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INTERACTION OF SELECTED FUNGICIDES WITH INSOLUBLE BOVINE SKIN COLLAGEN IN THE PRESENCE OF THE NON IONIC SURFACTANT TRITON X-100

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ABSTRACT

In the leather industry fungicides are often used for the protection of wet-blue leather. These fungicides are usually only sparingly soluble and are therefore formulated together with surfactants in order to increase their solubility and to ensure an even distribution over the surface of the hide after treatment.

Solutions containing both fungicides and surfactant are complex. The nature of these solutions was investigated. By means of UV/Vis spectroscopy and viscometry it was shown that the surfactant and fungicides form micelles and mixed micelles in solution. The nature of these micelles and mixed micelles was dependent on the solution temperature as well as on the concentrations of the surfactant and fungicides. At the higher temperatures and concentrations transition to large, possibly rod-shaped, mixed micelles occurred.

The interaction between the selected fungicides 2-(thiocyanomethylthio) benzothiazole and n-octyl-4-isothiazol-3-one with bovine skin collagen in the form of both limed and lightly chromed hide powder in the presence of the non ionic surfactant Triton X-100 was investigated. Fungicide uptake was determined by difference measurements on the float solutions at regular intervals during treatment. Binding was rapid with equilibrium being established within the first six hours even for the solutions with the highest surfactant concentration. Binding failed to follow a normal mass-action binding-type isotherm approaching a saturation limit, but increased continuously indicating a co-operative effect whereby binding site affinity actually increased with the amount of ligand bound. Binding was accompanied by a drop in the free surfactant in the solution at the higher biocide levels indicating the formation of complex mixed micelles which bind to the collagen fibres.

The uptake and antifungal activity of commercial fomulations of the fungicides on chrome-tanned wet-blue leather was investigated at various treatment temperatures. At lower fungicide treatment concentrations, binding tended to follow a typical mass-action type binding isotherm, increasing slightly with temperature. At higher float concentrations, an inflexion point was apparent beyond which uptake showed a marked increase with concentration. This inflexion point, signifying a change in binding characteristics, occurred at progressively lower concentrations with increasing temperature.

Antifungal activity in terms of storage periods to onset of fungal growth was determined on the wet-blue leather cuttings immediately after treatment and drainage and also on sample discs after exhaustive extraction of free fungicide using dichloromethane. Storage performance testing of the various treated wet-blue leathers was carried out by different methods. Residual protective periods showed a curvilinear increase with dosage offer and surface uptake. In the low dosage range treatment temperature had only a relatively slight effect in promoting uptake and improving storage protection. At higher dosages, the influence of temperature on uptake and storage protection was greater due to the increase in surface binding of the fungicides at the elevated temperatures. Only a portion of the fungicide uptake was recovered by direct solvent extraction of the treated wet-blue leather. Solvent extraction reduced storage margins. The storage response in relation to fungicide content was, however comparable after extraction, indicating that both irreversibly bound and physically associated fungicide offered effective protection.

Results of the study provide further insight into the mode of interaction of fungicide emulsion dispersion with bovine skin collagen, and the importance of the emulsion dispersions and its stability in determining the uptake of fungicide.

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GLOSSARY OF SYMBOLS AND ABBREVIATIONS

nm	-	Nanometre
gly	-	Glycine
Å	-	Angstrom unit ,10^{-18}cm
FLS	-	Fibrous long spacing
SLS	-	Segment long spacing
TCMTB	-	2-(Thiocyanomethylthio)benzothiazole
NOITZ	-	n-Octyl-4-isothiazol-3-one
C_{SAA}	-	Concentration of surface active agent
lqo	-	Scattered light intensity
K_{SP}	-	Specific conductance
γ	-	Surface tension
Λ	-	Interfacial area
A	-	Helmholtz free energy
σ	-	Donates surface quantities
μ	-	Chemical Potential
Γ_2^1	-	Relative absorption of component 2 with respect to component 1
μ_m°	-	Standard chemical potential in micellar state
μ_1°	-	Standard chemical potential in solution
a_1	-	Activity of the surfactant monomer
X_1	-	Mole fraction of monomer
F_1	-	Activity coefficient
ΔG_m°	-	Standard free energy of micellization
CMC	-	Critical micelle concentration
X1*	-	Mole fraction of monomer at the CMC
S	-	Monomer
N_{ag}	-	Aggregation number
Km	-	Equilibrium constant
$\mu_{mic,N_{ag}}$	-	Standard chemical potential per constituent surfactant molecule
ΔH_m°	-	Enthalphy of micellization
ΔS_m°	-	Entropy of micellization
NMR	-	Nuclear magnetic resonance

Triton X-100	-	Polydisperse preparation of p-(1,1,3,3 - tetramethylbutyl)phenoxypolyethylene glycols containing an average of 9,5 oxyethylene units per molecule
$\bar{\nu}$	-	The ligand-protein molal binding ratio (i.e., mol ligand bound per mol protein)
[L]	-	Ligand concentration
N	-	Number of binding sites on the protein molecule
k	-	Protein-ligand association constant
ΔG_B°	-	Standard free energy of binding
ΔH_B°	-	Enthphalpy of binding
ΔS_B°	-	Entropy of binding
UV/Vis	-	Ultraviolet/visible
PDA	-	Potato dextrose agar
Bulab 1077	-	A concentration surfactant free formulation of TCMTB
Kathon 893	-	A concentration surfactant free formulation of NOITZ
HPLC	-	High performance liquid chromatography
mM	-	millimole
mg/ℓ	-	milligrams per litre
(m/m)	-	Concentration on a mass per mass basis

CHAPTER ONE

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Overview

Organic fungicide formulations have found increasing use in the leather industry for the protection of wet-blue and wet-white leather. The use of these formulations raises queries as to the most efficient means of application of the relatively small amounts of the fungicide whilst ensuring a uniform distribution over the large surface area of the hide, the relative importance of bound and unbound fungicide, factors affecting residual activity and the role these play in the level of protection provided by the fungicide.

In leather manufacture, hide collagen interacts with a variety of compounds of varying molecular mass such as tanning agents, dyestuffs, biocides, bating agents and surfactants. These collagen-ligand interactions, because of their relevance to leather technology, are therefore of considerable economic importance. In the case of protective fungicide treatment, binding of active ingredient with skin collagen reduces the concentration of the free fungicide available to interact with fungal contaminants. This would lead to the reduction of fungicide activity unless the activity is unaffected by binding to collagen. In order to gain further insight into the possible effects of binding on fungicide activity, the interaction between various fungicides and skin collagen was investigated.

Specific binding interactions between hide collagen and fungicides can include hydrogen bonds, hydrophobic bonding and van der Waals attractions. Hydrogen bonds are electrostatic in nature and are formed when the hydrogen atom in the hydroxy, amino and peptide groups of the protein act as an electron acceptor, whilst oxy, peptide, carbonyl and other groups can act as electron donors. Van der Waals attractions are relatively short-range weak forces, resulting when two polarisable atoms are sufficiently close to each other such that the two electron clouds interact leading to an attraction between the different molecules. Hydrophobic bonds are formed between large portions of the fungicide

which are basically non-polar structures and aliphatic and aromatic side chains of the proteins. These interactions are inherently reversible depending on conditions and extent of interaction.

In the case of chrome tanned leather, a further possible interaction, depending on fungicide reactivity, is the coordination of fungicide active ingredient into the chrome complexes in the leather, resulting in strong, irreversible covalent binding of a portion of the fungicide active ingredient with possible inactivation resulting. In contrast with other types of physical interaction, covalently bound fungicide would not be extractable by the organic solvents used in the indirect analytical determination of fungicide in wet-blue leather.

The role and mechanisms of fungicide binding to skin collagen have as yet not been well documented nor has the effect that the various surfactants used, in commercial formulations of the fungicides have on binding. In addition, the role that bound and unbound (interstitial) fungicide plays on the effectiveness of fungal protection has also not been extensively investigated.

1.2 Collagen Chemistry

1.2.1 General Introduction

Collagens have been extensively studied resulting in a vast and comprehensive literature relating to collagen structure, function, biosynthesis, metabolism and pathology. Publications dealing with these aspects have become increasingly specialized. The 'Treatise on Collagen', Volume I, edited by Ramachandran (1967) contains a comprehensive account of the structure and the chemistry of collagen. Subsequent reviews include those by Bailey (1968), Traub and Piez (1971), Bailey (1975), Arridge (1977), Jackson (1978), Viidik and Vuust (1980) and Ramachandran (1988). The 'Treatise on Collagen', Volumes II, Parts A and B edited by Gould (1968) provide a broad coverage of the biology, biosynthesis and pathology of collagen. This has been supplemented by an extensive survey of collagen biochemistry edited by Ramachandran and Reddi (1976) and the review on the structure and function of collagen by Piez (1980). Sparrow (1982) briefly reviewed

aspects of collagen chemistry relevant to protein- ligand binding.

Collagens consist of a group of closely related proteins which occur widely throughout the animal kingdom. Collagens form the principal fibrous component of the skin, cartilage and tendon and are also found in the soft basement membrane and connective tissue. Calcified forms of collagens constitute the hard tissue of bone and dentine of the teeth. The role of the collagens in nature is essentially a structural one. Collagens thus display a variety of unusual properties which distinguishes them from the globular proteins and keratins.

In the skin collagen fibres are to be found in the corium minor or papillary layer, and in the fibrous network, of the corium major of the dermis. The corium minor consists of mainly collagen fibres, some reticulin and a loose network of elastin. In this layer, the collagen fibrils are very closely packed. The corium minor passes almost imperceptibly into the fibre network layer of the corium major which consists almost entirely of collagen fibrils. The collagen fibrils of the corium major are organised into wavy bundles which interpentrate to form a complex three dimensional weave. The fibrils are mostly cylindrical in form with a uniform diameter of 100nm. In the closely packed region of the papillary layer the fibrils broaden slightly and assume a hexagonal rather than a circular cross section.

It is relevant to review aspects relating to collagen structure, solubility and cross-link formation.

(i) Types of Collagen

There are species-differences in collagen amino acid sequences and content, as well as differences in collagens extracted from different tissues in the same organism. Genetically distinct forms are designated collagen I, II, III and IV (Miller and Matukas, 1969). Type I collagen is the major collagen of the skin, and the only collagen of tendon and bone. Type II collagen is specific to hyaline cartilage. Type III collagen (Miller et al, 1971) is a minor constituent of the skin, but a major component of the walls of large blood vessels. Type IV collagen differs markedly from the other three types as it has a large

non-collagenous component which contains both heteropolysaccharides and disulphide links (Olsen et al, 1973; Trueb et al, 1980).

(ii) Primary Structure

Early reviews of amino acid sequence studies dealing with the primary structure of various types of collagen are provided by Hannig and Nordwig (1967) and Miller and Matukas (1974). This sequencing relating to the different species of collagen is summarised in the 'Atlas of Protein Sequence and Structure' and its supplements (Dayhoff, 1972).

Molecular mass and amino acid analysis studies indicate that 'tropocollagen', the soluble monomer and associating unit of collagen fibrils, consists of about three thousand amino acid residues. Of these residues some 33% are glycine, 15% proline and usually about 14% hydroxyproline. Sequence studies on the peptide fragments indicate a consistent repetition of the gly-X-Y triplet, where X and Y are frequently the imino acids proline and hydroxy proline. The tripeptide units gly-pro-R, where R is an amino other than proline or hydroxy proline, make up 35% of the collagen structure, whereas 75% of the proline in collagen is found in gly-pro-R links (Grassman, et al, 1961). After assembly of collagen certain modifications to the side-chain residues of the amino acids occur. These include hydroxylation of proline and lysine, and glycosylation and aldehyde formation. (Undenfriend, 1970; Prockop, et al, 1973).

Chromatographic evidence showed that immature denatured collagen can be separated into three single-chain components. Amino acid analyses, which showed that every third amino acid to be a glycine residue. From these and X-ray diffraction studies, it was inferred that collagen had a triple helix structure (Rich and Crick, 1955; Ramachandran and Kartha, 1955), consisting of three non-coaxial helices, each with three residues per turn, arranged about a common central axes. Such an arrangement can accommodate a close-packing of the helical chains if the chains are staggered with respect to the glycine residues. The imino acids proline and hydroxyproline impart a stability to the structure by restricting the possibility of rotation about the bonds of the individual chains, and prevents the structure from collapsing. In addition, the

strategically situated proline and hydroxyproline imino acids, by virtue of their rigid structures, provide a necessary helical curvature to the individual strands. The three strands comprising the triple helix are considered to be held together predominantly by a regular sequence of hydrogen bonds.

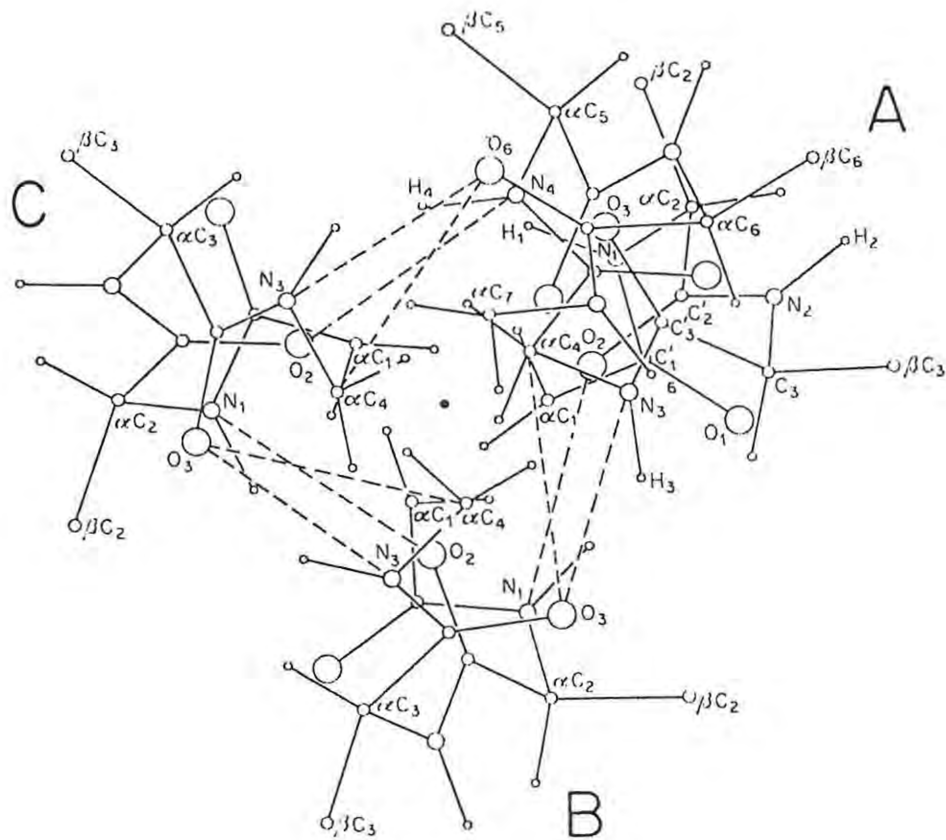


FIGURE 1 The atomic arrangement in the triple helix of collagen, projected down the central axis. (Note: All atoms occuring for a height of three residues (8,73 Å) are shown. The hydrogen bonds are shown by dotted lines. Two NH...O and one CH...O bonds occur for every three residues. (Reproduced from Ramachandran, 1967))

(iii) Single Chain Components

Collagen contains two types of single strand polypeptide chains, the $\underline{a}_1$ and $\underline{a}_2$ chains which occur in the ratio 2:1. Although not identical they are similar, each consisting of about one thousand residues and having molecular masses in the range 9,2 - 9,5 x 10^4. Besides the a-single chain components, double chain b components, triple-chain and even higher molecular mass components can be separated from mature denatured collagen indicating the presence of intra- and intermolecular covalent cross-links between the individual a-chains. These cross-links are formed by Mannich-type bond formation between lysine or hydroxylysine sub-units of one a-chain and aldehyde groups formed by deamination of lysine or hydroxylysine groups on a second a-chain. The isolation and chemistry of the cross-links in collagen are reviewed by Harlan and Fairheller (1977), McClain (1977) and Fujii et al, (1976).

(iv) Telopeptides

From amino acid sequence studies (Schmitt et al, 1964) it has been shown that in both $\underline{a}_1$ and $\underline{a}_2$ chains, the first ten to fifteen residues from the N-terminal end do not have glycine at every third position suggesting that this portion of the molecule is non helical. Similar evidence (Stark et al, 1971) shows that short segments at the C-terminal end of the tropocollagen molecule are also non helical. These regions, known as telopeptides, are believed to be involved in cross-link formation between the a-chains, and also to be responsible for the antigenic properties of collagen (Bornstein and Nesse, 1970; Furthmayr et al, 1971).

The collagen precursor containing additional peptide extensions is termed procollagen (Prockop, et al, 1973). These extensions are believed to render the procollagen soluble and to prevent fibril formation during its biosynthesis (Fessler et al, 1975).

(v) Fibril Structure

Tropocollagen molecules assemble into fibroblasts which form into cylindrical fibrils having fairly uniform diameters which can vary from

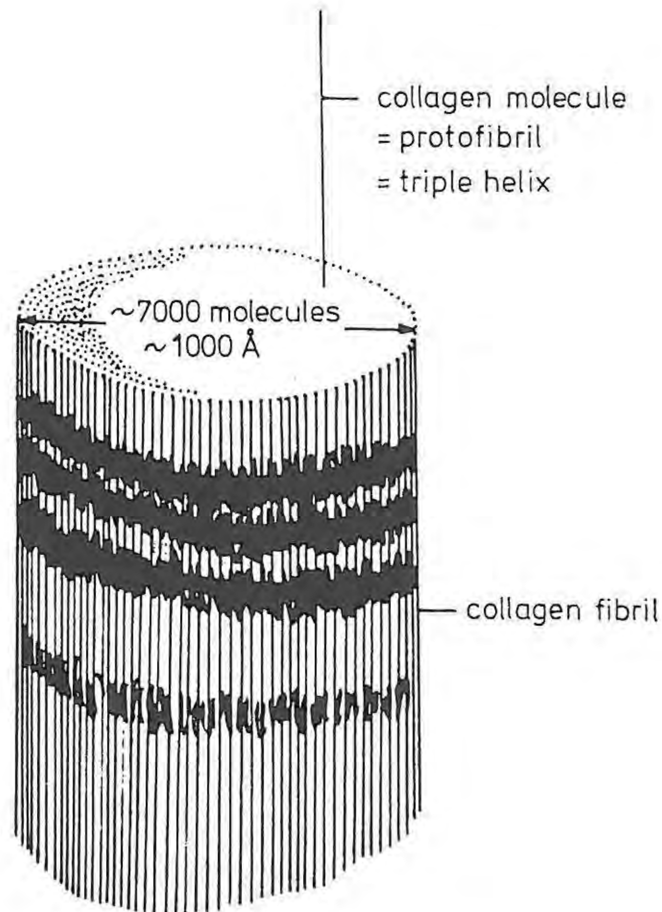


FIGURE 2 Schematic drawing of hide collagen (type 1) (Reproduced from Heidemann, 1982)

5nm to 200nm depending on the nature and age of the tissue (Figure 2).

Low-angle X-ray diffraction studies show the fibrils to have a repeat distance of 64nm for dry collagen and 70nm in the wet state, and show periodicity under the electron microscope when stained with phosphotungstic acid (Gross and Schmitt, 1948). Reconstituted collagen fibrils from acid solution show the same aggregation pattern and are referred to as native-type fibrils. In addition to the native-type fibril both fibrous long spacing (FLS) and segment long spacing (SLS) which have a repeat unit several times the length of the native fibril can be formed depending on the solution environment (Highberger et al, 1950; 1951; Schmitt et al, 1953). Both the FLS and SLS forms were found to have an amino acid composition characteristic of native collagen and to give the usual collagen wide-angle diffraction pattern. The three forms of fibrils are readily interconvertible by dissolution and

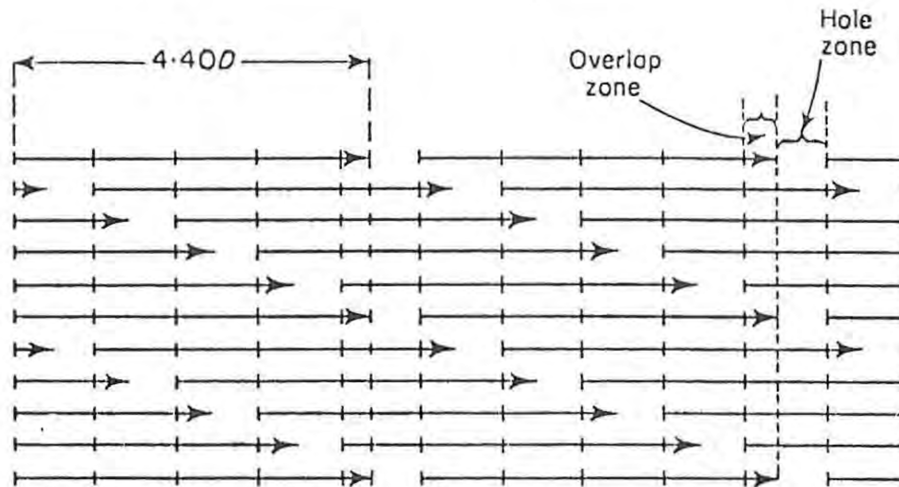


FIGURE 3 A two-dimensional representation of the packing arrangement of tropocollagen macro molecules in the native-type collagen fibril. (Reproduced from Hodge, et al, 1965)

re-adjustment of the solution environment (Gross et al, 1954). The banded pattern of stained native collagen indicate that the surface of the molecule is divided into alternating zones of ionic and uncharged side-chain residues. The bands represent the distribution of polar and apolar side-chain groups from the N- to C- terminal ends of the molecule. Detailed studies of the band pattern show that the length of the tropocollagen molecule to be 4,4 times the repeat period of the native fibril (Figure 3).

Hodge and Schmitt (1960) postulated that the FLS form consists of tropocollagen fibrils having coincident ends packed in antiparallel array (Figure 4), whereas the SLS form, also with coincident ends have a parallel orientation.

Veis et al (1967) proposed that native fibrils are composed of tropocollagen molecules which are displaced with respect to their nearest neighbours by one quarter of their length, known as the quarter stagger arrangement (Figure 5) (Cooper and Russell, 1969).

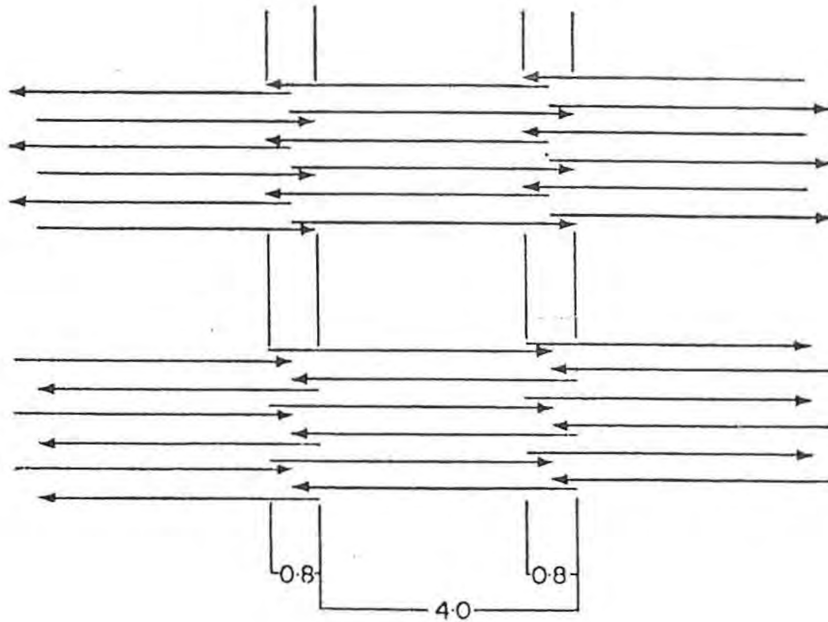


FIGURE 4 Diagram of packing arrangement of protofibrils in the FLS ordered aggregation state (Reproduced from Hodge, et al, 1965)

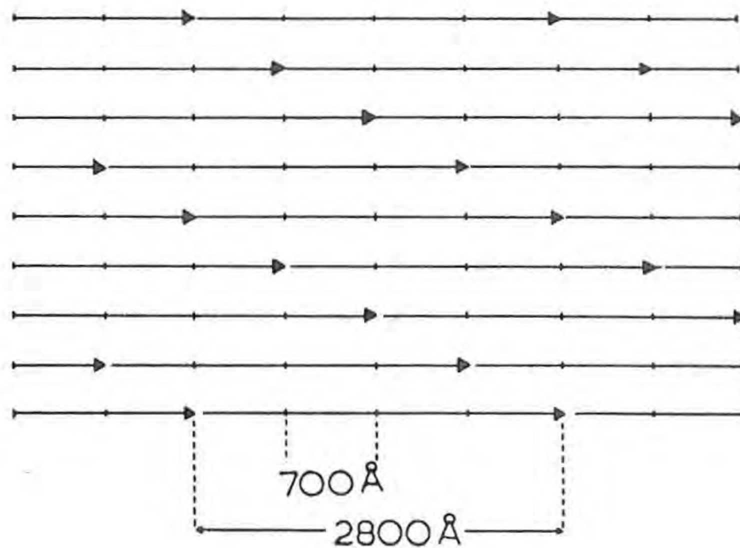


FIGURE 5 The original quarter-stagger concept of fibril packing with collagen macro molecules linked end to end and with a displacement of one-quarter of the macro molecular length (Reproduced from Cooper and Russell, 1969)

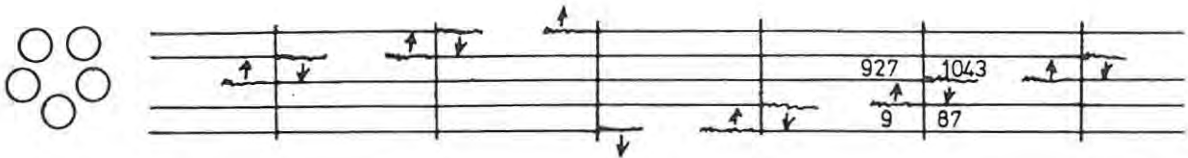


FIGURE 6 Left: Cross-section of the unit of five collagen molecules. Right: Line-drawing of collagen molecules, the five-membered unit cut open and laid down flat. (Note: The N-Terminal, non-helical end of the α 1 chain (Type 1) is composed of 16 amino acids, and allysin is situated in position N9. The C-Terminus contains 25 amino acids with C17 allysin for cross-links (Reproduced from Heidemann, 1982)

The fibrils are thus composed of tetrads of tropocollagen molecules in which there are regions of overlap interspersed with holes. In the fibrils these tetrads of tropocollagen molecules are linked together in a head to tail fashion by cross-links in the regions of overlap (Heidemann, 1982) (Figure 6).

(vi) Solubility and Stability in Solution

Although collagen is largely insoluble in water, it is possible to extract a proportion of collagen from the skin and connective tissue using acidic or neutral salt solutions. In terms of solubilization, collagen can be classified as acid-soluble (ASC), neutral-salt soluble (NSC) and insoluble (ISC). All three types of collagen are essentially similar to the soluble precursor form. Differences in solubility arise only with respect to the degree of incorporation into the insoluble fibres and to the extent of cross-link formation between molecular chains and between the individual monomeric soluble collagen units (Pinto and Bentley, 1974). The rodlike tropocollagen repeating units associating laterally to form insoluble fibrous collagen, with the

repeating units being held together in aggregates in the polymeric network of the fibrous tissue by a variety of covalent bonds. The decrease in the solubility of collagen with the age of the tissue can be attributed to an increase in the number of intermolecular covalent cross-links which form as the tissue ages (Pinto and Bentley, 1974).

1.2.2 Insoluble Skin Collagen

Hides and skins consist mainly of type 1 insoluble collagen. This is especially true in case of the corium major section of the skin. The insoluble skin collagen is essentially the same as the other forms of collagen described except that the greater degree of cross linking between the chains render the fibrous collagen insoluble. During the tanning process further crosslinking takes place to stabilize the molecule even further. Stabilization of the insoluble collagen matrix of the hide or skin constitutes the main feature of the process of conversion to leather.

(i) Limed hide powder

Hide powders are prepared from the corium major of bovine hides. They are usually prepared from salted hides which go through the process of soaking to recondition, machine fleshing and splitting, liming for depilation, followed by deliming, acetate dehydration and grinding. The details of various methods of preparation have been described (Atto *et al*, 1969; Atto and Nursten, 1971a; Lamb, 1981).

The main action of alkaline solutions on collagen during the liming process has been extensively reported (McLaughlin and Theis, 1945; Bowes *et al*, 1953; Blenkiewicz, 1983). The main reactions include:

(a) Hydrolysis of amide groups, by which the amount of free carboxyl groups is increased, and nitrogen is lost in the form of ammonia

(b) Modification of the guanidine groups (the arginine residues)

(c) Minor destruction of the aliphatic hydroxy acids (serine and threonine) and

(d) Some hydrolysis of keto-imide groups of the backbone of the collagen chains

(ii) Chromed hide powder and wet-blue leather

The conversion of hides into wet-blue leather is essentially a two stage process. Firstly in the liming process, hair, keratins and some fats and oils are removed. This is followed by a chrome tanning process to produce chrome-tanned leather commonly referred to as wet-blue leather. The tanning of collagen with chrome tanning salts has been intensively investigated and discussed (Atto and Nursten (1971b)) as has the preparation of chrome tanned hide powders.

In his earlier work Gustavson (1925; 1926; 1947) proposed that a divalent ion such as sulphate may be bound to the chromium and at the same time form a salt link with an amino group. He envisaged the coordination of the amino group as the primary factor in the case of anionic chrome tannage. A series of papers (Shuttleworth 1950; 1958; 1964; Shuttleworth and Sykes 1959; 1960;, Ellis and Sykes 1963; and Ellis _et al_ 1963) showed that the amino group does not co-ordinate with chromium under normal conditions of chrome tannage except possibly during the drying of the leather. Furthermore they showed that under similar conditions co-ordination compounds between chromium and the protein carboxyl groups of aspartic and glutamic acids, which occur in clusers, are formed. The main mechanism during this process is the co-ordination of chromium to the carboxyl groups of the protein, a proportion of which gives rise to bridges between the protein chains. This view was eventually also supported by Gustavson (1952a; 1952b; 1956), and confirmed by Heidemann and Fuhren (1962) and Heidemann (1982).

1.3 Fungicide Chemistry

1.3.1 General Introduction

Microbiocides have been used in the leather industry for many years. Uses range from bacterial protection during salt curing and temporary protection of green hides to fungal growth control on leather used in

moist hot conditions. Earlier preservatives were based on phenol derivatives or heavy metals such as mercury and organotin compounds which were often mixtures in order to provide a broad range of activity. With restrictions on the nature of chemicals entering waste treatment plants and the safety of employees handling chemicals, new and safer chemicals are becoming more important to the tanning industry. A large range of biocides are continually being evaluated for efficacy and safety, the results of which are regularly reported (Galloway and Cooper, 1974; Levy et al, 1974; Van Deren and Weiss, 1978; Van Deren, 1980; Russell et al, 1985; Bugby, 1987; Mitchell, 1987; Russell et al 1987).

Two of the active compounds commonly used for protection against fungi are investigated in this study namely:

2-(thiocyanomethylthio)benzothiazole and

n-octyl-4-isothiazol-3-one

1.3.2 2-(Thiocyanomethylthio)benzothiazole

One of the fungicides in general use contains 2-(thiocyanomethylthio)-benzothiazole, commonly referred to as TCMTB. This compound is the active ingredient found in various commercial fungicide formulations such as Busan 30L, Busan 72 and Leathergard.

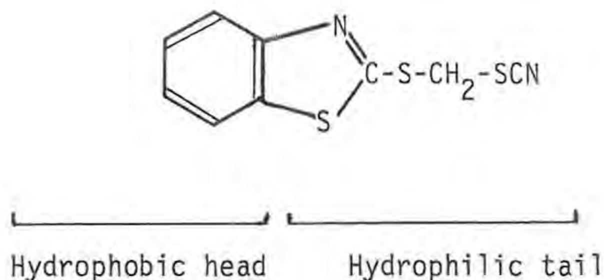


FIGURE 7 Structure of TCMTB showing possibility of the existence of a hydrophobic head and a hydrophilic tail

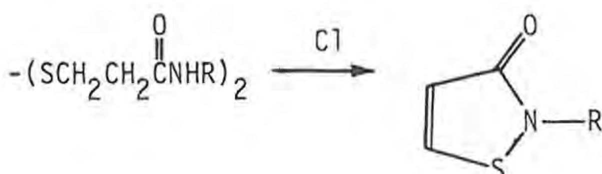
The effectiveness of TCMTB as a fungicide for leather has been confirmed by various workers (Galloway and Cooper, 1974; Van Deren and Weiss, 1978; Russell et al, 1985, Bugby, 1987; Fowler and Russell, 1990). TCMTB is stable in acid solutions (Van Deren and Weiss, 1978; Nattrass, 1986; Leppert, 1987), however when exposed to alkaline conditions it breaks down into harmless compounds (Sladen, 1977; Fowler et al, 1986).

Due to its low water solubility, TCMTB is normally formulated together with surfactants and diluents which give a relatively stable, white emulsion on dilution with water. Any interaction and binding with collagen thus takes place from the emulsion state rather than from true solution as would be the case with classical binding situations (Fowler and Russell, 1990; Fowler et al, 1990).

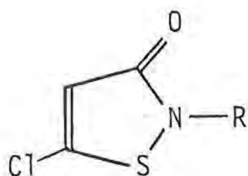
Due to the structure of the TCMTB molecule it is possible that it could act as an amphiphile or surfactant in solution as it could be considered as a molecule possessing both a hydrophobic head and hydrophilic tail. It could thus form micelles and mixed micelles with other surfactants.

1.3.3 n-Octyl-4-isothiazol-3-one

The synthesis and reactions of isothiazolones have been comprehensively discussed (Crow and Leonard, 1964; 1965; Crow and Gosney, 1966; 1967; 1969; Chan and Crow, 1968). They show that n-substituted isothiazolones are susceptible to nucleophilic attack at the ring sulphur atom. The binding of the isothiazolone to skin collagen components may thus be a covalent interaction between isothiazolone and skin proteins containing nucleophilic amino acids (De Bethizy et al, 1986). Miller et al (1971), Miller and Hausman (1971) and Lewis et al (1971a; 1971b) reported on the development of methods for the synthesis of a variety of alkyl substituted isothiazolones. The general synthesis of 4-isothiazol-3-ones was by the chlorine induced cyclization of 3,3'-dithiodipropionamides

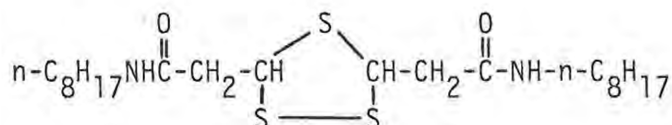


Miller *et al* (1971) showed that if R=CH_3 the 5-chloro substituted isothiazolone was a minor constituent of the reaction products



Levy *et al* (1974) investigated (both from a toxicology and environmental degradation aspect) these biological active compounds containing the isothiazole nucleus, especially the homologous series of n-alkyl-4-isothiazol-3-ones and found the n-octyl homologue to be an effective fungicide.

Like TCMTB, n-octyl-4-isothiazol-3-one, which will be referred to as NOITZ, is an active ingredient of fungicidal formulations used in the leather industry. Its effectiveness as a fungicide for wet-blue leather and other leather products has also been confirmed (Levy *et al*, 1974; Prentiss and Sigafoos, 1979; Bugby 1987; Galloway and Cooper 1974 and Russell *et al*, 1985). NOITZ has a low water solubility of about 700mg/ℓ (Levy *et al*, 1974) and when used as a fungicide in the leather industry is usually formulated as 9% active ingredient in 90% propylene glycol with 1% non ionic surfactant, under the trade name of Kathon LP. Levy *et al* (1974) showed that not all the isothiazolone is irreversibly bound to the collagen when treating leather as it was possible to water extract considerable amounts. He also showed that under alkaline conditions the HS^- ion catalyses the rapid degradation of NOITZ into innocuous by-products with the major one being



Due to its chemical structure it is possible, to regard NOITZ as being an amphiphile or surfactant. It contains a hydrophilic head and a hydrophobic tail and as such could form micelles and combination micelles with other surfactants

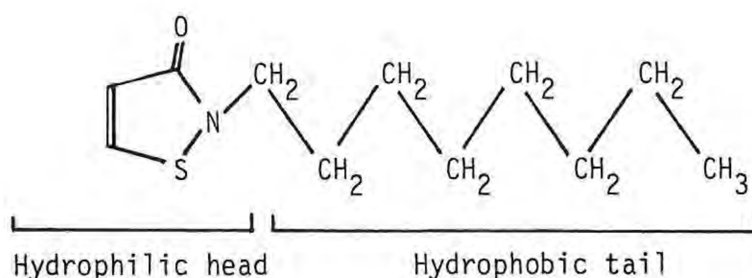


FIGURE 8 Structure of NOITZ showing possibility of the existence of a hydrophobic tail and a hydrophilic head

1.4 Surfactant Chemistry

1.4.1 General Introduction

The chemistry of surfactants and their use in biological systems has been studied extensively and a vast and comprehensive literature has been published on the subject. A series of reviews edited by Mittal (1977) Vol. 1 and 2; (1979) Vol 1 and 2; Mittal and Fendler (1982) Vol. 1 and 2 and Mittal and Lindman (1983) Vol. 1-3 provide a very comprehensive compilation of contemporary research and thinking anent surfactants in solution whereas Tanford (1980) presents a more basic text on the subject. A review on surfactants edited by Tadros (1984) provides a basic account of the physical chemistry, the behaviour at interfaces and their role on wetting and dispersing. Attwood and Florence (1983) deal with the pharmacological and biological aspects of surfactant chemistry.

A surfactant, an abbreviation for surface active agent (also referred to as amphiphile or tenside), when added to an aqueous liquid, changes the interfacial properties of that liquid. Surfactants can function as wetting, foaming, dispersing, emulsifying and/or penetrating agents. They are classified according to whether or not they ionise in solution and by the nature of the resulting ionic or electrical charges, yielding anionic (negatively-charged), cationic (positively-charged), non-ionic (uncharged), zwitterionic (can exhibit both positive and negative charges) and semipolar (in which charges can be induced) types.

1.4.2 Properties of solutions of surfactants

The properties of solutions of surfactants, micelle formation and the thermodynamics of micelle formation have been comprehensively discussed (Aranow, 1963; Attwood, 1968; Swarbrick and Daruwala, 1969; Attwood and Udeala (1975); Aniansson, et al 1976; Tanford, 1980; Corti and Deglorgio, 1981; Attwood and Florence, 1983; Ottewill, 1984; Couper, 1984; Tadros, 1984a; 1984b), whereas the mechanism of micelle formation and the shape and size of micelles has been reported on by Schulman et al (1959); Lindman (1984); Lindman and Wennerström (1982); Tanford (1972; 1974; 1980); Mukerjee (1972; 1980) Ribiero and Dennis (1976; 1977); Leibner and Jacobus (1977); Missel et al (1980; 1982). The adsorption of micelles onto solid surfaces is discussed by Giles (1982a; 1982b), and on microemulsions by Overbreek et al, (1984) while the microstructure within micelles (Goodwin, 1984) has also been reported. A very brief resumé of some of this work follows.

(i) Surface properties

The physical interactions which surfactant molecules undergo at liquid and solid interfaces are almost always rapidly reversible. A quantitative description of the corresponding equilibria can thus be given in thermodynamic terms. With pure materials a curve of surface tension against the logarithm of the molar concentration is obtained having the form as shown. (Figure 9a)

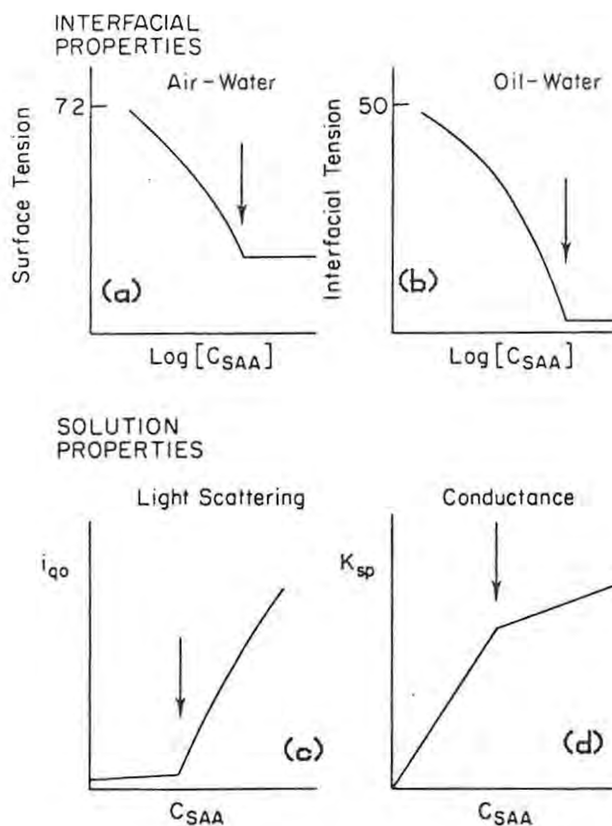


FIGURE 9 Interfacial and solution properties of surface active agents (Note: (a) air-water tension versus log concentration (Log[C_{SAA}]), (b) oil-water interfacial tension versus log concentration (c) scattered light intensity (i_{go}) versus concentration (C_{SAA}), (d) specific conductance (K_{SP}) versus concentration. (Reproduced from Ottewill, 1984)

An inflection occurs in the curve at a certain concentration and from this point up to higher concentrations surface tension remains essentially constant. The surface of a liquid is the boundary between the two bulk phases. Stability at the interface requires that the free energy should increase if the area of the interface increases thus leading to the definition of surface tension γ as the differential of the Helmholtz free energy A at constant temperature and composition with area Λ.

$$\gamma = \left(\frac{\partial A}{\partial \Lambda}\right)_{T,V,n_i} \tag{1}$$

For a single component, i.e. pure liquid, the excess surface free energy is identical to the surface tension and adopting the Gibbs model.

$$A^{\sigma} = \gamma \Lambda + \sum_i \mu_i n_i^{\sigma} \tag{2}$$

where σ denotes surface quantities, and thus for a multicomponent system, the surface excess free energy per unit area is equal to the surface tension γ only if the total adsorption is zero, leading to the Gibbs adsorption isotherm (Couper, 1984).

$$\Gamma_2^1 = - \frac{1}{RT} \left(\frac{\partial \gamma}{\partial \ln a_2} \right)_{P,T} \tag{3}$$

where Γ_2^1 is the relative adsorption of component 2 with respect to component 1. At concentrations just before the break point

$$\left(\frac{\partial \gamma}{\partial \ln a_2} \right)_{P,T} = \text{constant} \tag{4}$$

indicating that saturation adsorption of the surface has been reached and just above the break point

$$\left(\frac{\partial \gamma}{\partial \ln a_2} \right)_{P,T} = 0 \tag{5}$$

which gives on integration

$$\gamma = \text{constant} \times \ln a_2 \tag{6}$$

and since γ is constant in this region the activity of the surface active agent in the aqueous phase must remain constant. Therefore, even though more material is being added to the solution its activity in that solution remains constant. An effect which can only be explained if the surface active molecules are associating to form units with a low activity in solution.

ii) Bulk properties

Just as for surface tension, measurements of the intensity of light scattering at 90° to the incidence beam show an increase (Figure 9c) implying that association must be occurring in the solution phase to give larger units. Likewise conductivity measurements for ionic surfactants also show a change in gradient (Figure 9d) indicating a decrease in mobility.

Both interfacial properties and bulk properties of solutions of surfactants thus show that at a certain concentration the surfactant molecules (ions) in solution associate to form larger units, called micelles. The concentration at which this association phenomenon occurs is known as the critical micelle concentration CMC. Figure 10 illustrates the possible states of a surfactant in solution.

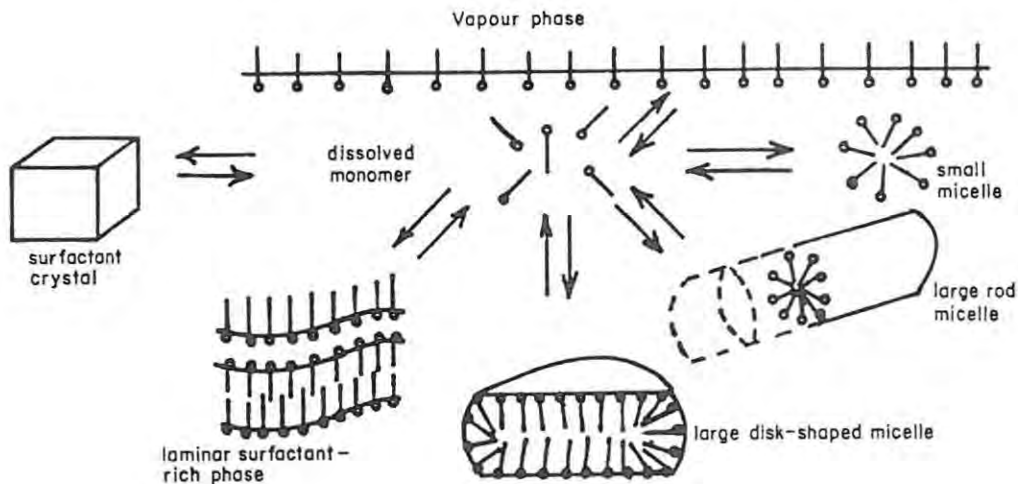


FIGURE 10 Possible states of an amphiphile in solution (Reproduced from Couper, 1984)

Micelles are often small aggregates nearly spherical in shape, but in some cases larger laminar or rod-shaped micelles may be formed. The mechanism of CMC formation can be described by various thermodynamic models (Couper, 1984; Lindman, 1984). The simplest model is that above the CMC phase equilibrium exists with constant activity in the micellar phase.

(a) Phase Separation Model

This model is formulated by assuming that the chemical potential of the surfactant in the micellar state is constant at a given temperature and may be adopted as the standard chemical potential μ_m°. Equilibrium with the surfactant in the solution is then represented by

$$\mu_m^\circ = \mu_1^\circ + RT \ln a_1 \quad (7)$$

where μ_1° is the standard chemical potential in solution and a_1 is the activity of the surfactant monomer.

Letting $a_1 = X_1F_1$ where X_1 is the mole fraction of monomers and if the activity coefficient F_1, is taken as unity the standard free energy of micellization per mole of monomer is given by

$$\Delta G_m^\circ = \mu_m^\circ - \mu_1^\circ = RT \ln X_1 \quad (8)$$

and at the CMC, X_1^* may be identified with X_1. For dilute solutions in water at 25°C, the concentration $X^* = C^*/55{,}5$ mole dm^{-3} (Couper, 1984) and

$$\Delta G_m^\circ = RT \ln C^* - RT \ln 55{,}5 \quad (9)$$

The phase separation model makes no provision for possible variation in the activity of the surfactant as the total concentration of the surfactant in the solution is increased, and an equation of state analogous to that used to describe absorbed films is required to refine the model.

b) Mass Action Law Model

This model postulates an equilibrium between monomers, S and a single micellar species of aggregation number, N_{ag}

$$N_{ag}S_1 \rightleftharpoons Mn \quad (10)$$

Thus $Km = X_n/X_1$

where Km is the equilibrium constant. The standard free energy of micellization per mole of monomer is then given by

$$\Delta G^\circ_m = -(RT/N_{ag}) \ln Km \quad (11)$$
$$= -(RT/N_{ag}) \ln Xn + RT \ln X_1$$

For large values of N_{ag} (>50) at total concentrations not much greater than the CMC the first term is negligible and thus the equation reduces to that of the phase separation model. It is an assumption inherent in the model that the association of monomers into the micelle is a cooperative process and that aggregates with smaller values of N_{ag} are unstable.

(c) Multiple Equilibrium Model

In reality micellar size is not restricted to a single aggregation number, but there is a micellar size distribution. An extension to the mass-action model allows for the existence of a range of values of N_{ag} by postulating a multiple equilibrium characterised by a series of equilibrium constants K_2, K_3, K_4 .... such that

$$\ln K_{Nag} = {}_2 \ln K_i \quad (12)$$

and associated with these equilibrium constants there will be a series of standard free energy changes which are represented by the distinct value for each micellar species of the standard chemical potential $\mu_{mic,N_{ag}}$, per constituent surfactant molecule. In the distribution of surfactant molecules between micelles of different size, N_{ag} will then be determined by the variation of $\mu_{mic,N_{ag}}$ with N_{ag}. The chemical potential of aggregation number, N_{ag}, is then given by

$$\mu_{mic,N_{ag}} = \mu_{mic,N_{ag}} + \frac{RT}{N_{ag}} \ln \frac{Xn}{N_{ag}} \quad \text{(Couper, 1984)} \qquad (13)$$

in which X_n is the mole fraction of surfactant in micelles of this size.

From these models Couper (1984) developed methods of predicting CMC values, and for non-ionic surfactants Ottewill (1984) showed that enthalpy of micellization is given by

$$- \Delta H_m^{\circ} = RT^2 \frac{d \ln[CMC]}{dT} \qquad (14)$$

and hence the value of ΔH_m° can be obtained from the variation of the CMC with temperature and, as ΔG_m° can be calculated from the same information, the entropy ΔS_m°, or $T \Delta S^{\circ}$, can also be obtained.

(iii) Properties of Micelles in Solution

Micelles are dynamic entities characterised by high intramicellar mobility, by frequent monomer exits and entrances and by a relatively short life time (Lindman, 1984). It is generally agreed that the micellar interior qualitatively resembles a liquid hydrocarbon "droplet" (Lindman and Wennerström, 1982; Lindman, 1984; Ribeiro and Dennis, 1975; 1976; 1977) but it has not been easy to quantify micelle size distribution, alkyl chain orientation and flexibility. Attwood and Florence (1983) point out that since micelles are dynamic structures with a liquid core it is probably unrealistic to regard them as rigid structures with a precise shape. It is, however, instructive to consider an average micellar shape. From carbon 13 labelling and proton NMR studies of the structure and mobility of micelles Ribeiro and Dennis (1977) show a restriction of the molecules from tumbling following micelle formation. A restriction of internal motion occurs at the hydrophobic-hydrophilic interface with progressively increasing fluidity occurring away from the interface in both the non-polar interior and polar exterior regions of a non-ionic micelle.

