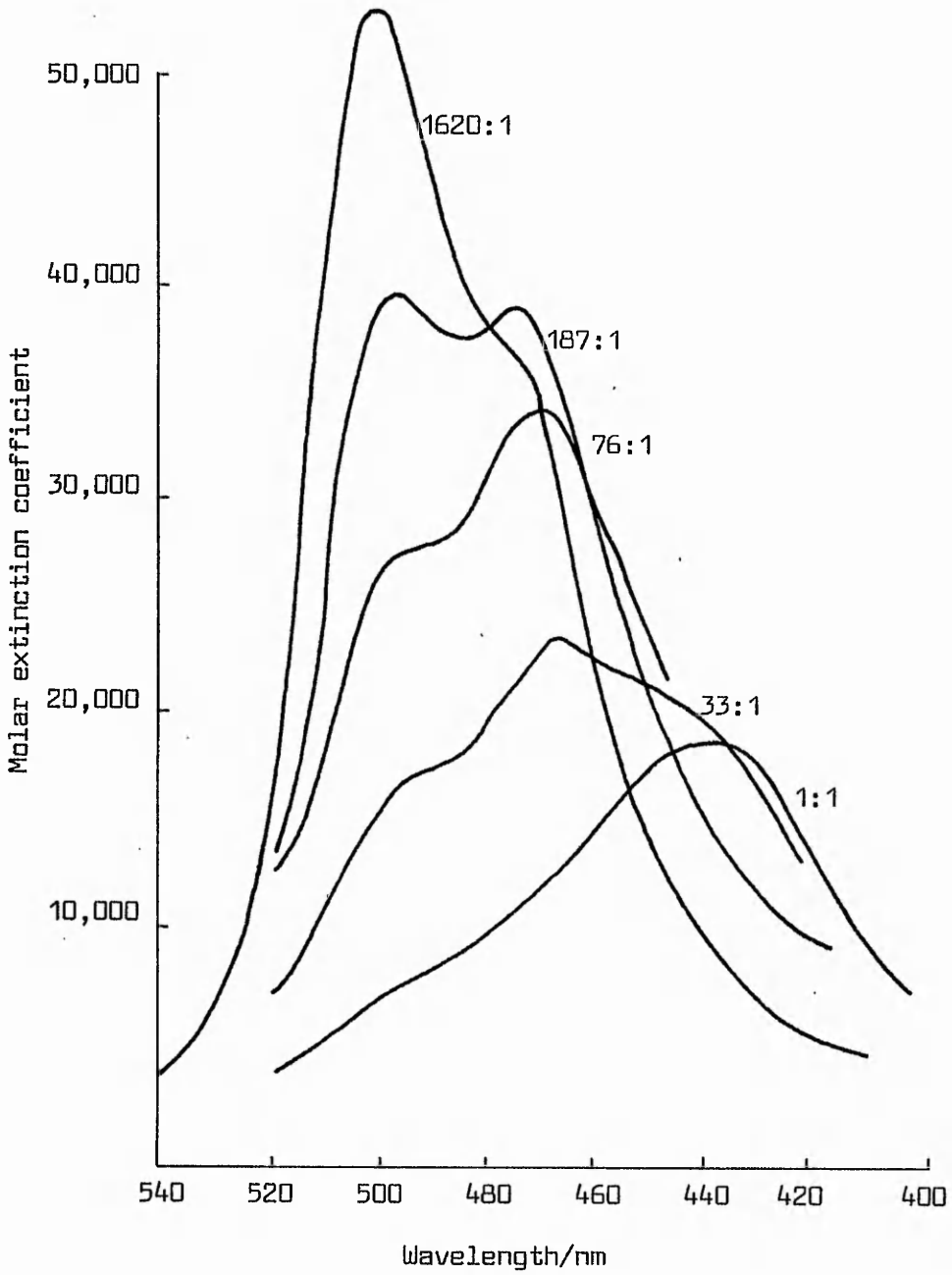


Figure 3. Example of reversal of Acridine Orange metachromasy by excess polymer[171]. Polymer - polyuridylic acid.

amounts of chromotrope to a solution of a metachromatic dye. At a fixed dye concentration, increasing chromtrope concentration generally produces a decrease in the α and β bands (for Azure A, Methylene Blue and Crystal Violet) and a rise in the extinction coefficient of the μ or γ band. This change continues until equivalence of chromotrope and dye is reached and the full metachromatic colour is seen. Further increase in chromotrope concentration reverses the changes in the absorbance bands; the μ or γ band intensity falls while the α and β band intensities increase to, or sometimes rather higher than their values in the absence of chromotrope. The failure of authors working with agar as the chromotrope to notice the sequential changes in absorption bands with increasing concentration of chromotrope was attributed to the formation of stiff gels by the agar as the concentration is increased, making the amount of chromotrope in true solution difficult to estimate, and probably not high[144]. Levine and Schubert used a purified agar sample and were able to show the onset of reversal of metachromasy[167]. Other chromotropes were also used in the same study, and the results of equilibrium dialysis experiments suggested that dye binding was not suppressed by chromotrope concentration increase, even when metachromasy had disappeared.

Bradley et al.[171,172] made a series of observations on the spectra produced by a number of chromotropes with Acridine Orange, and as a result introduced the stacking coefficient (K), which is a measure of the ease with which a particular chromotrope can reverse metachromasy when its concentration is raised to provide excess binding sites. When K=1, association of dye with polymer sites is random; ie. there is no promotion of stacking by the polymer. The P/D ratio required to produce a given degree of unstacking - or disassociation of dye molecules in the presence of the polyanion - was found to be charact-

eristic for each polyanion with a given dye. For DNA's, complete unstacking was observed at approximately P/D = 100. From a knowledge of the nature of the polymer in solution it was suggested that a high stacking coefficient corresponds to a flexible arrangement of binding sites, while a low stacking coefficient corresponds to a rigid arrangement, as found in a multistranded helix. Support for this idea came from results found for polyadenylic acid which was thought to undergo transition from a single stranded coil above pH 7 to a rigid, two stranded helix below pH 5. The stacking coefficient was found to be high at pH 7 but low at pH 5. Observations were later made on a series of native and denatured deoxyribonucleic acids[172,183] when it was found that the stacking tendency of native DNA is quite low (K approaching 1), but increases on denaturing. It was suggested that measurement of the stacking tendency could form the basis of a technique for assessing the extent of denaturation of DNA samples, both in solution and in tissue section[172]. These results were again related to the transition from a rigid helical strand to a structure of greater flexibility[181]. Stacking coefficients for native DNA's were found to be almost identical but a variation was found with denatured samples. When a variety of chromotropes was considered, a wide range of stacking coefficients was found for Acridine Orange, with DNA exhibiting the lowest value, and heparin and polyphosphate having values approximately 800 times higher. The same principle - the measurement of stacking tendency, or ease of reversal of metachromasy by increasing P/D ratio - was used to characterise plant sulphated polysaccharides[173].

In the course of their studies on the ultracentrifugal separation of Methylene Blue/chondroitin sulphate complexes, Pal and Schubert noted that the sediment deposited from metachromatic solutions at

high centrifugal fields (50,000 x g) always contained polymer and dye at a P/D ratio of 1:1, even when the P/D ratio of the original solution was as high as 20:1[169]. This finding was described as demonstrating the preferential saturation of some of the polyelectrolyte molecules while others remained almost free of dye cations. Pal and Schubert also used the absorbance of the metachromatic compound by insoluble calcium salts to study the effects of polyelectrolyte excess, and found that the amount of the metachromatic compound removed rose rapidly with chondroitin sulphate concentration to reach a plateau that was maintained up to a P/D ratio of 10:1[170]. At higher P/D ratios the amount of metachromatic compound removed began to fall. Centrifugation and extraction with trichloromethane were also used to estimate the amount of metachromatic compound removed, and gave similar results up to P/D = 10:1; at higher P/D values the curves deviated from each other. These results were, however, all subject to the major qualification that the presence of salt was necessary for sedimentation and for absorption of the metachromatic compound by the calcium salts.

In the pulse radiolysis studies carried out on Methylene Blue/heparin complexes[176,177], it was noted that the decay of e^-_{aq} was at a minimum at a P/D ratio of 100:1[177] but examination of a text figure suggests that the rate of decay was at a minimum at a P/D ratio of approximately 10:1. It is interesting that the amount of free dye estimated in the solution should be greater from spectral measurements than from radiolysis measurements, and that spectral measurements indicate that free Methylene Blue is at a minimum at a P/D ratio approaching 10:1 rather than 1:1, with a steady increase thereafter. The increase shown by spectral measurements is accompanied by a sharp

decrease followed by a steady increase in the amount of free dye estimated by radiolysis. The authors describe this finding as unequivocal evidence that the dye is bound at the anionic site[176,177].

2.9.5 Metachromasy in the presence of synthetic anionic polymers

Synthetic anionic polymers have been used to examine the mechanism of the metachromasy shown by Methylene Blue[184]. The effect of the structures of poly(vinyl sulphate) and its homologues on Methylene Blue metachromasy have shown that anionic site density and the flexibility of the polymer chain are important factors. The P/D range over which the α band absorbance was at a minimum varied among the polyanions examined, from 2-56 for a polymer of DP 1500 with 100% sulphation, to 1-7 for a polymer of DP 150 with 100% sulphation. The efficiency of aggregation of Methylene Blue also decreased with decrease in the degree of sulphation whilst the least flexible polymers also showed decreased efficiency in promoting aggregation. Another series of synthetic polymers having closely related structures was also studied for their effects on Methylene Blue metachromasy[185]. Again the variation of the intensity of the α band with P/D ratio was measured and found to vary with the nature of the polymer. For poly(sodium acrylate), the α band intensity reached a minimum at a P/D ratio of approximately 18:1, and apparently for none of the polymers was the minimum α band intensity reached below P/D = 8.0:1. The charge density and flexibility of the polymers were thought to be important here also but definite conclusions were not drawn. The metachromasy of Methylene Blue/poly(vinylphenol) in solutions of sodium hydroxide, chosen to give different degrees of dissociation of the phenolate anion residues, was investigated by Shirai, Nagaoka and Tanaka[186]. At each NaOH

concentration, the ratio of phenolate anion to dye for which the α band intensity was at a minimum was approximately 3. The metachromasy induced by poly(vinylphenol) was not destroyed appreciably by addition of sodium chloride, but was destroyed by addition of 4.8M urea.

The cyanine dye pinacyanol chloride and its interaction with synthetic polyanions was described by Pal and Ghosh, who drew parallels with findings for natural polymers[187]. Again the polymer conformation was felt to be of great importance in determining the metachromasy observed. Only poly(acrylate) and poly(vinyl sulphate) complexes showed 1:1 stoichiometry, whereas poly(methacrylic acid) and poly(styrene sulphonate) showed 2:1 stoichiometry. Poly(acrylate), poly(vinyl sulphate) and poly(methacrylate) (the latter only at P/D >3) had broad and multiple-banded spectra, whereas the spectra were sharp and single banded for poly(methacrylic acid) and poly(styrene sulphonate). The authors postulated that multiple-banded spectra were caused by chaotic aggregation of the dye due to its large size, when 'overcrowded' at a 1:1 stoichiometry as the random coil of the polymer became more compact with the reduction of repulsive electrostatic forces. Conversely, binding at alternate anionic sites (2:1 stoichiometry) allowed more regular aggregation of the bound dyes[187].

Shirai *et al.*[188] found that the metachromasy induced in Methylene Blue by poly(vinyl sulphate) and poly(p-styrene sulphonate) was destroyed by alkali metal chlorides, and by 1-alkyl-3-carbamoyl pyridinium bromides to an extent dependent on the size of the alkali metal cations and the hydrophobicity of the 1-alkyl group. The concentration of alkali metal cation required to destroy metachromasy decreased in the order $Li^+ > Na^+ > K^+ > Cs^+$, and in the order

$CH_3CH_2OH = CH_3(CH_2)_3OH > CH_3(CH_2)_7OH > CH_3(CH_2)_{11}OH$. Low dye concentrations were found to enhance the metachromasy shown by poly (p-styrene sulphonate).

A technique of spectral analysis, termed the extended principal component analysis method, has been applied to metachromatic dye/polyanion systems, and once again the conformation of the polymer and the nature of the anionic site have been found to be of great importance in determining the extent of metachromasy[189,190]. The dyes used, Crystal Violet and trypaflavine, were found to have metachromatic bands in their spectra at both shorter and longer wavelength than the peak wavelength for the free dye, when the spectra were analysed by the principal component method[189]. In addition, the analysis showed the presence of only one dye species, other than free dye, at $P/D \leq 1$, which was attributed to bound dye[189]. The fraction of this bound dye species was shown to decrease with increasing concentration of sodium chloride, even though the analysed spectra remained unchanged[190]. The principal component analysis method was also used to assess the performance of spin-labelled acridine dyes in producing metachromasy with polyelectrolytes, in comparison with their non-labelled counterparts, when the labelled dyes were shown to produce similar spectra to the unlabelled dyes[191].

2.9.6 Metachromasy in the presence of inorganic anions and detergents

The most frequently reported inorganic chromotropes are polyphosphates, although Schubert and Hamerman also mention silicates[144], and Ghosal et al. described dye aggregation on clay surfaces in very dilute suspension[192]. Wiame found that Toluidine Blue metachromasy was induced by sodium hexametaphosphate, but not by ortho-, pyro- or

triphosphate, nor by adenosine triphosphate[193]. Sodium trimetaphosphate gave a very slight metachromatic reaction attributed to contamination by hexametaphosphate. On the basis of E530nm/E630nm (the ratio of the molar extinction coefficient of the metachromatic band to that of the orthochromatic band) metachromasy was found to be strongest at a P/D ratio of 8:1, and decreased at very low and high concentrations. Pal and Biswas examined the possibility that small molecules might act as chromotropes, and concluded that the chromotrope need not be a macromolecule to induce metachromasia in a dye solution, and that the minimum number of charges per polyanion necessary to give it a chromotropic character varied with the nature of the polyanion[194]. Takatsuki and Yamaoka measured the absorption spectra of Crystal Violet and Trypaflavine in the presence of three sodium polyphosphates of different chain lengths[195]. The amount of bound dye was shown to increase with increasing chain length at a given P/D, although at low P/D values the spectra of the bound dyes were independent of chain length. These results were obtained by applying the method of extended principal component analysis. The effect of sodium chloride on sodium polyphosphate/dye complexes was somewhat similar, in that the analysis method showed that the fraction of bound dye decreased on addition of electrolyte, although the spectra remained unchanged[190].

Interest in detergent-induced metachromasy has centred around micelle formation, with the implications that this might have on general theories of metachromasy. Schubert and Hamerman list myristate, Duponol and phospholipids among the detergents studied[144], and Levine and Schubert showed that the two detergents myristate and Duponol induce metachromatic behaviour that is very similar to

that for anionic polysaccharides[167], with increase in intensity of metachromatic bands followed by a decrease in intensity as the chromotrope concentration continued to rise. Dialysis experiments suggested that at high chromotrope concentration, dye binding was still present when metachromasy was suppressed and the authors suggested that micelle formation could be important in detergent and polysaccharide solutions.

2.9.7 Metachromasy in the presence of polycations

In their review of 1956, Schubert and Hamerman state that 'All known chromotropes are anionic'[144]. However a few reports of polycationic chromotropes have been made, and again they have arisen from the interests of histologists in staining tissues of differing biochemical nature. Schubert and Hamerman also pointed out that a metachromatic system of an anionic dye and a cationic chromotrope would be of considerable interest as it would allow comparison of dyes which did and did not conform to Beer's Law. The elucidation of the mechanisms of vital dye staining of plasma led Gregerson and Gibson to examine the spectra of several anionic dyes in solution, in the presence and absence of sodium chloride or plasma (or serum)[196]. In some cases, for example a dye designated T.1824 and Niagara Sky Blue, the spectra appear to show hypo- and bathochromic shifts in the presence of plasma, but hypsochromic shifts in the presence of sodium chloride. Rawson[197] also used T.1824 and Niagara Sky Blue and again spectral shifts in the presence of plasma protein (in this case albumin) were hypo- and bathochromic, as were those for Trypan Blue. Kelly discussed these changes as non-metachromatic, as his definition of metachromasy required a hypsochromic shift, and described Congo Red as showing a

metachromatic shift in the presence of protamine sulphate[198]. Congo Red was known to aggregate in solution, but to obey Beer's Law, in contrast to a few anionic dyes related to Eosin which deviated from the Law. The spectral shift of Eosin with protamine was not thought to be metachromatic by Kelly, although the text figure indicates the development of a secondary absorbance band at shorter wavelengths than the main band[198]. Mataga and Koizumi[138] referred to spectral changes shown by Eosin - this time with chitosan hydrochloride as chromotrope - as being non-metachromatic, even though the spectrum showed a hypochromic and bathochromic shift, followed by some recovery towards the free dye spectrum on increasing the concentration of chromotrope. No similar change was observed when low molecular weight amine compounds - D-glucosamine hydrochloride or methylamino hydrochloride - were added to the dye solution. Erythrosin gave comparable results to Eosin, but Uranine showed marked spectral changes (hypo- and hypsochromic) with both high and low molecular weight compounds, with no recovery observed at the concentrations used[138].

One more recent report has suggested that metachromatic spectral changes may take place when solid substrates (cellulose polycations) are treated with anionic dyes[199].

2.9.8 Heteroaggregation

Pal and Schubert[200], and Pal, Ash and Ghosh[201] have reported an interesting phenomenon occurring when pairs of cationic dyes are in solution with one chromotrope. In the first study[200], the spectrum of each dye/chromotrope solution was prepared, as was the spectrum for the chromotrope in the presence of both dyes. By subtracting the

individual dye/chromotrope spectra from the spectrum of the mixture, Pal and Schubert were able to distinguish two types of metachromasy. In the first, the difference spectrum was almost zero, and showed that the solution contained a mixture of the two metachromatic complexes, while in the second, the residual spectrum showed high positive and deep negative peaks, suggesting that the two dyes had formed a mixed aggregate with a unique spectrum. The first condition was termed 'simple metachromasy' and the second 'compound metachromasy'. In compound metachromasy, the spectrum showed enhanced absorbance close to the μ-band of the dye whose μ-band was at shorter wavelength, while the absorbance was sharply decreased close to the μ-band at longer wavelength[200].

The chromotropes used in the second study were the synthetic polyanions poly(potassium styrene sulphonate) and poly(potassium vinyl sulphate) with the cyanine dyes pinacyanol and thiacarbocyanine[201]. In addition the spectrum of Methylene Blue and Acridine Orange in the presence of mercuric chloride was also examined. In each case, heteroaggregation was shown to occur, with the enhancement of the metachromatic band of the partner dye having λ max at relatively shorter wavelength, at the expense of the absorbance at the metachromatic band of the other dye[201].

2.9.9 The importance of water

That water plays a vital part in the metachromatic interaction of dyes and chromotropes has been recognised for many years; the exact nature of the role of water has been the subject of a great deal of conjecture. Many workers have observed that dehydration of histological specimens leads to loss of the metachromatic colour. Schubert and Hamerman[144] have reviewed this aspect of metachromasy and described

the early distinction made between 'true' and 'false' metachromasy, that is metachromatic staining that was stable to, or removed by, treatment with alcohol respectively. Critics of such a distinction pointed out that the extent of dehydration of tissue sections with alcohol was very variable, and highly dependent on individual technique[144]. Szirmai and Balazs noted the difficulties inherent in making quantitative assessments of the metachromatic reaction after fixation or dehydration of tissues, as for example on sections dehydrated with methyl benzoate and mounted in Canada Balsam, when the Azure A absorption maximum induced by rooster cartilage and comb material moved to longer wavelengths[153].

Padday[143] and Sheppard[158] both considered the fact that metachromatic dyes obey Beer's Law in organic solvents, and deviate from the Law in aqueous solution to be of great importance and to hold particular implications for the nature of dye aggregation in the metachromatic complex. McKay and Hillson studied the spectra of two metachromatic dyes in a variety of solvents of high dielectric constant, and were able to show that metachromasy was exhibited only in water and to a much smaller extent in glycerol solutions[162].

Recently, Lawton and Phillips have studied the effect of water on the metachromatic complexes of Methylene Blue/insoluble cellulosic polyanions by diffuse reflectance spectroscopy[202]. They were able to demonstrate that complete dehydration caused the complexes to become orthochromatic, and that rehumidification reversed the change. Under anhydrous conditions different types of cellulosic polyanions behaved similarly, suggesting that the type of site was not important, but that in the presence of water metachromatic behaviour decreased in the order:

sulphate > phosphate > carboxyl > carboxymethyl

for cellulosic polyanions whose site spacing was similar[202].

2.9.10 Optical properties related to metachromasy

Two major optical phenomena have been studied in conjunction with metachromasy - they are the variations in fluorescent emission exhibited by fluorescent metachromatic dyes, and the anomalous optical rotatory dispersion shown by some metachromatic complexes.

2.9.10.1 Fluorescence

In 1953, Koizumi and Mataga reported that the fluorescence intensity of Rhodamine 6G varied in a similar manner to the absorption intensity of the monomer band of the dye[203]. The chromotrope used was potassium poly(vinyl sulphate). A later study in the same series used a range of sodium and potassium alkyl sulphate esters of different chain length together with several fluorescent dyes[204]. Methyl and butyl sulphate had no effect on either the absorption or fluorescence spectra of the dyes, and amyl sulphate showed only a slight effect at high concentration. In the case of octyl sulphate, quenching of fluorescence occurred, accompanied by changes in the absorption spectrum; when the critical micelle concentration (CMC) was reached, the fluorescence recovered and the absorption spectrum became similar to that of the aqueous dye solution. Sodium lauryl sulphate showed similar effects to those of the octyl sulphate, but recovery of fluorescence began at a considerably lower concentration than the CMC. Changes in the fluorescence spectra of Eosin and Erythrosin were also noted when chitosan hydrochloride or dodecylamine hydrochloride were used as the chromotrope, and were thought to be analogous

to those observed for the basic dyes[138].

Pal used dilute aqueous solutions of the fluorescent dyes Acridine Orange and Rhodamine 6G, both of which become more fluorescent when urea or ethanol is added to the solution, and examined the effects of various polyanions on the fluorescence of the solutions[181]. He considered Rhodamine 6G to be only very weakly metachromatic, but observed that quenching and subsequent reappearance of fluorescence was found with both dyes. Experiments with addition of solvents of increasingly hydrophobic nature led Pal to conclude that micelle formation was implicated in the quenching of fluorescence, and in metachromasy[181]. The application of the fluorescence quenching of Acridine Orange in the presence of a polyanion in a quantitative assay for carrageenans was advocated by Cundall, Phillips and Rowlands[205,206]. They exploited the strong binding of Acridine Orange to polyanions and the fact that association with the polyanion induces a shift to red wavelengths of the green fluorescence shown by the unbound dye. The fluorescence intensity of the unbound dye was plotted against the concentration of carrageenan solution added, until an endpoint indicating 1:1 stoichiometry was reached, in an analogous manner to metachromatic titration. Carrageenans could be estimated in the presence of carboxymethylcellulose if the assay was performed at pH 3.0-3.5, as at this pH ionisation of the carboxyl group is suppressed[205,206]. The fluorescence of Acridine Orange was also used to investigate the binding strengths of six polyanions and the influence of simple electrolytes on the dye/polyanion complex. The order of effectiveness of the cations in disrupting the dye/polyanion complex was found to be:

$$Ba^{2+} > Sr^{2+} > Ca^{2+} > Mg^{2+} >> K^{+} > Na^{+} > Li^{+}.$$

Within the same valency group the most effective cation was that with the smallest hydrated radius[207]. The release of dye from the polyanion/dye complex on the addition of polycation was also studied by the fluorescence technique[208]. It was found that stoichiometric displacement of dye occurred with addition of polycations, but that the related monomers were not effective, even in great excess, in producing the release of free dye and hence increasing the intensity at the wavelength for unbound dye fluorescence. In another study the cationic binding strength of heparin fractions was examined using Acridine Orange and a correlation between anticoagulant activity and the linear charge parameter of the polyanion was implied[209].

2.9.10.2 Optical rotatory dispersion

The anomalous change in optical rotation with wavelength - optical rotatory dispersion or ORD - of metachrmoatic complexes, particularly of Methylene Blue/glycosaminoglycans complexes, has been studied by Stone and Moss[210], and by Stone[211]. On the basis of the ORD observed for heparin/Methylene Blue complexes at acidic pH a helical structure for the polyanion was proposed, which was thought to explain the induced Cotton effects[210]. The ORD correlated with pH rather than with hypochromism or frequency shifts, and the possibility of a conformational change in the polymer with change in pH was suggested. The importance of dye-dye interaction in producing the optical effects was emphasised, and it was observed that hydrolysis of about 33% of the N-sulphate groups, which is known to cause a loss of almost all the anticoagulant activity - implying changes in the secondary structure of heparin - led to a marked loss of the induced asymmetry of the bound Methylene Blue with relatively slight loss of metach-

romasy[210]. Work was extended to other glycosaminoglycans and the topic was reviewed by Stone in 1970[211]. In this, it was pointed out that the magnitude and signs of the extrinsic (or induced) Cotton effects (anomalous ORD) of a given dye varied greatly between the three classes of biopolymers - proteins, polynucleotides and polysaccharides, and disordered polypeptides showed no induced ORD. The cotton effects were seen to change with changes in dye:anionic site ratio, and this change varied with different dyes and polymers. The variability observed here is reflected by the differences in metachromasy exhibited by dyes and polyanions - the spectra of Acridine Orange and Methylene Blue with a range of acid polysaccharides were compared, and the Methylene Blue complexes were shown to have much greater variation than the Acridine Orange complexes. Acridine Orange/polymer complexes could not be used to study polymer conformations because of the changes in ORD and circular dichroism observed with changes in P/D ratio for different polymers, but Methylene Blue/polymer complexes gave results which could be predicted from conformational models[211]. Cleland also used Acridine Orange in a study of hyaluronic acid and chondroitin-4-sulphate, and found that increasing the ionic strength by addition of sodium chloride, and increasing P/D, had different effects on the ORD spectra in the metachromatic region[212]. At P/D = 1 the spectra for the two polymers were similar. At higher P/D ratios the optical rotation spectrum for chondroitin-4-sulphate increased in intensity although the metachromatic spectrum at the same P/D remained unchanged, whereas the ORD spectrum for hyaluronic acid was at a maximum at P/D = 1. Increasing the ionic strength of the Acridine Orange/chondroitin-4-sulphate system had little effect until quite high concentrations were reached, whereas 0.01M NaCl was

sufficient to remove both metachromatic and Cotton effects from the Acridine Orange/hyaluronic acid complex[212].

The complexing of Methylene Blue with different samples of sodium alginate was studied by Seely and Hart, who showed that the samples could be distinguished on the basis of their metachromatic spectra, and that only three of the samples showed circular dichroism (CD)[213]. Samples not showing CD had been subjected to very mild acid treatment and the authors postulated that conformational change, rather than disruption of primary bonds, was involved. Metachromatic spectra and CD effects were also found to vary with P/D, both the sign and the extent of CD altering as the P/D increased[213].

A recent review of the conformational studies carried out on glycosaminoglycans using ORD studies has been prepared by Kennedy[214].

2.9.11 Theories of metachromasy

Two papers are of particular interest in a review of the historical development of theories of metachromasy - that of Schubert and Hamerman[144] and one by Phillips[215]. Schubert and Hamerman stressed the earlier observations that all dyes known to exhibit metachromasy with tissues deviated from Beer's Law in aqueous solution, and also the long association of ideas of dye aggregation as responsible for such deviation. In addition they pointed out that aggregation of a dye in aqueous solution did not necessarily result in deviation from Beer's Law, and that in metachromatic dyes the permanent charge was part of the resonance system responsible for the colour of the dye. Schubert and Hamerman summarised the accumulated experimental data by saying that the deviations from Beer's Law observed in cationic dyes in aqueous solution were best interpreted by the assumption that

the dyes could exist as monomers, dimers and higher polymers, all in equilibrium, and that changes in dye concentration in aqueous solutions changed the proportions of the dye species in the equilibrium. When the polymerisation involved delocalised π electrons, changes in dye concentration were accompanied by changes in the absorption spectra. Schubert and Hamerman were anxious to qualify the assumption that the α , β and γ bands (with wavelengths $\alpha > \beta > \gamma$) could be assigned to monomeric, dimeric and polymeric forms of the dye cation respectively, by pointing out that monomeric forms could show all three bands, although the α band was usually the most intense[144].

In 1935 Lison suggested that metachromasy in solution in the presence of chromotropes could be explained by the presence in water of two forms of the dye, the normal and metachromatic forms, in reversible equilibrium[165]. The effect of chromotropes was to displace the equilibrium sharply in favour of the metachromatic form, but there was no stoichiometric relation between chromotrope and dye.

An idea that was to receive much greater investigation was that put forward by Bank and Bungenberg de Jong, who emphasised the importance of the charge density of the anionic colloids that they used as chromotropes for inducing metachromatic behaviour[166]. Levine and Schubert also considered detergents as chromotropes and compared the results obtained with results for anionic polysaccharides[167]. They suggested that at low concentrations ($< 10^{-6}$M) the individual polysaccharide molecules were linearly disposed and the detergent molecules were separate and ionized. In the concentration range 10^{-6}M to 10^{-4}M, in the presence of dye concentration of 1.25×10^{-5}M, micelle formation of the chromotrope occurred to produce micro-regions

of high anion density which were responsible for the metachromatic behaviour observed. Critical micelle concentrations for the detergents used were not quoted specifically, but were said to be in the range from 10^{-4}M to 5 x 10^{-1}M. From the text figures metachromatic bands for potassium myristate appear to develop between 10^{-5}M and 10^{-4}M, and to decrease at concentrations above 10^{-4}M[167]. However, Corrin, Klevens and Harkins found that increasing soap concentrations led to a sharp spectral change in favour of the free dye spectrum - this change was found to occur at the critical micelle concentration of the soap[216]. It was suggested that the basic dye ions were incorporated into micelles and polyanion aggregates in such a way that they were subjected to a uniform electrical field; the charge distribution and therefore the electronic configuration of the dye ion became the same as that of the unperturbed dye which then gave its normal absorption spectrum. Several attempts were made to demonstrate that direct interaction between dye ion and anionic site - the ion-pair model - was responsible for metachromasy. Schubert and Levine developed their findings with anionic polysaccharides into a qualitative theory of metachromasy, and made a series of predictions that could be tested quantitatively[217]. The qualitative theory consisted of four postulates based, in part, on work by earlier authors.

1) Chromotropes exist in solution as anionic globular clusters or micelles, whose charge density depends on the nature of the chromotrope and the solution environment; the micelles are separated by intermicellar spaces of small anion density.

2) Cations are reversibly, and more or less firmly, bound within the clusters or micelles, the relative firmness of binding

increasing with the charge of the cations.

3) The metachromatic dye exists in solution as a series of polymers in equilibrium, the proportions of the molecular species depending on the dye concentration.

4) The metachromatic colour is produced as a result of the selective and reversible binding of the polymeric dye cation of the highest charge available within the anionic cluster.

Binding of the dye cation of high charge caused a shift in the equilibrium of the dye species with a fall in the concentration of ions of lower charge, giving a relative increase in the concentration of dye cations of high charge even in dilute solution and thus a change in the absorption spectrum. Schubert and Levine drew a number of corollaries from their theory, which they proposed could be tested. Any given metachromatic dye should show similar colour changes for all chromotropes - differences among chromotropes would depend only on the differing extent to which they bound the dye cation of highest charge, which would in turn depend on the anionic density of the anionic cluster or micelle. The effectiveness of added salts or higher chromotrope concentration in destroying metachromasy must depend on the competitive action of their cations and would therefore depend on the cation concentration and charge and on the dye concentration. It should be possible to develop a quantitative theory of metachromasy in solution for dyes showing sufficient variation in α, β and μ bands to allow calculation of the equilibrium constants and identification of the μ-band-producing aggregate[217,144]. Later ultracentrifugation and specific adsorption studies on metachromatic compounds were held to support the theory of salt-like interaction between dye and chromotrope, when the adsorbed or precipitated compound was shown to

have a constant proportion of dye and chromotrope over a twenty-fold concentration range. In addition it was held that the precipitate composition showed that polyanions were preferentially saturated with dye molecules, rather than showing random distribution over all available sites[169,170]. Pal and Schubert later modified the theory by suggesting that the metachromatic compound formed by equivalent amounts of dye cations and polyanions was stabilised in aqueous solution by homopolymerisation of the dye and close electrostatic association of the homopolymer with the polyanion[200]. Sharing of π electrons by adjacent cations was considered to be responsible for the suppression of the α band and the appearance of the μ band in the metachromatic spectrum, while hydrophobic interaction between the dye molecules explained their existence in aqueous solution, and the effects of ethanol and urea in destroying the metachromatic compound[200]. Hillson and McKay also attributed metachromasy primarily to ionic interaction with substrates; aggregation was known to occur, and the uptake of counter-ions required to reduce electrostatic repulsion between aggregating dye ions was thought to have an additional effect on metachromasy[161]. Bean, Shepherd, Kay and Walwick thought that aggregation alone could not explain the complexity of the metachromatic spectra found for the dye which they studied (4,5,4',5'-dibenzo-3,3'-diethyl-9-methylthiacarbocyanine), but that the conformation of the dye and its interaction with the anionic site and with adjacent sites were all involved[175]. Pulse radiolysis and spectral studies were combined in an attempt to clarify the relative parts played by aggregation and dye binding in metachromasy[176-178,215] and it was concluded that there was a direct relationship between anionic site/dye binding and metachromasy, and that electronic exchange and partial or

complete neutralisation of the charge of the dye occurred. When all metachromasy was destroyed the existence of non-aggregated bound dye could still be detected in the solutions studied. In addition, the limiting salt concentration - the amount of salt required to remove dye/site interaction completely - was shown to increase with the energy of the metachromatic shift, and this was taken to indicate that the metachromatic shift was directly related to the strength of the site/dye interaction.

Philips[215] and Schubert and Hamerman[144] trace the origins of the aggregation theory of metachromasy to early work by Michaelis and Granick[149], and Michaelis[150]. By analogy with the behaviour of cationic dyes in solution it was suggested that polymerisation of the dye, either adsorbed on to the surface of the polyanion or bound at specific sites, was responsible for metachromasy, and that the behaviour of nucleic acids with basic dyes could be explained by the prevention, by the structure of the nucleic acid, of polymerisation of the dyestuff beyond the dimer[150]. From his work on hexametaphosphates, Wiame proposed that when many molecules of dye were combined with the same molecule of hexametaphosphate they behaved as if polymerised. In the presence of excess chromotrope the combined dye molecules became more scattered, with fewer on each chromotrope molecule, so that polymerisation did not occur and the metachromatic colour changed to the normal colour[193]. Sylven also supported the idea that electrostatic attraction between the substrate and dye was followed by dye/dye interaction, with the polymer acting as a pattern for orientation[152], and suggested that water molecules were intercalated between the dye molecules. Koizumi and Mataga proposed two ideal cases of metachromasy[203,204], with the states superimposed in

a general case. From their work with Rhodamine 6G, Trypaflavine and Acridine Yellow they concluded that the results could be adequately explained by changes in aggregation of the dye due to adsorption of the dye ion onto the chromotrope (their second case), rather than by a change in the electronic state of the dye ion as a result of interaction between the dye ion and the chromotrope.

A major exposition of the aggregation theory was provided by Bradley and Wolf in 1959, in which they introduced the concept of the dye-stacking coefficient. This numerical parameter expressed the characteristic tendency of a polymer to promote the reversible association, or stacking, of dye molecules bound to its surface[171]. Stacking coefficients were calculated for a number of chromotropes with the dye Methylene Blue, by studying the reversal of metachromasy on addition of excess polymer. A number of factors were thought to affect the stacking tendency of a dye on a particular polymer, including the extent to which the cationic charge of the dye was neutralised, the change in the dielectric constant in the vicinity of the dye, and the extent to which thermal agitation of the dye was reduced because of binding. However Bradley and Wolf suggested that the most important factor influencing promotion of association by different polymers could be relative orientation of dyes bound to adjacent sites and the rigidity with which this orientation was maintained. It was thought possible that polymers whose binding sites were free to assume optimal positions for the stacking of dye bound to them would have high stacking coefficients, while those whose binding sites were rigidly held in less than optimal positions would have lower stacking coefficients. Later work with native and denatured DNA confirmed that the polyanions assumed to be more flexible

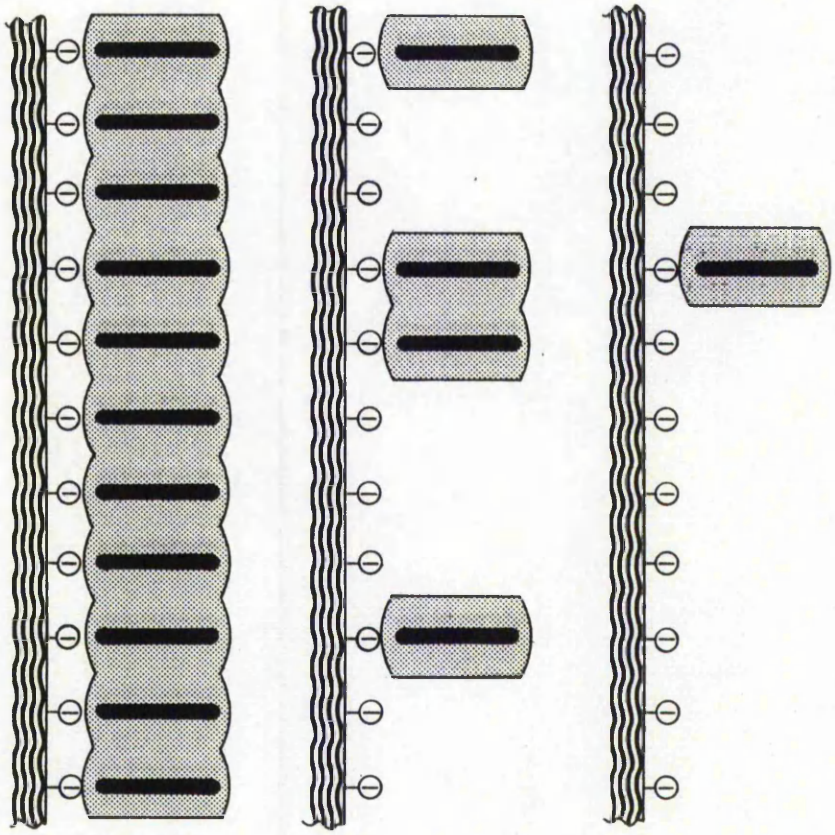
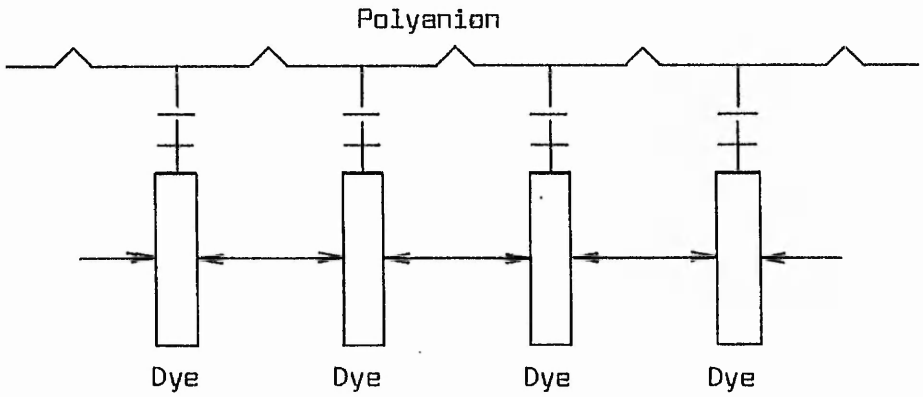


Figure 4. Schematic representation of the aggregation of dye molecules bound to the surface of a polyelectrolyte[171].



Representation of dye-dye hydrophobic bonds

Figure 5. Schematic model for metachromatic compound involving dye-polyanion electrostatic bonds and dye-dye hydrophobic bonds[182].

(the denatured samples) had higher stacking coefficients than the native rigid molecules[172,183].

From about 1970 onwards a variety of papers were published investigating the physical basis of metachromasy and incorporating or combining the ion-pairing and aggregation ideas to a greater or lesser degree. The results of conductimetric titrations on dye/chromotrope systems were held to support the view that aggregation of the dye ions rather than electrostatic bonding was responsible for the metachromatic spectral shift of the dye[182]. Polymerisation of the dye followed electrostatic attraction to polyanionic sites, and involved hydrophobic or other dye/dye bonds. Since conductimetric measurements showed that metachromasy was more sensitive than dye-binding to agents such as ethanol and dioxane it was concluded that the dye/dye forces disrupted by such agents must induce the spectral shift[182].

Scheibe used the concept of hydrophobic bonding to explain the promotion of aggregation of dyes at the polymer surface in response to the effects of structuring of water. The structuring was augmented by the hydrophobic surfaces of dye molecules and macromolecules, and the consequent lowering of entropy would tend to be compensated for by a reduction of surface area in contact with water through aggregation of the dye on the macromolecules, and of planar dye molecules with each other. The spectral shift was considered to result from the coupling of oscillator systems with the same resonant frequency and with dye molecules of high symmetry a new transition moment for the higher frequency oscillator would be produced[218].

Dye/dye interactions were also held to be important in studies

carried out on dye adsorption on solid cellulosic polyanions, but both papers stressed that dye/site interactions (and possibly dye/substrate interactions) could exert an influence on the spectral characteristics of the dye[154,202]. In particular the nature of the anionic site was shown to modify dye/dye interactions, even when the negative site spacing was the same for different anions. The overall conclusion drawn, however, was that site/dye binding did not in itself induce a metachromatic colour change, but that the strength of binding modified the metachromasy observed and was responsible for the differences observed between different dye/chromotrope complexes for the same dye, and for the different sensitivities of Acridine Orange and Methylene Blue. In apparent contrast with these findings is a paper, from the same group, in which it was considered that aggregation is a necessary prerequisite to dye binding, and that dye binding occurs most readily and stably at sites adjacent to an already bound dye cation, or that aggregated dye within the polymer domain is bound to the polyanion to form higher aggregates[207]. This second model was considered to be less likely than the first on the grounds of the marked differences shown by different polyanion/dye complexes in response to addition of electrolyte[207].

Wollin and Jaques proposed a combined theory of metachromasy involving ion-pairing and dye-stacking concepts[164]. On their model, metachromasy is produced by a dye-dimer reacting with a polyanion in a loose manner. The dimer unit is stabilised by the presence of water molecules, and the resonance of the dye molecules was thought to lead to a partial charge forming at each end of the dimer, allowing loose interaction with the polyanion. The metachromatic band shift was believed to be the result of this interaction involving the amino

groups forming part of the thiazine dye chromophore. The interaction reduces π electron delocalisation leading to absorbance at shorter wavelength, and the strength of binding of the N-group is reflected in the band shift.

Sculthorpe produced a brief review of metachromasy in 1978 in which he concluded that 'chromotrope metachromasia' involves electrostatic bond formation between closely spaced anionic groups of a substrate and cationic dye molecules[163]. The dye molecules should be sufficiently planar to stack down to intermolecular distances close to 0.3nm in order to allow overlap of π electrons, and should contain only one (terminal) positive charge so that coulombic repulsion forces between dye molecules is not too great. Where anionic sites are slightly more widely spaced a hydrogen bonded water molecule could be intercalated between each pair of dye molecules, and as few as 6-10 dye/substrate electrostatic bonds could be sufficient to allow metachromasy[163].

At about the same time a number of papers appeared which emphasised the importance of the flexibility of the chromotrope in determining the extent of metachromasy in synthetic polyanions. Shirai et al. considered that aggregation of Methylene Blue followed electrostatic binding of the dye to the polymer and that the greater degree of metachromasy shown by some polymers was due to their greater flexibility[184]. Random binding of the dye to the polyanion followed by aggregation induced by conformational change in the polymer chain was favoured over an alternative mechanism of preferential binding of Methylene Blue to sites adjacent to bound dye; spectral shifts due to aggregation were implied[219]. The effect of increasing P/D ratio was explained as also being due to conformational changes in

the polymer, the polymer coils extending as P/D increased, resulting in reduced aggregation[185]. Takatsuki and Yamaoka concluded that the peak positions of the metachromasy bands depend on the local conformation of the binding site of polymers, and that the configuration of the π electrons of the bound dye could be affected by the mean intersite distance[195]. The multiple-banded metachromasy observed with the dye pinacyanol chloride and chromotropes was again considered to be partly explained by polymer flexibility: as the random coil polyanion collapses to a more compact form due to reduction of repulsive forces by dye binding, the large bound pinacyanol cations would become overcrowded and form chaotic aggregates[187]. Where stoichiometry was shown to be 2:1 and dye molecules were assumed to bind to alternate anionic sites, the single metachromatic band was sharp and the dye aggregate assumed to be more regular. It was also thought possible that the polymer conformation could affect the non-electrostatic interaction of dye and polymer, again with effects on the metachromatic bands[187]. The geometry of dye aggregates in solution in the absence of chromotrope was considered by Pal and Ghosh who concluded that staggered aggregation should produce a bathochromic shift of the monomer λ max, and that the nature of the dye would determine the type of aggregation observed[220]. Thus, pseudoisocyanine shows a bathochromic shift with aggregation, while Methylene Blue aggregates in parallel stacks and shows a hypsochromic shift.

