# AN *IN VITRO* STUDY OF AN ALTERNATIVE ENDODONTIC BLEACHING SYSTEM

by

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## DECLARATION

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## DEDICATION

Στην αγαπιμενη μου μητερα, Ευγενια Παπουτση–Μαρουλη και στην μητερα της Σταυρουλα. Και στον πατερα μου, Νικολαο.

To my beloved mother Eugenia Papoutsis- Maroulis and her mother Stavroula.

And to my father, Nikolaos who would have been very proud.

## SUMMARY

Current techniques for intra-coronal bleaching of root filled teeth employ oxidative bleaching with hydrogen peroxide. However, concern over the potential for external cervical resorption in bleached root-filled has been expressed and recommendations have been made to limit the use of this agent (Friedman et al, 1988; Madison and Walton, 1990). In the textile industry conventional bleaching of wool and cellulose pulp also employs oxidative bleaching with hydrogen peroxide. There are many reports however of a combined oxidative-reductive bleaching process using hydrogen peroxide and thiourea, which produces improved whiteness of wool and cellulose pulp than hydrogen peroxide alone. In biological systems thiourea is a highly cell permeable scavenger of hydrogen peroxide and hydroxyl radicals (Stahl, Pan, and Longhurtst, 1993). The generation of hydroxyl radicals during bleaching of root-filled teeth has been demonstrated recently (Dahlstrom, Bridges and Heithersay, 1993). Hydroxyl radicals are extremely reactive and can destroy connective tissue products and may be a causative factor in bleaching related invasive cervical resorption (Dahlstrom, Bridges and Heithersay, 1993).

The development of a combined oxidative-reductive bleaching protocol for root-filled teeth using hydrogen peroxide and thiourea offers the possibility of producing superior bleaching and potentially may have the added benefit of removing damaging hydroxyl radicals.

The aim of the present study was to investigate the effectiveness of combining thiourea and hydrogen peroxide to bleach root-filled teeth discoloured by blood products.

In the first part of the study the effectiveness of various amine bleaching agents was compared with 30% hydrogen peroxide by quantitative and qualitative analysis of the absorption spectra of haemoglobin and methaemoglobin. The amine bleaching agents studied were 0.1 M aqueous thiourea, 0.1 M acidified thiourea and 0.1 M thiourea dioxide. The controls for the spectrophotometric studies were 0.01 M hydrochloric acid and 30% w/v hydrogen peroxide. The changes in the absorption spectra of haemoglobin and methaemoglobin produced by these agents were evaluated. In summary, 30% hydrogen peroxide rendered the haemoglobin and methaemoglobin colourless whereas the amine bleaching agents only partially decolourised these pigments in solution.

In the second part of the study extracted premolar teeth were discoloured by blood using an adaptation of the technique of Freccia and Peters (1982). Stained dentin samples were then subjected to 23 different bleaching regimens. The change in the colour of the blood-stained dentin samples was measured at each stage of the bleaching process with a photometer and a reflection densitometer. Comparisons of the different bleaching treatments was made using a method of Least Significant Difference and/or Analysis of Variance. These methods were applied separately to the data obtained using the photometer and that obtained using the reflection densitometer.

It was concluded that the bleaching regimens which employed the sequential use of acidified thiourea or aqueous thiourea and 30% hydrogen peroxide were at least as effective at bleaching blood-stained dentin as 30% hydrogen peroxide alone. Furthermore the reducing agent thiourea dioxide alone was as effective at bleaching blood-stained dentin as 30% hydrogen peroxide.

The recognition that bleaching discoloured teeth is a chemical process which can be achieved by both reducing agents and oxidising agents offers the possibility of developing new clinical bleaching protocols.

## ACKNOWLEDGEMENTS

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# 1 INTRODUCTION

Bleaching of both vital and root filled teeth has been practised for over 100 years. The first agent reported to bleach root filled teeth was chlorinated lime (Haywood 1992). This was superseded by hydrogen peroxide, introduced in 1877 by A.W. Harlan. In 1924 Prinz combined the use of hydrogen peroxide and sodium perborate (as cited in Mac Isaac and Hoen, 1994). In the following three decades the interest in bleaching appeared to wane but in the 1960's bleaching, as a treatment modality for discoloured root filled teeth, saw a resurgence.

In 1960, Nutting and Poe described the technique of sealing a cotton pellet saturated in 30% hydrogen peroxide into the pulp chamber. Subsequently Spasser (1961) substituted sodium perborate solution for hydrogen peroxide. Nutting and Poe (1963) in an attempt to improve the effectiveness of bleaching mixed 30% hydrogen peroxide with sodium perborate into a thick paste and sealed this into the pulp chamber. The paste was changed weekly until the desired lightening was achieved. Since the bleaching effect took place out of the dental office, this procedure became known as the 'walking bleach' technique. This is still widely practised today. A modification of this technique is the use of powdered urea peroxide mixed into a thick paste with water and sealed into the pulp chamber.

An alternative to the walking bleach technique is the thermocatalytic technique. In this technique bleaching is carried out in the dental office using 30% hydrogen peroxide activated by a heat source such as a photoflood lamp, UV light or a heated instrument.

Bleaching of root filled teeth has been held to be a safe and effective treatment. Its greatest advantage over restorative dentistry is conservation of tooth structure. This

belief was questioned in 1979 however, when Harrington and Natkin reported seven cases in which invasive cervical resorption was observed in root filled teeth which had been bleached with 30% hydrogen peroxide and heat. Other authors also reported bleaching related root resorption in root filled teeth (Montgomery, 1984; Cvek and Lindvall, 1985).

Harrington and Natkin (1979) suggested that the cause of invasive cervical resorption could be leakage of bleaching agents from the pulp chamber through patent dentinal tubules into the cervical periodontium. This could be facilitated by developmental or traumatically produced defects in the cementum layer in the cervical region of the tooth. Indeed, Rotstein, Tork, and Rivka (1991), in an *in vitro* study demonstrated the passage of hydrogen peroxide from the pulp chambers of root filled teeth to the external surface. Higher concentrations were reported when cementum defects were present in the teeth.