Although micelle radius may be computed using simple hydrodynamic

theory, Lindman (1984) found it more convenient to use the aggregate number rather than micelle size to compute the radius. This was due to difficulty in quantitatively determining the micelle size distribution curve, as intermediate sizes may be present in extremely low concentrations. While micelles of ionic or zwitterionic surfactants are spherical, ellipsoidal or oblong aggregates and contain 20 to 120 molecules (the aggregation number) those of non-ionic surfactants may contain from 100 to 1000 molecules (Lin and Zimmels, 1982). This is due to the electrostatic repulsion between ionic head groups which exceeds the steric counterpart between non-ionic head groups. Factors which govern the size, shape and aggregation number of micelles include: concentration, temperature, pressure, ionic strength, hydrocarbon chain length, nature of head group, type of counterion, mode of intermolecular action and type of additives. The transition from spherical to rod-shaped micelles may occur at higher concentrations which are well above the regular CMC thereby creating new additional CMC levels.

Evidence for various micelle shape and size distributions, both spherical and ellipsoidal and including the sphere-to-rod transition, has been widely documented (Tanford, 1972; 1974; 1980; Mukerjee, 1972; 1980). Leibner and Jacobus (1977) presented calculations showing that for charged molecules the shape of micelles incapable of attaining spherical geometry do not necessarily form oblate ellipsoids but rather hemisphere capped cylinders.

Although the dividing line between a micelle and a microemulsion is not very clear, Prince (1977) identified microemulsions as having droplet diameters in the range of 250nm down to 10nm and micellar solutions as having droplet diameters of less than 10nm.

(iv) Solubilization in Micelles and Microemulsions

Solubilization is usually described as the process of incorporation of a thermodynamically stable isotropic solution of an insoluble or very slightly soluble substance into surfactant micelles (Tadros, 1984b). There is, however, always doubt, particularly in the case of solids, whether there is conventional solubilization in the micelles or a breaking off of colloidal sized particles of the solubilizate, which are

stabilized in suspension by the adsorbed surfactant. Such particles would be small enough to pass through the commonly used filters as has been shown in the case of certain dye solutions (Heller and Klevens, 1946).

One can differentiate between four different locations of solubilization in micelles. Namely: (a) the hydrocarbon core (b) between surfactant chains in the micelle (palisade layer) with either deep or shallow penetration (c) in the outer hydrophilic region (d) adsorbed on the micelle surface and (e) in the polyethylene shell of the micelle of a non-ionic surfactant. These locations are illustrated (Figure 11).

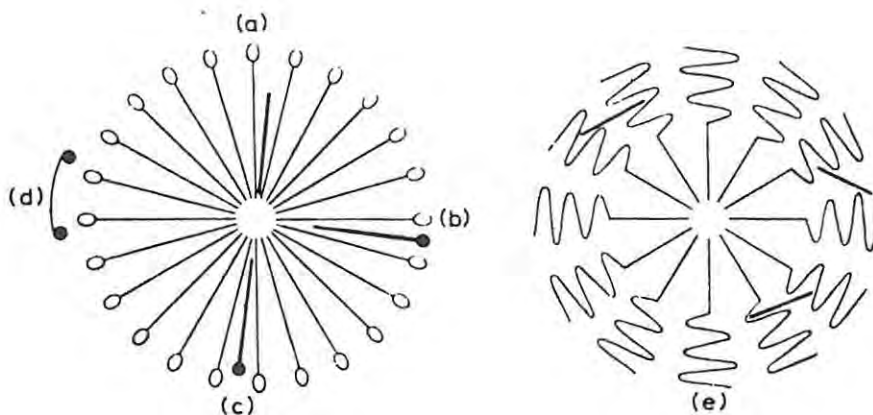


FIGURE 11 Possible sites of incorporation of a solubilizate in a micelle (Note: (a) in the hydrocarbon core; (b) short penetration of the palisade layer; (c) deep penetration of the palisade layer; (d) adsorption on the surface of the micelle; (e) in the polyoxyethylene shell of the micelle of a non-ionic surfactant. (Reproduced from Attwood and Florence, 1983)).

Although in many cases a particular location is preferred, the lifetime of a solubilizate within the micelle is long enough for a rapid interchange between different locations. Mukerjee (1971) discussed the solubilization of benzoic and derivatives by a series of polyoxyethelene surfactants in terms of an equilibrium between loci namely the polyoxyethylene core and the hydrocarbon core. He also discussed (Mukerjee, 1980) the effect of solubilizates on monomer-micelle equilibrium and micelle size distribution, including the sphere-to-rod transition and the location and distribution of solubilized species in terms of a two-state model. Several methods have been used to determine the location of the solubilizate. X-ray diffraction techniques have been used extensively, and although the method of interpretation of the data obtained has been subject to varying opinion (Philippoff, 1950; Fournet, 1951; Hartley, 1949) the information obtained shows an appropriate qualitative differentiation between various types of solubilizates. Other methods used include Absorption Spectrometry, NMR, Fluorescence Depolarization and Electron Spin Resonance. For instance Podo et al (1973) using NMR and spin-lattice relaxation studies on polyoxyethylated non-ionic surfactants failed to detect any significant water penetration of the micelle interior.

Studies indicate that, like the surfactant monomers themselves, the solubilized molecules are not rigidly fixed in the micelle but have a freedom of motion which is dependent to some extent on the solubilization site (Attwood and Florence, 1983). Not only does the solubilizate have freedom of movement, albeit restricted within the micelle but it is also in constant dynamic equilibrium with the bulk of the aqueous phase.

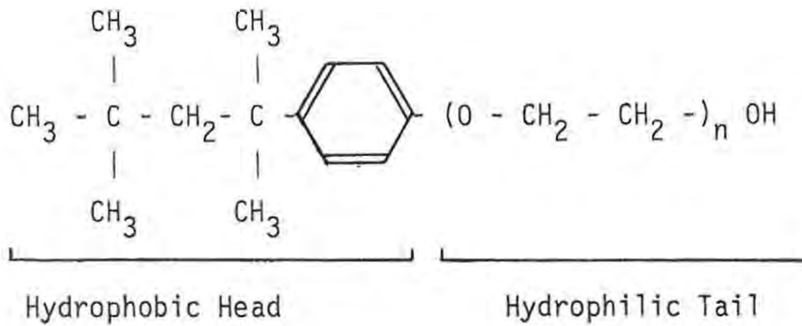
Several factors affect solubilization such as the structure of the surfactant and solubilizate, temperature and the addition of electrolytes and non-electrolytes (Attwood and Florence, 1983). Generalization about the manner in which the structural characteristics of the surfactant affect its solubilizing capacity are complicated by the existence of the different solubilization sites within the micelles. For solubilizates which are located either in the micelle core or in a position of deep penetration within the micelle, solubilization is

expected to increase with increase in the alkyl chain length of the surfactant. On the other hand, if the solubilizate occurs in the hydrophilic region, it will increase with increase in the size of that group. The solubilizate structure can also play a major role. Polarity and polarizability, chain branching, molecular size, shape and structure have been shown to have various effects. Temperature has an effect on the extent of micellar solubilization which is dependent on the structure of the solubilizate and of the surfactant (Attwood and Florence, 1983). In most cases the amount of solubilization increases with increase in temperature. This is reflected in the increase in solubility and increase of micellar size (particularly with non-ionic ethoxylated surfactants) (Tadros, 1984b). Addition of electrolytes to ionic surfactants usually cause an increase in micelle size and a reduction in the CMC, and hence an increase in the solubilizing capacity. Non-electrolytes, e.g. alcohols, also lead to an increase in the micelle size and hence to an increase in solubilization.

1.4.3 The Non-Ionic Surfactant Triton X-100

In biological work non-ionic surfactants have the advantage over ionic surfactants in that they are compatible with most other types of surfactant and their properties are generally unaffected by pH changes. Aqueous solutions of some non-ionic surfactants may, however, become turbid on warming as the cloud point is exceeded (Attwood and Florence, 1983). Non-ionic surfactants may also form microemulsions and when these microemulsions are compared with those from ionic ones two striking differences are found: (i) No cosurfactant is needed for their formation (often non-ionics are mixtures with a range of chain lengths) and (ii) temperature plays an essential role in the behaviour of microemulsions of non-ionic surfactants (Overbreek et al, 1984).

Triton X-100 is a commercial, polydisperse preparation of p-(1,1,3,3-tetramethylbutyl)phenoxypolyethylene glycols (Figure 12) containing an average of 9,5 oxyethylene units per molecule (Sukow and Sandberg, 1974; Sukow et al, 1980). It is an example of a non-ionic surfactant commonly used in biological work (Crook et al, 1963; Ray and Némethy, 1971; Mittal, 1977; Robson and Dennis, 1977; 1978; Mandal et al, 1980; Attwood and Florence, 1983).



where n averages at 9,5

FIGURE 12 Structure of Triton X-100 showing the existence of a hydrophobic head and a hydrophilic tail.

The formation of Triton X-100 micelles and the critical micelle concentration has been extensively investigated. Values for the CMC of between 0,2 and 0,25mM have been reported (Yedgar et al, 1974a). Mandal et al (1980) dealt with the characteristics of these micelles in aqueous solution both in the presence and absence of different additives, and Masullo et al (1986) using fluorimetric methods investigated the effects of urea, salts, organic solvents, pH and temperature on the CMC. They showed that for Triton X-100 the CMC increased with increase in urea and organic solvents, decreased with increase in temperature but remained fairly constant with increase in salt content and change in pH.

There is some controversy concerning the shape of larger micelles formed by non-ionic surfactants. Triton X-100 was considered by many of the earlier workers to be spherical. Kushner and Hubbard (1954) using viscometric and turbidimetric measurements on dilute aqueous solutions of non-ionic detergents found the molecular weight of the Triton X-100 micelle to be in the order of 90 000 in terms of a highly hydrated spherical micelle of radius equal to the length of a nearly completely extended detergent molecule. From geometrical consideration Robson and Dennis (1977) have shown, however, that a spherical micelle would be possible only if several oxyethylene chains were embedded in the hydrophobic core (Figure 13a) and consider that an oblate (Figure 13b) rather than prolate (Figure 13c) would be most consistent with intrinsic

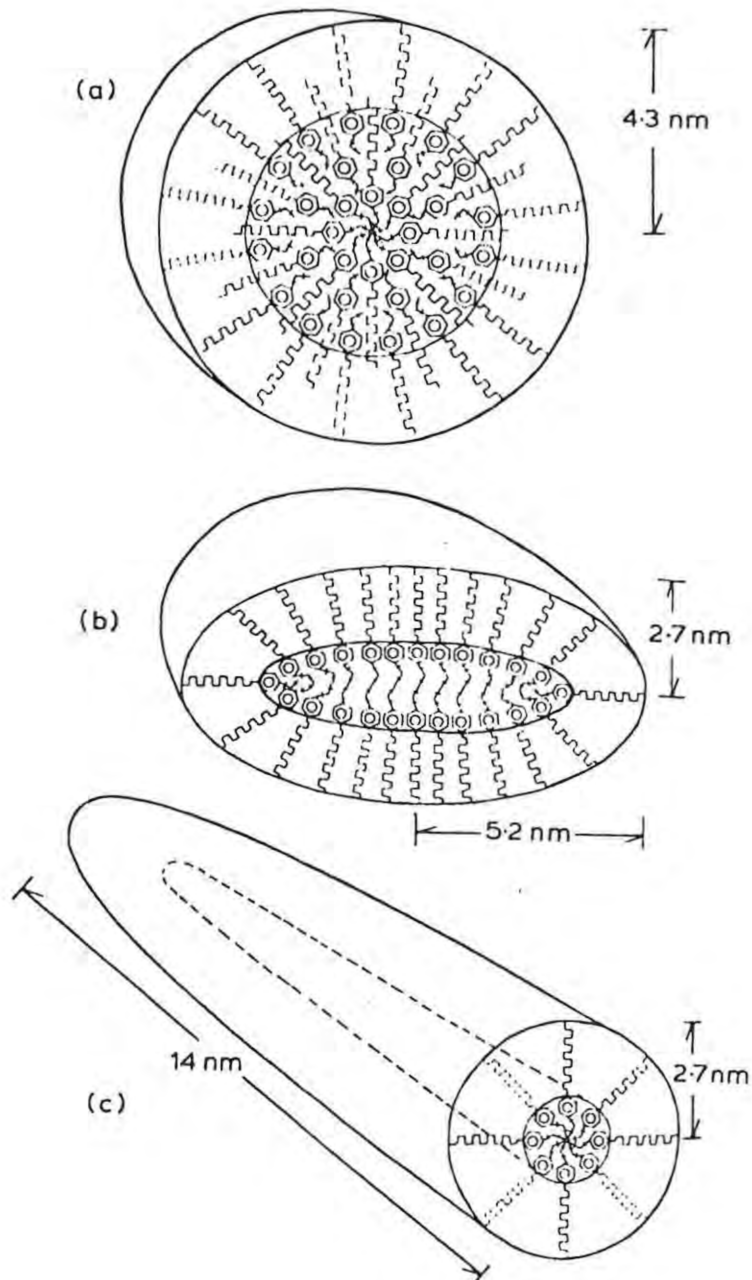


FIGURE 13 Schematic diagrams of one-half of the micelle of Triton X-100. (Note: Based on geometrical calculations by Robson and Dennis for (a) spherical, (b) oblate, and (c) prolate micelle models. The spherical model necessitates intrusion of the oxyethylene chains into the micellar core. (Reproduced from Attwood and Florence, 1983)).

viscosity measurements and volume calculations.

Small angle x-ray scattering measurements (Paradies, 1980), and conductivity and viscosity measurements (Mandal et al, 1980) were also more consistent with an oblate ellipsoid of revolution rather than a prolate equivalent. These and other workers thus conclude that micelles of Triton X-100 should be considered as oblate spheroids with a considerable degree of hydration (Attwood and Florence, 1983). The shape and size of micelle formation of Triton X-100 is treated statistically by Aranow (1963). Lin and Zimmels (1982), however, point out that transition from one structure to another may be induced by changes in the physicochemical conditions (external variables) such as temperature and pH and by the addition of a co-surfactant. They showed that cylindrical (with hemispherical ends) and lamellar (bilayer form) micelles are unrestricted in their growth and length, and are thus able to accommodate any number of amphiphile molecules.

In aqueous solutions two or more surfactants under suitable conditions may form mixed micelles. The formation of mixed micelles takes place more readily when the surfactant ions are similar or when they are either mixtures of ionic and non-ionic types or mixtures of only non-ionic types (Mandal and Moulik, 1982).

For Triton X-100 the resultant mixed micelles co-exist with the remaining micelles of pure Triton X-100 (Yedgar et al, 1974a; 1974b). It has been reported (Tanford and Reynolds, 1976) that mixed micelles of lipids with Triton X-100 may be formed with little change of shape or size of the pure micelle. The study of shape, size and hydration of pure Triton X-100 micelles is therefore important from a fundamental as well as a practical standpoint. NMR studies have also shown (Ribeiro and Dennis, 1975; 1976; 1977) that the presence of phospholipid in mixed micelles with Triton X-100 does not affect the micro-environment or motional behaviour of the micelle. Micelle-micelle interaction in aqueous media is treated mathematically by Chattoraj et al (1982) using second virial coefficients to confirm non-ionic micelles such as Triton X-100 to be oblate shaped rather than spherical ellipsoids.

1.5 Ligand-Collagen Binding Interactions

1.5.1 General Introduction

Protein-ligand interactions represent a prominent facet of protein chemistry. Some general aspects will be briefly reviewed including a discussion on the type of information supplied by protein-ligand binding studies, particularly with respect to the nature of the binding site and the forces involved in protein-ligand complex formation. Some of the mathematical models describing binding study data will be briefly examined.

Interest in protein-ligand interactions derives from the structure-activity relationships of pharmacologically active drugs (McElray and D'Arcy, 1979; 1980) and furthermore protein-ligand interactions provide the mechanisms for the control of biological activity at the molecular level. Binding studies are thus undertaken to provide insight into the mechanism of drug action, metabolism and drug elimination. Publications providing broad surveys of the field of protein-ligand binding are the reviews of Klotz (1953), Edsall and Wyman (1958), Tanford (1961), Feldman (1972), Klotz (1974), Heald and Sampat (1978), Whitehead (1980a; 1980b). Steinhardt and Reynolds (1969) provide a comprehensive survey of the subject. Sparrow (1982) examined a number of mathematical models, based on the protein-ligand model system, used to derive information from binding study data.

1.5.2 Binding Interactions

Protein-ligand binding studies attempt to characterise the protein-ligand interaction in terms of a binding isotherm. From the form of the isotherm it is possible to postulate a model to describe the binding, and to evaluate binding parameters such as the number of binding sites on the protein molecule or the protein-ligand binding constants. This provides information about the nature of the binding site, the energy changes associated with the protein-ligand complex. Interactions are usually studied in solution which contains, besides the solvents, a variety of salts or buffering agents and other additives. Thus protein structure and forces which stabilize protein and the ligand

conformation in solution also normally fall within the scope of ligand-protein interactions. Depending on conformational stability, binding may induce changes in the protein structure, or conversely changes in protein structure can bring about changes in ligand binding affinity. Measures of protein structural stability and propensity to undergo conformational change are reflected in the sensitivity of the binding isotherm to environmental changes such as pH, temperature and ionic strength.

Binding isotherms are usually expressed as a graphical plot of the quantity of ligand bound to the protein as a function of the concentration of unbound ligand which is in equilibrium with the protein-ligand complex, or a statistically fitted mathematical representation thereof. It is often more convenient to use equivalent transforms of the binding isotherm such as the Scatchard plot (Scatchard, 1949), the Lineweaver-Burke plot (Lineweaver and Burke, 1934), the linear-log plot (Bjerrum, 1941) the semi-reciprocal plot (Klotz, 1946) and log-log plots.

When a protein possesses only a single class of binding sites which act independently of each other the mass-balance relationship (Klotz *et al*,. 1946; Klotz, 1953) yields a hyperbolic relationship equation (15) where $\bar{v}$ is the quantity of ligand bound to a unit mass of the protein and [L] is the concentration of ligand in equilibrium with the protein-ligand complex.

$$\bar{v} = Nk[L]/(1 + k[L]) \tag{15}$$

where N is the number of binding sites on the protein molecule and k is the protein-ligand association constant. Linear transformations of this binding isotherm equation (16) are convenient because they enable the binding parameters N and k to be obtained directly from measurements of the slope and axial intercepts. For example in terms of the Scatchard variables $y = \bar{v}/[L]$ and $x = \bar{v}$ equation (15) reduces to:

$$\bar{v}/[L] = (N - \bar{v})k \tag{16}$$

i.e. $y = Nk - kx$ such that $x = N$ for $y = 0$ and $y = Nk$ for $x = 0$ with

slope equal to -k. Other equivalent linear transformations are the double reciprocal Lineweaver-Burke plot

$$1/\bar{v} = 1/Nk[L] + 1/N \tag{17}$$

and the semi-reciprocal plot (Klotz, 1946)

$$[L]/\bar{v} = 1/Nk + [L]/N \tag{18}$$

These graphs also provide graphical tests for differentiating between interactions involving a single class of binding sites and more complex modes of binding.

Curvature in these plots indicates that there may be binding site interaction and ligand induced conformational changes in the protein. Often curvature is interpreted as implying that the protein-ligand binding is heterogeneous with the protein possessing more than one class of binding sites, each with its own association constant. It is still possible, however, to use limiting slopes and extrapolate axial intercepts to derive estimates of binding parameters (Klotz and Hunston, 1971; Kirdani _et al_, 1979) (Figures 14, 15 and 16). This procedure can, however, lead to misinterpretation of the nature of the protein-ligand interaction due to the variety of factors which can contribute towards curvature.

Comparison of the efficacy of these graphical methods for determining binding parameters when the protein-ligand interaction cannot be simply represented, as well as the limitations of applying a simplistic model to more complex binding situations have been dealt with (Woosely and Muldoon, 1977; Klotz and Hunston, 1979; Peters and Pingound, 1979; Baldine _et al_, 1983; Klotz, 1985; Goldstein and Barrett, 1987).

Binding parameters are often obtained using least squares procedures which fit the binding isotherm data to an appropriate binding model incorporating the binding parameters. Whereas, as number of reports deal specifically with mathematical and regression procedures to extract binding isotherm data (Fletcher and Spector, 1968; Sprague _et al_, 1980; Thakur _et al_, 1980; Duggleby, 1980; Munson and Rodbard, 1980; Whitlam

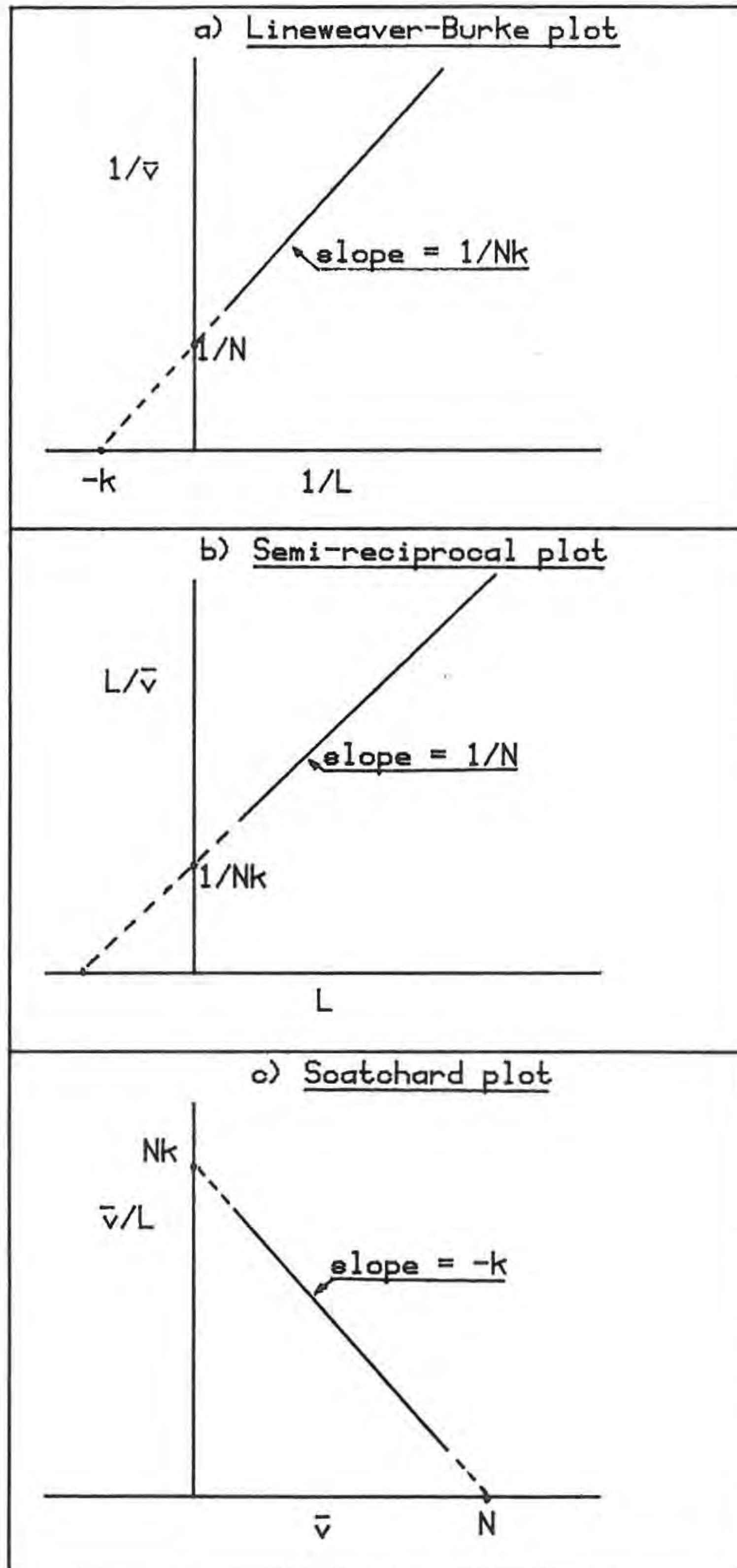


FIGURE 14 Graphical representation of the three commonly used linear transformations of the binding equation for a single site or single class of sites (Reproduced from Klotz and Hunston, 1971)

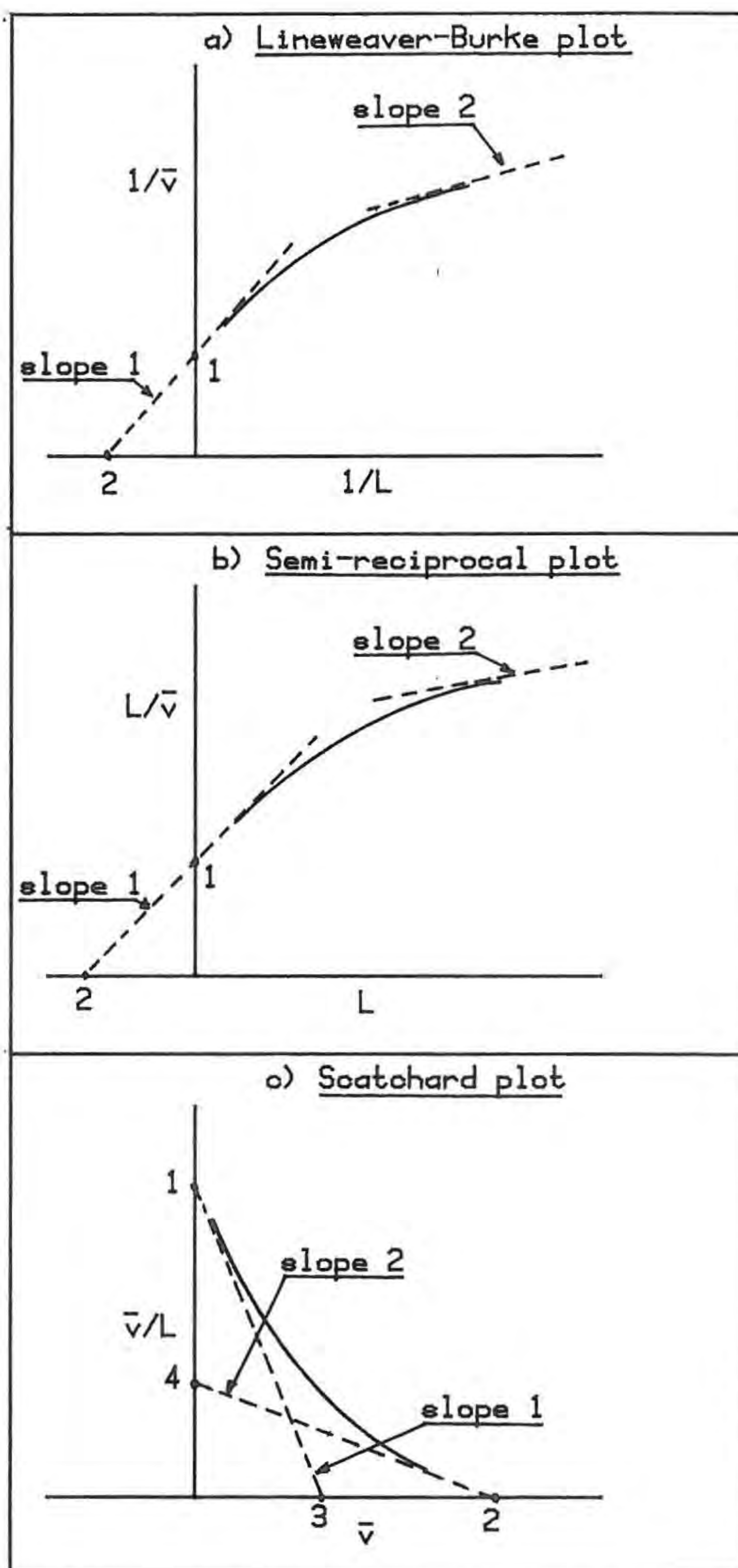


FIGURE 15 Schematic curves of each of the three pairs of transformed variables when m independent classes of binding sites are present (Reproduced from Klotz and Hunston, 1971)

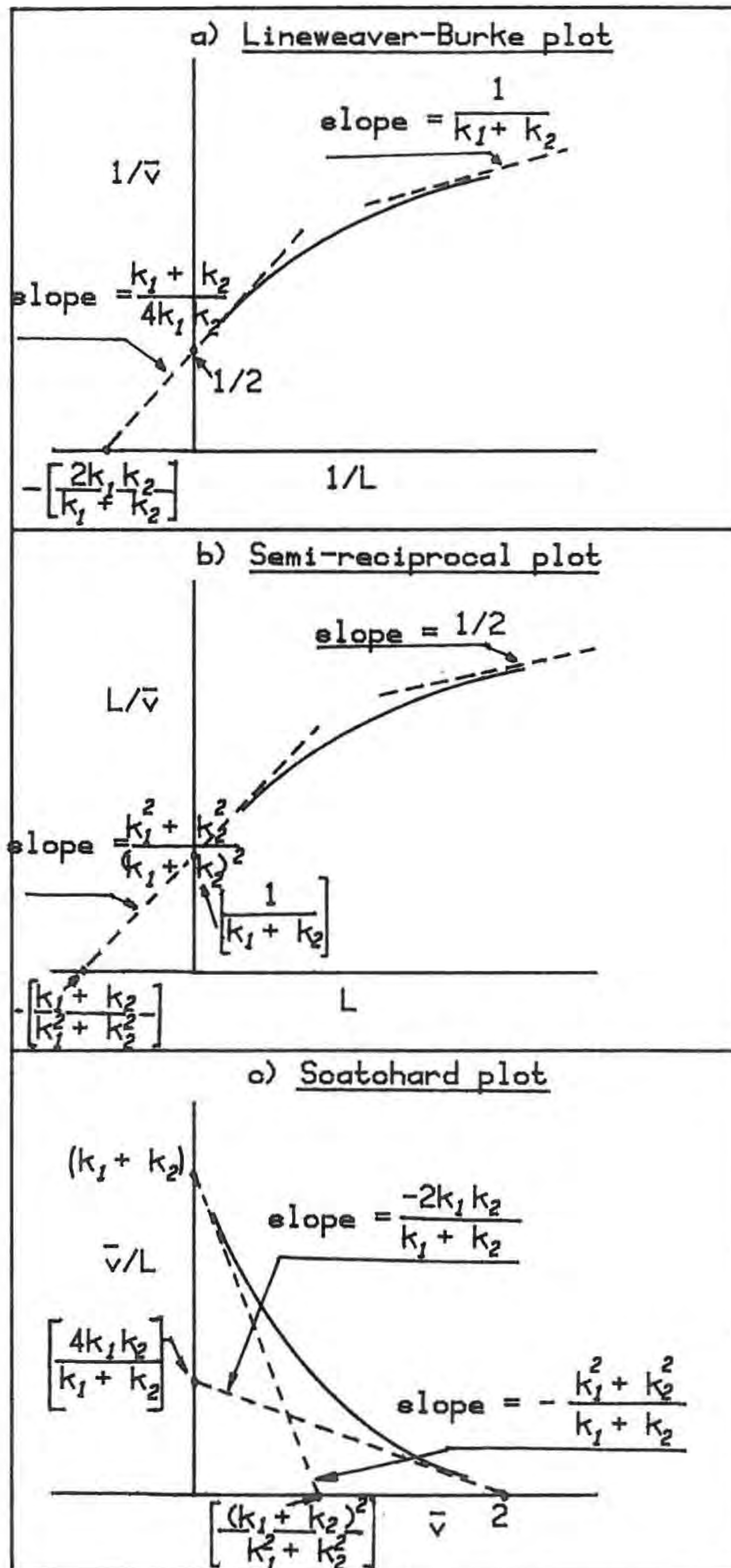


FIGURE 16 Schematic curves of each of the three pairs of coordinates for a system of only two independent binding sites. Intercepts and limiting slopes are indicated (Reproduced from Klotz and Hunston, 1971).

and Brown, 1980; Myers et al, 1987; Brodensen et al, 1987; Melikhova et al, 1988; Saroff, 1989). These consider both the statistical implications inherent in the methods, and discuss the reliability or otherwise of the parameters obtained in using such methods.

Enthalpy changes associated with the protein-ligand interaction can be obtained from the change in values of the association constants with temperature. The mode of binding in the protein-ligand complex can often be established from energy considerations, i.e. it may be possible to propose that binding is due to hydrogen bonding, hydrophobic, polar or electrostatic attractions, or even whether covalent links are formed.

The Gibbs function G° is given by

$$\Delta G_B^{\circ} = - RT \ln ki \qquad (19)$$

where k_i is the protein-ligand association constant.

$$\text{hence } \Delta H_B^{\circ} = R\, d\, [\ln ki]/d(1/T) \qquad (20)$$

$$\text{and } \Delta S_B^{\circ} = (\Delta H^{\circ} - \Delta G^{\circ})/T \qquad (21)$$

The energy aspects of protein-ligand binding have been reviewed by Weber (1975) as has the thermodynamics of ligand binding to macromolecules (Pohl, 1978; Eftink and Biltonen, 1980; Kuchel and Dalziel, 1980). When taken with energy-entropy changes, the variances in binding capacity and protein-ligand association constants with changes in ligand structure, provide criteria whereby the mode of binding may be determined. They also provide information relating to the structure of binding sites on the protein molecule.

Another aspect of protein-ligand binding is the investigation of the interaction between binding sites which may be the result of specific or induced ligand involvement. Ligand binding at one site on the protein molecule may enhance or attenuate the binding of the same or a different ligand at a second site referred to as the allosteric effect (Monad et al, 1963), and may be the result of direct steric interference or electrostatic repulsion. It may also be a consequence of ligand-induced

conformational change in the protein. Co-operativity is the term used to describe an increasing binding affinity with increasing site occupancy and is usually presumed to result from an unfolding of the protein to make additional binding sites available for further binding. Publications dealing with this aspect include those of Stankowski (1983; 1984), Kawalczykowski et al (1986) Brodersen et al (1987), Hubble (1987) Galley et al (1988) and Gill et al (1988).

A further aspect is the competitive binding between two or more ligands for the same site (Harris and Winzor, 1988; Lecureuil et al, 1986; Goldstein and Barrett 1987; Pederson and Pederson 1985; 1988).

1.5.3 Methods of Measuring Ligand Binding

Many techniques for measuring protein-ligand binding interactions in solution have been reported. Reviews which classify the methods available for protein-ligand binding and which discuss, in general terms, advantages and disadvantages of particular methods include publications by Klotz (1953), Steinhardt and Reynolds (1969), Bush and Alven (1973), Kanfer (1975), Liao and Oehme (1980), Sparrow et al (1982) and Barré et al (1985).

Experimental methods for measuring protein-ligand binding can usually be classified as either direct or subtractive methods. In direct methods the quantity of protein-ligand complex is measured directly from changes in a physico-chemical property of either the bound ligand or the macromolecule resulting from the binding process. Subtractive methods measure the change in concentration of the unbound ligand as a result of protein binding.

Direct methods utilize any property of the ligand or the macromolecule which changes on binding such as the measurement of light absorbance (Wang et al, 1981), fluorescence (Tejima and Ozeki, 1979; Bentley et al, 1985), optical rotation (Coleman, 1968), NMR (Botherby and Gassend, 1973), light scattering and spectroscopy (Amundsen et al, 1977; Nour and Vallner, 1984; Oberfelder and Lee, 1985; Sklar, 1987), titration calorimetry (Sukow et al, 1980), X-ray diffraction (Blake and Oatley, 1977), Raman resonance (Yu, 1986) and scintillation proximity

(Undenfriend et al, 1987). Direct methods are, in general, more accurate than subtractive methods where the protein-ligand affinity is small and where subtractive methods would require the measurement of small differences in ligand concentration. Where the protein-ligand affinity is large direct methods offer no advantage in accuracy over subtractive methods.

Subtractive methods usually depend on the distribution of the ligand between two phases, only one of which can accommodate the protein. Subtractive methods include partition equilibria between phases, gel filtration (Braumbaugh and Ackers, 1971; Andreu, 1985; Kido et al, 1985), ultrafiltration (Mihailova and Russeva, 1978; Sophianopoulous and Sophianopoulous, 1985; Wolfer and Rippon, 1987), affinity chromatography (Nakano et al, 1979; Dunn, 1984; Bergman and Winzor, 1986), electrical conductivity and polarography (Tanford, 1961) as well as various dialysis methods - both equilibrium and dynamic dialysis.

The most generally applicable subtractive methods are dialysis methods which are essentially partition methods in which the distribution of the ligand between the protein and the solvent is achieved by means of a semipermeable membrane. (Crank, 1956; Carr 1961; McPhie, 1971). Friedman and McCally (1972) dealt with quantitative aspects of dialysis while Barrie et al (1975) provided mathematical descriptions for the diffusional processes occurring within the diffusion membrane.

Dialysis is the separation of a mixture of macromolecules and low molecular mass compounds by diffusion. A semi-permeable membrane permits diffusion of the small molecules (referred to as the dialysate), and retains the macromolecules. The diffusion of the small molecules occurs under the influence of a concentration gradient. A complete separation of the macromolecule and the low-molecular mass solute can be achieved by continually replacing the solution in the sink side of the membrane by fresh solvent to maintain the concentration gradient.

The most commonly used dialysis procedure is the classical equilibrium dialysis method (Klotz et al, 1946; Bush and Alvin, 1973) which is still considered to possess several advantages over some of the more sophisticated methods. It consists of separating the protein in the

sample compartment from a solution of the ligand in the sink compartment by a membrane permeable to all components of the system except the protein. When the system attains equilibrium, the activity of all components except the protein will be the same on either side of the membrane and the activity of the ligand in equilibrium with the protein-ligand complex is determined by assaying the solution in the protein free compartment. Concentration determinations are valid only if concentrations have the same relationship to chemical potential on each side of the membrane. Disadvantages of this method is that it is subject to a number of sources of error (Steinhardt and Reynolds, 1969; Behm and Wagner, 1979), and requires a long time in order to obtain equilibrium.

Most of the other dialysis techniques which have been used to investigate protein-ligand binding rely on the measurement of the rate of ligand diffusion rather than the measurement of equilibrium ligand concentration. These procedures constitute kinetic or dynamic dialyses methods. (Hoch and Muller, 1966; Silhavy et al, 1975; Meyer and Guttman, 1968; 1970a; 1970b; Colowick and Womack, 1969; Lecureuil et al, 1973; Robertson and Madsen, 1974; Rachidy and Niazi, 1978). Based on the Meyer and Guttman model Sparrow (1982) developed an improved method of continuous-flow dynamic dialysis whereas Macheras and Koupparis (1986) developed an automatic flow-injection serial dynamic dialysis technique.

The gel-filtration method (Hummel and Dreyer, 1962) uses a Sephadex column equilibrated with ligand into which the protein-ligand mixture is introduced, and the column eluted with ligand solution. In principle the method is similar to that of dialysis in that the surface of the gel is used as the interface to effect a distribution of ligand between the protein phase and the bulk solution. Modern methods of various gel-filtration techniques have been described by Andreu (1985) and Kido et al (1985).

The ultrafiltration method closely resembles the equilibrium dialysis method. In this technique, the long time required to attain equilibrium between the protein and the ligand is reduced by using high pressure membrane filtration. High pressures are used to separate a fixed volume

of protein-free solution from the protein-ligand mixture which is then analysed to determine its free ligand concentration. Ultrafiltration is theoretically equivalent to equilibrium dialysis but much simpler to carry out (Sophianopoulos et al, 1978) and appears to be more appropriate because it can be carried out rapidly without storage or addition of potentially competitive buffer components and electrolytes (Whittam and Brown, 1981). Limitations of the ultrafiltration technique are the polarization of protein on the membrane, the uptake of small molecules by the membrane and the change in the protein concentration with the volume filtered. Continuous ultrafiltration or diafiltration techniques have been developed which are analogous to dynamic dialysis methods (Roy and Miles, 1982; Claeyssens et al, 1985). The differences in the results obtained between using ultrafiltration techniques and dialysis methods have been compared (Blatt et al, 1968; Barré et al, 1985; Wolfer and Rippon, 1987).

1.6 Binding interactions in the presence of surfactants

In most biological systems protein-ligand interactions do not occur in isolation. Besides the effects of temperature and pH, additives such as buffering salts, surfactants, extenders and other impurities found in commercial formulations, can play a major role in the binding mechanisms. These factors can lead to the situation where the normal mass action binding theory does not apply but must be drastically modified to describe these interactions. In these instances the models serve only to indicate the complex nature of the mechanisms involved. Many poorly soluble drugs, biocides and pesticides are administered in solubilized form using micellar solutions in order to increase the bio-availability of the chemical. Moreover, the use of surfactants as emulsifying agents, suspension stabilizers, wetting agents and dispersing agents can coincidentally lead to significant changes in the biological activity of the active agent in the formulation. Surfactants may even be used in order to target a drug or pesticide to the site of action (Tadros, 1984b) and are also used for the control of various surface phenomena such as adhesion promotion or prevention, spreading or deposit formation.

Although surfactants can and do have a major influence on protein-ligand

binding interactions, this aspect has not been very widely reported in the literature. Some reports dealing with the interaction between various proteins and certain surfactants are those by Fishman and Eirich (1971) and Tipping et al (1974) who reported on the interaction of the anionic surfactant sodium n-dodecyl sulphate (SDS), Aoki and Hiramatsu (1974) and Hiramatsu et al (1977) on the interaction of cationic surfactants and Sukow and Sandberg (1974), Yedgar et al (1974a; 1974b) and Sukow et al (1980) on the interaction of non-ionics including Triton X-100. The binding of Triton X-100 to proteins is apparently very selective and favours lipophilic proteins (Sukow and Sandberg, 1974). The complexity of these interactions was illustrated by Sukow et al (1980) who showed that in the binding of the Triton X series of non ionic surfactants to bovine serum albumin (BSA) that the first 2 ligands bind co-operatively to high-affinity sites and 15 additional ligands bind at lower affinity, thermodynamically identical and independent sites. The thermodynamic parameters for formation of this complex indicate a large entropic contribution to binding which is consistent with a predominantly hydrophobic interaction.

Only limited reference has been made to the effects of surfactants on protein-ligand reactions. Some references dealing with the effects of surfactants, micelle formation in surfactant solutions and mixed ligand surfactant micelles on the interaction between protein and ligand are those of Day and Powell (1973) who showed that the uptake of radiolabelled low density lipoprotein by fibrous collagen and elastin is inhibited by the presence of non-ionic surfactants and Mandal et al (1983) who investigated effects of micelle and mixed micelle formation on the interaction of chromium-aluminium with leather.

In the presence of surfactants binding isotherms tend to show co-operative effects (Sukow and Sandberg, 1974; Hiramatsii et al, 1977; Sukow et al, 1980; Ishida et al, 1988). On the formation of mixed micelles the ligand can often be considered as a self-associating ligand which results in a deviation from all normal hyperbolic binding curve and the appearance of apparent co-operativity in the Scatchard plot (Ishida et al, 1988). Cobinding of the ligand and surfactant can also result possibly in more than two molecules being bound at the same time (Sato et al, 1988). The ligand/surfactant mixed micelle can also bind

in clusters (Kowalczykowski *et al*, 1986) with the shape of the mixed micelle playing a predominant role (Stankowski, 1983; 1984).

In the study presented in this thesis the effect of the non-ionic surfactant Triton X-100 on the binding interaction of two commonly used commercial fungicides TCMTB and NOITZ to insoluble bovine skin collagen was investigated. It was shown that over the lower fungicide concentration range the fungicide uptake follows a normal mass-action type binding isotherm. At the higher concentration levels, however, binding interactions deviated drastically from the classical binding theory with sudden massive ligand and surfactant uptake. This was interpreted as being due to the formation of large rod like mixed micelles binding to individual binding sites. The formation of these mixed micelles was confirmed by both UV/Vis spectroscopy and viscosity studies on solution mixtures of the fungicides and Triton X-100. The effect of these binding interactions on the fungistatic activity of the fungicide when applied to wet-blue leather was also investigated.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

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2.1 Materials

2.1.1 Insoluble Collagen (Hide Powders)

The preparation of hide powders has been described by Lamb (1981) who compared the methods of preparation used by Meunier with that of the British Leather Manufacturers Research Association. Atto and Nursten (1971b) described methods for the chrome tanning of hide powders.

Limed hide powder (Society of Leather Technologists and Chemists - Official Hide Powder Batch B5) was purchased from the British Leather Manufacturers Research Association and was used without any further purification. It was, however, analysed for moisture, nitrogen (collagen) and ash content and the pH value was measured by standard Society of Leather Technologists and Chemists methods (SLTC, 1965). The physical character of this hide powder was white and powdery with a few glassy heat denatured particles.

Lightly chromed hide powder was purchased from the Institut Für Macromolekulare Chemie Der Hochschule, Darmstadt. This was likewise analysed for moisture, nitrogen (collagen), chrome content and ash content and the pH value was measured. The hide powder was also used without any further purification.

2.1.2 Insoluble Collagen (Wet-blue leather)

Freshly flayed hides from the local abattoir were thoroughly washed and after proper drainage, the hides were sided down the line of the backbone and then processed through to the wet-blue condition by standard chrome tanning methods (Boast, 1989), using locally produced chrome tanning salts. After draining for two hours the wet-blue leathers were analysed for moisture and chrome content and the pH value was measured. Rectangular samples (125mm x 225mm) were then cut from the drained wet-blue leather sides from adjacent the line of the

backbone. The neck and belly sections were discarded. The rectangular samples were numbered sequentially and were allocated at random to the various fungicide treatments with samples kept as controls and for analyses (Fowler and Russell, 1990).

2.1.3 Fungicides

A crystalline sample of 2-(thiocyanomethylthio)benzothiazole (>99,9% purity) was kindly donated by Buckman Laboratories, Hammarsdale, R.S.A. Buckman Laboratories also kindly donated commercial formulations namely Busan 30L and Bulab 1077.

Busan 30L, commonly used in the leather industry to protect wet-blue leather, is a formulation of approximately 30% TCMTB as active ingredient in an inert aromatic high boiling point solvent and contains surfactant/s as dispersing/solubilizing agent/s. This sample of the formulation was analysed for active ingredient and found to contain 30,0% TCMTB on a mass per mass basis (m/m).

Bulab 1077 is a formulated, surfactant free, concentrated form of TCMTB in the same aromatic solvent as used in Busan 30L. On analysis it was found that this particular sample of Bulab 1077 contained 35,0% (m/m) TCMTB. A sample of the solvent (a mixture of high boiling point fraction of aromatic solvents from the petrochemical industry) was donated together with the sample of Bulab 1077. This was used together with Bulab 1077 in all the experiments to maintain a consistent solvent concentration.

A sample of pure n-octyl-4-isothiazol-3-one could not be obtained. Rohm and Haas (U.K.) Limited however donated a sample of Kathon 893, Lot No. 27142, which contained 47,0% NOITZ in propane-1,2-diol. This particular formulation of NOITZ was surfactant free. Hopkin and Williams C.P. propane-1,2-diol was purchased and was used in all the experiments together with Kathon 893 in order to maintain a consistent solvent concentration.

A commercial formulation Kathon LP (Levy, 1974) containing 9%(m/m) NOITZ 1% (m/m) Triton X-100 and 90% propane-1,2-diol was prepared using the

sample of Kathon 893, propane-1,2-diol and the non ionic surfactant Triton X-100.

2.1.4 Surfactant Triton X-100

A polydisperse preparation of p-(1,1,3,3-tetramethylbutyl)-phenoxypolyethylene glycols (Triton X-100) (Scintran grade) was purchased from BDH Chemicals and was used without any further purification.

2.1.5 Fungi

The fungi used in the activity experiments was a suspension containing seven of the fungal spores common to local wet-blue leather (Galloway and Cooper, 1974; Russell _et al_, 1985).

Aspergillus niger
Aspergillus wentii
Aspergillus terreus
Trichoderma viride
Penicillium frequentans
Paecilomyces sp.
Cladosporium sp.

These had been maintained on potato dextrose agar (PDA) slopes at 22°C. Prior to the fungicide evaluation each individual fungal culture was streaked onto separate PDA plates and incubated at 22°C for 7 days to ensure active sporulation.

2.1.6 Other Reagents

All other reagents used during analysis were commercially available analytical grade chemicals. Deionized water was used during all experiments.

2.2 Methods

2.2.1 Ligand Binding Methods

As various forms of insoluble collagen were used for the binding interactions simple filtration methods could be adopted in order to separate the free ligand from the collagen ligand complex. In principle the techniques used consisted of variants of the equilibrium ultrafiltration method where filtration is performed under pressure (Mihialova and Russeva, 1978). Certain limitations, such as

(i) Changes in collagen concentration
(ii) Equilibrium not achieved in time allowed
(iii) Ligand and/or Ligand-Surfactant complex being physically retained by the filter
(iv) Ligand and/or Ligand-Surfactant complex being adsorbed by the filter and
(v) Collagen being solubilized and passing through the filter

effecting the measured free ligand/ligand-collagen complex equilibrium might be expected to arise when using this technique. Preliminary determinations and analytical techniques were used to overcome these problems.

(a) Binding interactions of NOITZ to hide powders in the presence of Triton X-100

Four sets of five experiments were conducted in 250mℓ flat-bottomed flasks with ground glass stoppers. These were mounted on a mechanical shaker encased in a constant temperature chamber maintained at 25,0 ± 0,5°C (Figure 17).

In the first five experiments the binding interaction of NOITZ with limed hide powder in the presence of the non-ionic surfactant Triton X-100 was investigated. Hide powder (0,617g) which had a moisture content of 19% H_2O (that is 0,50g on a dry basis) was weighed into each of the flat-bottomed flasks and wet back with deionized water (25mℓ). After two hours the pH was adjusted to 4,0 (0,5mℓ 0,01M HCl), and the

FIGURE 17 Photograph showing apparatus used for binding of fungicides to hide powders in the presence of Triton X-100

flasks were shaken in the chamber overnight to equilibrate. Measured amounts of Kathon 893 to give 12 final concentrations ranging from 0 to 11,0mM NOITZ, propane-1,2- diol to give a final concentration of 34,8mM and Triton X-100 to give final concentrations of 0; 0,16; 0,80; 1,20 and 1,60mM were weighed into 20mℓ volumetric flasks which were then made up to volume with deionized water. These volumetric flasks were also equilibrated overnight and the contents added, after further vigorous shaking, to the flat-bottomed flasks at the start of each experiment. The empty volumetric flasks were retained and any residual fungicide was analyzed for NOITZ in order to adjust the initial fungicide concentrations.