Finally, Thiery and Outracht carried out electronmicroscopy studies of isolated alginate fibres treated with metachromatic dyes and described the periodic structure observed as support for metachromatic theories requiring that dye molecules are regularly arranged at the chromotrope surface[221].

2.9.12 Summary

The shift in absorption spectra shown by some cationic dyes in solution with chromotropes (metachromasy) can be shown to be stoichiometric. The metachromatic complex may be partly or wholly destroyed by heating, addition of electrolytes or addition of solvents of varying hydrophobicity.

Chromotropes vary in the extent to which they can induce metachromatic behaviour in a particular dye, and dyes differ similarly. The nature of the binding site and of the intersite distance, and the flexibility of the polyanion, or factors correlated with these, have all been shown to affect metachromasy.

Three broad types of theory of metachromasy have been proposed:

1) that metachromasy is due to the electrostatic interaction between polymer and aggregate (ion-pairing);
2) that metachromasy is due to the dye aggregates that are formed as a result of individual dye ions binding to a suitable surface;
3) that metachromasy is due to the combined effects of aggregation and ion-pairing.

It is not yet clear which, if any, of these three theories offers the best explanation of metachromasy.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Enzyme studies on chitosan

3.1.1 Introduction

The methods of preparing chitosan by deacetylation of chitin most commonly in use all require relatively harsh conditions and cause reduction in molecular weight of the polymer to a greater or lesser extent (Section 2.5.2). Enzymic deacetylation of chitin has been reported[50], but no reference to a commercial deactylation application has been found, even though the specificity of enzyme-substrate interactions and the mild conditions generally required would appear to offer a means of obtaining high molecular weight chitosan.

In an attempt to deacetylate chitosan to a very low residual degree of N-acetylation, two enzymes were studied - an extract of Pseudomonas aeruginosa of high amidase activity, and a commercially available preparation of papain.

3.1.2 The effect of Pseudomonas aeruginosa extract on chitosan

Extracts of Pseudomonas aeruginosa cultured with acetanilide as the sole source of carbon were incubated with solutions of chitosan hydrobromide and assayed for amidase activity. Control enzyme aliquots incubated with acetanilide or acetamide and hydroxylamine were highly active as soon as assayed, but the chitosan solutions showed no amidase activity even after 24 hours. Furthermore the chitosan solutions showed evidence of precipitation on addition of the enzyme extract. It seems possible that the enzyme complexed irreversibly

with the chitosan substrate, leading to loss of activity and precipitation.

3.1.3 The effect of papain on chitosan

3.1.3.1 Introduction

The use of extracts of the papaya tree fruit, Carica papaya, for cheese making was recorded in Roman times, and was described for flesh tenderising from the middle of the Eighteenth Century[222]. Part of the activity of the papaya extracts is now known to be due to the enzyme papain, which has been extensively studied[223,224], and shown to be a sulfhydryl enzyme with proteolytic, esterase and amidase activity. Optimal activity of papain preparations is found in the presence of mild reducing and chelating agents - these ensure that the disulphide bridges are reduced to the free sulfhydryl group and that trace heavy metal ions do not inhibit enzyme activity. Standard assay conditions require a medium containing 0.005M cysteine and 0.001-0.002M EDTA. The molecular weight of the enzyme molecule is estimated to be approximately 22,000[223].

Papain was introduced to solutions of chitosan and chitosan (HBr salt) in the presence of sodium metabisulphite and EDTA, in order to investigate the possibility of deacetylation of the polymer by the enzyme. Although films cast from the chitosan/enzyme solution showed no difference in degree of N-acetylation, when compared with films cast from the control solution, the two solutions showed marked differences in viscosity. No reference to this effect of papain on chitosan solutions could be found in the literature, and the change in viscosity on addition of enzyme was therefore investigated.

3.1.3.2 Change in viscosity of concentrated polymer solutions

A Brookfield viscometer (Model LVF) was used to follow the change in viscosity of chitosan solutions after the addition of papain. The viscosities of chitosan (Kytex H) solutions were also measured with and without the activators used - sodium metabisulphite and EDTA. The chitosan was subsequently precipitated, thoroughly washed, and redissolved, and the viscosity remeasured. The results are presented in Tables 1-2 and in Figure 6.

The large change in viscosity in the presence of the activators is presumably due to the reduction in polymer volume in the increased ionic concentration due to the introduction of sodium metabisulphite. More interesting is the steady fall in the viscosity of the polymer solution with time after the introduction of the enzyme. The continuation of this fall in viscosity over at least two hours, coupled with the low enzyme concentration (approximately 18,800 monomer units per enzyme molecule) would appear to rule out the possibility that the enzyme is merely altering the polymer conformation by binding irreversibly to the polymer chain. It seems much more likely that the enzyme is interacting with the polymer substrate in a reversible manner, and is reducing the chain length, thus reducing the viscosity of the substrate solution. This view is further supported by the viscosity of the redissolved polymer, which remained very low.

3.1.3.3 Change in viscosity of dilute polymer solutions

Preliminary measurements showed that the change in viscosity of chitosan in the presence of papain could be followed readily by using a suspended level capillary viscometer. The viscometer was placed in a viscometer bath (Bridge Control Instrument Series III) the temperature

Table 1. Brookfield viscosity measurements.

Solution	Temperature/^{O}C	Viscosity/centipoise
A	20	626
A	37	359
B	37	131

Solution A - Kytex H (1g) in acetic acid (1% v/v, 100cm^3).

Solution B - Kytex H (0.88g) in acetic acid (0.88% v/v, 100cm^3), sodium metabisulphite (0.0048M), EDTA (0.0019M).

Table 2. Brookfield viscosity measurements in the presence of papain.

Time elapsed/min	Viscosity/cps	Time elapsed/min	Viscosity/cps
0	131	60	58
5	114	65	55
10	103	70	54
15	98	75	51
20	94	80	48
25	89	85	44
30	86	90	44
35	81	95	42
40	74	100	40
45	72	105	38
50	65	110	38
55	63	24 hours	20

Solution B as in Table 1, 37^{O}C, 0.5cm^3 enzyme solution containing 0.0139g protein.

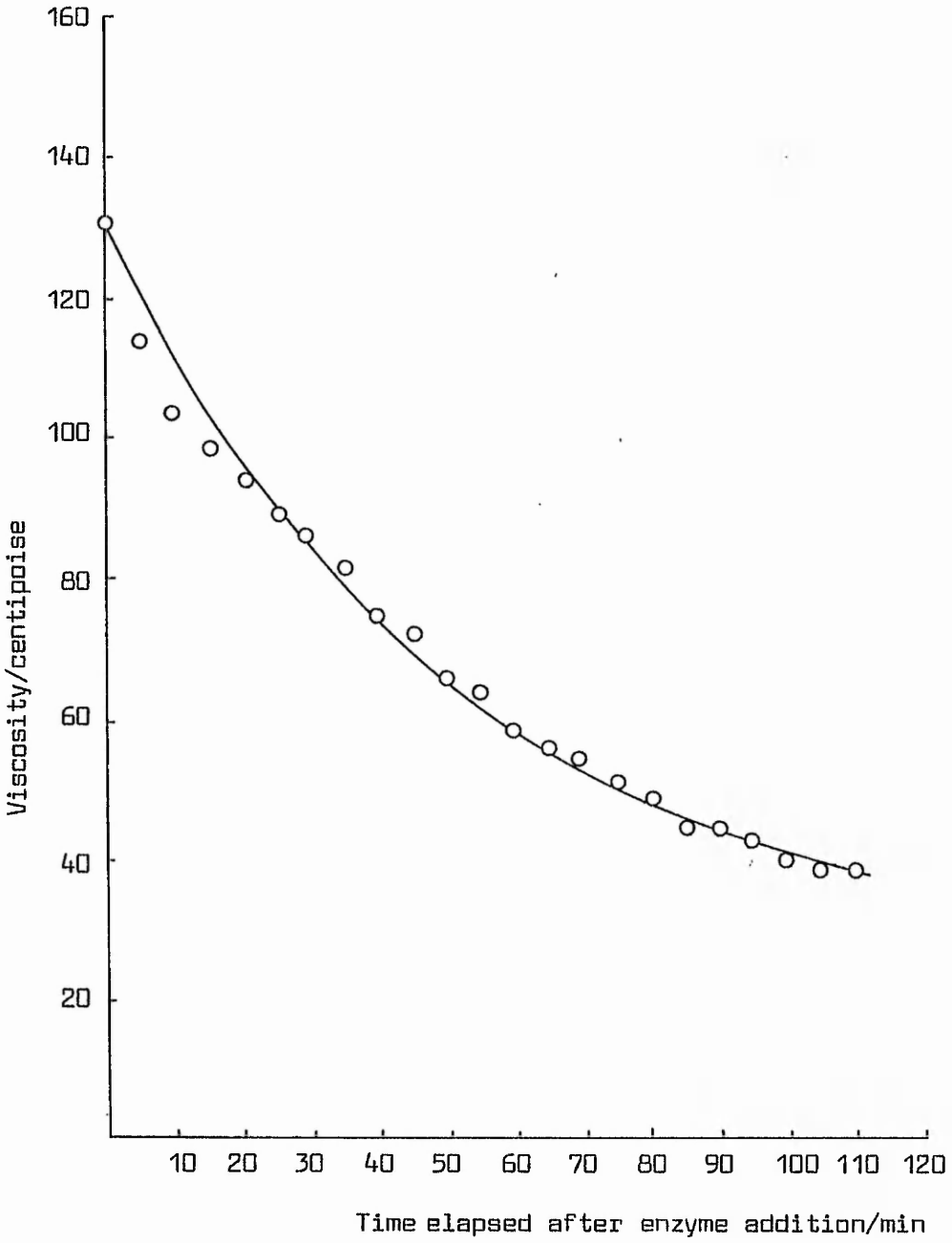


Figure 6. Graph of viscosity of chitosan versus time elapsed after enzyme addition. Solution B in Table 1, 37°C, 0.0139g protein.

of which could be maintained accurately to within 0.1 degree. The capillary viscometer affords more precise measurement of the change in viscosity of solutions than the Brookfield instrument, and also allows the use of smaller volumes. Initially, the effect of papain suspension on the flow times of chitosan solutions was explored in the presence of low concentrations of activators (Figure 7), and as with the measurements carried out on concentrated solutions, a marked decrease in viscosity was observed - the change in viscosity being rapid at first and then falling gradually and becoming steady at the reduced level.

3.1.3.4 Measurement of the rate of change of viscosity at fixed enzyme concentration

The chitinolytic activity of a chitinase extract of black koji mould, Aspergillus niger, was studied by Otakara using a substrate of glycol chitin[52]. The time required by different extracts to halve the viscosity increment was used as a measure of enzyme activity. Viscosity increment was defined as relative viscosity -1, so that

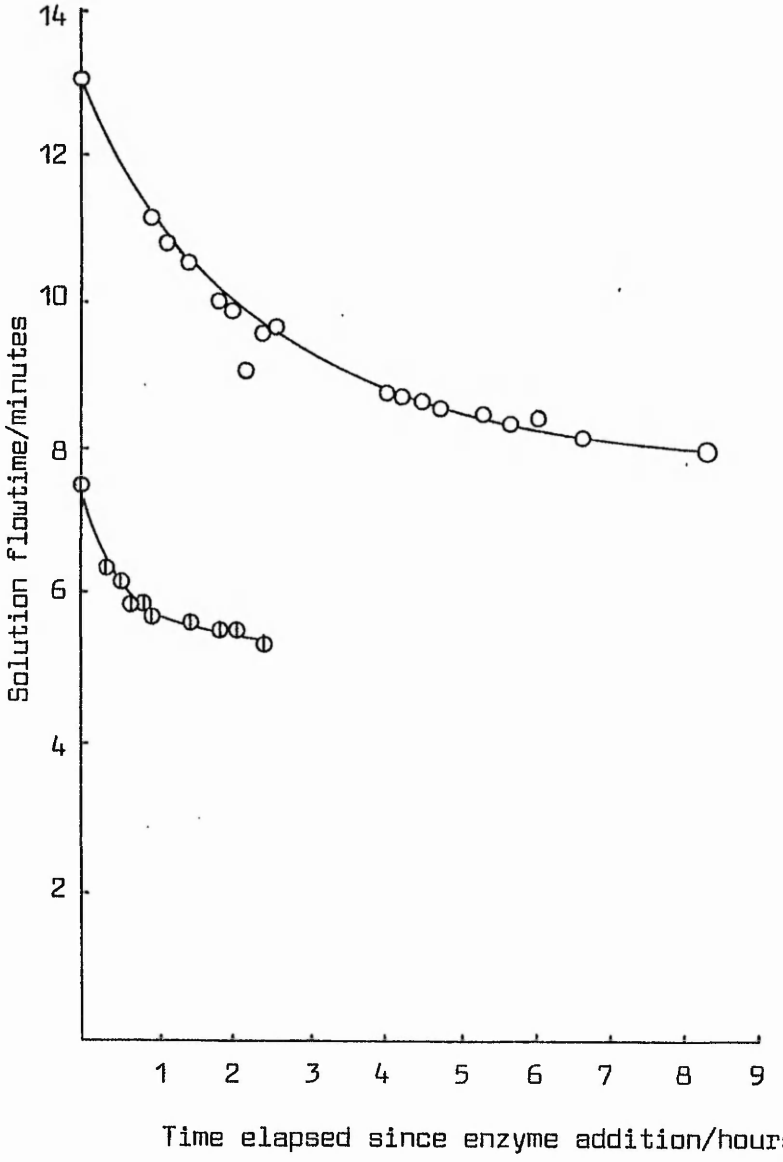
$$0.5 \times \text{viscosity increment} = 0.5\ (t/t_o - 1)$$

where t = polymer flowtime in the absence of enzyme

t_o = solvent flowtime.

Otakara used the observation that the time required to halve the viscosity of the reaction mixture was inversely proportional to the amount of enzyme used, to assess the relative strengths of Aspergillus extract fractions. The time required to halve the viscosity increment was called the 'half-life time' and was used as the basis of comparison for the fractions.

Figure 7. Change in viscosity of polymer solutions.



○ chitosan (8×10^{-3}g) in acetic acid (1% v/v, $25cm^3$), sodium metabisulphite (2×10^{-4} moles dm^{-3}), EDTA (2×10^{-4} mole dm^{-3}), papain (1.4mg).

Φ chitosan (2×10^{-2}g) in acetic acid (1% v/v, $125cm^3$), sodium metabisulphite (1×10^{-4} moles dm^{-3}), EDTA (4×10^{-5} moles dm^{-3}), papain (13.9mg).

In order to study the rate of change of viscosity for the enzyme/substrate system papain/chitosan, dilute enzyme suspensions were added to a range of chitosan solutions of different concentrations, and the change in the viscosity increment (η_{sp}^{225}, t/t_o-1) was plotted against time elapsed since enzyme addition (Figure 8). The half-life times for the solutions were then compared (Figure 9), and it was found that at constant enzyme concentration, the half-life time for the solution increases as the polymer concentration increases. This relationship would be expected if cleavage of glycoside bonds was taking place within the polymer chain - that is, if the enzyme was acting in an "endo" manner.

3.1.3.5 Measurement of rate of change in viscosity at fixed polymer concentration

The change in viscosity of polymer solutions of the same concentration in the presence of different concentrations of papain was followed by measuring the change in flowtime in a capillary viscometer as before. Again, results were plotted as change in specific viscosity versus time elapsed from the addition of the enzyme solution, and half-life times were estimated from the graph (Figure 10). Half-life times were also plotted as a function of enzyme concentration and of the logarithms of enzyme concentration (Figures 11 and 12). The graphs suggest strongly that half-life time is inversely proportional to enzyme concentration for this enzyme/substrate system. Again, this relationship would be expected if cleavage of the polymer chains was taking place in an "endo" manner.

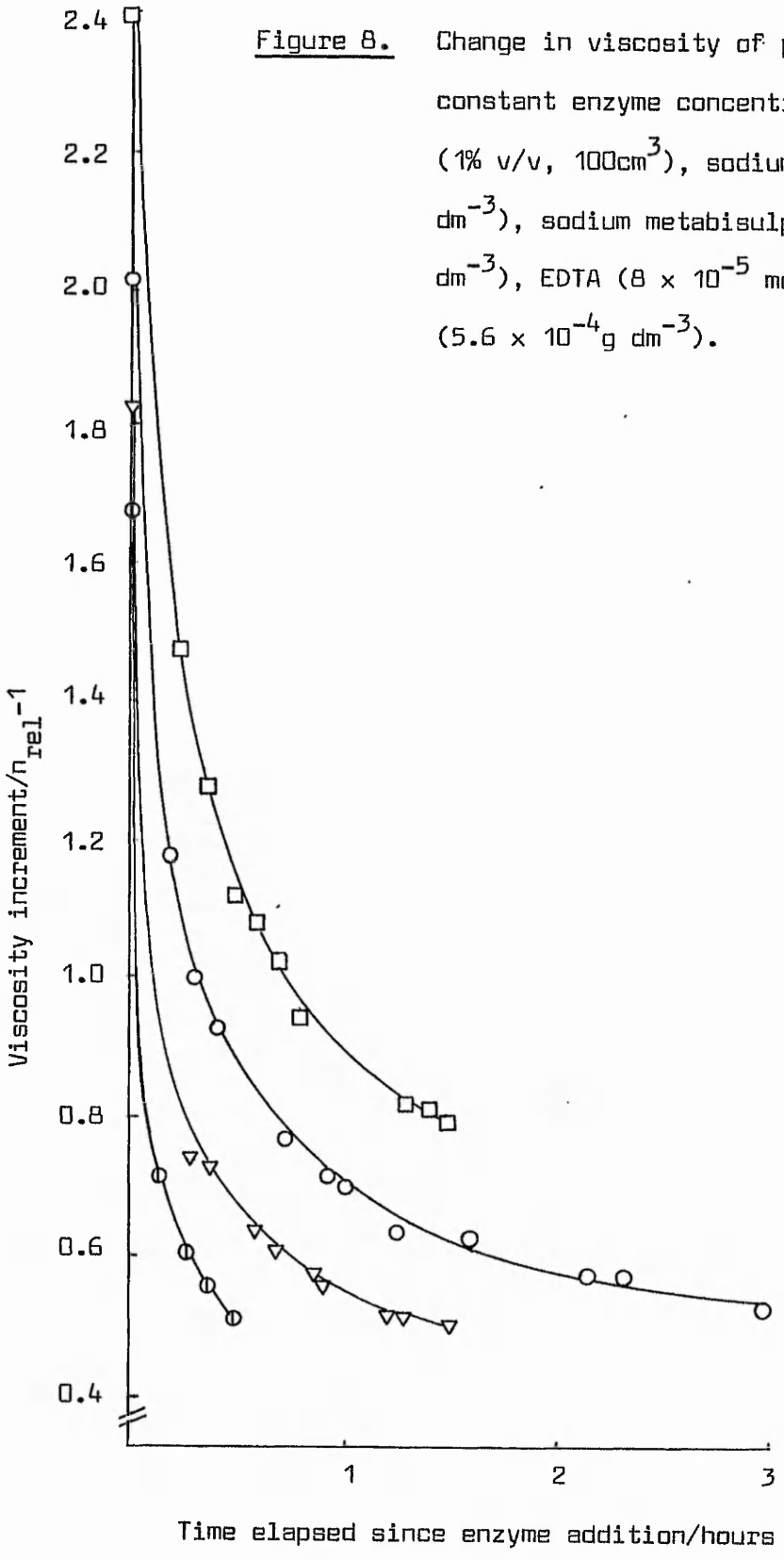


Figure 8. Change in viscosity of polymer solutions - constant enzyme concentration. Acetic acid (1% v/v, 100cm^3), sodium chloride (0.1 mole dm^{-3}), sodium metabisulphite (1×10^{-4} mole dm^{-3}), EDTA (8×10^{-5} mole dm^{-3}), enzyme (5.6×10^{-4}g dm^{-3}).

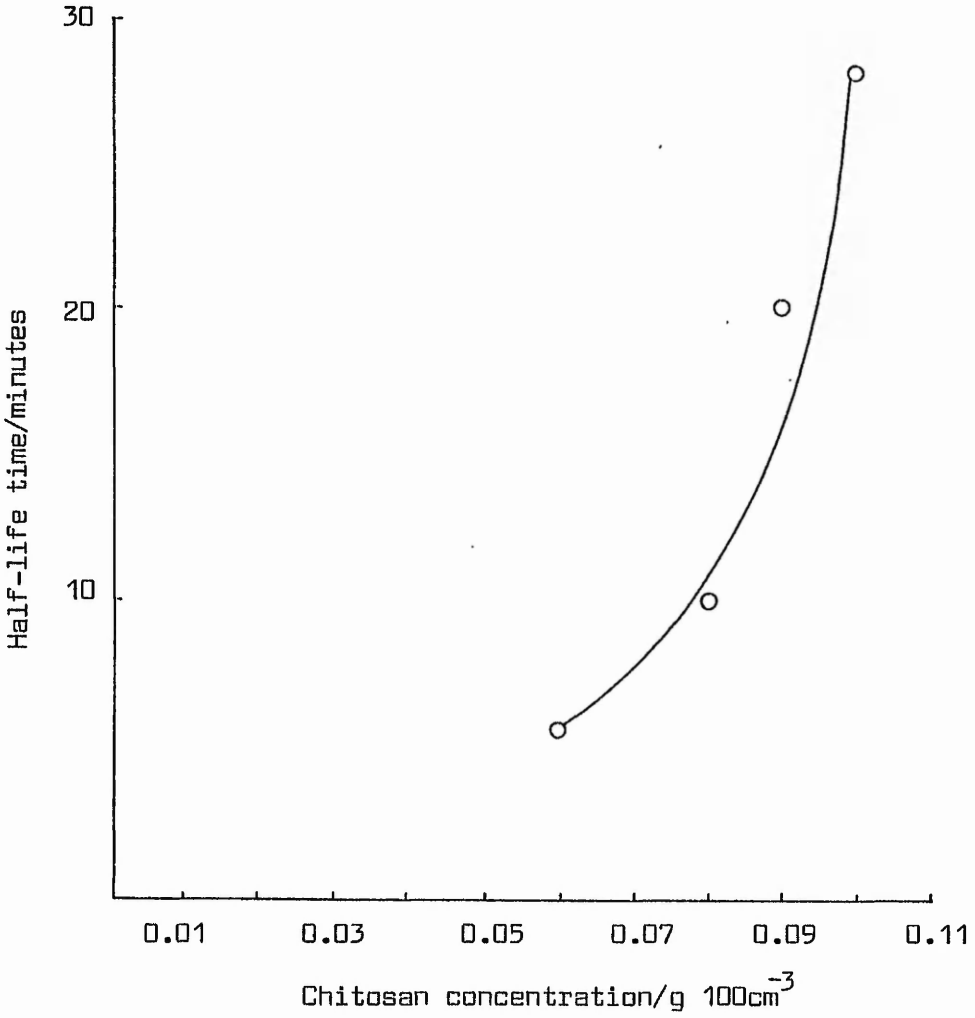


Figure 9. Half-life time versus polymer concentration - constant enzyme concentration (solutions as in Figure 8).

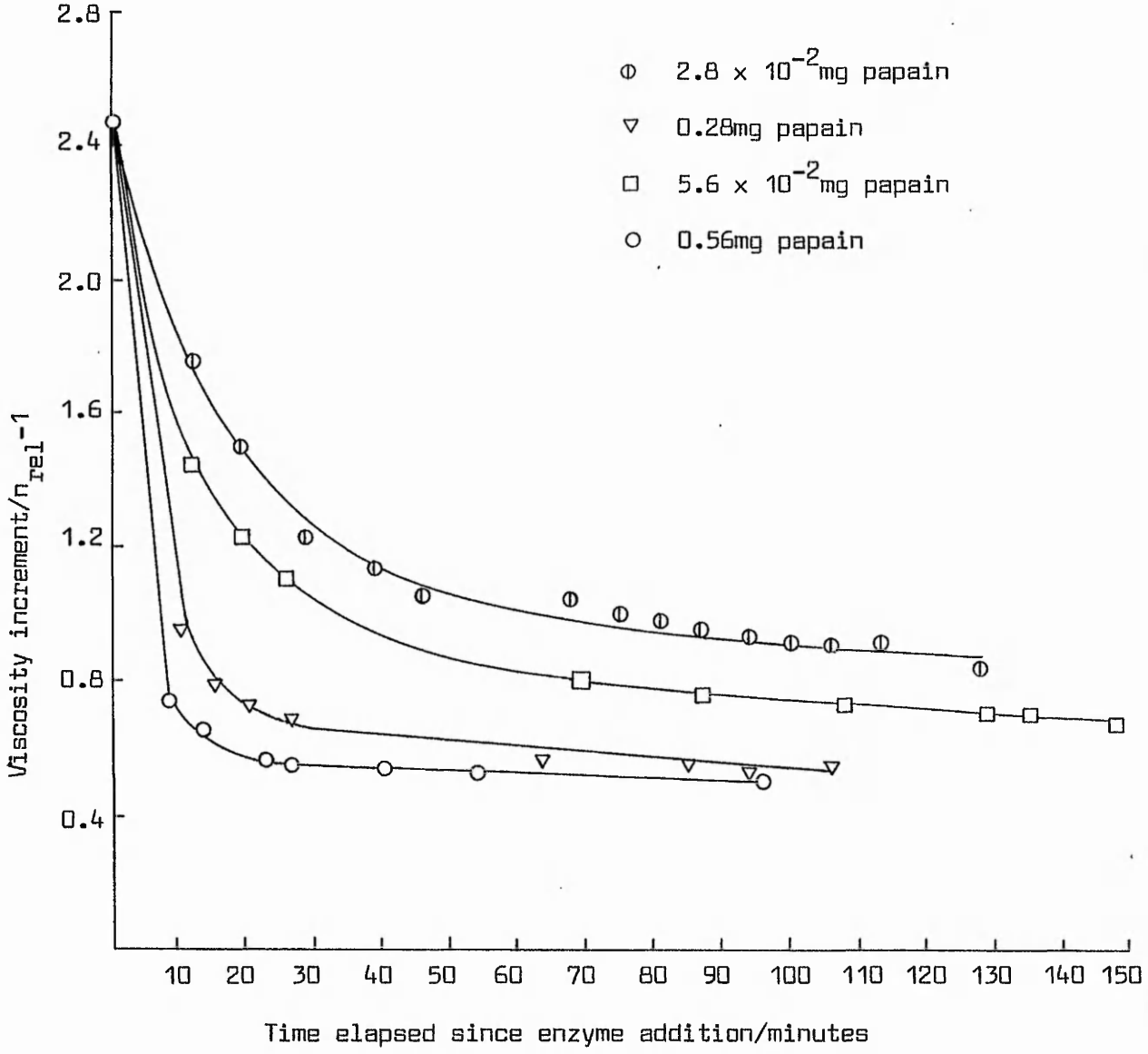


Figure 10. Change in viscosity of polymer solutions - constant polymer concentration. Chitosan (0.1g) in acetic acid (1% v/v, 100cm^3), sodium chloride (0.1 moles dm^{-3}), sodium meta-bisulphite (1 x 10^{-4} moles dm^{-3}), EDTA (8 x 10^{-5} moles dm^{-3}).

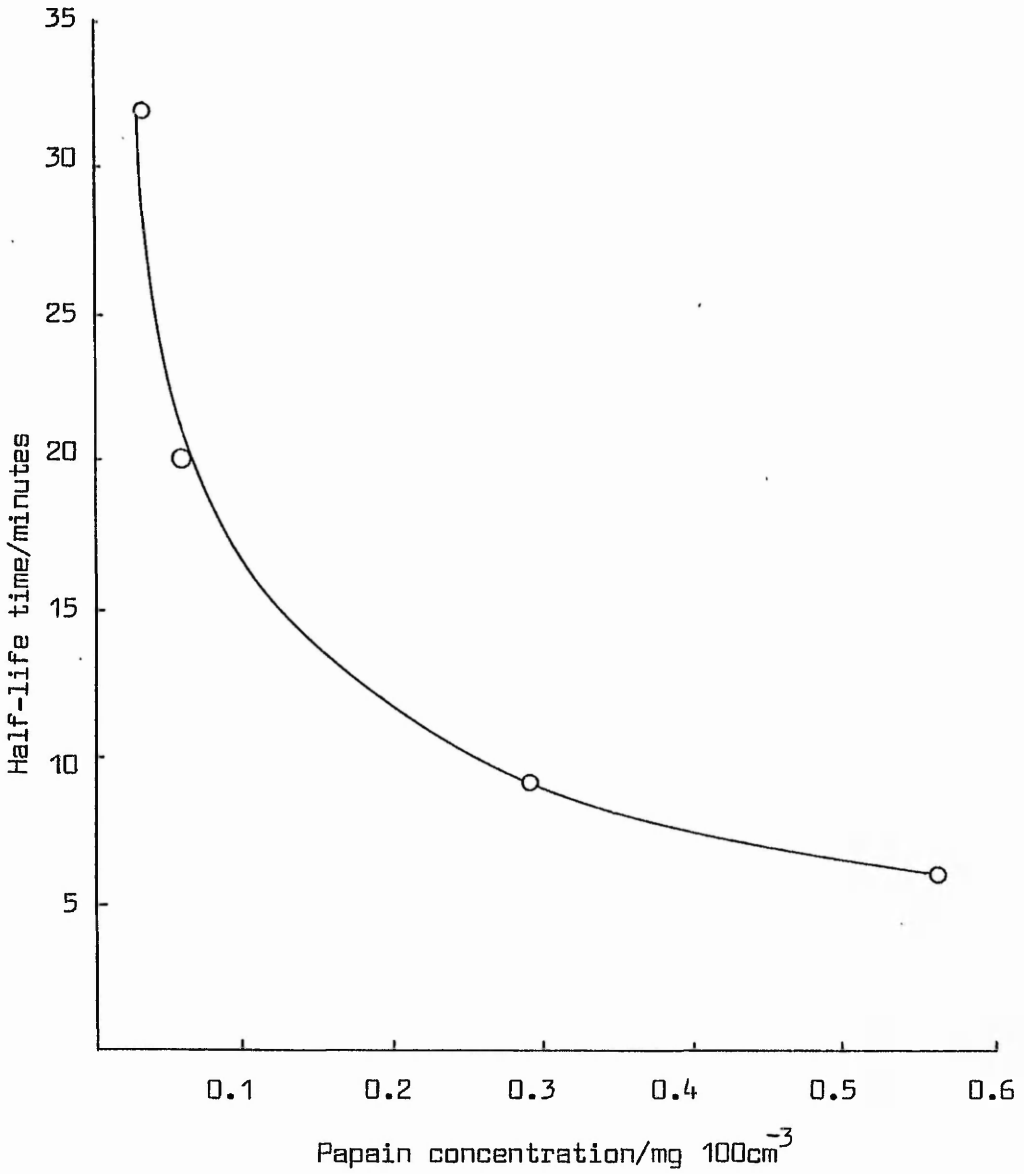


Figure 11. Half-life time versus enzyme concentration (solutions as in Figure 10).

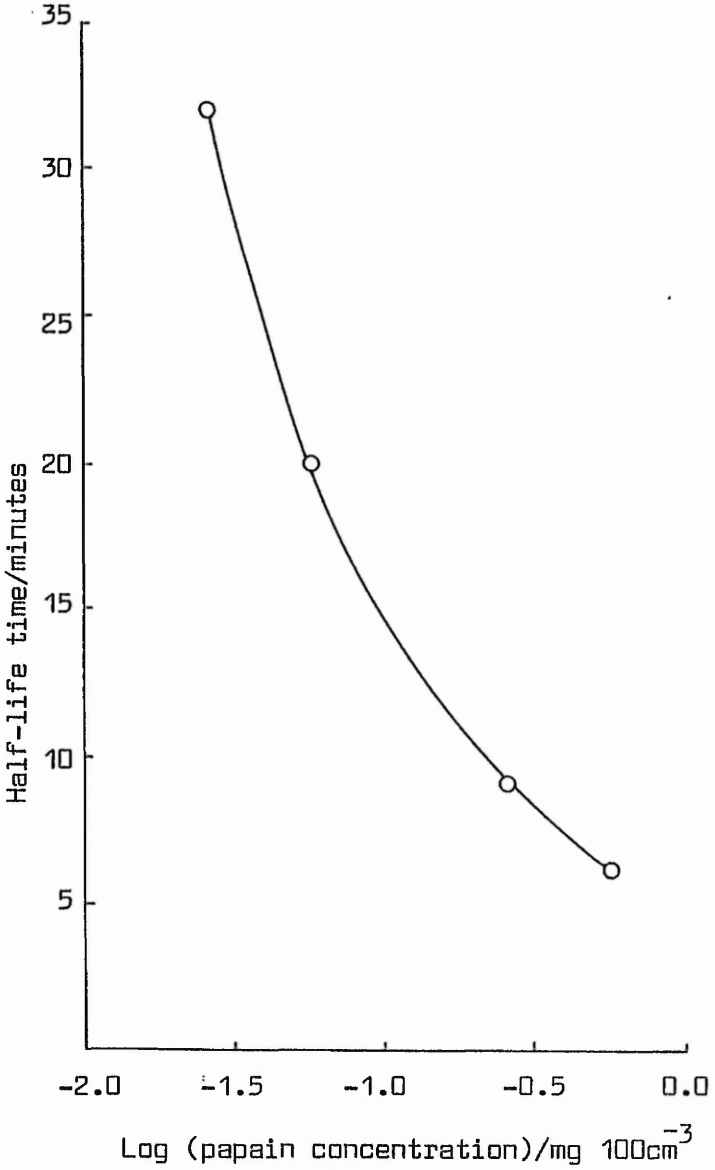


Figure 12. Half-life time versus log (enzyme concentration) - constant polymer concentration (solutions as in Figure 10).

3.1.3.6 The effect of papain on chitosan solutions of different degrees of N-acetylation

Six samples of chitosan of different degrees of N-acetylation were prepared for viscosity measurements in the presence of papain. Activator-free solutions were used for these measurements, as it had previously been shown that, surprisingly, the enzyme did not require the presence of activators with the chitosan substrate (Table 3). Flowtimes were recorded for each solution in the absence and presence of enzyme, and the half-life times were compared in order to determine whether the activity of the enzyme was affected by deacetylation of the polymer. Furthermore a correlation of enzyme activity with the degree of N-acetylation of the chitosan should indicate whether the classification of chitinase or chitosanase was appropriate. The results are presented in Figures 13 and 14.

It is apparent from Figure 13 that there is relatively little change in viscosity for the samples of very low degree of N-acetylation. As the degree of N-acetylation increases, the rate of reduction of the viscosity of the polymer solutions also increases. When the results are plotted as half-life time versus degree of N-acetylation, a direct relationship between degree of N-acetylation and enzyme activity is indicated. The two samples of polymer which approached the idealised chitosan structure did not show sufficient change in viscosity to give a half-life time within the duration of the measurements (up to 1 week for sample S10). By contrast, the sample of highest degree of N-acetylation, S30 (55% N-acetylation) showed a very rapid change in viscosity, even at the low enzyme concentration used (Figure 14).

Solvent type	Solvent flowtime/sec	Polymer flowtime/sec	Polymer + enzyme flowtime/sec
Standard	205.8	386.5	270.1
No sodium metabisulphite	206.1	423.1	269.2
No EDTA	205.8	458.1	273.4
No NaS_2O_5 or EDTA	206.1	482.2	269.0
Standard	205.8	386.5	365.4*

* Heat denatured protein

Solutions: Chitosan (0.06g) in acetic acid (0.8% v/v, 100cm^3), papain (1.4mg), sodium chloride (0.1 moles dm^{-3})

Activators (where present): EDTA (8×10^{-5} moles dm^{-3}), sodium metabisulphite (1×10^{-4} moles dm^{-3})

Table 3. Change in flowtime of chitosan solutions with papain, in the absence of activators.

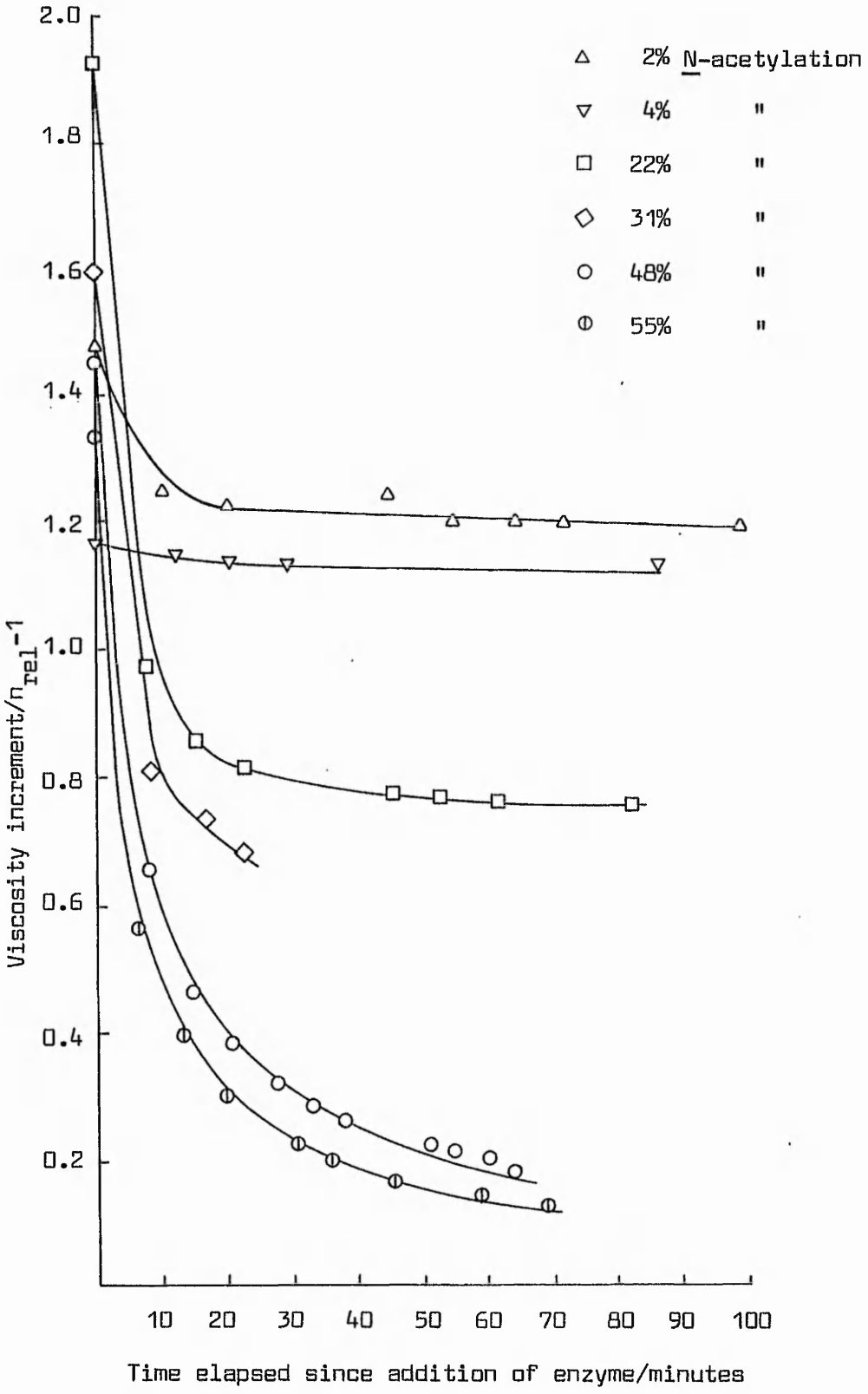


Figure 13. Change in viscosity of polymer solutions of different degrees of N-acetylation. Chitosan (0.1g) in acetic acid (1% v/v, 100cm^3), sodium chloride (0.2 moles dm^{-3}), papain (0.56mg).

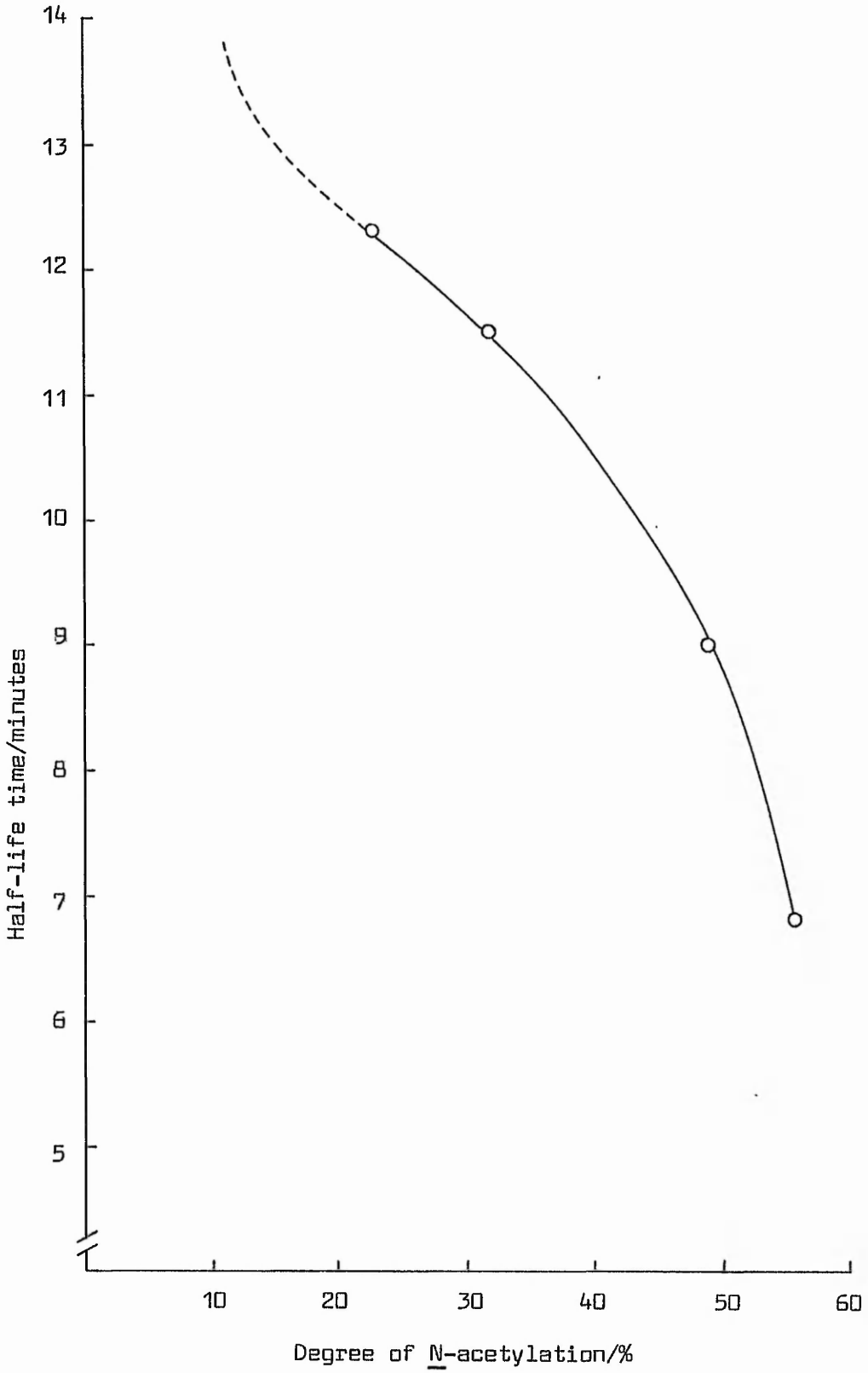


Figure 14. Half-life time versus degree of N-acetylation (solutions as in Figure 13).

It may be deduced from these findings that the papain acts as a chitinase rather than a chitosanase, and that the presence of N-acetyl groups on the polymer chain is important in promoting activity in the enzyme.

3.1.3.7 The addition of fresh substrate to a papain-treated chitosan solution

A fresh untreated solution of chitosan was added to a solution that had previously been treated with papain, and the change in the flow-time of the solution through a capillary viscometer was followed.

Whereas the viscosity of the treated solution appeared to have reached a stable value, the addition of fresh substrate produced an overall increase in solution viscosity, followed by a decrease in viscosity (decrease in flowtime). Half-life time was reached in approximately $3\frac{1}{2}$ hours.

Enzyme activity was still, therefore, present, and measurable, in spite of the very low dilution of the enzyme, and the very high ratio of monomer units to enzyme molecules ($\simeq 1 \times 10^6$ to 1).

3.1.3.8 The effect of papain on hydroxyethyl cellulose

To test whether papain acts as a glycosidase for the structurally related polysaccharide cellulose, hydroxyethyl cellulose solution was treated with papain suspension in the same way in which chitosan solutions were treated (activator present in the solvent), no change in viscosity (no change in flowtime) was observed.

3.1.3.9 Summary

The absence of enzyme activity with hydroxyethyl cellulose supports

the view that the observed effects of papain on chitosan solutions were a result of a true enzyme/substrate interaction which is promoted by the presence on N-acetyl groups on the polymer chain. The activity of this sulfhydryl enzyme in the absence of activators was surprising, and the mode of substrate/enzyme interaction was not fully elucidated, although hydrolysis of the $\beta(1\rightarrow4)$ linkage in the vicinity of an N-acetylated repeat unit seems indicated.