A pH drop which has been observed at the root surface when hydrogen peroxide is used to bleach root filled teeth has also been suggested as a probable cause (Kehoe, 1987).

Further research by Dahlstrom (1992) and Dahlstrom, Heithersay and Bridges (1996) demonstrated that hydrogen peroxide can act on haem pigment to generate hydroxyl radicals. Hydroxyl radicals are extremely reactive and can destroy connective tissue components, collagen, and hyaluronic acid as well as cell membranes and can alter deoxyribonucleic acid (DNA) (Greenwald, 1981). Hence the production of hydroxyl radicals during bleaching of teeth discoloured by blood products with 30% hydrogen peroxide may be a causative factor in the development of cervical resorption in traumatised teeth.

Whilst the factor or factors that constitute the aetiology of bleaching related resorption have not been identified, it is generally accepted that bleaching root filled teeth, particularly if heat is used to activate the hydrogen peroxide, may pose a risk to the development of invasive cervical resorption. This risk however has not been quantified and has been questioned in the literature (Heithersay, Dahlstrom and Marin, 1994).

The association between cervical resorption and bleaching root filled teeth, has led many authors to caution against the use of the thermocatalytic technique (Friedman et al, 1988, Madison and Walton, 1990; Torabinejad and Walton, 1996). Others (Smith, Cunningham and Montgomery, 1992), have condemned the use of hydrogen peroxide completely and advocate the use of sodium perborate mixed with water (as originally described by Spasser). In contrast Goldstein and Garber (1995) suggest that the use of the thermocatalytic technique is permissible provided that a suitable protective base, e.g. glass ionomer cement, is placed over the root filling in order to exclude hydrogen peroxide from the cervical periodontal tissue.

Controversy over the use of bases was raised by Mac Isaac and Hoen (1994) who in a review of the materials that have been proposed for use as intermediate bases, concluded that none is ideal and that all have a considerable potential for microleakage.

Whilst research into the aetiology of bleaching related cervical resorption is inconclusive, and the ideal base may not exist, it may be beneficial to explore alternative bleaching regimens to those traditionally used. Reports in the dental literature of research into alternative bleaching regimens is sparse. Marin (1993) in an *in vitro* study of non-traditional bleaching agents concluded that none were effective bleaching agents for blood stained dentin. The agents tested were

"Desferal", Ciba-Geigy Pharmaceuticals, and "Enzymatic Cleaner", Dr Thilo & Co. GmbH.

The primary aim of bleaching, including bleaching of root filled teeth, is the removal of unwanted colour. Chemical bleaching can be achieved by both reducing and oxidative agents. The traditional bleaching agents currently used in dentistry are hydrogen peroxide, sodium perborate or urea peroxide. Urea peroxide and sodium perborate release hydrogen peroxide. Hence irrespective of the technique used, bleaching in dentistry relies on oxidative bleaching with hydrogen peroxide.

Interestingly, the reducing agent sulphur dioxide was used in the past to succcesfully bleach root filled teeth when oxidative bleaching had failed (Prinz (1920).

In the textile industry conventional bleaching of wool and cellulose pulp also employs oxidative bleaching with hydrogen peroxide. There are many reports however of a combined oxidative-reductive bleaching process using hydrogen peroxide and thiourea, which produces a better whiteness of wool and cellulose pulp than hydrogen peroxide alone.

Thiourea also forms addition compounds with metallic salts and for this reason has been used in silver tarnish removers. In biological systems, thiourea is a highly cell permeable scavenger of hydrogen peroxide and hydroxyl radicals (Stahl, Pan and Longhurst, 1993).

The development of a combined oxidative-reductive bleaching protocol for root filled teeth using hydrogen peroxide and thiourea offers the possibility of producing

superior bleaching and potentially may have the added benefit of removing damaging hydroxyl radicals.

The purpose of the present study was to test the effectiveness of a combined thiourea hydrogen peroxide bleaching regimen for root filled teeth discoloured by blood products.

The main aims of this project were to:

1. Determine the effectiveness of various amines as bleaching agents, *in vitro,* compared with 30% hydrogen peroxideby quantitative and qualitative photometric analysis of the absorption spectra of haemoglobin and methaemoglobin.

2. Determine the efficacy of various bleaching protocols compared with 30% hydrogen peroxide in decolourising blood-stained dentin using a photometer and reflection densitometer.

# 2 COLOUR

## 2.1 What is colour?

Colour is the way that the brain recognises the different qualities of light falling on the retina (Griffiths, 1976). A discussion of colour must therefore begin with an understanding of the properties of light.

The electromagnetic spectrum includes radiation ranging from very short wavelengths such as X-rays and $\gamma$ rays to radiation of very long wavelengths such as radio waves (Fig 1). Only a very narrow portion of the total spectrum can produce a visual sensation when the radiation is incident on the eye. The limits of the visible region extend from about 400 nm to 800 nm. In the restricted sense of the word we refer to radiation in this region as light. The different qualities of light responsible for the sensation of colour are its wavelength, or equivalently, its photon energy.

If a reasonably homogeneous mixture of all wavelengths of light between 400 and 800 nm is incident on the retina of the eye, then the sensation of white is manifested. White, like black or grey is termed an achromatic colour. When white light is passed through a suitable prism or diffraction grating the beam is split up into a continuum of colours, the dominant hues occurring in the well known order: red, orange, yellow, green, blue and violet (Fig 2). The wavelength of radiation giving rise to these colours decreases in the same order, from violet to red. Thus a low energy photon gives the sensation of red, whereas a high energy photon gives the sensation of violet (Griffiths, 1976).