In the following five experiments, the binding interaction of NOITZ with lightly chromed hide powder, the procedure was repeated using 0,595g hide powder (0,50g on a dry basis) without adding any hydrochloric acid (at equilibrium pH of solutions was naturally 4,0). The fungicide, surfactant and propane-1,2-diol was made up to volume in 25mℓ volumetric flasks.

Approximately 2,5mℓ samples of a homogenous mixture of hide powder and solution (ensuring a constant collagen concentration) were taken from each of the flasks after ½, 1, 2, 4, 6, 8, 12 and 24 hours (except in the two experiments where the Triton X-100 concentration was 1,20mM when samples were only taken after 24 hours) by means of hypodermic syringes. The samples were then filtered through 25mm Whatman glass microfibre (GF/C) filters in a Millipore Polypropylene Swinnex Filter Unit and the filtrate analysed for NOITZ and where possible for Triton X-100 by of HPLC. At the end of each binding experiment the final pH of each flask and the concentration of nitrogen and chromium in each flask was determined.

Note:- In a preliminary experiment varying amounts of mixtures of NOITZ and Triton X-100 were filtered through various filter media and it was found that only with glass microfibre filters that no significant retention of either NOITZ or Triton X-100 resulted.

(b) Binding interactions of TCMTB to hide powders in the presence of Triton X-100

The above ten experiments were repeated using the formulation Bulab 1077. The twelve initial TCMTB concentrations ranged from 0 to 7,4mM in each of the ten experiment. Varying amounts of aromatic solvent was added to maintain a constant solvent concentration of 3250mg/ℓ.

Samples were taken after ½, 1, 3, 6, 12 and 24 hours for the experimental binding to limed hide powder with Triton X-100 concentrations of 0 and 0,80mM. The binding experiments using limed hide powder in the absence of and at a concentration of 1,60mM Triton X-100 were sampled after 6, 12 and 24 hours, as was the binding experiment using chromed hide powder in the absence of Triton X-100. The frequency of sampling was reduced as the earlier experiments had shown that equilibrium was rapidly achieved. All the remaining experiments were sampled only after 24 hours when equilibrium had, effectively, been reached.

The filtered samples were analysed for TCMTB and Triton X-100 concentrations. In addition the final sampling (after 24 hours) was analysed for Nitrogen and in the case of the chromed hide powder experiments for chrome content. The pH value of these solutions was measured.

(c) Binding interactions of the commercial formulation Kathon LP to wet-blue leather at various temperatures

A total of 28 rectangular samples (approximately 125mm x 225mm) cut from one of the drained wet-blue leather sides were allocated at random to the various fungicide samples with 4 samples kept as control and for analysis. The fungicide treatment of the wet-blue leather was carried out at 10°C, 25°C and 40°C. The mass of each rectangular sample of wet-blue was determined and a 100% float containing the calculated amount of fungicide to cover a pre-set range of concentrations was added into each of eight glass Wacker tumbrils. The wet-blue leather samples were then drummed in these solutions for two hours (Figure 18).

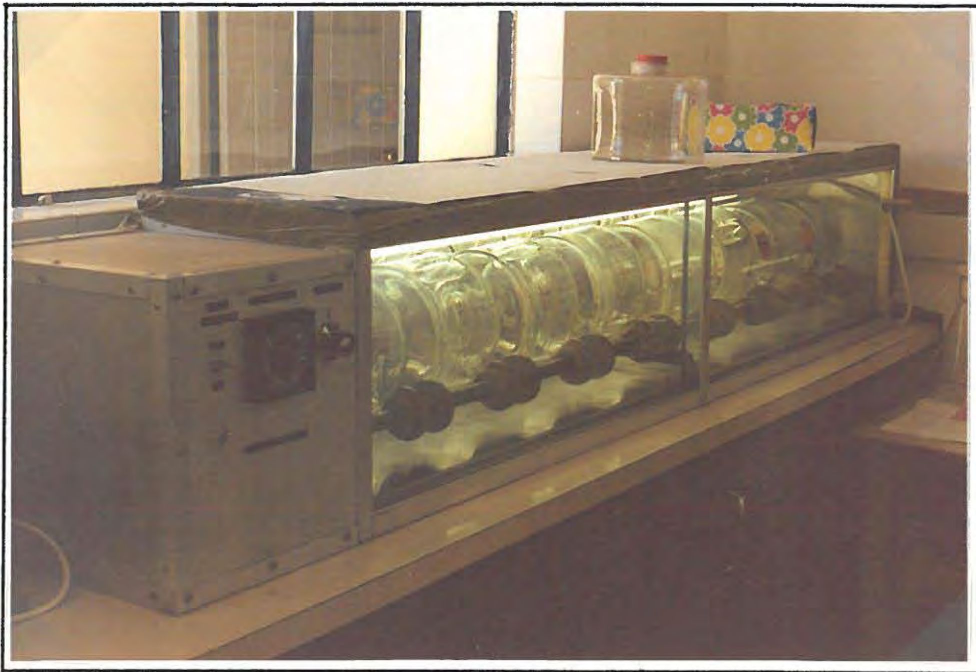

FIGURE 18 Photograph showing glass Wacker tumbril apparatus used in the binding of commercial fungicide formulations to wet-blue leather

Thereafter, the samples were removed and allowed to drain for two hours. This treatment with fungicide was applied using eight different concentrations, ranging from 0 to 0,50% (m/m) Kathon LP. The solution in the Wacker tumbrils at the end of the two hour treatment was kept for pH value measurement and for analyses of residual active ingredient (NOITZ) concentration and chrome content. After draining for two hours each rectangular wet-blue leather sample was again weighed in order to determine any change in moisture content and eleven circular 38mm diameter discs were punched from each of the treated samples for use in fungicide activity tests and for solvent extraction. All specimens were kept at 4°C until analysed or used in biocide activity tests.

(d) Binding interactions of the commercial formulation Busan 30L to wet-blue leather at various temperatures

The above experiment was repeated with Busan 30L in place of Kathon LP at four different temperatures, namely 10°C, 25°C, 40°C and 55°C. After draining fifteen circular discs were cut from each of the rectangular wet-blue leather pieces. All samples were stored at 4°C until analysed or used for biocide activity tests.

2.2.2 Analytical Methods

(a) High Performance Liquid Chromatography Determinations

(i) Simultaneous determination of NOITZ and Triton X-100

The method used for the simultaneous determination of both NOITZ and Triton X-100 was an adaptation of methods described for isothiazolones such as NOITZ by Matissek et al (1985), Matissek and Lehnguth (1984; 1988) and Matissek and Wittkowske (1984) and for Triton X-100 by Ahel and Giger (1985a; 1985b) and for TCMTB by Fowler et al (1987).

The concentrations of NOITZ and Triton X-100 were determined in all the filtered solutions by means of HPLC using Waters Associates Modules viz. Model 6000A solvent delivery system, Model 440 absorbance detector which monitored the eluate at 280nm and a 250mm stainless steel reverse phase column packed with 10μm Techsil C18 and fitted with a guard column

packed with the same material. Retention times and chromatograms were recorded with a Waters Data Module which has the facility of integrating the absorption count. Chromatograms were produced using 20μℓ samples from each of the filtrates. The samples were eluted with 80:20 Methanol: 0,4% Acetic Acid solution at a flow rate of 2mℓ/min.

(ii) Simultaneous determination of TCMTB and Triton X-100

The methods used were the same as for the NOITZ experiments. The reverse phase column, however, deteriorated towards the end of the analyses hence the TCMTB concentration in the samples taken after 24 hours from the experiments using 1,60mM Triton X-100 and limed hide powder and 0,16; 0,80; 1,20 and 1,60mM Triton X-100 and chromed hide powder were determined by first extracting into dichloromethane and then passing them through a Waters Radial-PAK B column (Fowler et al, 1987), using dichloromethane as eluant, at a flow rate of 3mℓ/min.

(iii) Determination of NOITZ concentrations in solutions and leather pieces

The concentration of free NOITZ in solution at the completion of the binding experiment was determined by using both the reverse phase column as above and by first extracting NOITZ into dichloromethane and then HPLC determination using the Radial-PAK B column. Quadruplicate discs from the wet-blue leather rectangular pieces, at the completion of the experiment, were extracted with dichloromethane, as described for TCMTB (Fowler et al, 1987; Fowler and Russell, 1990; Cooper, 1991), and the concentration of extractable NOITZ determined by HPLC using the Radial PAK B column. The extracted discs were retained at 4°C for biocide activity tests.

(iv) Determination of TCMTB concentrations in solutions and leather pieces

TCMTB was extracted from the discs of wet-blue leather at the completion of the experiment (Fowler et al, 1987; Fowler and Russell, 1990). Six of the wet-blue leather discs were separately immersed in excess dichloromethane in beakers and sonicated in the boiling solvent for 30

minutes. The discs and solvent were then quantitatively transferred to a Soxhlet apparatus and extracted for a further 7,5 hours. The extract was concentrated to 15mℓ and then filtered through phase separation paper. The phase separation paper was rinsed with dichloromethane and the filtrate and washings were made up to 25mℓ in volumetric flasks. The extracted discs were retained at 4°C for biocide activity tests.

The TCMTB in the filtered solutions at the end of the experiment was extracted into dichloromethane. The extractions were carried out in triplicate where a suitable aliquot was extracted with three successive solvent aliquots of 7; 7 and 6mℓ. These extracts were filtered through phase separation paper into 25mℓ volumetric flasks and the extracted TCMTB determined by means of HPLC.

The quantity of TCMTB in each of the extractions (both from the wet-blue leather discs and the float solutions) was determined using the HPLC method described above using dichloromethane as eluant.

(b) Other Analytical Procedures

(i) pH determinations

The pH of all solutions (at the end of the experiments) was measured using a Digital Data Systems (Pty) Ltd dds 200 pH/conductivity meter fitted with a combination electrode.

(ii) Nitrogen determinations

The total nitrogen (on both the limed and chromed hide powders and also on all of the final filtered sample solutions) was determined by means of the standard Kjeldahl method. This was done to determine the quantity (if any) of solubilized hide powder in the filtrate in order to correct the measured amounts of unbound (free) fungicide found when analysing the extract.

(iii) Chrome determinations

The chrome content of the wet-blue leather, chromed hide powder and all filtered solutions sampled at the end of the experiments using wet blue leather or chromed hide powder was determined by means of Atomic Absorption Spectroscopy after wet acid ashing.

(iv) Moisture determinations

In order to determine the moisture content of the wet-blue leather samples, the mass of three discs from each treatment was determined. As drying in an oven causes the outer layer of the wet-blue leather to become case hardened with entrapped moisture the discs were each dried in a microwave oven for approximately 1 minute, then in a drying oven at 100°C for a further 30 minutes, cooled in a desiccator, and the mass again determined.

The moisture content of the hide powders was determined using a standard Brabender apparatus set at 100°C.

2.2.3 Antifungal Activity Measurements

Fungistatic testing was conducted using three different methods, namely the potato dextrose agar embedding, the moist incubation (wet filter paper) and the humidity chamber (only for TCMTB fungistatic testing of the 10; 25 and 40°C treatments) methods. The testing was conducted on duplicate wet blue discs from each treatment concentration both before and after extraction with dichloromethane.

(a) Moist Incubation Method (Wet Filter Paper)

This method has been described (Galloway and Cooper, 1974; Russell _et al_, 1985). All media and equipment were sterilized before use by autoclaving at 121°C for 20 minutes. Fungistatic tests were carried out in 90mm Petri dishes using sterile Whatman No. 1 filter paper. The "7-mix" fungal spore suspension was used. To each plate was added 15mℓ of 0,85% saline and the spores were suspended in the media by means of gentle rubbing with a sterile loop. Each of the resulting suspensions

were diluted to 50mℓ with sterile saline solution. An equal volume (25mℓ) of each fungal spore suspension thus prepared was added to a 250 flask to form the composite "7-mix" suspension. Although the suspension can be maintained and used for up to 2 months, a freshly prepared spore suspensions were used to ensure viability. In each case the viability was checked by using untreated wet blue leather as control.

One mℓ of the "7-mix" was added to the surface of each wet blue disc on filter paper in a petri dish, the excess liquid was run off onto the filter paper and to ensure 100% relative humidity the petri dish was sealed with insulation tape. The petri dishes were stored in an incubator at 22°C.

(b) Potato Dextrose Agar Plate

To one side of each petri dish, containing a wet blue disc, was added 15mℓ of sterile molten and cooled (45°C) PDA and the dish rocked gently to ensure even distribution of the agar without covering the surface of the disc. After allowing to cool 0,7mℓ of the "7-mix" fungal suspension was added onto the centre of each wet blue disc. The plate was gently rocked to ensure an even distribution and a sterile "hockey stick" was used to spread the remaining suspension evenly across the agar surface. The petri dishes were sealed with insulation tape and stored in the incubator at 22°C.

The plates in both methods were examined for fungal growth at regular intervals up to 120 days. Fungal growth at three levels was recorded. (Figure 19)

slight - fungal growth just on edge of disc
moderate - fungal growth covering $<$ 1/3 of disc
heavy - fungal growth covering $>$ 1/3 of disc

Plates showing no fungal growth either on the paper, agar or wet blue disc were reinoculated at monthly intervals and tests were terminated after heavy fungal growth was noted.

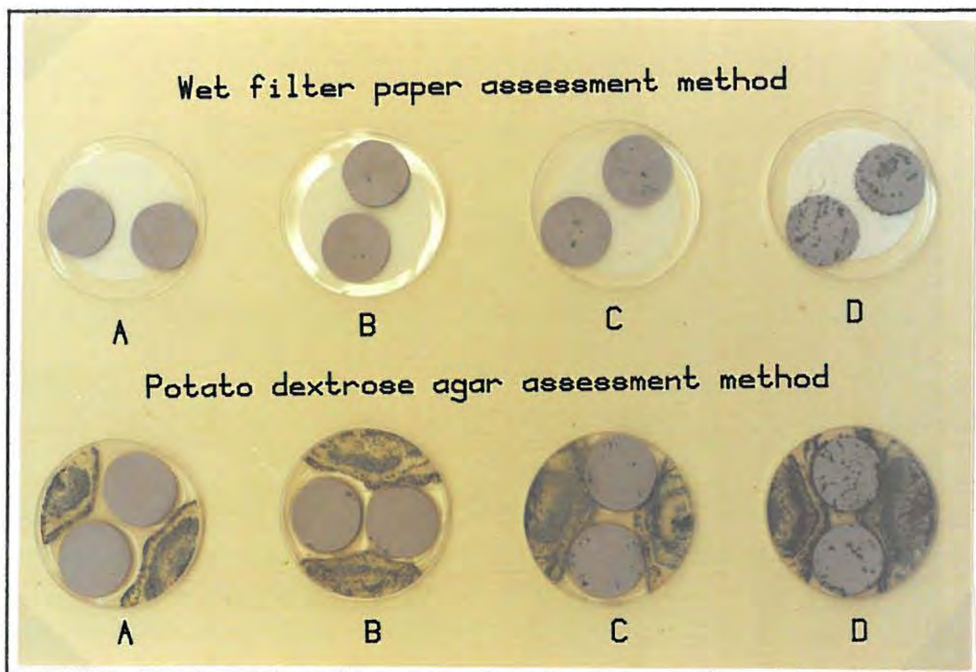

FIGURE 19 Photograph showing fungal spore growth on wet-blue leather discs (Note: A-No Growth, B-Slight growth, C-Moderate growth, D-Heavy growth)

(c) Buckman Humidity Chamber

Duplicate sample wet blue leather discs (both drained and dichloromethane extracted) from each Busan 30L treatment in the 10°, 25° and 40°C binding experiments were sent to the Buckman Laboratories at Hammersdale for testing in their humidity chambers. The samples were placed in the humidity chamber at 30°C for an exposure period of 8 weeks. Every week the samples were examined for fungal growth and the extent of fungal proliferation scored accordingly.

2.2.4 Micelle and Emulsion Studies

Studies were conducted on various mixtures of solutions of the two fungicides and Triton X-100 in order to better understand the dynamic solution system and the different micelle species present for possible binding interactions with collagen. The solutions were examined by means of UV/Vis Spectroscopy, Viscosity and Conductivity methods.

The following range of solution mixtures were prepared in 25mℓ volumetric flasks which were stored in a thermostatic water bath and the physical measurements conducted at 10°, 25° and 40°C.

(a) Solutions containing NOITZ

Five lots of fifteen solutions containing from 0 to 4,41mM NOITZ and (0; 0,16; 0,80; 1,20 and 1,60mM) Triton X-100 were made up in 25mℓ volumetric flasks. Varying amounts of propane-1,2-diol were added to each flask to adjust the concentration to 13,9mM.

(b) Solutions containing TCMTB

Five sets of fifteen solutions containing from 0 to 2,9mM TCMTB and the same amounts of Triton X-100 as above were made up in 25mℓ volumetric flasks. The aromatic solvent concentration in all of these solutions was adjusted to 1300mg/ℓ.

(c) UV/Vis Spectroscopy

The solutions were allowed to equilibrate overnight and then after vigorous shaking the UV/Vis spectrum of all the solutions was determined on a Hewlett Packard Model 8452 A Diode Array Spectrophotometer between 190 and 400nm. The absorbance at 376nm in the visible range was noted in order to measure solution turbidity. The spectrum were measured at 10°; 25° and 40°C for NOITZ solutions and at 25°C for TCMTB solutions.

(d) Viscometry

The kinematic viscosity of all the solutions was measured using a Cannon-Fenske size 50 viscometer (Specification BS 188 and IP 77) in a Gallenkamp Viscometer Bath set at 10°, 25° and 40°C for solutions containing NOITZ and at 25°C for solutions containing TCMTB.

(e) Conductivity

The conductivity of all the solutions was measured using a Digital Data Systems (Pty) Ltd dds 200 pH/conductivity meter, at 10°, 25° and 40°C for the solutions containing NOITZ and at 25°C for solutions containing TCMTB.

CHAPTER 3

MICELLE AND EMULSION STUDIES

CHAPTER 3

MICELLE AND EMULSION STUDIES

3.1 n-Octyl-4-isothiazol-3-one/Triton X-100 Solutions

3.1.1 Results

The UV/Vis spectrographs (Figure 20) for increasing concentrations of Triton X-100 in water at 25°C show shifts in wavelength of the absorbance maxima and the formation of shoulders. These shifts in wavelength are illustrated (Figure 21).

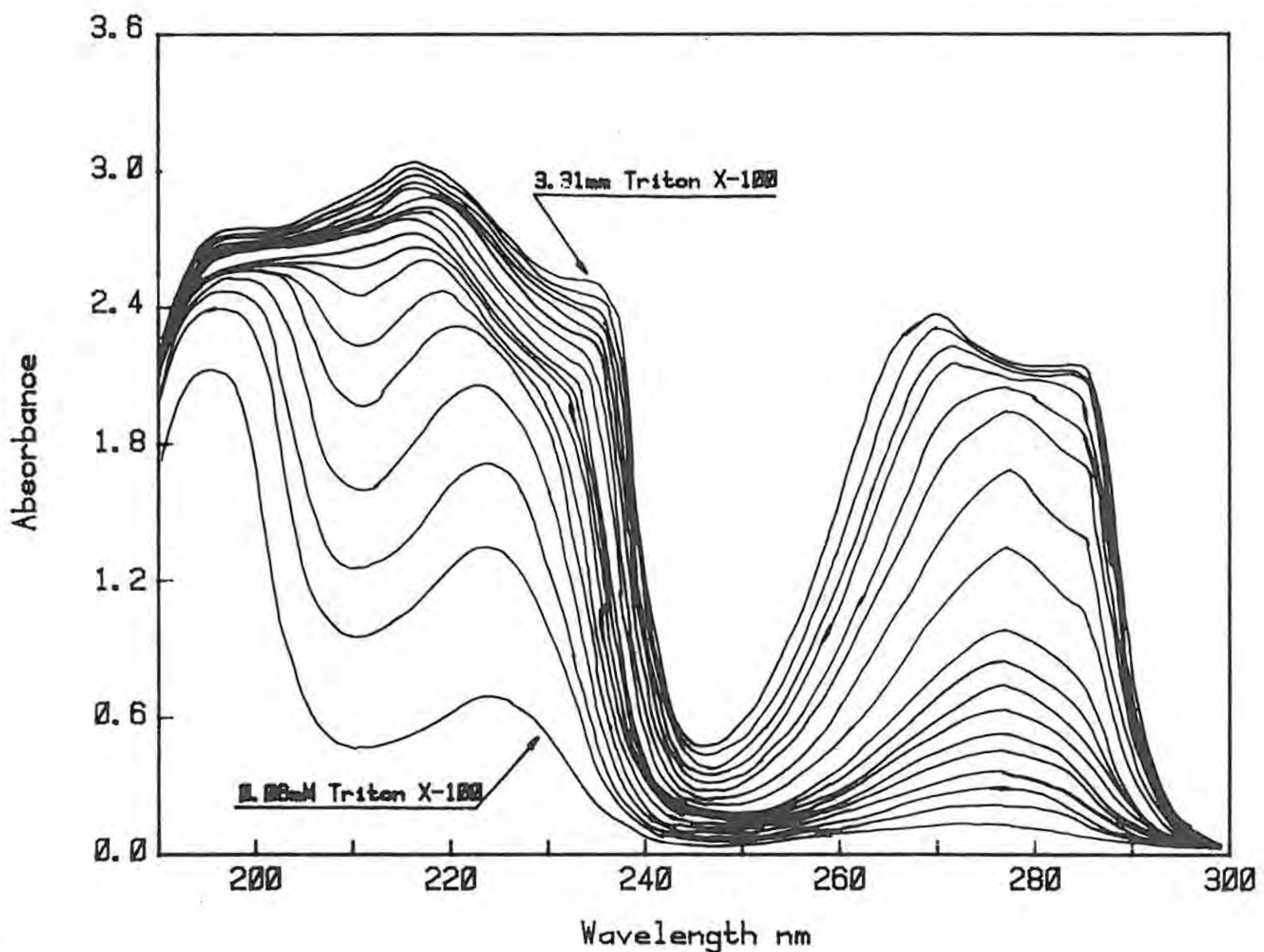

FIGURE 20 UV/Vis spectrographs for increasing concentration of Triton X-100 at 25°C (Note: Each spectrograph represents a different concentration of Triton X-100 increasing from 0,06 to 3,31mM)

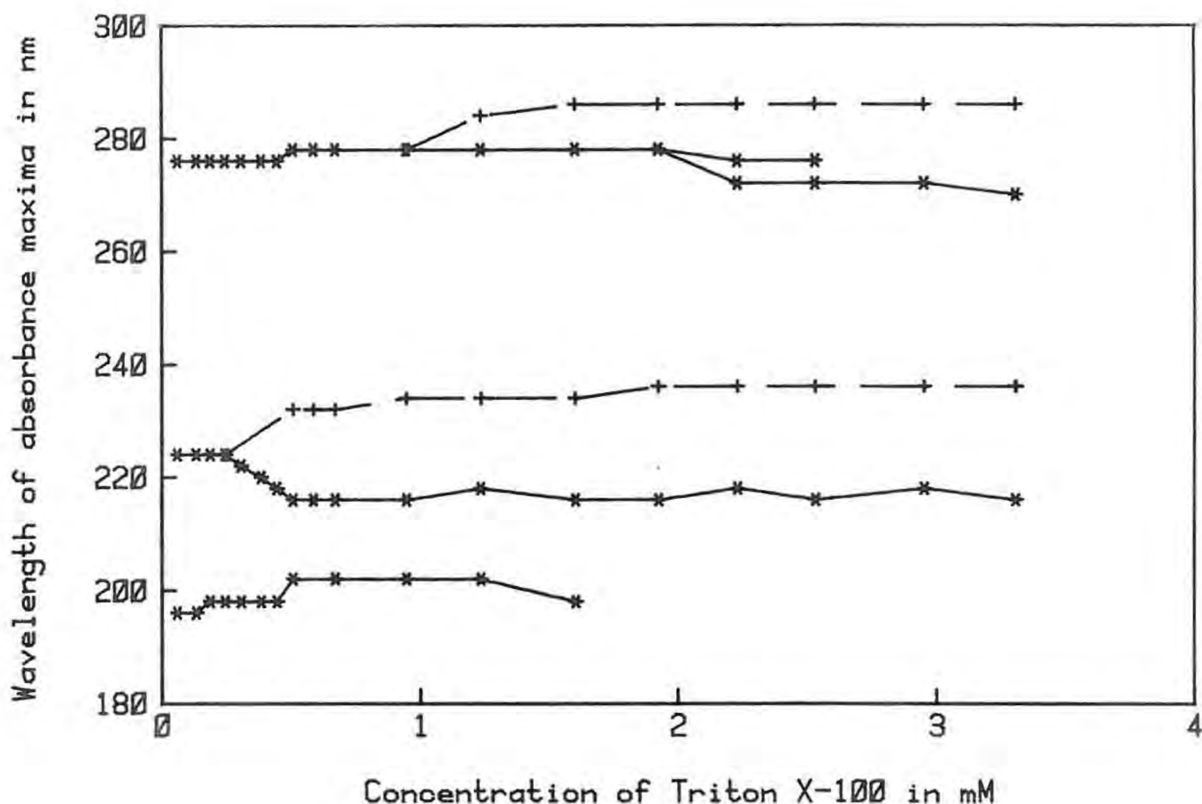

FIGURE 21 Shifts in the wavelength of the absorbance maxima with increasing concentrations of Triton X-100 (Note: *—* absorbance peak, +—+ absorbance shoulder)

It was previously found by Cardinal and Mukerjee (1978) that there is a shift in the wavelength of absorption maxima at 220nm between 0,25 and 0,30mM Triton X-100. This shift in wavelength has been interpreted as an indication of the formation of a second or different species in the solution such as the formation of micelles. Between the same concentrations a shoulder formed at a wavelength of 240nm. At a much higher Triton X-100 concentration of 2mM a similar absorption maximum wavelength shift occurred at 280nm. As with the shifts in wavelenth of absorption maxima at 220nm and at lower Triton X-100 concentrations these could also indicate the formation of additional species in the solution such as the transition to a different micelle species.

A set of UV/Vis spectrographs for increasing concentrations of NOITZ in the absence of Triton X-100, is illustrated (Figure 22) whereas shifts in wavelength of the absorption maxima at 10°C, 25°C and 40°C are indicated (Figure 23).

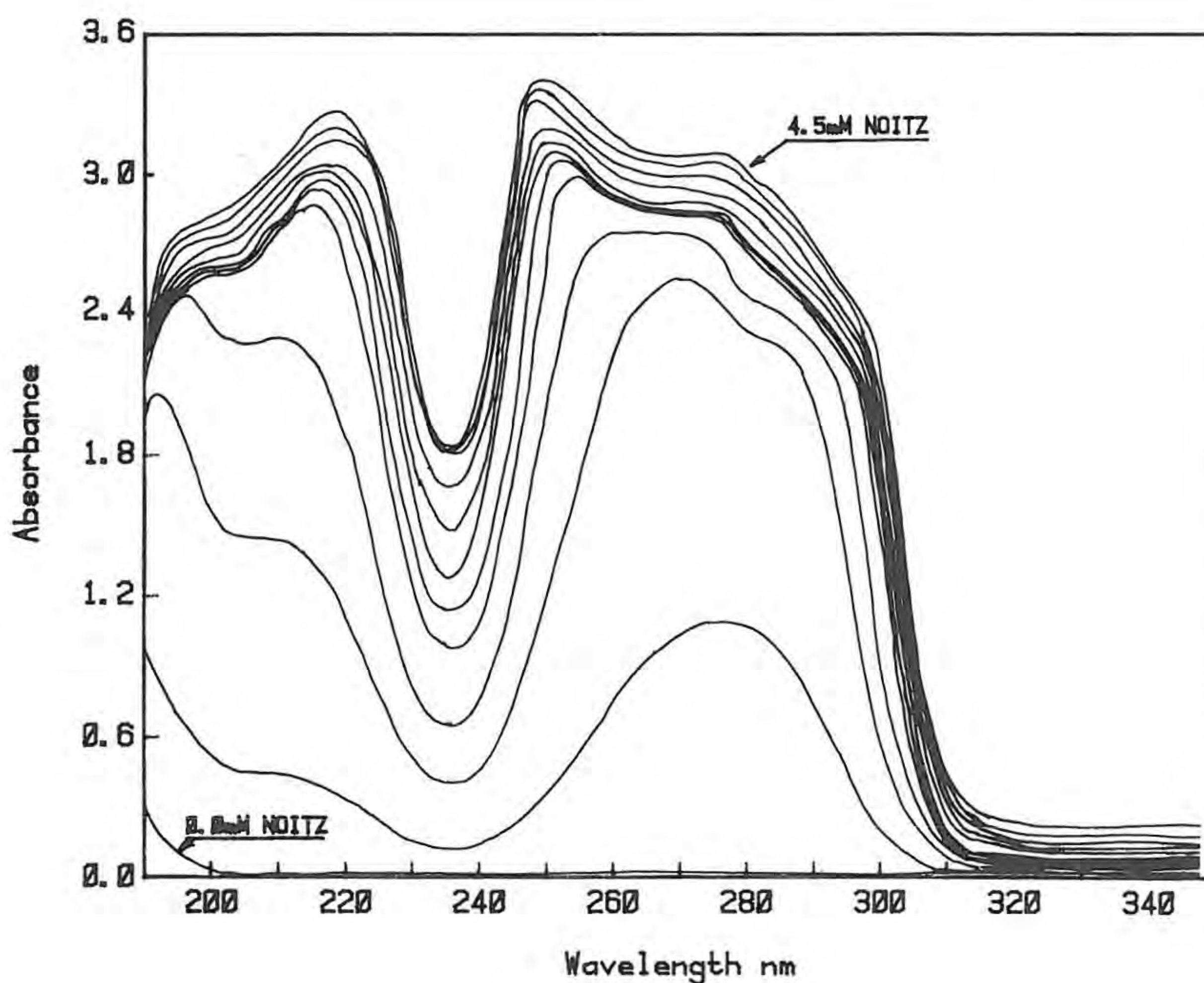

FIGURE 22 UV/Vis spectrograph for increasing concentration of NOITZ in the absence of Triton X-100 (Note: NOITZ concentration increases from 0 to 4,5mM. Spectrographs measured at 25°C)

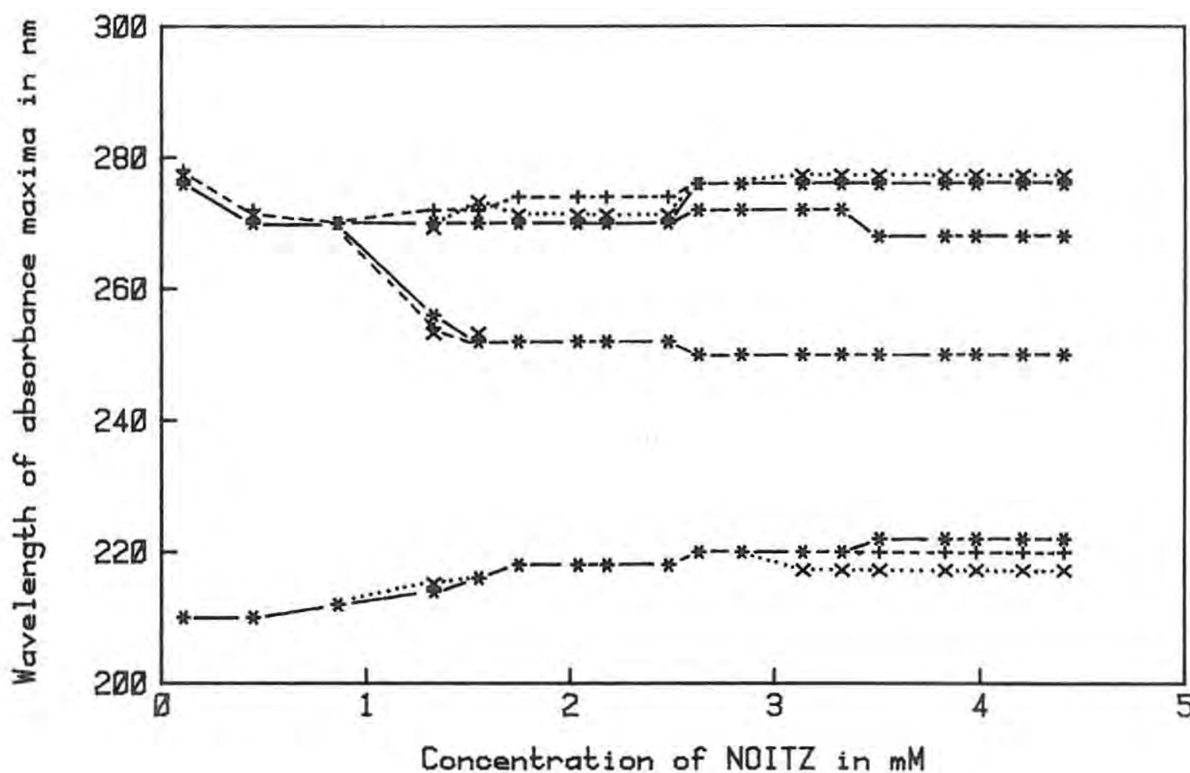

FIGURE 23 Shifts in wavelength of the absorbance maxima with increasing concentration of NOITZ (Note: *—* 10°C, +--+ 25°C, x·····x 40°C)

These figures illustrate the following:

i) There was very little or no change in the spectra when the temperature was increased from 10°C to 40°C.

ii) Shifts occurred in the wavelength of absorption maxima similar to those found for Triton X-100 accompanied by the formation of a shoulder and additional peaks. These occurred at concentrations of between 1,0 and 1,4mM NOITZ. As for Triton X-100 these could indicate the possible formation of biocide micelles.

iii) At no concentration did the solution become turbid as is indicated by the absorption in the visible range at a wavelength of 376nm. This indicates that the solubility of NOITZ was not exceeded.

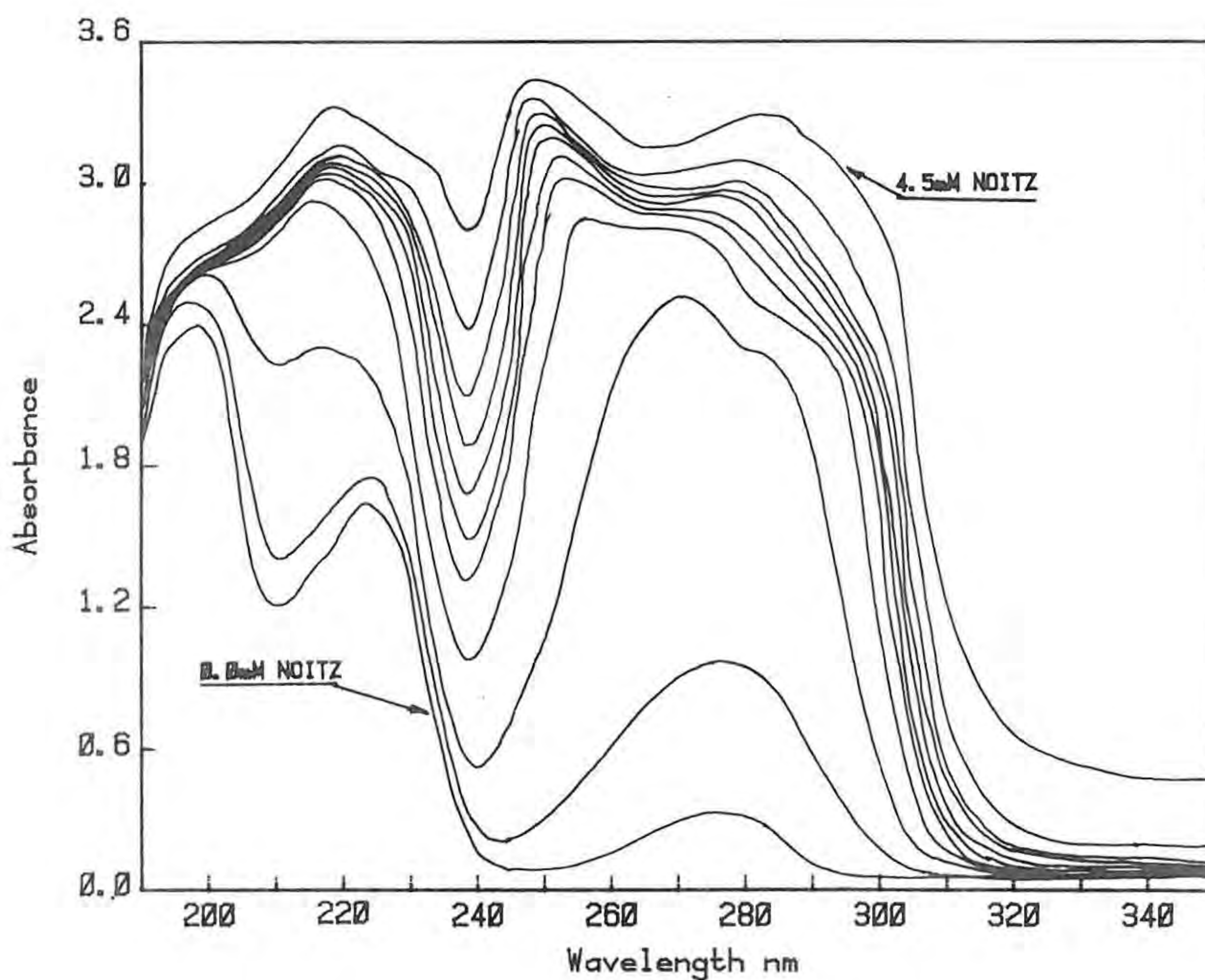

FIGURE 24 UV/Vis spectrograph for increasing concentrations of NOITZ in the presence of 0,16mM Triton X-100 (Note: NOITZ concentration increases from 0 to 4,5mM. Spectrograph measured at 25°C)

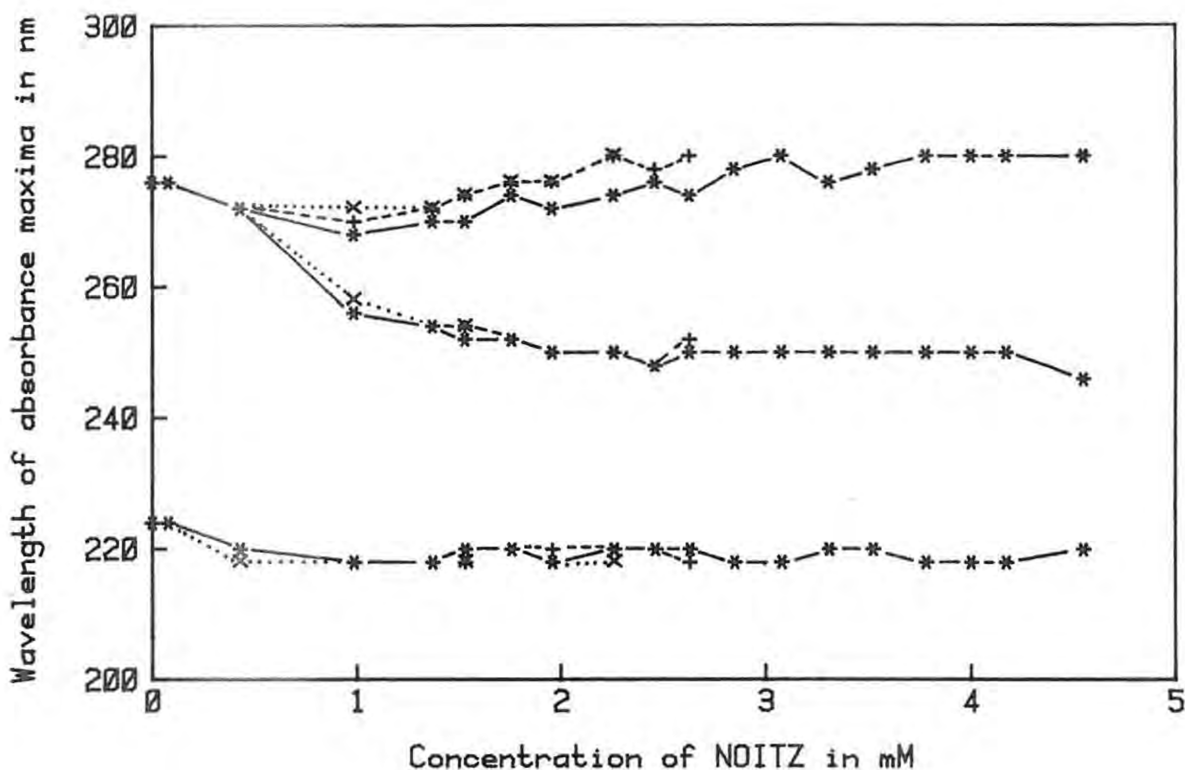

FIGURE 25 Shifts in wavelength of the absorbance maxima with increasing concentration of NOITZ in the presence of 0,16mM Triton X-100 (Note: *—* 10°C, +—+ 25°C, x......x 40°C)

The UV/Vis spectrographs of various mixed dispersions of NOITZ and Triton X-100 at the three selected temperatures of 10°C, 25°C and 40°C were determined. A typical set for Triton X-100 concentration of 0,16mM at 25°C is illustrated (Figure 24).

These spectrographs depict two major features. Firstly in the UV range there are shifts in the wavelength of the maxima of various absorption peaks. Secondly at certain temperatures and concentrations of NOITZ and Triton X-100 the solutions become turbid as measured by the absorbance at 376nm indicating that the solubility of the NOITZ in the micelle/mixed micelle complex was exceeded. The turbidity point is

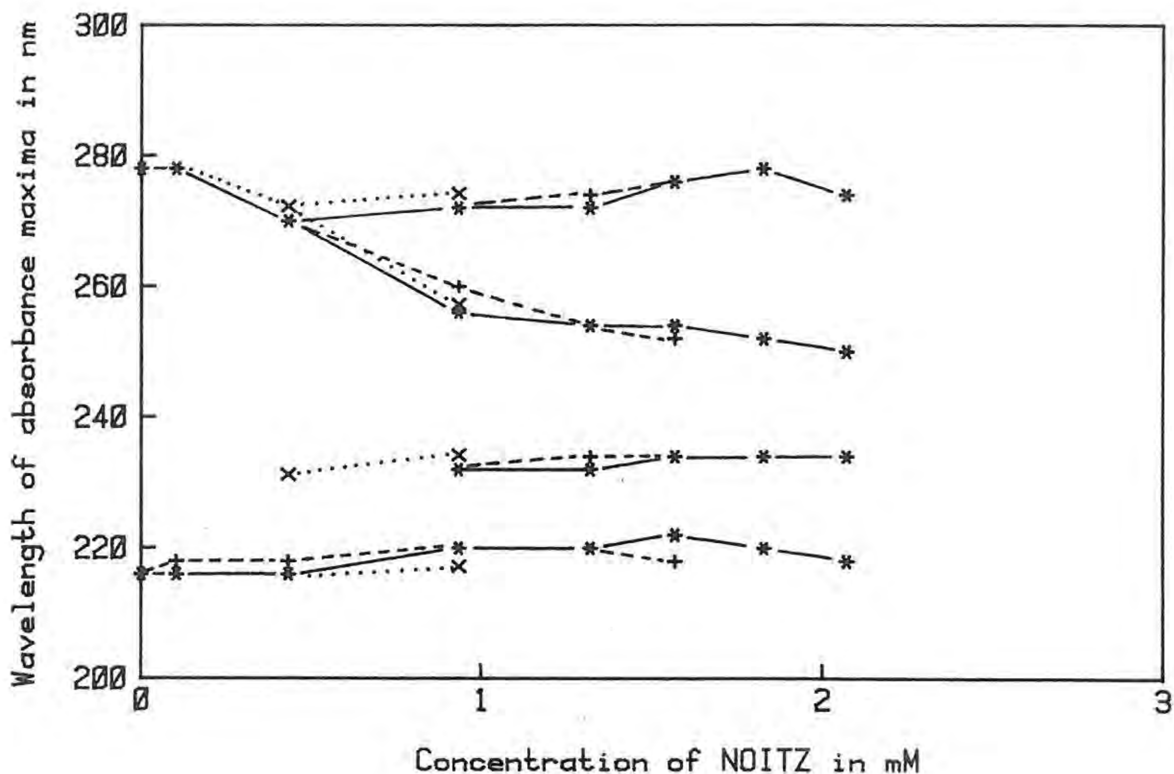

FIGURE 26 Shifts in wavelength of the absorbance maxima with increasing concentration of NOITZ in the presence of 0,80mM Triton X-100 (Note: *—* 10°C, +– –+ 25°C, x.....x 40°C)

highly temperature dependent. As NOITZ concentration increases the turbidity decreases and the solutions become transparent. This phenomenon indicates the formation of a new soluble species.

The wavelength shifts of the absorption peaks are illustrated (Figures 25 to 28). As previously indicated for pure Triton X-100 and NOITZ these wavelength shifts can be interpreted as being caused by the formation of micelles, different forms of micelles and mixed micelle species.

The changes in the turbidity of the solutions are clearly illustrated in

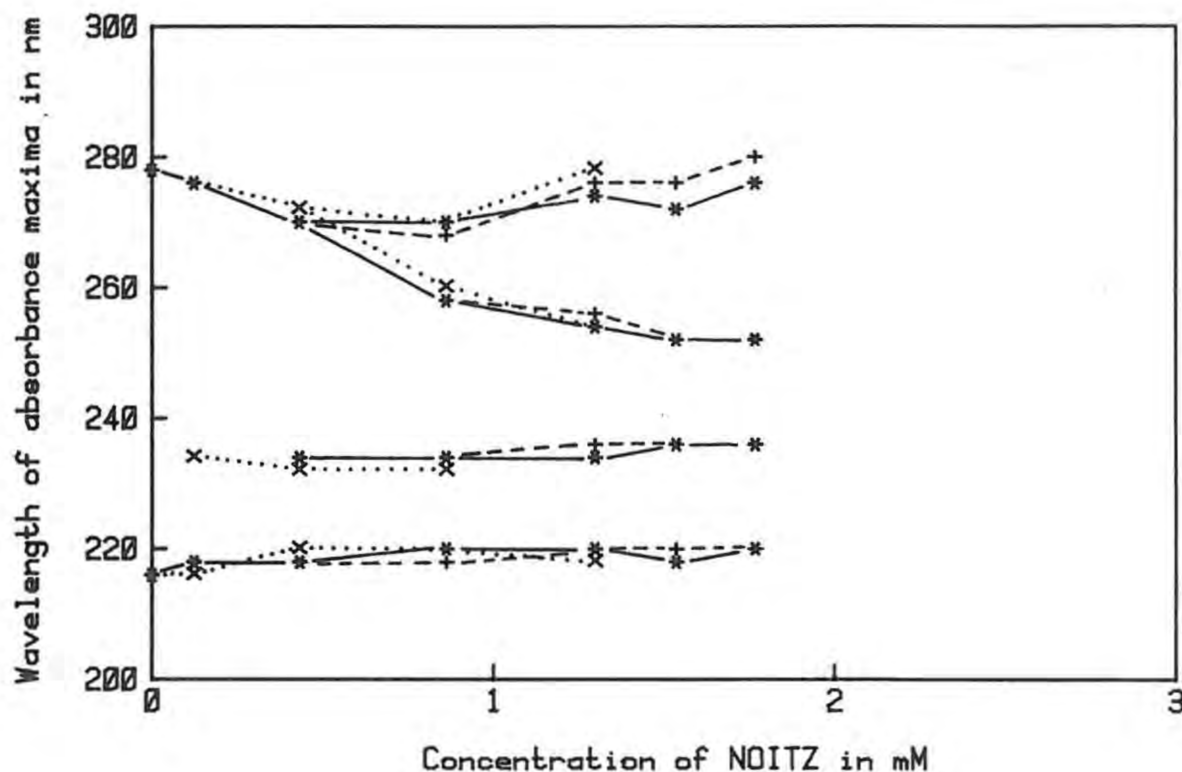

FIGURE 27 Shifts in wavelength of the absorbance maxima with increasing concentration of NOITZ in the presence of 1,20mM Triton X-100 (Note: *—* 10°C, +---+ 25°C, x····x 40°C)

the (point to point) plot of absorbance at 376nm with increasing NOITZ concentrations at 10°C, 25°C and 40°C (Figures 29 to 31).

The figures indicate that in the presence of Triton X-100 when NOITZ concentration is increased.

(i) There is a shift in the wavelength of absorption maxima and the formation of an additional peak at 274nm at a lower concentration. This shift occurs at a concentration of between 0,5 and 1mM NOITZ compared with a concentration of between 1 and 1,25mM NOITZ in the absence of Triton X-100

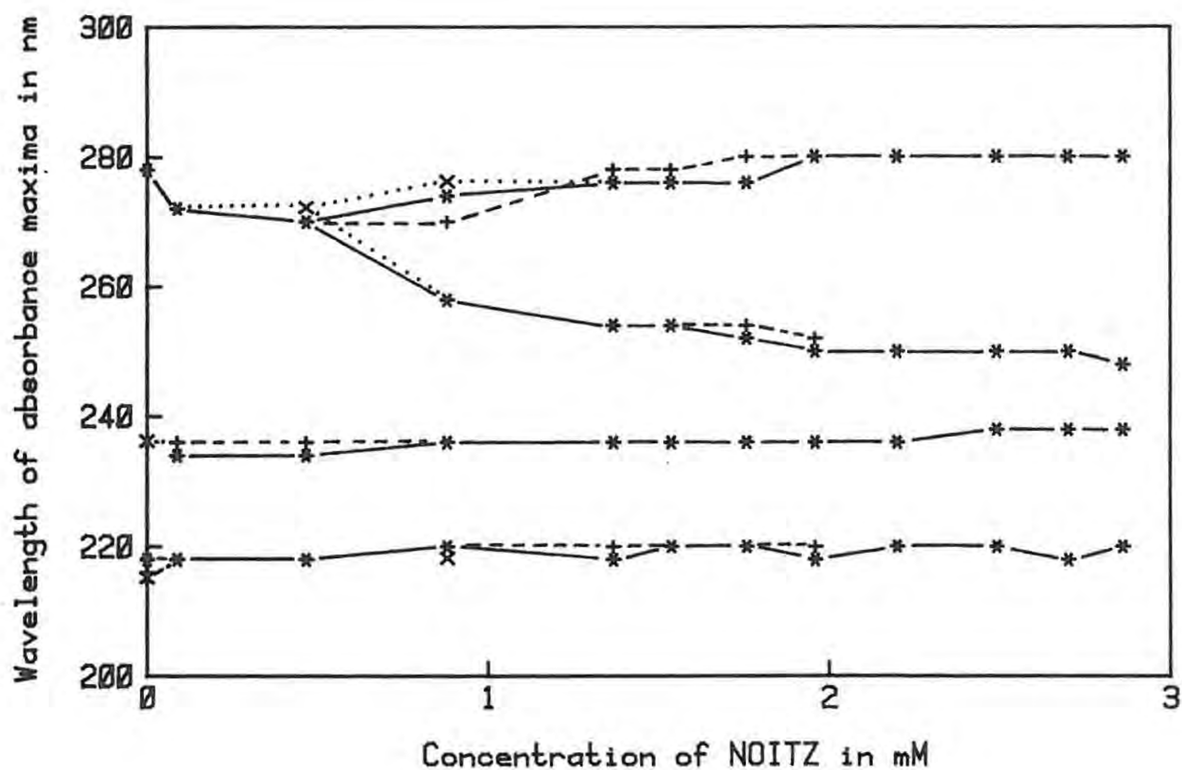

FIGURE 28 Shifts in wavelength of the absorbance maxima with increasing concentration of NOITZ in the presence of 1,60mM Triton X-100 (Note: *—* 10°C, +---+ 25°C, x····x 40°C)

indicating the formation of micelles/mixed micelles at lower concentrations. The formation of a second absorption peak and shift in wavelength maxima is not very temperature dependent as only small differences could be detected between the spectral shifts in this lower concentration range at the three temperatures.