It is possible that the observed chitinase activity of lysozyme extracts from Papaya and Ficus[57] was due to traces of papain present in the lysozyme, and not primarily to the lysozyme.

3.2 Deacetylation studies on chitosan

3.2.1 Introduction

The problems associated with obtaining high molecular weight chitosan by de-N-acetylation of chitin have been outlined in Section 2.5.2. It has been observed that deacetylation under alkaline conditions proceeds rapidly until the polymer is approximately 70% deacetylated[83]. After this stage is reached deacetylation proceeds slowly until the product is about 20% N-acetylated. Further alkaline treatment does not produce a significant increase in the degree of deacetylation, but does degrade the polymer chain. Since this work was carried out, Mima, Miya, Iwamoto and Yoshikawa have reported a method for obtaining chitosan of virtually 100% deacetylation; the molecular weight of this product was found to be 5×10^5 ($\bar{M}w$), which suggests that some degradation has occurred[226].

It is possible that N-acetyl groups which are folded within the configuration of the polymer chain of the solid parent material are not accessible for chemical de-N-acetylation; if this were so,

solution of the polymer after an initial alkaline deactylation treatment, followed by reprecipitation and further alkaline treatment, should redistribute the remaining N-acetyl groups relative to the particle surface, and enable a product of low degree of N-acetylation and high molecular weight to be produced.

3.2.2 Deacetylation of chitosan

In order to test this hypothesis, a series of deacetylation treatments were carried out on a commercially available chitosan sample. Each group of treatments included the solution and reprecipitation of the products, and the degree of N-acetylation and the molecular weight of the samples were determined at each stage. Strongly alkaline conditions have been used to prepare chitosan for many years (Section 2.5.2) and two of the treatments described here used 50% w/w sodium hydroxide as both reaction medium and deacetylating agent. In two other treatments approximately 96% of the sodium hydroxide was replaced by tertiary butyl alcohol as the reaction medium - this system is known to give good results[227]. Sodium borohydride was introduced into two treatment systems (one all sodium hydroxide, and one tertiary butyl alcohol plus sodium hydroxide): it has been shown that the presence of sodium borohydride can prevent the degradation, from the reducing end-groups, of cellulose in alkaline processes by reduction of the terminal aldehyde group to a hydroxyl, and it was hoped that it would be possible similarly to protect the chitosan polymer chain, giving a higher molecular weight product.

The four deacetylation systems used are given in Table 4. Three samples were retained from each system for molecular weight and degree of N-acetylation determinations: after the first alkali

Table 4. De-N-acetylation systems used

System	tertiary butyl alcohol	sodium hydroxide	sodium borohydride
A	✓	✓	–
B	–	✓	–
C	✓	✓	✓
D	–	✓	✓

treatment (eg. A1), after solution and reprecipitation (eg. A2) and after a further alkali treatment (eg. A3). The starting material was commercial chitosan (Kypro) which had been dissolved in 1% acetic acid, filtered to remove insoluble fragments and reprecipitated. Experimental details are given in Section 5. The results obtained from this series of de-N-acetylation treatments are presented in Table 5.

Molecular weights were determined from viscosity measurements using the viscometric constants for chitosan reported by Roberts and Domszy[228]. The degree of N-acetylation was determined by a metachromatic titration method described fully in Section 3.5.

The samples in all four systems were deacetylated to minimum levels while molecular weights remained very high. One interesting and unexpected finding was that intermediate solution and reprecipitation of a sample was associated with a considerable decrease in N-acetylation - for samples B2 and D2 virtually no residual N-acetyl groups could be detected. When sodium borohydride was present in the system, molecular weights decreased further, relative to the starting material, than when sodium borohydride was absent, in contrast to what was anticipated. System A and system C were

Table 5. Molecular weight and degree of N-acetylation for the chitosan samples

De-N-acetylation system	Sample	Limiting Viscosity Number	Molecular weight, $\times 10^{-6}$	% N-acetylation
-	S*	1320	2.01	22
A	A1	1233	1.87	10
	A2	1040	1.56	4
	A3	975	1.45	2.4
B	B1	1180	1.79	11
	B2	1180	1.79	0
	B3	1085	1.63	1
C	C1	1118	1.69	7
	C2	1109	1.67	4
	C3	870	1.29	2
D	D1	1120	1.69	9
	D2	1008	1.51	1
	D3	892	1.32	0

* starting material

relatively more effective for de-N-acetylation of the samples than system B and system D respectively; that is, a low concentration of sodium hydroxide in the presence of tertiary butyl alcohol was as effective as a very high concentration of sodium hydroxide alone. In an industrial process where solvent recovery was feasible, an alcohol/alkali system could be cheaper to operate than one based on alkali.

Infrared spectra confirmed the very low degree of N-acetylation of the chitosan samples obtained by de-N-acetylation after solution and reprecipitation - Figures 16 and 17 show the spectrum of the starting material and that of sample C3. In Figure 17, the fully de-N-acetylated sample has no amide I band at 1655cm^{-1}, whereas the spectrum for the starting material shows a well-developed amide I band.

It would therefore appear that reprecipitation of chitosan results in the increased availibility of residual N-acetyl groups for chemical modification, through alteration of the morphology of the polymer.

Since this work was completed, Domard and Rinaudo have reported a method for preparing fully deacetylated chitosan of high molecular weight[229]. In calculating viscosity-average molecular weight from the Mark-Howink equation, they used the values calculated by Lee[230] for the parameters K and a, and noted the disparity between the viscosity-average molecular weight obtained and the $\bar{M}n$ calculated by membrane osmometry. However, if the constants calculated by Roberts and Domszy[228] are substituted into the equation, the viscosity-average molecular weight obtained is slightly greater than twice

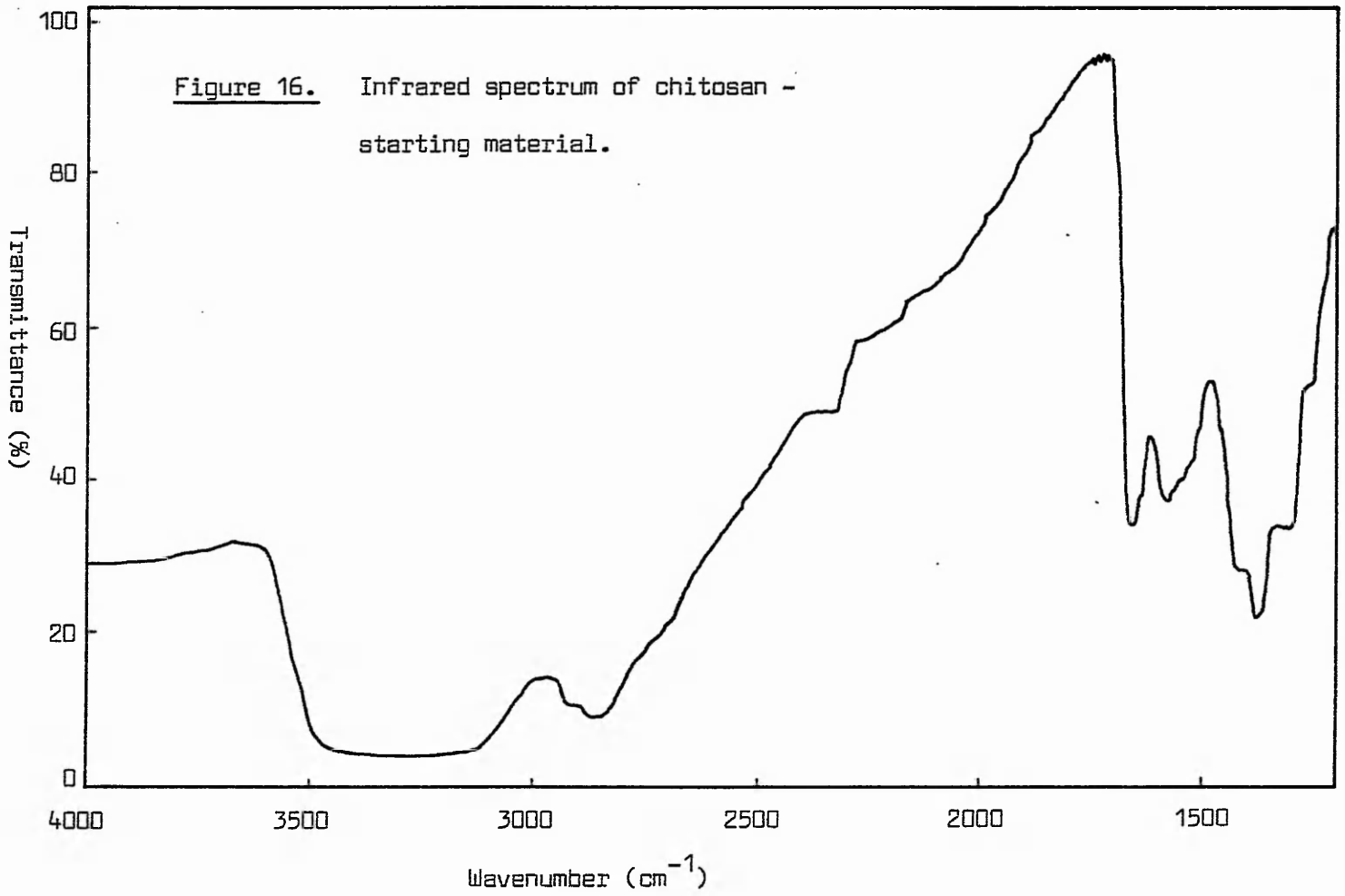


Figure 16. Infrared spectrum of chitosan - starting material.

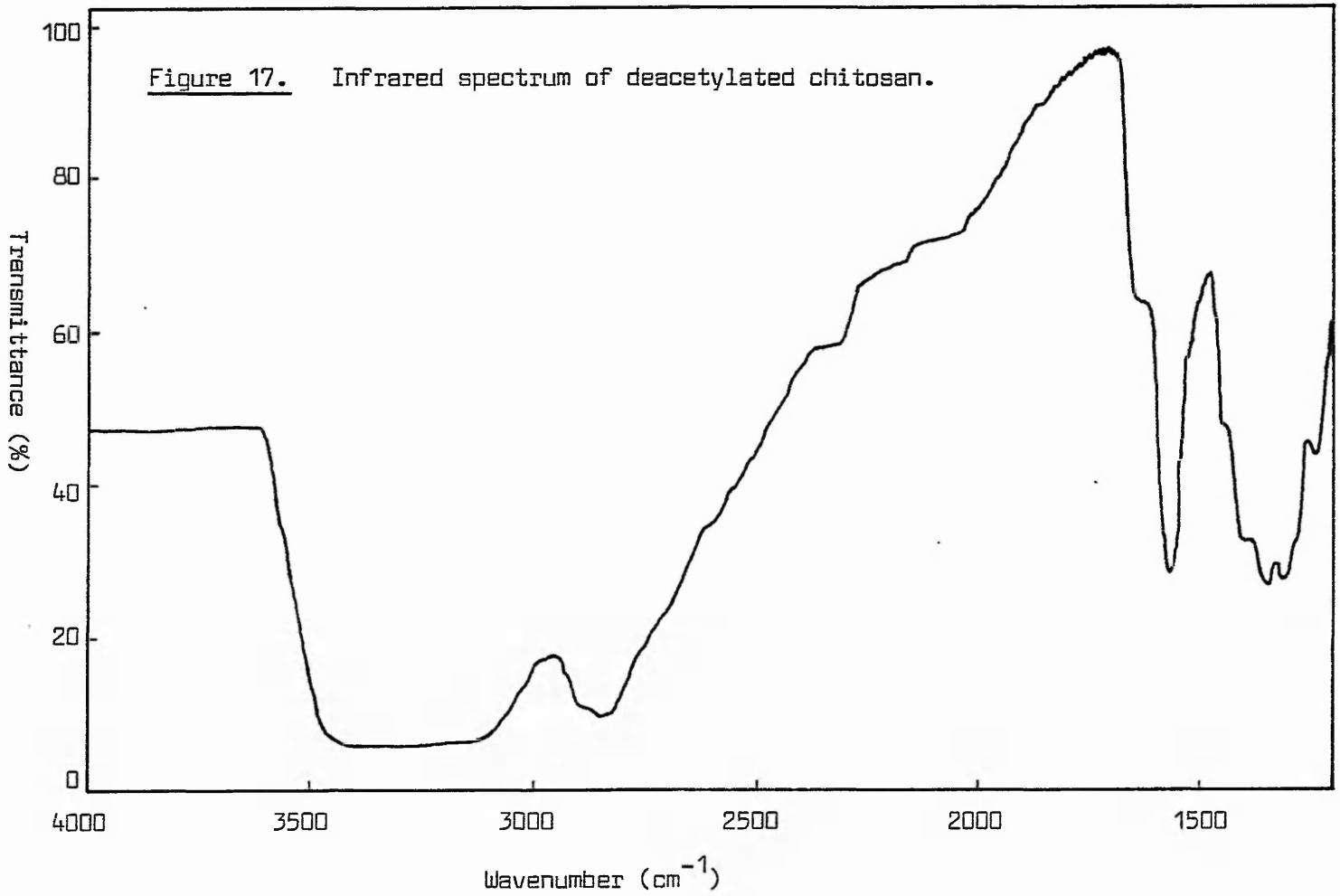


Figure 17. Infrared spectrum of deacetylated chitosan.

the number average molecular weight, as would be expected for a randomly degraded sample showing a normal molecular weight distribution.

The molecular weights of samples prepared by this method[229] are lower than those prepared by the method described in this section but the N-acetyl content of samples would seem to be similarly low.

3.3 Adsorption of Cu(II) ions by chitosan

The adsorption of metal ions, including Cu(II) ions, from dilute aqueous solutions has been studied by a considerable number of workers (see Section 2.7). However the actual method of complex formation - ionic interaction, ligand bond formation, chelation - has not been established. Furthermore much of the work has been carried out on uncharacterised chitosan hence comparison between the results of different workers is difficult. In the work reported here a chitosan sample of known free amine group content was used. In addition, the chitosan substrate was used in the form of film in an attempt to determine rates of uptake of Cu(II) ions without the problems arising from particle size variation in chitosan flake.

One advantage of using chitosan in film form is that the substrate can be examined directly by infrared and uv/visible spectroscopy. If a suitable absorption band could be found the concentration of Cu(II) ion in the film could be measured repeatedly, as a function of time, on the same sample.

3.3.1 Detection of Cu(II) ions in chitosan film

Initial trials with solutions of various Cu(II) salts indicated that cupric acetate is a suitable salt for adsorption studies. Chitosan

films treated with aqueous solutions of cupric acetate showed increased absorption at about 260nm but the band is too intense to be of use even for thin films and low Cu(II) ion uptakes. The blue colour of the films is due to a broad and very shallow absorption band centred around 660nm, but this was not distinct enough to be used for monitoring ion uptake, with the instrument then available, even at relatively high Cu(II) ion concentrations (Figures 18,19).

The infrared spectra of cupric acetate-treated films show a broadening of the band at 1360cm^{-1} and a shift in the amide II band from 1590cm^{-1} to 1550cm^{-1}. This shift is similar to that found with increasing N-acetyl content[107] and may also be due to a reduction in the extent of involvement of the amide groups in hydrogen bonding (Figures 20,21). In an attempt to find an infrared band that could be used for monitoring Cu(II) ion uptake the spectrum of a treated film was recorded down to 200cm^{-1}. Comparison with the spectrum of an untreated film failed to indicate a suitable band. In view of these negative results the use of an aftertreatment with copper complexing reagents was investigated.

3.3.1.1 Aftertreatment with sodium diethyldithiocarbamate

Chitosan films treated with sodium diethyldithiocarbamate (NaDDC) show absorption bands at about 260nm and 293nm, whilst the infrared spectrum does not differ significantly from that of the film prior to treatment with NaDDC. In contrast to this, both the infrared and the uv/visible spectra of cupric acetate-treated chitosan films show marked changes after treatment with aqueous solutions of NaDDC. The uv/visible spectrum shows bands at 280nm and 450nm on treatment with NaDDC, the latter band being of suitable intensity for use in

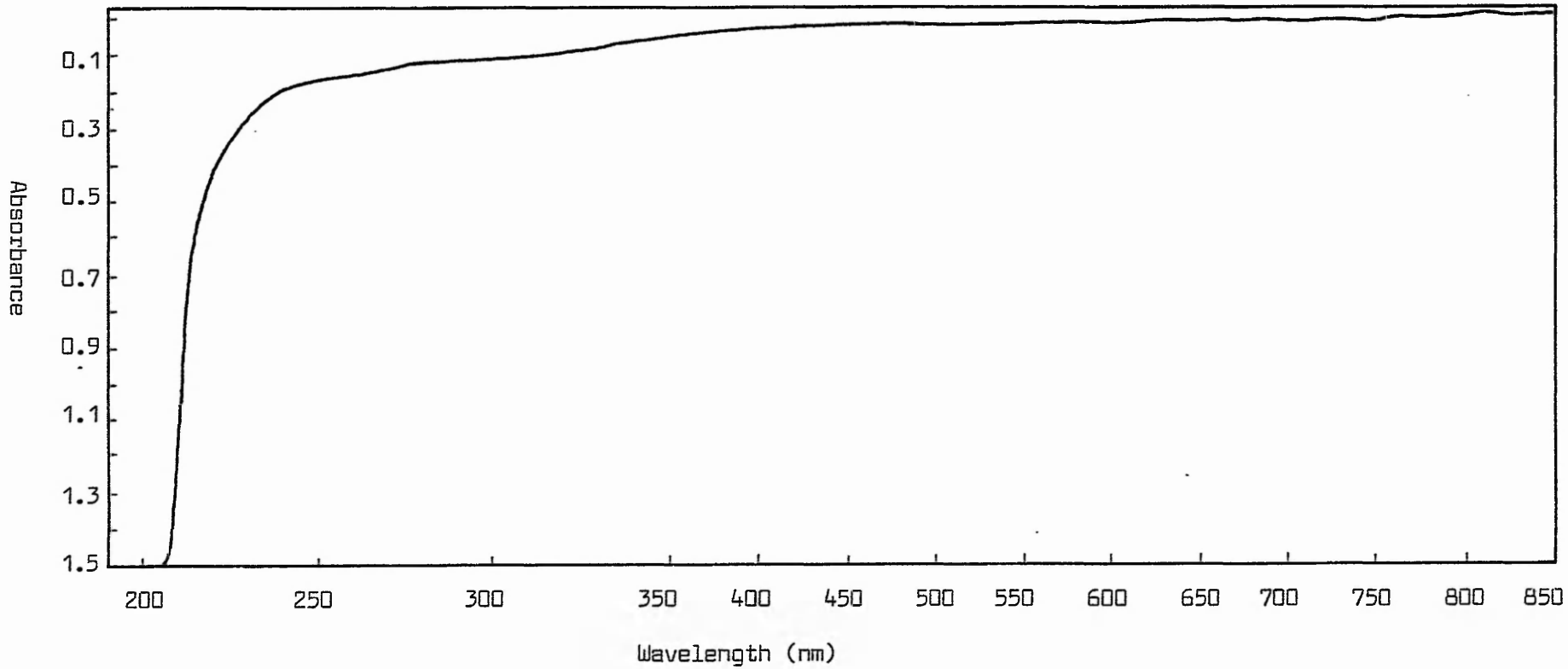


Figure 18. Uv/visible spectrum of chitosan film.

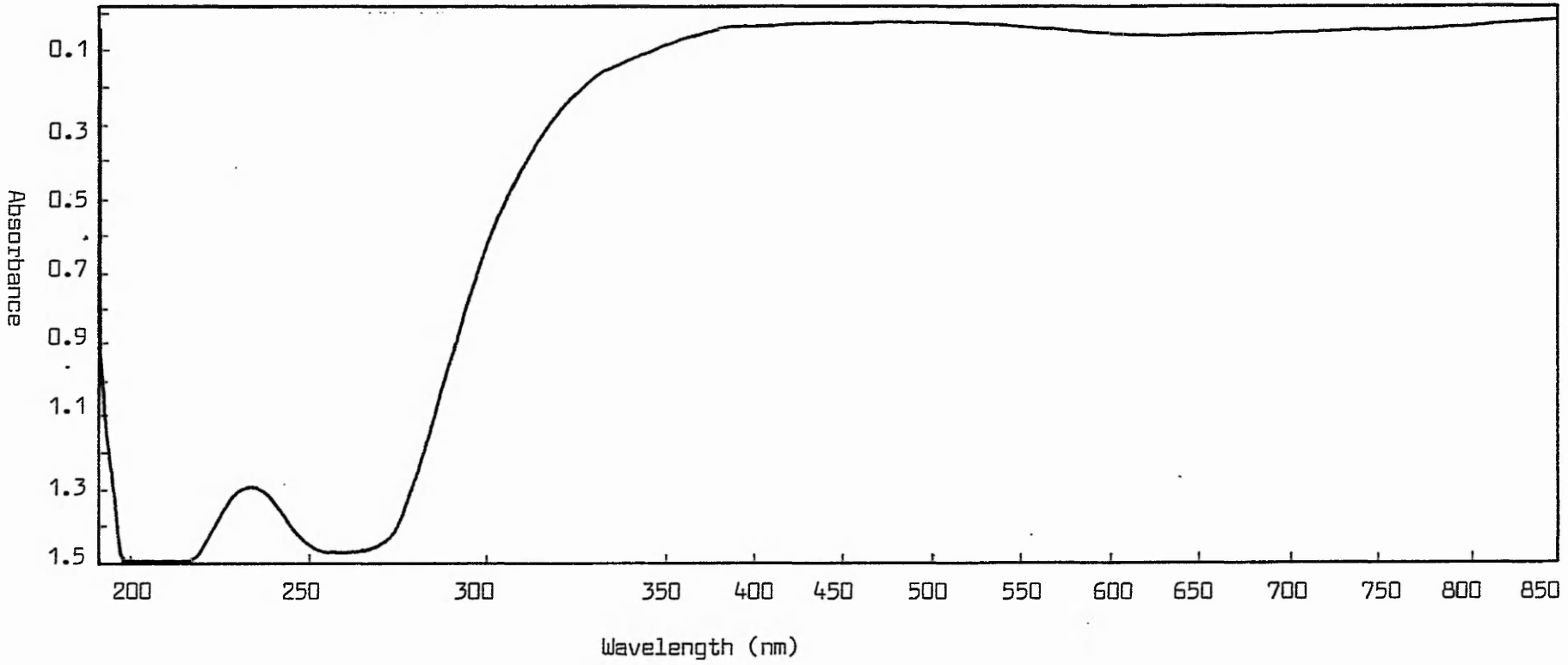


Figure 19. Uv/visible spectrum of chitosan film after treating with dilute cupric acetate solution.

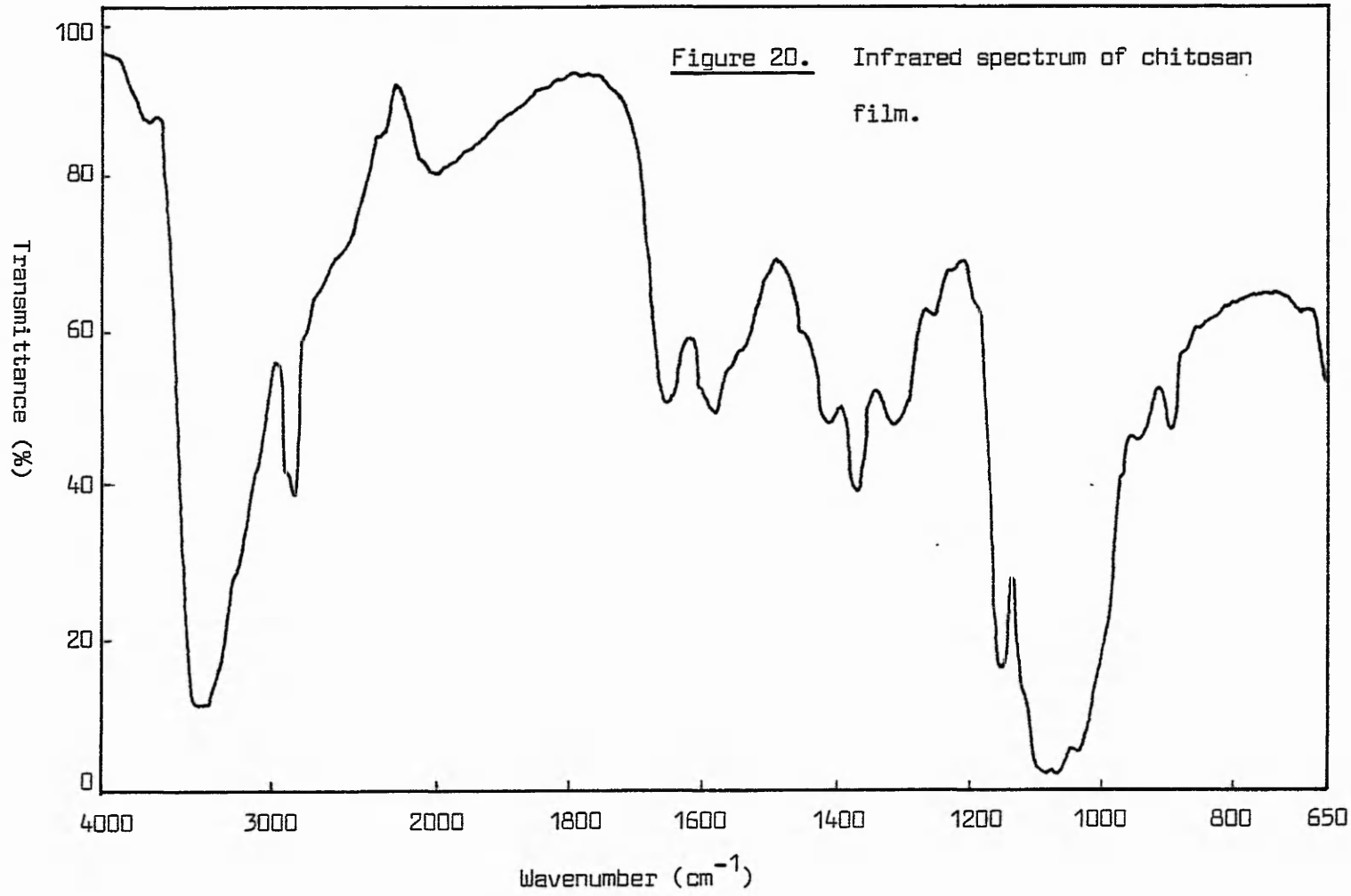


Figure 20. Infrared spectrum of chitosan film.

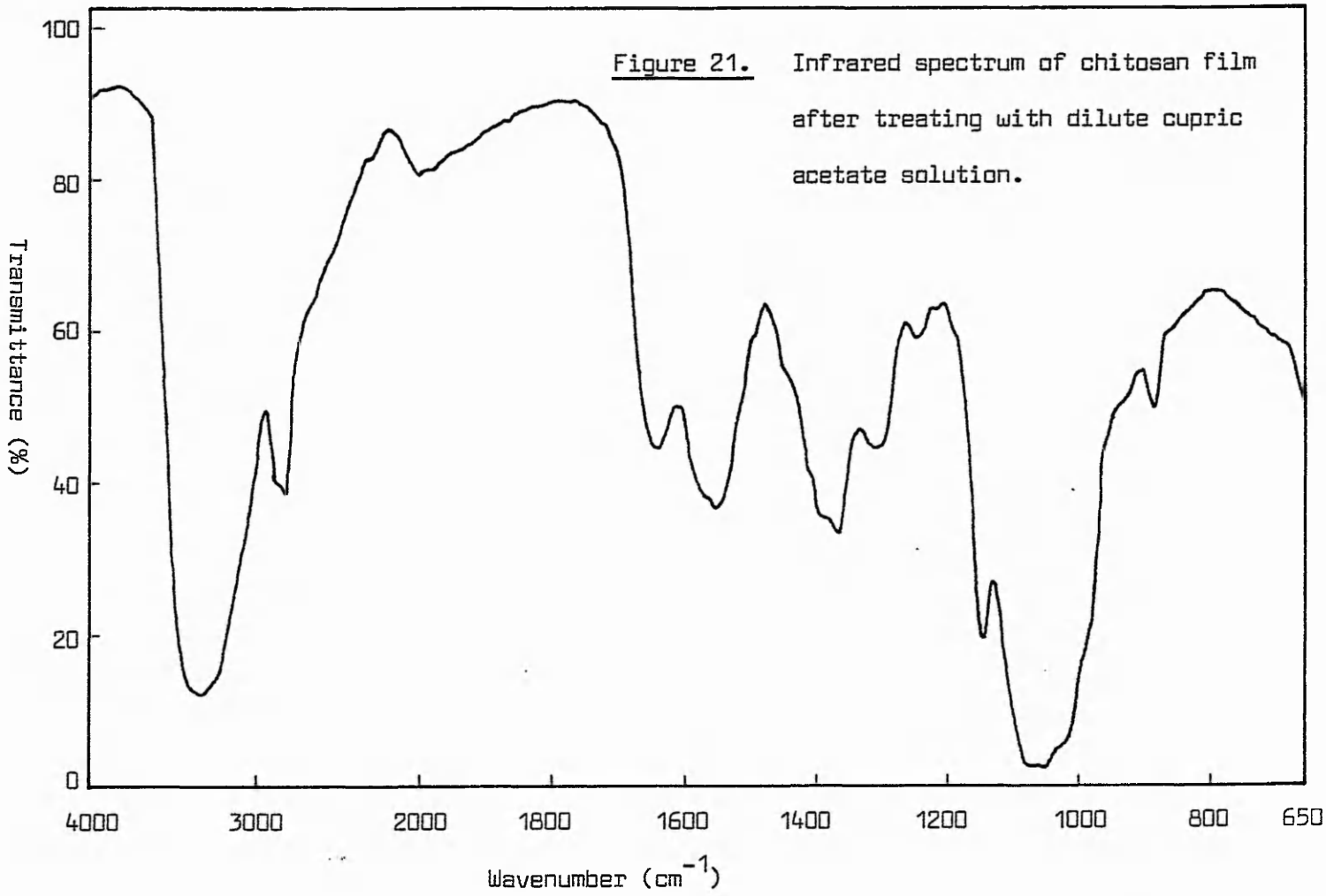


Figure 21. Infrared spectrum of chitosan film after treating with dilute cupric acetate solution.

monitoring Cu(II) ion uptake (Figure 22). The infrared spectrum (Figure 23) shows a number of additional sharp absorption bands, the most notable being at 1500cm^{-1}, 1260cm^{-1} and 1200cm^{-1}. The fact that the complexed copper-containing film was deep brown in colour, whilst the treatment liquor remained colourless, indicates that no Cu(II) ions are removed from the film during the NaDCC treatment. Although this method gave excellent results at very low concentrations of Cu(II) ion uptake, at higher concentrations the absorbance at 450nm became too intense whilst a less intense band at 650nm was too broad for quantitative measurements.

3.3.1.2 Aftertreatment with ammonium thiocyanate/pyridine

Treatment with ammonium thiocyanate and pyridine gives no appreciable change in the uv/visible spectrum of copper-containing chitosan films whilst the infrared spectrum shows a sharp absorption band at 2000cm^{-1} after treatment with the reagent and washing with water. However a similar band, though less intense, is obtained on treating copper-free chitosan film (Figures 24,25).

3.3.1.3 Aftertreatment with dimethylglyoxime

Treatment of copper-containing chitosan films with dimethylglyoxime in methanol results in the solution becoming intensely coloured, suggesting that the treatment is leeching out the Cu(II)/dimethyl-glyoxime complex. This was confirmed by giving a dimethylglyoxime-treated film a subsequent treatment with NaDCC. None of the absorption bands characteristic of the Cu(II)/NaDCC complex were observed in the infrared spectrum showing that the Cu(II) ions were no longer present in the film in detectable amounts.

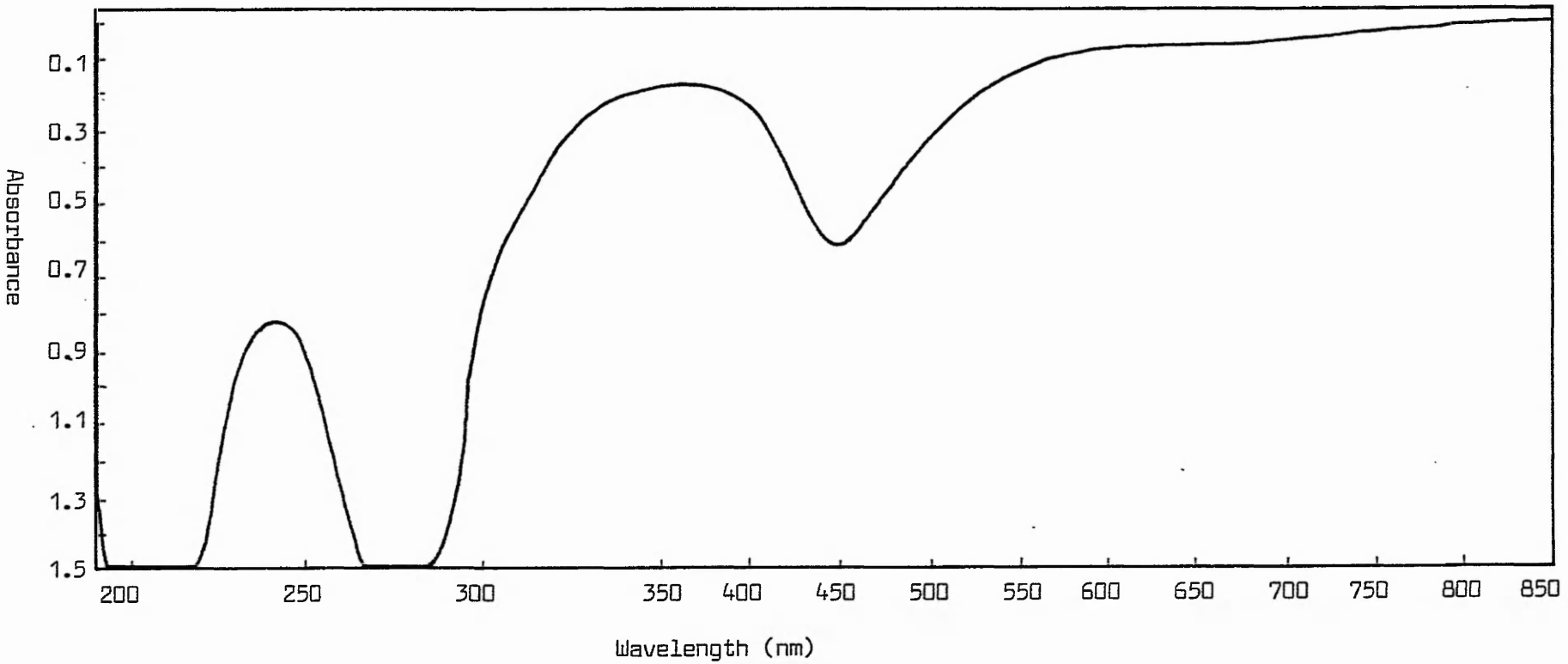


Figure 22. Uv/visible spcetrum of chitosan film treated with dilute cupric acetate solution, then treated with NaDDC.

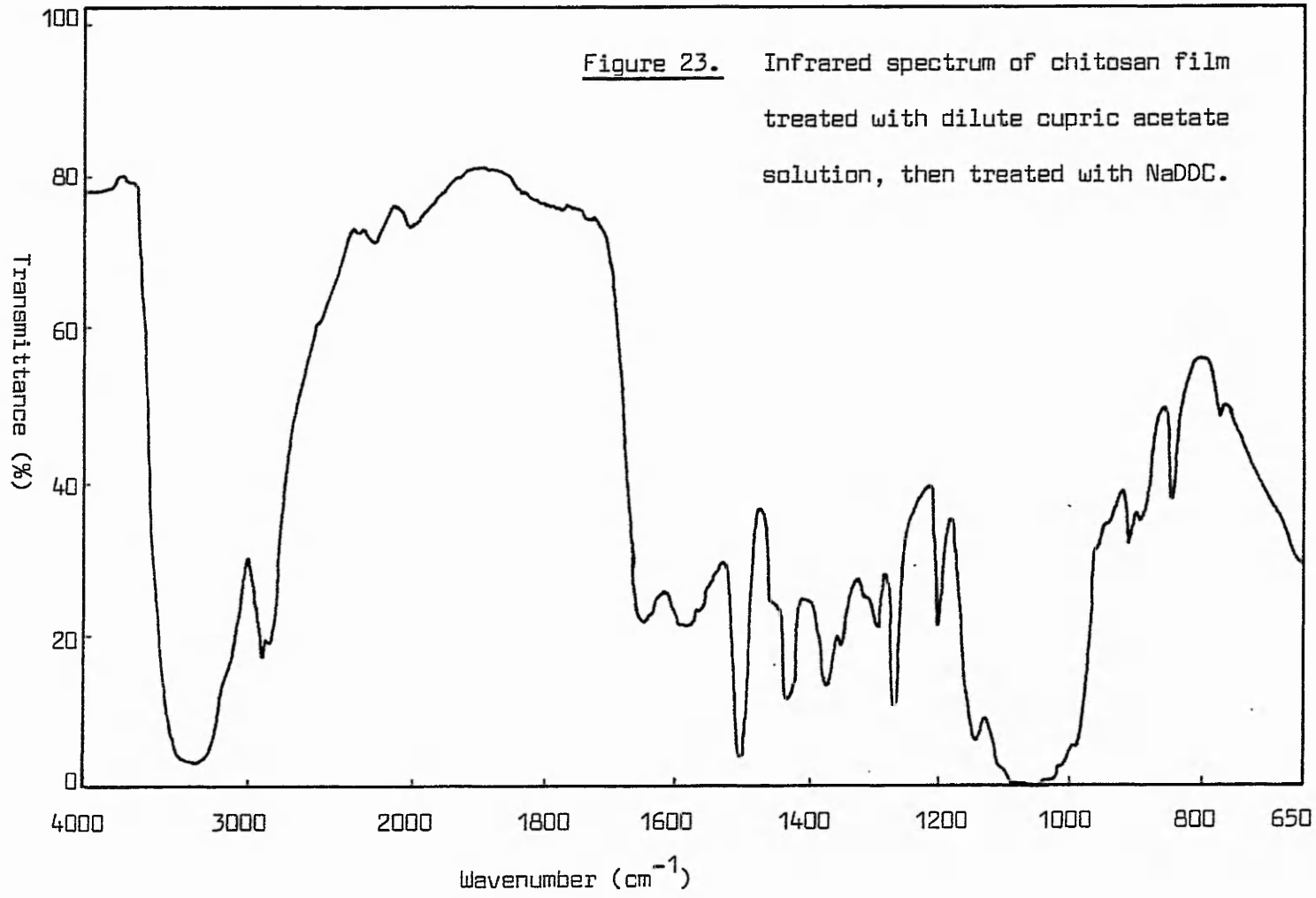


Figure 23. Infrared spectrum of chitosan film treated with dilute cupric acetate solution, then treated with NaDDC.

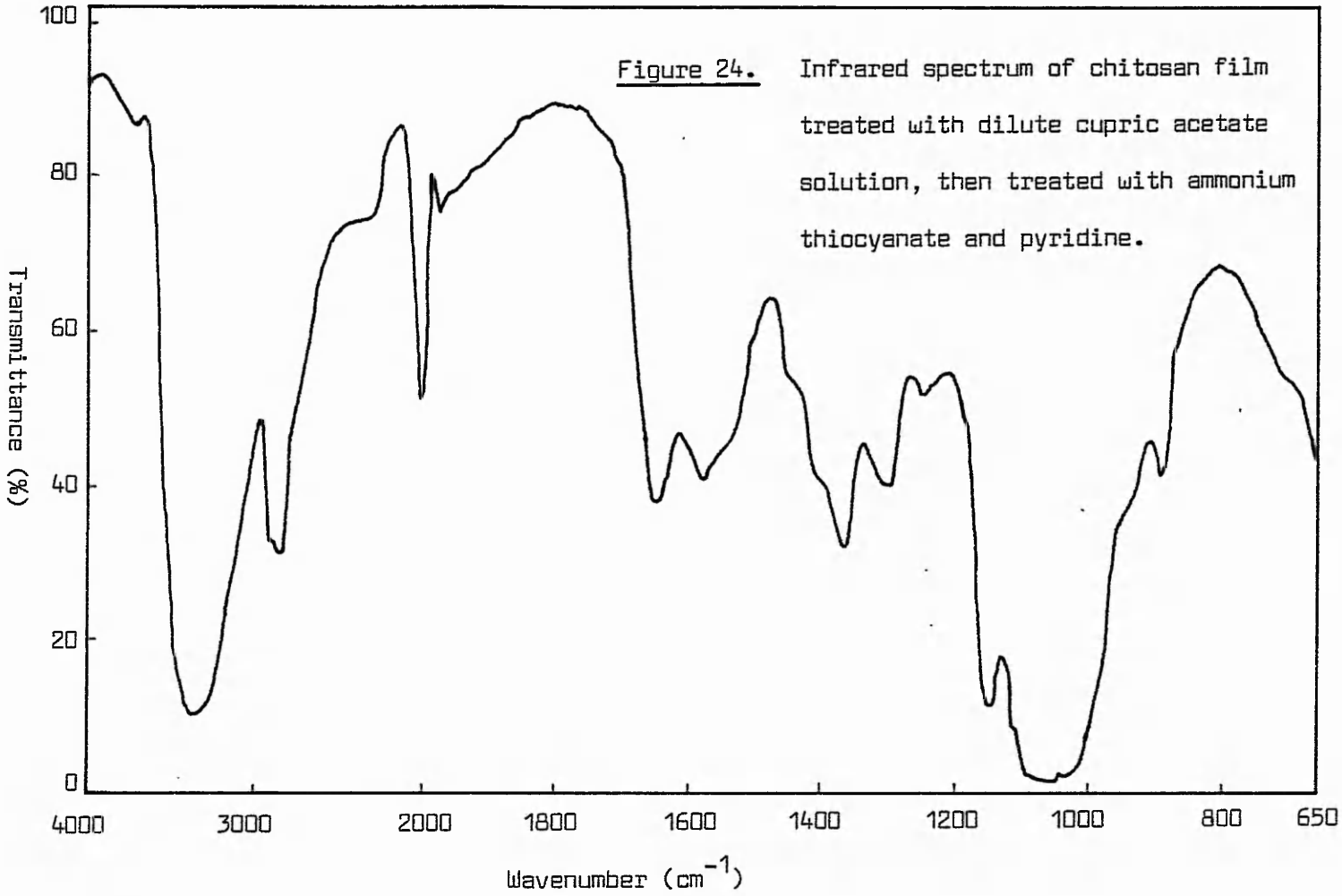


Figure 24. Infrared spectrum of chitosan film treated with dilute cupric acetate solution, then treated with ammonium thiocyanate and pyridine.

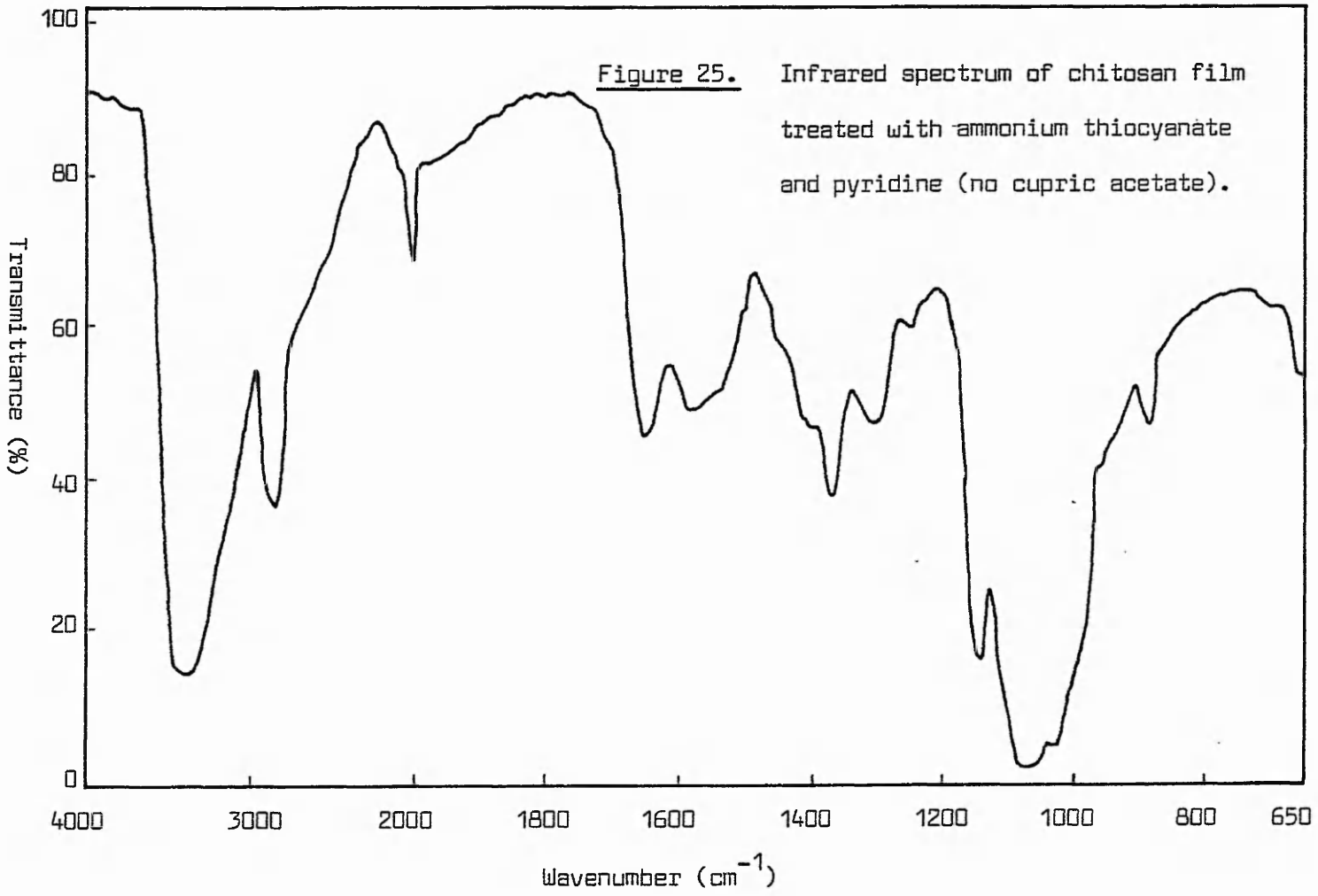


Figure 25. Infrared spectrum of chitosan film treated with ammonium thiocyanate and pyridine (no cupric acetate).