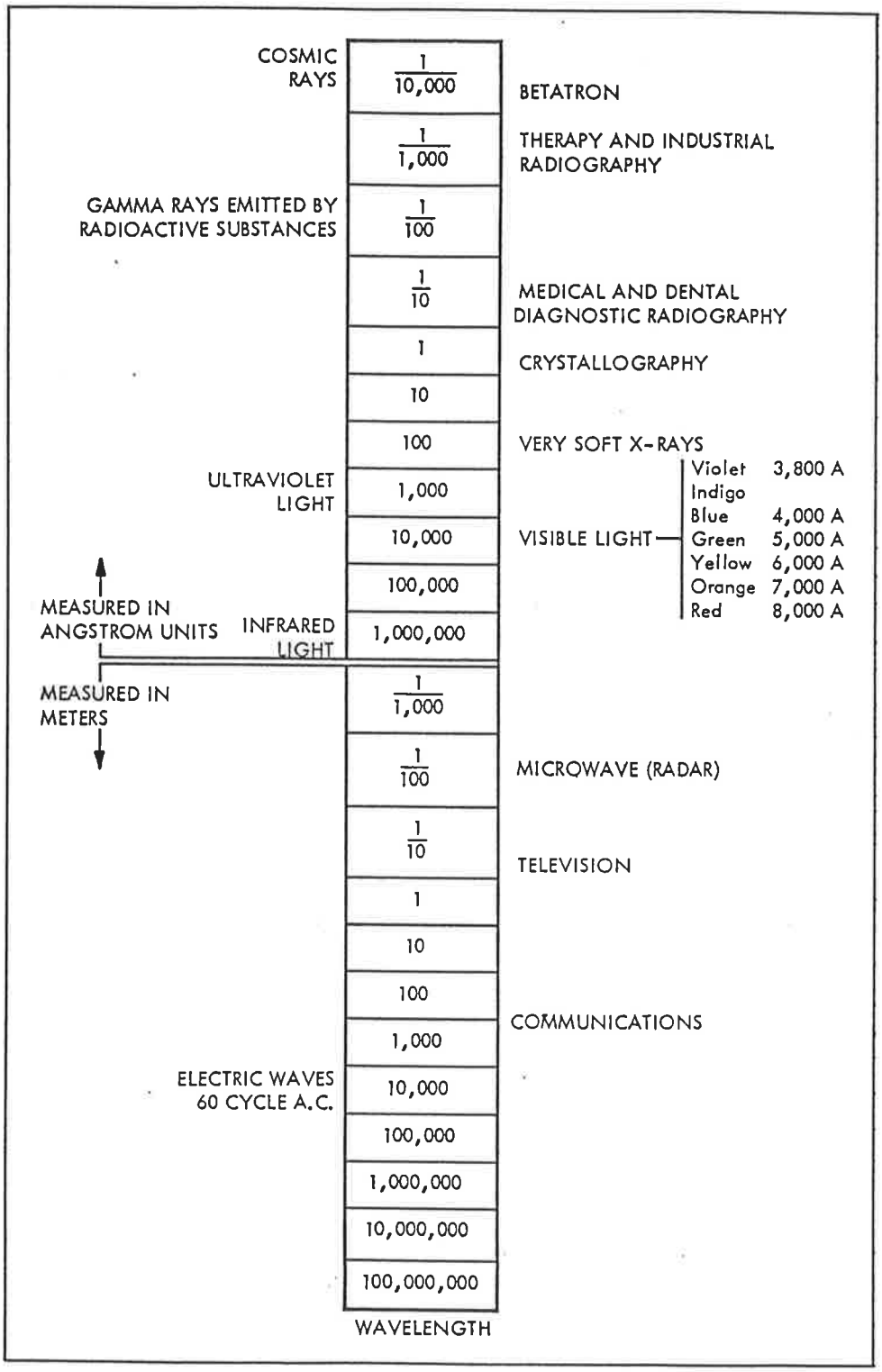



Fig 1 The electromagnetic spectrum.

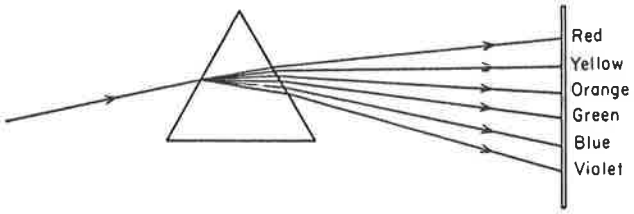



Fig 2 The dispersion of white light into the visible spectrum.

For normal purposes the visible spectrum is divided into nine broad regions, each readily distinguishable from the others, and these can be depicted in the form of a colour circle (Fig 3).

All the colours of the circle if mixed in the correct amounts give white light. White light, at least as far as the human eye is concerned, can also be produced by mixing two monochromatic radiations from any pair of opposite sectors.

A pure spectral hue can be produced by a monochromatic light wave. Alternatively the same hue can be accurately duplicated by mixing two different monochromatic radiations *e.g.* orange can be duplicated by mixing red and yellow. If three monochromatic radiations are selected so that they are well separated on the colour circle, it is possible to reproduce every possible hue by additive mixing of colours. In colour television for example the colour reproduction depends on three phosphors, giving the primary colours red, green and blue. The colour yellow on a television screen is due to hundreds of closely spaced red and green spots. The eye blends these two colours additively to give the sensation of yellow. The human eye can therefore register the same colour sensation for a wide variation in the quality of radiation (Griffiths, 1976).

Pure spectral (ie monochromatic) colours or additively mixed colours are rare in nature. The vast majority of colours in our environment arise from what is known as a subtractive colour mixing process. If one wavelength or a narrow band of wavelengths is removed from white light, the colour registered by the eye is the complementary colour of the radiation removed.