(ii) With increase in NOITZ concentration at a certain point the solutions became turbid, as indicated by the absorption at 376nm. This turbidity occurred even at the lowest Triton X-100 concentration of 0,16mM which is below the CMC of pure

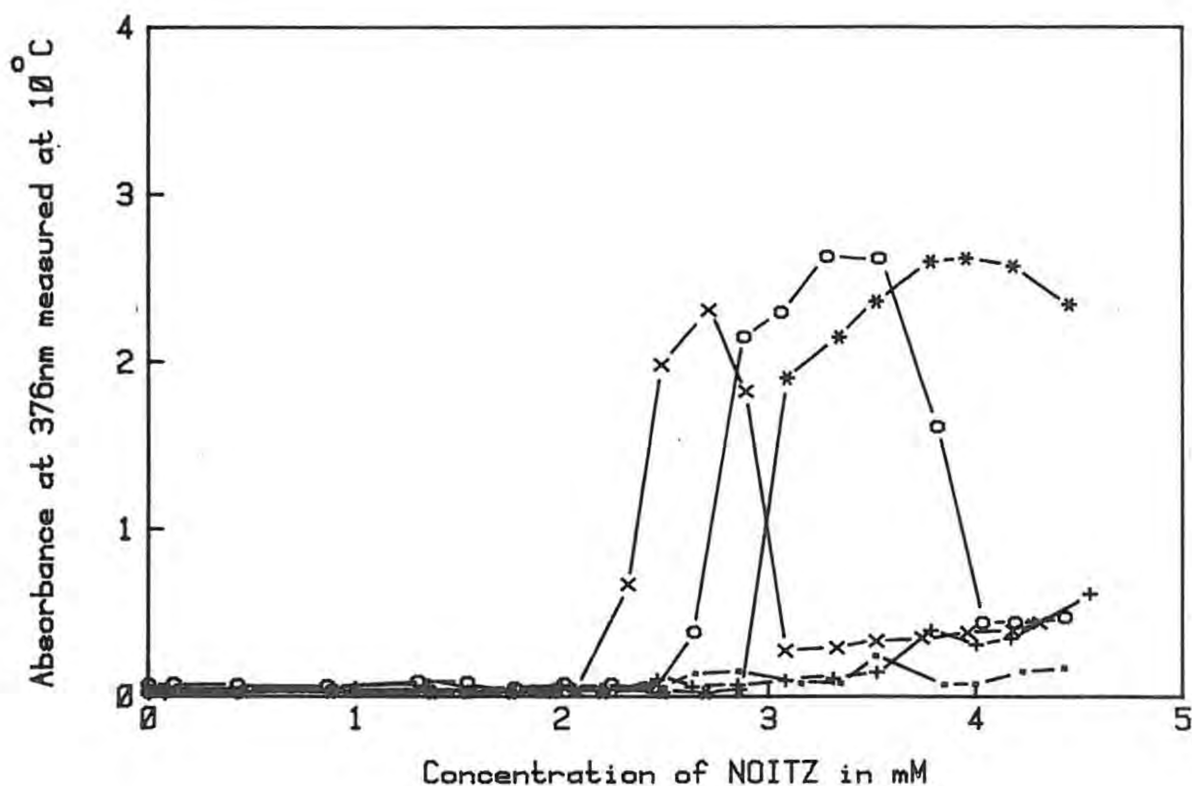

FIGURE 29 Change in absorbance at 376nm with increase in NOITZ concentration as measured at 10°C (Note: .___. 0,00mM Triton X-100, +——+ 0,16mM Triton X-100, x——x 0,80mM Triton X-100, o——o 1,20mM Triton X-100, *——* 1,60mM Triton X-100)

surfactant. The turbidity point is extremely temperature dependent.

(iii) Of greater importance, (as will be indicated later during the binding studies), was the observation that as the NOITZ concentration is increased, at Triton X-100 concentrations above the surfactant CMC, a point is reached where the turbidity of the solution suddenly decreases indicating the possible formation of a different soluble species.

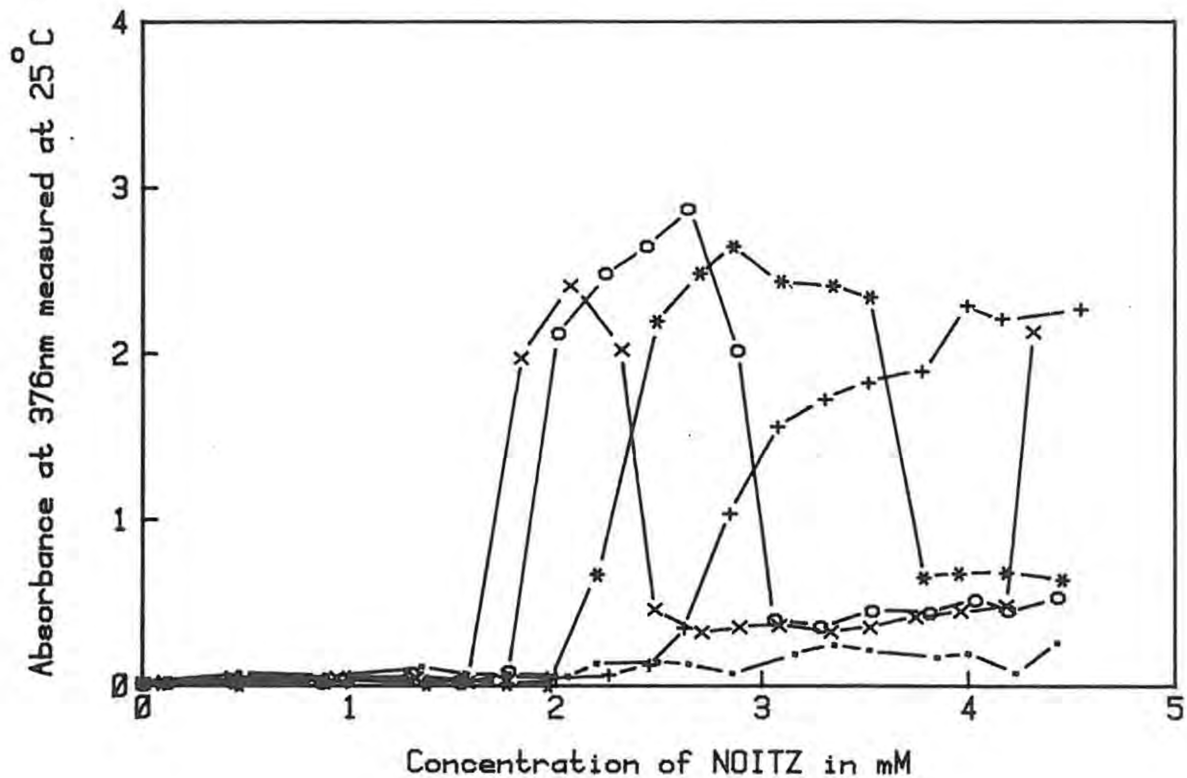

FIGURE 30 Change in absorbance at 376nm with increase in NOITZ concentration as measured at 25°C (Note: .—. 0,00mM Triton X-100, +—+ 0,16mM Triton X-100, x—x 0,80mM Triton X-100, o—o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

These figures (Figures 29, 30 and 30), depicting the point to point plots of absorbance in the visible range at 376nm with increase in NOITZ concentration at 10°C, 25°C and 40°C respectively, further show that:

i) The increase in turbidity (cloud point) is dependent on both the Triton X-100 concentration and on the temperature.

ii) the decrease in turbidity at the higher NOITZ concentration is also dependent on both Triton X-100 concentration and on temperature.

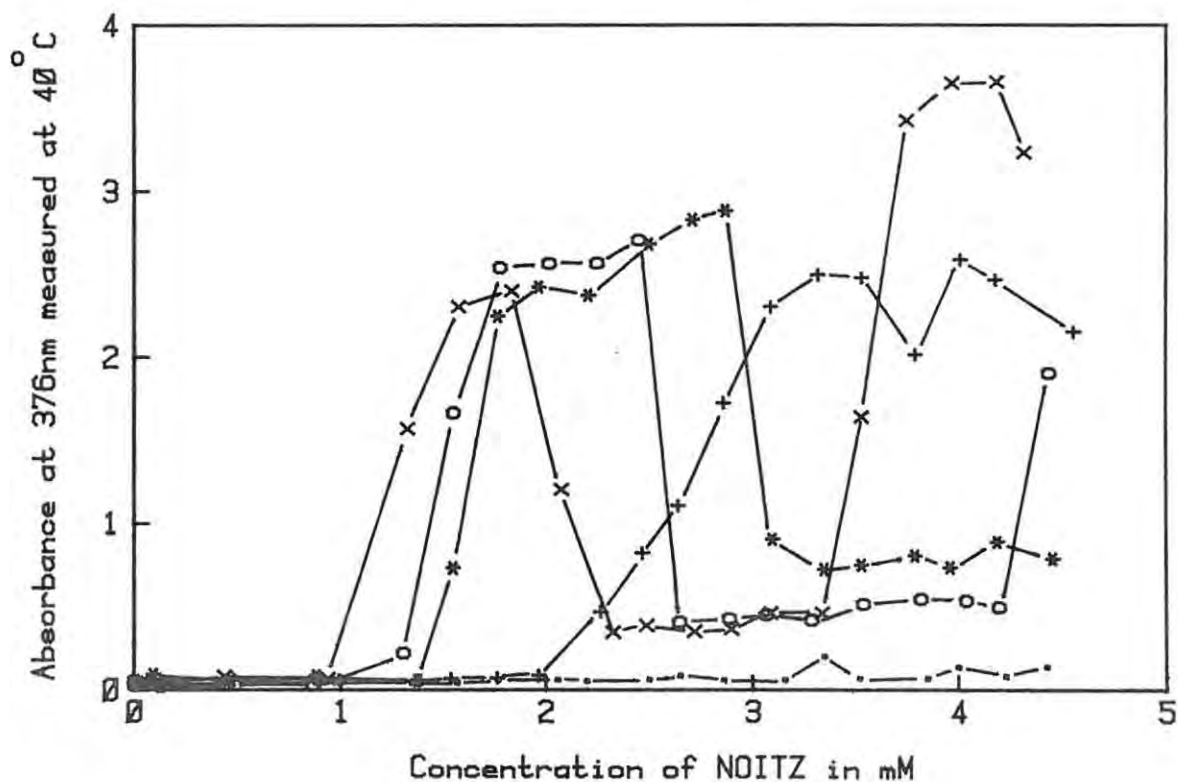

FIGURE 31 Change in absorbance at 376nm with increase in NOITZ concentration as measured at 40°C (Note: .—. 0,00mM Triton X-100, +—+ 0,16mM Triton X-100, x—x 0,80mM Triton X-100, o—o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

iii) Solutions without Triton X-100 do not show the above phenomena in the concentration range measured.

iv) The solutions containing 0,16mM Triton X-100, which is below the CMC of a pure solution of the surfactant, only show sharp increases in turbidity at the higher NOITZ concentrations at 25°C and 40°C but not at 10°C.

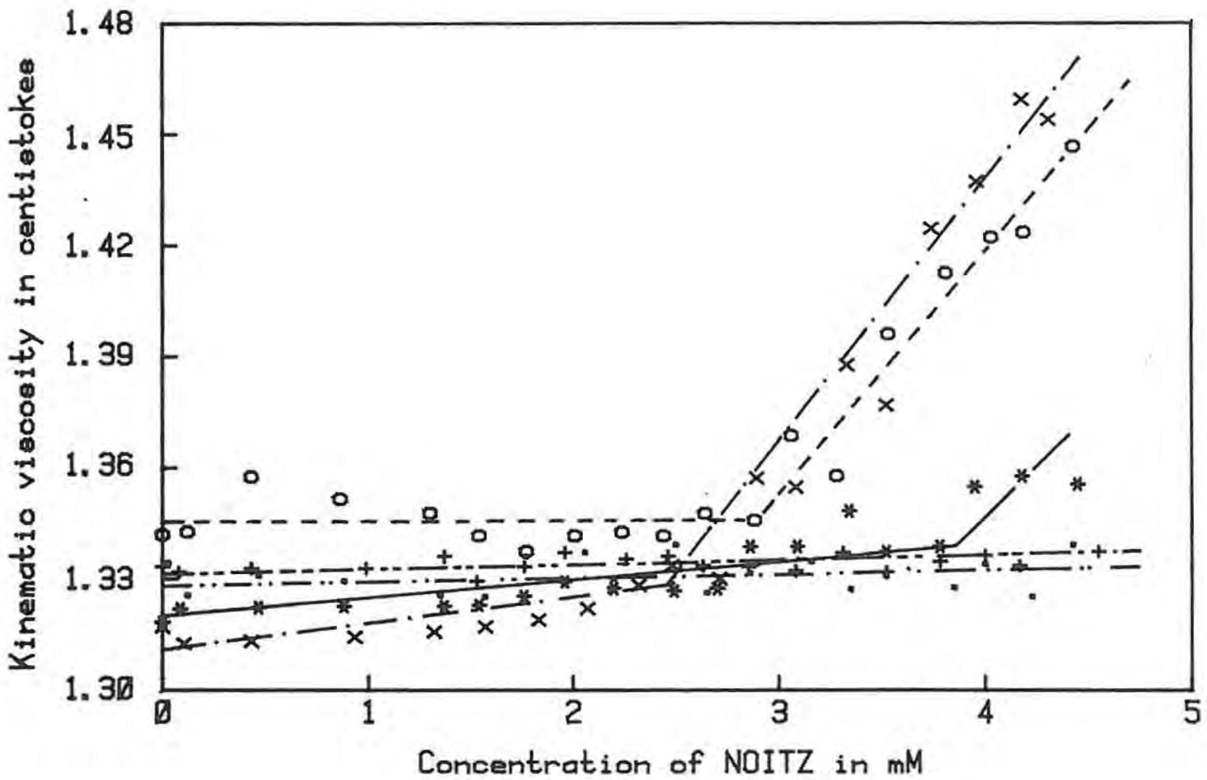

FIGURE 32 Change in viscosity with increase in NOITZ concentration as measured at 10°C (Note: .—..—. 0,00mM Triton X-100, +—.--+ 0,16mM Triton X-100, x-•-x 0,80mM Triton X-100, o---o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

v) The molar ratios of NOITZ to Triton X-100 where turbidity occurs are temperature dependent and are as follows:

a) at 10°C (Figure 29) they are 2,75:1 for 0,80mM Triton X-100; 2,17:1 for 1,20mM Triton X-100 and 1,73:1 for 1,60mM Triton X-100.

b) At 25°C (Figure 28) they are 15,6 for 0,16mM Triton X-100; 2,0:1 for 0,80mM Triton X-100; 1,45:1 for 1,20mM Triton X-100 and 1,24:1 for 1,60mM Triton X-100.

c) At 40°C (Figure 29) they are 12,5:1 for 0,16mM Triton X-100, 1,19:1 for 0,80mM Triton X-100; 1,04:1 for 1,20mM Triton X-100 and 0,87:1 for 1,60mM Triton X-100. In addition there is a second cloud point at a ratio of 4,19:1 for the 0,80mM Triton X-100 concentration.

vi) Of more interest is the phenomenon of the decrease in turbidity at the higher NOITZ concentrations (which is also dependent on Triton X-100 concentration and on temperature). The molar ratios at which this phenomenon occurs are as follows:

a) At 10°C (Figure 29) this decrease occurs at the molar ratios of NOITZ to Triton X-100 of 3,5:1; 2,7:1 and 2,6 for 0,80; 1,20 and 1,60mM Triton X-100 respectively.

b) At 25°C (Figure 30) the corresponding ratios are 3,0:1; 2,4:1 and 2,3:1 respectively and

c) At 40°C (Figure 31) they are 2,5:1; 2,1:1 and 1,9:1 respectively.

The measurement of absolute viscosity of the various solution mixtures of NOITZ and Triton X-100 is illustrated in (Figures 32 to 34 for temperatures of 10°C, 25°C and 40°C respectively.

The lines drawn in the figures are linear regressions of the points on either side of the "break point" or discontinuity. These diagrams indicate quite clearly that at a similar concentration where the turbidity of the solutions decrease there is a marked increase in the viscosity of the solution. This is illustrated by the increase in the rate of change of viscosity with increased NOITZ concentration for the solutions at 10°C and 25°C and, by not only a change in the rate of increase in viscosity, but by a marked discontinuity in the curve showing the viscosity increase in the solutions at 40°C. This increase in viscosity could also be explained by the formation of a massive, possibly rod shaped, mixed micelle.

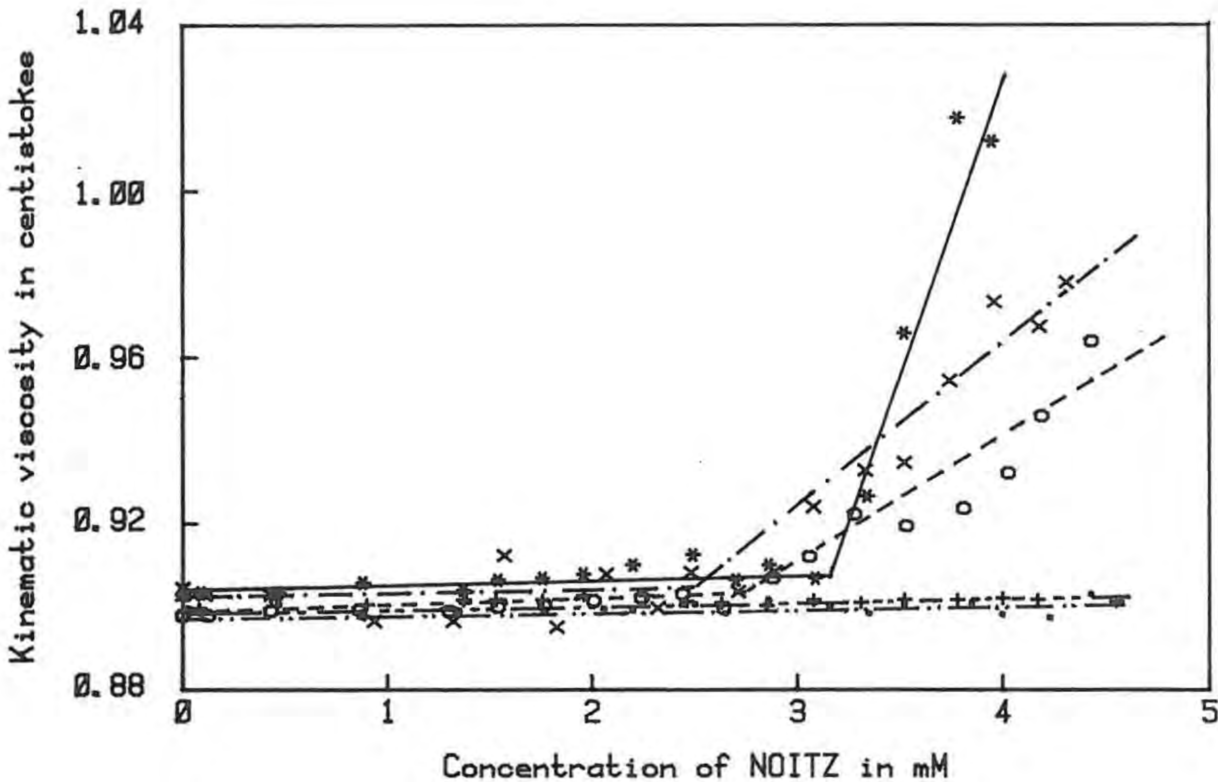

FIGURE 33 Change in viscosity with increase in NOITZ concentration as measured at 25°C (Note: .—..—. 0,00mM Triton X-100, +----+ 0,16mM Triton X-100, x—·—x 0,80mM Triton X-100, o---o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

The change in conductivity of the solutions at the three temperatures is depicted in (Figure 35). Although an increase in the overall conductivity was measured corresponding to the increase in NOITZ concentration no changes in the rate of this increase could be measured. This was due mainly to the extremely low conductivity values measured and to the variability in individual measurements which could have been caused by small amounts of contaminants. This line of investigation was not continued.

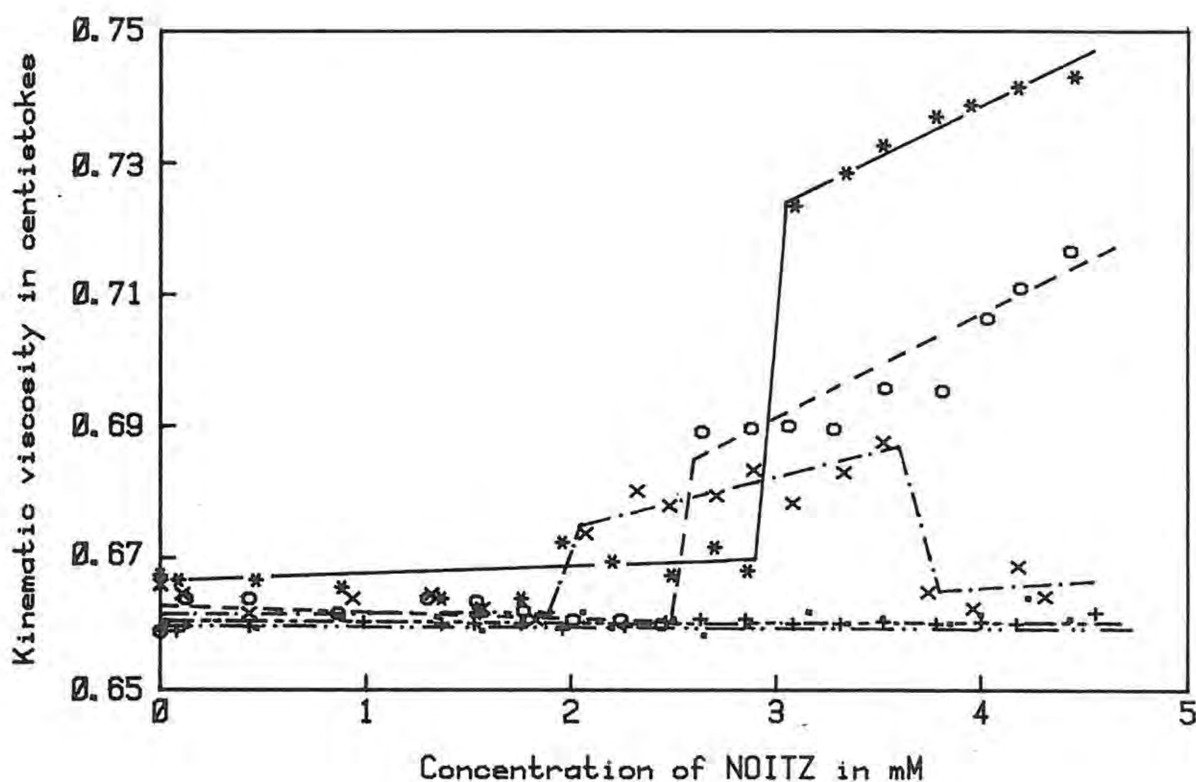

FIGURE 34 Change in viscosity with increase in NOITZ concentration as measured at 40°C (Note: .-..-. 0,00mM Triton X-100, +-·-·+ 0,16mM Triton X-100, x-·-x 0,80mM Triton X-100, o---o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

3.1.2 Discussion

Solubilization in micellar systems is intimately related to the structure and properties of micelles and also the molecular structure of the solubilized species (Mukerjee,1980). The location and distribution of solubilized species can be studied in terms of the two state model which postulates an equilibrium distribution between a "dissolved state" associated with the hydrocarbon core, and an "absorbed state" arising from the interfacial activity of the dissolved species. A solubilizate usually increases the sizes of micelles not only by the incorporation of the solubilizate itself, but also by causing an increase in the average

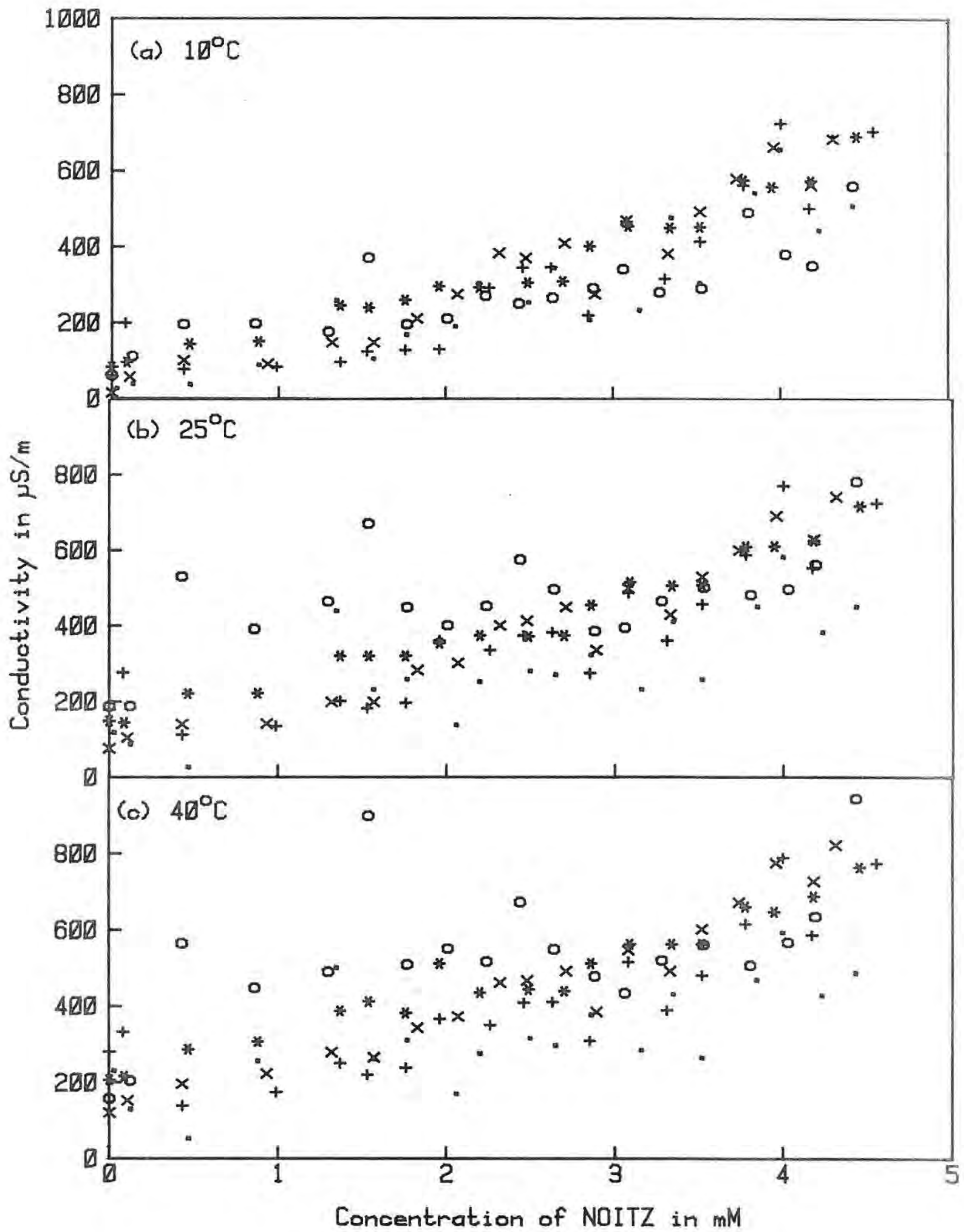

FIGURE 35 Change in conductivity with increase in NOITZ concentration (Note: . . 0,00mM Triton X-100, + + 0,16mM Triton X-100, x x 0,80mM Triton X-100, o o 1,20mM Triton X-100, * * 1,60mM Triton X-100)

number of surfactant molecules in the micelle. Viscosity studies can indicate that in some micellar systems the formation of very large asymmetric micelles may be induced. In the association model for surfactants in many systems only "small" micelles are present which are the same size as a spherical micelle with radius equaling the length of a streched out monomer. For such a system the size distribution is determined by the cooperativity in the self-association process in the early stages of the growth of the micelles followed by an anticooperativity for larger micelles arising out of a sphere-to-rod transition. In some cases, this anticooperative region of growth is not pronounced enough to prevent the formation of "large" micelles, presumably rod-like. A solubilizate is likely to give greater stability to larger micelles than smaller ones as the former incorporate a relatively greater number of the solubilizate molecules.

Work on the location of solubilizates has been done at high concentration of the solubilizate, the mole ratios of the solubilizates to surfactant in the micelles being as high as 2 (Cardinal and Mukerjee 1978). At such concentrations micellar structures are likely to be modified substantially and pronounced changes in solution viscosity and UV spectral changes (Murkerjee & Cardinal, 1978) have been observed indicating the presence of asymmetric micelles. Depending on the solubilization site, solubilization is expected to have different effects on counterion binding, hydration and the size and shape of micelles (Lindmann, 1984). Solubilization may not only have a marked influence on micelle size and shape but may induce the formation of another phase, generally a lamellar liquid crystal. The growth of micelles to long rods is associated with solubility in the head group region and may have dramatic effects on induced viscoelasticity. Ribeiro and Denis (1975) showed that upon mixed micelle formation the phospholipid molecule appeared to be intercalated into the Triton X-100 micelle in such a manner as to place its hydrophobic groups in the apolar core, and its polar groups into the polar exterior palisade layer. It is likely that NOITZ would behave in the same way. They subsequently showed, however, that the presence of the phospholipid in mixed micelles with Triton X-100 did not affect the microenvironment or motional behaviour of the micelles (Ribiero and Denis, 1976).

Both light scattering and viscosity methods used by Attwood (1968) to determine changes in the size and shape of micelles, showed an increase in viscosity at the same concentration as increase in light scattering and concluded that this is the concentration at which large micelles are first observed. He found non Newtonian behaviour (using Cannon-Fenske No 50 and 100 viscometers) due to disturbance of the aggregation process under high shear rates. This is possibly the reason for not detecting any rate of increase in viscosity when the cloud point was reached in the present study. According to Fishman and Eirich (1975a; 1975b), however, flow times in excess of 120 seconds would make kinetic energy corrections unnecessary. In the present study flow times of between 190 and 390 seconds were used. Mandal et al (1980) working on lipids solubilized by Triton X-100 also showed increase in viscosity indicating that the shape of the mixed micelles had changed towards more asymetry.

Thus in the present study we note that in the UV range the various shifts in wavelength of absorbance maxima illustrates the very dynamic nature of these mixed fungicide surfactant solutions with the formation of micelles, mixed micelles and various different micelle species.

Of more significance is the change in turbidity of the solutions as measured by the absorbance at 376nm. At any fixed temperature at a certain concentration the turbidity point is reached indicating that the fungicide NOITZ is no longer fully solubilized by the surfactant micelle, however at higher biocide concentration a level is reached where the solution again becomes transparent indicating that the fungicide is resolubilized in presumably a much larger mixed micelle, possible rod-shaped. A very similar result was achieved by Yedgar et al (1974a; 1974b) in their studies on the size and shape of micelles dependent on the ratio of Triton X-100 to lipid in the solubilization of Sphingomyelin. They measured the turbidities by recording the optical densities at 350 and 450nm and found rapid decrease in the optical densities as the lipid formed mixed micelles with Triton X-100.

The measurement of viscosity confirms the turbidity data. The viscosity showed an increase at the same or similar concentrations as the sudden decrease in turbidity. This also indicates (as has been reported by many authors) the formation of rod-shaped large micelles (Attwood, 1968;

Tanford et al, 1977; Mandal et al, 1987). The size and shape of micelles may be inferred from measurements of the viscosity of the system and comparison of one system with another can yield evidence of the micellar structure (Corti and Deglorgio, 1981).

As the non-ionic surfactant Triton X-100 imparts negligible conductance to the solution (Mandal et al, 1980) and also NOITZ being a non ionizable molecule, conductance in the solution was extremely low. As there were large variations in the resulting measured conductance of the solution, this parameter could not be used to gain information on micelle and mixed micelle formation.

The results above clearly indicate the very complex nature of solutions containing both biocide and surfactant. The very dynamic nature of these solutions with the formation of micelles/mixed micelles including various different species and forms (shapes) of micelles and mixed micelles complicates any study of the biocide binding reactions in these solutions. The detailed investigation of the dynamic nature of the various mixed micelle species formed falls outside the scope of this thesis. Of more interest is the point of the sudden decrease in turbidity and increase in viscosity as it appears that this is the concentration at which a new large mixed micelle species is formed. This species could be considered as a separate ligand and could bind to a single binding site resulting in what would appear in the binding curve to be a massive cooperative effect. This type of effect was noted during the binding studies.

3.2 2-(Thiocyanomethylthio)benzothiazole/Triton X-100 Solutions

3.2.1 Results

The UV/Vis spectrographs for increasing concentrations of TCMTB in water at 25°C are illustrated (Figure 36) whereas shifts in wavelength of the absorbance maxima are indicated (Figure 37).

As was observed in the case of NOITZ a shift in wavelength of the absorbance maxima with increasing concentrations of TCMTB was noted. This shift from 266nm to 278nm occurred at TCMTB concentration of

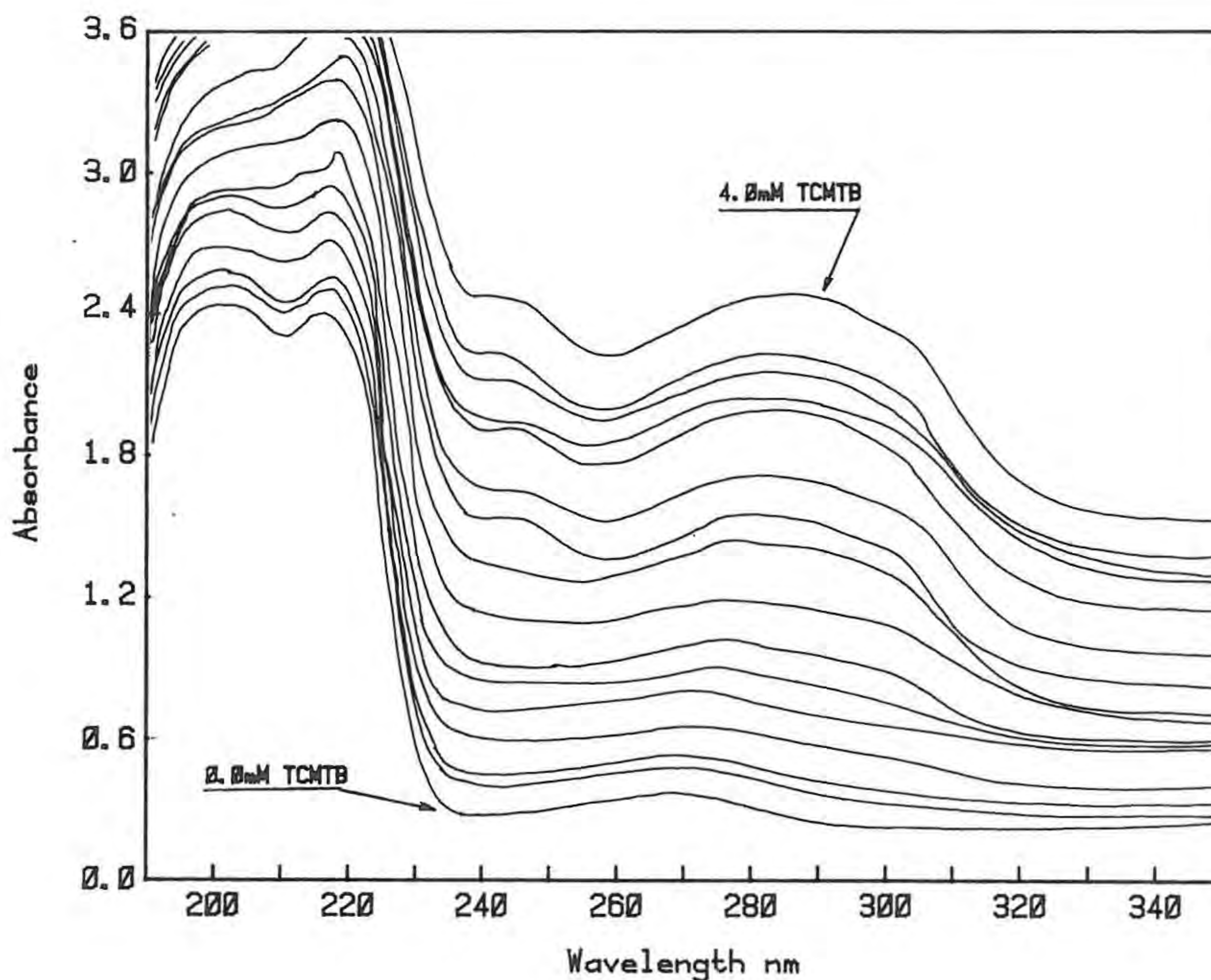

FIGURE 36 UV/Vis spectrograph for increasing concentration of TCMTB in the absence of Triton X-100 (Note: TCMTB concentration increases from 0 to 4,0mM. Spectrographs measured at 25°C

between 0,31mM and 0,45mM and can be attributed to be caused by the formation of a separate or different species in solution such as micelle formation. Unfortunately the aromatic high boiling point solvent also showed absorbance in the UV range. This is shown by the spectrum obtained even when no TCMTB was present (Figure 36). The absorbance caused by the solvent tended to mask other effects such as shifts in wavelength of absorbance maxima in the wavelength region of less than 240nm. In addition in the presence of Triton X-100 the solution became turbid and no UV/Vis spectrographs could be obtained for these mixtures.

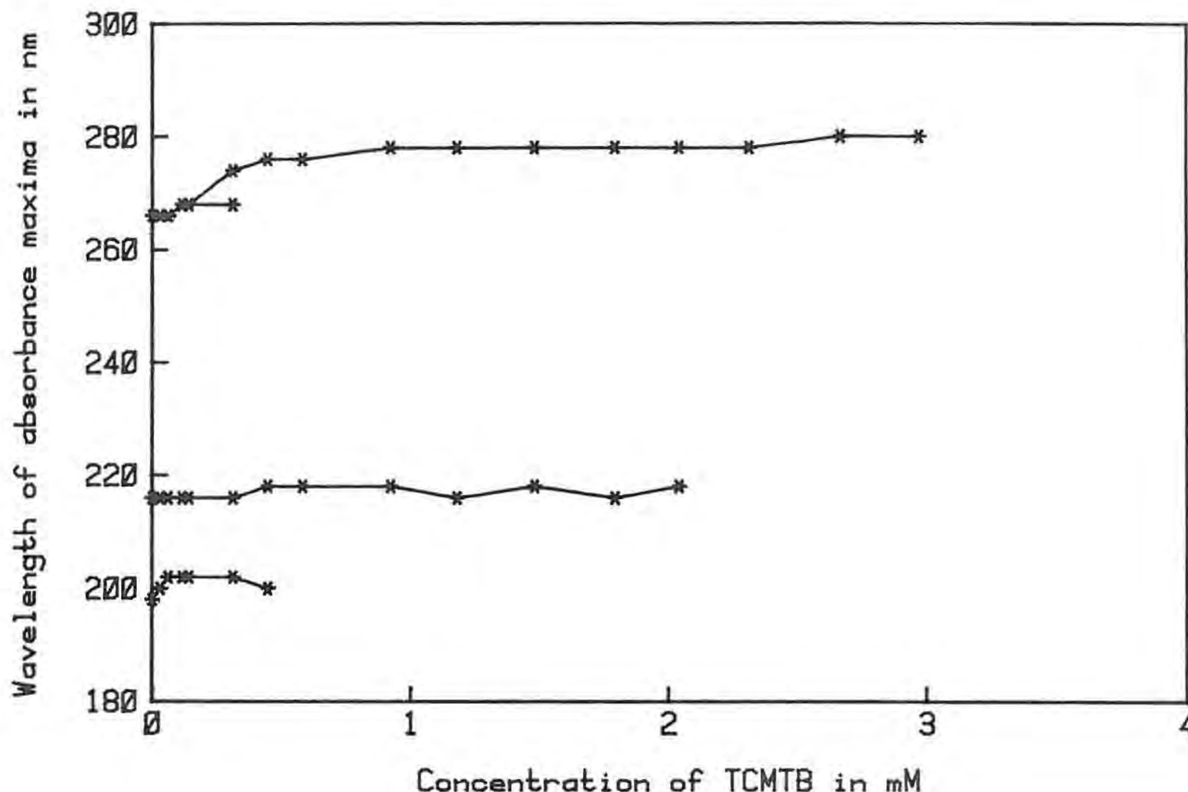

FIGURE 37 Shifts in wavelength of the absorbance maxima with increasing concentrations of TCMTB

The absorbance in the visible range at 376nm (Figure 38) showed a steady increase with no abnormalities or sudden changes in the rate of increase. The deviation from linearity, especially in the lower range between 0,2 and 0,4mM, could signify self-association and micelle formation. Due to the variation between the individual absorbance values in this low concentration range, this deviation from linearity might not be significant. Of note, however, is the fact that even in the absence of TCMTB there was still positive absorbance caused by the presence of the high boiling point aromatic solvent present in these solutions.

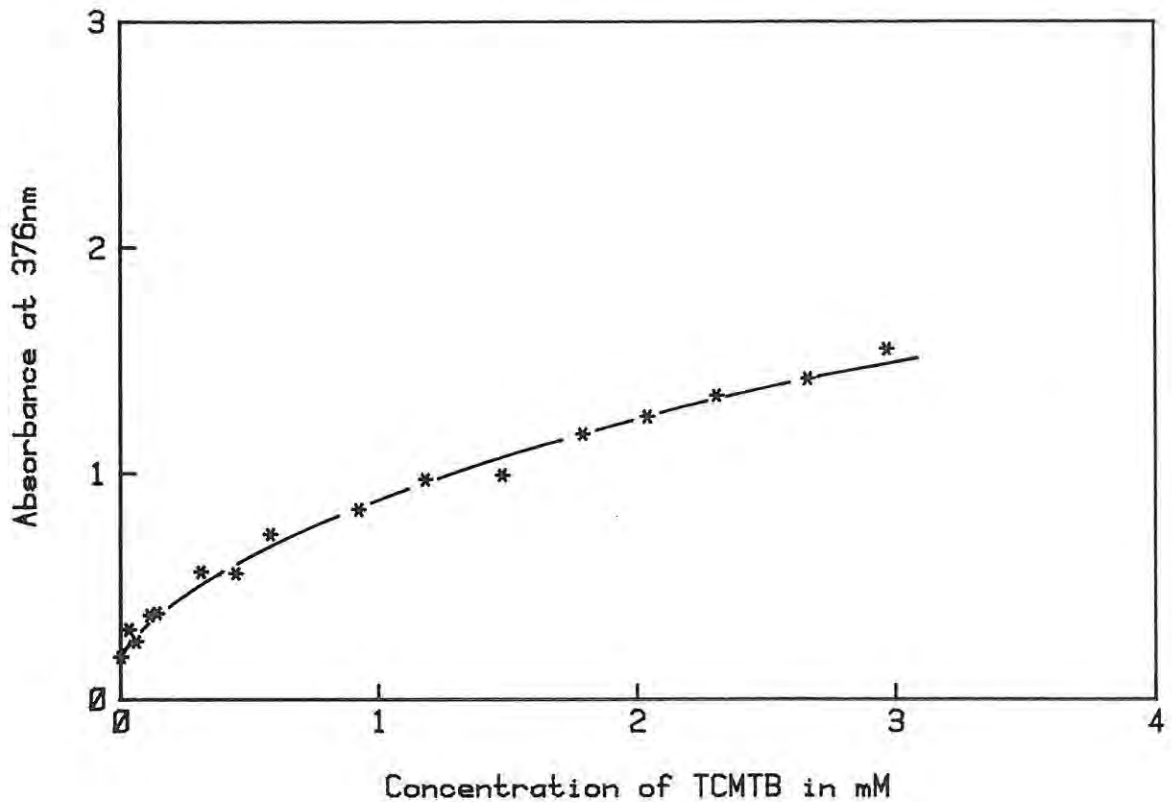

FIGURE 38 Change in absorbance at 376nm with increase in TCMTB as measured at 25°C

Viscosity measurements of solutions of TCMTB (Figure 39) show a similarity to solutions of NOITZ in that there was a sudden increase in viscosity at the higher TCMTB concentration at Triton X-100 levels of 0,80mM and 1,60mM. These increases take place at a molar ratio of TCMTB to Triton X-100 of between 2,6:1 and 2,8:1 for the 0,80mM Triton X-100 level and of between 1,6:1 and 1,9:1 for the 1,60mM Triton X-100 level, and could also indicate the formation of massive, possible rod-like, mixed micelles. The solutions containing 1,20mM Triton X-100 did not show any marked increases in viscosity. These inflexion points in the viscosity curves correspond to abnormalities in binding curves produced when TCMTB binds with bovine skin collagen as will be shown in a later chapter.

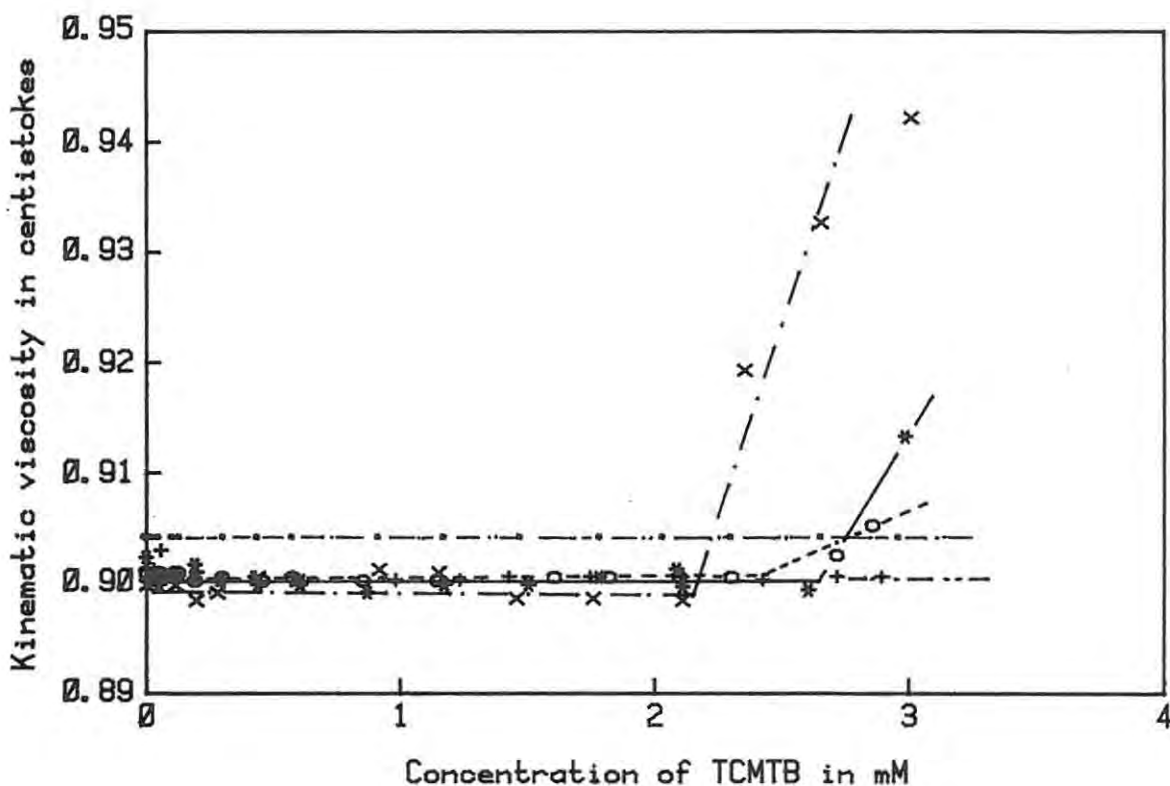

FIGURE 39 Change in viscosity with increase in TCMTB concentration as measured at 25°C (Note: —·— 0,00mM Triton X-100, +—·—+ 0,16mM Triton X-100, x—·—x 0,80mM Triton X-100, o– –o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

As in the case of NOITZ the conductivity measurements show too large a variation at the extremely low conductivity values to illustrate any significant trends.

3.2.2 Discussion

As in the case with NOITZ the solutions containing both TCMTB and Triton X-100 are complex in nature. Unfortunately the presence of the high boiling point aromatic solvent complicated the results and only in the case of the viscosity measurements were there any indications of the formation of large mixed micelles. In the binding experiments to be

reported in a later chapter there were also similar abnormalities in the binding isotherms which indicated large cooperative type binding. There was also a sudden drop in free surfactant concentrations in solution indicating that the Triton X-100 was also binding to the collagen. This observation could be caused by mixed micelles being formed and the mixed micelles binding as single ligands to individual binding sites on the collagen.

CHAPTER 4

BINDING INTERACTIONS OF n-OCTYL-4-ISOTHIAZOL-3-ONE WITH COLLAGEN (HIDE POWDERS)

CHAPTER 4

BINDING INTERACTIONS OF n-OCTYL-4-ISOTHIAZOL-3-ONE WITH COLLAGEN (HIDE POWDERS)

4.1 Analyses of Hide Powders

The results of the hide powder analyses are shown in Table 1. The values found for the limed hide powder were the same as reported by British Leather Manufacturers Research Association. The analytical values found for the chromed hide powder are similar to other analyses of batches reported in the literature (Atto and Nursten, 1971a; 1971b). These figures show that:-

(i) Using a factor of 5,62 for the conversion of total nitrogen to collagen that the collagen content on a dry basis is 90% for both hide powders.