3.3.1.4 Aftertreatment with α-benzoinoxime

Treatment of copper-containing chitosan films with α-benzoinoxime (ABO) gives no observable changes in the infrared spectrum, but does give a sharp absorption band at 444nm in the uv/visible spectrum (Figure 26). Films treated in this way were green in colour whilst the treatment liquor was colourless on visual inspection. However spectroscopic analysis indicated that a small amount of the Cu(II)/ABO complex had been extracted from the film during the treatment.

3.3.1.5 Summary

The above results indicate that sodium diethyldithiocarbamate is the most suitable of the complexing agents studied as there is no evidence of any removal of Cu(II) ions from the film during the complexation stage, whilst the complex formed gives rise to suitable absorption bands in both the uv/visible and infrared spectra. α-Benzoinoxime also gives a complex with a suitable band in the uv/visible spectrum but with some indication of a slight amount of extraction of the Cu(II) ions during the treatment. Use was therefore made of these two reagents in studying the uptake of copper ions by chitosan films.

3.3.2 Adsorption of Cu(II) ions by chitosan film

Preliminary studies indicated that no appreciable increase in Cu(II) ion uptake occurred after 24 hours immersion in solutions of cupric acetate and so this treatment time was selected for use in subsequent investigations. The first factor chosen for study was the effect of film thickness on the Cu(II) ion uptake, using the infrared absorption band at 890cm^{-1} as an internal standard to correct for film thickness.

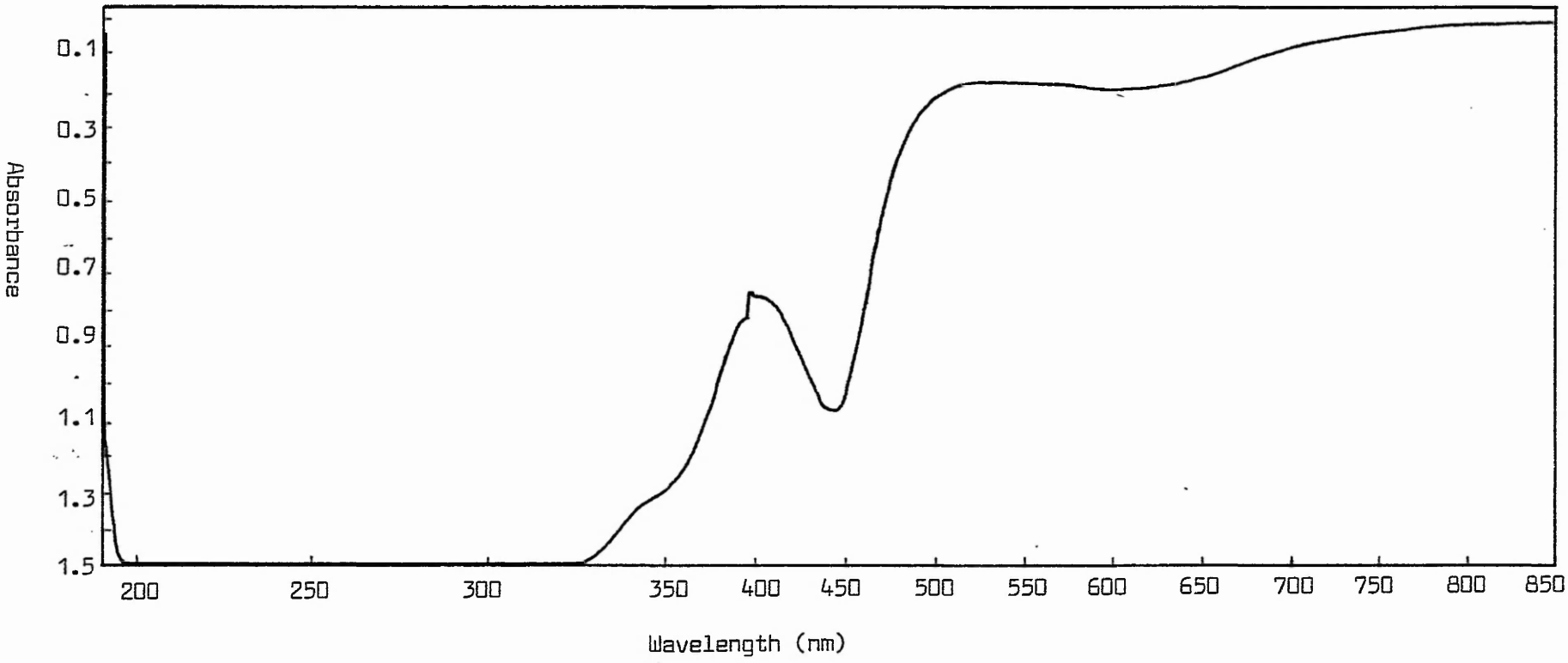


Figure 26. Uv/visible spectrum of chitosan film treated with dilute cupric acetate solution, then treated with α-benzoinoxime.

A preliminary check confirmed that there was good correlation between the film thickness, as measured using a Mercer film thickness gauge, and the absorbance of the 890cm^{-1} band.

Samples of chitosan film of varying thicknesses were suspended in well stirred solutions of cupric acetate covering a range of concentrations. After 24 hours the samples were removed, washed to remove excess cupric acetate solution and treated with either NaDCC or ABO. The absorbance values of the films were measured at 450nm (NaDCC) or 444nm (ABO), together with the absorption values of the 890cm^{-1} in the infrared. The results are shown in Figures 27 and 28 and clearly demonstrate that with all the solution concentrations used there is a decrease in the absorbance, corrected for film thickness, with increase in film thickness. Furthermore, films steeped in more concentrated cupric acetate solutions became brittle and showed considerable contraction, and developed relatively less colour on treatment with complexing agents. This inverse relationship between corrected absorbance and film thickness is in agreement with the results of Blair and Ho[116] who found that the equilibrium adsorption of Cu(II) ions decreased with increase in film thickness, and also commented on the greater rigidity and brittleness of cupric sulphate-treated chitosan film compared with untreated film.

Assuming that equilibrium uptake has been reached within the 24 hour treatment time, these results suggest either that the Cu(II) ion concentration is not uniform throughout the film but instead there is a concentration gradient from surface to centre, or that cross-linking of the chitosan chains by the Cu(II) ions prevents the chelating reagent penetrating fully into the film. The results of Blair and Ho[116] support the former conclusion as they determined

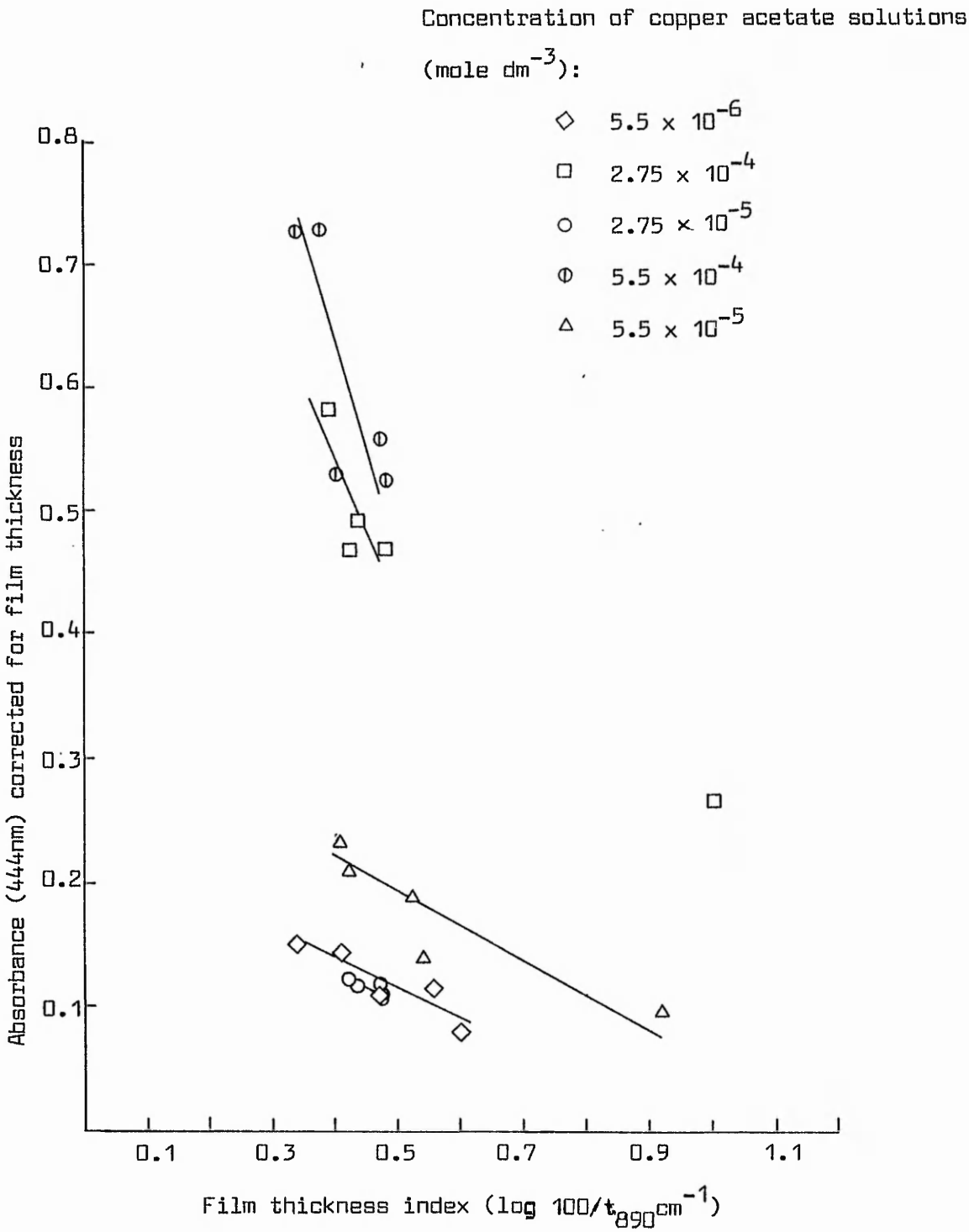


Figure 27. Absorbance of chitosan films treated with copper acetate and aftertreated with α-benzoinoxime.

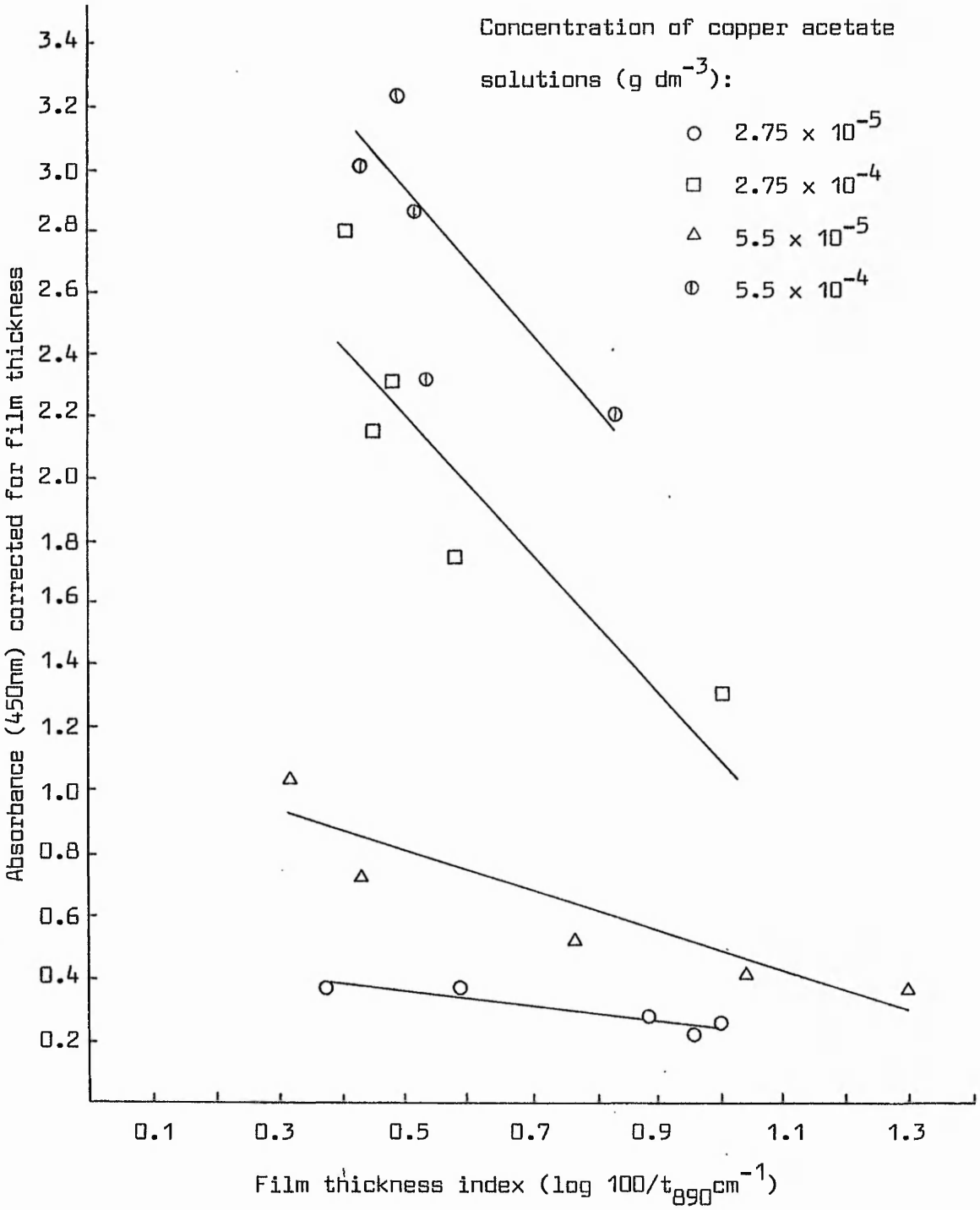


Figure 28. Absorbance of chitosan films treated with copper acetate and aftertreated with sodium diethyldithiocarbamate.

the Cu(II) ion uptake by change in concentration of the cupric sulphate solution and so their results did not depend on subsequent penetration of the film by a complexing reagent. Furthermore, even the films with the greatest Cu(II) ion uptake allowed the diffusion of a dye (C.I. Acid Orange 10) through them[116]; therefore it is reasonable to expect that the smaller complexing reagent molecules could also penetrate.

Establishing unequivocably the presence of a concentration gradient within the film would require the use of specialised techniques, such as microdensitometry, that can scan a cross-section of the film. No such technique was available but subsequent to this work Averbach[231] reported the determination of the distribution of Cu(II) ions within chitosan films using scanning electron microscopy. In his work the films were treated in solutions of cupric sulphate for up to 200 hours but even with these treatment times uniform Cu(II) ion distributions were not obtained, the concentration decreasing with increase in the distance from the film surface. Despite the non-uniformity of Cu(II) ion distribution the sulphate ions were found to have a uniform distribution throughout the film.

3.3.3 Adsorption of complexed Cu(II) ions by chitosan film

In view of the possibility that cross-linking of chitosan polymer chains by Cu(II) ions occurs, the adsorption of Cu(II) ions that were already complexed was studied. For this the Cu(II) ions were complexed with iminodiacetic acid, disodium salt (IDA), a tridentate ligand. In this form the Cu(II) ions require only a single additional ligand to complete their co-ordination number so chitosan can only function as a monodentate ligand - see Figures 29 and 30.

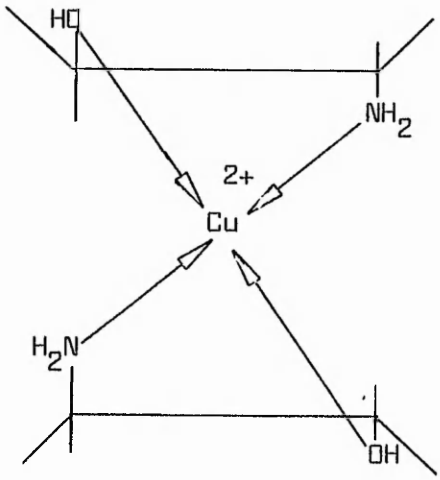


Figure 29. Possible structure for chitosan cross-linked by Cu(II) ions.

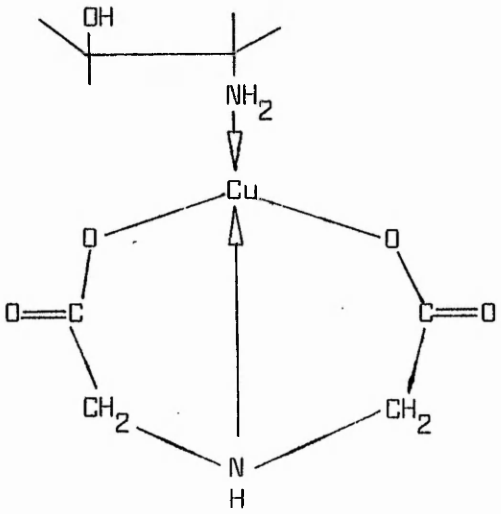


Figure 30. Cu(II)/IDA complex bound to chitosan.

Preliminary studies using cupric sulphate showed that the values of λ max and εmax of Cu(II)/IDA solutions are highly dependent on the Cu(II):IDA ratio, with λmax moving towards lower wavelengths with increase in IDA (Figure 31). . For this reason the studies were carried out using a Cu(II):IDA ratio of 1:1 although these solutions showed some instability, leading to the formation of a

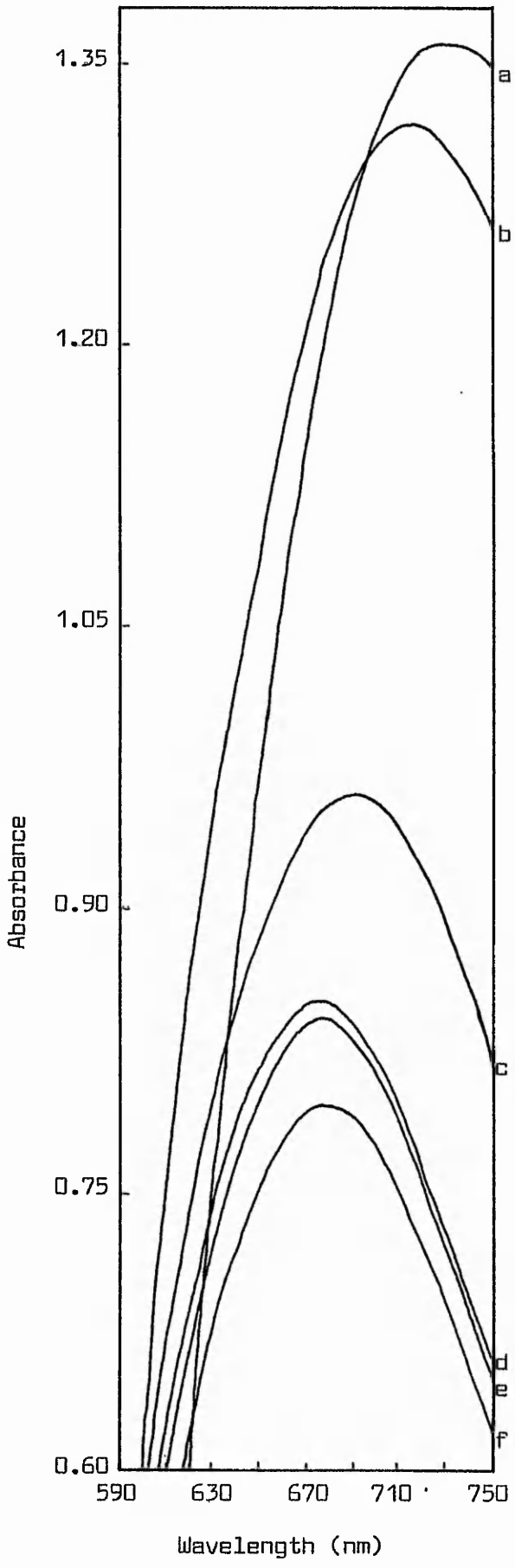


Figure 31. Visible spectra of cupric sulphate/IDA solutions (constant cupric sulphate concentration).

Cupric sulphate:/IDA ratios:

a 1:1

b 1:1.5

c 1:2

d 1:4

e 1:5

f 1:6

precipitate on standing. A separate calibration plot was required for the cupric sulphate:IDA solution which had a much higher extinction coefficient than solutions of cupric sulphate alone.

A sample of film was immersed in an aliquot of the solution of Cu(II)/IDA and the concentration of complexed Cu(II) ions remaining after 24 hours determined spectrophotometrically. The uptake of Cu(II), measured in this way, was calculated to be 64.5g/free amine equivalent weight, which within experimental error is very close to a Cu(II):amine group ratio of 1:1. This demonstrates that when conditions are chosen so as to restrict the role of chitosan to that of a monodentate ligand, complete reaction of the amine groups takes place.

3.4 Dyeing of chitosan films

Studies of the uptake of dye by chitosan films are relatively rare (Section 2.8) and the extent of N-acetylation of the polymer chains has not been adequately characterised in the reports of dyeing experiments. As an initial step towards an examination of this variable, chitosan film, N-acetylated chitosan film and N-acetylated/de-O-acetylated chitosan films were dyed with the mono-sulphonate dye C.I. Acid Red 88 (C.I. AR88, Structure I in Figure 41).

The free acid form of the pure dye (C.I. Constitution No. 15620) was used in the absence of additional electrolyte. Samples of each of the three types of chitosan film (undyed) were also subjected to the Rimini test[40] or to Schiff's base formation with salicylaldehyde - both tests give positive results in the presence of free amine groups. The results of the tests are summarised in Table 6, and in Figures 32 and 33.

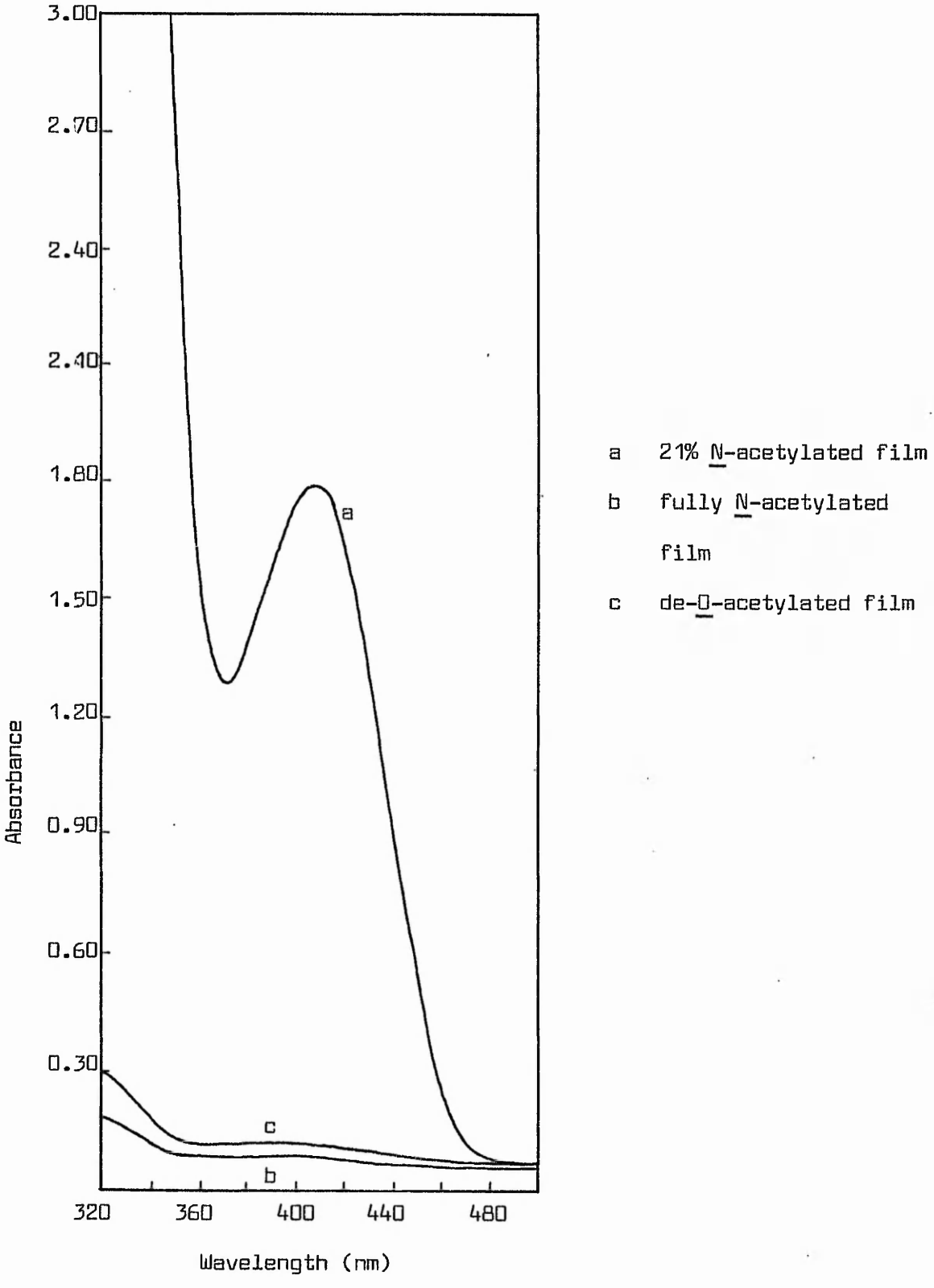


Figure 32. Uv/visible spectra of chitosan films treated with salicylaldehyde.

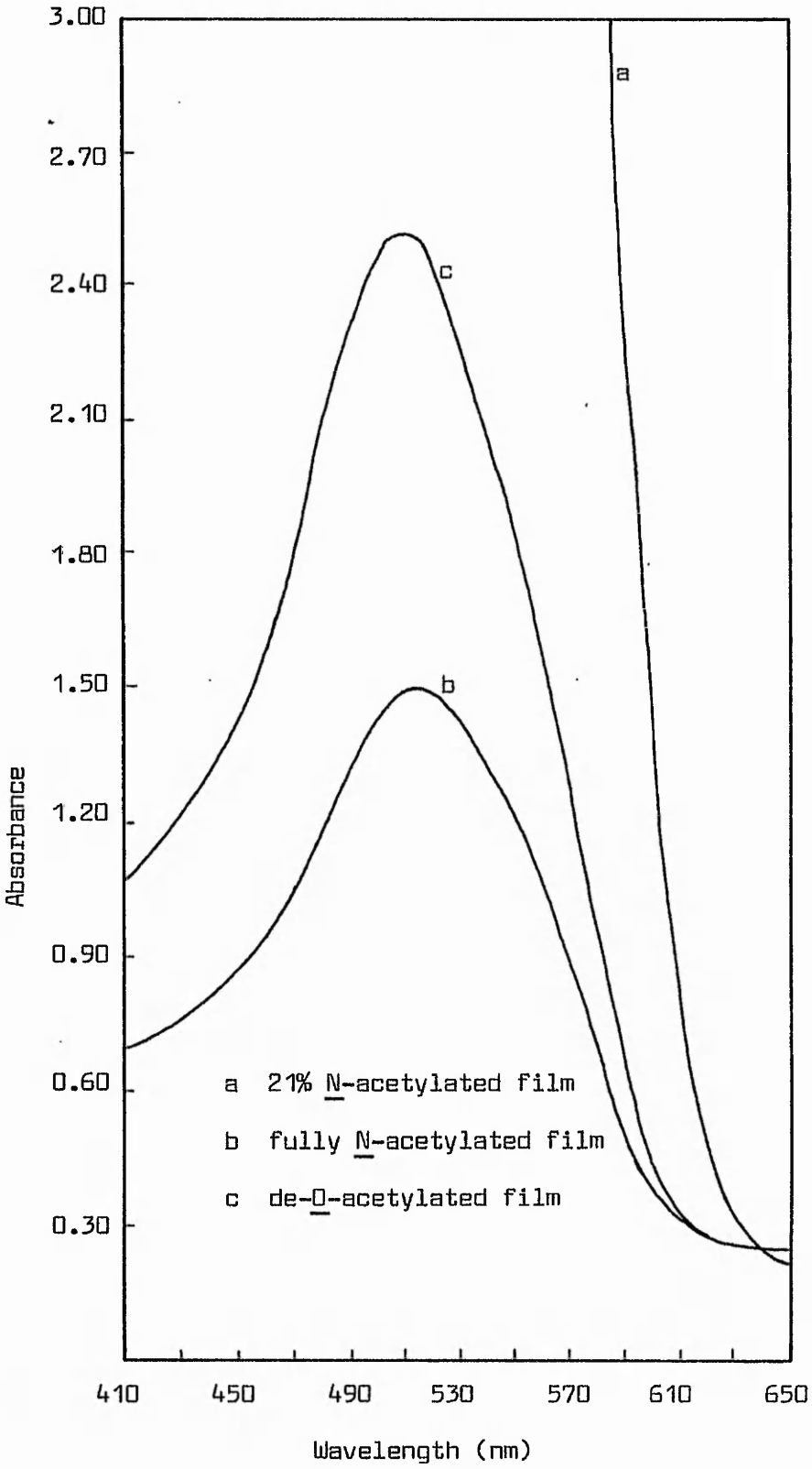


Figure 33. Visible spectra of chitosan films treated with AR88

	chitosan film	N-acetylated chitosan film	N-acetylated/ de-O-acetylated chitosan film
C.I. Acid Red 88	deep red	pale pink	pink
Rimini test	+	-	-
Schiff's base	+	-	+

Table 6. Effect of N-acetylation of chitosan film.

The peak at 405nm in the spectrum in Figure 32 is due to the Schiff's base formed by reaction of free amine groups with salicylaldehyde. Although pronounced in the spectrum from the chitosan film, the band is barely detectable in the spectra from the N-acetylated and N-acetylated-de-O-acetylated films. Similarly the absorption band at 510nm in the spectra in Figure 33, due to adsorbed dye, is much less pronounced in the spectra from the two treated chitosan films than in the spectrum from the chitosan film. However in each case, salicylaldehyde treatment and C.I. Acid Red 88 treatment, the relevant absorption band is more pronounced after the de-O-acetylation treatment, showing that some de-N-acetylation also occurs even though it is not detectable using the Rimini test[232], whilst there is a low amine group concentration in the acetylated chitosan film even prior to the de-O-acetylation treatment.

Further dyeings were carried out on N-acetylated chitosan film using the free acid form of dye (I). It was found that uptake of dye was reduced by the addition of formic acid (1% o.w.f.), a 2% dyeing in the presence of formic acid showing lower absorbance than a 1% dyeing in the absence of formic acid, even though the

dyebath pH was lower in the former (pH 3.8-4.0) than the latter (pH 5.1) - Figure 34. This suggests that the formate anions compete with the dye anions for the few available dye sites, thereby reducing the dye uptake. However if dye (I) was used in the sodium salt form the behaviour was reversed and dye uptake was increased by addition of formic acid (Figure 35).

A series of dyeings was carried out using a range of dye concentrations based on the weight of film being dyed (o.w.f.), and uptake of dye was calculated from measurement of the absorbance of the dyebath before and after dyeing. The films adsorbed from 4-7 times as much dye from the dyebaths of high dye concentration as they adsorbed from the dyebath of lowest concentration, even though the latter was not exhausted, only approximately 38% being adsorbed. A plot of Dye adsorbed versus % Dye applied (Figure 36) shows an initial curve followed by a linear portion. These results suggest that there are two adsorption mechanisms operating:

i) specific site adsorption onto the protonated amine groups;

ii) diffuse adsorption at random points along the chain.

In order to study the site adsorption mechanism in the absence of the second mechanism, dyeings were carried out on the unmodified film since the concentration of dye sites is so great it is reasonable to assume that all the dye taken up will be adsorbed at specific sites. For the first set of dyeing experiments an acetic acid/sodium acetate buffer was used to control the pH of the solution of the free acid form of (I). In one of the early runs the film dissolved and this was accompanied by a visual change in the colour from red to orange. When measured spectrophotometrically it was found that

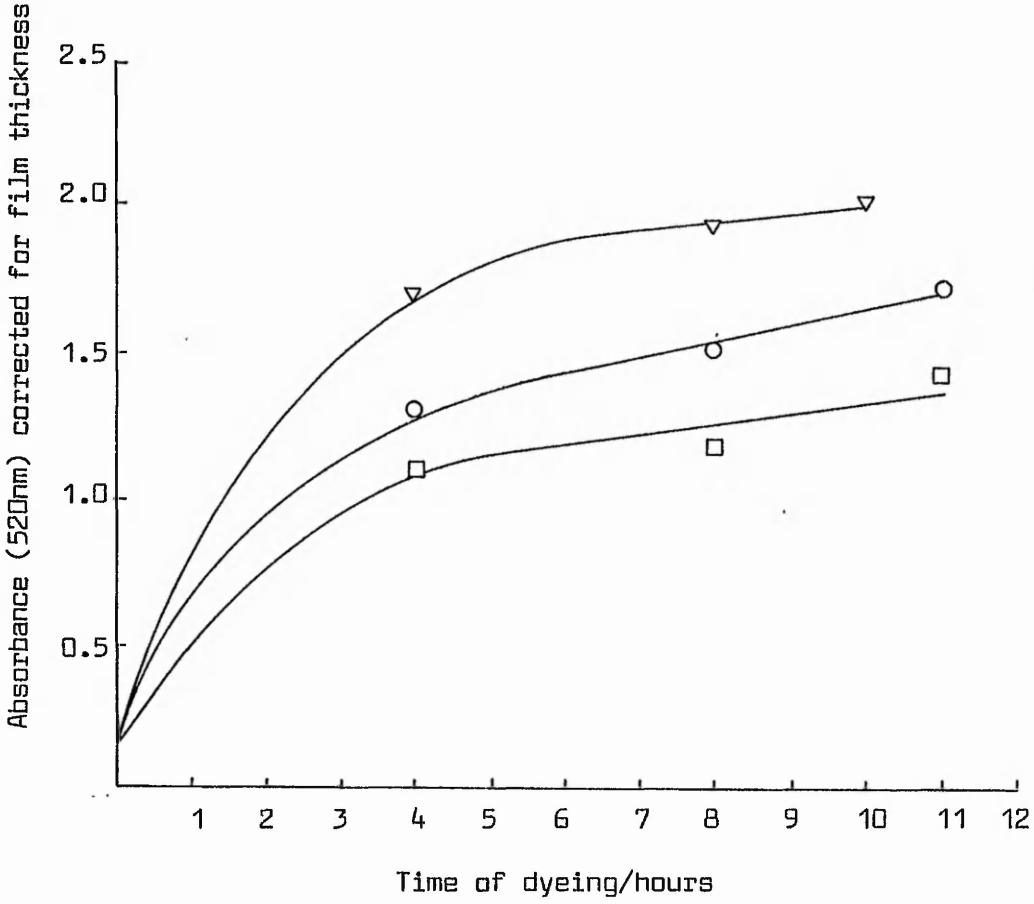


Figure 34. Absorbance of chitosan films dyed with C.I. Acid Red 88 (free acid form).

□ 1% owf AR88, 1% owf formic acid

○ 2% owf AR88, 1% owf formic acid

▽ 1% owf AR88, no formic acid

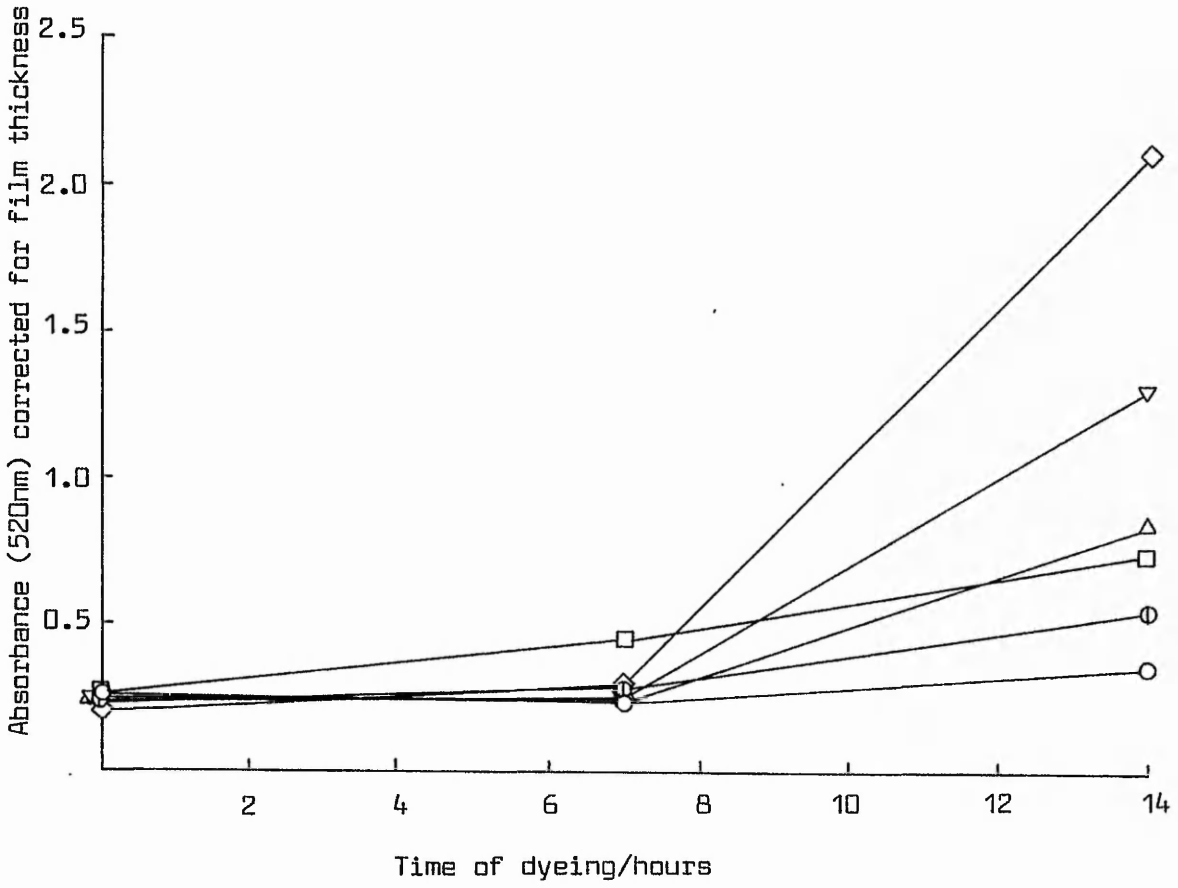


Figure 35. Absorbance of chitosan films dyed with C.I. Acid Red 88 (sodium salt), 2% owf formic acid added after dyeing for 7 hours.

○ 0.5% owf AR88

⊕ 1.0% owf AR88

□ 2.0% owf AR88

◇ 3.5% owf AR88

▽ 5.0% owf AR88

△ 10.0% owf AR88

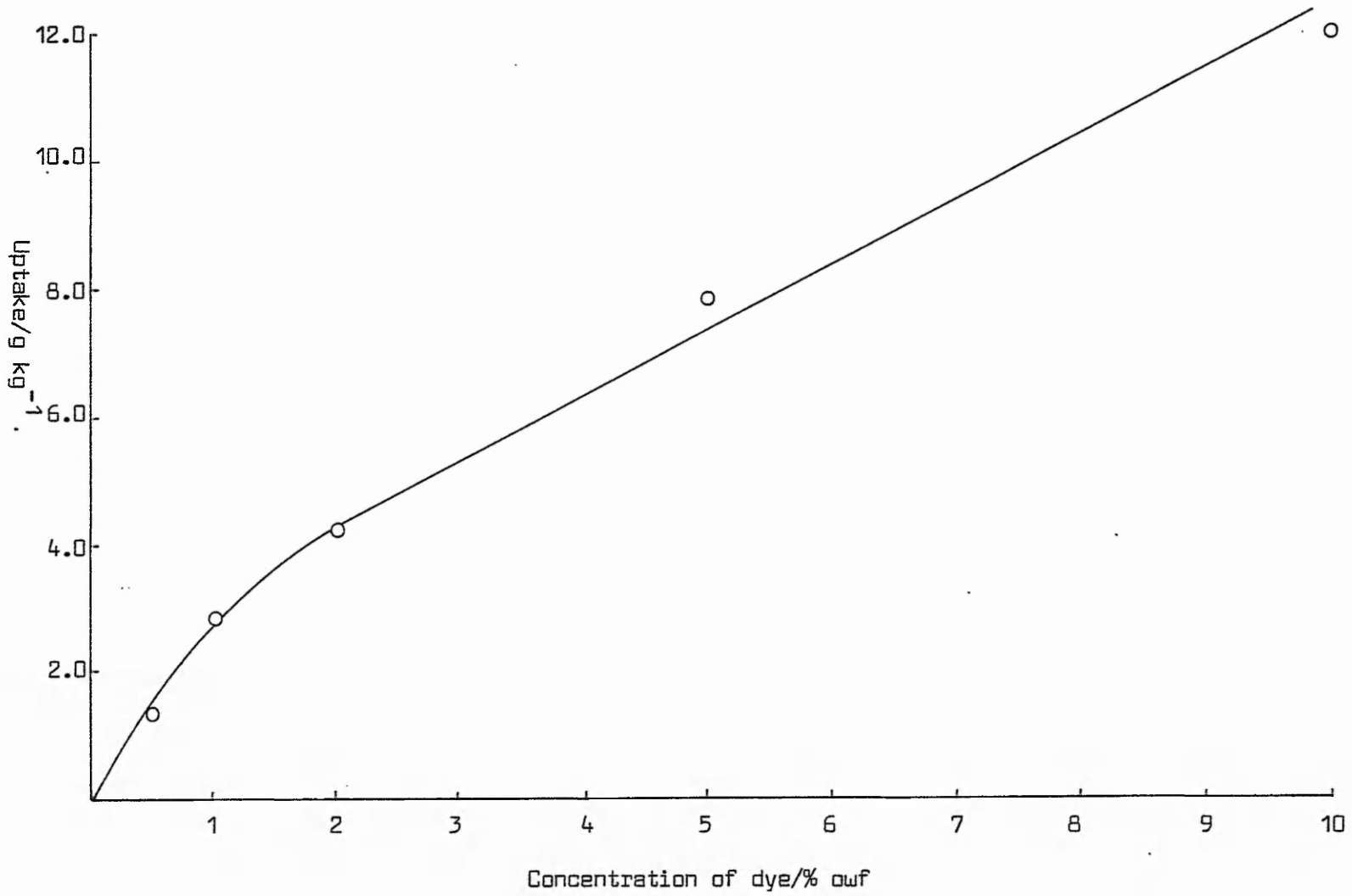


Figure 36. Uptake of C.I. Acid Red 88 by chitosan film.

dissolution of the chitosan film had caused a hypsochromic shift in λ max from 505nm to 450nm. This shift was reproducible and was also demonstrated on mixing a solution of chitosan with a solution of (I). The mixed solution was orange but reverted to red as the film formed on drying.

In view of the importance of this finding and its potential usefulness in studying metachromasy, all further work was directed towards investigating the metachromasy-inducing behaviour of chitosan.

3.5 Metachromatic studies on chitosan

3.5.1 Introduction

It was indicated in Section 2.9.7 that reports of metachromatic reactions between polycations and anionic dyes are rare, and the observed change in colour of the anionic dye C.I. Acid Red 88 described in Section 3.4 was thus of great interest. Furthermore, the possibility of using the metachromatic interaction between chitosan and the dye as a novel, facile means of measuring the degree of N-acetylation of the polymer - within the soluble range - was of prime importance. The wide range of types of anionic dyes gave an opportunity to study the relationship between dye structure and metachromatic behaviour, and the behaviour of chitosan/dye solutions in the presence of electrolytes, urea and ethanol was of interest in view of the work carried out on anionic polymer/cationic dye solutions. Finally, it was hoped that the effect of the molecular weight of the polymer, and the behaviour of some dibasic dyes in the presence of chitosan, would support a new theory of metachromasy.