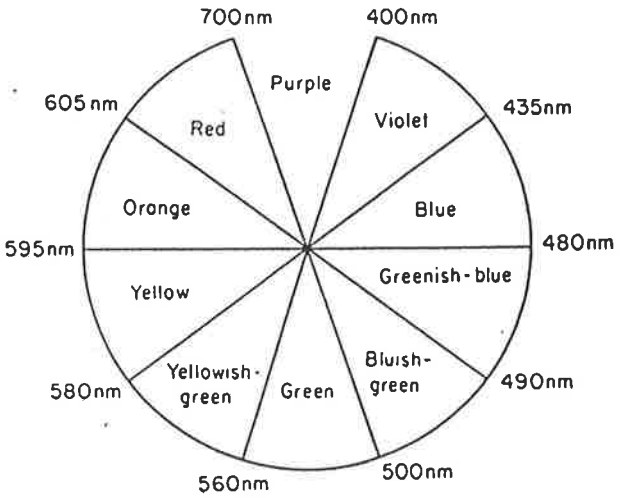



Fig 3 The colour circle. Each sector corresponds to the wavelengths of monochromatic light giving a particular hue.
(Adapted from Griffiths, 1976)

Dyes, pigments and other coloured substances appeared coloured because of the phenomenon of subtractive colour mixing; the molecules selectively filtering certain wavelengths from normal daylight (Nassau, 1983).

## 2.2 The Measurement of Colour

The complete visible absorption spectrum of a coloured object can be measured either by transmission or reflectance. Alternatively, the colour of a test object can be matched by additive colour mixing of three light primaries red, green and blue (Nassau, 1983). The test object is viewed under a standard white light source and an adjacent white surface is brought to match the test colour by irradiating it with an appropriate mixture of primary lights.

The relative intensities of the three primaries required to give the match thus afford a set of three numbers that define the colour of the test colour. These parameters are called the tristimulus values of the colour. The widely accepted international system of colour measurement, C.I.E. *(Commission Internationale de l'Eclairage)*, is essentially based on this approach.

Three properties of colour emerge from the measurement of colour in this way, hue, saturation and intensity.

*Hue*

The hue is that property which distinguishes, for example, the red from the orange and corresponds to the dominant primary or pair of primaries in the additive mixture.

*Saturation*

The saturation of colour is the measure of the proportion of the total light intensity from all three primaries that is provided by the dominant primary. For example if the red primary was completely dominant, and the green and blue non-existent, then a fully saturated red would be observed. If the background intensities of the green and blue primaries are slowly increased, relative to the red, the overall effect would be a dilution of the red colour with white, producing a pink. Thus pink would be described as a red of low saturation. This procedure would not alter the hue.

*Intensity*

If the intensity of all three lights were lowered by equal amounts, the hue and saturation would remain unaltered, but the result would be a darker colour. The lightness of the colour is then altered. In the present example, a red of low lightness would be effectively brown.

## 2.3 Coloured Objects

When light interacts with a block of partly transparent substance, part of the incoming beam is reflected. The reflected light normally carries some of the colour of the object, since the light penetrates slightly into the surface. If the surface is very smooth, then a *specular* or "mirror-like" reflection occurs. If the surface has some roughness to it, there will be some *diffuse* reflected light scattered from the surface. This diffuse reflected light originates from a more intimate interaction with the object and reveals its colour more strongly than does the specular light (Nassau, 1983).

Some of the light that enters the block of partly transparent substance is absorbed, some is scattered inside the material and some is reflected at point B (Fig 4) where the residual beam exits. Conservation of energy dictates that:

Light entering = (light scattered) + (light reflected) + (light absorbed) + (light transmitted)

Some absorbed light may be converted to heat, while some may be re-emitted as fluorescence. This process of fluorescence may add to the colour.

## 2.4 The interactions of light with molecules, atoms and electrons

After part of a beam of light has been absorbed in a solid, some of the energy lost from the light may be found in increased atomic or molecular vibrations and rotations. This type of selective absorption is number 3 in the list of 15 causes of colour assembled by Nassau (1983) in Table 1. The other 14 causes of colour of Table 1 whether occuring as natural phenomena or otherwise all involve the excitation of electrons; this involves the selective absorption and emission of light, as well as its reflection, deflection and scattering.

The classification of all colour to the 15 groupings in Table 1 is to some extent arbitrary and is structured to present the essential features most simply ( Nassau, 1983).

## 2. 5 Colour caused by transition metals

The best known cause of colour is derived from transition metal compounds or impurities (see Appendix 1). This provides the colour of many of our gems, ores, paints, and pigments. The absorption of light is due to the presence of unpaired d-electrons in the ions (Fremantle, 1987).

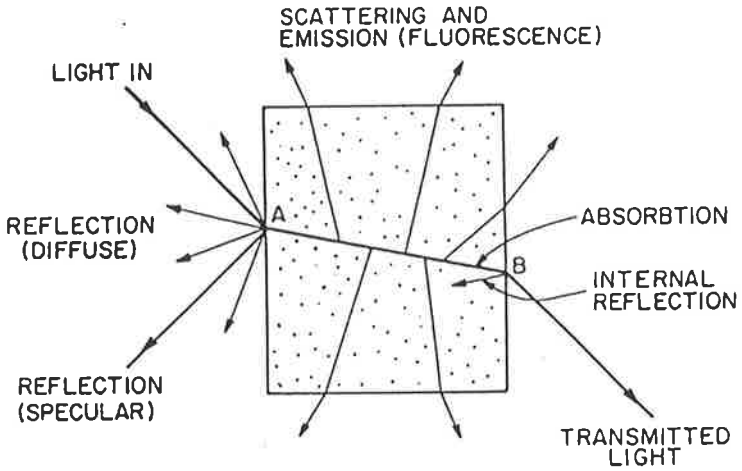



Fig 4 The behaviour of a beam of light passing through a partly transparent substance. (Adapted from Nassau, 1983)

TABLE 1

Examples of the fifteen causes of colour

*Vibrations and Simple Excitations*

1. *Incadenscence*: Flames, lamps, carbon arc, limelight
2. *Gas excitations*: Vapor lamps, lightning, auroras
3. *Vibrations and rotations*: Water, ice, iodine, blue gas flame

*Transitions Involving Ligand Field Effects*

4. *Transition metal compounds*: Pigments, turquoise, some fluorescence, lasers
5. *Transition metal impurities*: Ruby, emerald, red iron ore, some fluorescence