(ii) The moisture contents of the hide powders were 16% for chromed and 19% for limed hide powder. These moisture contents were taken into consideration in all mass measurements.

(iii) The natural pH for chromed hide powder was 3,9 and for limed hide powder 6,3. In the experiments using limed hide powder the pH of the solution was adjusted to 4,0 with hydrochloric acid. The experiments using lightly chromed hide powder were carried out without any pH adjustments being made.

(iv) The chrome content of the chromed hide powder (0,32%) is lower than reported by Atto and Nursten (1971a) from their studies on the reaction of dyes with chromed hide powder. The chrome content is much lower than found in full-chrome leather.

(v) The ash content of the chromed hide powder was slightly higher than that of the limed hide powder. This is to be expected due to the presence of chrome salts.

TABLE 1 ANALYSIS OF HIDE POWDER

	CHROMED	LIMED
Ash (%) (dry basis)	0,76	0,26
Chrome as Cr (%) (dry basis)	0,32	-
Nitrogen expressed as N (%) (dry basis)	16,1	16,4
Nitrogen expressed as Collagen(%) (dry basis)	90,5	92,1
Moisture (%)	16	19
pH (25°C)	3,9	6,3

4.2 pH, Nitrogen and Chrome Determinations

The final pH of all the solutions at the end of the 24 hour binding period did not vary significantly and no trend either with increase in the concentrations of NOITZ or Triton X-100 could be discerned. All the solutions in the limed hide powder experiment had final pH values of 4,0 ± 0,1 while those in the chromed hide powder experiment had values of 3,8 ± 0,1.

Nitrogen determination on the blank solutions during the limed hide powder experiments showed a mean nitrogen concentration of 4mg/ℓ representing 1,2mg hide powder. On the filtered solutions during the experiment using chromed hide powder the mean nitrogen concentration was 12mg/ℓ representing 3,7mg of chromed hide powder. Chrome determinations showed that these latter solutions contained 0,23mg/ℓ chrome which represented 3,5mg of chromed hide powder, thus confirming the nitrogen results. These quantities of dissolved hide powder were taken into account when calculating the free and bound ligand during the binding experiments.

4.3 Variation in Float Residual NOITZ and Triton X-100 with time.

The time course measurements of free ligand NOITZ in the various treatments are depicted in log/linear form in Figures 40 and 41. They show that for both limed and lightly chromed hide powder there was an initial rapid drop in free ligand concentration with equilibruim values

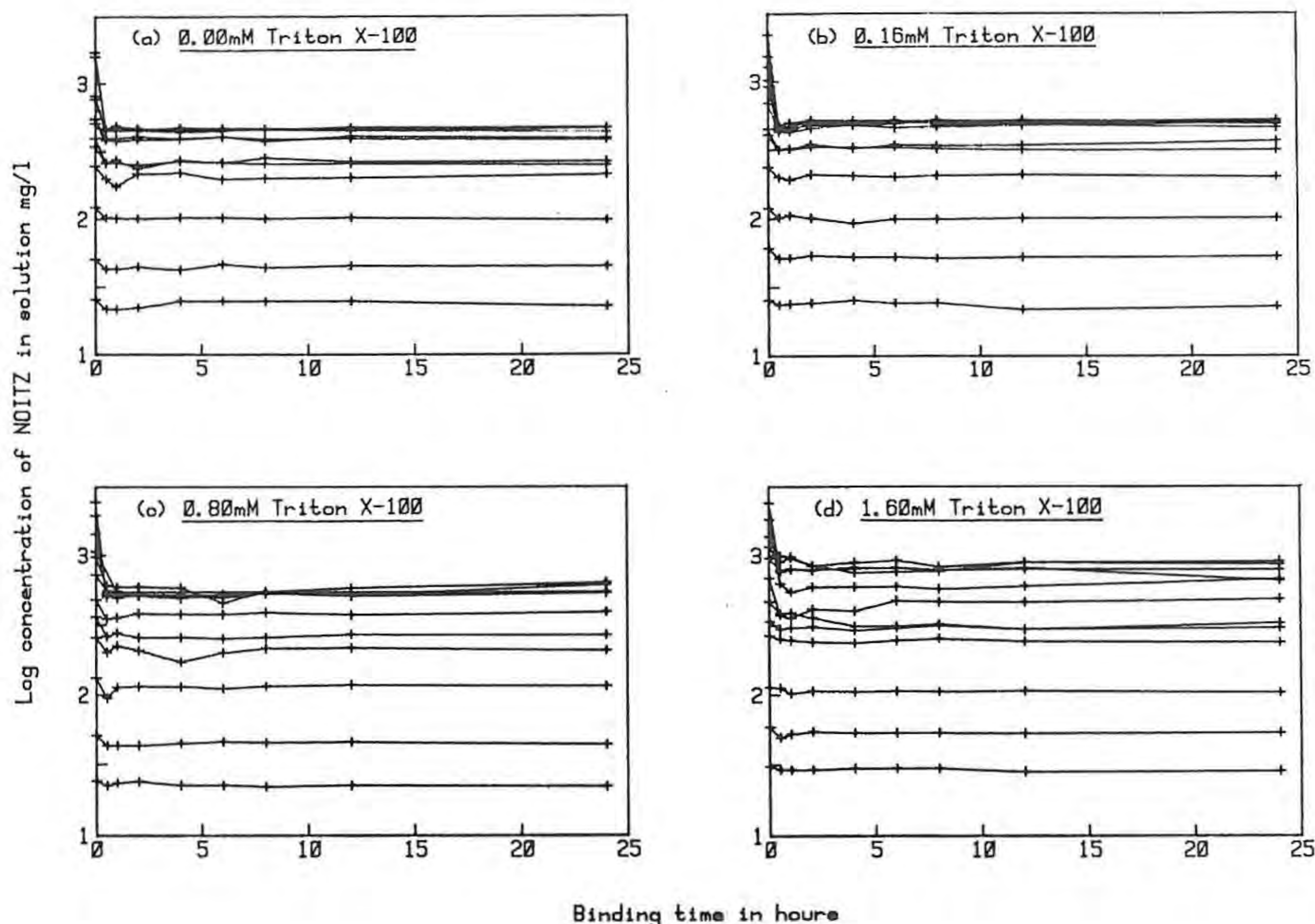

FIGURE 40 Free ligand (NOITZ) in solution during binding experiments using limed hide powder (Note: At twelve different initial concentrations of NOITZ)

being approached within the first half hour for the majority of the treatments at the lower surfactant concentrations. In the treatments with higher surfactant and ligand concentrations the initial drop in free ligand concentration was not as rapid. Equilibrium was however reached within six hours. Thus most of the binding took place within a relatively short period, however, due to the role played by the surfactant, especially in the higher concentration range, equilibrium was only reached after several hours. Although there was still a small variation in concentration measured between twelve and twenty four hours, the differences were within the limits of accuracy for the analytical procedure. It can thus be assumed that the concentration of free ligand NOITZ after twenty four hours was a true representation of

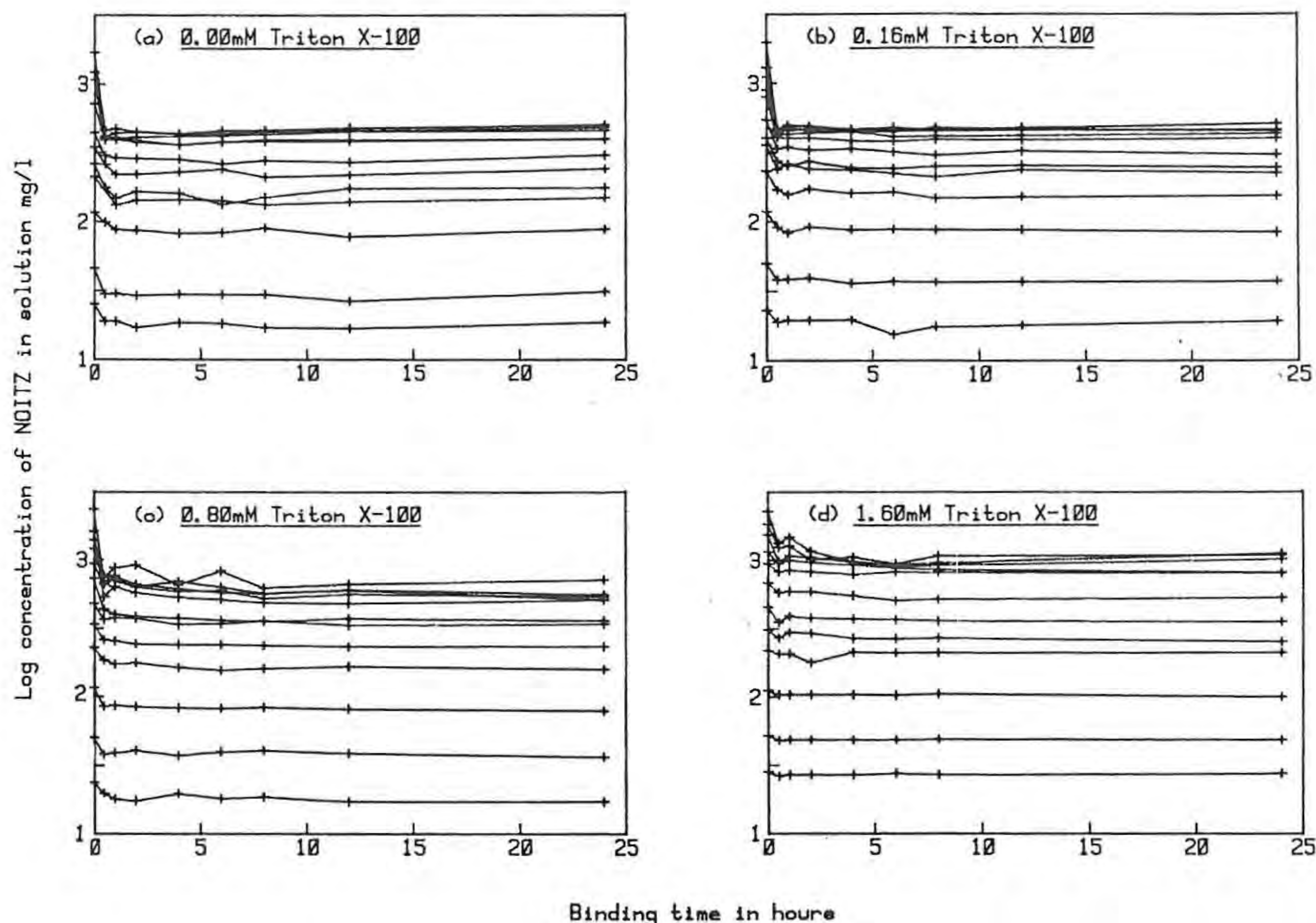

FIGURE 41 Free ligand (NOITZ) in solution during binding experiments using to chromed hide powder (Note: At twelve different initial concentration of NOITZ)

the equilibruim value. This was despite the fact that various workers have shown for certain systems that it requires long periods of time to reach equilibrium. Sukow et al (1980) found that in the binding of Triton X-100 to bovine serum albumin that time course measurements indicated equilibrium only after thirty six to forty eight hours. Attwood and Florence (1983) report that various workers found that the time required varies from hours to months for solubilizates in surfactant systems to reach equilibrium.

The original and final concentrations of both NOITZ and Triton X-100 are given in Tables 2 and 3. The tables show that not only was there a decrease in the NOITZ concentration, but also in that of Triton X-100,

TABLE 2 BINDING OF NOITZ TO LIMED HIDE POWDER AT 25°C

		CONCENTRATION OF FREE LIGAND mg/ℓ											
NOITZ	ORIGINAL	25,4	46,0	116	211	261	348	440	713	721	1 067	1 205	1 674
	FINAL	18,6	31,0	88,4	149	176	242	302	395	399	456	481	502
TRITON X-100	ORIGINAL	0	0	0	0	0	0	0	0	0	0	0	0
	FINAL	0	0	0	0	0	0	0	0	0	0	0	0
NOITZ	ORIGINAL	22,9	50,0	118	232	356	402	539	792	892	1 103	1 293	1 963
	FINAL	19,4	37,5	84,1	154	226	246	306	401	434	455	460	510
TRITON X-100	ORIGINAL	100	100	100	100	100	100	100	100	100	100	100	100
	FINAL	93	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
NOITZ	ORIGINAL	23,5	50,2	117	229	338	479	730	938	1 202	1 396	1 613	2 349
	FINAL	17,0	36,4	79,0	159	234	341	361	560	572	544	511	720
TRITON X-100	ORIGINAL	500	500	500	500	500	500	500	500	500	500	500	500
	FINAL	296	385	185	280	257	389	114	335	207	138	55	104
NOITZ	ORIGINAL	21,6	48,5	109	219	332	464	735	887	1 117	1 255	1 746	2 148
	FINAL	19,7	44,5	103	210	286	386	628	698	776	842	1 053	1 173
TRITON X-100	ORIGINAL	720	790	706	730	734	756	708	698	812	702	782	748
	FINAL	707	800	636	627	720	740	680	680	720	507	493	542
NOITZ	ORIGINAL	28,2	52,2	112	219	309	456	685	947	1 148	1 533	1 842	2 272
	FINAL	27,6	48,6	101	211	254	355	534	806	814	1 020	1 089	1 124
TRITON X-100	ORIGINAL	1 040	1 000	972	976	984	1 086	970	980	976	990	1 002	966
	FINAL	1 120	1 120	1 040	1 040	1 120	960	880	1 040	1 000	840	760	558

TABLE 3 BINDING OF NOITZ TO CHROMED HIDE POWDER AT 25°C

		CONCENTRATION OF FREE LIGAND mg/ℓ											
NOITZ	ORIGINAL	25,6	50,6	121	245	339	394	508	548	765	811	1 551	1 683
	FINAL	22,8	45,0	98,7	212	248	264	383	397	437	468	474	472
TRITON X-100	ORIGINAL	0	0	0	0	0	0	0	0	0	0	0	0
	FINAL	0	0	0	0	0	0	0	0	0	0	0	0
NOITZ	ORIGINAL	25,4	61,1	119	236	408	452	698	920	1 021	1 271	1 517	2 171
	FINAL	22,7	52,6	101	199	313	366	457	504	506	524	486	498
TRITON X-100	ORIGINAL	108	92	92	92	104	108	102	104	98	108	114	102
	FINAL	93	75	56	52	56	34	< 20	<20	< 20	< 20	< 20	< 20
NOITZ	ORIGINAL	24,0	51,0	113	257	361	477	725	974	1 066	1 423	1 938	2 429
	FINAL	22,0	44,0	116	209	269	392	537	635	543	541	535	614
TRITON X-100	ORIGINAL	526	492	486	476	522	544	500	522	500	528	510	632
	FINAL	502	475	475	391	390	446	422	327	158	149	59	59
NOITZ	ORIGINAL	28,7	55,0	119	247	350	523	706	949	1 164	1 555	1 910	2 331
	FINAL	27,8	45,3	97,5	196	248	388	570	761	851	645	743	572
TRITON X-100	ORIGINAL	782	750	940	746	708	700	760	806	816	700	790	716
	FINAL	787	598	1 038	788	566	472	582	614	714	189	260	118
NOITZ	ORIGINAL	30,6	58,7	114	267	338	467	688	954	1 156	1 372	1 825	2 405
	FINAL	28,4	53,5	104	239	305	329	486	793	910	863	678	662
TRITON X-100	ORIGINAL	954	976	1 174	984	920	958	940	1 132	996	954	936	1 080
	FINAL	967	1 016	1 204	981	892	558	619	1 115	991	768	281	223

especially at the higher NOITZ values. This indicated that both NOITZ and the surfactant Triton X-100 were binding simultaneously onto the hide powder, possibly in the form of a complex mixed micelle. No drop in Triton X-100 concentration in the absence of NOITZ was measured indicating, as has been previously shown (Sukow and Sandberg, 1974; Sukow et al, 1980) that the surfactant Triton X-100 does not bind onto collagen to any appreciable degree. This drop in the surfactant concentration in solution was more apparent in the case of the chromed hide powder illustrating the greater affinity of the mixed fungicide/surfactant micelle for the chromed hide powder collagen fibres than for the untanned collagen. Similar results on the increased affinity of certain dyes for chromed hide powder with increase in chrome content have also been reported (Atto et al, 1969; Atto and Nursten, 1971a; 1971b)

4.4 Binding Isotherms

4.4.1 Limed Hide Powder

The binding isotherms are illustrated in Figures 42 and 43. In Figure 42 the isotherms are shown using different scales for the x- and y-axes for the experiments at different surfactant concentrations in order to emphasise the shape of the individual binding isotherm curves. In Figure 43 the same five isotherms are drawn to the same scale in order to demonstrate any differences between the isotherms.

Other than at the lower biocide concentration levels, the binding curves did not follow the normal mass-action binding type isotherms approaching a definite saturation limit, but continued to show increased ligand binding with increasing concentration of the biocide. When there was no surfactant present or when the surfactant concentration was below the CMC of Triton X-100 of between 0,2 and 0,25mM (Yedgar et al, 1974; Masullo et al 1986), the concentration of free biocide in solution reached a constant value of approximately 2,2mM which was well below its published water solubility level of 3,3mM (Levy et al, 1974) and then remained fairly constant. Increasing the amount of surfactant progressively reduced the initial fungicide uptake, thereby increasing the quantity of free ligand in solution to beyond the normal solubility

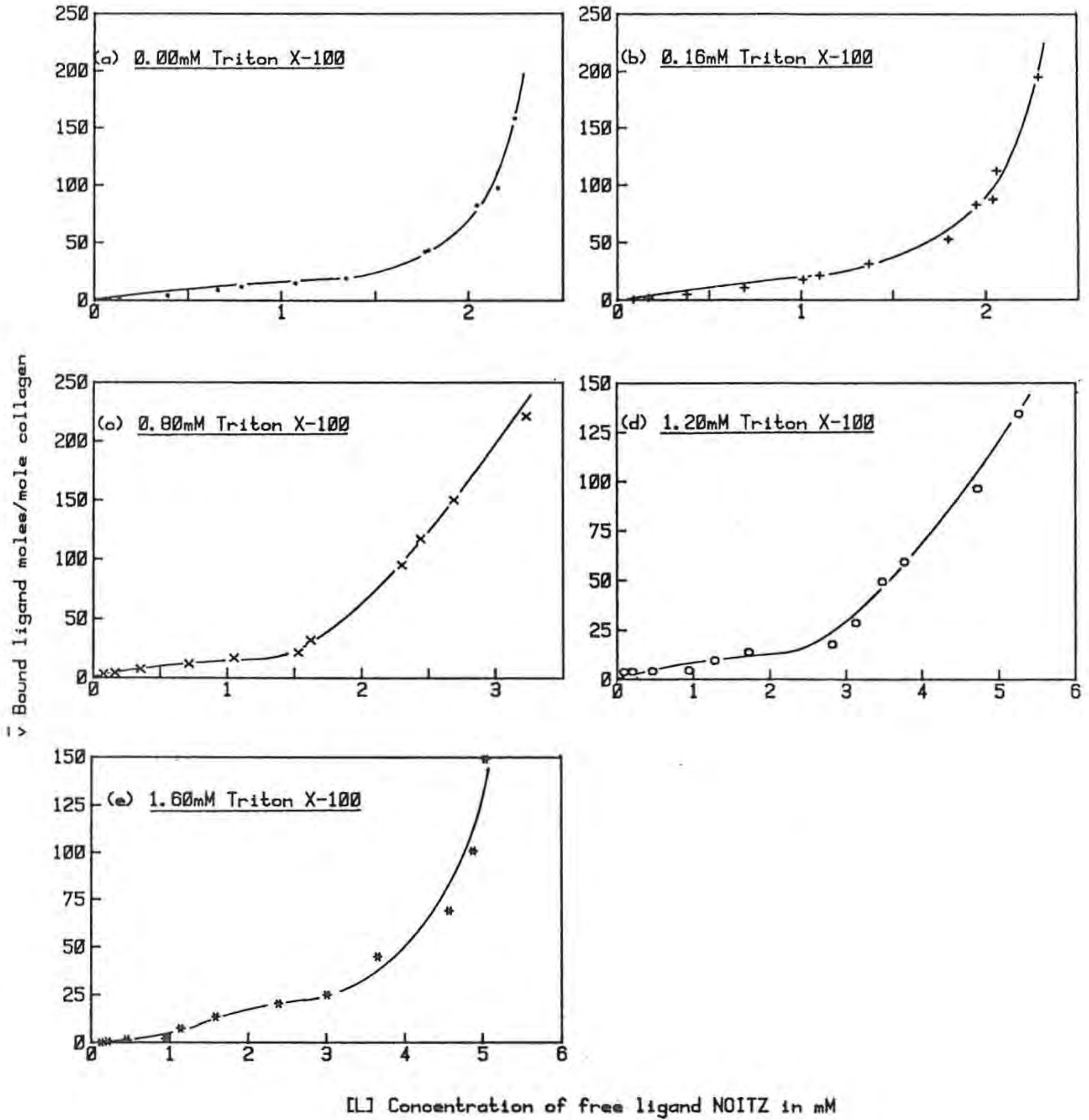

FIGURE 42 Individual binding isotherms for NOITZ with limed hide powder at 25°C (Note: The scale of the x- and y-axes vary in order to emphasise the shape of each of the individual binding isotherms)

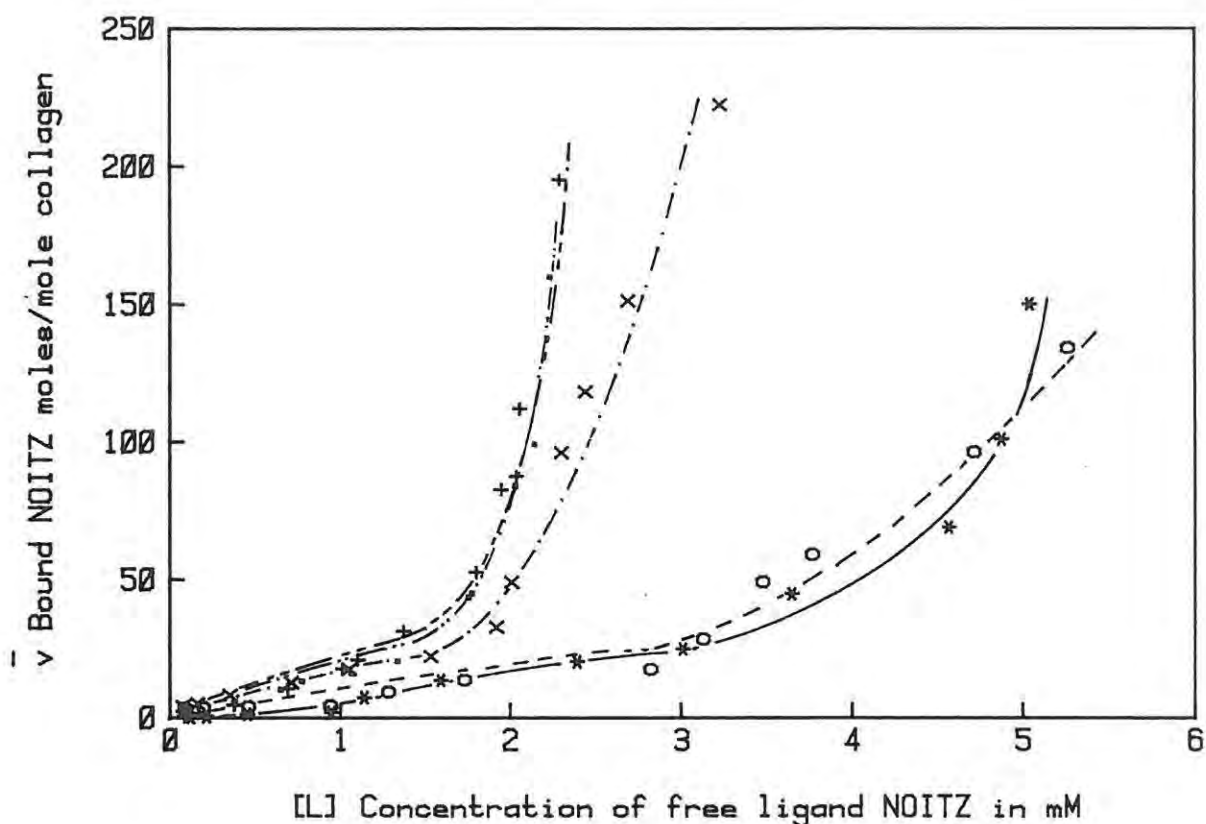

FIGURE 43 Binding isotherms for NOITZ with limed hide powder at 25°C (Note: .—..—. 0,00mM Triton X-100, +—---+ 0,16mM Triton X-100, x—.—x 0,80mM Triton X-100, o— — —o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

limit. The binding isotherms were thus markedly dependent on surfactant concentrations. Similar curves for cooperative type of binding in the presence of surfactants have been reported in the literature. Hiramatsu et al (1977) in the interaction of BPA attributes the cooperative effect to the unfolding of the BPA molecule whereas Kresheck et al (1982) concludes that cooperative type curves point to evidence of the formation of mixed surfactant phospholipid micelles after CMC is reached. Sato et al (1988) in studies on the multiple binding of bilirubin to human serum albumin and cobinding of bilirubin and laurate showed that more than two molecules of either ligand can be bound at the same time and found that saturation levels were not reached.

These forms of binding curves however, preclude the use of Klotz-type isotherms (Klotz, 1974; 1985), or modifications thereof, as a valid

representation of the more common types of binding. In the binding of NOITZ to collagen in the presence of Triton X-100, this type of binding behaviour can be attributed to complexes involving more than one molecule binding to any one site. Although the curves showed an increase in binding with increase in fungicide concentration there was a sharp increase in the rate of binding at fungicide to surfactant ratios of approximately 2,8:1; 2,3:1 and 2,3:1 for Triton X-100 concentrations of 0,80; 1,20 and 1,60mM respectively. These ratios corresponded closely to those for the increase in viscosity and the decrease in turbidity of 3,0:1; 2,3:1 and 2,3:1 at 25°C as reported in the previous chapter thus indicating the formation of a large mixed micelle binding to the collagen fibre.

4.4.2. Chromed Hide Powder

The binding isotherms are illustrated in Figures 44 and 45. In Figure 44 the isotherms are again shown using different scales for the x- and y-axes to show the shape of the individual curves whereas in Figure 45 all five isotherms are drawn to the same scale in order to demonstrate any differences between the isotherms.

As found when using limed hide powder the binding curves did not follow normal mass-action type isotherms other than at the lower fungicide concentration levels. The binding affinity also continued to increase with increase in fungicide concentration.

Increasing the amount of Triton X-100 increased the level of free ligand in solution as expected. However, after a certain critical concentration there was a sudden increase in binding accompanied by a drop in free ligand concentration. As is shown in Table 2, this was accompanied by a drop in the solution surfactant concentration. This drop in free ligand concentration occurred at NOITZ to Triton X-100 ratios of approximately 3,0:1; 2,9:1 and 2,5:1 for Triton X-100 levels of 0,80; 1,20 and 1,60mM respectively. This again approximates the ratios at which viscosity increases and turbidity decreases and can be attributed to the binding of a large mixed micelle complex onto the chrome collagen surface. (The exact nature of these mixed micelles falls outside the scope of this thesis).

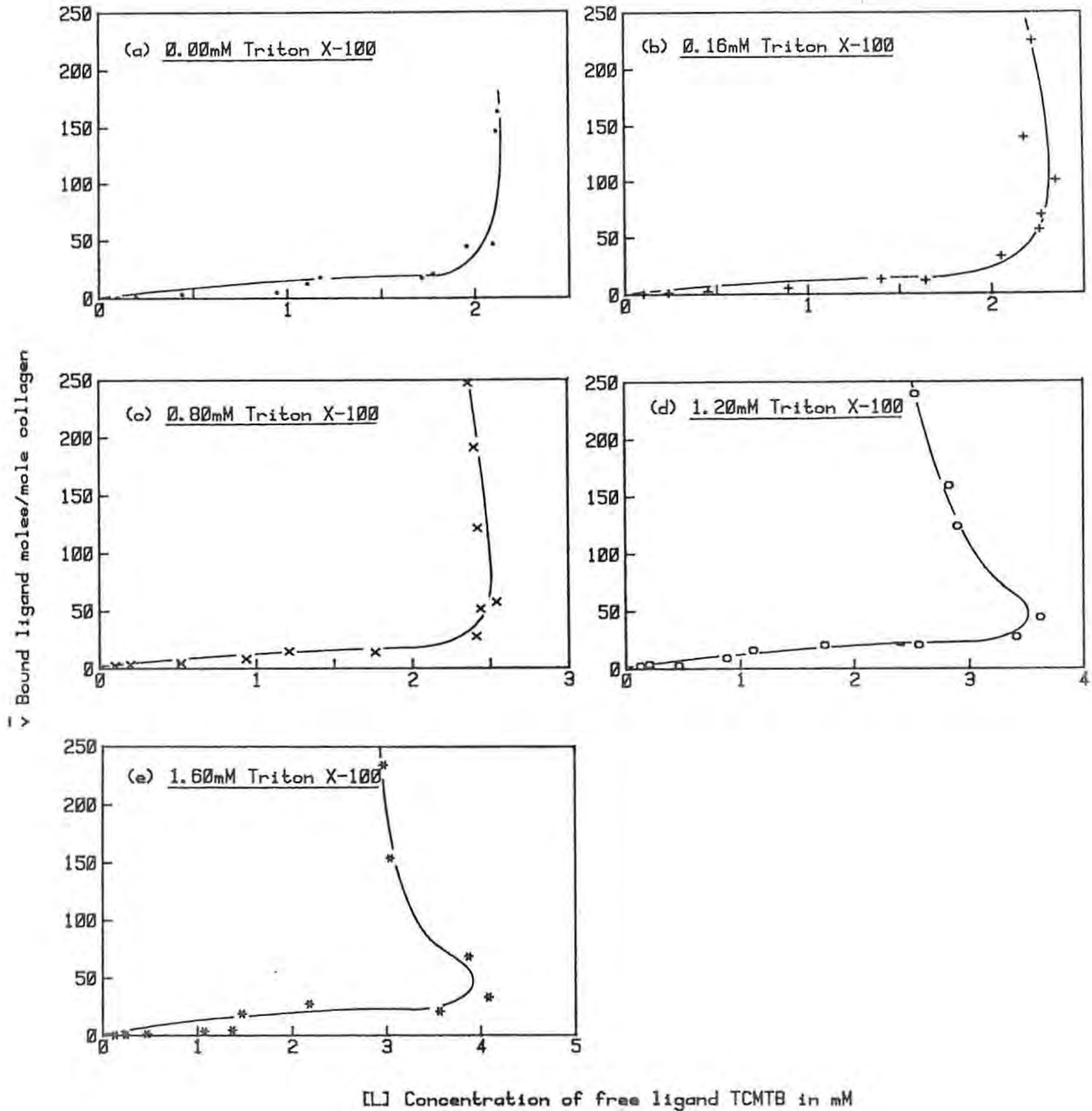

FIGURE 44 Individual binding isotherms for NOITZ on chromed hide powder at 25°C (Note: The scale of the x- and y-axes vary in order to emphasise the shape of each of the individual binding isotherms)

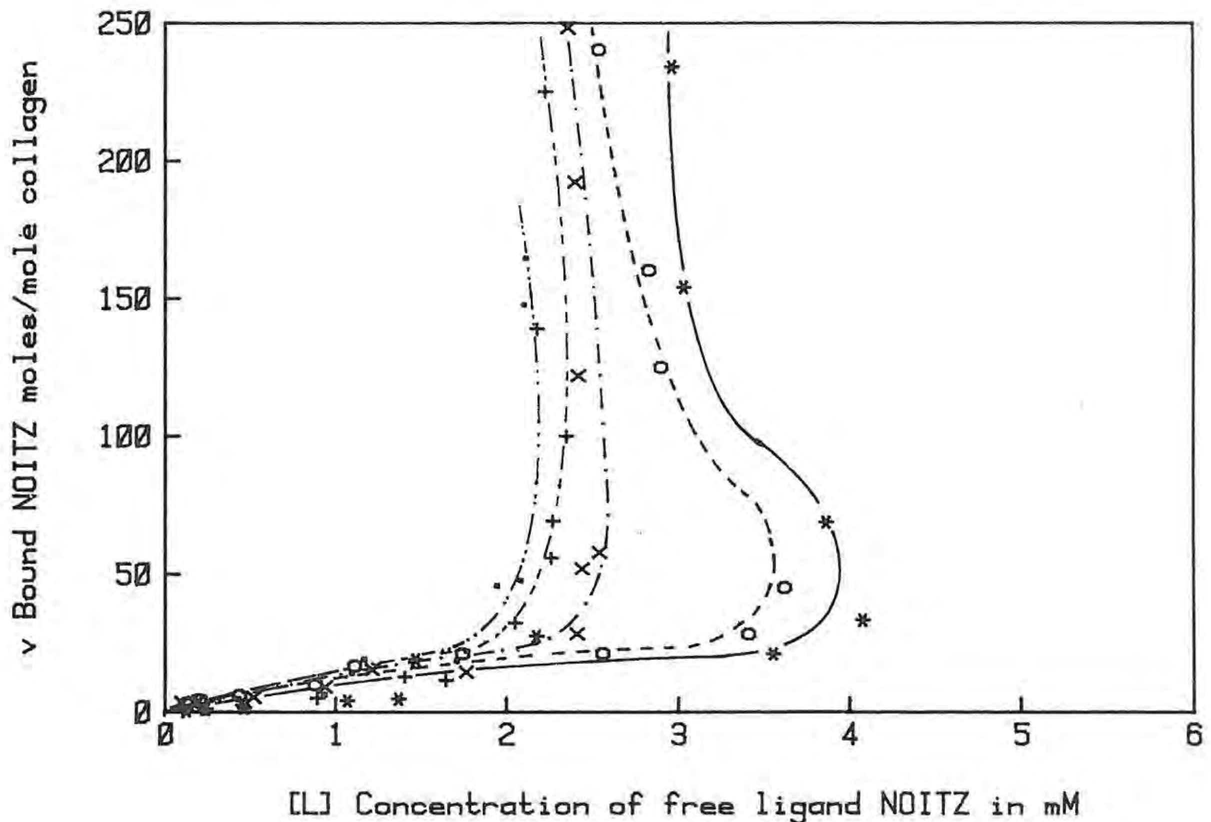

FIGURE 45 Binding isotherms for NOITZ with chromed hide powder at 25°C (Note: .—..— 0,00mM Triton X-100, +—..—+ 0,16mM Triton X-100, x—.—x 0,80mM Triton X-100, o---o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

4.5 Scatchard Plots

A Scatchard plot as an alternate method of representing binding data are also shown. In Figure 46, the Scatchard plots for the binding of NOITZ onto limed hide powder and, in Figure 47, onto lightly chromed hide powder are shown. In all cases the plots have positive slopes. Although the form of these curves precludes their use as a valid representation of the more common types of binding, the positive slopes do however indicate an increase in binding with increase in concentration consistent with a co-operative effect (Sukow et al, 1980). This is most probably due to the formation of combined

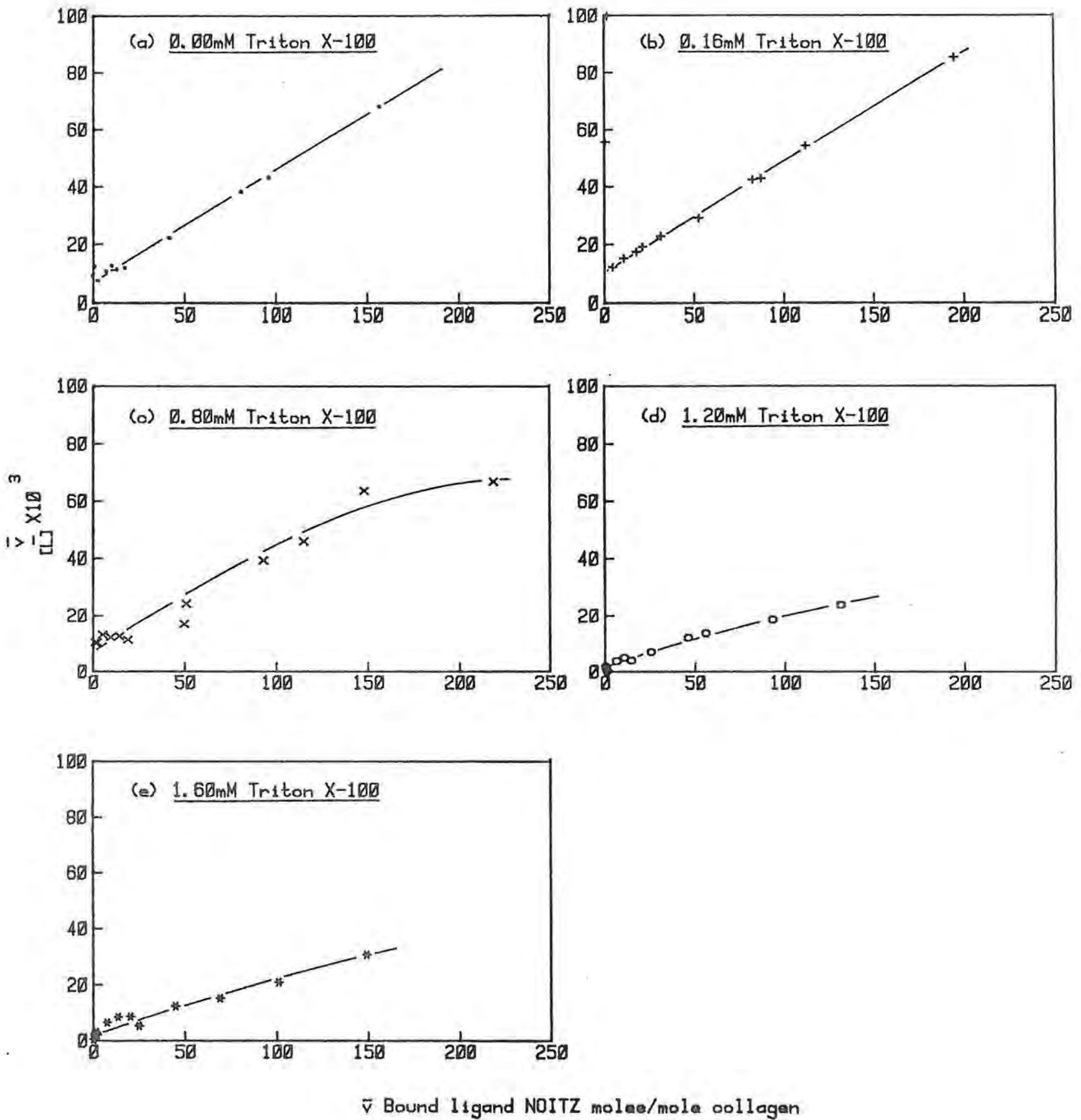

FIGURE 46 Scatchard plots of binding of NOITZ to limed hide powder at 25°C

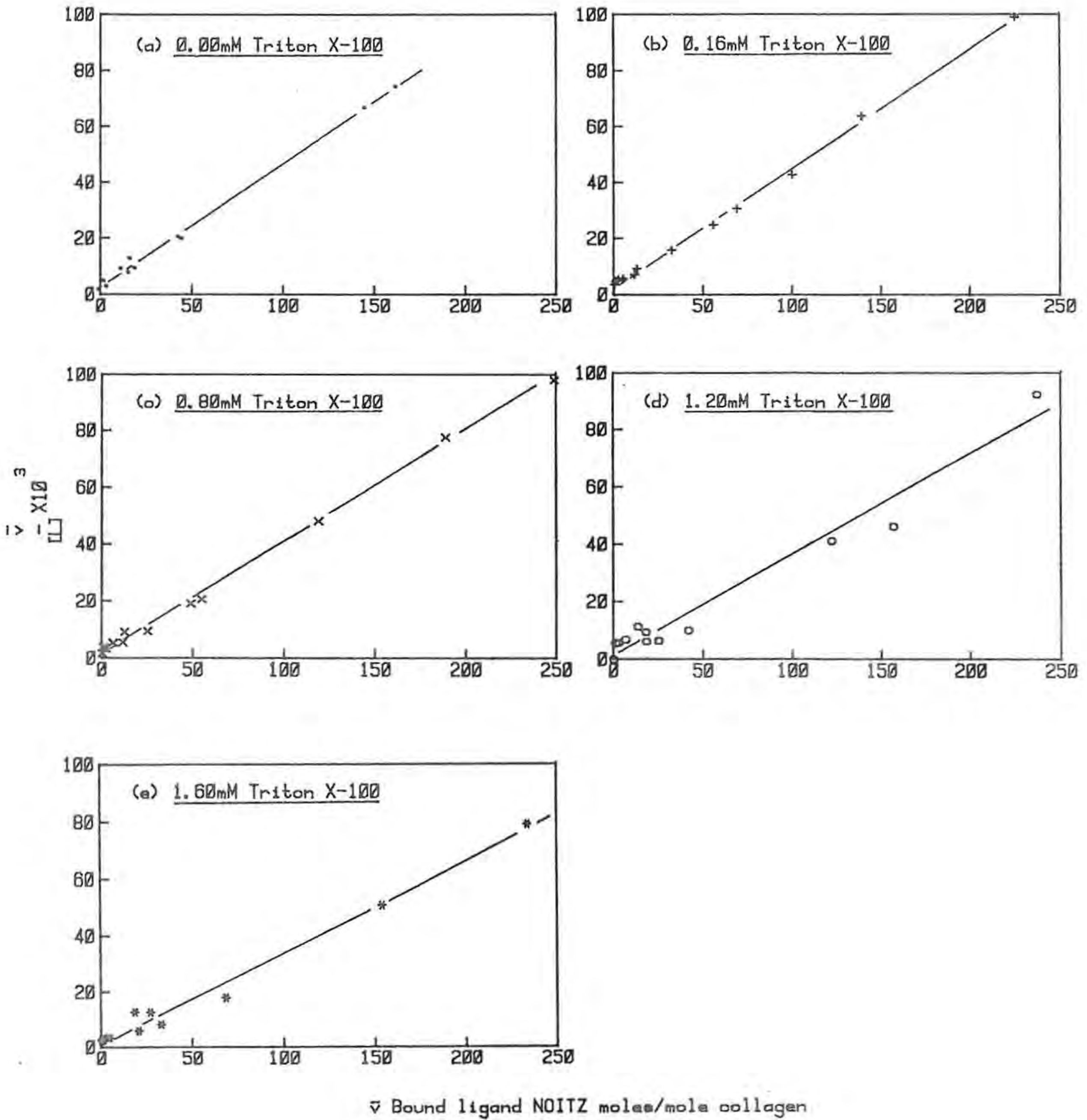

FIGURE 47 Scatchard plots of binding of NOITZ to chromed hide powder at 25°C

fungicide/surfactant micelles with increased hydrophobicity. The binding of these mixed micelles results in additional hydrophobic areas being formed on the collagen surface and further association of additional mixed micelles onto these hydrophobic areas on the hide powder surface. Alternatively it could be due to the binding of larger mixed micelles onto the limited number of binding sites.

The slopes of the binding curves for limed hide powder were slightly less than those for the chromed hide powder indicating that these bonds are promoted by the presence of chrome. This was especially true at the higher surfactant concentrations.

4.6 Conclusions

It was thus not possible to obtain any detailed information relating to the magnitude of the association constants for the interactions between NOITZ and skin collagen. The binding capacity of hide powder collagen for these fungicide ligands appears to be extremely large. In the case of NOITZ when the free ligand concentration was 2,5mM the number of ligand molecules bound to a collagen molecule was approximately 150.

The implications are that the binding is relatively non-specific. The binding of these ligands to the collagen fibres is not dependent on any particular amino acid sequence or protein configuration at the binding site. It is thus very likely that the binding of these ligands to collagen is by hydrophobic bonds involving large possibly rod-shaped mixed NOITZ/Triton X-100 micelles in the regions of the apolar side chain residues which are present in collagen.

CHAPTER 5

BINDING INTERACTIONS OF 2-(THIOCYANOMETHYL)BENZOTHIAZOLE WITH COLLAGEN (HIDE POWDERS)

CHAPTER 5

BINDING INTERACTIONS OF 2-(THIOCYANOMETHYLTHIO)BENZOTHIAZOLE WITH COLLAGEN (HIDE POWDERS)

5.1 Analysis of Bulab 1077 for TCMTB

Five solutions ranging from 0,01 to 0,50% of the commercial product Bulab 1077 were analysed by means of HPLC for TCMTB content by injecting directly onto the reverse phase column (Techsil C18) and also by first extracting into dichloromethane and injecting onto the Waters Radial-PAK B column. The quantities of TCMTB detected in the sample of Bulab 1077 are shown in Table 4.

TABLE 4 % TCMTB IN SAMPLE OF BULAB 1077

	% TCMTB Detected	
Original Bulab 1077 %	Techsil C18	Radial-PAK B
0,010	28,4	36,7
0,025	36,7	33,8
0,050	38,4	34,8
0,100	37,1	34,4
0,250	36,3	35,4
0,500	33,6	35,0
Mean	35,1	34,9
Standard Deviation	3,6	1,1

Although the percentage of TCMTB found for the sample of Bulab 1077 was slightly lower after extraction into dichloromethane and determination using the Waters Radial-PAK B column the standard deviation over the range measured was less than when using the Techsil C18 reverse phase column. Previous work has shown that more than 99% of TCMTB is extracted from aqueous solutions using dichloromethane (Fowler et al, 1987). The mean amount of TCMTB in the sample of Bulab 1077 using both methods was 35,0 ± 2,5.

5.2 pH Determinations

The final pH of all the solutions at the end of the 24 hour binding period did not vary significantly and no trend with increase in either TCMTB or Triton X-100 could be discerned. All of the solutions had final pH values of 4,0 ± 0,2. Due to the difficulty experienced in filtering the samples and the small amounts of filtrate recovered the solutions in these experiments were not analysed for chrome and nitrogen content.

5.3 Variation in Float Residual TCMTB and Triton X-100 with Time

For the experiments using limed hide powder, the experiments with initial Triton X-100 concentrations of 0,00mM and 0,80mM were sampled and analysed after binding for 1/2, 1, 3, 6, 12 and 24 hours. For the experiment using limed hide powder and 1,60mM initial Triton X-100 concentrations samples were taken and analysed after binding for 6, 12 and 24 hours. For the rest of the experiments analyses were only carried out on the samples taken after 24 hours of binding. In the case of the experiments using chromed hide powder difficulty was experienced during the filtration of the samples. The 24 hour samples were the only samples that had a large enough volume for dichloromethane extraction and HPLC determination of TCMTB.

The variation in free ligand (TCMTB) concentration with time for the four sets of experiments are depicted in semilog plot (Figure 48) and show that:

i) In the Triton X-100 free solutions using limed hide powder there is an initial, rapid drop in free ligand concentration, contrary to the classical type of binding where a steady exponential decrease would be expected. The concentration of free ligand was initially lower in the first few hours, followed by a slight increase before finally stabilising at a fairly constant equilibrium value. A similar drop in free ligand TCMTB concentration was found when conducting experiments on the binding of TCMTB in the commercial

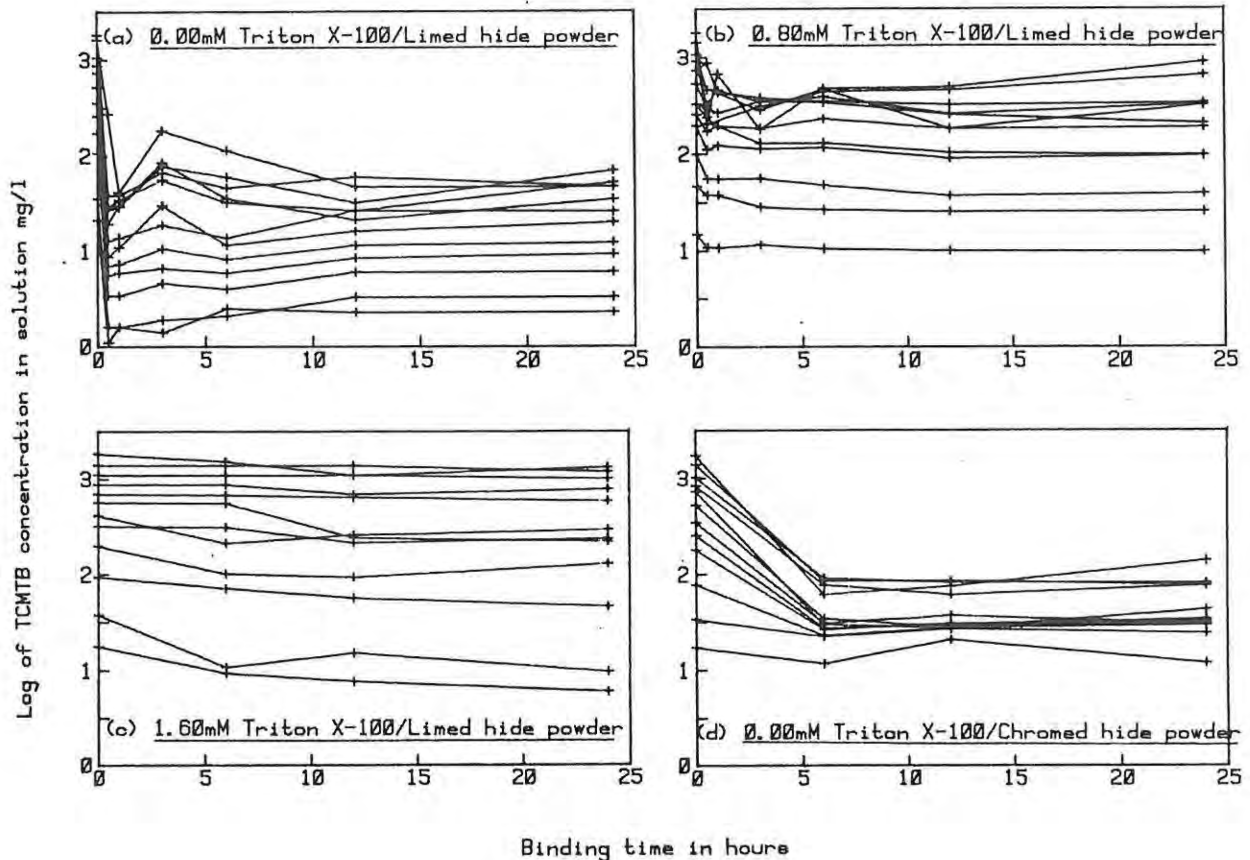

FIGURE 48 Free ligand (TCMTB) in solution during binding experiments at twelve different initial concentrations of TCMTB

formulation Busan 30L to hide powders at various temperatures (Fowler et al, 1990) and was attributed to the binding of associated molecules to the hide powder collagen which subsequently dissociated releasing ligand back into the solution. As the Triton X-100 free experiment of the binding of TCMTB to chromed hide powder was sampled only after 6 hours the above trend was not observed. When Triton X-100 was present in the original solution at 0,80mM only the higher concentrations of ligand showed a similar trend while at 1,60mM Triton X-100 concentration there was regular decrease in free ligand concentration with time. These trends seem to indicate that the TCMTB molecule is self-associating but this interaction decreases as the concentration of the surfactant is increased.