3.5.2 The metachromatic titration of chitosan

Figure 37 shows the spectra of C.I. Acid Red 88 (AR88) and of AR88 + chitosan. Whereas the peak absorbance for the dye alone occurs at 505nm, in the presence of the polymer the maximum shifts downfield to 450nm, and visually the solution changes from red to orange.

When aliquots of dye of known concentration were mixed with various concentrations of chitosan and diluted to standard volume with 0.1M acetic acid, the series of spectra shown in Figure 38 were obtained. The absorbance values at 450nm and 505nm were plotted to show the shift in λmax in relation to polymer concentration (Figure 39), and both peaks were found to reach steady absorbances at the same point. This point coincided with a 1:1 molar ratio of AR88 to chitosan free amine. The fact that the metachromatic reaction between chitosan and AR88 is stoichiometric suggested that it could be used either to determine the concentration of chitosan of known degree of N-acetylation, or to determine the degree of N-acetylation of a chitosan sample.

3.5.2.1 Determination of chitosan concentration

A sample of chitosan (Kypro) of known degree of acetylation was dissolved in 0.1M acetic acid and the solution used to titrate a solution of AR88 of known molarity, using the decrease in the absorbance of the 505nm band to follow the metachromatic behaviour. From the volume of chitosan solution required to reach a steady absorbance, the number of moles of dye present and the equivalent weight of the amine groups in the chitosan sample, the concentration

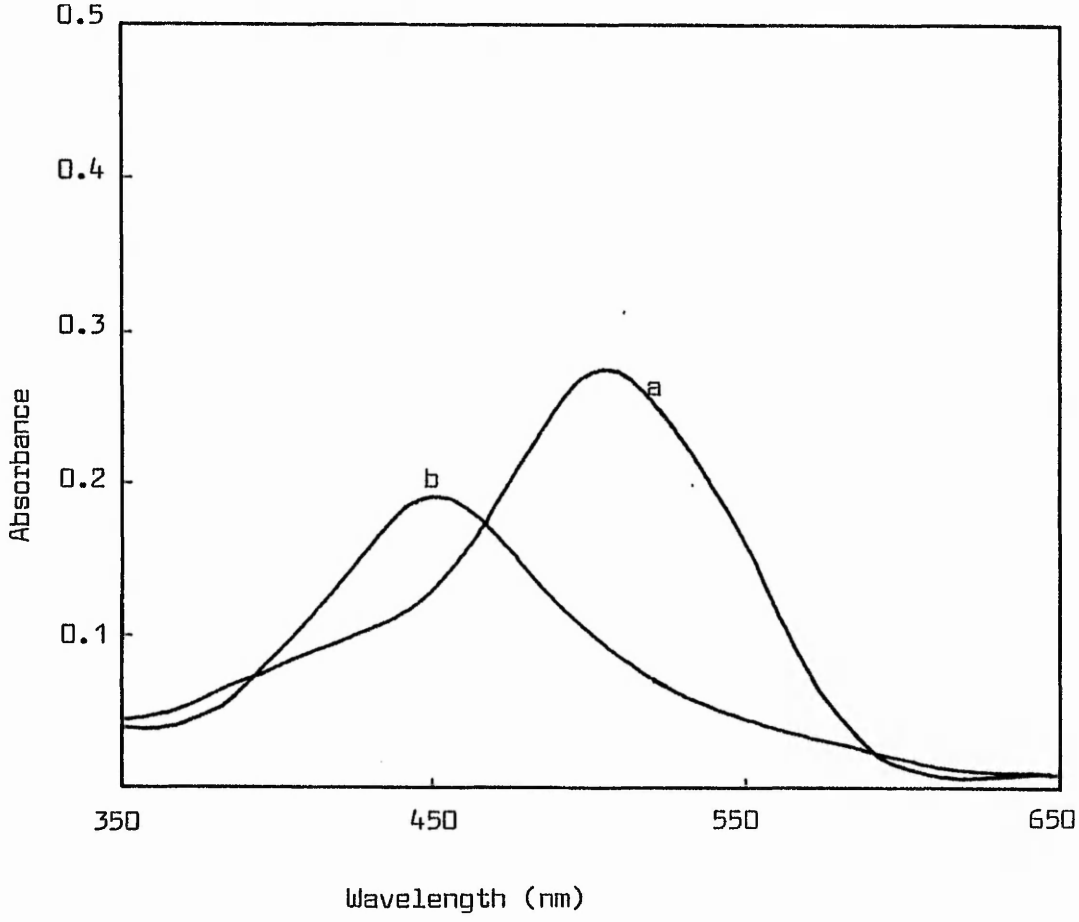


Figure 37. Metachromasy of AR88 in the presence of chitosan.

a spectrum of dye solution (1.5×10^{-5} M)

b spectrum of dye + polymer (P/D = 1:1)

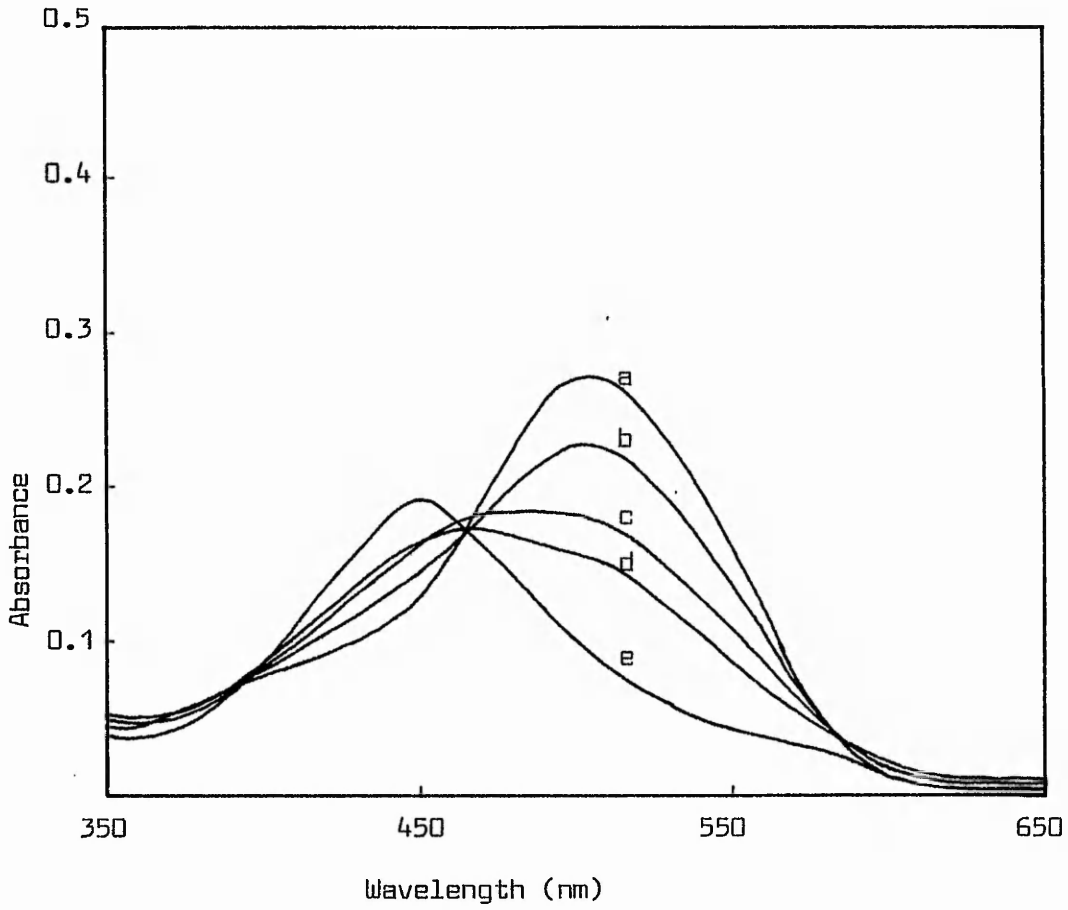


Figure 38. Metachromasy of AR88 in the presence of chitosan, showing the hypsochromic shift in λmax with increasing P/D ratio.

P/D ratios:

a 1:1

b 0.25:1

c 0.5:1

d 0.75:1

e 1:1

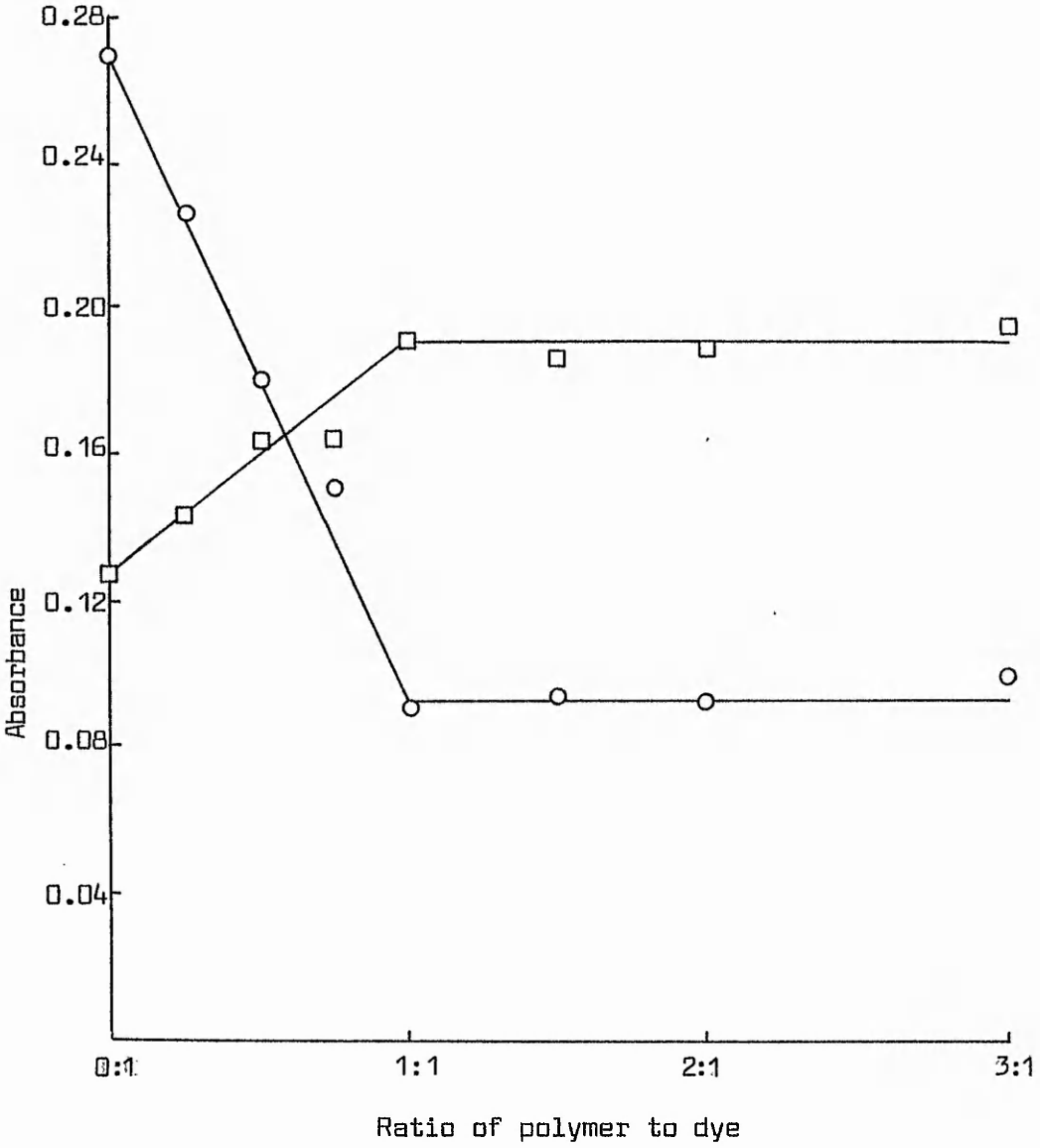


Figure 39. Change in absorbance of C.I. Acid Red 88 in the presence of chitosan solution (1.5×10^{-5}M dye solution).

○ Absorbance at 505nm

□ Absorbance at 450nm

of chitosan in solution was calculated. This was carried out for four solutions and the results are given in Table 7.

Table 7

Actual and measured concentrations of chitosan in solution.

Actual conc./g dm^{-3}	Measured conc./g dm^{-3}
0.887	0.915
0.0583	0.0586
0.0389	0.0395
0.0194	0.0196

3.5.2.2 Determination of degree of N-acetylation of chitosan

Samples of chitosan covering a range of degrees of N-acetylation were prepared by homogeneous N-acetylation of chitosan in solution in aqueous methanol[103]. Films were cast from each sample and analysed for degree of N-acetylation by infrared spectroscopy using the absorbance at 1655cm^{-1} as a measure of the amide group content and that at 3450cm^{-1} as an internal reference to correct for film thickness[107]. The samples were also analysed for free amine content, and from this for degree of N-acetylation, by metachromatic titration. The results are given in Table 8 and in graphical form in Figure 40, from which it can be seen that there is a good correlation between the results from the two techniques.

The accuracy of the infrared spectroscopic technique has recently been demonstrated by its correlation with other techniques[103,234] so that Figure 40 confirms the accuracy of the metachromatic titration method. The metachromatic method is now in regular use in this

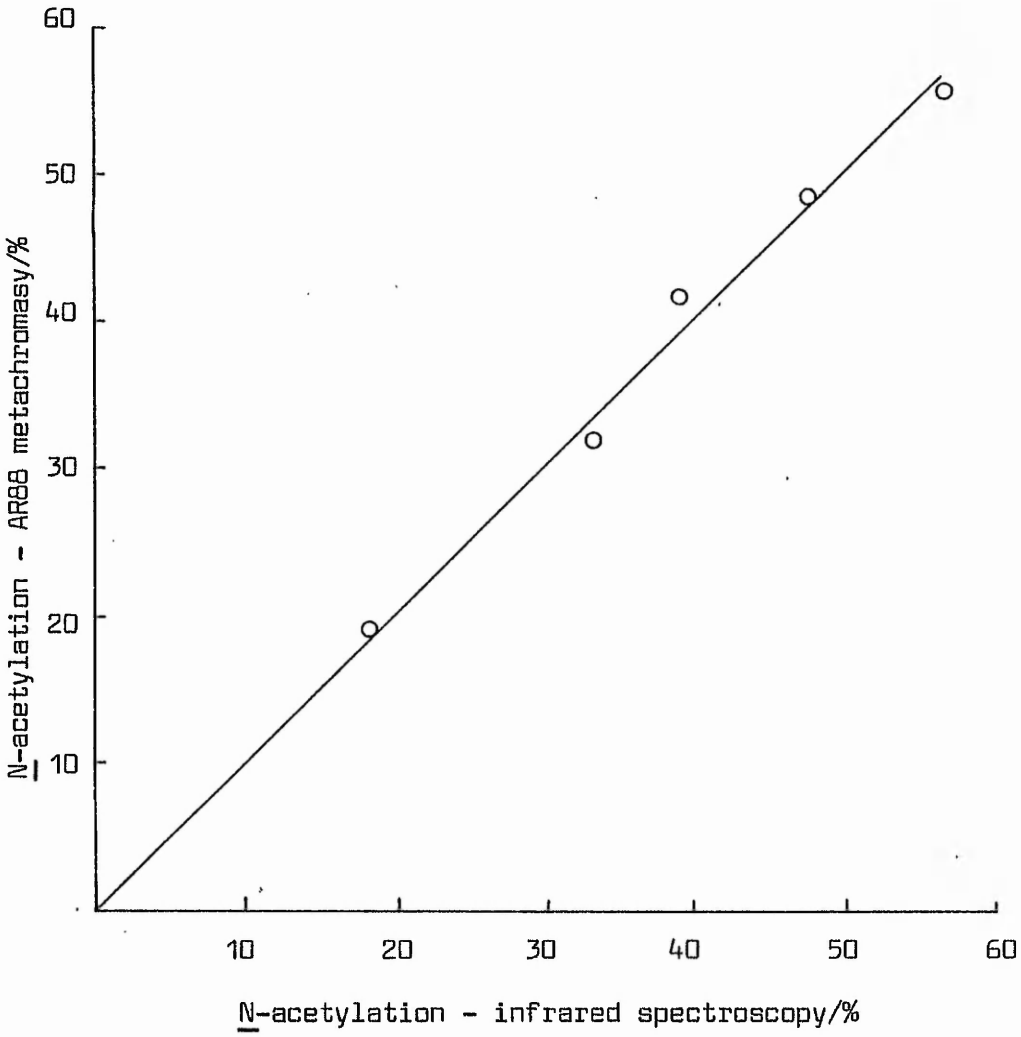


Figure 40. Comparison of measurements of N-acetylation of chitosan by infrared spectroscopy and metachromasy.

Table 8

Comparison of percent N-acetylation of chitosan samples calculated from infrared spectroscopy or metachromatic titration.

	percent N-acetylation	
Chitosan sample	IR	AR88
A	18.20	19.0
B	33.04	31.7
C	38.67	41.6
D	47.31	48.3
E	56.59	55.5
F	59.20	58.7

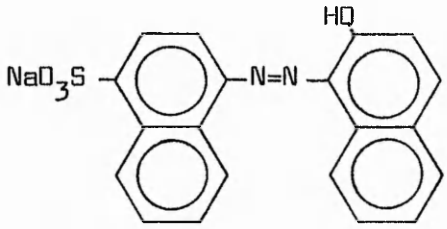
laboratory, giving reproducible and rapid results. It is also used to calculate the concentration of a solution of known degree of N-acetylation. The method is given in full in Section 5, together with the method of calculating the free amine equivalent weight from the percentage N-acetylation and the conversion graph derived from this calculation. A sample calculation is given in Appendix I.

3.5.3 Survey of anionic dyes

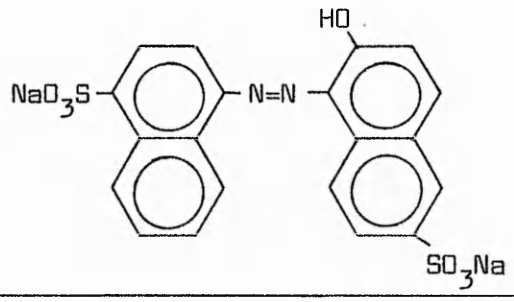
Having established that chitosan induces metachromatic change in the uv/visible spectrum of AR88, it was decided to carry out a survey of a number of other anionic dyes. This was done in order to determine whether the behaviour of AR88 in the presence of chitosan is typical of anionic dyes in general, and if any correlations between dye structure and metachromatic behaviour could be established. The dyes examined are listed in Figure 41 and Table 9 - 6 cationic

Figure 41. Dye Structures[233]

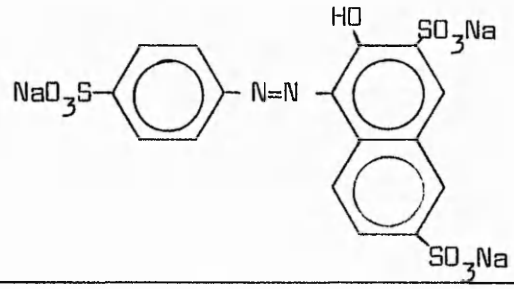
I C.I. Acid Red 88 C.I. 15620



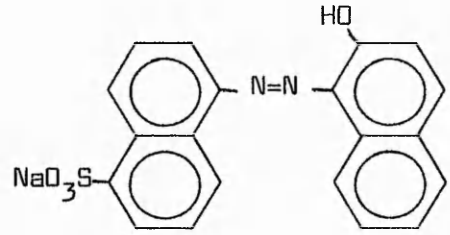
II C.I. Acid Red 13 C.I. 16045



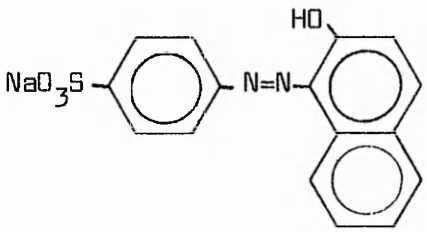
III C.I. Acid Red 27 C.I. 16185



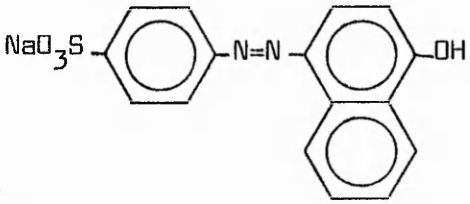
IV C.I. Acid Red 141 C.I. 15625



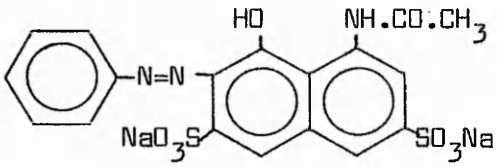
V C.I. Acid Orange 7 C.I. 15510



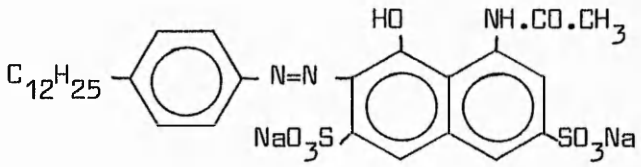
VI C.I. Acid Orange 20 C.I. 14600



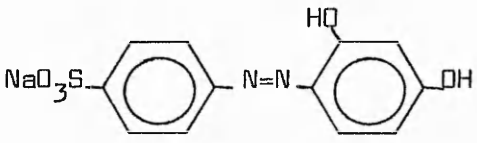
VII C.I. Acid Red 1 C.I. 18050



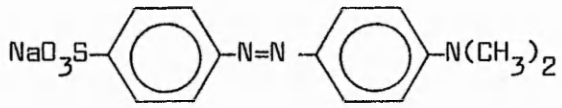
VIII C.I. Acid Red 138 C.I. 18073



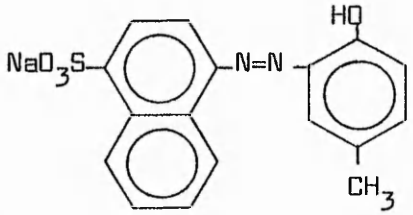
IX C.I. Acid Orange 6 C.I. 14270



X C.I. Acid Orange 52 C.I. 13025



XI Dye A C.I. -



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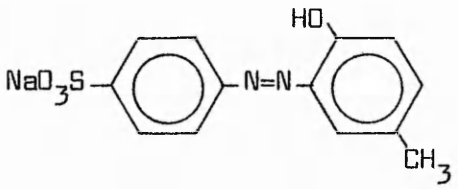
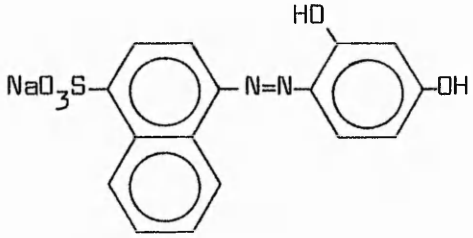
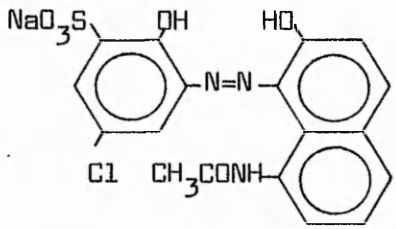


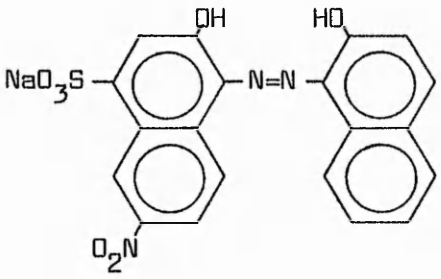
XIII Dye C C.I. -



XIV C.I. Mordant Black 38 C.I. 18160



XV C.I. Mordant Black 1 C.I. 15710



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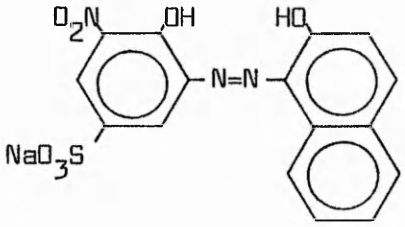

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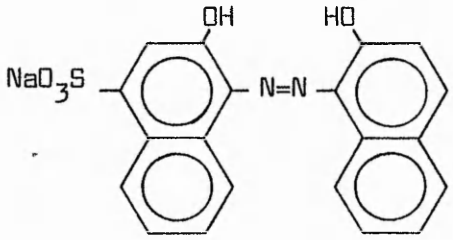

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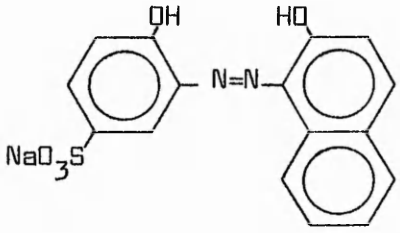

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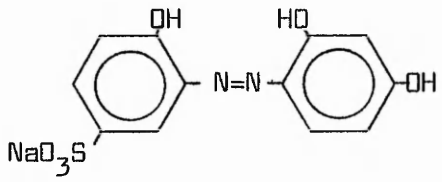

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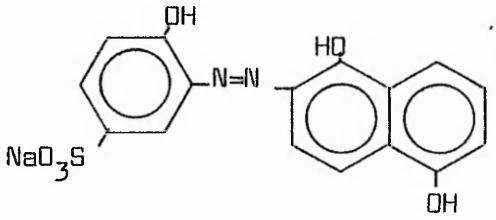

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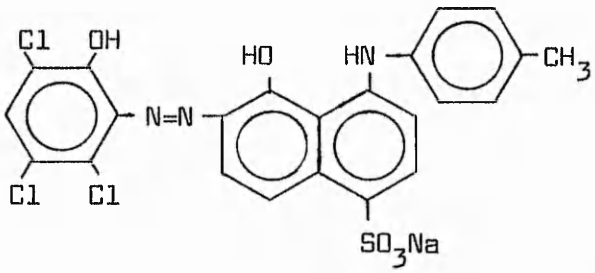

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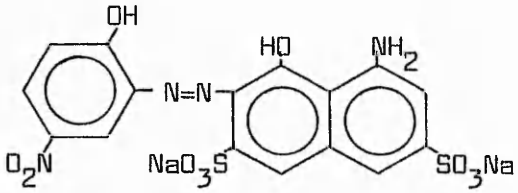

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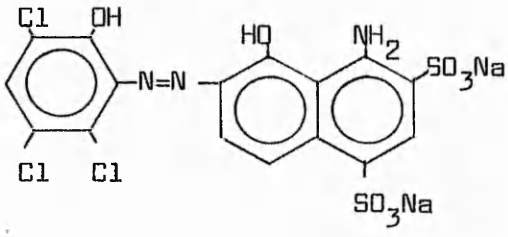

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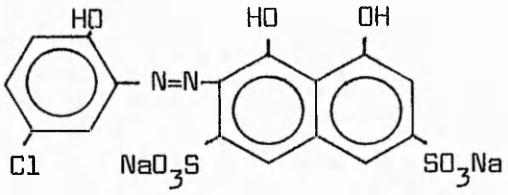

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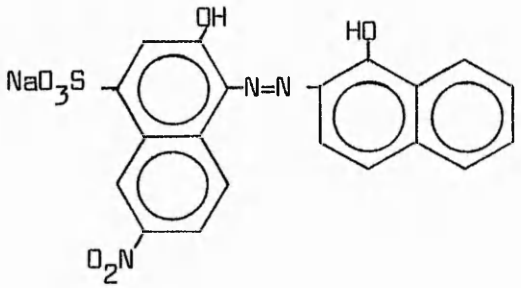

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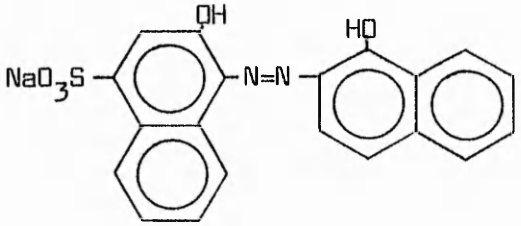

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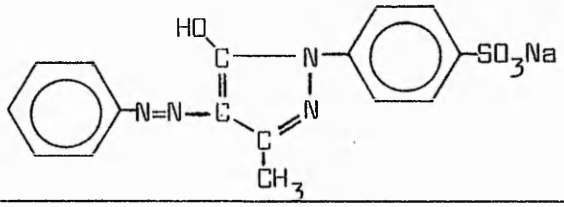

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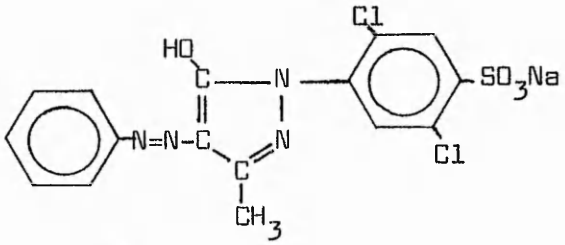

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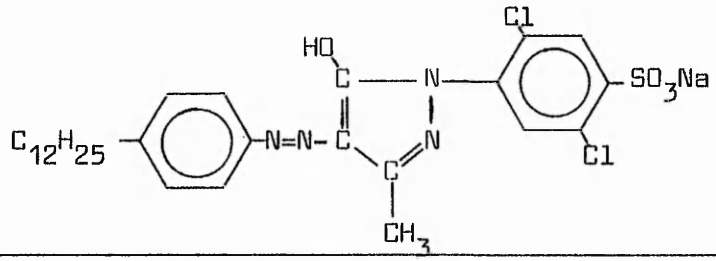

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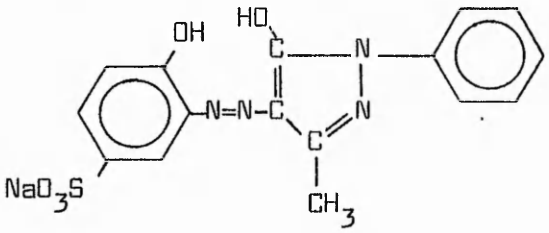

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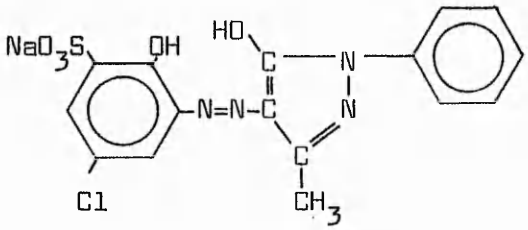

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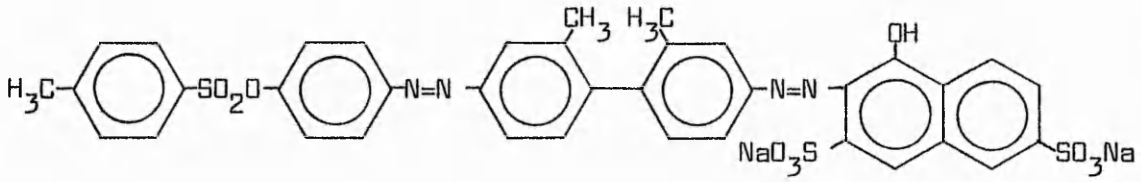

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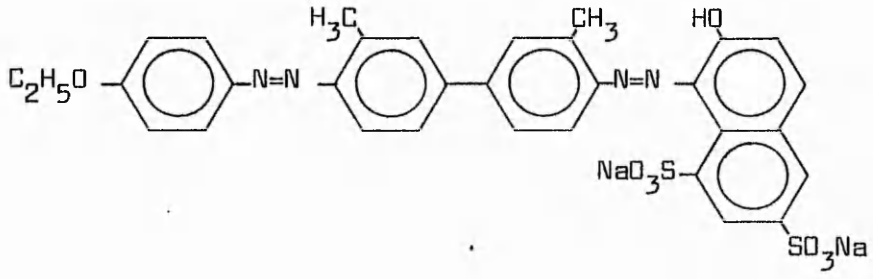

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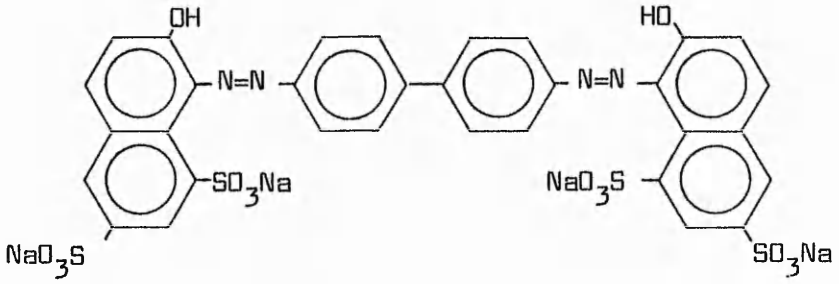

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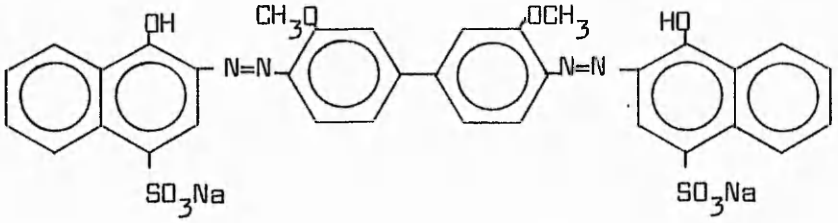

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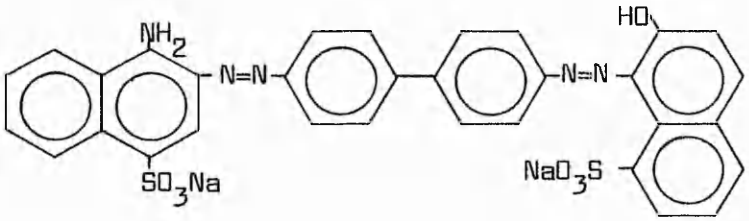

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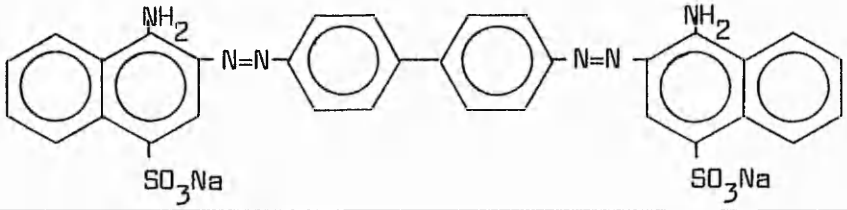

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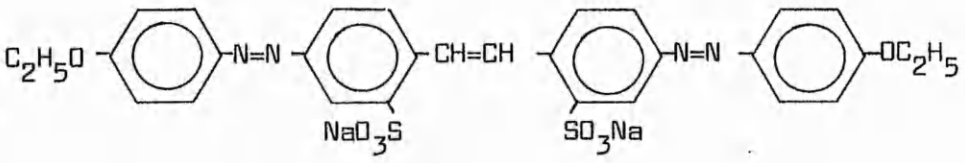

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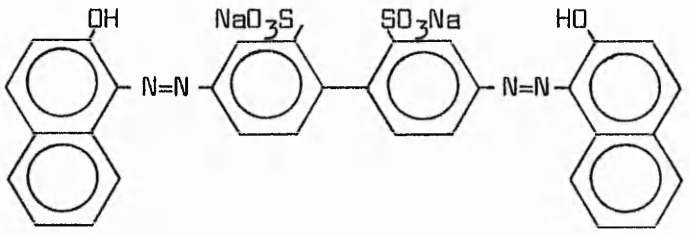
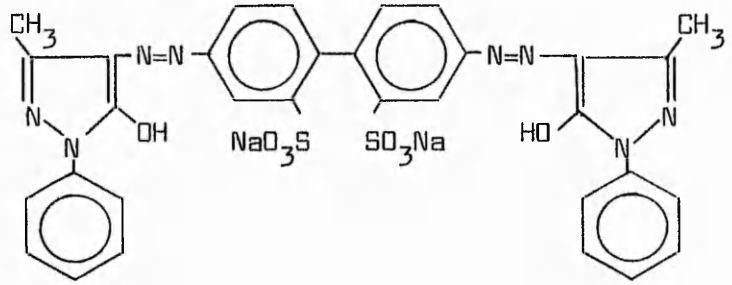
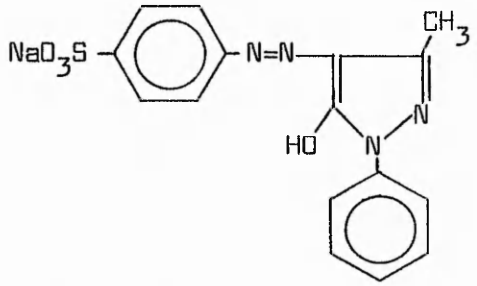


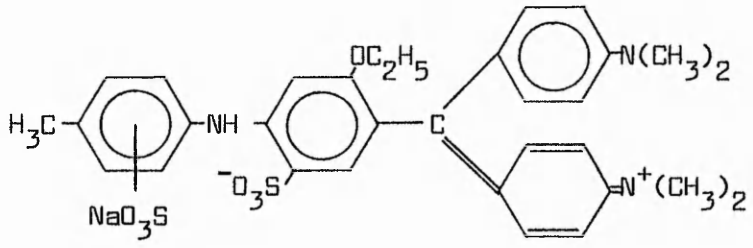
XL C.I. Acid Yellow 42 C.I. 22910



XLI C.I. Acid Yellow 42 - half unit**



XLII C.I. Acid Violet 15 C.I. 43525



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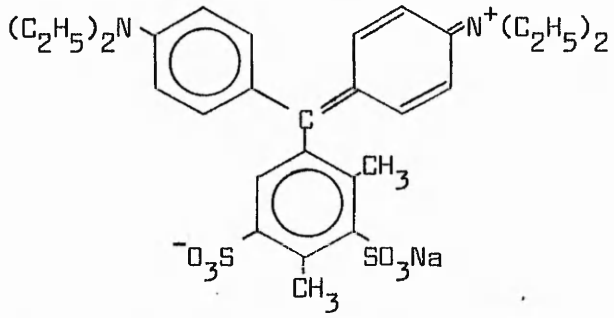

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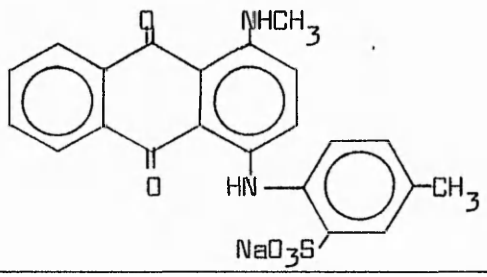

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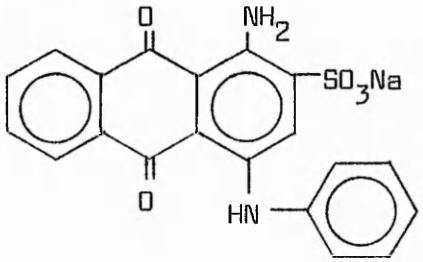
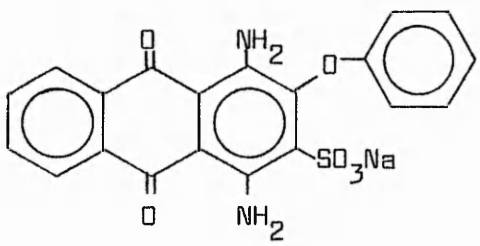


XLVI C.I. Acid Violet 41 C.I. 62020



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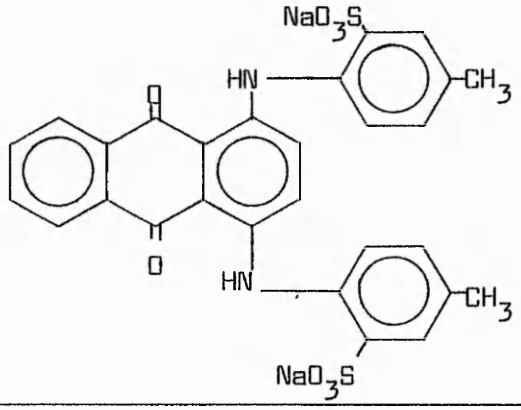

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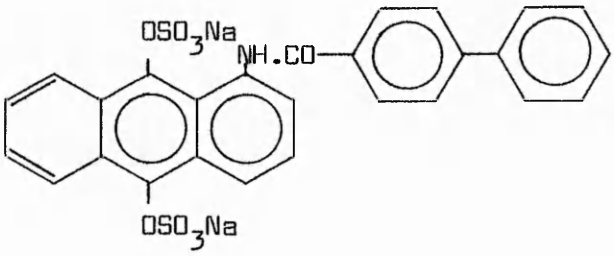

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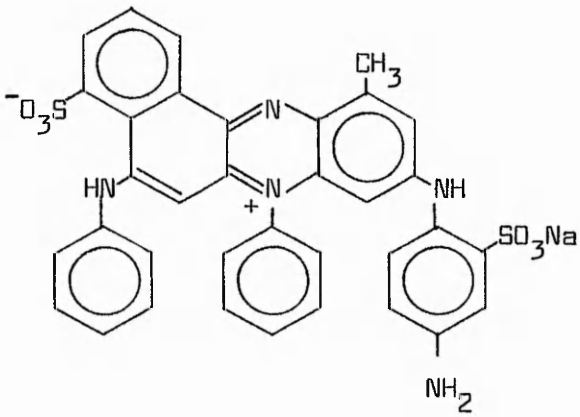
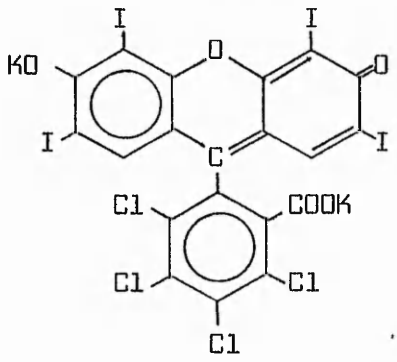
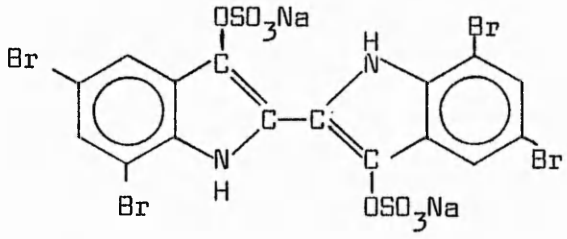


L C.I. Acid Red 94 C.I. 45440



LI C.I. Solubilised Vat Blue 5 C.I. 73066



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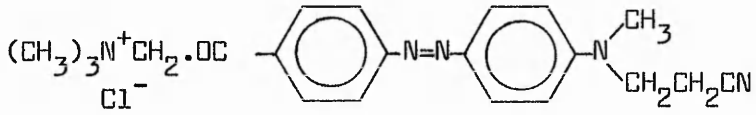

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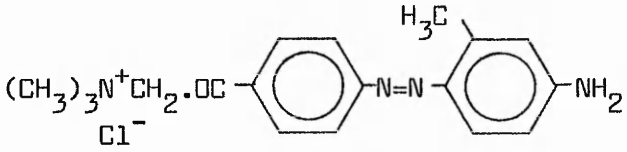
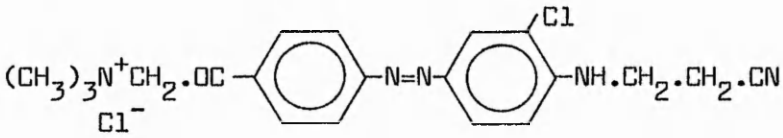
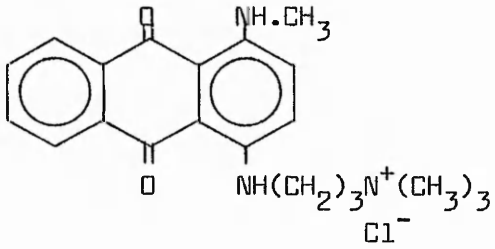


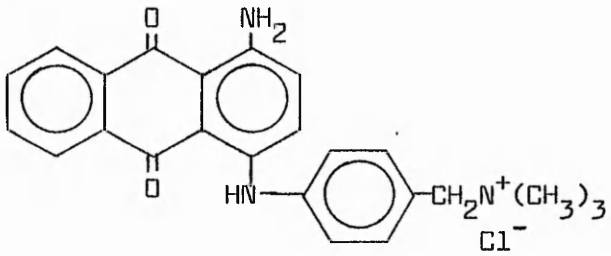
LIV C.I. Basic Yellow 15 C.I. 11087



LV C.I. Basic Blue 22 C.I. 61512

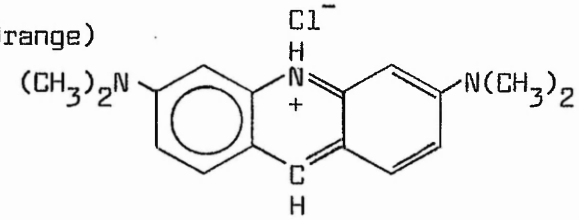


LVI C.I. Basic Blue 47 C.I. 61111



LVII C.I. Basic Orange 14 C.I. 46005

(Acridine Orange)



** Prepared by Dr G.A.F. Roberts.

Table 9. Change in absorbance of dyes in the presence of polymer

	C.I. Name	Shift(nm)
I	Acid Red 88	-55
II	Acid Red 13	-25
III	Acid Red 27	-5 (+70)
IV	Acid Red 141	-50
V	Acid Orange 7	-55
VI	Acid Orange 20	-27
VII	Acid Red 1	x*
VIII	Acid Red 138	+5 +20
IX	Acid Orange 6	x
X	Acid Orange 52	-10
XI	Dye A	+5**
XII	Dye B	+5**
XIII	Dye C	-20**
XIV	Mordant Black 38	-10
XV	Mordant Black 1	+5
XVI	Mordant Black 15	+38
XVII	Mordant Black 17	(+40)
XVIII	Mordant Violet 5	(+40)
XIX	Mordant Red 5	x
XX	Mordant Black 9	-5
XXI	Mordant Green 34	-5
XXII	Mordant Green 17	-2 (+40)
XXIII	Mordant Blue 44	x
XXIV	Mordant Blue 13	-55

	C.I. Name	Shift (nm)
XXV	Mordant Black 11	-18
XXVI	Mordant Black 3	-30
XXVII	Acid Yellow 11	-7
XXVIII	Acid Yellow 14	x
XXIX	Acid Yellow 72	+10
XXX	Mordant Orange 37	-10
XXXI	Mordant Red 19	x
XXXII	Acid Red 111	+10
XXXIII	Direct Red 39	-2
XXXIV	Direct Violet 45	(+50)
XXXV	Direct Blue 8	-15 (+75)
XXXVI	Direct Red 17	-45
XXXVII	Direct Red 28	x
XXXVIII	Direct Yellow 12	+35
XXXIX	Acid Red 97	+2.5 (+38)
XL	Acid Yellow 42	+20
XLI	Acid Yellow 42 - half unit	-5**
XLII	Acid Violet 15	x
XLIII	Acid Green 7	x
XLIV	Acid Blue 27	+20 +50*
XLV	Acid Blue 25	+20
XLVI	Acid Violet 41	+10 +10
XLVII	Acid Green 25	+10 +15
XLVIII	Solubilised Vat Yellow 7	+18
XLIX	Acid Blue 61	+5
L	Acid Red 94	x†

	C.I. Name	Shift (nm)
LI	Solubilised Vat Blue 5	-5
LII	Basic Orange 24	x
LIII	Basic Orange 25	x
LIV	Basic Yellow 15	-5
LV	Basic Blue 22	+3 +3*
LVI	Basic Blue 47	+5 +20
LVII	Basic Orange 14	-15*

Key:

- \- Shift of dye/polymer solution to shorter wavelength (hypsochromic shift) relative to dye solution.
- \+ Shift of dye/polymer solution to longer wavelength (bathochromic shift) relative to dye solution.
- ( ) Shift in parenthesis refers to a shoulder on the spectrum.
- * Peak ratios changed when polymer was present.
- † Very great increase in intensity in the presence of polymer.
- ** Prepared by Dr G.A.F. Roberts.

dyes are also included, and were tested in the presence of carboxymethyl cellulose. It should be noted that for the purposes of this survey, and unless specially prepared, all dyes were used as supplied by the manufacturers, and contained unknown quantities of impurities, of which sodium chloride was likely to be the most common. It was felt, however, that the concentration of salts and other impurities was not likely to be sufficiently high to interfere with any metachromatic interaction taking place. Furthermore, the structures given represent those of the most likely isomers present, as commercial dyes are not purified prior to sale. In only a few cases would the precise structures have been determined. In Table 9, shifts in λ max of the dye/polymer solution relative to the dye solution are shown - a minus sign indicates a shift to shorter wavelengths (hypsochromic shift) and a plus sign indicates a shift to longer wavelengths (bathochromic shift). In a few cases, λ max remained similar for both solutions but a shoulder either developed, or changed position in the spectrum. A shift of this type is shown in parenthesis, and an example of such a spectrum is given in Figure 42.