*Transitions between Molecular Orbitals*

6. *Organic Compounds*: Most dyes, most biological colouration
7. *Charge Transfer*: Blue sapphire, many pigments

*Transitions Involving Energy Bands*

8. *Metals*: Copper, silver, gold, iron, brass
9. *Pure Semiconductors*: Silicon, diamond
10. *Activated Semiconductors*: light emitting diodes, some lasers and phosphors
11. *Colour centres*: Amethyst

*Geometrical and Physical Optics*

12. *Disperse Refraction*: Rainbow, halos, "fire" in gemstones
13. *Scattering*: Blue sky, red sunset, blue eyes and other biologic colours
14. *Interference*: Oil slick on water, soap bubbles some biological colours
15. *Diffraction*: Opal, some biological colors, most liquid crystals

(Adapted from Nassau, 1983)

There are some conventional colour attributions such as green is caused by copper, dark blue originates from cobalt and so on. Chromium on the other hand can produce almost any colour. As Nassau (1983) states "generalisations are meaningless and detailed study is needed to establish the specific cause of colour of any unknown material."

The colour of cations of d-block metals are listed in Table 2. The colour of the salt of a d-block metal depends on the interaction of the cation and the anion. The salt may be coloured even though the anion is colourless. A good example of this is copper (II) salts. They have a variety of colour even though the anion in each case is colourless (Table 3).

All the transition metals can form complexes. Ligands in complexes can be halide ions, or many other ions and compounds such as, $CN^-$, $NH_3$, CO, $NO^+$, and even $R^-$.

The unpaired electrons in d- or f-orbitals absorb light energy by becoming protonated from their ground state energy levels to their excited state energy levels. The wavelength of the light absorbed depends on the energy difference ($\Delta E$) between the ground state and excited state (Fig 5).

The energy difference between the ground state and excited state in turn depends on the nature of the ligands in the coordination sphere of the d-block metal and the structure of the complex ion. The colour of a d-block metal ion thus also depends on the nature of the ligand and the structure of the ion. Table 4 illustrates the variety colours possible for the transition metal chromium in a ligand field.

TABLE 2

Colour of d-block ions

| Ion | Colour |
|---|---|
| $Ti^{3+}$ (aq) | purple |
| $V^{3+}$ (aq) | green |
| $Cr^{3+}$ (aq) | violet |
| $CrO_4^-$ (aq) | yellow |
| $Cr_2O_7^-$ (aq) | orange |
| $Mn^{2+}$ (aq) | pink |
| $Mn^{3+}$ (aq) | violet |
| $MnO_4^+$ (aq) | purple |
| $Fe^{2+}$ (aq) | pale green |
| $Fe^{3+}$ (aq) | yellow |
| $Co^{2+}$ (aq) | pink |
| $Ni^{2+}$ (aq) | green |
| $Cu^{2+}$ (aq) | blue |

(Adapted from Fremantle, 1987)

TABLE 3

Colours of copper salts

| Copper (II) salt | Formula | Colour |
|---|---|---|
| copper(II) ethanoate | $Cu(CH_3COO)_2$ | green |
| copper(II) sulphate | $CuSO_4$ | white |
| copper(II) sulphate-5-water | $CuSO_4.5H_2O$ | blue |
| copper(II) chloride | $CuCl_2$ | yellow |
| copper(II) chloride-2-water | $CuCl_2.2H_2O$ | blue-green |
| copper(II) nitrate-6-water | $Cu(NO_3)_2.6H_2O$ | blue |
| copper(II) sulphide | $CuS$ | black |

(Adapted from Fremantle, 1987)

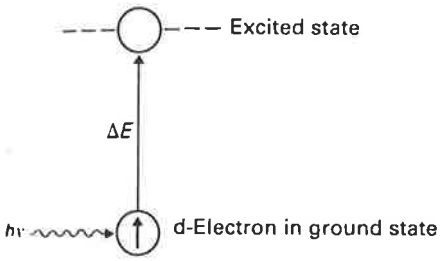



Fig 5 Excitation of a d-electron.
(Adapted from Fremantle, 1987)

TABLE 4

Ligand field colours of some chromium salts

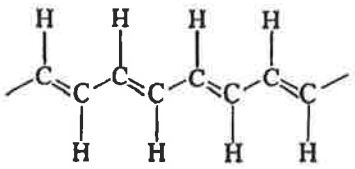
| Anion | Divalent $Cr^{II}$ | Trivalent $Cr^{III}$ |
|---|---|---|
| Bromide | White | Olive green |
| Chloride | White | Violet |
| Fluoride | Green | Green |
| Iodide | Gray | Black |
| Oxalate hydrate | Yellow | Red |
| Sulphate hydrate | Blue | Violet |

(Adapted from Nassau, 1983)

## 2.6 Colour in organic molecules

Organic compounds tend to be colourless unless a chromophore is present. A chromophore is the part of the molecule responsible for the colour of a compound.

Chromophores include carbon double bonds, particularly in conjugated systems containing alternating single and double bonds as in the carbon chain structure, shown below.



Structure 1

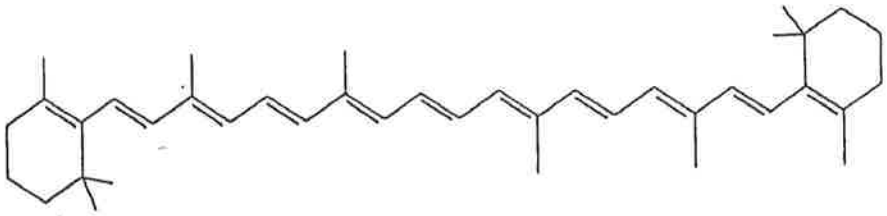
In addition there are groups which if present in a molecule will always cause colour. These are, the azo group, thio group and nitroso group the structures of which are illustrated below.

| | | |
|---|---|---|
| Azo- | —N=N— | Structure 2 |
| Thio- | >C=S | Structure 3 |
| Nitroso- | —N=O | Structure 4 |

To these basic 'colour bearing groups' can be attached auxochromes which include groups such as $-NH_2$, $NR_2$, $-NO_2$, $CH_3$, OH, OR, Br, Cl and so on. These have the property of intensifying colour if it is already present.