TABLE 5 BINDING OF TCMTB TO LIMED HIDE POWDER AT 25°C

		CONCENTRATION OF FREE LIGAND mg/ℓ											
TCMTB	ORIGINAL	21,3	35,7	105	169	257	351	512	720	862	1 043	1 598	1 798
	FINAL	2,3	3,3	6,0	9,3	12,3	20,0	26,0	34,8	52,0	68,6	46,8	46,8
TRITON X-100	ORIGINAL	0	0	0	0	0	0	0	0	0	0	0	0
	FINAL	0	0	0	0	0	0	0	0	0	0	0	0
TCMTB	ORIGINAL	17,2	64,1	95,2	168	235	378	557	767	892	1 018	1 390	1 773
	FINAL	3,7	*26,2	20,2	51,4	78,5	78,5	*258	91,6	*311	90,8	206	361
TRITON X-100	ORIGINAL	96	*192	124	92	102	96	*224	100	*262	98	124	104
	FINAL	96	192	-	91	106	91	234	96	260	91	-	-
TCMTB	ORIGINAL	14,7	46,2	99,1	195	255	326	529	722	908	1 064	1 414	1 776
	FINAL	9,8	25,4	41,5	95,0	96,3	186	204	314	323	333	649	885
TRITON X-100	ORIGINAL	552	498	514	492	510	498	510	532	518	510	512	472
	FINAL	425	225	250	281	437	350	-	-	-	-	-	-
TCMTB	ORIGINAL	21,1	37,5	101	207	288	381	517	698	877	1 045	1 371	1 716
	FINAL	19,8	33,3	77,1	139	250	266	318	488	451	432	823	1 150
TRITON X-100	ORIGINAL	740	802	734	734	772	776	760	758	746	746	770	744
	FINAL	713	750	563	563	712	675	-	-	-	-	-	-
TCMTB	ORIGINAL	17,7	37,8	93,5	195	315	408	562	690	877	1 104	1 394	1 819
	FINAL	6,0	10,0	46,4	93,9	236	291	457	586	787	1 005	1 192	1 326
TRITON X-100	ORIGINAL	992	1 170	1 022	1 014	1 118	1 180	1 004	1 028	1 000	1 204	1 084	1 064
	FINAL	770	1 209	1 060	385	-	-	-	-	-	-	-	-

* Due to the high concentration of Triton X-100 in these samples they were discarded when plotting the binding isotherms.

TABLE 6 BINDING OF TCMTB TO CHROMED HIDE POWDER AT 25°C

		CONCENTRATION OF FREE LIGAND mg/ℓ											
TCMTB	ORIGINAL	17,2	33,8	77,7	177	251	342	522	725	833	1 040	1 385	1 712
	FINAL	12,0	24,6	29,8	32,8	34,7	30,8	32,1	43,5	77,2	82,2	77,2	140
TRITON X-100	ORIGINAL	0	0	0	0	0	0	0	0	0	0	0	0
	FINAL	0	0	0	0	0	0	0	0	0	0	0	0
TCMTB	ORIGINAL	17,2	36,4	98,0	155	245	387	550	697	840	1 076	1 358	1 769
	FINAL	7,4	13,1	31,3	26,6	45,6	32,3	40,7	40,9	*75,5	52,3	56,3	243
TRITON X-100	ORIGINAL	98	100	114	120	114	100	98	94	*158	138	96	124
	FINAL	51	51	26	-	-	-	-	-	-	-	-	-
TCMTB	ORIGINAL	18,2	35,7	97,3	154	291	391	539	700	851	1 115	1 407	1 782
	FINAL	6,3	17,3	38,6	58,0	108	148	121	181	212	311	306	474
TRITON X-100	ORIGINAL	516	502	510	474	496	526	500	484	470	560	508	558
	FINAL	385	332	230	153	-	-	-	-	-	-	-	-
TCMTB	ORIGINAL	19,6	38,9	102	183	252	365	565	726	836	1 068	1 422	1 728
	FINAL	5,8	19,3	61,5	87,9	184	208	380	398	389	368	452	546
TRITON X-100	ORIGINAL	750	764	740	764	746	756	750	776	768	776	768	782
	FINAL	539	578	385	-	-	-	-	-	-	-	-	-
TCMTB	ORIGINAL	15,4	50,4	85,1	162	245	403	530	666	892	1 036	1 368	1 740
	FINAL	4,7	34,2	55,6	96,3	150	274	367	397	654	785	814	912
TRITON X-100	ORIGINAL	1 006	1 048	1 018	1 006	1 194	992	1 006	1 069	996	1 012	992	1 024
	FINAL	770	924	539	-	-	-	-	-	-	-	-	-

* Due to the high concentration of Triton X-100 in these samples they were discarded when plotting the binding isotherms.

ii) In most of the experiments equilibrium was established within six hours. Thus most of the binding takes place within a relatively short period. However, due to the role played by the surfactant, especially in the higher concentration range, equilibrium is only reached after several hours. Although there was still a small variation in the concentration of the free ligand measured between the twelve and twenty four hour binding samples, the differences in most cases were within the limits of accuracy for the analytical procedure. Thus, for all the binding isotherms in this study, the concentration of the free ligand TCMTB after twenty four hours was assumed to be an accurate indication of the equilibrium value. Consequently the concentration of free ligand TCMTB was only measured after twenty four hours in the remainder of the experiments.

The original and final concentrations of both TCMTB and Triton X-100 are given (Tables 5 and 6). The tables show that not only was there a decrease in the TCMTB concentration, but also in that of Triton X-100. This indicates that Triton X-100 and TCMTB bind simultaneously to hide powder collagen, possibly in the form of a mixed micelle. At the higher TCMTB concentrations the TCMTB interfered with the analyses of Triton X-100. As is illustrated (Table 7) Triton X-100 in the absence of TCMTB did not bind to the hide collagen.

TABLE 7 DROP IN TRITON X-100 CONCENTRATION IN THE ABSENCE OF TCMTB DURING BINDING STUDIES TO HIDE POWDERS

		Concentration of free Triton X-100 mg/ℓ				
Limed Hide	Original	0	124	492	722	1008
Powder	Final	0	124	450	800	1106
Chromed Hide	Original	0	96	472	766	1020
Powder	Final	0	96	410	654	1001

As was the case for NOITZ the drop in the surfactant and TCMTB concentrations was more apparent in the presence of chromed hide powder than with limed hide powder. This illustrates the greater affinity of the mixed fungicide/surfactant micelle for the more highly charged tanned hide powder collagen than for the untanned collagen.

5.4 Binding Isotherms

5.4.1. Limed Hide Powder

The binding isotherms are illustrated (Figures 49 and 50). In Figure 49 the isotherms are shown using different scales for the x- and y-axes for each of the experiments at different surfactant concentrations in order to emphasise the shape of the individual binding curves whereas in Figure 50 all five isotherms are drawn on the same scale in order to demonstrate any differences between the isotherms.

Other than in the lower concentration range the binding isotherms do not follow the normal mass-action binding type isotherm approaching a definite saturation limit but show a sudden increase in the binding or an inflexion in the binding curve. When there is no surfactant present or when the surfactant level is below its aqueous CMC of between 0,2 and 0,25mM the binding seems to simulate the classical binding situation up to approximately 0,25mM free ligand concentration. At higher concentrations a dramatic increase in binding takes place. This is especially the case in the absence of Triton X-100 when the free ligand concentration appears to decrease in solution with possible ligand self association and binding of TCMTB micelles onto the collagen hide fibres. At higher Triton X-100 concentrations more of the TCMTB is solubilized within the surfactant micelle, however, the same pattern is followed. At a particular free ligand concentration there is a sudden increase in binding. This sudden increase is usually coupled with a decrease in solution surfactant level indicating that the now self associating ligand is possibly a complex mixed fungicide/surfactant micelle. The concentration of free ligand at which this sudden increase in binding occurs is higher the greater the Triton X-100 level which could indicate that only after the hydrocarbon interior of the Triton X-100 becomes saturated with TCMTB does the self association mechanism come into play.

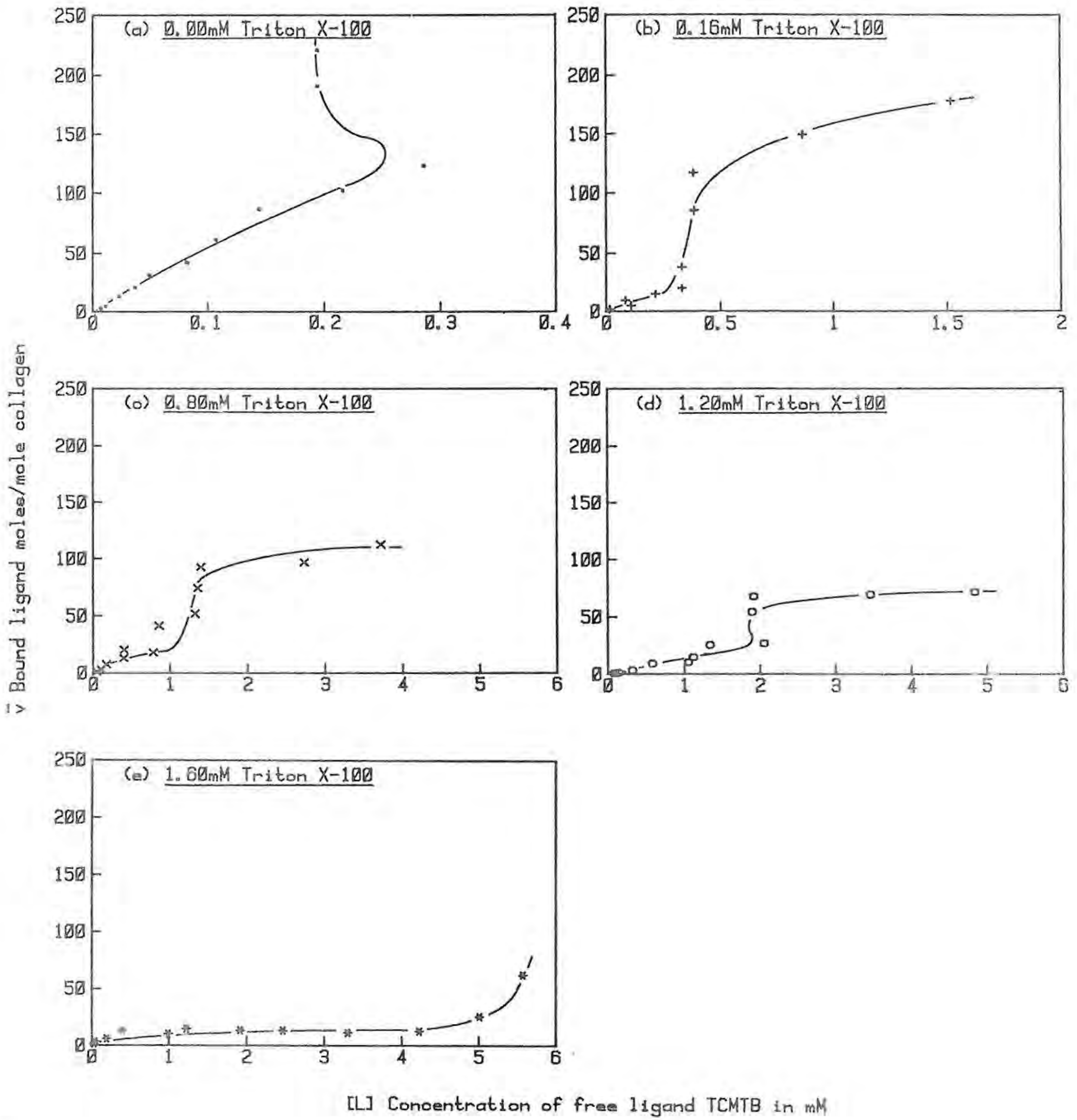

FIGURE 49 Individual binding isotherms for TCMTB with limed hide powder at 25°C (Note: The scale of the x- and y-axes vary in order to emphasise the shape of each of the individual binding isotherms)

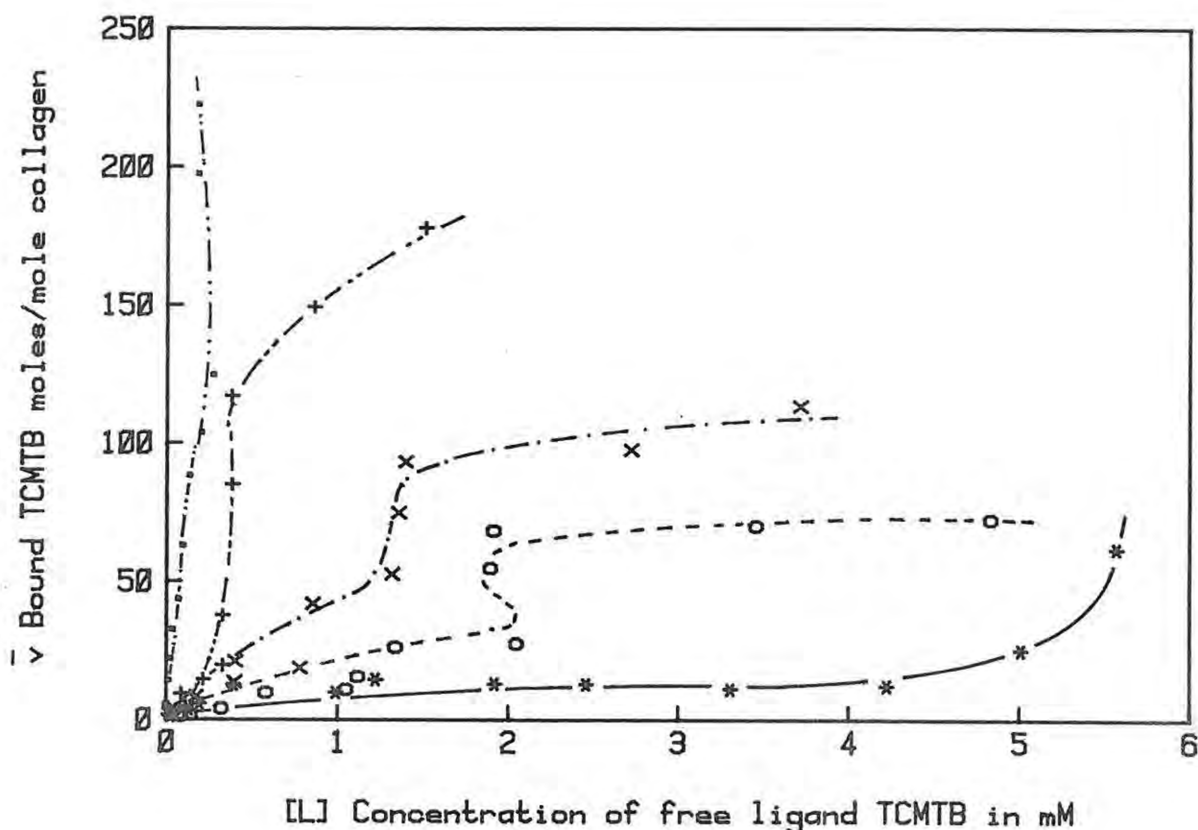

FIGURE 50 Binding isotherms for TCMTB with limed hide powder at 25°C (Note: .—..—. 0,00mM Triton X-100, +-·-·+ 0,16mM Triton X-100, x—·—x 0,80mM Triton X-100, o---o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

A phenomena not observed in previous work (Fowler et al, 1990) with TCMTB in the commercial formulation Busan 30L, and in the binding of NOITZ as described in the previous chapter is that after a certain extreme level the hide powder appeared to become saturated and the level of free ligand TCMTB in solution increased. This phenomena was observed when the initial Triton X-100 concentration was 0,16 and 1,20mM. Unfortunately at these concentrations of TCMTB in solution the amount of free Triton X-100 in solution could not be measured. A simultaneous

increase in Triton X-100 concentration would have indicated a disintergration of part of the fungicide/surfactant micelle bound to the hide powder collagen fibres with the release of both TCMTB and Triton X-100 into solution.

These forms of binding curves preclude the use of Klotz-type plots or modifications thereof as a valid representation of the more common type of binding. In the binding of TCMTB to hide powder collagen this type of binding behaviour could be attributed to complexes involving the binding of more than one molecule to any one site. The role of the surfactant however has a major effect on the shape of the curves.

The molar ratios of TCMTB to the surfactant Triton X-100 where these sudden increases take place are approximately 1,9:1; 1,8:1; 1,7:1 and 2,6:1 for original Triton X-100 concentrations of 0,16mM; 0,80mM; 1,20mM and 1,60mM respectively. Although these ratios do not correspond to those found for the sudden increase in viscosity of 2,8:1 for 0,8mM and 1,9 for 1,60mM Triton X-100 they nevertheless are still an indication of the complex nature of the binding of both TCMTB and Triton X-100 complexes to hide powder collagen fibres.

5.4.2. Chromed Hide Powder

The binding isotherms are illustrated in Figure 51 and 52. In Figure 51 the isotherms are again shown using different scales for the x- and y-axes in order to highlight the shape of the individual curves whereas in Figure 52 the same scale is used for all five isotherms in order to demonstrate the difference between the isotherms.

Figure 51 shows that other than at the lower concentration range in the presence of Triton X-100 above its CMC, the binding isotherms do not follow the general shape as experienced in the classical binding situation. As was the case when binding to limed hide powder they demonstrate that at certain free ligand concentrations there is a sudden increase in binding. This increase takes place at lower free ligand concentrations than for the corresponding curves for the binding on limed hide powder collagen, indicating that any mixed fungicide/surfactant micelle present has a higher affinity for the more

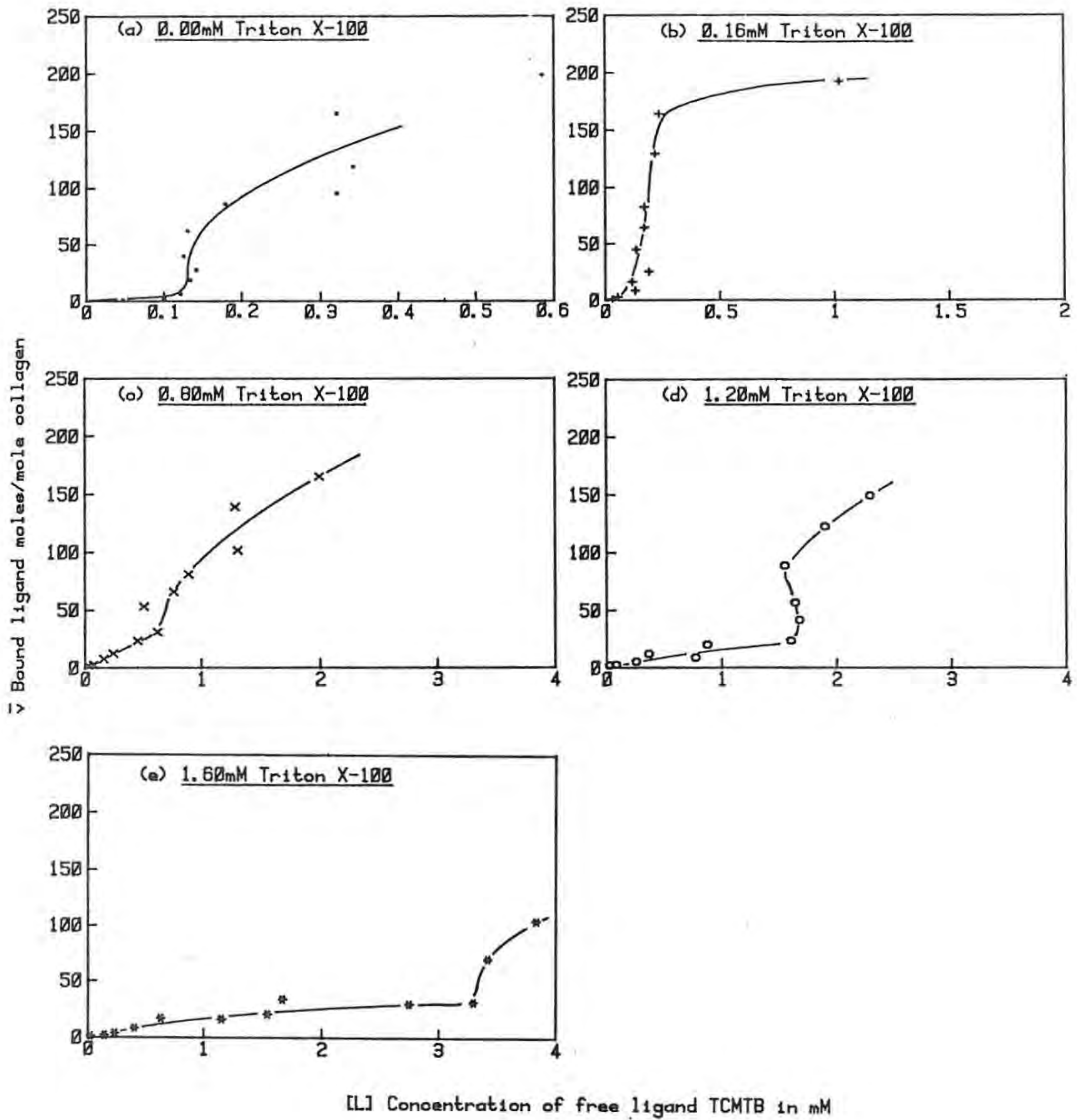

FIGURE 51 Individual binding isotherms for TCMTB onto chromed hide powder at 25°C (Note: The scale of the x- and y-axes vary in order to emphasise the shape of each of the individual binding isotherms)

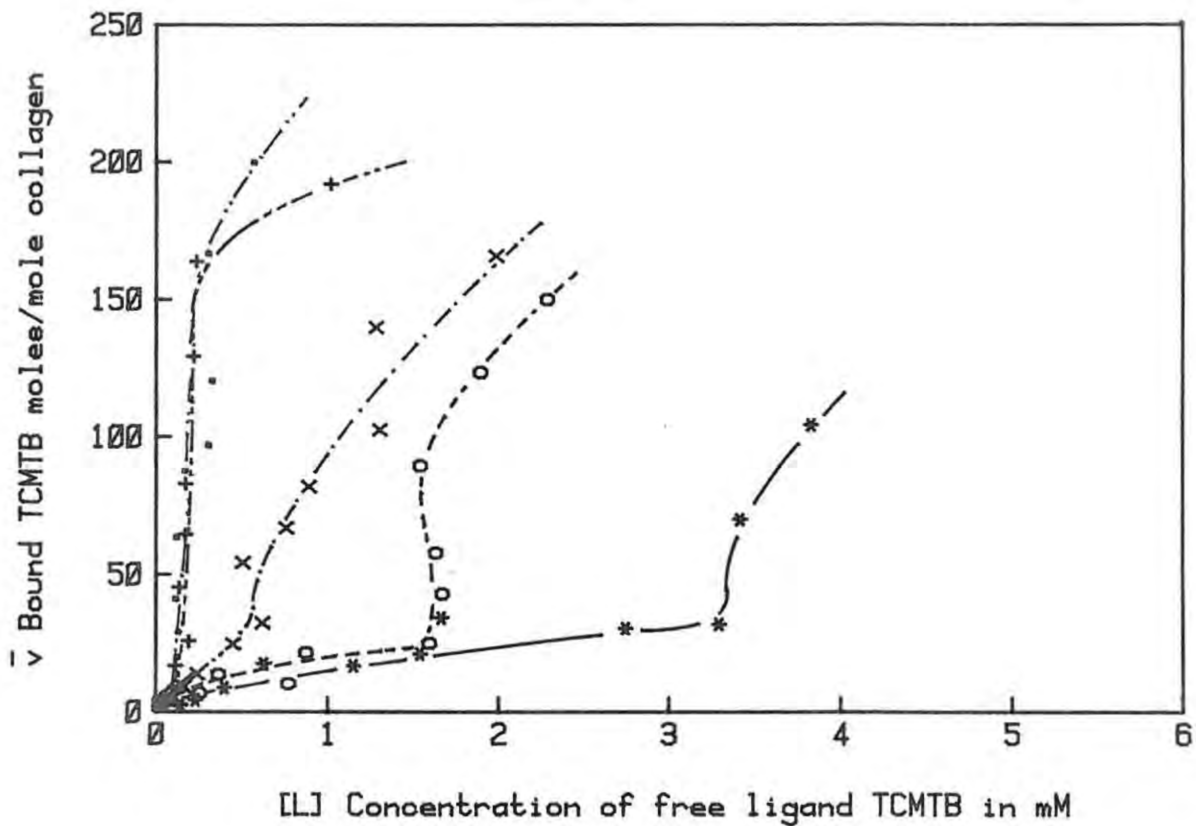

FIGURE 52 Binding isotherms for TCMTB with chromed hide powder at 25°C (Note: .—..—. 0,00mM Triton X-100, +-.-+ 0,16mM Triton X-100, x—.-x 0,80mM Triton X-100, o---o 1,20mM Triton X-100, *—* 1,60mM Triton X-100)

highly charged chrome collagen fibres than for the untanned collagen.

The molar ratios of TCMTB to surfactant where these sudden increases in binding take place are approximately 1,25:1; 1,7:1; 1,4:1 and 2,0:1 for original Triton X-100 concentrations of 0,16mM; 0,80mM; 1,20mM and 1,60mM respectively. The latter ratio of 2,0:1 for 1,60mM Triton X-100 corresponding to the ratio found for the increase in viscosity.

5.5 Scatchard Plots

The Scatchard plots for the binding of TCMTB to limed hide powder are illustrated in Figure 53, whereas those for lightly chromed hide powder are shown in Figure 54. In all cases, except at the highest Triton X-100 concentration when binding to limed hide powder, the plots are unusual. In contrast to the more familiar linear plots or hyperbolae with negative slopes, when binding involves more than a single class of binding site, the plots for these fungicide collagen interactions tend to be hyperbolae with positive slopes. Due to their positive slopes these Scatchard plots, which usually provide initial estimates of the binding parameters for subsequent least squares refinement are not very informative.

Scatchard plots with positve slopes are not unprecedented and have been reported by Shen and Gibaldi (1974), and by Judis (1980) for alkaloid-albumin interactions, by Sukow et al (1980) for Triton X-100/BSA interactions, and by Sparrow (1982) for monomolecular phenols - soluble collagen interactions. Protein-ligand systems which exhibit positive slopes in the Scatchard transformation of the binding isotherms have been reviewed by Bowmer and Lindup (1978), and a variety of explanations have been proposed to account for the phenomenon. These explanations have invoked both cooperatively and ligand induced self-association of the protein. Steiner (1980) reviewed a variety of model systems which report on the influence of the ligand on self-association of the protein, while Cann (1978) generated curves to simulate protein-ligand interactions for a series of protein monomer-dimer systems.

Although it is possible to account for the positive curvature of the Scatchard plot by means of model systems invoking ligand mediated dimerization of the protein Sparrow (1982) showed that errors in measurement at low ligand concentrations can lead to the same results. Care must therefore be exercised in the extraction of binding mechanisms from the form of the Scatchard plot.

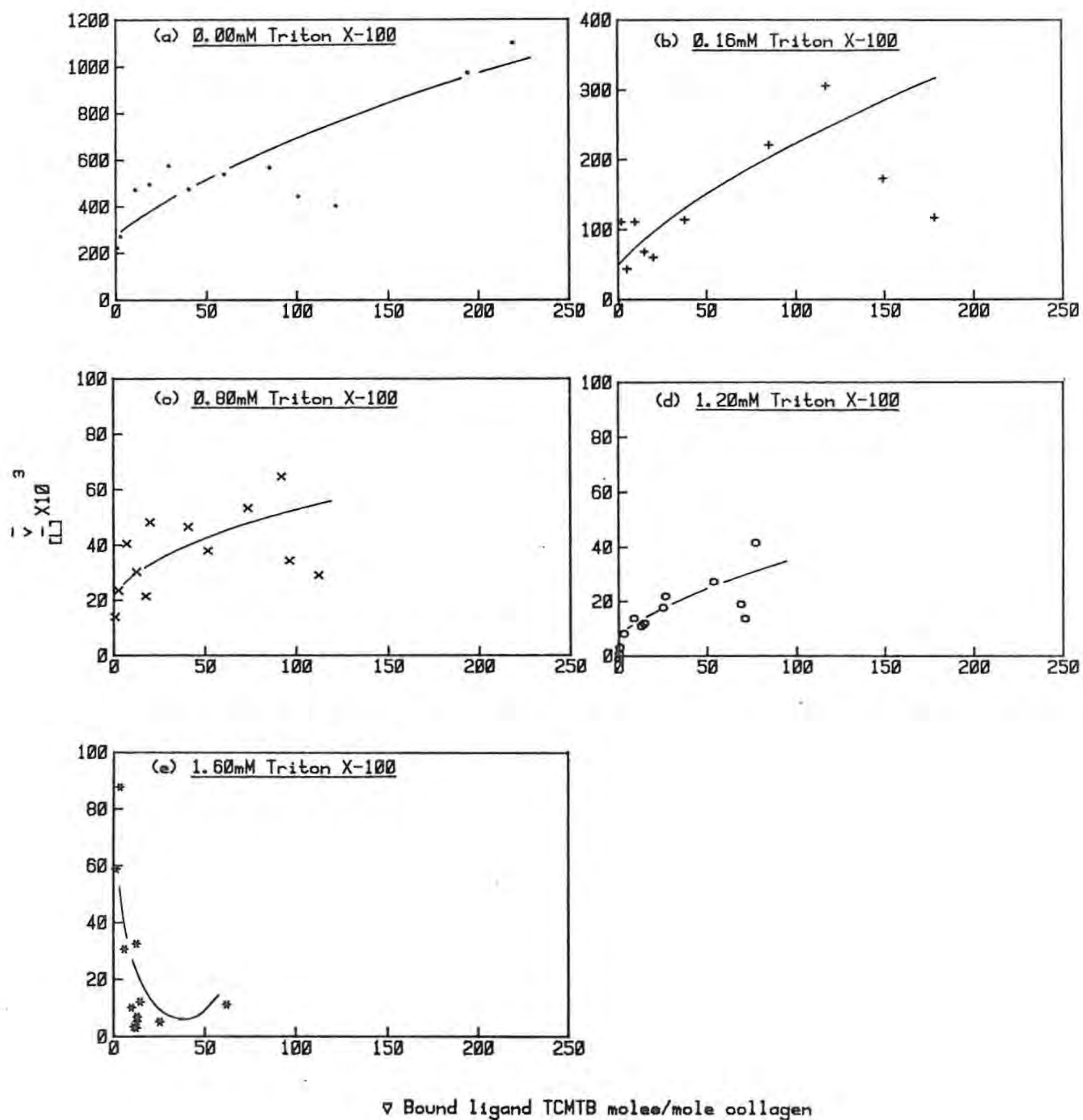

FIGURE 53 Scatchard plots of binding of TCMTB with limed hide powder at 25°C

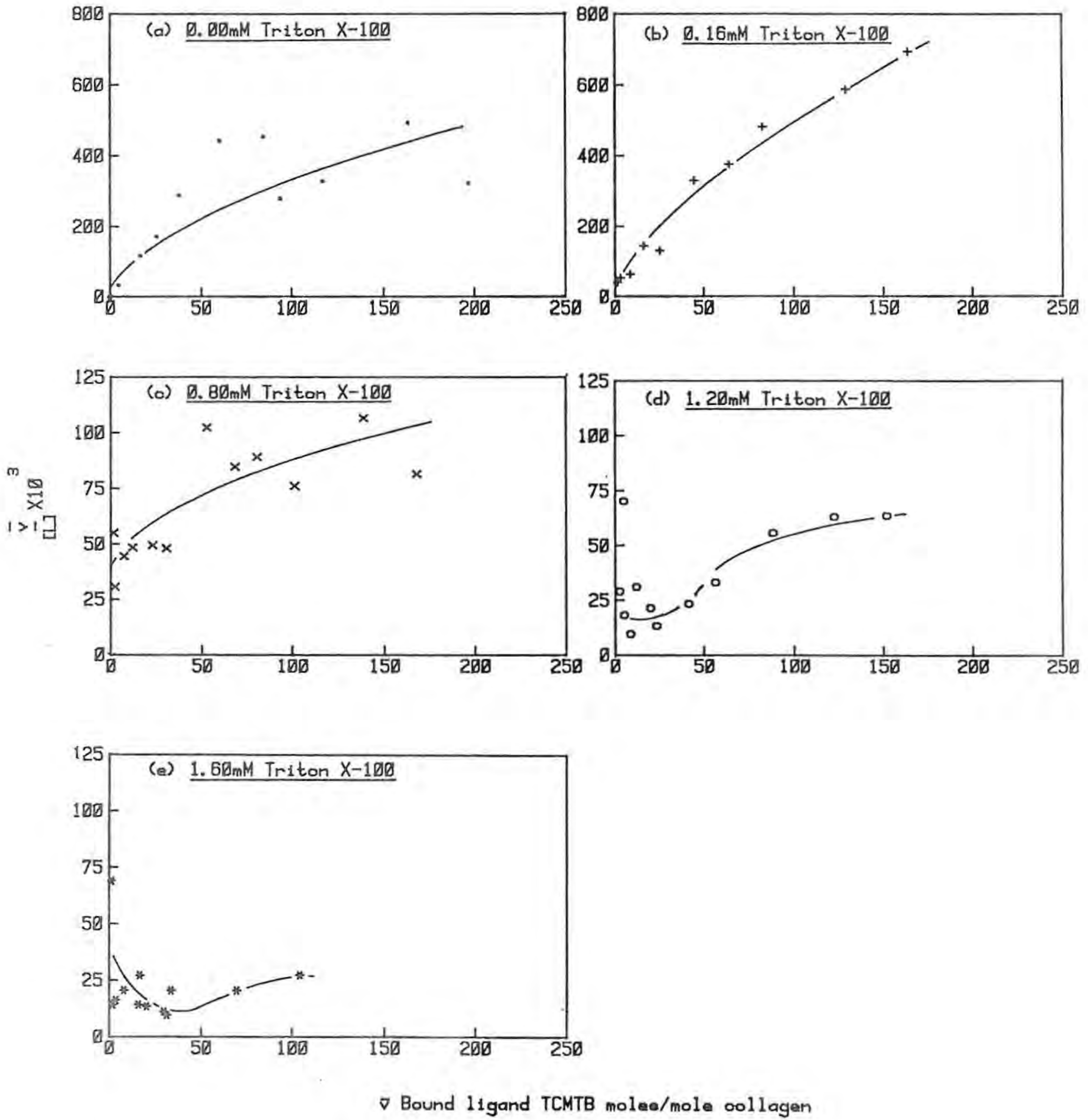

FIGURE 54 Scatchard plots of binding of TCMTB with chromed hide powder at 25°C

5.6 Conclusions

It was thus not possible to obtain any detailed information relating to the magnitude of the association constants for these interactions. The binding capacity of hide powder collagen for these fungicide ligands does however appear to be extremely large. In the case of TCMTB when the free ligand concentration is 0,25mM the number of ligand molecules bound to a collagen molecule is about 100. In contrast Sparrow (1982) found that in the case of catechin only at the higher free ligand concentration of 2,5mM was the number of ligand molecules bound to soluble collagen 100. This compares with about six phenol red molecules to BSA at the same ligand concentration. Collagen thus appeared to have an extremely high capacity for these fungicide molecules.

The implications are that the binding is relatively non specific. The binding of these ligands to the collagen fibres is not dependent on any particular amino acid sequence or protein configuration at the binding site. It is thus very likely that the binding of these ligands to collagen is by hydrophobic bonds involving large mixed fungicide/surfactant micelles in the region of the apolar side-chain residues which are present in collagen.

CHAPTER 6

BINDING INTERACTIONS OF A COMMERCIAL FORMULATON OF NOITZ WITH WET-BLUE LEATHER AND THE RESULTING ANTIFUNGAL ACTIVITY

CHAPTER 6

BINDING INTERACTIONS OF A COMMERCIAL FORMULATION OF NOITZ WITH WET-BLUE LEATHER AND THE RESULTING ANTIFUNGAL ACTIVITY

6.1 Binding Interactions of NOITZ with wet-blue leather from float analysis

The binding interaction between the active ingredient NOITZ in the commercial fungicidal formulation Kathan LP and wet-blue leather was determined by float difference analyses from the concentration of free ligand remaining in solution after two hours (Table 8). The table shows that the decrease in free ligand concentration is greater at the higher temperatures at all original float concentration offers.

TABLE 8 CONCENTRATION OF FREE NOITZ IN mg/ℓ IN SOLUTION AFTER TWO HOURS TREATMENT

Treatment	Kathon LP Offer in Float (%)							
Temp °C	0,01	0,025	0,05	0,10	0,25	0,50	1,00	2,50
10	3,04	7,21	13,8	28,9	67,1	172	275	736
25	1,69	5,19	11,5	19,2	44,0	72,5	121	320
40	1,01	2,59	5,64	11,3	34,9	57,1	130	296

The decrease in the free ligand concentration at the different temperatures is depicted more clearly on a log scale (Figure 55).

The percentage of NOITZ bound to the wet-blue leather is given (Table 9). This shows that in all the treatments carried out at 40°C between 82% and 90% of the NOITZ was bound, whereas at the lower treatment temperature of 10°C only between 62% and 72% was bound.

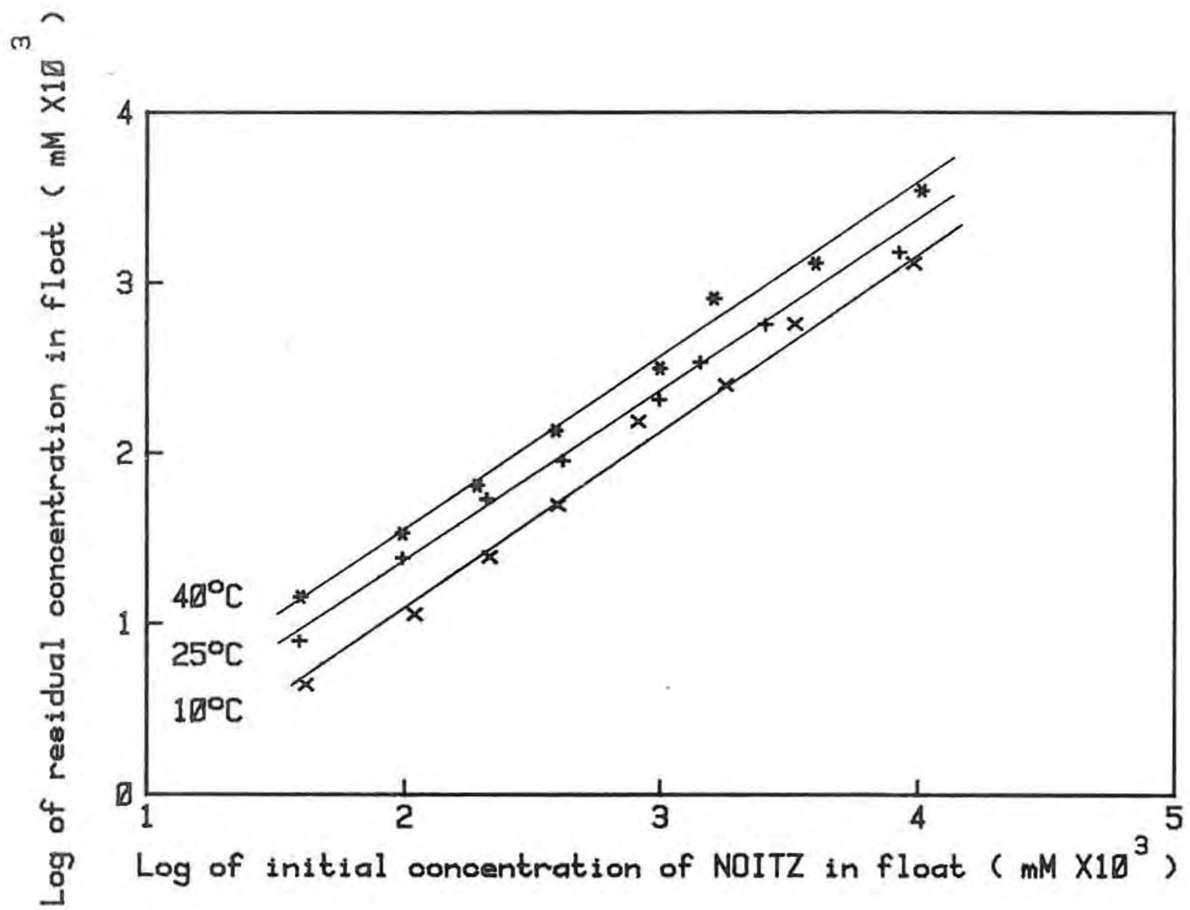

FIGURE 55 Log of concentration of free NOITZ after two hours treatment at different temperatures and float offer

TABLE 9 PERCENTAGE OF NOITZ BOUND WITH WET-BLUE LEATHER AFTER TWO HOURS TREATMENT

Treatment	Kathon LP Offer in Float (%)							
Temp °C	0,01	0,025	0,05	0,10	0,25	0,50	1,00	2,50
10	66	71	71	72	71	62	70	67
25	82	78	74	79	80	83	83	84
40	89	90	87	88	83	85	82	86

The amount of NOITZ bound to the wet-blue leather on an area basis is shown (Table 10). These amounts are derived from the free ligand in solution, the total NOITZ originally present in the float, the final mass of the wet-blue leather pieces, and the average mass and moisture content of the individual 38mm diameter plugs cut from the wet-blue leather pieces. Surface area rather than mass was used to determine the binding curves due to the variations in mass between the individual pieces of wet-blue leather (210g to 313g) arising from differences in hide substance (thickness). Using surface area in this fashion assumes that all the biocide is bound to the surface and does not penetrate throughout the wet-blue leather. This table also shows greater binding of the fungicide to the wet-blue leather at the higher temperature.

TABLE 10 AMOUNT OF NOITZ BOUND IN mg/m² BOUND WITH WET-BLUE LEATHER SURFACE AFTER TWO HOURS TREATMENT

Treatment	Kathon LP Offer in Float (%)							
Temp °C	0,01	0,025	0,05	0,10	0,25	0,50	1.00	2,50
10	19,1	45,2	97,4	203	444	988	1945	4908
25	23,9	55,1	133	248	571	911	1837	5136
40	23,0	58,6	130	230	644	1038	1955	6032

The binding isotherms for NOITZ with wet-blue leather are illustrated (Figures 56 and 57). Figure 56 shows the binding isotherm over the full range of NOITZ concentrations whereas Figure 57 limits the curve to the concentration range usually used for production applications. These binding curves show a linear increase in binding indicating that at no stage is a saturation level reached. There is however a marked increase in binding with increase in temperature.

The increase in binding at the higher temperature could be due to:-

i) An increased binding rate. The solutions were sampled after two hours and equilibrium conditions might not have been reached in the experiments at the lower temperatures. Equilibrium was, however, rapidly achieved during the binding

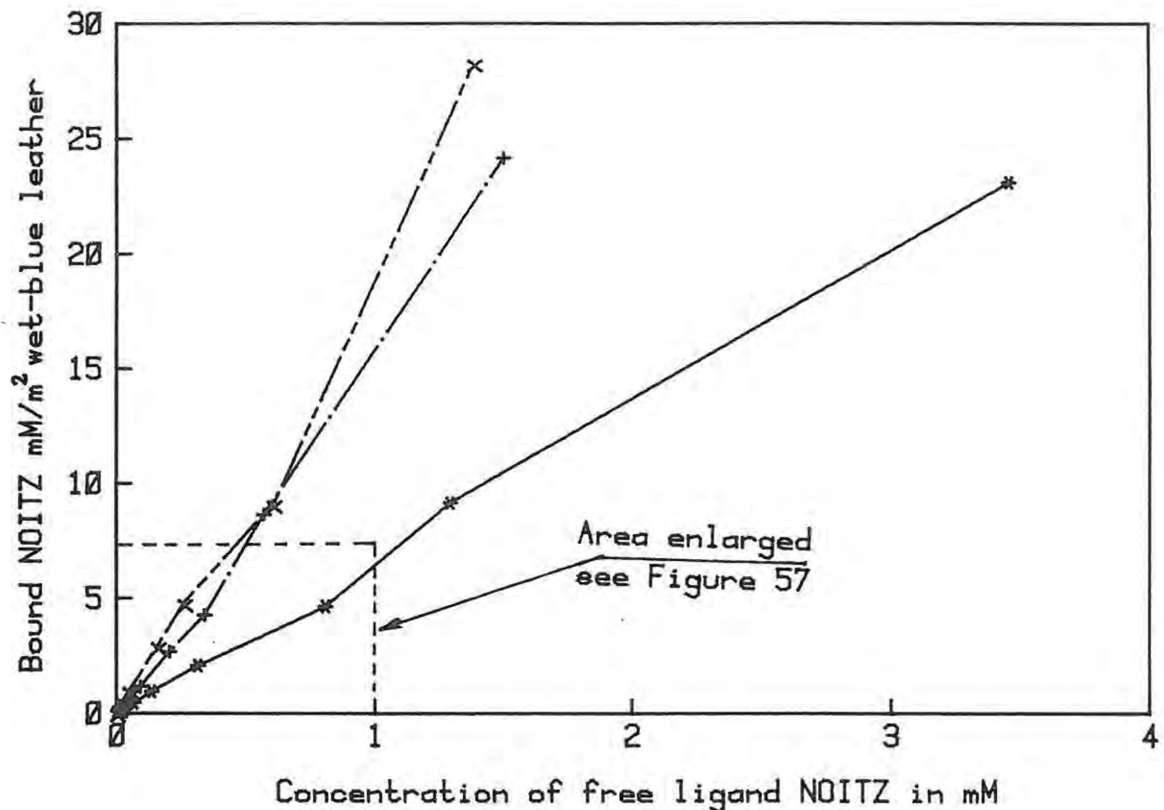

FIGURE 56 Binding isotherms of NOITZ with wet-blue leather (Note: *—* 10°C, +-·-+ 25°C and x---x 40°C)

of NOITZ to hide powders. It is therefore reasonable to assume that after two hours a state of equilibrium existed between the free ligand and the ligand bound to the wet-blue leather surface.

ii) Greater binding affinity as a result of the availability of more binding sites at the higher temperatures or alternatively the binding of larger biocide/surfactant micelles to the fixed number of available sites. The surfactant concentration at a product (Kathon LP) offer of 2,5% is 0,40mM which is almost double the aqueous CMC of Triton X-100. At the same Kathan LP concentration there was 10,6mM NOITZ in solution. The formation of large mixed micelles could thus be expected.

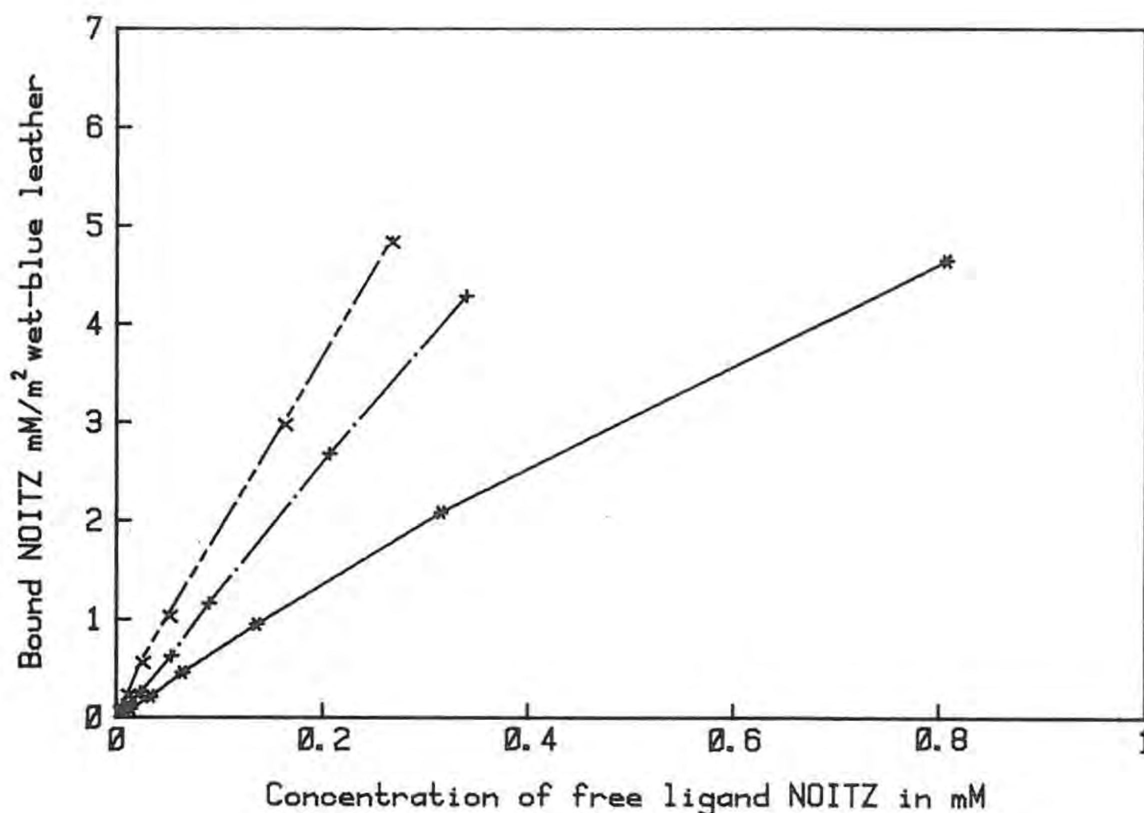

FIGURE 57 Binding isotherms of NOITZ with wet-blue leather (at concentration in production application range) (Note: *—* 10°C, +-·-+ 25°C and x---x 40°C)

6.2 Recoveries of NOITZ from treated wet-blue leather by solvent extraction

The mean percent recovery values of the NOITZ extracted from the four plugs in each of the treatments are given (Table 11). The results show that for most treatments a similar amount was extracted from the pieces for the different temperatures. However, at the higher concentrations less NOITZ was extracted.