A number of conclusions may be drawn from the results in Figure 41 and Table 9, although in some cases the conclusions are only tentative and would require the examination of a considerable number of additional dyes before being generally accepted.

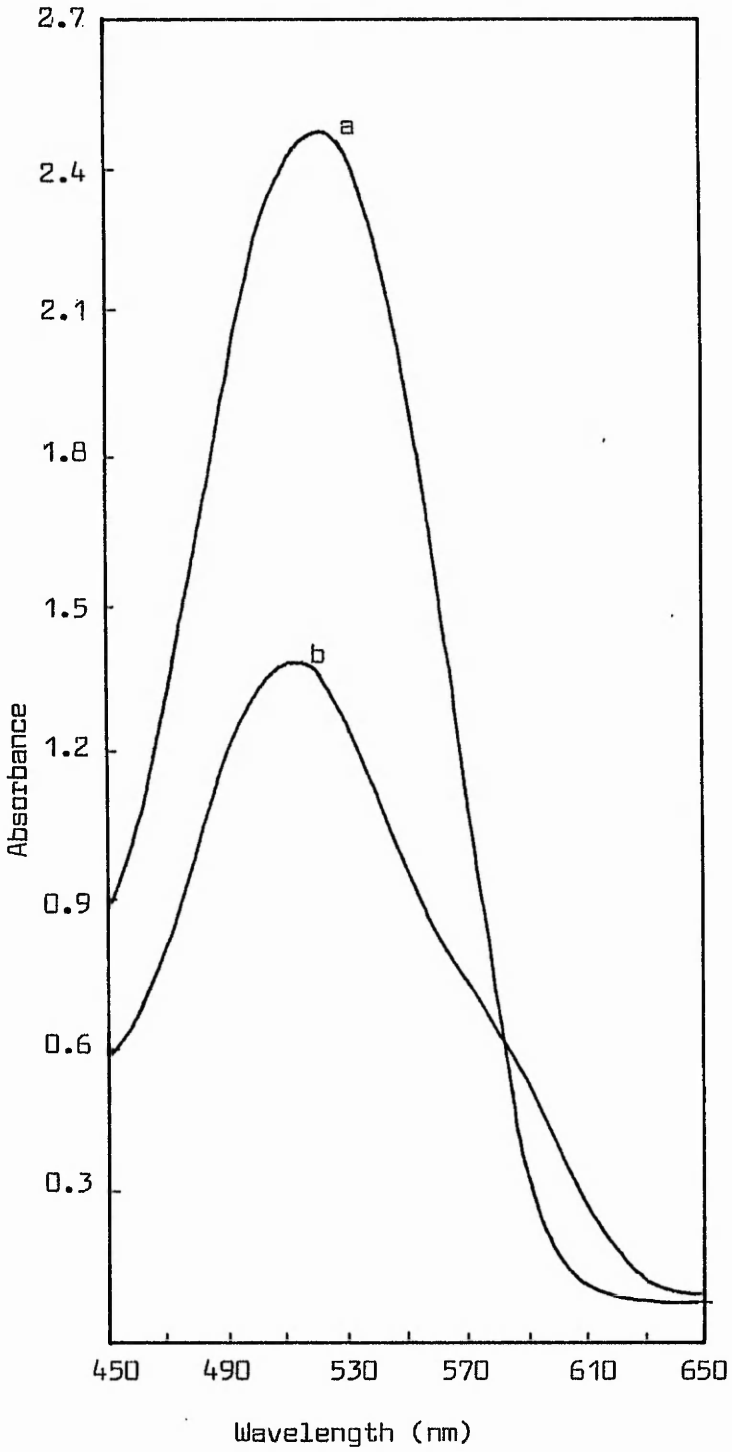


Figure 42. Example of metachromatic shift in the presence of chitosan, showing development of a shoulder bathochromic to the uncomplexed dye λ max (C.I. Acid Red 27).

a AR27 solution alone

b AR27 + polymer

3.5.3.1 Direction of metachromatic shift

Both hypsochromic and bathochromic shifts in λmax have been found. This contrasts with the findings of other workers, who have only reported hypsochromic shifts in their studies of polyanionic/cationic dye systems. In particular, the anthraquinone-based dyes, both anionic (XLV-XLVIII) and cationic (LV-LVI) show bathochromic shifts in the presence of polyelectrolytes of opposite charge. The correlation with structure is not so clear with other dyes showing bathochromic shifts, although two points are of interest. In a series of mordant dyes (o,o'-dihydroxyazo derivatives) based on naphthalene ring systems (XV-XVIII) the shift is bathochromic, although related monohydroxyazo derivatives (I, II, IV), show hypsochromic shifts. However, the two dyes XXV and XXVI show hypsochromic shifts although the structures given in the Colour Index[233] are o,o'-dihydroxyazo structures. Normally diazonium ions would be expected to couple with 1-naphthol in the 4-position rather than the 2-position, but it is suggested in the Colour Index[235] that with these dyes coupling takes place at the 2-position because of the strongly alkaline coupling conditions used. However the hypsochromic shifts that both dyes exhibit with chitosan suggest that coupling occurs in the 4-position as would normally be expected, so that the dyes do not contain the o,o'-dihydroxyazo structure, and hence do not give bathochromic shifts similar to those given by XV-XVIII.

There are two pairs of dyes (VII/VIII and XXVIII/XXIX) in which the only difference between the two dyes in each pair is that one of them contains a large hydrocarbon "weighting" group. Although the dye in each pair that is unweighted shows no shift in either

direction, both weighted dyes show bathochromic shifts.

3.5.3.2 The importance of delocalised charges

Theories of metachromasy state that the change in metachromasy is due to a change in distribution of the delocalised charge over the chromophore when the dye ion associates with the ionic sites on the polymer[144]. This concept presumably arose because most previous work on metachromasy has been carried out using a few basic (cationic) dyes which have delocalised charges. The negative charge of the ionised sulphonic acid group, which is the solubilising group for most of the dyes in Figure 41, cannot be delocalised over the molecule in the same way as can the positive charge in Acridine Orange (LVII) or Methylene Blue, and this would tend to disprove the suggestion given above. However, it could be argued that dye ion/ionic site interaction might affect electron delocalisation in the aromatic ring bearing the sulphonic acid group. The behaviour of a number of dyes in which the ionic group is insulated from the rest of the molecule - so that even this possibility is excluded - was therefore studied. Both anionic dyes such as LI and XLVIII and cationic dyes such as LIV, LV and LVI showed metachromatic shifts with the appropriate polyelectrolyte, clearly demonstrating that metachromasy does not require the dye ion to contain a delocalised charge.

3.5.3.3 Basicity of the dye

Dyes I to III form a series in which the number of sulphonic acid groups increases, although the remainder of the molecule remains

constant. The shift in λ max decreases with increase in the number of sulphonic acid groups and this can be attributed to two factors, both of which would reduce the tendency of the dyes to aggregate on going from I → III, and hence might be expected to decrease the metachromatic shift. As the number of sulphonic acid groups increases, the dye ions become more water soluble and the electrostatic repulsion between them increases.

It is obvious that the relationship between dye structure and the nature and extent of the shift in absorbance produced is complex. The sample of dyes used in this brief survey forms only a small part of the vast range of anionic dyes available, and it would be unwise to draw conclusions that were other than general and qualified. However, it is quite clearly seen that the metachromatic shift can be to both higher and lower wavelengths in cationic dyes as well as anionic dyes. With a wider sample, and with the choice of closely related structures varying in one characteristic at a time, it should be possible to produce a clearer picture of the effect that dye structure has on the metachromatic interaction of dye and polymer.

3.5.4 The effect of electrolyte, urea and ethanol on dye/chitosan solutions

3.5.4.1 Introduction

Section 2.9.4.3 summarises the work carried out on the reversal of the metachromatic shift in cationic dye/polyanion systems. It was of considerable interest to determine whether a similar reversal of anionic dye/chitosan metachromasy could be induced by comparable agents, and the effect of a variety of electrolytes,

and of urea and ethanol was assessed spectrophotometrically. For most of this work the monoazo dye C.I. Acid Orange 7 (AO7, structure V in Figure 41) was used, as the survey described in Section 3.5.3 had shown that the shift induced in the spectrum of this dye was of the same order as that shown by AR88, whilst the smaller size of the AO7 molecule relative to AR88 suggested that reversal of metachromasy would be more readily achieved.

3.5.4.2 The reversal of metachromasy by electrolytes

For each of the electrolytes used, the measurement technique was the same. The effect of a range of concentrations of the electrolyte on the absorbance of a standard concentration of the dye was first recorded; the same electrolyte concentrations were then added to dye/polymer solutions containing a standard amount of dye and sufficient polymer to give equivalence of free amine sites to dye ions, plus a slight, and constant, excess to ensure that the metachromatic shift was complete. By taking the dye/polymer solution absorbance in the absence of electrolytes as zero percent free dye, and the dye/electrolyte absorbance for the particular electrolyte concentration as 100 percent free dye, the percent free dye at each electrolyte concentration could be calculated. Examples of the initial absorbance plot, and of the derived free dye plots, are given in Figures 43-46. The electrolytes used were:

sodium chloride

sodium iodide

sodium bromide

benzenesulphonic acid (sodium salt)

benzene m-disulphonic acid (sodium salt)

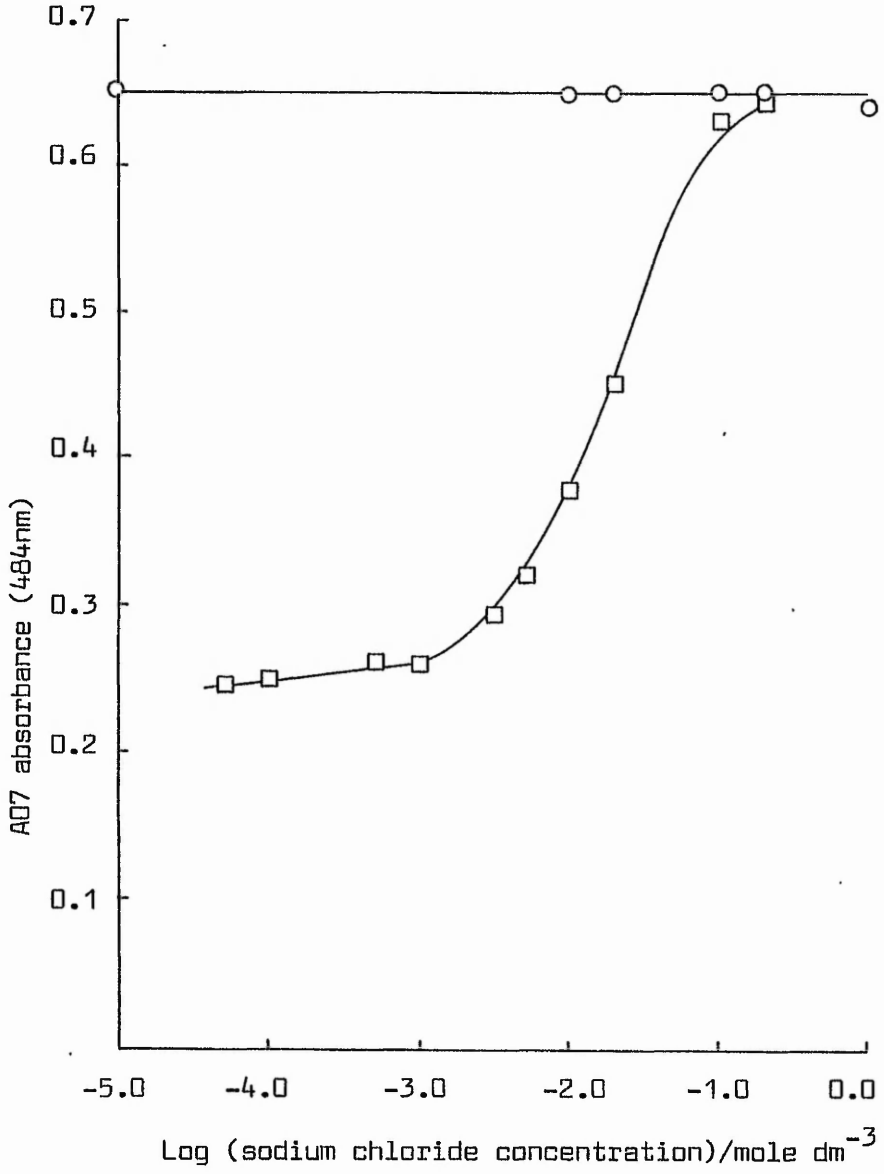


Figure 43. Absorbance of polymer/dye solution in the presence of sodium chloride (3×10^{-5}M AO7 solution)

○ dye solution

□ polymer/dye solution (P/D = 1:1)

lithium chloride	barium chloride
tetramethylammonium chloride	calcium chloride
potassium chloride	strontium chloride
	magnesium chloride

In each of the free dye plots (Figures 44-46) the concentrations shown are those of the monovalent ion; eg. for $CaCl_2$ the concentrations shown are those of the $Cl^{\ominus}$ ion.

The pattern of release of free dye from the metachromatic polymer/dye complex was similar for all the electrolytes used in the study. Initially the change in absorbance with change in concentration was quite slow, but thereafter the change was very rapid and linearly related to the concentration of the electrolyte. The rate of change then decreased as the proportion of free dye in solution approached 100 percent. With the exception of the benzene disulphonic acid salt, all the electrolytes produced a reversal of metachromasy within a narrow concentration range. For a dye concentration of 3×10^{-5}M, the reversal effect was first observed when the salt concentration was approximately 3×10^{-3}M, and 50% free dye was present in solution when the electrolyte concentration was about 1.8×10^{-2}M. Complete reversal was achieved when the molarity of the monovalent electrolyte ion was approximately 0.2M. The narrow concentration for this effect (for example, $2.8 - 1.3 \times 10^{-2}$M electrolyte ion to produce 50 percent free dye) suggests that the nature of the cation or anion present may not be of great importance in this dye/polymer system, but that the presence of greatly increased electrolyte strength is the controlling factor. The similarity of the curves suggests that the same mechanism is operating in each case (with the possible exception of the

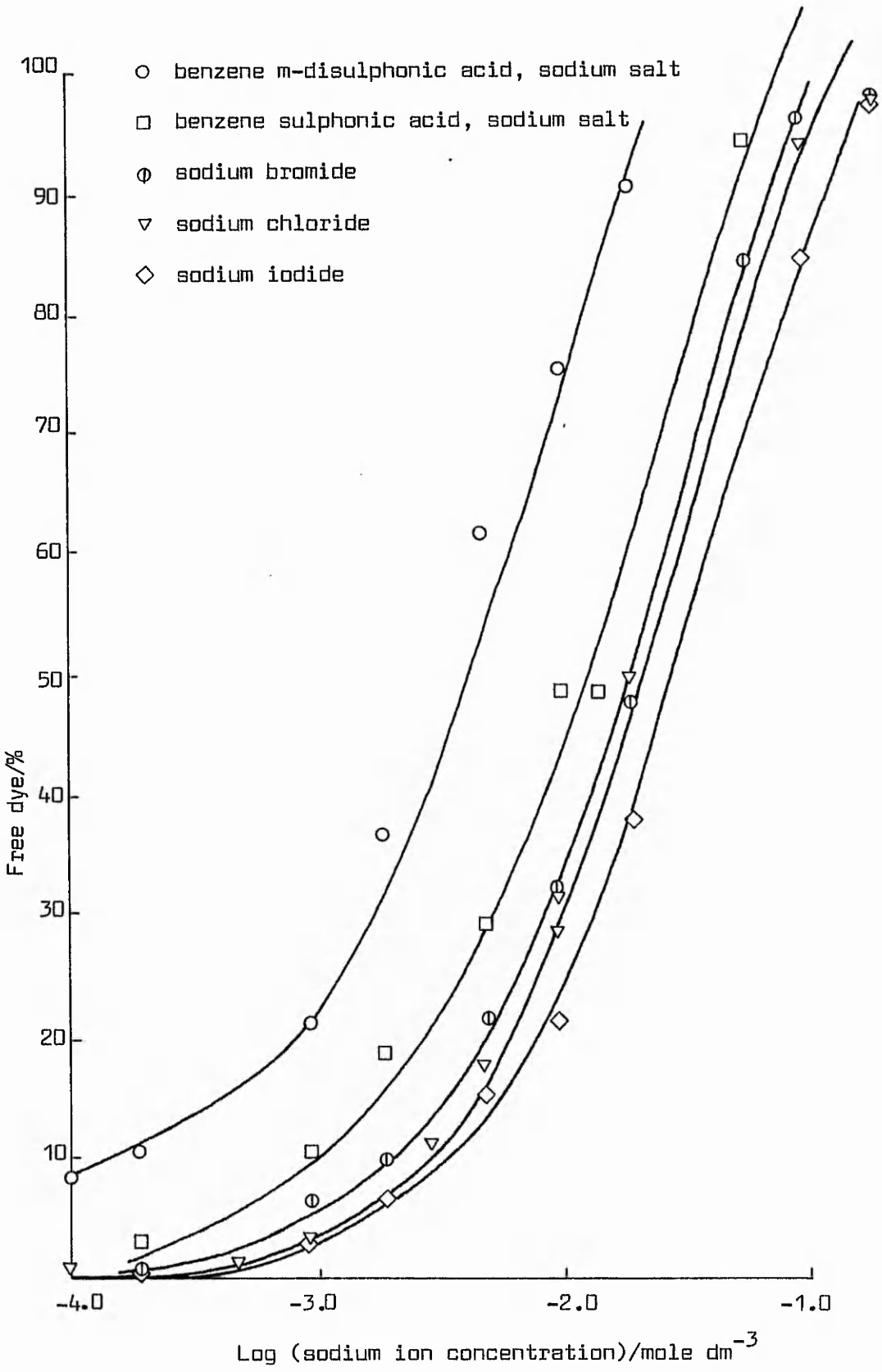


Figure 44. Reversal of metachromasy by sodium salts.

(3×10^{-5}M A07 solution, P/D = 1:1)

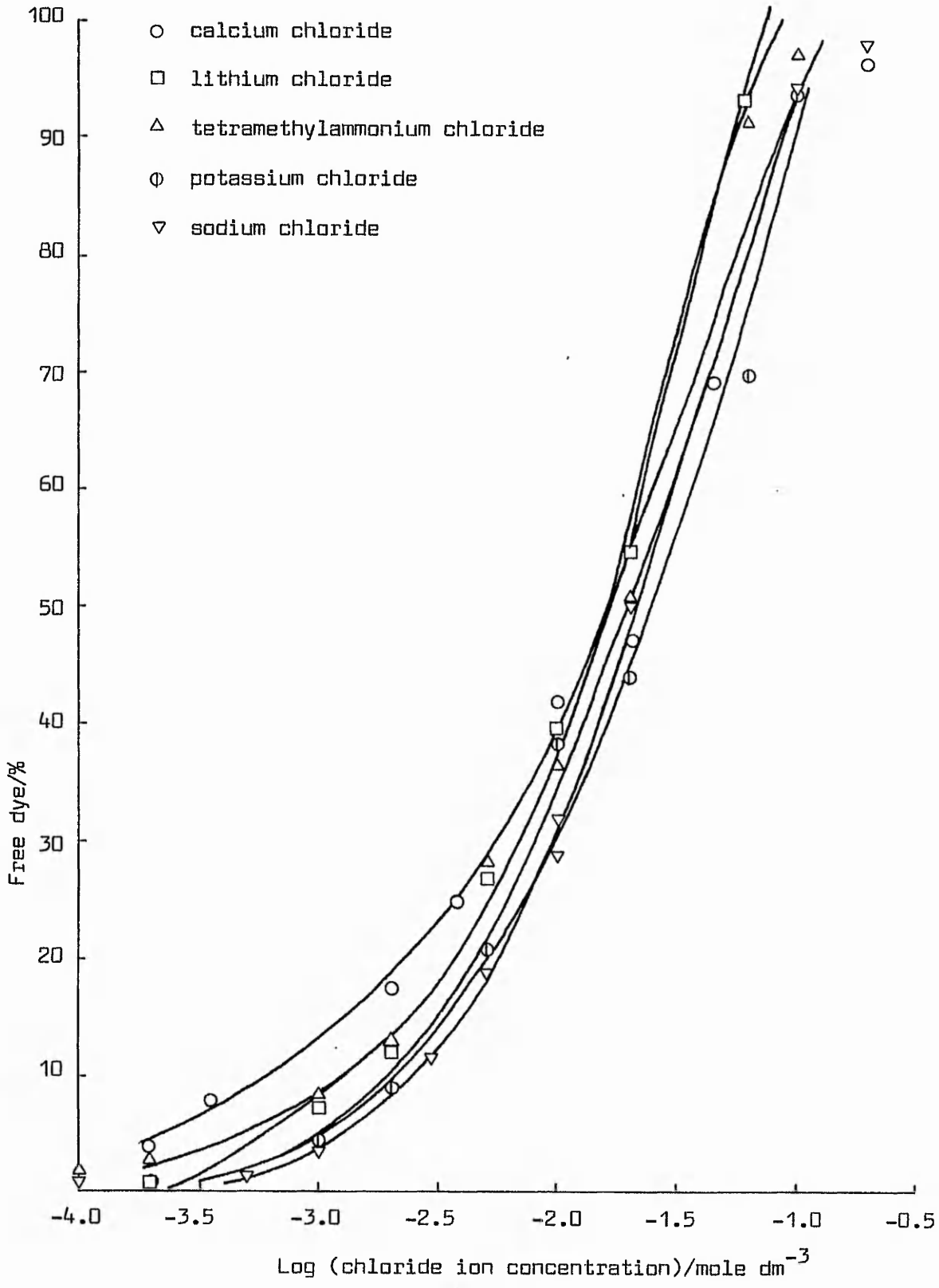


Figure 45. Reversal of metachromasy by chloride salts

(3×10^{-5}M AO7 solution, P/D = 1:1)

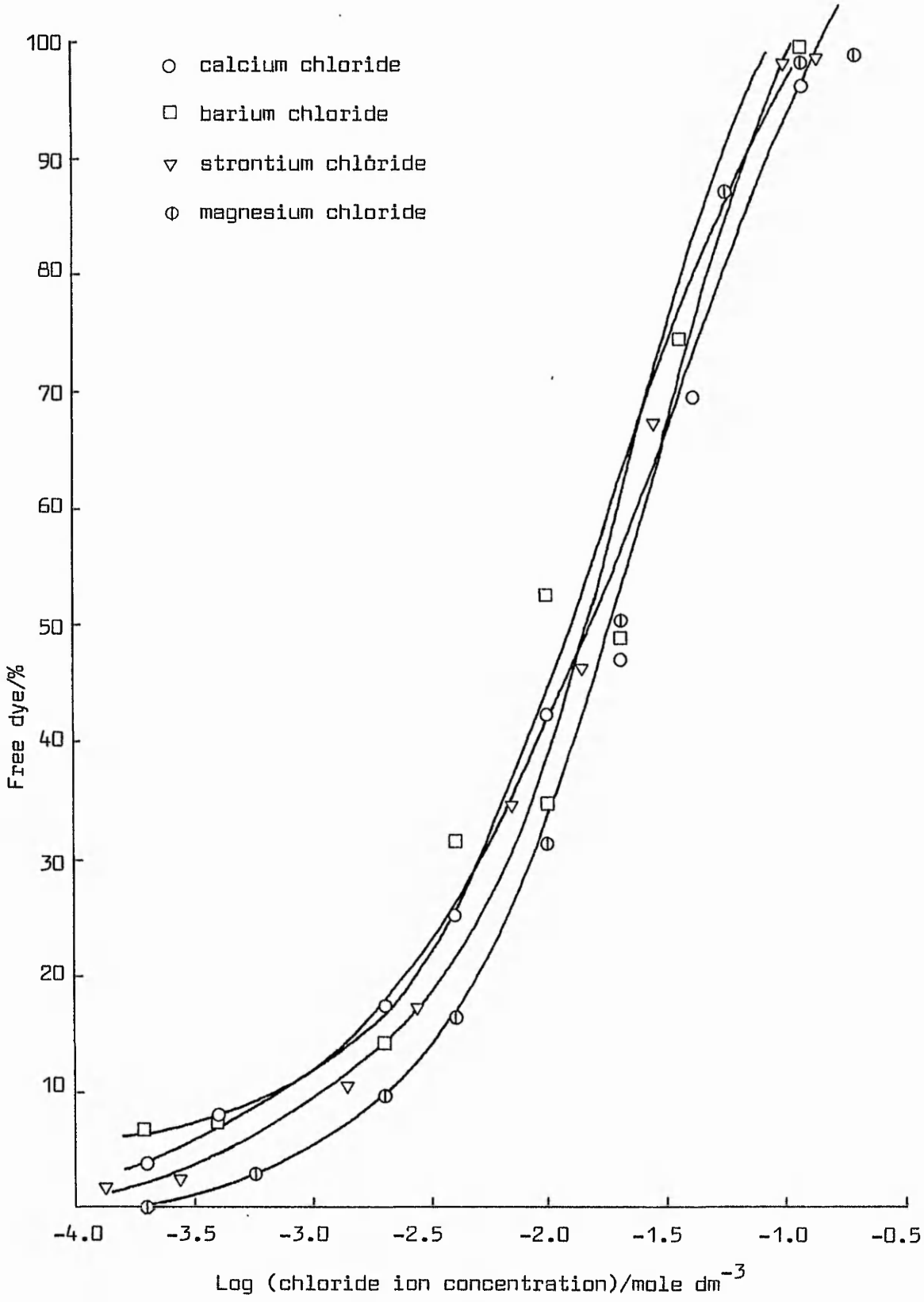


Figure 46. Reversal of metachromasy by divalent salts

(3×10^{-5}M A07 solution, P/D = 1:1)

disulphonate). It was notable that the complete reversal of metachromasy was achieved at electrolyte concentrations well below those required for aggregation of the dye in solution in the absence of polymer. This was not true for the AR88/chitosan complex which required higher concentrations of sodium chloride (approximately 20,000 times the concentration of the dye) than the A07/chitosan complex before the level of free dye in solution reached 50 percent. This disparity in the relative concentrations required was expected in view of the higher molecular weight and lower solubility of AR88 which would increase the tendency of the dye to aggregate.

3.5.4.3 The reversal of metachromasy by urea and ethanol

Section 2.9.4.3c gave a brief review of the work carried out on the reversal of metachromasy in cationic dye/polyanion systems by the introduction of urea or alcohol to the dye/polymer solution. Of the alcohols previously examined, only ethanol was used in the study described here, and the technique for both urea and ethanol was similar to that described in Section 3.5.4.2. The results are plotted in Figure 47, together with the plot for sodium chloride.

It is immediately apparent that there is little difference between the efficiency of urea and of ethanol in promoting the reversal of metachromasy. Whereas a concentration of sodium chloride of 2.0×10^{-2}M is sufficient to give 50 percent free dye in solution, the concentration of urea required is 2.2M (for a dye concentration of 3×10^{-5}M).

One apparently hitherto unreported aspect of the reversal of metachromasy was the importance of the point at which the electrolyte or urea was added to the solution. If the salt or urea was added

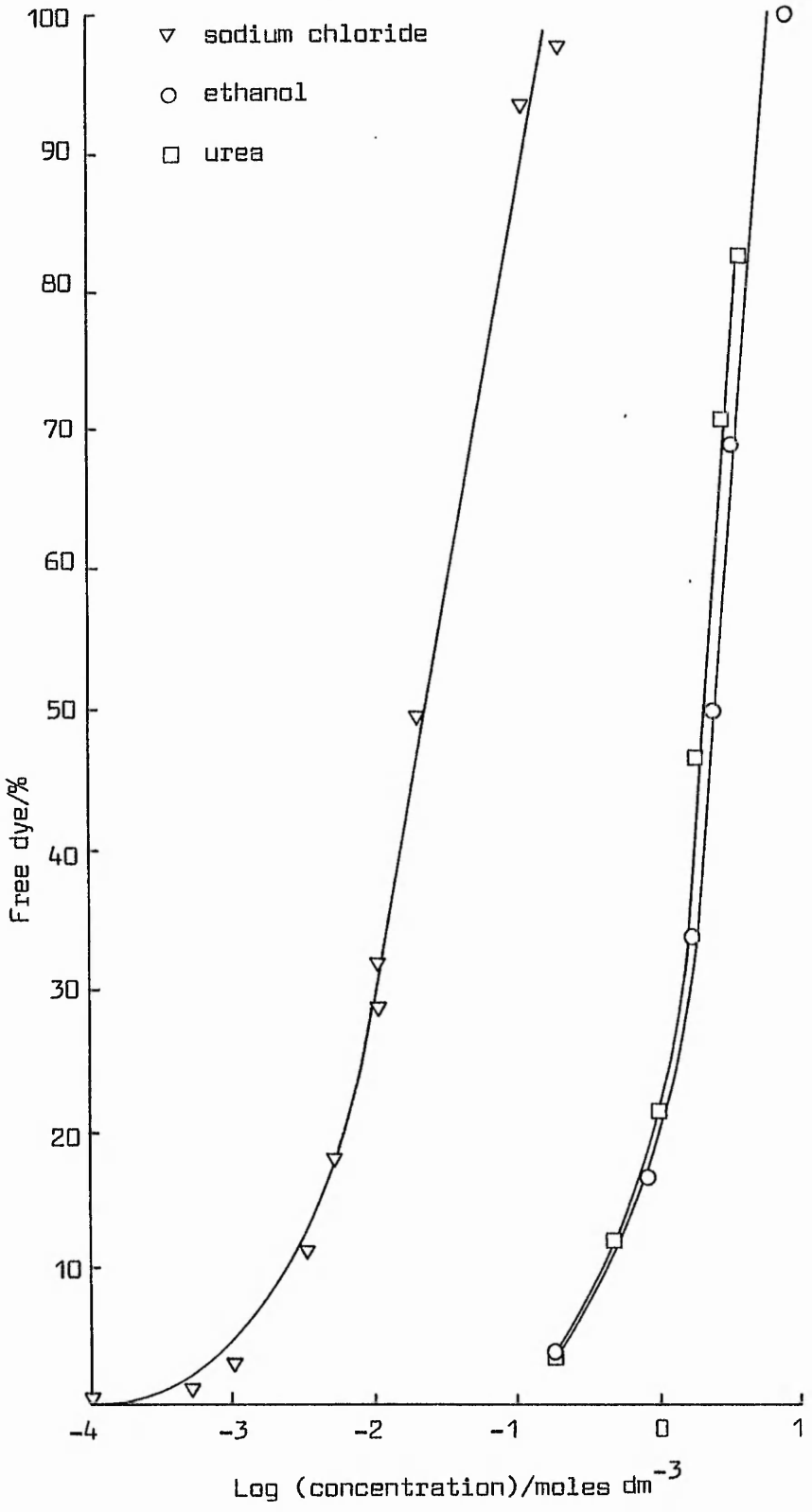


Figure 47. Reversal of metachromasy by sodium chloride, ethanol and urea (3×10^{-5}M AO7 solution, P/D = 1:1)

to the dye solution before the addition of the polymer, reversal of metachromasy was observed at a slightly lower concentration of the additive than if the polymer/dye complex was formed first. This effect was reproducible, and held for sodium chloride and urea with AO7, and also for sodium chloride with AR88 solutions at concentrations of salt below those causing extensive precipitation of the dye in solution. If the concentration of sodium chloride was further increased, to the point at which the aggregation of AO7 in solution took place, it was found that aggregation of the dye occurred first in solution in the absence of polymer, then in solutions in which the salt was added last (Figure 48). It would be useful to discover whether this effect held with other additives, and whether it was also present in polyanion/cationic dye systems. A further point worthy of investigation is the possibility of a synergistic effect between two additives promoting reversal of metachromasy by different means. Preliminary tests showed no evidence of synergism in the action of different sodium chloride concentrations when a constant concentration of urea was maintained. On the contrary, for 0.1M and 0.01M urea (between approximately 10,000 and 1000 times the concentration of the dye) no difference in the efficiency of sodium chloride was found from that in the complete absence of urea. It would, however, be worthwhile to extend the range of concentrations studied.

3.5.4.4 The reversal of metachromasy by increase in temperature

a) In the absence of additional electrolyte

Balazs *et al.* recognised that the metachromatic shift lost on heating a polyanion/cationic dye solution was completely restored

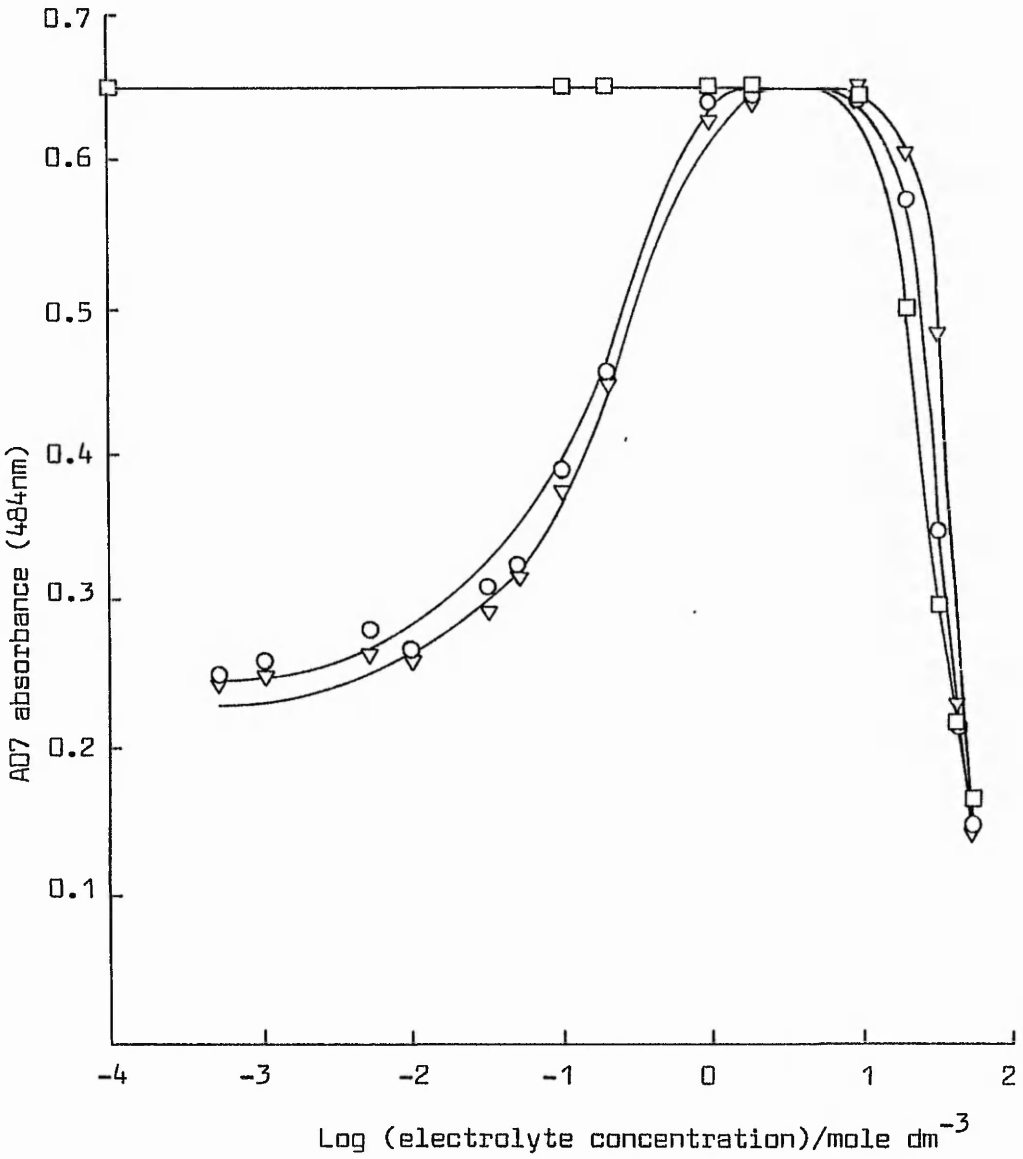


Figure 48. Effect of sodium chloride on the metachromasy of AO7 (3×10^{-5}M AO7 solution, P/D = 1:1)

○ electrolyte added before polymer

▽ electrolyte added after polymer

□ dye solution (no polymer)

by cooling[177], and that the nature of the polyanion affected the change in metachromasy on heating. When AO7/polymer solutions were heated, and the change in absorbance at 484nm was monitored, a linear relationship between absorbance and temperature was found; the system returned to the original absorbance levels on cooling to room temperature (Figure 49). AR88/polymer solutions did not reverse so readily, and at the highest temperature at which results were recorded for this system (90^{o}) the absorbance was still quite low. A cooling curve was recorded for AR88/chitosan, and showed a steady and almost linear return to the original absorbance level.

b) In the presence of additional electrolyte

The change in absorbance of AO7/polymer solution on heating in the presence of electrolyte was studied for three salts - calcium chloride, sodium chloride and sodium iodide. All three salts promoted the reversal of metachromasy at a given temperature when compared with the absorbance of the solution in the absence of salts, and in all three the extent of reversal was dependent upon the concentration of salt present - the greater the concentration of salts the greater the reversal of metachromasy (Figures 50,51). When the rate of change in absorbance per unit change in temperature was plotted against concentration, the efficiency of the three salts was in the order

$$CaCl_2 > NaI > NaCl.$$

The order was the same when the calcium chloride concentration was expressed as chloride ion concentration, but differs from that for the effects of salts at constant temperature, where sodium chloride was more efficient than sodium iodide.