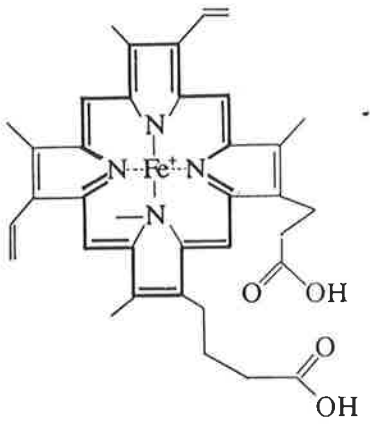
2.6.1 Noncyclic polyenes

A system of conjugated double bonds (a polyene) if large enough, will absorb light in the visible region of the spectrum and appear coloured. β carotene (responsible for the orange colour of carrots) is a typical non-cyclic conjugated polyene, (Structure 5).



Structure 5

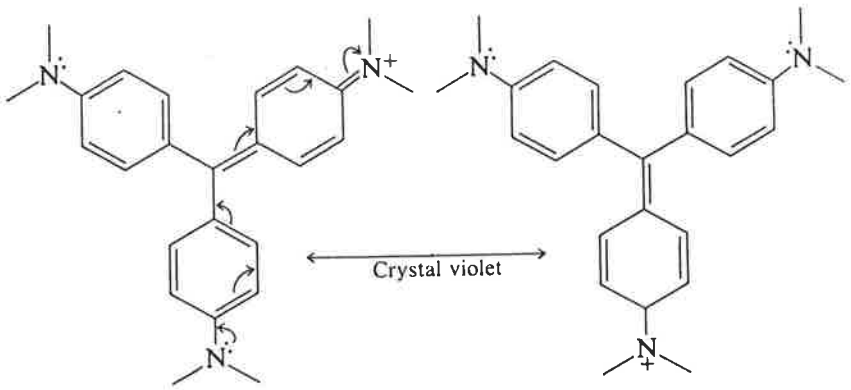
Other organic colourants comprise benzenoid systems and nonbenzenoid ring systems. Chlorophyll and the porphyrins are cyclic nonbenzenoid conjugated systems. The red colour of haem arises from the 18 member conjugated π system[1] shown by heavy lines in Structure 6 (Nassau, 1983).




Structure 6

1 The $p_z$ orbital of each carbon atom overlaps with the $p_z$ orbital of an adjacent carbon atom to form a cloud of π electrons. Hence the π component of a double bond consists of delocalised electrons which form an electron cloud that is shared by many atoms.

The origin of colour in benzenoid systems is also due to the existence of resonance forms as illustrated for crystal violet (Structure 7). The shifting of 2 non-bonding electrons from the bottom nitrogen to form a double bond with the benzene ring as shown by the lowest curved arrow results in a sequence of electron movements along the conjugated path resulting in Structure 8.




Structure 7 Structure 8

Simple organic molecules have electron transitions that absorb in the ultraviolet region of the spectrum and therefore have no colour. The presence of conjugated systems and/or the addition of auxochromic groups can move the absorption bands into the visible.

## 2.7 The measurement of tooth colour.

The human tooth cannot be considered an optimum sample for colour measurement. The surfaces of a natural tooth have variable gloss, texture and shape, making positioning of the sample for colour measurement difficult ( Lemire and Bruce, 1975).

Nevertheless in 1974 the J. M. Ney company undertook a study of the distribution and frequency of natural tooth colours. The complete visible absorption spectrum of natural anterior teeth was measured by reflectance using a General Electric Recording Spectrophotometer. It was found that natural teeth occupy a small portion of the total colour space if the latter is represented by a sphere (Fig 6).

More recent reports on the colour range of teeth include those of MacEntee and Lakowski, 1987; Solheim, 1988; Rosenstiel, Gegauff, McCafferty and Johnson, 1991. Other researchers have undertaken a study and measurement of tooth colour in an effort to develop optical methods for determining the presence or absence of dental caries (Angmar-Mansson and ten Bosch, 1987; Brinkman, ten Bosch, and Borsboom, 1988; Sündstrom, Fredricksson, Montan, Hafstrom, Jorkman, Ström, 1985).

ten Bosch and Coops (1995) measured the colour of extracted teeth using two scattering instruments; the scattering meter for bulk materials and the optical caries meter. The scattering meter uses white light and has a 45°/0° geometry *i.e.,* the sample is illuminated with two small light beams at 45° and the light returned is measured perpendicular to the surface. The optical caries meter uses monochromatic light ($\lambda$= 560 nm) and parallel fibres or illumination and measurement (0°/0° geometry). The results of this study indicate that tooth colour is predominantly dependent on the properties of dentin. Fluorescence does not contribute to normal tooth colour at all and enamel scattering only plays a minor role in the lightness of a tooth.