The NOITZ extractability thus decreased progressively with binding. This is consistent with irreversible fixation of an increasing proportion of the fungicide uptake due to the possible formation of

chrome complexes. The extraction results were lower than for TCMTB (Fowler et al, 1987). This could be due to the combined effects of factors such as variable extraction efficiency due to the high moisture content of the wet-blue leather, greater penetration of the fungicide into the wet-blue leather matrix, and complexation causing a proportion of the NOITZ to be irreversibly bound to the fixed chrome.

TABLE 11 PERCENTAGES OF BOUND NOITZ EXTRACTED FROM WET-BLUE LEATHER BY DICHLOROMETHANE

Treatment	Kathon LP Offer in Float (%)							
Temp °C	0,01	0,025	0,05	0,10	0,25	0,50	1,00	2,50
10	84	79	38	42	37	13	28	29
25	84	70	30	28	28	25	19	24
40	100	69	56	24	26	36	29	32

6.3 Storage-dosage response in wet-blue leather testing

Anti-fungal activity in terms of storage period versus fungicidal offer at various treatment temperatures as determined by the two storage assessment methods are shown in bar type format for treated wet-blue leather (Figure 58) and for treated and subsequently solvent extracted wet-blue leather (Figure 59). These figures indicate that except for the unextracted wet-blue leather pieces using the paper assessment method there was less protection at the higher treatment temperatures. This is consistent with the greater surface binding of larger fungicide/surfactant mixed micelles onto the surface of the wet-blue leather at the higher temperatures. In the agar assessment method NOITZ could more easily migrate from the surface to the surrounding agar thus providing less fungal protection. This surface bound fungicide could also be more easily removed during solvent extraction. If greater penetration into the wet-blue leather matrix was the major reason for the greater binding at the higher temperatures, better protection at the higher treatment temperatures when using the paper assessment method on the unextracted pieces would not have been obtained.

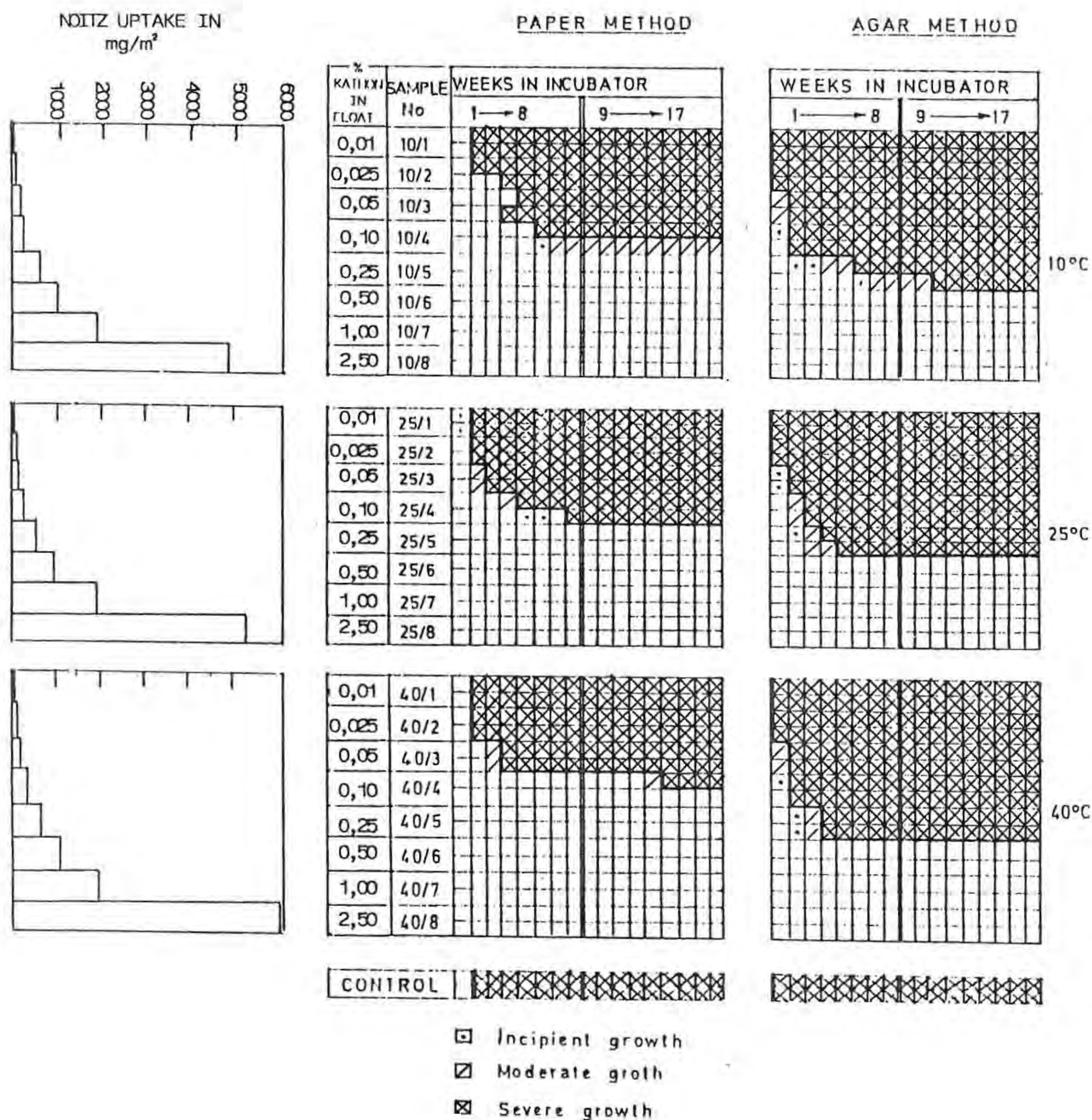

FIGURE 58 Variation in protective storage (in weeks) with dosage offer and treatment temperature in incubation tests carried out at 22°C on treated wet-blue leather.

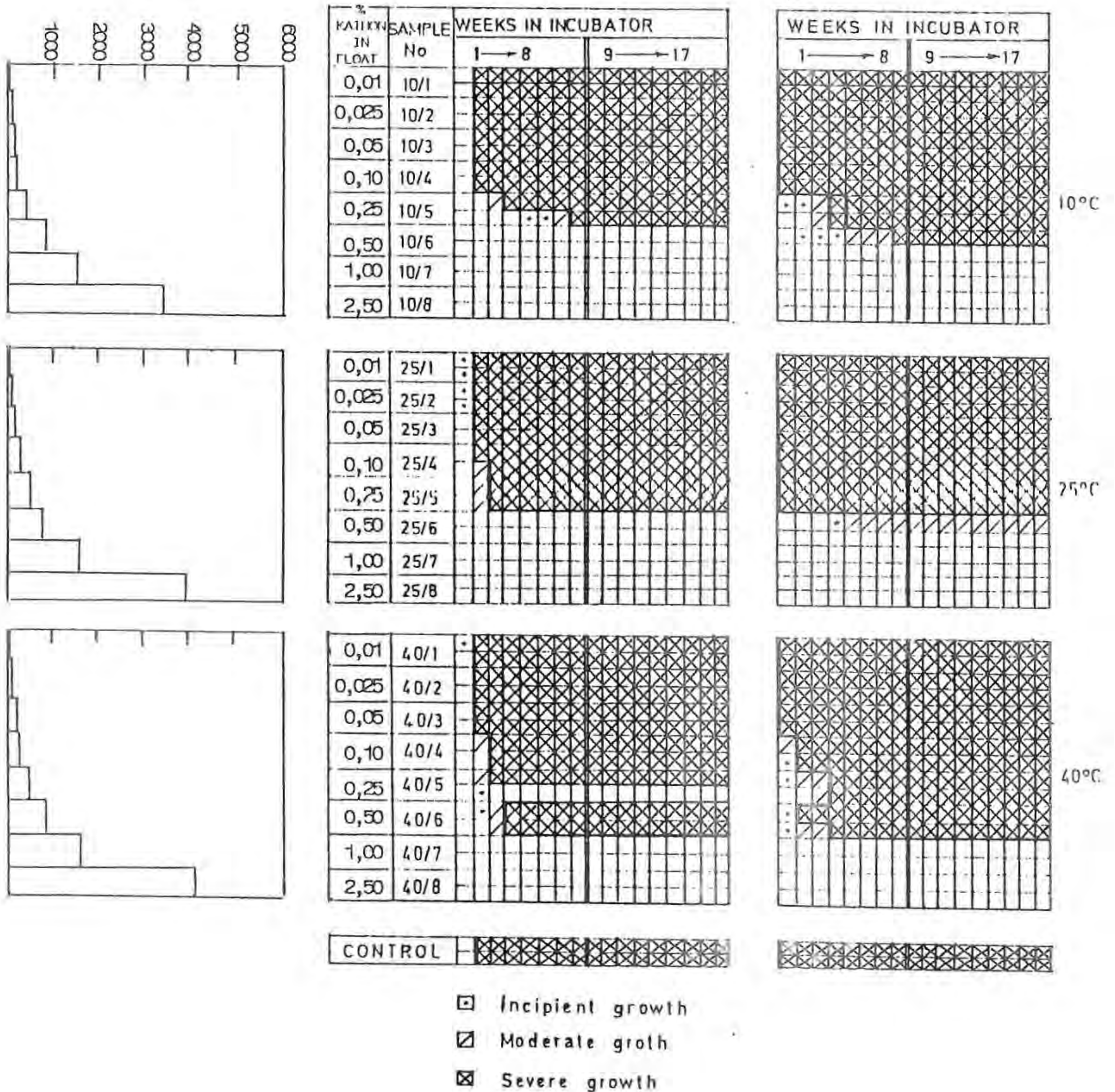

FIGURE 59 Variation in protective storage (in weeks) with dosage offer and treatment temperature in incubation tests carried out at 22°C on treated and solvent-extracted wet-blue leather

Minimum inhibitory dosage levels for 8 week fungal-free protection (Table 12) showed a tendency to decrease between the 10°C and higher temperature treatments in the storage assessment method on the drained plugs when using the paper method. This is consistent with the greater binding at the higher temperatures. This greater protection at the higher treatment temperatures was however not observed in any of the other assessment methods indicating that the biocide was less permanently bound to the surface of the wet-blue leather and could thus migrate into the agar and also be more easily extracted.

TABLE 12 MINIMUM INHIBITORY DOSAGES OF KATHON LP FOR EIGHT WEEKS PROTECTIVE STORAGE OVER A RANGE OF TREATMENT TEMPERATURES

Treatment	Treated wet-blue			Treated/solvent extracted		
Temperature	Paper	Agar	Means	Paper	Agar	Means
10°C	0,50	0,50	0,50	0,50	1,00	0,75
25°C	0,25	0,50	0,38	0,50	1,00	0,75
40°C	0,10	0,50	0,30	1,00	1,00	1.00
Means	0,28	0,50	0,39	0,67	1,00	0,83

Solvent extraction of interstitial free and physically bound NOITZ resulted in a 2 to 4 fold increase in the minimum inhibitory dosage necessary for 8-week protection. Substantial residual protection remaining after extraction, however, indicated that both the physically associated fungicide component as well as the bound NOITZ within the treated wet-blue leather matrix make a substantial contribution to the overall wet-blue leather protection.

6.3.1 Storage response in relation to NOITZ uptake

Storage response relative to the actual surface NOITZ content, as determined by difference analysis of treatment float concentration show an inverse relationship between uptake and fungal growth and activity. Protective storage periods averaged over the treatment temperature range for both treated and solvent-extracted wet-blue leather samples are shown (Figure 60) depicting the curvilinear increase in storage with NOITZ uptake.

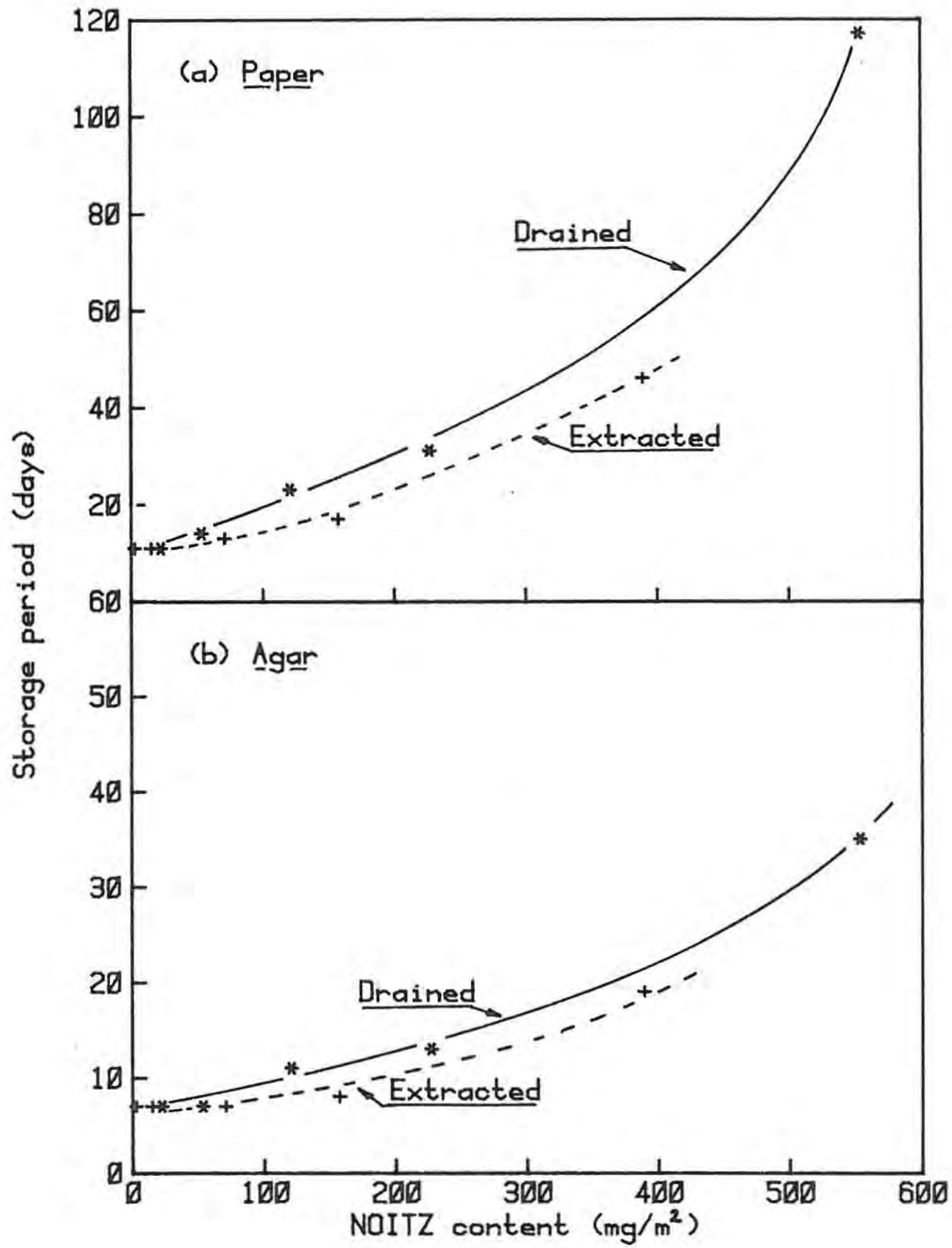

FIGURE 60 Storage period (averaged over temperature range) in relation to wet-blue leather surface uptake of NOITZ

In both storage tests, no marked difference in storage response versus NOITZ content before and after extraction was apparent. This again indicated that both the extractable and bound NOITZ components appeared to offer comparable protection (or only marginally reduced protection in the case of residual bound NOITZ) for a given range of up to 600mg/m^2. Storage increased relatively gradually up to the region of 300 to 400mg NOITZ/m^2 and then at a rapidly increasing rate, exceeding the experimental storage limit (of four months) in the region of 500 to 600mg NOITZ/m^2.

6.3.2 Effect of treatment temperature on storage

In the lower dosage range up to 0,25% Kathon LP offer, corresponding with practical application levels, treatment temperature had the effect of increasing NOITZ uptake. This increased uptake, only corresponded to an increase in protective storage on the drained wet-blue leather samples when measured by the paper storage assessment method. The samples showed a decrease in protective storage with increase in temperature in the case of the potato dextrose agar method of assessment (Figure 61). The solvent extracted samples showed only a slight increase in protection with increase in treatment temperature. This again indicates that at the higher temperature the fungicide is deposited in a form that in can easily migrate onto the PDA surface and is also more readily extracted.

Temperature effects on storage margins could not be readily compared quantitatively at the higher levels of fungicide offer as the resulting storage period exceeded the experimental observation limit of four months.

The role of temperature during treatment in influencing uptake and storage seemed to be twofold. At the higher temperatures more fungicide is bound to the surface of the wet-blue leather leading to enhanced storage. This additional fungicide is, however, more easily removed which could negate any beneficial protection effects.

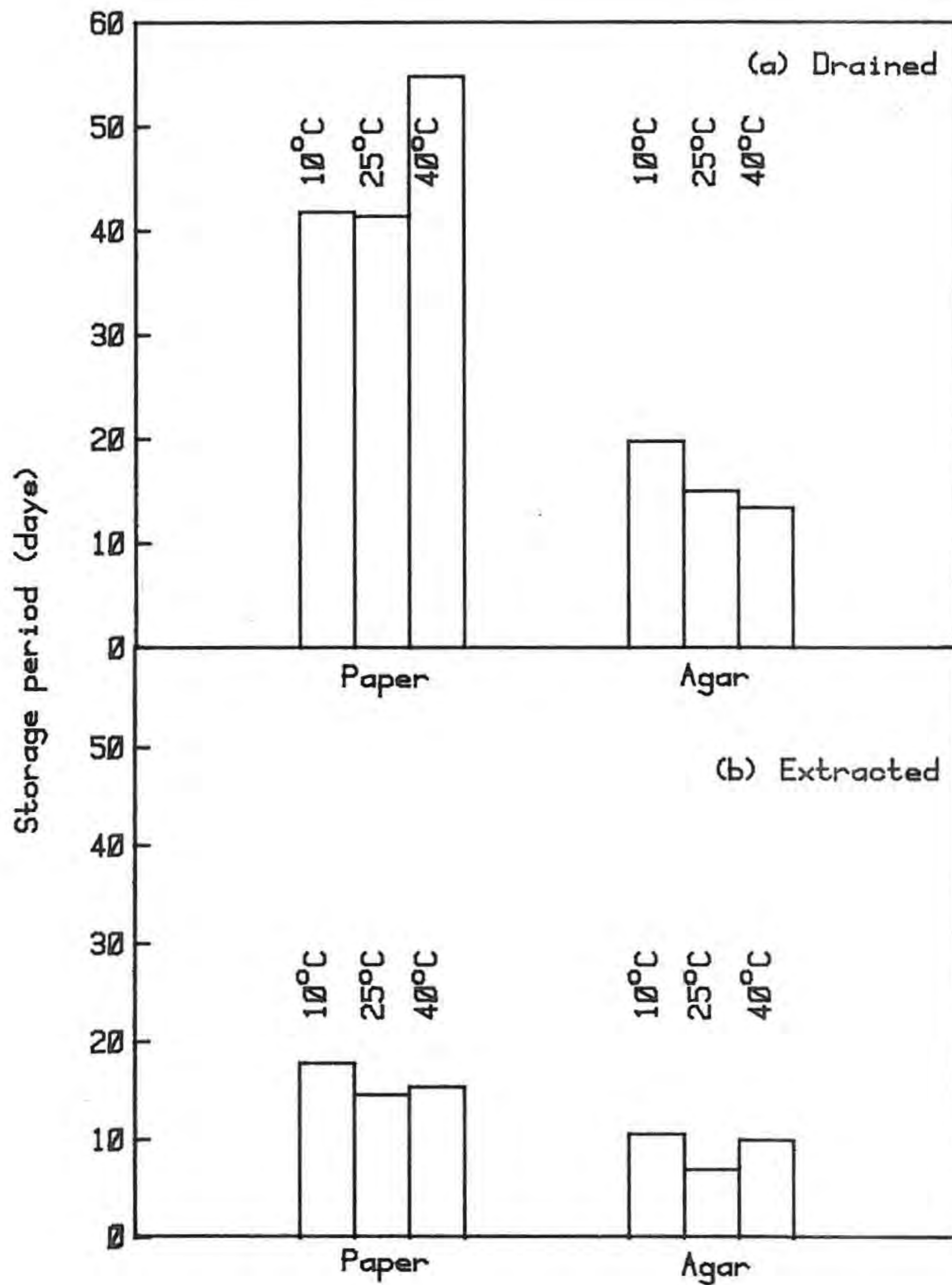

FIGURE 61 Storage period (averaged up to 0,25% Kathon LP offer) in relation to treatment temperature and solvent extraction

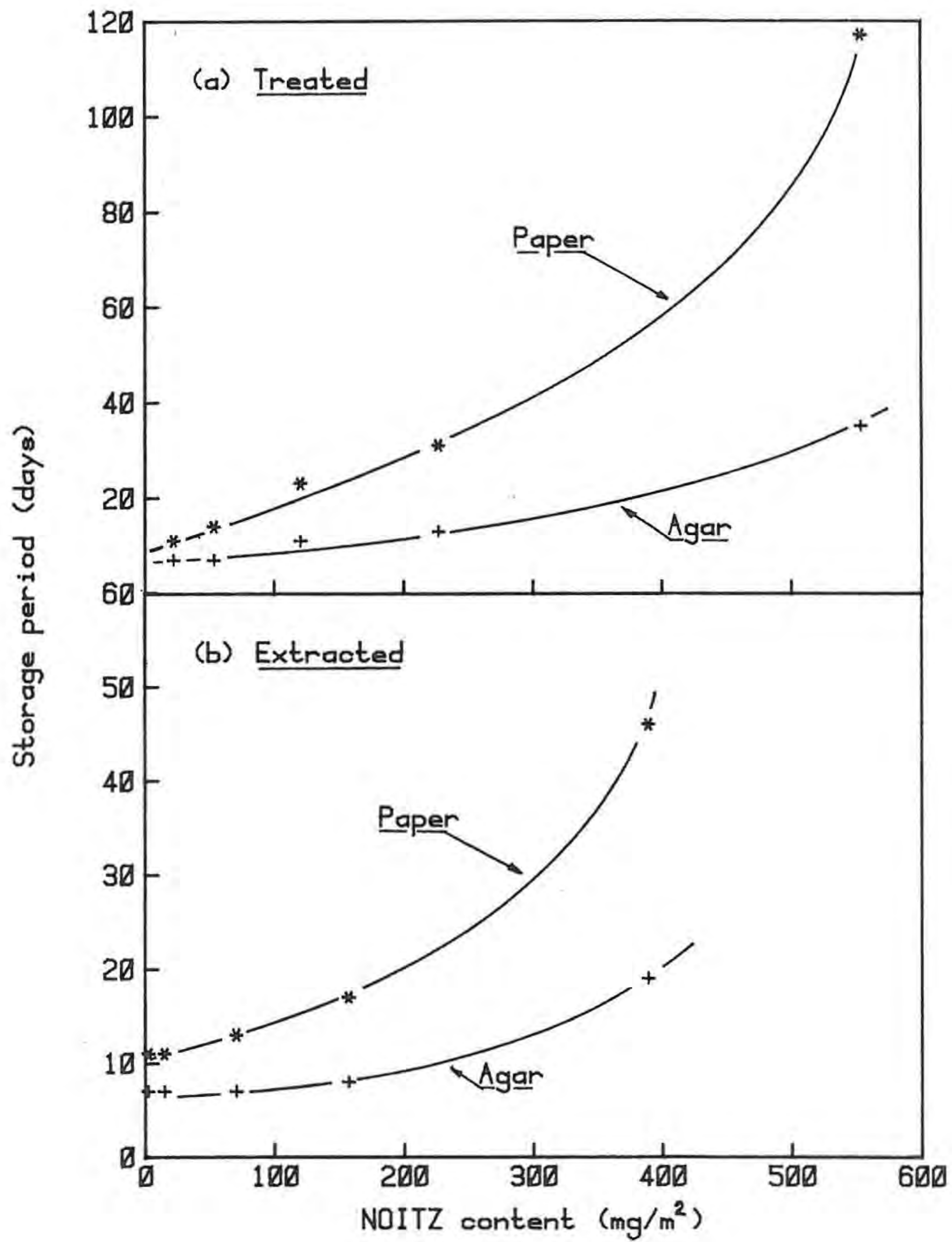

FIGURE 62 Storage period (averaged over temperature range) in various storage assessment tests on treated and solvent extracted wet-blue leather

6.3.3 Comparison of storage assessment methods

A qualitatively similar curvilinear storage response in relation to NOITZ content was noted in both incubation tests on treated samples (Figures 60 and 62).

The paper assessment method, however, took a longer period to establish a definite, heavy fungal growth. Parallel storage testing after solvent extraction of NOITZ, showed only a slight drop in storage response in the case of both test protocols (Figure 59).

Storage performance testing by the two protocols thus indicated that while solvent extraction reduced storage response at various dosage offers, variation in storage margins with actual NOITZ content were only slightly lower after extraction. This indicated that both bound and extractable NOITZ conferred more or less comparable protection to the wet-blue leather.

CHAPTER 7

BINDING INTERACTIONS OF A COMMERCIAL FORMULATION OF TCMTB WITH WET-BLUE LEATHER AND THE RESULTING ANTIFUNGAL ACTIVITY

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7.1 Binding interactions of TCMTB with Wet-Blue Leather from Float Analysis

The binding of TCMTB and wet-blue leather was calculated by float difference analysis from the amounts of free ligand remaining in solution. (Fowler *et al*, 1987)

Table 13 shows the analysis of the free ligand (TCMTB) concentration. Each determination was done in triplicate and from the mean CV (coefficient of variation) of only 3,5%, it can be concluded that there was good replication of results. The results show that there is not much difference in free TCMTB levels between the different temperature treatments at the lower concentration levels. At higher concentrations however there is greater binding at the higher temperatures resulting in lower free ligand levels.

TABLE 13 CONCENTRATION OF FREE TCMTB IN mg/ℓ IN SOLUTION AFTER TWO HOURS TREATMENT

Solution Temp. °C	Busan 30L Offer in Float (%)							
	0,0025	0,005	0,010	0,025	0,050	0,100	0,250	0,500
10	1,40	2,76	4,84	13,2	24,8	77,0	269	553
25	1,04	1,82	3,23	8,95	18,6	72,8	254	398
40	1,56	1,85	3,50	8,95	19,4	32,1	181	342
55	1,23	2,61	4,12	10,4	26,8	54,2	183	221

Mean C.V. = 3,50% Range of C.V.'s 0,0 - 9,49%

The percentage TCMTB bound after two hours drumming is also shown (Table 14). It is seen that in most cases over 80% is bound and only at the

higher concentration and lower temperatures is less than 75% bound.

TABLE 14 PERCENTAGE OF TCMTB BOUND WITH WET-BLUE LEATHER AFTER TWO HOURS TREATMENT

Solution Temp. °C	Busan 30L Offer in Float (%)							
	0,0025	0,005	0,010	0,025	0,050	0,100	0,250	0,500
10	82	81	84	83	84	75	71	63
25	87	88	90	88	88	76	66	75
40	80	89	88	88	87	90	76	78
55	83	82	86	86	82	81	75	85

The amounts of TCMTB bound to the wet-blue leather on an area basis (Table 15) taking into account the total of grain and flesh surface areas are derived from the free ligand in solution, the total amount of TCMTB originally present and the average mass of individual 38mm diameter plugs cut from the wet-blue pieces. Surface area rather than a mass basis was used due to the significant variations in mass between the different pieces of wet-blue leather (152g to 257g) arising from the differences in hide substance (thickness). As in the case of free ligand, there was very little difference between treatments at various temperatures except at the higher concentrations where there was greater binding of TCMTB.

In an earlier experiment, carried out in these laboratories (unpublished), stratigraphic analyses of wet-blue leather split showed little or no TCMTB penetration indicating that TCMTB is predominantly bound to the surface of the wet-blue. The mean substance of the test pieces was 5,5mm, thus the edges of the pieces account for some 6,9% of the total area (varying between 5 and 8,7%). As the pieces were randomly allocated to the treatments the differences in area would not contribute significantly to any observed trends but only affect individual values.

TABLE 15 AMOUNT OF TCMTB IN mg/m^2 BOUND WITH THE WET-BLUE LEATHER SURFACE AFTER TWO HOURS TREATMENT

Solution Temp. °C	Busan 30L Offer in Float (%)							
	0,0025	0,005	0,010	0,025	0,050	0,100	0,250	0,500
10	16,7	36,6	68,6	194	311	604	1 215	2 480
25	14,7	40,6	71,4	188	304	644	1 380	2 742
40	17,9	33,3	70,3	184	379	625	1 522	3 133
55	16,2	38,9	71,5	175	357	714	1 669	3 493

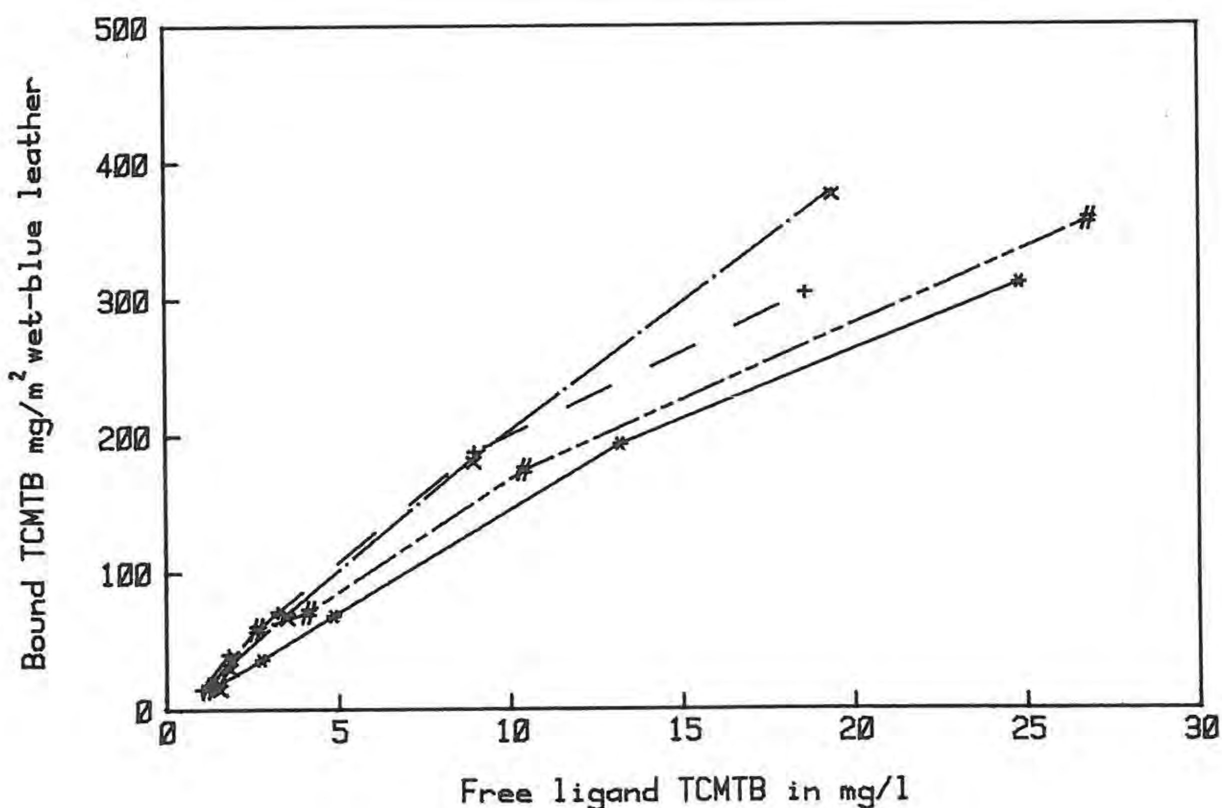

FIGURE 63 Binding isotherms of TCMTB with wet-blue leather (at concentration in production application range) (Note: *——* 10°C, +– –+ 25°C, x–·–x 40°C, #---# 55°C)

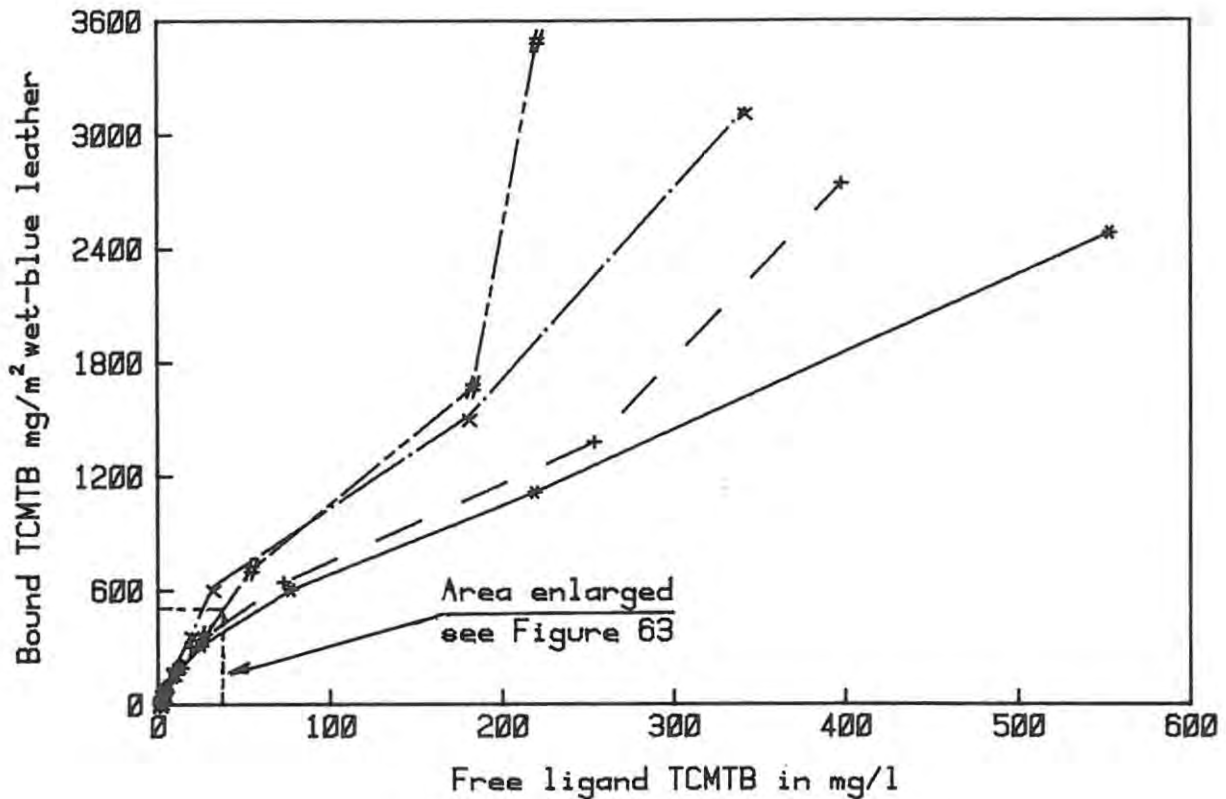

FIGURE 64 Binding isotherms of TCMTB with wet-blue leather (Note: *—* 10°C, +- -+ 25°C, x-·-x 40°C, #---# 55°C)

The binding isotherms for TCMTB on wet-blue are illustrated (Figures 63 and 64). Where the concentration of biocide was in the normal production application range (Figure 63) the binding of TCMTB followed a normal mass-action type curvilinear uptake curve. There was a fair amount of scatter about the mean with an increase in binding from 10°C to 40°C, but a decrease at 55°C. These differences may not be significant. However, when the concentration of TCMTB (and surfactant) was increased there was a marked increase in binding with increase in temperature (Figure 64). There was also an inflexion in the curve where binding suddenly increased rapidly. This inflexion occurred earlier at higher temperatures and was probably due to onset of mixed micelle formation where interfacial mechanisms came into play, resulting in

enhanced binding of TCMTB/surfactant micelles onto the leather fibres, particularly in apolar regions of the fibrils.

The enhanced uptake at the higher concentrations may be directly related to the formation of large mixed fungicide/surfactant micelles. Since TCMTB was added in formulated form as Busan 30L containing a fixed proportion of surfactant, both the concentration of TCMTB and the amount of surfactant increased. At higher concentrations (in excess of the critical micelle concentrations) surfactant/fungicide aggregation to form large mixed micelles occurred. This led to binding of both surfactant and TCMTB. This aggregation and mixed micelle formation takes place at lower concentrations with increase in temperature. (Masullo et al (1986)

7.2 Recoveries of TCMTB from Treated Wet-Blue Leather by Solvent Extraction

The mean percent recovery values of the TCMTB extracted from six plugs in each of the treatments is given (Table 16). The results show that for most treatments a similar amount was extracted from the pieces for all the different binding temperatures. However, for treatments at the higher fungicide concentrations less TCMTB was extracted in the the case of the lower temperature treatments. TCMTB extractability initially decreased progressively with binding particularly at the lower treatment temperatures, consistent with irreversible fixation of an increasing proportion of the fungicide uptake due to formation of chrome complexes. TCMTB extractability remained relatively high at the higher fungicide offer and treatment temperatures due to the effect of binding of TCMTB/surfactant mixed micelles arising under these conditions. These larger mixed micelles being more readily extractable by dichloromethane than bound TCMTB molecules. These results which are lower on average than found previously (Fowler _et al_, 1987) could be due to the combined effects of factors such as variable extraction efficiency due to the high moisture content of the wet-blue, greater binding than found previously due to formation of smaller mixed micelles as well as complexation causing a proportion of the TCMTB to be irreversibly bound to the fixed chrome.

TABLE 16 PERCENTAGE OF BOUND TCMTB EXTRACTED FROM WET-BLUE LEATHER BY DICHLOROMETHANE

Solution Temp. °C	Busan 30L Offer in Float (%)							
	0,0025	0,005	0,010	0,025	0,050	0,100	0,250	0,500
10	71	65	44	28	35	25	18	15
25	74	51	43	47	54	38	35	48
40	64	38	50	28	35	47	37	37
55	69	63	54	38	46	43	39	44

7.3 Storage-Dosage Response in Wet-Blue Leather Testing

Minimum inhibitory dosage levels for 8-week fungal-free (Table 17) protection showed a tendency to decrease between the 10°C and higher temperature treatments, particularly in the unextracted wet-blue leather samples, reflecting the enhanced uptake due to increasing TCMTB binding to the leather fibres with increasing temperature and float concentration. A qualitatively similar temperature response was apparent in the paper and agar storage assessment methods although higher inhibitory dosages were necessary in the agar assessment method to achieve the same storage response.

TABLE 17 MINIMUM INHIBITORY DOSAGES OF BUSAN 30L FOR EIGHT WEEKS PROTECTIVE STORAGE OVER A RANGE OF TREATMENT TEMPERATURES

Treatment Temperature	Treated Wet-Blue				Treated Solvent extracted			
	Paper	Agar	Chamber	Means	Paper	Agar	Chamber	Means
10°C	0,10	0,25	0,05	0,25	0,25	0,50	0,25	0,33
25°C	0,05	0,10	0,05	0,07	0,25	0,50	0,25	0,33
40°C	0,05	0,10	0,10	0,08	0,10	0,25	0,50	0,28
55°C	0,05	0,10	0,10	0,08	0,10	0,50	0,50	0,37
Means	0,06	0,14	0,08	0,09	0,18	0,44	0,38	0,33

Solvent extraction of interstitial free and physically bound TCMTB resulted in a 3 to 4-fold increase in the minimum inhibitory dosage necessary for 8-week protection. Substantial residual protection remaining after extraction, however, indicated that both the physically associated fungicide component as well as the bound TCMTB within the treated wet-blue leather matrix make a substantial contribution to overall wet-blue leather protection.

TCMTB minimum inhibitory dosage at 25°C and higher treatment temperatures (0,05%m/m for 8 weeks storage, paper method) in the present study was slightly higher than found in previous laboratory studies (Galloway and Cooper, 1974; Russell et al, 1985; Bugby, 1985) but compared favourably with production dosage offers.

Antifungal activity in terms of storage period versus fungicide offer at the various treatment temperatures as determined by the three storage tests are shown in bar-type format in Figures 65 (treated wet-blue leather) and 66 (treated, extracted wet-blue leather).

7.3.1 Storage Response in Relation to TCMTB Uptake

Storage response relative to actual surface TCMTB content, as determined by difference analysis of treatment float concentrations, is also illustrated in Figures 65 and 66 which show the inverse relationship between uptake and fungal growth and activity. Protective storage periods averaged over the experimental treatment temperature range for both treated and solvent-extracted wet-blue leather sample series are shown in Figures 67 and 68, depicting the curvilinear increase in storage with surface TCMTB uptake.

In the moist paper storage test, no marked difference in storage response versus TCMTB content before and after extraction was apparent. This indicates that both extractable and "bound" TCMTB components appeared to offer comparable protection (or only marginally reduced protection in the case of residual bound TCMTB) for a given uptake in the range up to 500 mg/m^2. Storage response increased relatively gradually up to the region of 200mg $TCMTB/m^2$ and then at a rapidly

increasing rate, exceeding the experimental storage limit for four months in the region of 400 to 500mg TCMTB/m^2. Extended storage protection at these higher TCMTB contents coincided with progressive emulsion instability and TCMTB and TCMTB/surfactant mixed micelle binding onto the leather fibre as previously noted.

In the case of the humidity chamber and agar nutrient tests, a qualitatively similar uptrend with increasing TCMTB surface content was noted. A lower storage response was, however, observed after solvent extraction, particularly in the case of the agar test. This altered response following solvent removal of TCMTB may be attributable to absorption of nutrient components by the embedded wet-blue leather sample from the surrounding agar in contact with the sample, thereby supporting more rapid development of fungal growth. In addition, a clear zone of fungal growth inhibition was established immediately around the treated sample. This was due to diffusion loss of active ingredient from the sample. Fungal growth occurred on the remaining medium surface. Both the agar and chamber methods represent more stringent tests since the samples are continuously exposed to fungal spore contamination during storage.

7.3.2 Effect of Treatment Temperature on Storage

In general, in the lower dosage range up to 0,1% fungicide offer corresponding with practical application levels, treatment temperature had only a relatively slight effect in increasing TCMTB uptake and the associated protective storage period (Figures 65 and 69).

In the higher dosage range above 0,1% fungicide offer, temperature effects on TCMTB uptake were more marked with a progressive increase in storage margin due to the increased binding of TCMTB/mixed micelle binding that occurred at elevated temperatures. Temperature effects on storage margins could not be readily compared quantitatively at the higher levels of fungicide offer, however, as resulting storage periods exceeded the experimental observation limit of four months.