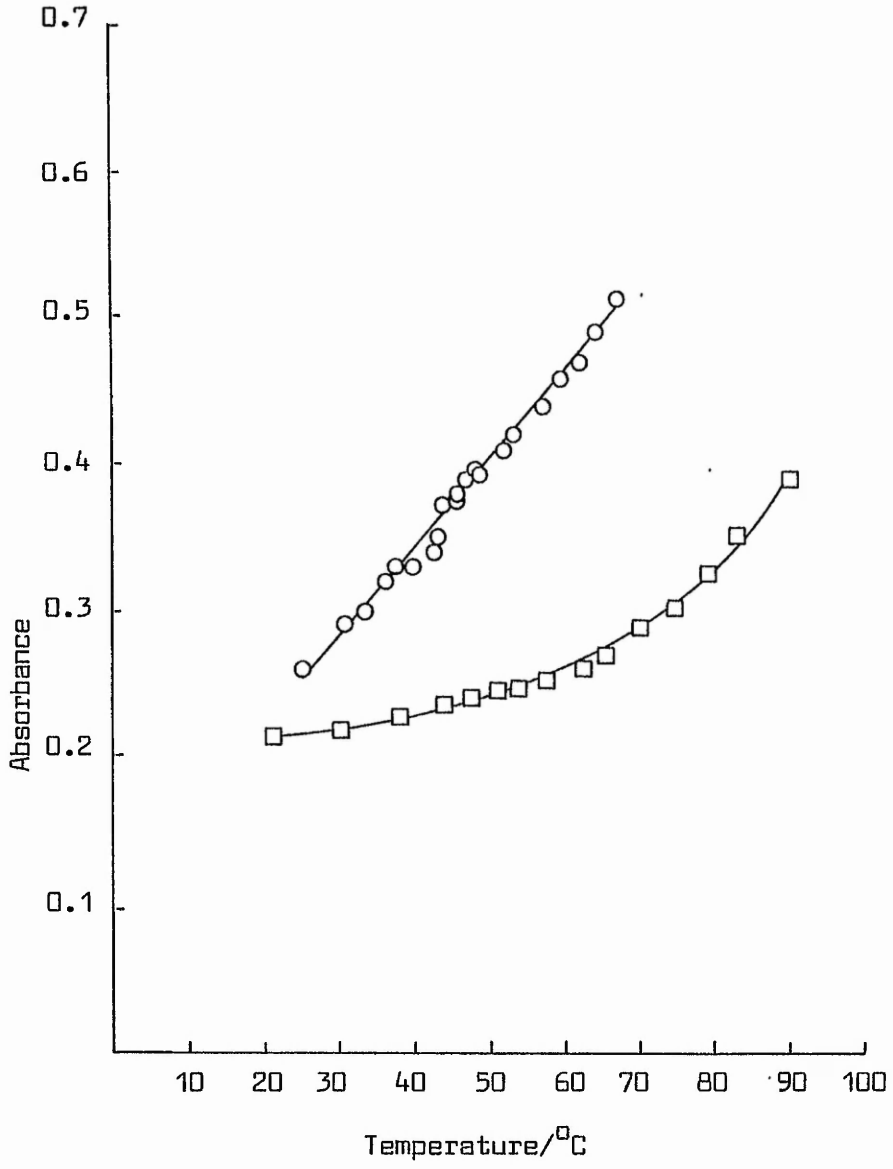


Figure 49. Reversal of metachromasy by heating

(3×10^{-5}M dye solution, P/D = 1:1)

○ AO7/polymer solution (484nm)

□ AR88/polymer solution (505nm)

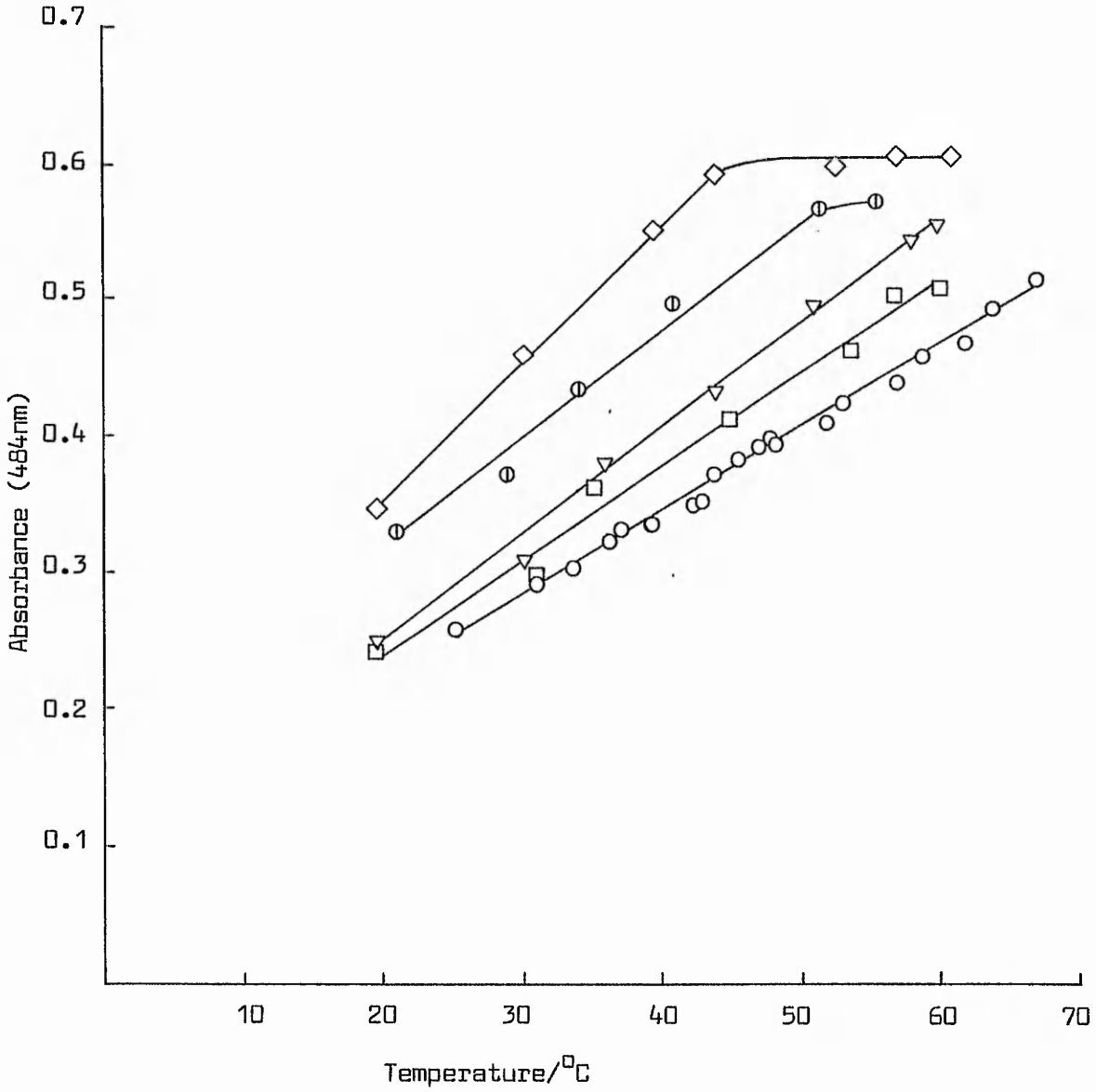


Figure 50. Reversal of metachromasy by heating in the presence of sodium chloride (3×10^{-5}M AO7 solution, P/D = 1:1)

Concentration of sodium chloride (moles dm^{-3}):

- ○ 0
- ⊕ 5×10^{-3}
- □ 5×10^{-4}
- ◇ 1×10^{-2}
- ▽ 1×10^{-3}

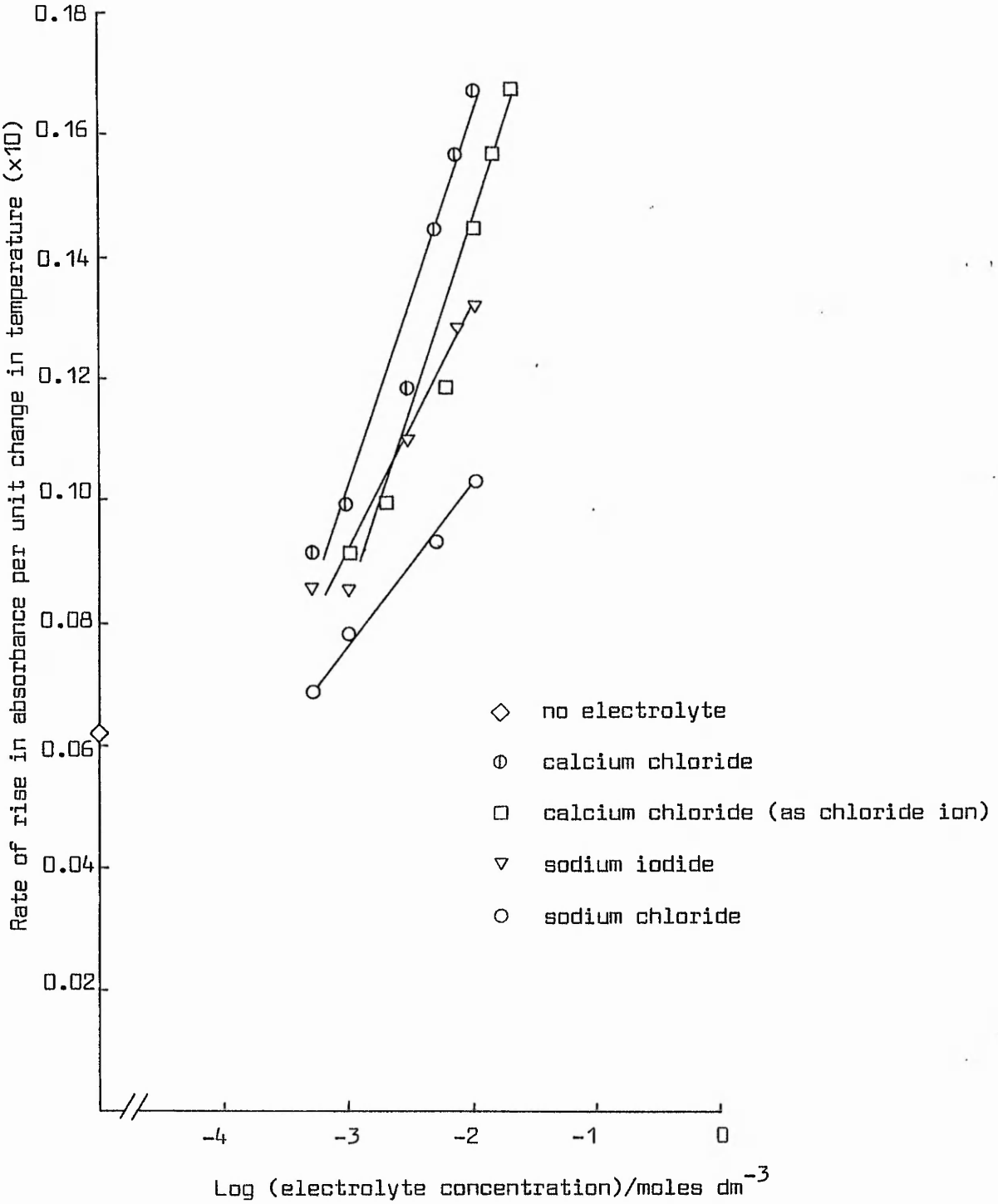


Figure 51. Rate of reversal of metachromasy by heating in the presence of electrolytes (3×10^{-5}M A07 solution, P/D = 1:1)

3.5.5 The effect of increasing the polymer/dye ratio

In view of the similarity of the metachromasy of the polycation/anionic dye system to that of the polyanion/cationic dye systems previously described (Section 2.9.4) the effect of increasing the ratio of polymer cationic sites to dye ions in solution was of interest. Section 2.9.4.3d reviews the work published on increasing the proportion of chromotrope present in a solution of metachromatic dye to well beyond the concentration required for equivalence of polymer anionic sites and dye. As the polymer to dye concentration increases (P/D > 1) the spectrum of the solution gradually shifts back towards that of the dye solution in the absence of polymer. In order to discover whether a similar effect could be produced in the chitosan/AO7 system, the absorbances of a series of solutions of constant dye concentration, but increasing P/D ratio were recorded (Figure 52). As the amount of excess chromotrope present in solution increased, the metachromatic shift of the solution was reversed. The absorbance of the solution at the wavelength of the free dye rose steadily until reversal was virtually complete - the high molecular weight of the sample precluded following of the change in absorbance further than shown in the figure.

Again, the metachromasy exhibited by AO7 and the polycationic chromotrope chitosan seems to be equivalent to that shown by cationic dyes in the presence of polyanionic chromotropes.

3.5.6 The mechanism of metachromasy

There are three main objections to the theory of adsorption at sites on the polymer leading to aggregation of the dyes, as suggested by,

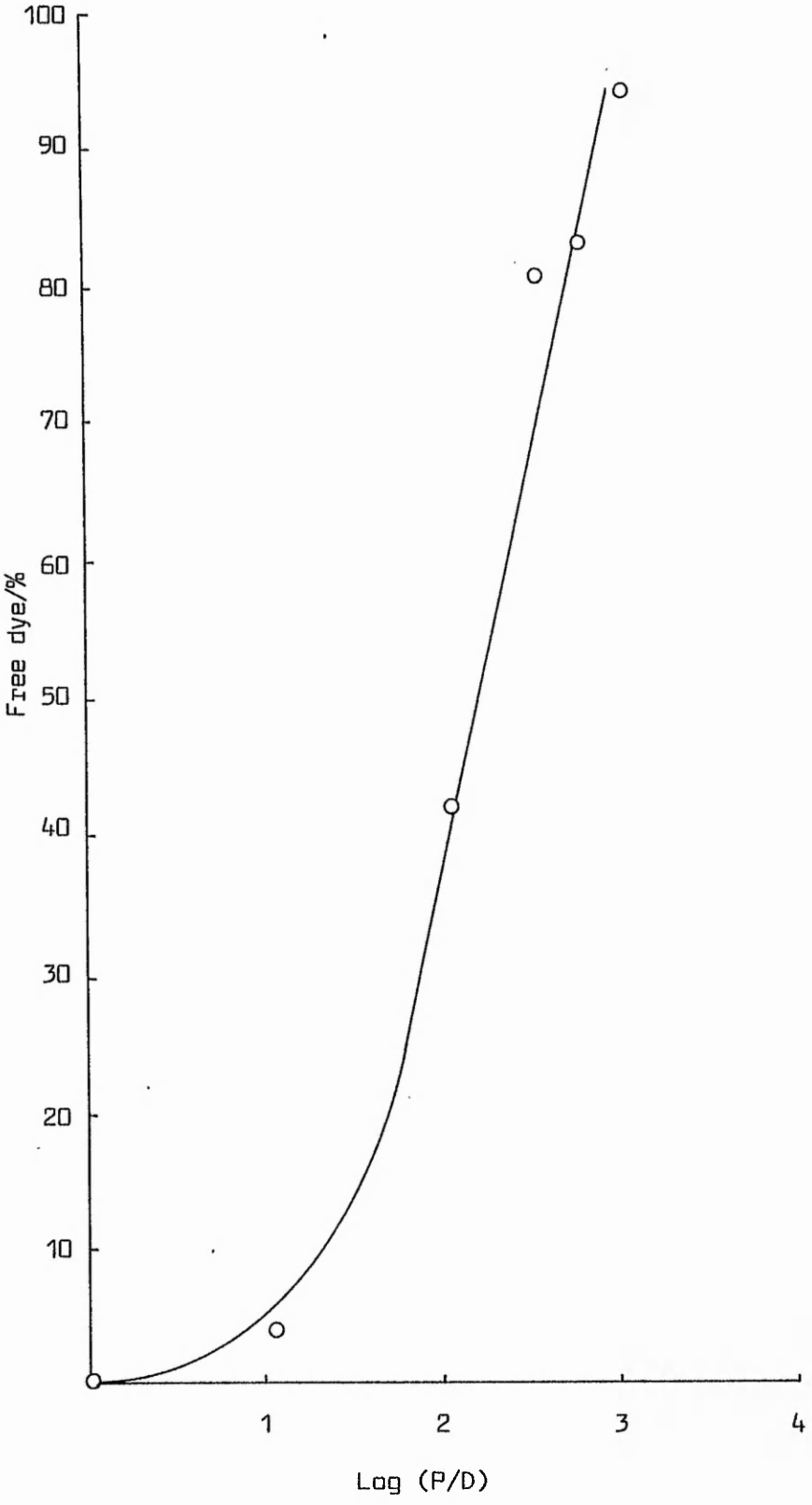
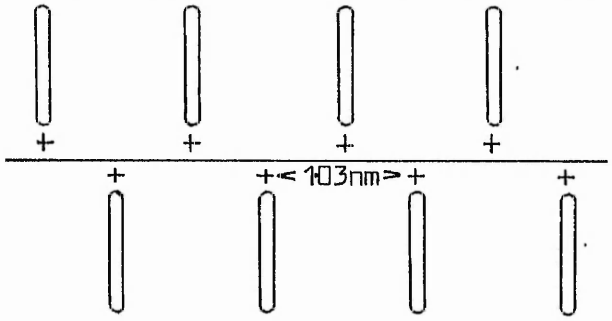


Figure 52. Reversal of metachromasy by addition of excess polymer (3×10^{-5}M AO7 solution)

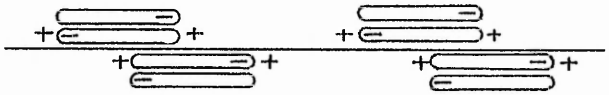
for example, Phillips[215], Shirai et al.[184] and Pal and Chaudhuri[182].

i) The intersite distance in fully de-N-acetylated chitosan is approximately 1.03nm, since the sites alternate on each side of the chain, and will be even greater when the chitosan is partially N-acetylated. Thus, the stacking model suggested by previous workers[183] is unlikely to lead to aggregation since the dye molecules



(A)

would be too far apart. Furthermore, a cationic starch with an average of only 1 charged group for every 15 anhydroglucose units has been shown to induce metachromasy in AO7, despite the even greater separation of sites[236]. Another model which might be invoked to overcome this objection in part, is one in which the dye ions are adsorbed at specific sites but lie along the chain rather than perpendicular to it.



(B)

ii) Polymers in solution have a very high degree of conformational entropy due to rapid conformational changes caused by segmental motion. Such changes would be impossible with aggregation of the type in model (A) which would prevent segmental motion since

this would require continual disruption of the dye aggregates. Thus aggregation of the type depicted in (A) would convert the polymer from a flexible chain to a rigid one with consequent loss of considerable conformational entropy. Aggregation of the type depicted in (B) would not prevent segmental motion completely but would reduce it considerable, hence reducing the conformational entropy.

iii) In the case of the chitosan/AR88 system the metachromatic shift can be observed visually as the colour changes from red to orange. Precipitates which formed in the metachromatic solutions in some instances, on standing, were red, although it is reasonable to assume that in the precipitate there is close ion pairing between the positively charged sites on the polymer chain and the sulphonate group of the dye. Evidence in support of specific site interactions in the solid state have been adduced from electron microscope studies[221] but these would have been carried out on a sample under vacuum and therefore in the absence of water. Under these conditions, site-dye interactions would be expected and a regular periodic structure observed, but such a structure would not necessarily occur in solution. Change in colour can also be observed on casting a film from the metachromatic chitosan/AR88 solution. As the solvent evaporates and the film forms, the colour reverts from the metachromatic orange to the red colour typical of the dye in the absence of chitosan. Conversly, a solution of AR88 undergoes no metachromatic change in the presence of hydroxethyl cellulose but a film cast from the solution shows bands of orange. These results suggest that in the latter case dye aggregation occurs as the solvent evaporates to give a more concentrated solution, but in the former case the dye is aggregated in solution, the aggregates breaking up as the solvent evaporates to give ion paired structures between charged sites on

the polymer and the dye ions.

The phenomenon of metachromasy can be explained by a mechanism that does not involve aggregation of the dye ions on the polymer. In polyelectrolyte solutions the counterions are not distributed uniformly throughout the solution but are concentrated within the electrostatic domain of the individual polyelectrolyte chains (Figure 53).

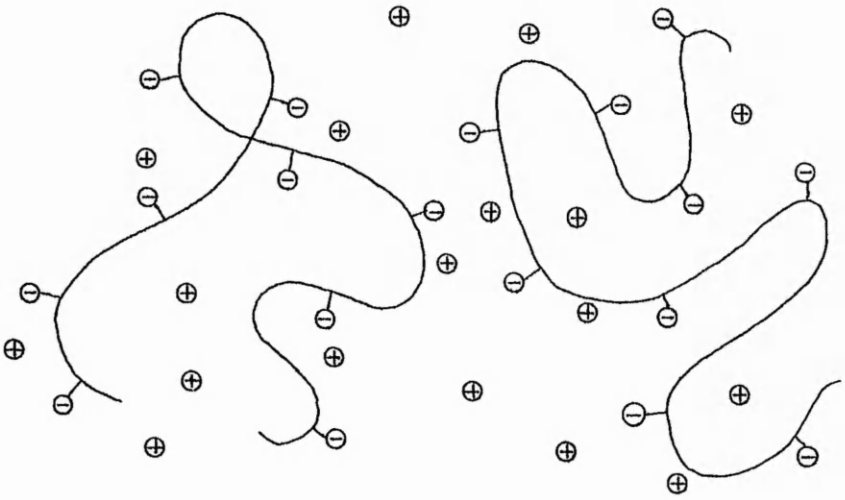


Figure 53. Distribution of counterions within a solution of a polyelectrolyte[237].

This distribution is reflected in the very low activity coefficients for the counterions[237]. On addition of a dye to the solution the dye ions are exchanged for the counterions in the electrostatic domain of the polyelectrolyte.

$$P\text{-}NH_3^{\oplus} + Xp^{\ominus} + D^{\ominus} \rightleftharpoons P\text{-}NH_3^{\oplus} + Dp^{\ominus} + X^{\ominus}$$

where $P\text{-}NH_3^{\oplus}$ = protonated amine group on chitosan chain

$Xp^{\ominus}$ = counterion within the electrostatic domain of a polyelectrolyte chain

$X^{\ominus}$ = counter ion outside the electrostatic domain of a polyelectrolyte chain

$D^{\ominus}$ = dye ion outside the electrostatic domain of a polyelectrolye chain

$Dp^{\ominus}$ = dye ion within the electrostatic domain of a polyelectrolyte chain

This exchange means that a highly concentrated dye solution is produced within the electrostatic domain of each of the polyelectrolyte chains, leading to aggregation of the dye ions and to the observed spectral changes. Aggregation lowers the total energy of the system by reducing the extent of contact between the hydrophobic surfaces of the dye molecules and water molecules. Furthermore, according to the theory of hydrophobic bonding, such aggregation will result in an overall increase in the entropy of the system. The reduction in energy and increase in entropy are the driving forces that move the equilibrium completely over to the right hand side and indeed ensure that dye ions enter the domain in excess of the amount of counter ion initially present, the limit being electrostatic equivalence between charged sites on the polymer and ionic groups on the dye molecules.

The ability of added neutral electrolyte to reverse metachromasy is due to a mass action effect shifting the equilibrium over towards the left hand side whilst the effect of ethanol and urea is due to the ability of these compounds to reduce or prevent aggregation of dyes, possibly by disrupting the structure of water and hence reducing hydrophobic bonding effects. Similarly, increasing the temperature will reduce the aggregating tendency of the dye ions, as

can be observed in bulk solutions. Reducing the tendency or ability of the dye ions to aggregate will reduce the driving force for dye ions to enter the polymer domain except by simple ion exchange with the counter ions. Reversal of metachromasy by increasing the P/D ratio above the stoichiometric ratio is due to the increase in the volume of the total electrostatic domain and hence the decrease in dye concentration, within this volume, until the concentration is below that required for aggregation.

Two main experimental results that have been interpreted as supporting specific site-dye interactions being important for the production of metachromatic shifts in the absorption spectra of dyes are i) the pulse radiolysis work of Phillips et al.[178,215] and ii) the ORD work of Stone et al.[210,211]. In the former works it was found that the rate of destruction of hydrated electrons , by interaction with cationic dyes, was reduced in the presence of an anionic polyelectrolyte. This was interpreted as being caused by a reduction in the accessibility, to the hydrated electrons, of the charges on the dye ions due to ion pair formation between the polymer sites and the dye ions. However the critical assumption in the argument, that the dye concentration is the same in the dye solution and in the dye/polymer solution, is open to question. Certainly, the overall concentrations in the two solutions are the same but the concentration distributions will not be. The dye ions will be uniformly distributed throughout the dye solution but this will not be the case in the dye/polymer solution. In the dye/polymer solution the dye ions will be concentrated within the electrostatic domains of the polymer chains. The hydrated electrons will be generated uniformly through both the dye solution and the dye/polymer solution,

but the rate of interaction between hydrated electrons and dye ions will be reduced in the latter solution because of the non-uniform distribution of dye ions within it.

The ORD results of Stone et al.[210,211] can be explained in terms of the proposed mechanism of metachromasy by assuming that a small fraction of the dye aggregates is adsorbed on the polyelectrolyte chain so that there is an equilibrium between dye aggregates in solution and adsorbed dye aggregates, with the equilibrium position lying well over on the side of aggregation in solution (Figure 54).

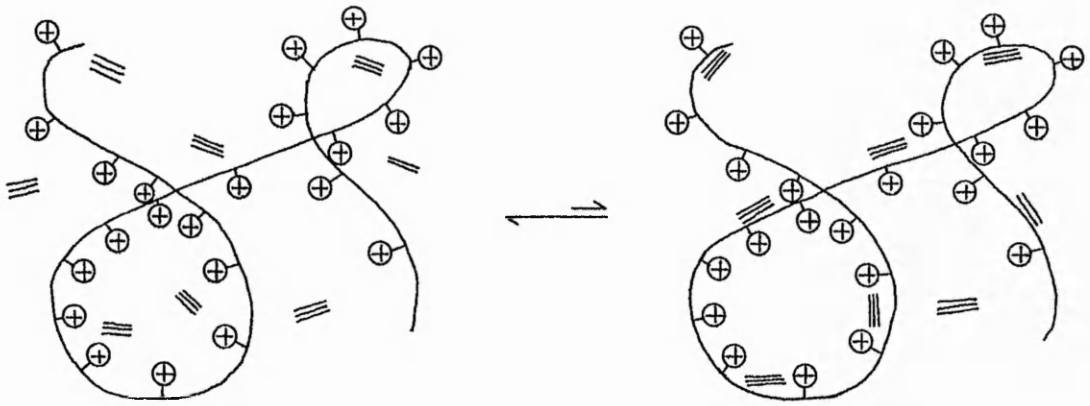


Figure 54. Distribution of aggregates between the solution and adsorption on the polymer chain.

The above theory eliminates the problem of intersite distance, and the problem of the loss of conformational entropy that would of necessity occur with dye/polymer site binding followed by aggregation as in the stacking model. Furthermore it offers an explanation of why denatured DNA is a more efficient chromotrope than native DNA[172], despite both forms having the same number of anionic sites per unit weight of polymer. Native DNA is a much stiffer polymer than

denatured DNA and hence its effective volume in solution will be greater. This means that the dye concentration within the electrostatic domain will be greater for the more flexible, more compact, chains of denatured DNA - hence reversal of metachromasy will be more difficult than with native DNA.

The theory also allows an explanation of metachromasy induced by detergents. A micelle formed by ionic detergent molecules will have a highly charged surface surrounded by a layer of water in which there is a high concentration of counter ions (Figure 55).

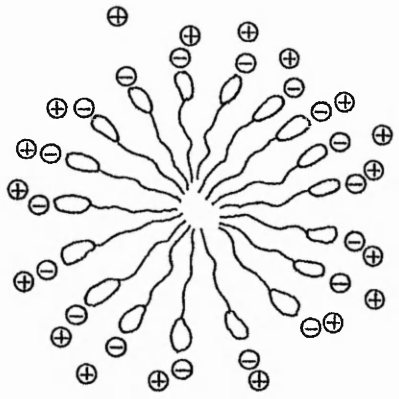


Figure 55. Detergent micelle structure.

Exchange of these counter ions for similarly charged dye ions will produce a concentrated dye solution around the micelle surface, leading to a high degree of dye aggregation and hence a change in the uv/visible spectrum. Again the driving forces for the ion exchange are the lowering of energy due to the decrease in water/hydrophobic surface interactions and the increase in entropy due to release of structured water molecules from around the individual dye molecules.

Two experimental tests were devised to distinguish between the stacking theory and the theory proposed above.

3.5.6.1 The interaction of dibasic anionic dyes with chitosan

The existing theories of the mechanism of metachromasy all involve specific interaction between the charged sites on the polymer and the dye ions, combined with aggregation of the dye. The order in which ion-pairing and aggregation take place, and their relative importance are discussed by several authors (see Section 2.9.11) but the general model of metachromæsy is one involving the regular arrangement of aggregated dye ions along the polymer backbone. For such a model the polymer site:dye ion stoichiometry (P/D ratio) for the metachromatic interaction with dibasic dye ions would have to be 1:1 despite the dye ions being divalent. Obviously it would be extremely unlikely, if not impossible, for the polyelectrolyte to remain in solution if each dye ion was bonded to two dye sites, as this would require either cross-linking between extended chains, or intra-molecular cross-links in folded chains (Figures 56a, 56b).

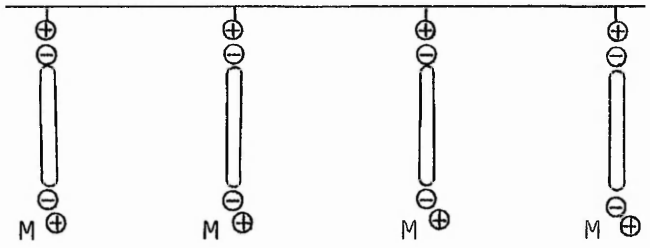


Figure 56a. Possible model for divalent dye/polyelectrolyte interaction involving 1:1 polymer site:dye molecule stoichiometry.

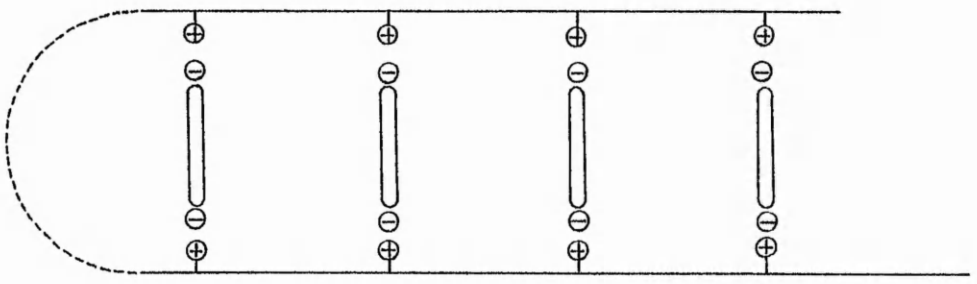


Figure 56b. Possible model for divalent dye/polyelectrolyte interaction involving interchain or intrachain cross-linking.

However, if specific site binding is not involved, and if aggregation of dye molecules in solution within the polymer domain is responsible for metachromasy, then the stoichiometry at the metachromatic equivalence point should indicate a P/D ratio of 2:1 (a polymer free amine group:dye anionic group ratio of 1:1).

Four dibasic dyes were included in this study. These were C.I. Direct Yellow 12 (DY12), C.I. Acid Red 97 (AR97), C.I. Acid Yellow 42 (AY42) and C.I. Direct Blue 8 (DB8) - structures XXXVIII, XXXIX, XL and XXXV in Figure 41. Metachromatic titrations were carried out with these dyes as for AO7 and AR88, and the results were plotted so that the equivalence point could be determined. The spectra and the plot of the ratio of peak heights at the initial and final λ max are shown for one dye (AY42) in Figures 57 and 58.

All four dyes gave similar results in that the analysis of their spectra - whether by peak height ratio or two-component mixture analysis - suggested that the metachromatic shift was complete when one dye anionic site was present for each polymer cationic site. This was confirmed by subjective visual assessment of the solutions,

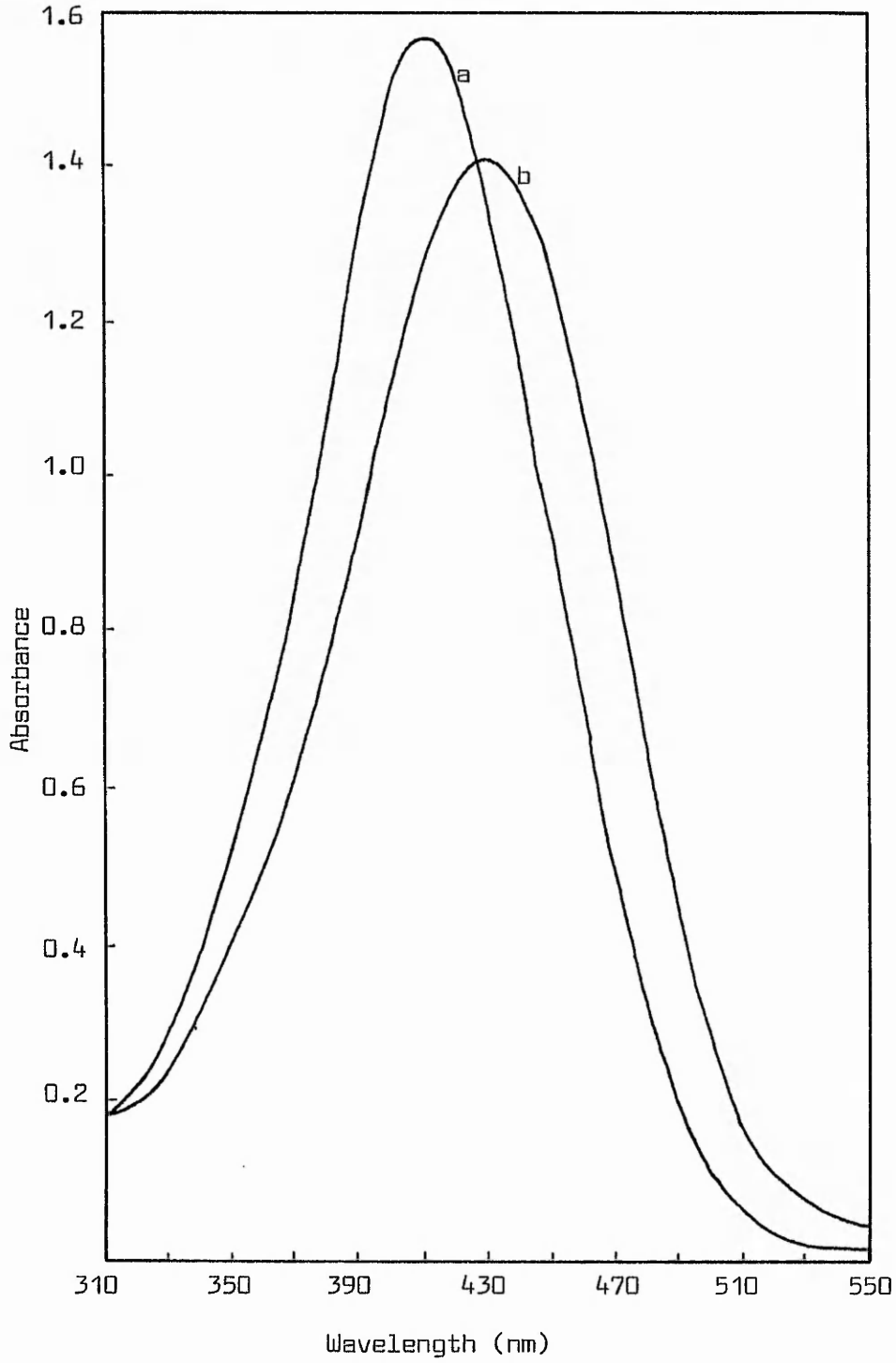


Figure 57. Metachromatic spectrum of the dibasic dye C.I. Acid Yellow 42.

a dye solution alone

b dye + polymer solution

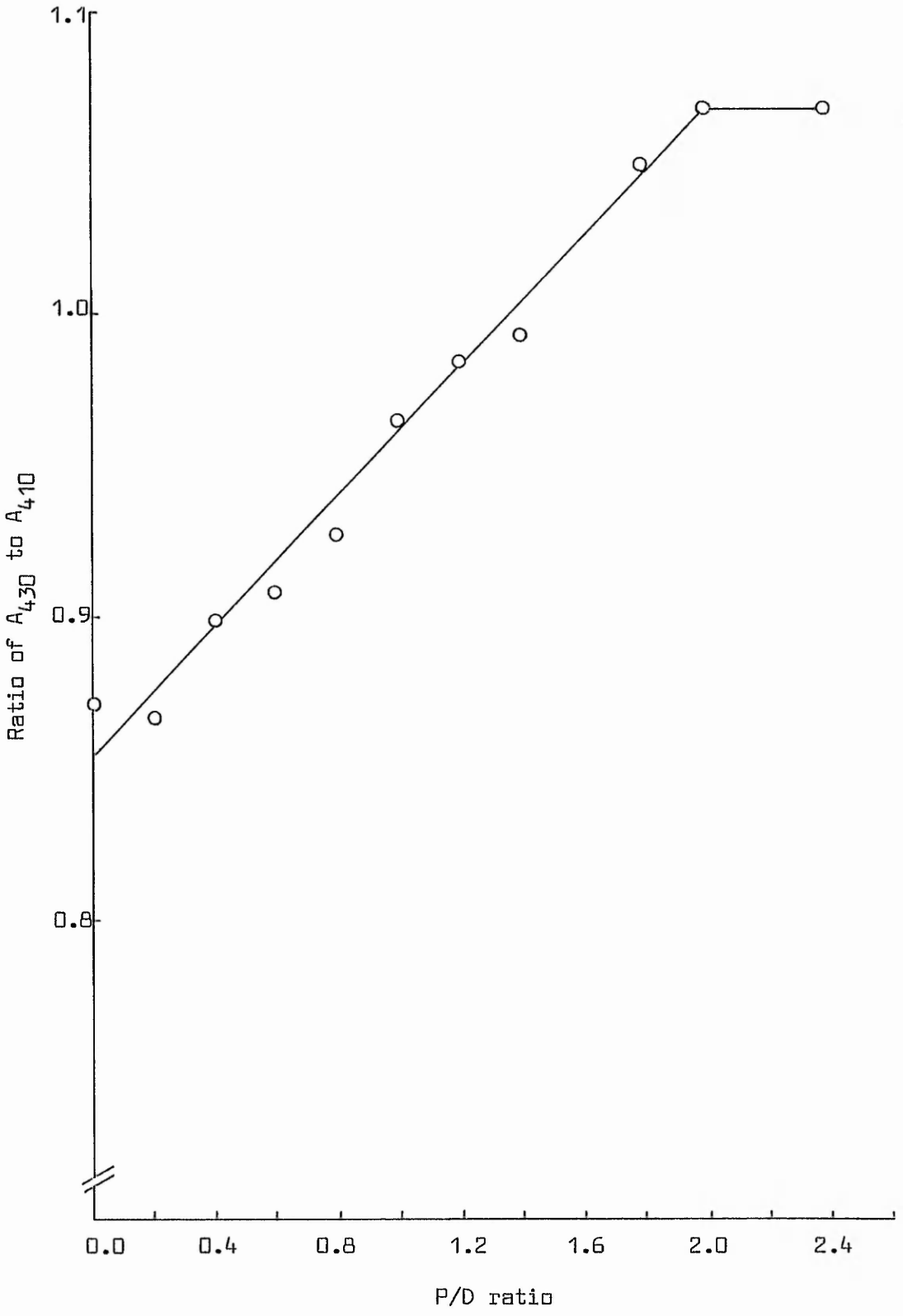


Figure 58. Metachromasy of C.I. Acid Yellow 42.

and spectral shifts, and also by a plot of the absorbance of the solution against the P/D ratio, when the equivalence point was found to be at a P/D ratio of 2:1. The titration plots were not so straightforward as those for the monobasic dyes, and in all four cases passed through a minimum at a P/D ratio of approximately 1.5:1 (Figure 59). The presence of precipitate was also noted in the solutions at this point. Interpretation of the spectra was complicated by the relatively small separation of the peaks (20nm for AY42) giving an apparent increase in free dye at the equivalence point for AY42. The spectra, however, showed that the λ max moved steadily towards the metachromatic wavelength (430nm, bathochromic shift) and that after the minimum was passed the solution peaks gained rapidly in intensity.

It is difficult to explain the metachromatic equivalence of dibasic dyes at a P/D ratio of 2:1 if the site-site binding of conventional models is maintained. However, if aggregation alone is considered, then the physical constraints imposed by ion-pairing are no longer a consideration, and equivalence will occur at the point at which the number of dye anionic groups is equal to the number of free amine groups. Within the polymer domain, aggregation of the dye molecules would take place to reduce the area of hydrophobic surface exposed to water and the metachromatic shift in the spectrum would be observed.

3.5.6.2 The influence of chitosan molecular weight on the ease of reversal of metachromasy

There are two models used in studies of polyelectrolytes in solution;

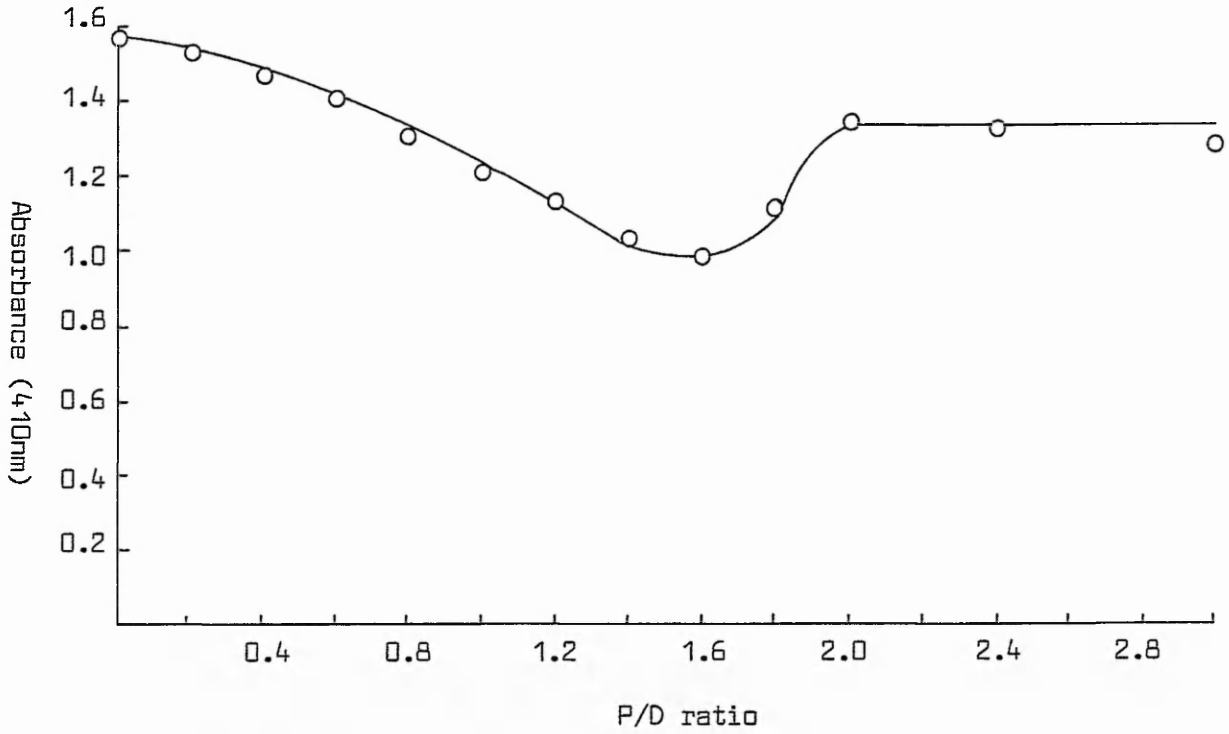


Figure 59. Metachromatic titration of C.I. Acid Yellow 42.

neither model has been found to be applicable in all situations. One model - the Strauss-Ander model[238], envisages the polymer domain as a cylindrical volume surrounding the polymer chain (Figure 60a). In this model the domain volume is directly proportional to the polymer chain length. The second model, the 'equivalent sphere' model[239], considers the polymer domain to be a roughly spherical volume surrounding the coiled polymer chain as a whole (Figure 60b). In this model the domain volume is proportional to $D.P.^{3/2}$ and would therefore increase more rapidly than the chain length with increasing degree of polymerisation.

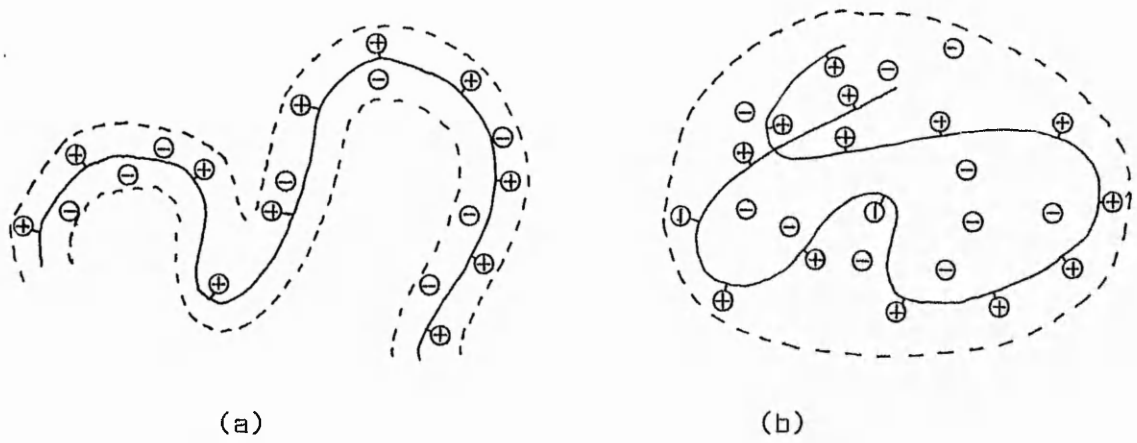


Figure 60. a) the Strauss-Ander model[238], and
b) the 'equivalent sphere' model[239] of polymer volume.

It would be predicted from the accepted model of metachromasy - involving aggregation of dye through binding at specific sites - that differences in the molecular weight of the polyelectrolyte would have no effect on the ease with which metachromasy could be reversed by increasing the P/D ratio, regardless of which of the models of polymer volume is correct. In the case of the theory proposed in Section 3.5.6 the ability of excess polymer to reverse

metachromasy would similarly be independent of molecular weight if the Strauss-Ander model for the polymer domain is correct since the total polymer domain volume for a given concentration of polymer in solution will be the same regardless of the molecular weight. However, if the 'equivalent sphere' model is correct then the total polymer domain volume for a given concentration of polymer in solution will be larger the greater the molecular weight of the polymer. Thus at any P/D ratio the concentration of dye within the polymer domain will be lower the greater the molecular weight of the polymer and hence a lower P/D ratio would be required to reduce the dye concentration to a low enough level that aggregation would not take place.