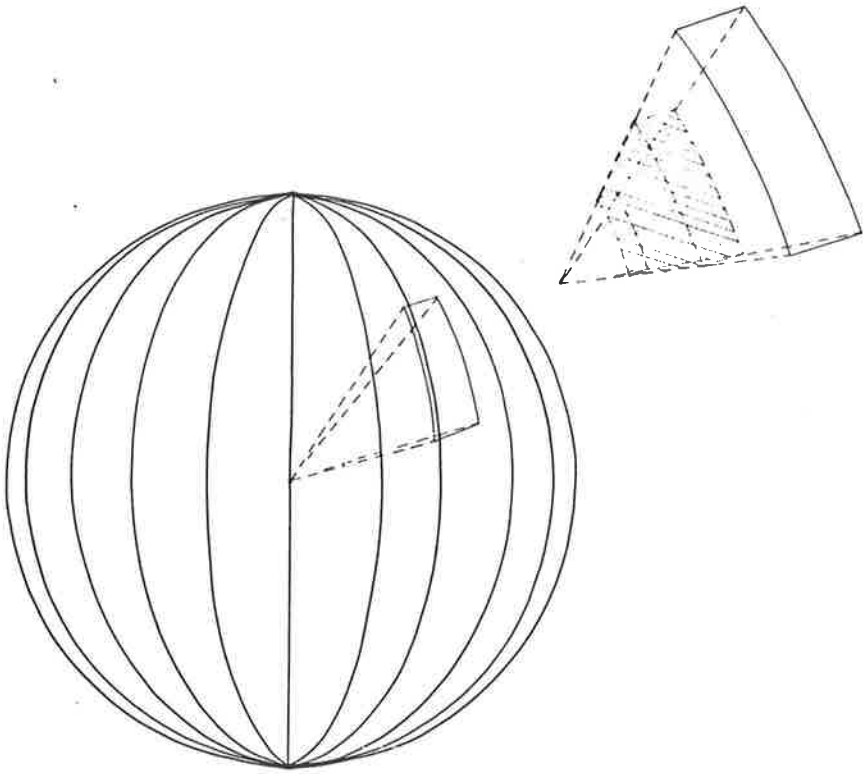


Fig 6. The sphere is a graphic representation of total colour space. A portion of the wedge (dotted lines) illustrates the portion of colour space occupied by natural teeth. (Adapted from Lemire and Bruce, 1975).

# 3 TOOTH DISCOLOURATION

Tooth discolouration is due to either extrinsic or intrinsic factors. Extrinsic discolouration results from the deposition of a film, pigment, or calculus on the surface of enamel, exposed dentin or cementum, while intrinsic discolouration refers to changes in colour affecting the internal calcified tissues of the teeth which may be of local or systemic origin (Vogel, 1975)

## 3.1 Extrinsic staining

Extrinsic stains can usually be removed by abrasive agents. The most common causes of extrinsic staining of the teeth are dark-coloured foods, beverages or tobacco.

Tobacco smoking causes a yellowish brown to black discolouration, usually in the cervical part of the teeth especially in the lingual surfaces. Chewing tobacco on the other hand frequently penetrates the enamel to produce even darker stains. Marijuana may also stain the cervical part of the teeth with characteristically well delineated slightly greenish rings (Goldstein, Haywood, Heymann, Steiner, West, 1995).

Chan, Hormati and Kerber, (1981) in an *in vitro* study of the staining capability of food substances found that coffee and soy sauce stained the dental tissues more than cola beverage and tea. The discolouration of enamel was superficial whereas that of dentin and cementum was ingressive and penetrated up to 0.5-1 mm into both of these tissues. The degree of staining also increased with time.

The discolouring effects of wine are also well known. The acid in the wine causes erosion and the exposure of dentin which is then more prone to the uptake of stain.

In addition wine contains a high concentration of tannin. Tannins may be divided into two groups: (1) derivatives of flavanols and (2) hydrolysable tannins, which are esters of a sugar, with one or more dihydroxybenzenecarboxylic acids. It is believed that penetration of tannin in complex form into exposed dentinal tubules and carious enamel may produce discolouration (Schuurs, Abraham-Inpijn, van Straalen, Sastrowijoto, 1987)

In betel chewers polyphenols play a major role in the staining of the dental hard tissues (Reichardt, Lenz, Konig, Becker, Mohr, (1985).

Extrinsic stains in enamel can be removed readily by scaling and polishing.
However stains become more resistant if the teeth have pits, grooves or enamel defects. Microcracks in the enamel may also allow the stain to enter dentin and make stain removal more difficult .

When changes in colour occur within the body of the enamel, dentin or cementum of teeth the discolouration is no longer extrinsic but is intrinsic. These discolourations can not be removed by simple polishing to restore natural tooth colour.

## 3.2 Intrinsic Discolouration

During odontogenesis, teeth may become discoloured from changes in the quality or quantity of enamel or dentin, or as a result of the incorporation of discolouring agents into the hard tissues. Discolouration of the teeth may also occur post -eruption by agents entering the hard tissues form the pulp cavity or tooth surface.

3.2.1 Intrinsic tooth discolourations with an endogenous origin are rare but occur in such conditions as phenylketonuria, porphyria, haemolytic anaemia, amelogenesis imperfecta and dentinogenesis imperfecta.

3.2.2 Common causes of intrinsic tooth discolouration with an exogenous origin:

*Systemic drugs or chemicals such as tetracycline or fluoride*

Dental fluorosis is an enamel hypoplasia found in communities where the fluoride in the drinking water is greater than one part per million. The degree of fluorosis is directly proportional to the amount of fluoride ingested. The enamel may appear with white flecks or spots in the more mild forms to mottled or pitted in more severe forms. Tooth discolouration by tetracyclines was first reported by Schwashman and Schuster in 1956. Tetracycline is able to cross the placental barrier and can affect the deciduous as well as the permanent teeth. During mineralisation tetracycline binds to the calcium to form a calcium phosphate-tetracycline complex which is most concentrated in the dentin (Hayes, Full and Pickham, 1986). The severity of the discolouration depends on the dose, duration, time of administration and type of drug administered. Tetracycline stained teeth show a bright yellow fluorescence under ultraviolet light.

*Dental caries*

Caries can appear as opaque, white halo, or grey discolouration. Bacterial degradation of food debris in areas of tooth decay may cause a deeper brown to black discolouration (Godstein and Feinman, 1995)

*Dental medicaments and materials*

Degraded tooth coloured restorations such as acrylics, glass ionomers, or composites as well as corrosion products from amalgam restorations can cause teeth to look grey and discoloured. In addition materials containing therapeutic agents eg oils, iodines, nitrates, and root canal sealers are all able to penetrate dentinal tubules and produce tooth discolouration (Goldstein and Feinman, 1995).