The role of temperature in influencing uptake and storage thus appeared to be twofold in nature. At moderate dosages, temperature exerted a

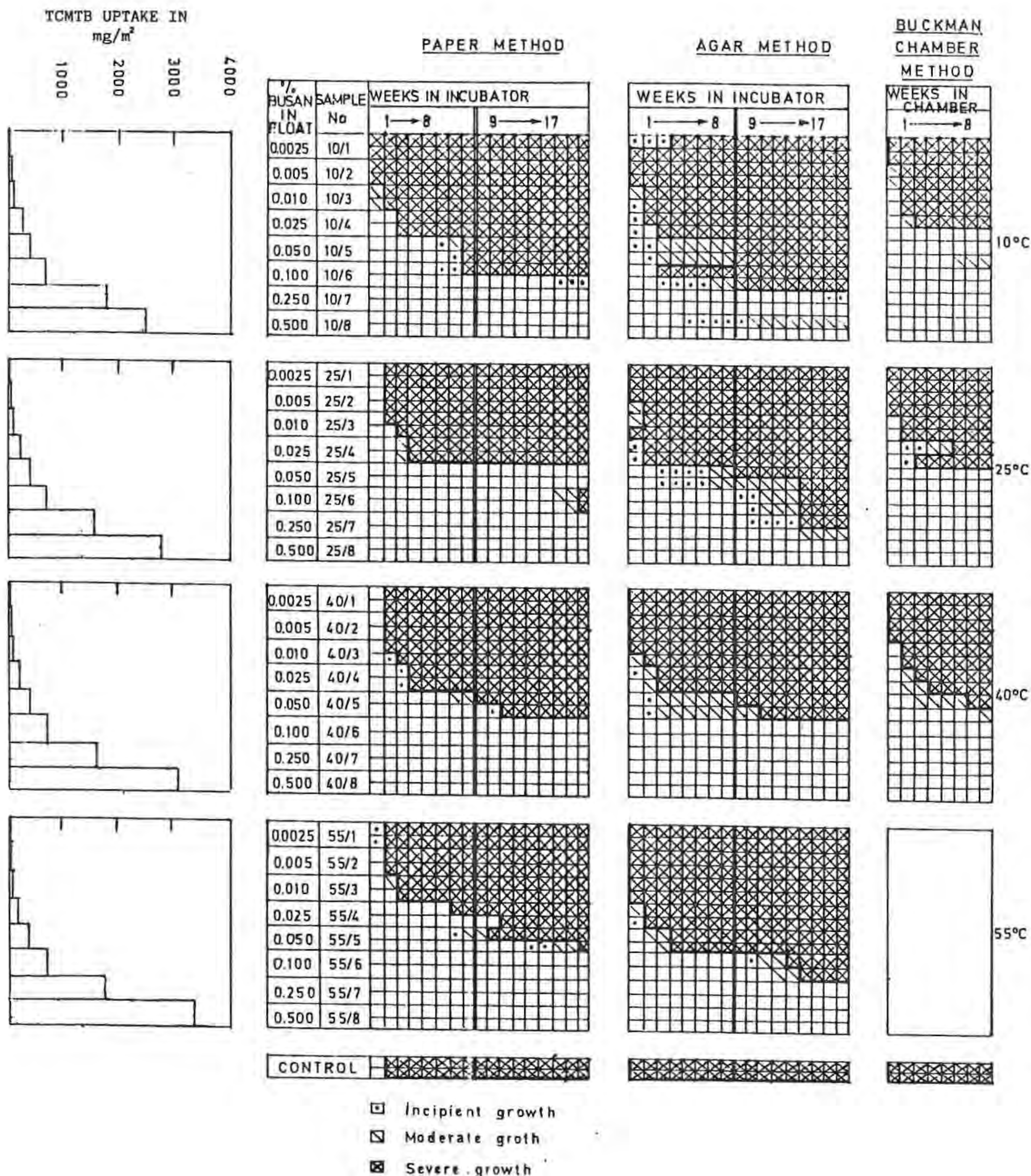

FIGURE 65 Variation in protective storage period (in weeks) with dosage offer and treatment temperature in incubation tests at 22°C (paper and agar) and 30°C (humidity chamber) on treated wet-blue leather

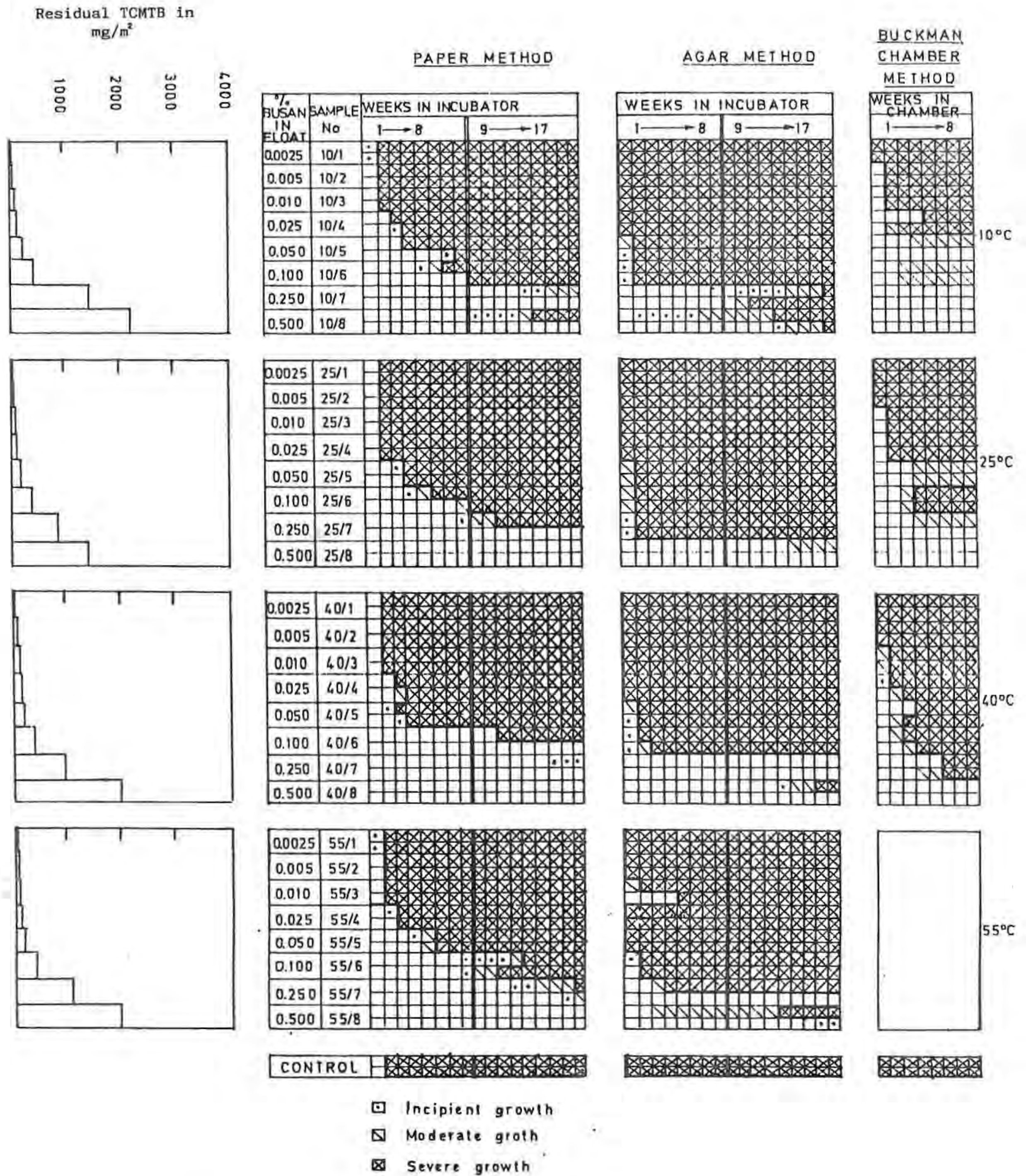

FIGURE 66 Variation in protective storage period (in weeks) with dosage offer and treatment temperature in incubation tests at 22°C (paper and agar) and 30°C (humidity chamber) on treated and solvent extracted wet-blue leather

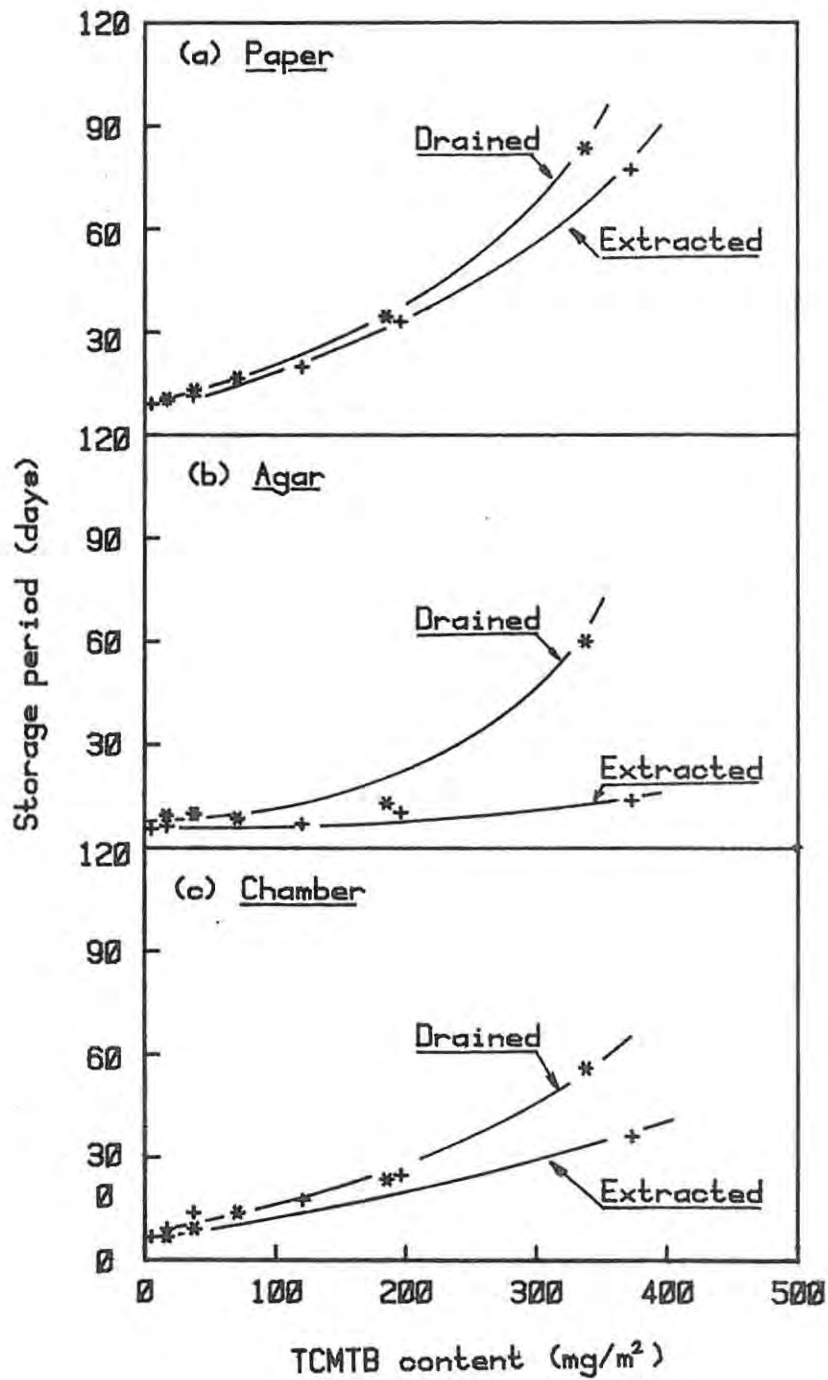

FIGURE 67 Storage period (averaged over temperature range) in relation to wet-blue leather surface uptake of TCMTB

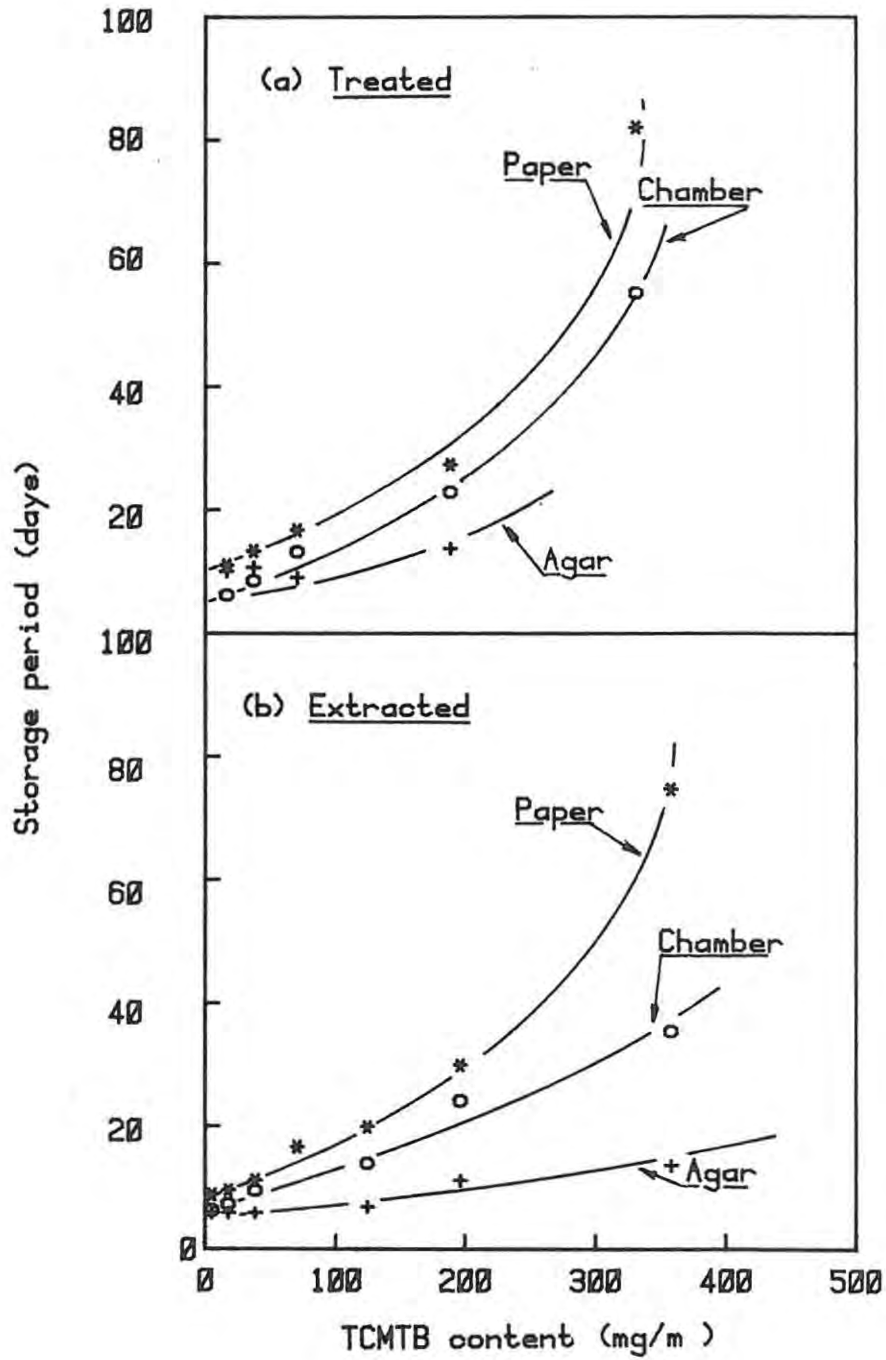

FIGURE 68 Storage period (averaged over temperature range) in various storage tests on treated and solvent extracted wet-blue leather

direct, but small effect in promoting mass-action controlled interaction and binding of TCMTB in the chromed collagen fibre. At higher fungicide dosage and application temperatures, an indirect effect of temperature in promoting the formation of larger mixed micelles which lead to increased uptake on the hide fibre surface and improved storage protection.

In the production situation, the importance of ensuring good initial emulsion dispersion of TCMTB for uniform distribution in production drum loads is underlined by current findings as well as the role of the surfactant component present in formulations in maintaining emulsion stability at elevated temperatures. Temperatures approaching 40°C to 50°C may arise from frictional heating effects in large production drums, particularly when using short floats to promote chrome exhaustion.

Present results indicate that where initial TCMTB dispersion is adequate, subsequent progressive destabilisation of the emulsion, accelerated by heat build-up in the drum, can be beneficial in promoting final uptake and exhaustion of TCMTB for enhance storage protection.

7.3.3 Comparison of Storage Assessment Methods

A qualitatively similar curvilinear storage response in relation to TCMTB content was noted in all three incubation tests on treated samples (Figure 67 and 68). The order of response in terms of storage period to establishment of definite, heavy growth on the samples was as follows:-

Paper $>$ Chamber $>$ Agar

Parallel storage testing after solvent extraction of TCMTB, showed a slight drop in storage response in the case of the paper test, with a progressively larger decrease in the case of the chamber and agar tests, respectively. The order of storage response thus remained the same after extraction, but with more marked differentiation between the test protocols.

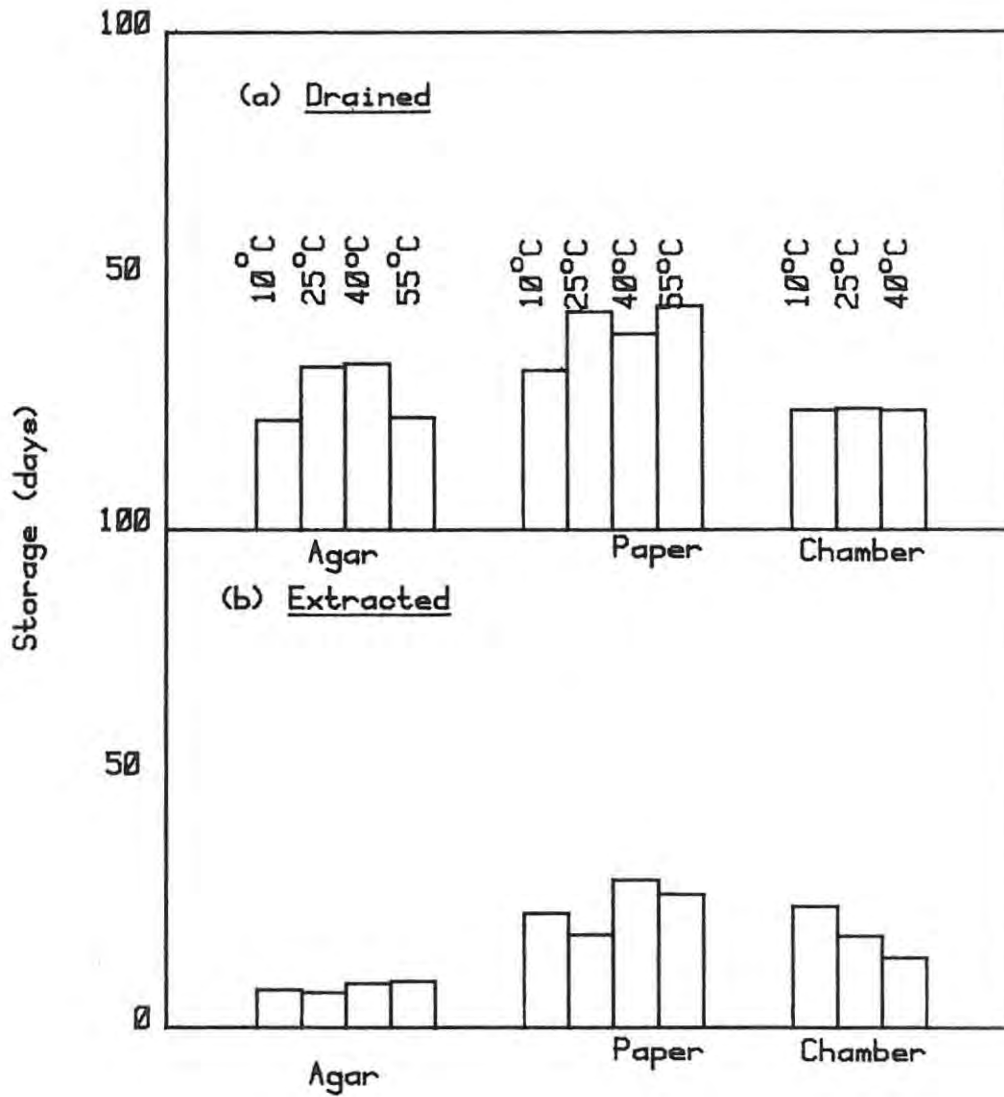

FIGURE 69 Storage period (average up to 0,1% Busan 30L offer) in relation to treatment temperature and solvent extraction

Storage performance testing by the three protocols thus indicated that while solvent extraction reduced storage response at various dosage offers, variation in storage margins with actual TCMTB content were only slightly lower after extraction in the moist paper test. This indicated that both bound and extractable TCMTB conferred more or less comparable protection. In the case of the chamber and agar tests, however, in which the samples are constantly challenged by the presence of fungal spores, removal of the extractable TCMTB component by extraction appeared to increase the susceptibility of the material to fungal growth. Greater susceptibility of the extracted wet-blue leather in the agar test may be attributable to uptake of nutrients from the agar by the embedded sample, thereby further supporting establishment of earlier fungal growth.

Various studies to date (Galloway and Cooper, 1974; Russell et al, 1985; Bugby, 1985) indicate that moist storage with re-inoculation (paper method) gives minimum dosage offers which are close to practical production requirements. From the present comparative study both the humidity chamber (30° incubation) and agar embedding procedures represent accelerated tests giving qualitatively similar storage response curves, but higher minimum dosage requirements, particularly after solvent extraction in the case of TCMTB.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The binding of the fungicides NOITZ and TCMTB to insoluble bovine skin collagen from solutions containing the non-ionic surfactant Triton X-100 is complex and showed deviations from the normal classical mass-action binding situation. At the higher concentrations of surfactant and fungicide the surfactant/fungicide mixed micelles also became bound. As the two fungicides can be regarded as polar molecules which have the potential to behave as surfactants the nature of solutions of Triton X-100/fungicide was also complex. The fungicide and surfactants in these solutions formed micelles and mixed micelles. At different relative concentrations and temperatures transition from one form of micelle/mixed micelle to other forms took place. Generally with both increase in temperature and increase in fungicide concentrations at a fixed surfactant concentration the transition from small uniform spherical or near spherical micelles to larger rod-shaped micelles occurred. The transition was dependent on the fungicide surfactant molar ratios.

The dynamic nature of the mixed micelles formed from NOITZ and Triton X-100 was illustrated by various shifts in the wavelength of the absorbance maxima in the UV range. Sudden changes in both turbidity and absolute viscosity indicated that at fixed concentrations of the fungicide and surfactant formation of large rod shaped micelle occurred. This transition point was temperature dependent.

Due to the nature of the solutions containing both TCMTB and Triton X-100 the shifts in absorbance maxima in the UV range and changes in turbidity could not be measured. Solutions of TCMTB without any Triton X-100 did, however, show absorbance wavelength shifts illustrating that TCMTB can also behave as a surfactant type molecule and form micelles. Viscosity curves which showed sudden increases at inflexion points confirmed the formation of large rod-shaped micelles of greater hydrodynamic volume in the TCMTB/Triton X-100 solutions. These transitions were also highly temperature dependent.

As these surface active rod-like mixed micelles became available for binding with the solid collagen surface due to hydrophobic effects changes in binding mechanism were observed.

The binding of both NOITZ and TCMTB from aqueous emulsion solution, as measured by difference analysis of the treatment float with both chromed and limed hide powder, was relatively rapid. Most of the fungicide was bound in the first hour, when surfactant concentration was low and, equilibrium being established within the first six hours when the concentrations were high.

The fungicidal active ingredients NOITZ and TCMTB uptake failed to follow normal mass-action binding type isotherms approaching definite saturation limits, but increased continuously, indicating a co-operative type interaction. This increased binding was also accompanied by a drop in free surfactant in solution at the higher biocide levels which indicated that complex mixed type micelles were formed which bound to the collagen fibres. This was more pronounced in the case of the chromed hide powder demonstrating a greater affinity for the more highly charged chromed tanned collagen surface.

The binding was relatively non specific and not readily attributable to any particular amino acid sequence or protein configuration at the binding site. The binding was probably hydrophobic involving the apolar side chain residues present in collagen.

Wet-blue leather was treated with commercial formulations of the two fungicides and the storage response was assessed. NOITZ showed a linear increase in binding with wet-blue leather and at no stage was a saturation level reached. There was increased binding with increased temperature. This was due to the formation and binding of larger mixed micelles at the higher temperatures. Although the proportion of solvent extractable NOITZ decreased with increased offer, the temperature effects on extractability were relatively small.

Although there was greater binding at the higher temperatures only the paper assessment method showed increased storage protection. This was due to the presence and greater mobility of fungicide from the large

mixed micelles which could more easily migrate onto the PDA surface leaving the wet-blue leather surface less protected. The larger mixed micelles were also more easily removed by solvent extraction resulting in less protection of the leather treated at higher temperatures. This was the case no matter which assessment method was used. When expressed on the actual surface concentration of fungicide, no marked difference in storage response before and after solvent extraction was noted. This indicated that both the irreversibly bound and the physically associated fungicide gave comparable protection. Storage protection increased relatively gradually up to a surface area concentration of 300 to 400mg NOITZ/m^2 and then rapidly thereafter.

The role of temperature during treatment in influencing uptake and storage protection was thus twofold. The higher treatment temperature promoted greater uptake of the fungicide by the wet-blue leather surface leading to enhanced storage. However the fungicide could be more easily removed which could negate any beneficial protection effects. The two assessment methods were found to be qualitatively similar although the paper method took longer to establish definite fungal growth.

For TCMTB over the lower fungicide concentration range (up to 0,1% m/m fungicide offer in 100% float) uptake followed a normal mass-action type binding isotherm increasing progressively with fungicide offer, whilst at the same time, the proportion of solvent extractable TCMTB in the wet-blue leather decreased, due in part to increasing levels of irreversible chrome complex formation. In this range temperature effects were relatively small.

At higher fungicide offers a marked increase in TCMTB uptake was observed with destabilisation of float dispersion and surface binding of TCMTB and TCMTB/surfactant mixed micelles. With increase in temperature this occurred at lower concentration and also resulted in a higher level of solvent extractability. This indicated that increased temperature promoted the formation of larger mixed fungicide/surfactant micelles.

Protective storage margins for TCMTB treated wet-blue leathers showed a curvilinear increase with dosage offer and surface uptake of TCMTB. No marked improvement in storage response due to increased treatment

temperatures was apparent for fungicide offers up to 0,05%. At higher fungicide offers (0,1% to 0,5%) a marked improvement in storage response was observed with protective periods exceeding the four month experimental limit.

Solvent extraction of the treated wet-blue leather resulted in a general reduction in surface TCMTB. The results indicated that both extractable and residual bound TCMTB components contribute to storage protection. Solvent extractability of TCMTB and resultant changes in storage protection tended to be lower in the 10°C treatments, but increased with temperature due to the greater amount of reversibly bound TCMTB and TCMTB/surfactant mixed micelles on leather fibres.

Storage response in relation to surface content of TCMTB after treatment showed a qualitatively similar curvilinear increase in the three test methods, but storage periods at various TCMTB uptakes decreased in the order,

Paper › Chamber › Agar

Storage response in relation to surface content of TCMTB after solvent extraction showed a slight decrease in the paper test, but progressively larger decreases in the chamber and agar tests, probably related to continuous sample exposure to fungal spores and nutrient diffusion into the leather in the agar test.

Results of these studies suggest that the course of fungicide uptake from emulsion dispersion is progressively dominated by interfacial phenomena, underlining the importance of the surfactant component in formulations to ensure adequate emulsion stability over the practical treatment range. Elevated temperatures in the region of 40°C can occur during the tanning process, due to frictional heating in large scale production drums. These can lead to progressive destabilisation of the dispersion before uniform distribution has occurred over the hide surface during production. Provided emulsion stability is sufficient to allow for good initial distribution and uniform uptake however,

subsequent slow emulsion destabilisation could be beneficial in promoting float exhaustion and further absorption of fungicides by the wet-blue leather surfaces.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ahel, M. and Giger, W. (1985a) Anal. Chem. 57 1577.

Ahel, M. and Giger, W. (1985b) Anal. Chem. 57 2584.

Amundsen, A.R., Whelan, J. and Bosnich, B. (1977) J. Am. Chem. Soc. 99 (20) 6370.

Andreu, J.M. (1985) Meth. Enzymol. 117 346.

Aniansson, E.A.G., Wall, S.N., Almgren, M., Hoffman, H., Kielmann, W.V., Zana, R., Lang, J. and Tondre, C. (1976) J. Phys. Chem. 80 905.

Aoki, K. and Hiramatsu, K. (1974) Anal. Biochem. 60 213.

Aranow, R.H. (1963) J. Phys. Chem. 67 556.

Arridge, R.C.G. (1977) Spectrum (U.K.) 15 (1) 47.

Atto, A.T. and Nursten, H.E. (1971a) J. Soc. Leather Technol. Chem. 55 84.

Atto, A.T. and Nursten, H.E. (1971b) J. Soc. Leather Technol. Chem. 55 163.

Atto, A.T., Fish, J.A., Olga Flint, F., Kittmer, R.A. and Nursten, H.R., (1969) J. Soc. Leather Technol. Chem. 53 400.

Attwood, D. (1968) J. Phys. Chem. 72 339.

Attwood, D. and Florence, A.T. (1983) "Surfactant Systems Their Chemistry Pharmacy and Biology", Chapman and Hall, London and N.Y.

Attwood, D. and Udeala, O.K. (1975) J. Phys. Chem. 79 889.

Bailey, A.J. (1968) Biochem. Biophys. Acta 1960 447.

Bailey, A.J. (1975) Colston Papers 26 115.

Baldine, G., Succi, M. and Varani, G. (1983) IL Nuovo Cimento 2 (4) 1965.

Barré, J. Chamouard, J.M., Houin, G. and Tillement, J.P. (1985) Clin. Chem. 31 (1) 60.

Barrie, J.A., Spencer, H.G. and Quig, A. (1975) J. Chem. Soc. Faraday Trans. 1 71 (12) 2459.

Behm, M.L. and Wagner, J.G. (1979) Ress. Comm in Chem. Path. and Pharmacol. 26 (1) 145.

Ben-Naim, A.Y. (1982) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 27.

Bentley, K.L., Thompson, K., Klebe, R.J. and Horowitz, P.M. (1985) Biotechniques 3 (5) 356.

Bergman, D.A. and Winzor, D.J. (1986) Anal. Biochem. 153 380.

Bjerrum, J. (1941) "Metal Ammine Formation in Aqueous Solution", P Haase and Sons, Copenhagen p 660.

Blake, C.C.F. and Oatley, S.J. (1977) Nature (London) 268 115.

Blatt, E. (1987) Aust. J. Chem. 40 851.

Blatt, W.F., Robinson, S.M. and Bixler, H.J. (1968) Anal. Biochem. 26 151.

Blenkiewicz, K.J. (1983) "Physical Chemistry of Leather Making", Krieger Publishing Company, Florida.

Boast, D.A. (1989) J. Soc. Leather Technol. Chem. 73 164.

Bornstein, P and Nesse, R. (1970) Arch. Biochem. Biophys. 138 443.

Botherby and Gassend (1973) Ann. N.Y. Acad. Sci. (USA) 222 668.

Bowes, J.H., Elliott, R.G. and Moss, J.A. (1953) In "Nature and Structure of Collagen", (Ed. Randall, J.T.) Butterworths, London p 199.

Bowmer, C.J. and Lindup, W.E. (1978) J. Pharm. Sci. 67(8).

Braumbaugh, E.E. and Ackers, G.K. (1971) Anal. Biochem. 41 543.

Brodersen, R., Nielsen, F., Christiansen, J.C. and Andersen, K. (1987) Eur. J. Biochem. 169 487.

Bugby, A. (1985) "Paper Presented to 1st North European Group Conference of IULTCS - Harrogat, 1985".

Bugby, A. (1987) J. Soc. Leather Technol. Chem. 71 138.

Bush, M.T. and Alvin, J.D. (1973) Ann. N.Y. Acad. Sci. (USA) 226 36.

Cann, J.R. (1978) Meth. Enzymol. 48 299.

Cardinal, J.R. and Mukerjee, P. (1978) J. Phys. Chem. 82 1614.

Carr, C.W. (1961) Phys. Methods Chem. Anal. 4 1.

Chan, A.W.K. and Crow, W.D. (1968) Aust. J. Chem. 21 2967.

Chattoraj, D.K., Birdi, K.S. and Dalsager, S.U. (1982) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 505.

Claeyssens, M., van Tilbeurgh, H. and De Bruyne, C.K. (1985) Bull. Soc. Chim. Belg. 94 2.

Coleman, J.E. (1968) J. Biol. Chem 243 4574.

Colowick, S.P. and Womack, F.C. (1969) J. Biol. Chem 244 774.

Cooper, D.R. and Russell, A.E. (1969) Clin. Orthop. 67 188.

Cooper, Von. S.M. (1991) Das Leder 8 42(6) 109.

Corti, M. and Deglorgio, V. (1981) J. Phys. Chem. 85 1442.

Couper, A. (1984) In "Surfactants", (Ed. Th. F. Tadros,) Academic Press, London p 19.

Crank, J. (1956) "The Mathematics of Diffusion", Second Ed. Claredon Press Oxford.

Crook, E.H., Fordyce, D.B. and Trebbi, G.F. (1963) J. Phys. Chem. 67 1987.

Crow, W.D. and Leonard, N.J. (1964) Tetrahedron Letters 23 1477.

Crow, W.D. and Leonard, N.J. (1965) J. Org. Chem. 30 2660.

Crow, W.D. and Gosney, I. (1966) Aust. J. Chem. 19 1693.

Crow, W.D. and Gosney, I. (1967) Aust. J. Chem. 20 2729.

Crow, W.D. and Gosney, I. (1969) Aust. J. Chem. 22 765.

Day, C.E. and Powell, J.R. (1973) Int. J. Biochem. 4 117.

Dayhoff, E. (Ed.)(1972) "Atlas of Protein Chemistry and Supplements", (1972-1976) Natl. Biomed. Research Foundation, Washington D.C.

De Bethizy, J.D., Longacre, S.L., Steigerwalt, R.B., Deckert, F.W., Moss, J.N., Hayes, A.W., Smith, J.M. and Scribner, H.E. (1986) Fd. Chem. Toxic. 24 43.

Duggleby, R.G. (1980) Eur. J. Biochem. 109 93.

Dunn, B.M. (1984) Appl. Biochem. Biotechnol. 9 261.

Edsall, J.T. and Wyman, J. (Eds.) (1958) In "Biophysical Chemistry", Vol. 1 Academic Press, N.Y. Ch 5 and 9.

Eftink, M. and Biltonen, R. (1980) Biol. Microcalorium (Ed. A.E. Beezer) 343.

Ellis, M. and Sykes, R.L. (1963) J. Am. Leather Chemists Assoc. 58 346.

Ellis, M., Shuttleworth, S.G. and Sykes, R.L. (1963) J. Am. Leather Chemists Assoc. 58 358.

Feldman, H.A. (1972) Anal. Biochem. 48 (2) 317.

Fessler, L.I., Morris, N.P., Fessler, J.H. (1975) Proc. Natl. Acad. Sci. (USA) 72 (12) 4905.

Fishman, M.L. and Eirich, F.R. (1971) J. Phys. Chem. 75 1971.

Fishman, M.L. and Eirich, F.R. (1975a) J. Phys. Chem. 25 2740.

Fishman, M.L. and Eirich, F.R. (1975b) J. Phys. Chem. 25 3135.

Fletcher, J.E. and Spector, A. (1968) Comput. Biomed. Res. 2 164.

Fournet, G. (1951) Disc. Faraday Soc. 11 121.

Fowler, W.M. and Russell, A.E. (1990) J. Soc. Leather Technol. Chem. 74 107.

Fowler, W.M., Russell, A.E., Kruger, I.H. and Pinchuck, S.C. (1987) J. Soc. Leather Technol. Chem. 71 100.

Fowler, W.M., Russell, A.E. and Whiteley, C.G. (1990) J. Am. Leather Chemists Assoc. 85 243.

Fowler, W.M., Shuttleworth, S.G. and Cooper, D.R. (1986) J. Soc. Leather Technol. Chem. 70 22.

Friedman, M.H. and McCally, R.L. (1972) Science 175 556.

Fujii, K., Tanzer, M.L. and Cooke, M. (1976) J. Mol. Biol. 106 (1) 223.

Furthmayr, H., Beil, W. and Timpl, R. (1971) FEBS Lett. 12 341.

Galley, W.C., Bouvier, M., Clas, S.D., Brown, G.R. and St-Pierre, L.E. (1988) Biopolymers 27 79.

Galloway, A.C. and Cooper, D.R. (1974) J. Soc. Leather Technol. Chem. 58 67.

Giles, C.H. (1982a) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 113.

Giles, C.H. (1982b) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 123.

Gill, S.J., Connelly, P.R., Cera, E.D. and Robert, C.H. (1988) Biophys. Chem. 30 133.

Goldstein, A. and Barrett, R.W. (1987) Mol. Pharm. 31 603.

Goodwin, J.W. (1984) In "Surfactants", (Ed. Th. F. Tadros,) Academic Press, London p 133.

Gould, B.S. (Ed.) (1968) "Treatise on Collagen" Vol. 2, Academic Press, London.

Grassman, W., Nordwig, A. and Hörmann, H. (1961) Hoppe Seyler's Z. Physiol. Chem. 323 48.

Gross, J. and Schmitt, F.O. (1948) J. Am. Leather Chem. Assoc. 43 658.

Gross, J., Highberger, J.H. and Schmitt, F.O. (1954) Proc. Natl. Acad. Sci (USA) 40 679.

Gustavson, K.H. (1925) J. Am. Leather Chemists Assoc. 20 382.

Gustavson, K.H. (1926) J. Am. Leather Chemists Assoc. 21 22.

Gustavson, K.H. (1947) J. Am. Leather Chemists Assoc. 42 201.

Gustavson, K.H. (1952a) J. Soc. Leather Technol. Chem. 47 425.

Gustavson, K.H. (1952b) J. Am. Chem. Soc. 74 4608.

Gustavson, K.H. (1956) "The Chemistry and Reactivity of Collagen", Academic Press, N.Y.

Hannig, K. and Nordwig, A. (1967) in "Treatise on Collagen", (Ed. G.N. Ramachandran) Academic Press, London Ch 2.

Harlan, J.W. and Fairheller, S.H. (1977) Adv. Exp. Med. Biol. 86A 425.

Harris, S.J. and Winzor, D.J. (1988) Anal. Biochem. 169 319.

Hartley, G.S. (1949) Nature 163 767.

Heald, A.F. and Sampat, M.S. (1978) Annals. Clin. Libr. Sci. 8 239.

Heidemann, E. (1982) J. Soc. Leather Technol. Chem. 66 21.

Heidemann, E. and Fuhren, P. (1962) Das Leder 13 108.

Heller, W. and Klevens, H.B. (1946) J. Chem. Phys. 14 567.

Highberger, J.H., Gross, J. and Schmitt, F.O. (1950) J. Am. Chem. Soc. 72 3321.

Highberger, J.H., Gross, J. and Schmitt, F.O. (1951) Proc. Natl. Acad. Sci. (USA) 37 286.

Hoch, H. and Miller, P.O. (1966) Anal. Chem. 38 658.

Hodge, A.J. and Schmitt, F.O. (1960) Proc. Natl. Acad. Sci. (USA) 46 186.

Hodge, A.J., Petruska, J.A. and Bailey, A.J. (1965) in "Structure and Function of Connective and Skeletal Tissue", (Eds. S. Fitton-Jackson, R. Harkness, S. Partridge and G. Tristram) Butterworth, London.

Hiramatsu, K., Ueda, C., Iwata, K., Arikawa, K. and Aoki, K. (1977) Bull. Chem. Soc. Japan 50 363.

Hubble, J. (1987) Biotechnol. and Bioeng. 30 208.

Hummel, J.P. and Dreyer, W.J. (1962) Biochem. Biophys. Acta 63 530.

Ishida, T., Horiike, K., Tojo, H. and Nozaki, M. (1988) J. Theor. Biol. 130 49.

Jackson, S.D. (1978) Int. Rev. Biochem. 18 233.

Judis, J. (1980) J. Pharm. Sci. 66 802.

Kanfer, I. (1975) Ph.D Thesis, Rhodes University, Grahamstown, South Africa.

Kawalczykowski, S.C., Paul, L.S., Lonberg, N., Newport, J.W., McSwiggen, J.A. and von Hippel, P.H. (1986) Biochemistry 25 1226.

Kido, H., Vita, A. and Horecker, B.L. (1985) "Meth. Enzymol. 117 342.

Kirdani, R.Y., Priore, L., Murphy, G.P. and Sandberg, A.A. (1979) Prog. in Clinical and Biol. Res. 33 145.

Klotz, I.M. (1946) Arch. Biochem. 9 109.

Klotz, I.M. (1953) Ann. N.Y. Acad. Sci. (USA) 226 18.

Klotz, I.M., Walker, F.M. and Pivan, R.B. (1946) J. Am. Chem. Soc. 68 1486.

Klotz, I.M. and Hunston, D.L. (1971) Biochemistry 10 (16) 3065.

Klotz, I.M. (1974) Accounts of Chemical Research 7 162.

Klotz, I.M. and Hunston, D.L. (1979) Arch. Biochem. Biophys. 193 (2) 314.

Klotz, I.M. (1985) Quarterly Rev. Biophys. 18 (3) 227.

Kresheck, G.C., Kale, K. and Erman, J. (1982) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 677.

Kuchel, P.W. and Dalziel, K. (1980) J. Theor. Biol. 85 (3) 497.

Kushner, L.M. and Hubbard, W.D. (1954) J. Phys. Chem. 58 1163.

Lamb, N.C.J. (1981) J. Soc. Leather Technol. Chem. 65 120.

Lecureuil, N. Lejeunne, B. and Crouzat-Reynes, G. (1973) J. Chimie Physique 70 (5) 782.

Lecureuil, N., Trochet, J., Garreau, L. Crouzat-Reynes, G. and Lecureuil, M. (1986) Anal. Biochem. 159 396.

Leibner, J.E. and Jacobus, J. (1977) J. Phys. Chem. 81 130.

Leppert, A. (1987) Private Communication.

Levy, J.F., Lewis, S.N. and Prentiss, W.C. (1974) J. Am. Leather Chem. Assoc. 69 280.

Lewis, S.N., Miller, G.A., Hausman, M. and Szamborski, E.C. (1971a) J. Heterocyclic Chem. 8 571.

Lewis, S.N., Miller, G.A., Szamborski, E.C. and Hausman, M. (1971b) J. Heterocyclic Chem. 8 587.

Liao, J.F. and Oehme, K. (1980) Vet. and Human Toxicol. 22 (3) 151.

Lin, I.J. and Zimmels, Y. (1982) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 455.

Lindman, B. (1984) In "Surfactants", (Ed. Th. F. Tadros,) Academic Press, London p 83.

Lindman, B. and Wennerström (1982) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 3.

Lineweaver, H. and Burke, D. (1934) J. Am. Chem. Soc. 56 658.

Macheras, P.E. and Koupparis, M.A. (1986) Analytica Chemica Acta 185 65.

Mandal, A.B. and Moulik, S.P. (1982) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 521.

Mandal, A.B., Ray, S., Biswas, A.M. and Moulik, S.P. (1980) J. Phys. Chem. 84 856.

Mandal, A.B., Kanthimathi, M., Govindaraju, K. and Ramaswamy, D. (1983) J. Soc. Leather Technol. Chem. 67 147.

Mandal, A.B., Ramesh, D.V. and Dhar, S.C. (1987) Eur. J. Biochem. 169 617.

Masullo, M., Tecce, M.C., Palumbo, G., De Vendittis, E. and Bocchini, V. (1986) Bull. Mol. Biol. Med. 11 93.

Matissek, R. and Wittkowski, R. (1984) Fres. Z. Anal. Chem. 318 414.

Matissek, R., Harper, B. and Lehnguth, R. (1985) Fres. Z. Anal. Chem. 322 465.

Matissek, R. and Lehnguth, R. (1987) Fres. Z. Anal. Chem. 328 108.

Matissek, R. and Lehnguth, R. (1988) Fres. Z. Anal. Chem. 332 270.

McClain, P.E. (1977) Adv. Exp. Med. Biol. 86B 603.

McElray, J.C. and D'Arcy, P.F. (1979) J. Pharm. Methods 2 315.

McElray, J.C. and D'Arcy, P.F. (1980) Int. J. Pharm. 6 34 205.

McLaughlin, G.D. and Theis, E.R. (1945) "The Chemistry of Leather Manufacture", Reinhold Publ. Scranton, PA U.S.A.

McPhie, P. (1971) Meth. Enzymol. 22 23.

Melikhova, E.M., Kurochkin, I.N., Zaitsev, S.V. and Varfolomeev, S.D. (1988) Anal. Biochem. 175 507.

Meyer, M.C. and Guttman, D.E. (1968) J. Pharm. Sci. 57 1627.

Meyer, M.C. and Guttman, D.E. (1970a) J. Pharm. Sci. 59 33.

Mihialova, D. and Russeva, V. (1978) Proc. 11th Int. Symp. IUPAC Chemistry of Natural Products Series 1 49.

Miller, E.J. and Matukas, V.J. (1969) Proc. Natl. Acad. Sci. (USA) 64 1264.

Miller, E.J. and Matukas, E.D. (1974) Fed. Proc. 33 1197.

Miller, G.A. and Hausman, M. (1971) J. Heterocyclic Chem. 8 657.

Miller, G.A., Weiler, E.D. and Hausman, M. (1971) J. Heterocyclic Chem. 8 581.

Missel, P.J., Mazer, N.A., Benedek, G.B., Young, C.Y. and Carey, M.C. (1980) J. Phys. Chem. 84 1044.

Missel, P.J., Mazer, N.A., Carey, M.C. and Benedek, G.B. (1982) In "Solution Behavior of Surfactants", (Eds. K.L. Mittal and E.J. Fendler) Plenum Press, N.Y. and London p 373.

Mitchell, J.W. (1987) J. Am. Leather Chem. Assoc. 82 372.

Mittal, K.L. (Ed.) (1977) "Micellization Solubilization and Micro-emulsions", Vols. 1 and 2, Plenum Press, New York.

Mittal, K.L. (Ed) (1979) "Solution Chemistry of Surfactants", Vols. 1 and 2, Plenum Press, New York.

Mittal, K.L. and Fendler, E.J. (Eds) (1982) "Solution Behaviour of Surfactants", Vols. 1 and 2, Plenum Press, New York.

Mittal, K.L. and Lindman, B. (Eds) (1983) "Surfactants in Solution", Vols. 1 - 3, Plenum Press, New York.

Monad, J., Changeaux, J.P. and Jacob, F. (1963) J. Mol. Biol. 6 306.

Mukerjee, P. (1971) J. Phys. Chem. 75 1528.

Mukerjee, P. (1972) J. Phys. Chem. 76 565.

Mukerjee, P. and Cardinal J.R. (1978) J. Phys. Chem. 82 1620.

Mukerjee, P. (1980) Pure and Appl. Chem. 52 1317.

Munson, R.J. and Rodbard, D. (1980) Anal. Biochem. 107 220.

Myers, A.C., Kovach, J.S. and Vuk-Pavlović, S. (1987) J. Biol. Chem. 262 6494.

Nakano, N.I., Takayuki, O., Sato, N., Shimamori, Y. and Yamaguchi, S. (1979) Chem. Pharm. Bull. 27 (9) 2048.

Nattrass, M. (1986) Private Communication.

Nour, S. and Vallner, J.J. (1984) Analytica Chimica Acta 162 323.

Oberfelder, R.W. and Lee, J.C. (1985) Meth. Enzymol. 117 381.

Olsen, B.R., Alpwer, R. and Kefalides, N.A. (1973) Eur. J. Biochem. 38 220.

Ottewill, R.H. (1984) In "Surfactants", (Ed.Th. F. Tadros.) Academic Press, London p 1.

Overbreek, J.T.G., De Bruyn, P.L. and Verhoeckx, F. (1984) In "Surfactants", (Ed. Th. F. Tadros) Academic Press, London p 111.

Paradies, H.H. (1980) J. Phys. Chem. 84 599.

Pederson, J.B. and Pederson, S.M. (1985) Biophys. Chem. 22 255.

Pederson, J.B. and Pederson, S.M. (1988) Biophys. Chem. 32 79.

Peters, F. and Pingoud, A. (1979) Int. J. Biomed. Comput. 5 401.

Philippoff, W. (1950) J. Colloid Sci. 5 169.

Piez, K.A. (1980) Dev. Biochem. 15 143.

Pinto, J.S. and Bentley, J.P. (1974) Biochem. Biophys. Acta 354 254.

Podo, F., Ray, A. and Némethy, G. (1973) J. Am. Chem. Soc. 95 6164.

Pohl, F.M. (1978) FEBS Symp. 58 3.

Prentiss, W.C. and Sigafoos, C.R. (1979) J. Am. Leather Chem. Assoc. 74 322.

Prince, L.M. (Ed) (1977) "Microemulsions: Theory and Practice", Academic Press, New York.

Prockop, D.J., Dehm, P., Olsen, B.R., Berg, R.A. and Grant, M.E. (1973) In "Biology of the Fibroblast," (Eds. Kulonen, E. and Pikkarainen, J.) Academic Press, Londonp 311.

Rachidy, E. and Niazi, S. (1978) J. Pharm. Sci. 67 (7) 967.

Ramachandran, G.N., Kartha, G. (1955) Nature (London) 176 593.

Ramachandran, G.N. (Ed.) (1967) "Treatise on Collagen," Academic Press, London.

Ramachandran, G.N. and Reddi, A.H. (Eds.) (1976) "Biochemistry of Collagen", Plenum Press, London.

Ramachandran, G.N. (1988) Int. J. Peptide Prot. Res. 31 1.

Ray, A. and Némethy, G. (1971) J. Am. Chem. Soc. 93 6787.

Ribeiro, A.A. and Dennis, E.A. (1975) Biochem. 14 3746.

Ribeiro, A.A. and Dennis, E.A. (1976) J. Phys. Chem. 80 1746.

Ribeiro, A.A. and Dennis, E.A. (1977) J. Phys. Chem. 81 957.

Rich, A. and Crick, F.H.C. (1955) Nature (London) 176 593.

Robertson, J.S. and Madsen, B.W. (1974) J. Pharm. Sci. 62 234.

Robson, J.R. and Dennis, E.A. (1977) J. Phys. Chem. 81 1075.

Robson, J.R. and Dennis, E.A. (1978) Biochem. Biophys. Acta 508 513.

Roy, K.B. and Miles, H.T. (1982) Biochemistry 21 57.

Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. (Eds.) (1987) "Principles and Practice of Disinfection, Preservation and Sterilisation", Blackwell Sc. Publ., Oxford.

Russell, A.E., Pinchuck, S. and Cooper, D.R. (1985) J. Soc. Leather Technol. Chem. 69 135.

Saroff, H.A. (1989) Anal. Biochem. 176 161.

Sato, H., Honoré, B. and Brodensen, R. (1988) Arch. Biochem. Biophys. 260 (2) 811.

Scatchard, G. (1949) Ann. N.Y. Acad. Sci. (USA) 51 660.

Schmitt, F.O., Gross, J. and Highberger, J.H. (1953) Proc. Natl. Acad. Sci. (USA) 34 459.

Schmitt, F.O., Levine, L., Drake, M.P., Rubin, A.L., Pfahl, D. and Davidson, P.F. (1964) Proc. Natl. Acad. Sci. (USA) 51 493.

Schulman, J.H., Stoeckenius, W. and Prince, L.M. (1959) J. Phys. Chem. 63 1677.

Shen, D. and Gibaldi, M. (1974) J. Pharm. Sci. 63 (11) 1698

Shuttleworth, S.G. (1950) J. Soc. Leather Technol. Chem. 34 410.

Shuttleworth, S.G. (1958) In "The Chemistry and Technology of Leather", A.C.S. Monograph No. 134, 281.

Shuttleworth, S.G. (1964) "Symposium on Recent Advances in Mineral Tannages", Central Leather Research Institute p 12.

Shuttleworth, S.G. and Sykes, R.L. (1959) J. Am. Leather Chemists Assoc. 54 259.

Shuttleworth, S.G. and Sykes, R.L. (1960) J. Am. Leather Chemists Assoc. 55 124.

Silhavy, T.J., Smelcman, S., Boos, W. and Schwartz, M. (1975) Proc. Natl. Acad. Sci. (USA) 72 (6) 2120.

Sklar, L.A. (1987) Ann. Rev. Biophys. Biophys. Chem. 16 479.

SLTC (1965) (Society of Leather Technicians and Chemists) Official Methods of Analyses. Method SLT 3 Testing of Hide Powder. 4th Revised Edition.

Sladen, J.B. (1977) J. Soc. Leather Technol. Chem. 61 17.

Sophianopoulous, J.A., Durham, S.J., Sophianopoulous, A.J., Ragsdale, H.L. and Cropper, W.P. (1978) Arch. Biochem. Biophys 187 (1) 132.

Sophianopoulous, A.J. and Sophianopoulous, J.A. (1985) Meth. Enzymol. 117 354.

Sparrow, N.A. (1982) Ph.D Thesis, Rhodes University, Grahamstown, South Africa.

Sparrow, N.A., Russell, A.E. and Glasser, L. (1982) Anal. Biochem. 123 255.

Sprague, E.D., Larabee, C.E. and Halsall, H.B. (1980) Anal. Biochem. 101 75.

Stankowski, S. (1983) Biochimica et Biophysica Acta 735 341.

Stankowski, S. (1984) Biochimica et Biophysica Acta 777 167.

Stark, M., Rauterberg, J. and Kühn, K. (1971) FEBS Lett. 13 101.

Steiner, R.F. (1980) Molecular and Cellular Biochemstry 31 5.

Steinhardt, J. and Reynolds, J.A. (Eds.) (1969) "Multiple Equilibria in Proteins", Academic Press, N.Y.

Sukow, W.W. and Sandberg, H.E. (1974) FEBS Lett. 42 36.

Sukow, W.W., Sandberg, H.E., Lewis, E.A., Eatough, D.J. and Hansen, L.D. (1980) Biochem. 19 912.

Swarbrick, J. and Daruwala, J. (1969) J. Phys. Chem. 73 2627.

Tadros, TH.F. (1984a) In "Surfactants", (Ed. Th. F. Tadros,) Academic Press, London p 197.

Tadros, TH.F (1984b) In "Surfactants", (Ed. Th. F. Tadros,) Academic Press, London p 323.

Tadros, TH.F. (Ed) (1984) "Surfactants", Academic Press, London.

Tanford, C. (1961) "Physical Chemistry of Macromolecules", John Wiley and Sons, N.Y. p 526.

Tanford, C. (1972) J. Phys. Chem. 76 3020.

Tanford, C. (1974) J. Phys. Chem. 78 2469.

Tanford, C. (1980) "The Hydrophobic Effect Formation of Micelles and Biological Membranes", 2nd Edition John Wiley and Sons, N.Y.

Tanford, C. and Reynolds, J.A. (1976) Biochim. Biophys. Acta 457 133.

Tanford, C., Nozaki, Y. and Rohde, M.F. (1977) J. Phys. Chem. 81 1555.

Tejima, K. and Ozeki, S. (1979) Chem. Pharm. Bull. 28 (2) 585.

Thakur, A.K., Munson, P.J., Hunsten, D.L. and Rodbard, D. (1980) Anal. Biochem. 103 240.

Tipping, E., Jones, M.N. and Skinner, H.A. (1974) J. Chem. Soc. Faraday 70 1306.

Traub, W. and Piez, K.A. (1971) Adv. Prot. Chem. 25 243.

Trueb, B. Odermatt, B.F., Sahu, A.P., Spiess, M., Ruettner, J.R. and Winterhalf, K.M. (1980) Renal. Physiol. 3 23.

Undenfriend, S. (1970) "Chemistry and Molecular Biology of the Intercellular Matrix", Vol. 1 (Ed. A.E. Balazs) Academic Press, N.Y. p 371.

Undenfriend, S., Gerber, L. and Nelson, N. (1987) Anal. Biochem. 161 494.

Van Deren, J.M. and Weiss, E.F. (1978) J. Am. Leather Chem. Assoc. 73 498.

Van Deren, J.M. (1980) Private Communication.

Veis, A., Anesy, J. and Mussel, S. (1967) Arch. Biochem. Biophys. 98 104.

Viidick, A. and Vuust, J. (1980) Proc. Aarhus Symposium Fibrous Proteins, Academic Press, London.

Wang, C.A., Leavis, B.C., Horrock, W. and Gergely, J. (1981) Biochemistry 20 2439.

Weber, G. (1975) Adv. Prot. Chem. 29 1.

Whitehead, E.P. (1980a) J. Theor. Biol. 86 (1) 45.

Whitehead, E.P. (1980b) J. Theor. Biol. 87 (1) 153.

Whitlam, J.B. and Brown, R.F. (1980) Int. J. Pharmaceutics 5 49.

Whitlam, J.B. and Brown, R.F. (1981) J. Pharm. Sci. 70 (2) 146.

Wolfer, G.K. and Rippon, W.B. (1987) Clin. Chem. 33 (1) 115.

Woosely, J.T. and Muldoon, T.G. (1977) J. Steroid Biochemistry 8 625.

Yedgar, S., Barenholz, Y. and Cooper, V.G. (1974a) Biochim. Biophys. Acta 363 98.

Yedgar, S., Hertz, R. and Gatt, S. (1974b) Chem. Phys. Lipids 13 404.

Yu, N. T. (1986) Meth. Enzymol. 130 350.