Samples of high molecular weight chitosan were hydrolysed in dilute hydrobromic acid solution to yield a medium and a low molecular weight chitosan sample, the molecular weights for both of these samples and a high molecular weight sample being determined by dilution viscometry[228]. The effects on the percentage of free dye of altering the P/D ratio for each of these samples is shown in Figure 61. It is clear that the metachromasy of the solutions is reversed more readily by the addition of excess high molecular weight chitosan than by the addition of excess medium molecular weight chitosan, and that the low molecular weight chitosan required a higher P/D ratio than either of the other two samples in order to reverse the metachromasy. This is the behaviour that is predicted on the basis of the new theory of metachromasy proposed in Section 3.5.6. It also shows that the 'equivalent sphere' model is more appropriate than the Strauss-Ander model for the discussion of the metachromasy-inducing behaviour of polyelectrolytes. The percent

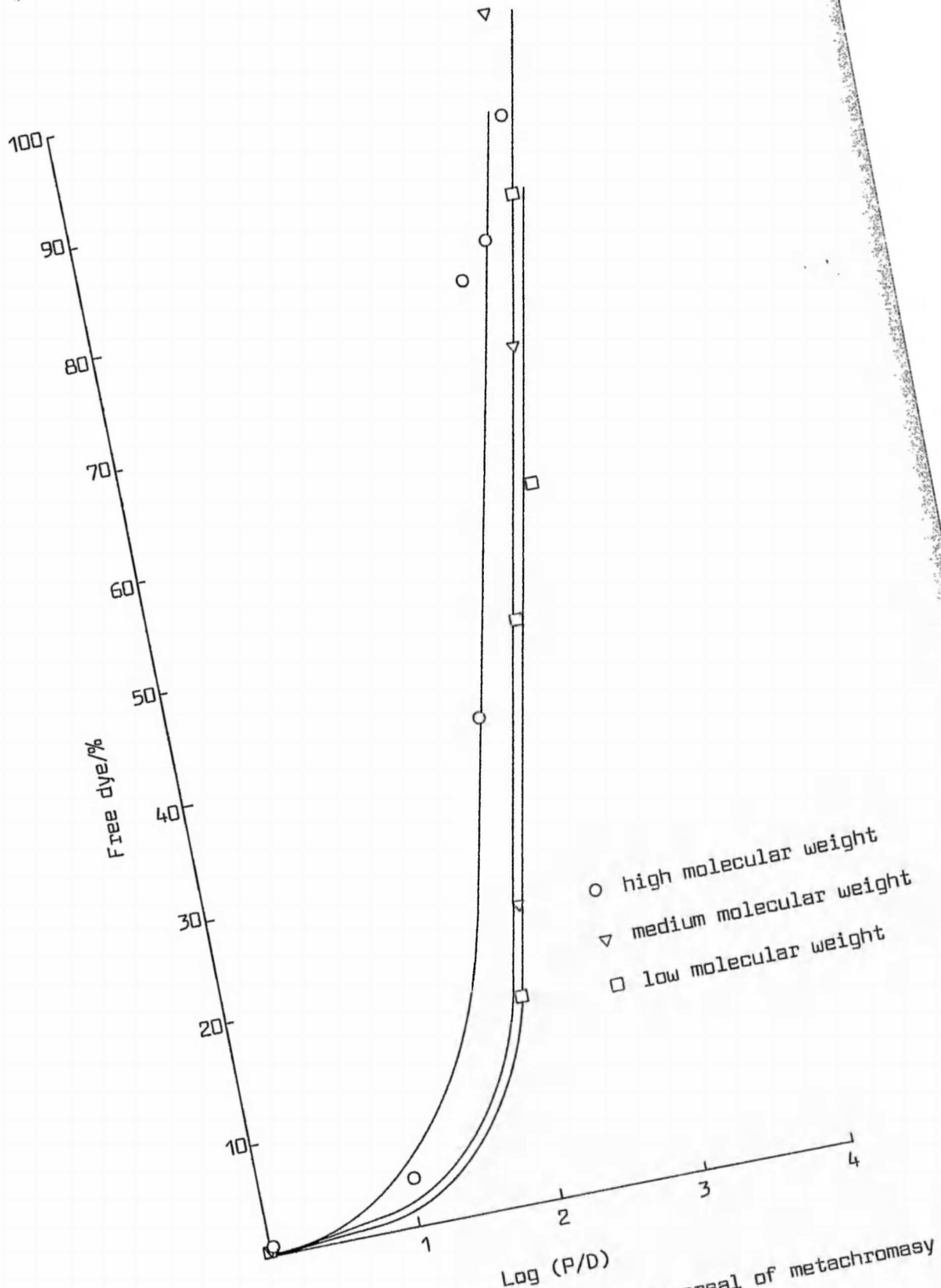


Figure 61. Effect of molecular weight on reversal of metachromasy (3×10^{-5}M AO7 solution)

N-acetylation of these samples was not identical, being 17.5% for the high molecular weight and 11.5% for the medium molecular weight sample. It does not seem likely, however, that this difference, which reflects free amine group contents of 82.5% and 88.5% respectively, would significantly affect the ability of the samples to reverse metachromasy. Furthermore, the percent N-acetylation of the low molecular weight sample was the same as that for the medium molecular weight sample.

These two tests - the behaviour with dibasic anionic dyes and the effect of polymer molecular weight on metachromasy reversal - support the proposed mechanism for metachromasy induced by the presence of polyelectrolytes, namely that the spectral changes are due to the formation of dye aggregates in the highly concentrated dye solution produced within the polymer domain by exchange of dye ions for the polymer counter ions.

CHAPTER 4

CONCLUSIONS

1) Copper (II) ions are readily adsorbed from solution by chitosan films and the extent of adsorption can be followed by treating Cu(II)-containing films with a number of copper-complexing reagents. There is an inverse relationship between film thickness and the quantity of Cu(II) ions adsorbed per unit weight of chitosan, suggesting that a concentration gradient exists across the film from the surface to the centre.

2) Chitosan having an enhanced degree of deacetylation may be obtained by giving two deacetylation treatments, with dissolution and reprecipitation of the chitosan between the two alkaline treatments. The chitosan produced in this way has a very low residual N-acetyl content (~ 1%) but high molecular weight. This supports the concept that deacetylation of chitin under heterogeneous conditions is limited by the lack of accessibility of a portion of the N-acetyl groups due to some morphological factor such as chain folding.

3) The enzyme papain (E.C. 3.4.22.2) does not hydrolyse residual N-acetyl groups of chitosan under homogeneous conditions. Instead the main polymer chain is hydrolysed leading to a reduction in chain length of the chitosan. The rapid rate of reduction of the solution viscosity, and the fact that the rate of reduction in viscosity increases with increase in the degree of N-acetylation of the chitosan substrate, indicates that the enzyme acts on chitosan in an 'endo'

manner and behaves as a chitinase rather than a chitosanase.

4) The adsorption from solution of C.I. Acid Red 88 by N-acetylated chitosan film proceeds by more than one mechanism. The mechanisms involved are most probably specific site adsorption at the protonated amine groups and diffuse adsorption at random points along the chain.

5) De-O-acetylation of N-acetylated chitosan film by treatment with ethanolic potassium hydroxide solutions causes some de-N-acetylation of the film as well. The degree of de-N-acetylation is too low to give a positive Rimini test but is apparent from the increased adsorption at 405nm, after treatment with a solution of salicylaldehyde in methanol, or at 510 nm after treatment with an acidified aqueous solution of C.I. Acid Red 88.

6) Chitosan has been shown to be capable of inducing metachromasy in a large number of anionic dyes covering a range of chemical structures. The induced metachromasy can be reversed by addition of neutral electrolytes, ethanol, urea or excess polymer, and by increase in temperature. This behaviour shows that the chitosan-induced metachromasy is similar to the metachromasy induced in cationic dyes by anionic polyelectrolytes.

7) The results show that contrary to previous statements in the literature the induced spectral shift may be either hypsochromic or bathochromic, depending on the particular dye, and that there is no requirement for the charge on the dye ion to be delocalised.

8) The metachromatic interactions between chitosan and C.I. Acid Red 88, and between chitosan and C.I. Acid Orange 7, are stoichiometric; equivalence being reached when there is one protonated polymer amine group for each dye sulphonic acid group. Thus the interaction can be used to assay the percentage of amine groups in a chitosan sample or to determine the concentration in solution of a chitosan sample of known degree of N-acetylation.

9) The results of the chitosan/anionic dye studies have been interpreted in terms of a new model for the mechanism of metachromasy. This attributes metachromasy to the aggregation of dye ions in solution within the polymer domain, due to the very high dye ion concentration. Specific ion pairing between dye ions and polymer ionic sites is not necessary for metachromasy and may actually prevent aggregation and hence preclude the induction of metachromasy.

10) The stoichiometry of the metachromatic interaction between chitosan and a number of disulphonic acid dyes is such that equivalence is reached when there is one protonated polymer amine group for dye sulphonic acid group. Furthermore reversal of the metachromatic behaviour of C.I. Acid Orange 7 on addition of excess chitosan occurs at a decreasing P/D ratio with increasing molecular weight of the chitosan. These results cannot be explained in terms of a mechanism for metachromasy that involves specific ion pairing between dye ions and ionic sites on the polymer, but support the new mechanism outlined in (9).

CHAPTER 5

EXPERIMENTAL

5.1 Materials used

5.1.1 Chitosan

The chitosan used in this work was supplied by Hercules Incorporated, Delaware,(Kytex H, Lot SPX 5350) and by the Kypro Company, Seattle, Washington.

5.1.2 Chemicals

The chemicals and solvents used were of General Purpose Reagent grade. The dyes used were commercial samples, purified, where necessary, as described in Section 5.11, except for XI, XII, XIII and XLI which were prepared by Dr G.A.F. Roberts. The water used was singly distilled. The papain used was supplied by Sigma [papainase, E.C. 3.4.22.2, lot P3125, twice recrystallised, 100mg enzyme suspended in 3.60cm^3 sodium acetate (0.05M) containing thymol (0.01%)]. Tris buffer [tris(hydroxymethyl)aminomethane], 'Trizma' base, was supplied by Sigma.

5.2 Spectroscopic methods

Infrared spectra were recorded on a Perkin Elmer 137 sodium chloride spectrophotometer, and on a Perkin Elmer 683 spectrophotometer, recording to 200cm^{-1}.

Uv/visible spectra and single wavelength measurements were carried out on a Perkin Elmer 551S spectrophotometer, and a Pye

Unicam SP500 single beam spectrophotometer.

5.3 Viscometry

Solution viscosities were measured using a Brookfield LVF viscometer, or for dilute solution a suspended level viscometer with sintered glass filter (Polymer Consultants Ltd.). All dilute solution viscometry was carried out in a specially designed thermostatically controlled waterbath at $25^{o}C \pm 0.05^{o}C$ (Bridge Instruments Ltd.) and flow times were measured by a stopwatch accurate to $\pm$ 0.2 seconds.

5.4 Film thickness

The thickness of chitosan films was measured using a Mercer film thickness gauge (Thomas Mercer Ltd., Model 54) graduated to 0.002mm. The absorbance of films at $890cm^{-1}$ was used as an internal reference for film thickness for the correction of absorbance measurements in Cu(II) and dyeing experiments. Good correlation was found between the two methods.

5.5 Characterisation of chitosan

5.5.1 Molecular weight

The molecular weight of chitosan samples was determined by measurement of their Limiting Viscosity Number (LVN) in a 0.1M acetic acid/0.2M sodium chloride solvent, using the suspended level viscometer. Repeat measurements of flowtimes were made until readings agreed to within 0.2 seconds. The Limiting Viscosity Numbers were related to molecular weight using the viscometric constants determined by Roberts and Domszy[228]. LVN's were determined from plots of Viscosity

number versus Concentration/g cm^{-3}, extrapolated to zero concentration. The molecular weight of Kytex H had been determined previously, using the same solvent and constants, to be 1.72×10^6g[103]. The molecular weight of the Kypro chitosan was found to be 2.01×10^6g.

5.5.2 Degree of N-acetylation

The degree of N-acetylation of Kypro chitosan was determined by metachromatic titration with AR88 (Section 5.16) to be 22%. This method has been shown to correlate well with the infrared method of Moore and Roberts[107]. The degree of N-acetylation for Kytex H had previously been shown to be 21%[103].

5.6 Preparation of chitosan

5.6.1 Powder form

Chitosan (1g) was dissolved in 1% aqueous acetic acid ($100cm^3$). The solution was filtered through a monofilament polyester cloth and poured into methanol/0.880 ammonia (7/3 v/v, $200cm^3$). The white precipitate was stirred thoroughly and then filtered off. It was then repeatedly slurried with methanol and filtered until neutral to litmus, and was then washed with ether ($100cm^3$). The product was then partially dried in air, ground to a powder, and dried completely.

5.6.2 Film form

Chitosan (1g) was dissolved in 1% aqueous acetic acid ($100cm^3$), and then filtered through a monofilament polyester cloth. Portions of the viscous solution were poured onto clean glass plates and spread as thinly and evenly as possible. The plates were then covered and

left to dry in a level, draught-free position. When all the solvent had evaporated, the plates were immersed in a large tank containing methanol/0.880 ammonia (7/3 v/v) for 16 hours. The films were then removed from the plates by soaking in a 10% solution of sodium hydroxide for 3 hours. The films were then washed in water and methanol before being dried between filter papers.

5.6.3 Low molecular weight chitosan

Chitosan (5g) was dissolved in hydrobromic acid (1% v/v, 500cm^3), and then filtered through a monofilament polyester cloth. The solution was divided into two portions, both of which were kept at 70^{o}C. One portion was reprecipitated after 48 hours and the second portion was reprecipitated after 168 hours. Reprecipitation was achieved by pouring each chitosan solution into methanol/0.880 ammonia solution (7/3 v/v, 200cm^3), stirring the precipitate thoroughly and then filtering. The precipitate was washed repeatedly with methanol until the filtrate was neutral to litmus, and was then washed with ether and air dried.

5.7 Preparation of enzyme solutions

5.7.1 Brookfield viscosity measurements

Chitosan (1g) was dissolved in dilute acetic acid (1% v/v, 220cm^3) and the solution viscosity was measured at both 20^{o}C and 37^{o}C. To the solution was then added sodium metabisulphite (1% w/v, 25cm^3) and EDTA (0.02M, 25cm^3), and the viscosity was remeasured at 37^{o}C. Papain suspension (0.5cm^3) was then added, and the viscosity of the solution was measured at intervals of 5 minutes at a temperature of 37^{o}C.

5.7.2 Dilute solution viscosity measurements

Details of individual solutions are given on the appropriate Figures. All solutions were prepared approximately one hour before being measured so that the solution could equilibrate to the temperature of the waterbath. Papain suspension was added immediately before measurements commenced, and measurement of elapsed time began at the time of enzyme addition. If no enzyme was added an equivalent amount of an appropriately diluted sodium acetate solution was added instead. For dilute enzyme concentrations the enzyme suspension ($0.5cm^3$) was diluted to $10cm^3$ with distilled water. The dilute suspension was freshly prepared daily.

Viscometers were thoroughly rinsed before measuring a new solution, first with acetic acid (10% v/v, three rinses), then with solvent (two rinses), and then with the new solution (two rinses). If activators were used, a stock solvent containing the desired amounts of the activators was prepared, and was used to dissolve and subsequently dilute the chitosan.

5.7.3 Assay of Pseudomonas aeruginosa

Pseudomonas aeruginosa cultures (strain AI3, 4 x $100cm^3$) provided with acetanilide as the sole source of carbon were supplied by Dr A. Skinner, Department of Life Sciences, Trent Polytechnic. The cultures were centrifuged in 2 x $200cm^3$ aliquots at 10,000 rpm for 5 minutes, and the supernatant was poured off. The cells were resuspended in 2 aliquots in 'tris' buffer (0.05M, pH 7.2, $10cm^3$) and were recentrifuged. The pellets were combined and resuspended in 'tris' buffer ($5cm^3$) and kept on ice. The cells were then

ruptured by sonication (3 x 20 seconds), while the temperature of the cells was kept low in an ice bath. Finally, the culture was centrifuged at $5^{o}C$ (15,000 rpm, 15 minutes), the ruptured cells were discarded and the supernatant retained for assay of amidase activity.

Pseudomonas aeruginosa is an 'accidental pathogen' - although a normal part of the gut flora it can infect cuts, wounds and burns. As it is very resistant to antimicrobial agents, strict attention should be paid to hygiene in the handling and disposal of suspensions of this bacterium.

When cultured in the presence of acetanilide as the sole carbon source, Pseudomonas aeruginosa (AI3), produces an amidase which will hydrolyse several amides, such as acetamide (1), and will also catalyse the formation of acyl hydroxamates from amides in the presence of hydroxylamine (2).

$$CH_3.CO.NH_2 + H_2O \longrightarrow NH_4^+ + CH_3COO^- \quad \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

$$CH_3.CO.NH_2 + NH_2OH + H^+ \longrightarrow CH_3.CO.NHOH + NH_4^+ \quad \ldots\ldots\ldots(2)$$

The hydrolysis of p-nitroacetanilide, and the formation of an acyl hydroxamate can both be used as convenient assays of amidase activity.

a) The hydrolysis of p-nitroacetanilide

To p-nitroacetanilide ($2.9cm^3$, $5 \times 10^{-4}M$) in 'tris' buffer (0.05M) is added cell-free extract ($0.1cm^3$) and a similar blank solution with no extract is prepared. The solutions are incubated at $37^{o}C$, and the appearance of p-nitroaniline monitored by measurement of the uv/visible absorbance at 400nm.

b) The formation of hydroxamate

A solution of mixed substrates was prepared containing acetamide (0.4M):hydroxylamine hydrochloride (2.0M, freshly neutralised): 'tris' buffer (0.1M, pH 7.2), in the ratio 1:1:2. The mixed substrate solution was kept on ice until required in order to avoid the spontaneous reaction of acetamide with hydroxylamine. A portion ($3.8cm^3$) of this solution was equilibrated in a waterbath (37^oC) for a few minutes, then the cell-free extract ($0.2cm^3$) was added. A blank was prepared similarly, but with additional 'tris' buffer ($0.2cm^3$) in place of the enzyme extract. Aliquots ($1cm^3$) were removed from the solutions at 0, 5 and 10 minute intervals, and added to ferric chloride/hydrochloric acid reagent ($2cm^3$). The solution was shaken, and the absorbance measured at 500nm against the extract-free blank. Ferric chloride gives a dark brown complex in the presence of hydroxamates.

c) Chitosan assay

A mixed substrate was prepared from chitosan hydrobromide (1% w/v, $2cm^3$), hydroxylamine hydrochloride (2.0M, $2cm^3$) and 'tris' buffer (0.1M, $4cm^3$). Similar mixed substrates were prepared from chitosan hydrobromide (0.1% w/v, $2cm^3$) or from N-acetylglucosamine (0.1% w/v, $2cm^3$). All substrates were dissolved in 'tris' buffer. From each mixed substrate solution, aliquots ($3.1cm^3$) were taken to provide an enzyme system and a blank. The solutions were equilibrated to 37^oC. Enzyme extract ($0.2cm^3$, previously shown to be highly active) or 'tris' buffer ($0.2cm^3$) was then added as appropriate. Aliquots ($1cm^3$) were withdrawn from each system at 0, 5 and 15 minute intervals

and added to ferric chloride/hydrochloric acid reagent ($2cm^3$). Sampling was also carried out after 24 hours.

5.8 De-acetylation of chitosan

5.8.1 Sodium hydroxide system

Chitosan (4g) was slurried with sodium hydroxide (50% w/v, $80cm^3$), heated to 75^oC in a waterbath, and maintained at this temperature for 1 hour. The slurry was stirred throughout the procedure. The chitosan was then filtered, washed well with water, and slurried with methanol/water (4:1 v/v). Small amounts of acetic acid were added to neutralise the alkali in solution, to facilitate the extraction of alkali from the sample. The addition of acid was monitored carefully by pH meter to ensure that the mixture did not become acidic. When all the sodium hydroxide had been extracted, the sample was filtered, washed thoroughly with water, then methanol and finally ether, before being air dried and weighed. Samples were taken at this stage for molecular weight and degree of N-acetylation determination. The chitosan was then redissolved in acetic acid (1% v/v, $250cm^3$), reprecipitated in methanol/0.880 ammonia (7:3, $450cm^3$), filtered on a monofilament cloth, and then repeatedly slurried in methanol and filtered until the filtrate was neutral to litmus. The chitosan was then washed with ether, and air dried. Samples were again retained for molecular weight and degree of N-acetylation determinations. The alkali treatment was then repeated (2g chitosan, $40cm^3$ sodium hydroxide) and final samples were taken.

5.8.2 Alcohol/sodium hydroxide system

This system was essentially the same as that described in Section 5.8.1, but the quantity of alkali was greatly reduced. Chitosan (4g) was slurried with 2-methylpropan-2-ol (tert-butanol, 80cm^3) and heated with stirring to 75^{0}C on a waterbath. Sodium hydroxide (50% w/w, 5cm^3) was added to the flask and the temperature maintained at 75^{0}C for one hour. The chitosan was then filtered, washed, redissolved and precipitated as before, with samples retained at appropriate stages. The alcohol/alkali treatment was then repeated (2g chitosan, 40cm^3 2-methylpropan-2-ol, 2.5cm^3 alkali).

5.8.3 Deacetylation in the presence of sodium borohydride

a) Sodium hydroxide system

Sodium borohydride (2g) was added to the chitosan/NaOH slurry immediately after the addition of the alkali. When the solution and reprecipitation step of the process was completed, a second deacetylation process was carried out and sodium borohydride (1g) was added to the slurry immediately after the addition of alkali.

b) Alcohol/sodium hydroxide system

Sodium borohydride (0.5g) was added to the chitosan/NaOH slurry immediately after the addition of the alkali. When the solution and reprecipitation step of the process was completed, a second deacetylation process was carried out and sodium borohydride (0.25g) was added to the slurry immediately after the addition of alkali.

5.9 The treatment of chitosan films with copper acetate

Pieces of chitosan film (≃ 0.025g) were mounted in plastic slide mounts (35mm transparency size) and suspended in copper acetate solutions ($50cm^3$) of the required concentration. Washing of the film with distilled water, and the subsequent treatment of the film with copper-complexing agents were carried out without removing the film from the slide mount. The slide mounts also offered a convenient means of presenting the chitosan film for infrared and uv/visible spectrophotometric measurement. The sample areas of the instruments were adapted with suitable slide holders built from sheet polystyrene and painted matt black.

5.10 Detection of Cu(II) ions

5.10.1 Sodium diethyldithiocarbamate[240]

Well-washed films were treated with aqueous sodium diethyldithiocarbamate solution (0.2% w/v, $20cm^3$). A yellow-brown to dark-brown colour develops in the film in the presence of copper. Treatment solutions remain clear.

Spectrophotometric details (differences from untreated film):

in the absence of copper acetate, λ max (uv/vis) 260nm, 293nm;

in the presence of copper acetate, λ max (thin film) 280nm, 450nm;

ν max (thin film) $1500cm^{-1}$, $1260cm^{-1}$, $1200cm^{-1}$.

5.10.2 Ammonium thiocyanate/pyridine[241]

Chitosan film was immersed in water ($20cm^3$), pure pyridine ($2cm^3$)

and solid ammonium thiocyanate (0.5g) were added to the solution - chitosan films containing copper became bright green at this point. The film was then washed and dried and the spectrum recorded.

Spectrophotometric details (differences from chitosan film):

in the absence of copper, ν max 2000cm^{-1};

in the presence of copper, ν max 2000cm^{-1}, intense.

5.10.3 Dimethylglyoxime[242]

Chitosan film was immersed in a solution containing methanolic dimethylglyoxime (1% w/v, 25cm^3) and 0.880 ammonia (1% v/v, 5cm^3). In the presence of copper-treated films, the solution became intensely coloured.

5.10.4 α -Benzoinoxime[243]

Chitosan film was treated with a solution containing 0.880 ammonia (1% v/v, 5cm^3), α-benzoinoxime (1% v/v in methanol, 5cm^3) and water (25cm^3). Film pretreated with copper became deep green.

Spectroscopic details (differences from chitosan film):

in the presence of copper, λ max 444nm.

5.11 Purification and preparation of dyes

5.11.1 Purification of C.I. Acid Red 88

The free acid of dye (I), previously purified by the method of Nursten and Williams, was available as a solution, as the dry free acid is known to be unstable to heat[244]. The sodium salt of the dye was prepared by titrating the dissolved free acid (0.0508% w/v,

500cm^3) against dilute sodium hydroxide solution (0.001M), until the neutral point was reached (pH meter). The dye was then evaporated to dryness under vacuum in a rotary evaporator and solutions of the sodium salt in distilled water prepared. The concentrations of dye solutions were checked from the published extinction coefficient for the sodium salt[244], and were then expressed as % w/v so that concentrations in relation to the weight of chitosan film (percent on weight of film, % owf) could be readily calculated:

$$\% \text{ owf} = \frac{V \times S}{W}$$

where V = volume of stock solution taken, cm^3

W = weight of film, g

S = concentration of stock solution, % w/v

5.11.2 Purification of C.I. Acid Orange 7 and dibasic dyes

C.I. Acid Orange 7 and the dibasic dyes (structures XXXV, XXXVIII, XXXIX, XL) were purified by the following method. Dye (1g) was soxhlet-extracted with ethanol (150cm^3) for 4 hours. The alcoholic dye solution was then evaporated on a steam bath to about half of the original volume and the dye recrystallised. The solid was filtered, redissolved in hot ethanol and again recrystallised.

5.12 Dyeing of chitosan films with C.I. Acid Red 88

Chitosan film ($\simeq$ 0.025g) was dyed from a solution containing sufficient dye (0.0508% w/v free acid or 0.0513% w/v sodium salt) to give the required concentration (% owf). Formic acid (0.1% w/v) was added where appropriate to give the required concentration (% owf).

The total solution volume was adjusted with distilled water to give a liquor ratio (LR) of 2200:1 (approximately 50cm^3). The film was dyed in a stoppered conical flask in a shaking waterbath (65^{0}C) or under reflux at the boil.

Acetate buffer was prepared from acetic acid (0.2M) and sodium acetate (0.2M) to give the following pH's:

acetic acid/cm^3	sodium acetate/cm^3	pH
6.0	4.0	4.45
4.0	6.0	4.80
2.0	8.0	5.23
0.5	9.5	5.89

5.13 N-acetylation of chitosan

5.13.1 In film form[40]

Chitosan film (0.025g) was steeped in acetic anhydride in methanol (1% v/v) overnight, then washed thoroughly in methanol. Where subsequent de-O-acetylation was carried out, the N-acetylated film was soaked in ethanolic potassium hydroxide (0.5% w/v) then washed thoroughly with ethanol.

5.13.2 Homogeneous N-acetylation[40]

Chitosan (1.25g) was dissolved in acetic acid (1% v/v, 125cm^3) and diluted with methanol (150cm^3). Acetic anhydride in methanol (2% v/v, 3.5cm^3) was added, the solution was thoroughly mixed and then allowed to stand for 1 hour. After this time the reaction was stopped by pouring the solution into methanol/0.880 ammonia (7:3).

The precipitate was filtered, washed thoroughly with methanol until the filtrate was neutral to litmus, then washed with ether, and finally air dried. Other chitosan samples were N-acetylated by the same technique with 15, 20 and 30cm^3 methanolic acetic anhydride (2% v/v) respectively.

5.14 Formation of the Schiff's base of chitosan with salicylaldehyde

Chitosan film (0.025g) was refluxed in methanol (50cm^3) in the presence of salicylaldehyde (1cm^3) for one hour. The film was then washed thoroughly with methanol and allowed to dry, before the uv/visible spectrum was obtained (λ max 407nm).

5.15 Survey of dyes for metachromatic behaviour

5.15.1 Acid, mordant and direct dyes

A small amount of the unpurified dye (approximately 0.01g) was dissolved in a test tube in acetic acid (0.6% v/v, 30cm^3). The solution was heated gently to encourage dissolution of the dye when necessary. The solution was then divided into two parts (15cm^3 each); to one half was added chitosan solution [1% w/v in acetic acid (0.6% v/v), 15cm^3], and the other portion was then diluted to the same extent with acetic acid (0.6% v/v, 15cm^3). The uv/visible spectra of the two solutions were then compared.

5.15.2 Basic dyes

The technique for basic dyes was essentially the same as that for acid, mordant and direct dyes, but all solutions were prepared in distilled water, and the polymer used was carboxymethyl cellulose (2% w/v in distilled water).

5.16 Metachromatic titration of chitosan

5.16.1 Preparation of stock dye solution

C.I. Acid Red 88 (0.3g) was dissolved in aqueous acetic acid ($1000cm^3$) to give a solution of approximately $7.5 \times 10^{-4}M$. Aliquots ($4cm^3$) were then used in the metachromatic titration in a total volume of $100cm^3$, giving a dye concentration of approximately $3 \times 10^{-5}M$, and an absorbance (1cm cells, 505nm) of $\simeq$ 0.60 absorbance units in the absence of polymer. The exact solution concentration was determined using the published extinction coefficient for AR88 of 20,350[244]. Stock solutions of C.I. Acid Orange 7 were prepared in a similar way and calibrated using ε max 22,500[244]. The absorbance of this solution in the absence of polymer (1cm cells, 484nm) was approximately 0.64 absorbance units.

Note: Initial work with C.I. Acid Red 88 used the dye at $1.5 \times 10^{-5}M$ in the final solutions, giving absorbances in the absence of polymer of approximately 0.29 absorbance units. Reproducibility in this range was good, but the concentration was increased to allow greater independence from minor variations in experimental technique.

5.16.2 Preparation of chitosan solutions

Chitosan (0.16g) was dissolved in aqueous acetic acid (0.6% v/v, $100cm^3$). A portion ($10cm^3$) of this solution was then diluted to $250cm^3$ with aqueous acetic acid; $10cm^3$ of this stock solution were approximately equivalent to $4cm^3$ of the stock dye solution.

5.16.3 The titration of chitosan solution with a metachromatic dye

Aqueous acetic acid (0.6% v/v, 20cm^3) was placed in a volumetric flask (100cm^3 capacity) and an aliquot of stock dye solution (4cm^3) added. The flask was stoppered and inverted to ensure that all the dye was diluted. The required amount of dilute chitosan stock solution was then added to the flask, and the solution made up to the mark with acetic acid (0.6% v/v). The contents of the flask were mixed thoroughly by repeated inversion before the absorbance of the solution was read at 484nm (AO7) or 505nm (AR88) in 1cm cells against a distilled water blank. [It had previously been established that there was no difference in the absorbance values obtained in comparison of solutions made up with distilled water or with acetic acid (0.6% v/v) blanks]. Suitable volumes of dilute chitosan stock solution (for a 21% N-acetylated sample) were 2, 5, 8, 10, 15 and 20cm^3. A control flask without polymer was also prepared. The recorded absorbances were then plotted against the volume of chitosan added, so that the volume of polymer solution required for equivalence could be determined.

5.16.4 Determination of the degree of N-acetylation of chitosan

5.16.4.1 Construction of a free amine equivalent weight/degree of N-acetylation conversion graph

The free amine equivalent weight at any degree of N-acetylation can be calculated from the following equation:

$$\text{free amine equivalent weight} = \frac{(N \times C) + (100-N) \times P}{100-N}$$

where N is the degree of N-acetylation (percent)

C is the repeat unit weight for chitin (100% N-acetylated) = 203

P is the repeat unit weight for polyglucosamine (no N-acetylation) = 161

Thus, the free amine equivalent weight for a 20% N-acetylated sample of chitosan is given by:

$$\text{free amine equivalent weight (20\% N-acetylated)} = \frac{(20 \times 203) + (80 \times 161)}{80}$$

$$= 211.75$$

Similar calculations for different degrees of N-acetylation were used to construct Figure 62. This graph could then be used to convert a calculated equivalent weight of free amine to % N-acetylation, or conversely, to convert the degree of N-acetylation of a sample to the relevant free amine equivalent weight.

5.16.4.2 Calculation of degree of N-acetylation from the equivalence point

The equivalence point for the metachromatic titration was determined by interpolation of the horizontal plot obtained (when P/D > 1) to the slope obtained as P/D approaches 1. It was then possible to calculate the weight of chitosan that was equivalent to $4cm^3$ of dye of known concentration, and thus to determine the free amine equivalent weight of the sample, given that the stoichiometry for the titration is

1 mole free amine $\equiv$ 1 mole dye (monobasic dye)

An example of the calculation is given in Appendix I.

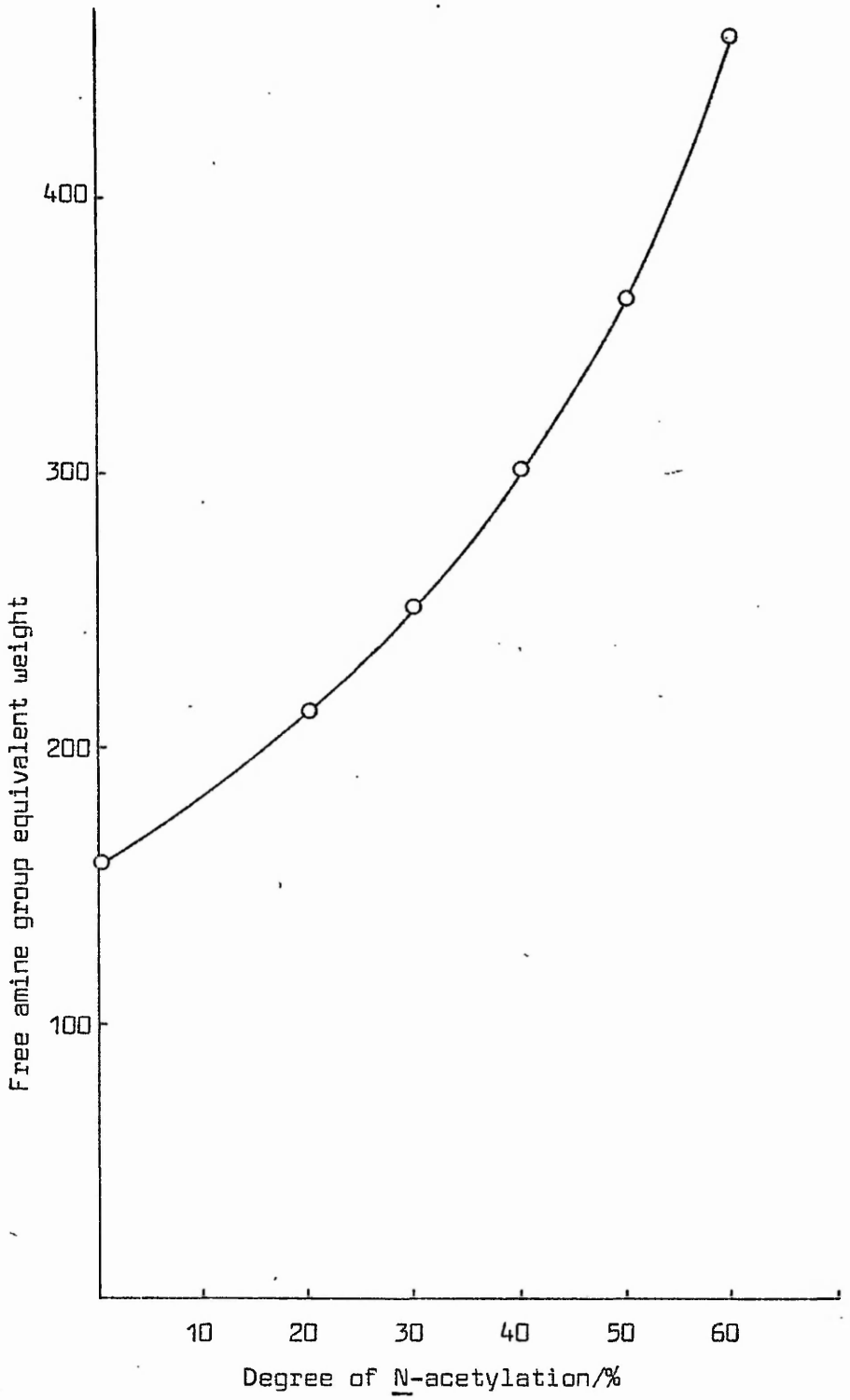


Figure 62. Conversion graph - Free amine group equivalent weight versus Degree of N-acetylation of chitosan.

5.16.4.3 Calculation of the concentration of chitosan

The calculation followed the steps set out in Section 5.16.4.2, but here the degree of N-acetylation of the sample was known, and could be converted to free amine equivalent weight (Figure 62). It was then possible to calculate the concentration of the chitosan solution from the volume required for equivalence between the polymer and a known concentration of the dye, taking into account the dilution used to obtain the working solutions.

5.17 Metachromatic titration in the presence of electrolytes and urea

Stock chitosan solution was first titrated against the standard dye solution (A07) as described in Section 5.16 in order to determine the equivalence point for the polymer. A series of volumetric flasks were then prepared, each of which contained aqueous acetic acid (0.6% v/v, $25cm^3$), dye (7.5×10^{-4}M, $4cm^3$) and chitosan solution (3×10^{-4}M free amine equivalent, $10cm^3$). The solutions were mixed well, and the required volumes of electrolyte or urea solution (2M; 0, 0.5, 1, 5, 10 or $25cm^3$; 0.02M; 0.5, 1, 5, 10 or $25cm^3$). All electrolyte or urea solutions were prepared by dissolving the dry solid in aqueous acetic acid (0.6% v/v). The flasks were then made up to the mark with aqueous acetic acid (0.6% v/v) and the contents were thoroughly mixed by inversion of the flasks, before absorbances were read (484nm, 1cm cells, distilled water reference).

A similar routine was followed for a series of control solutions, which were prepared in the absence of polymer so that the effect of the electrolyte or urea on the absorbance of the dye could be measured.

Determinations were also carried out:

a) using AR88 (7.5×10^{-5}M, $4cm^3$ or $2cm^3$) measured at 505nm;

b) using higher concentrations of sodium chloride or urea, when sufficient dry solid was added to the flasks to give the desired concentration;

c) with the addition of the electrolyte or urea preceding the addition of the polymer solution.

5.18 Metachromatic titration in the presence of ethanol

Chitosan solution (5×10^{-4}M free amine equivalent, $6cm^3$) was placed in a volumetric flask ($100cm^3$ capacity) and glacial acetic acid ($0.55cm^3$) was added. A07 stock solution (7.5×10^{-5}M, $4cm^3$) and distilled water ($84.5cm^3$) were also added. The flask contents were mixed thoroughly, and then ethanol ($5cm^3$) was added, to bring the solution up to the mark. Similar flasks were prepared containing 0, 1, 10, 15, 20, 50 or $89.5cm^3$ ethanol, with the amount of distilled water required to make a total of $100cm^3$ being added in each case. The absorbance of the solutions was measured at 484nm (1cm cell, distilled water reference). Control flasks were also prepared in the absence of polymer.

A similar series was also carried out using a lower molecular weight chitosan (3×10^{-4}M free amine equivalent, $10cm^3$).

5.19 Metachromatic titration in the presence of excess polymer

Chitosan solution (3×10^{-2}M free amine equivalent) was added to volumetric flasks ($50cm^3$) to give final P/D ratios ranging from 1:1 to 1000:1. The flasks were stoppered and heated gently in a

waterbath to 70°C. Dye solution (AO7, 7.5×10^{-4}M, $2cm^3$) was then added to the flasks and the contents were made up almost to the mark with aqueous acetic acid (0.6% v/v). The samples were then allowed to cool to room temperature before being diluted with acetic acid to the mark. The absorbance of the solutions was measured at 484nm (1cm cells, distilled water blank).

It was necessary to warm the concentrated polymer solution before adding the dye in order to avoid precipitation.

5.20 The effect of temperature on the metachromasy of chitosan

Jacketed, stoppered cells (both sample and reference) were connected in parallel to a constant temperature waterbath and circulating pump (Grant Instruments Model FH15). The temperature difference between the cells was less than 1°C. The change in absorbance of polymer/dye solutions at a P/D ratio of 1:1 (prepared from solutions standardised as in Section 5.16.3) was followed with change in temperature, at 484nm (AO7) or 505nm (AR88).

Similar measurements were carried out in the presence of sodium chloride, sodium iodide and calcium chloride, with solutions prepared as in Section 5.17. Again the P/D ratio was 1:1. Electrolyte concentrations ranged from 5×10^{-4}M to 1×10^{-2}M.

5.21 Dibasic dye titrations

Purified dibasic dye (0.3g) was dissolved in aqueous acetic acid (0.6% v/v, $500cm^3$). Aliquots of the stock dye solution ($4cm^3$) were then titrated against previously standardised chitosan solution, following the method set out in Section 5.16.3. The equivalence point was defined as the volume of polymer at which the plot of

Polymer volume versus Absorbance became horizontal. The λ max for each of the dyes in the absence of polymer was:

C.I. Direct Yellow 12	395.5nm
C.I. Acid Yellow 42	410nm
C.I. Direct Blue 8	565nm
C.I. Acid Red 97	497.5nm.

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APPENDIX I

Example of the calculation of the degree of N-acetylation of a chitosan sample by metachromatic titration.

Chitosan solution/cm^3	absorbance, 505nm (results plotted in Figure AI)
1	0.550
2	0.516
4	0.438
6	0.346
8	0.265
10	0.210
14	0.195
16	0.193

Dye concentration = 2.95×10^{-5}M

Chitosan concentration = $0.0650 gdm^{-3}$

Equivalence point = $9.8cm^3$ (from Figure A1)

ie. $9.8cm^3$ chitosan solution are equivalent to $100cm^3$ of dye, when the dye concentration is 2.95×10^{-5}M

$\therefore$ $98cm^3$ chitosan solution $\equiv$ $1000cm^3$ 2.95×10^{-5}M dye

But the chitosan concentration is $0.0650 gdm^{-3}$

$\therefore$ $98cm^3$ chitosan solution contains $\frac{0.0650}{1000} \times 98$g chitosan

$\equiv 2.95 \times 10^{-5}$ mole free amine.

$\therefore$ chitosan free amine equivalent weight = 215.93.

From the conversion graph (Figure 62) the degree of N-acetylation of the chitosan is 21.5%.

APPENDIX II

Two component mixture analysis[245]

For a mixture of two dyes of different λ max, the absorbance at any wavelength will depend upon the additive absorbance values of each of the two dyes, which will in turn depend on the extinction coefficient for each dye at that wavelength, and on the concentration of the dye. Thus:

$$A_x = Ea_xCa + Eb_xCb$$

$$A_y = Ea_yCa + Eb_yCb$$

where A_x = absorbance at x nm

A_y = absorbance at y nm

$Ea_x Eb_y$ = extinction coefficients of the two dyes a and b at x nm or y nm

Ca,Cb = concentrations of the two dyes a and b.

If a single dye in two states is considered (free dye and metachromatic complex) then it should be possible to analyse the spectra produced at various stages by the two component mixture analysis, in order to determine the relative proportions of free dye and complex present in solution.

The assumption is made that in the absence of polymer the absorbance spectrum will be due entirely to uncomplexed dye, and the extinction coefficients for the uncomplexed dye at the orthochromatic and metachromatic wavelengths can thus be determined, using the known concentration of the dye in solution and the measured absorbances. Similarly it is assumed that in the presence of excess

polymer (when no further change in absorbance value is observed) the absorbance spectrum will be due entirely to complexed dye that the relevant extinction coefficients can be obtained, again using the known concentration of the dye in solution. The two component mixture equation can then be used to give the concentrations of complexed and uncomplexed dye present at different P/D ratios.

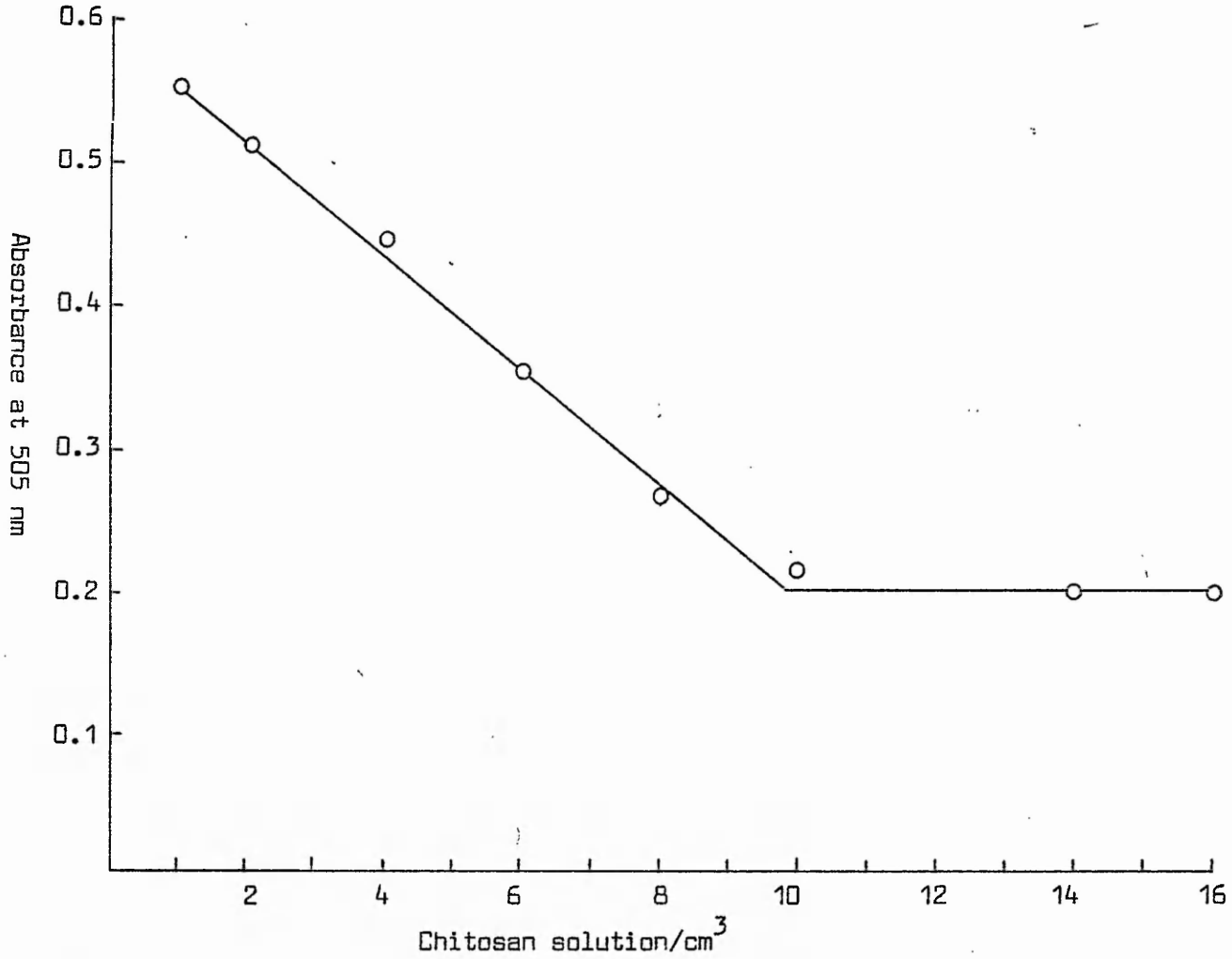

Figure AI. Metachromatic titration of a chitosan sample.

1 mole chalk from = 161 g

161 g in 1,000 cm³

161 g in 1 mole

[illegible]

[illegible]