*Pulpal death and decomposition*

In 1920 Prinz stated that death of the pulp was the principal source of tooth discolouration and that this leads to progressive staining of the entire dentin structure. This premise has been repeated many times in the literature, including Walton, (1995), "Coloured products from the disintegration of pulpal tissue are believed to permeate the dentinal tubules and stain the dentin." The products of pulp disintegration causing the discolouration remain unidentified.

*Trauma, with extravasation of blood*

Colour changes in traumatised teeth were observed by Prinz (1920). He noted an initial pinkish hue which would turn to yellow brown and after some considerable time the tooth would take on a permanent slate grey or black colour. Prinz believed that gradual decomposition of haemoglobin and changes in its constitution produced the observed colour changes. Prinz's observations have been verified by more recent writers (Jacobsen, 1980, Andreasen, 1986). In addition, it is now recognised that in instances of moderate trauma such as luxation or extrusion transient tooth discolouration occurs and that this gradually reverses with the revascularisation of the pulp (Andreasen, 1986). Prinz (1920) also suggested that bacterial decomposition of the pulp released hydrogen sulphide which would react with the iron in haemoglobin to yield black iron sulphide. Thus in these instances iron was the most important element causing discolouration of the teeth. The release

of iron from haemoglobin and the formation of iron sulphide has been widely accepted as a cause of tooth discolouration and has been quoted often in the literature (Grossman, 1943; Frank, 1980; Howell, 1980; Freccia and Peters, 1982; Walton, 1989; Rotstein, Zalkind, Mor, Tarabeah and Friedman, 1991. However there is a lack of scientific evidence to support this belief.

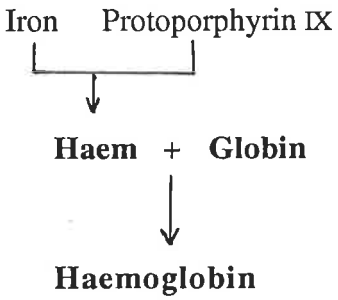
Intrinsic discolourations may be treated by one or a combination of the following techniques: full coverage restorations, labial veneering with resin or porcelain, external bleaching or internal bleaching. In a society that places a high value on appearance, tooth discolouration can have a tremendous psychological effect on the individual (Weyman and Porteus, 1963).

*"The dental profession has an obligation to treat tooth discoloration in the most conservative manner possible. In many cases, tooth bleaching is the method of choice based on conservation of tooth structure and its effectiveness in lightening tooth discoloration." (Fasanaro, 1992).*

# 4 THE CHEMISTRY OF HAEMOGLOBIN

Haemoglobin (or reduced haemoglobin) is the porphyrin-iron(II)-protein compound which gives blood its red colour. The porphyrin portion of haemoglobin is protoporphyrin IX which, combined with Fe(II), forms haem. Haem plus the protein, globin, constitutes haemoglobin (Fig 7). Haemoglobin consists of four polypeptide chains which are identical in pairs ($\alpha_1\alpha_2$ and $\beta_1\beta_2$) and four haem groups each with one atom of iron (II).

Fig 7




## 4.1 The structure of haem

As shown in Fig 8, haem or ferrous protoporphyrin is composed of an iron atom coordinated to the four rings of the porphyrin through the nitrogen atom. Haem is readily oxidised to ferric protoporphyrin. The ferric complex has one residual positive charge and is isolated as a halide, most commonly as the chloride. Haem in its isolated form is usually haemin (chloride) which becomes haematin when dissolved in alkaline solution. Ferrous protoporphyrin can form complexes with nitrogenous bases which are called haemochromes or haemochromogens (Fig 9).

Naked 'haem', the Fe-porphyrin complex without the accompanying polypeptide, is irreversibly oxidised to Fe(III) by molecular $O_2$. This reaction is prevented by the protein environment in which the haem is embedded in the haemoglobin molecule.

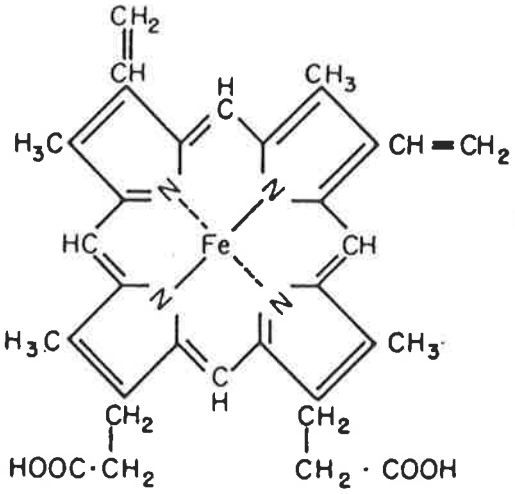



Fig 8 The structure of haem (Adapted from Ranney and Sharma, 1990)

N = Porphyrin Nitrogens which contribute 2 negative charges to the complex. N′ = Non-porphyrin Nitrogens

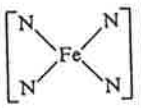
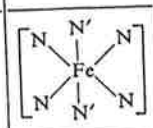
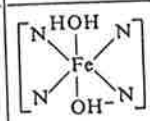
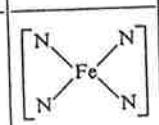
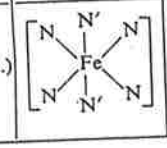
| | *Structure* | *Charge* | *Iron Atom* | |
|---|---|---|---|---|
| | | | *Valency* | *Ferrous or Ferric* |
| Haem (reduced haematin) |  | 0 | 2 | Ferrous |
| Haemochromogens |  | 0 | 2 | Ferrous |
| Haematin |  | 0 | 3 | Ferric |
| Haemin (chloride, etc.) |  | +1 | 3 | Ferric |
| Parahaematin (chloride, etc.) |  | +1 | 3 | Ferric |

Fig 9 Forms of iron protoporphyrin IX.
(Adapted from Datta and Ottoway, 1972)

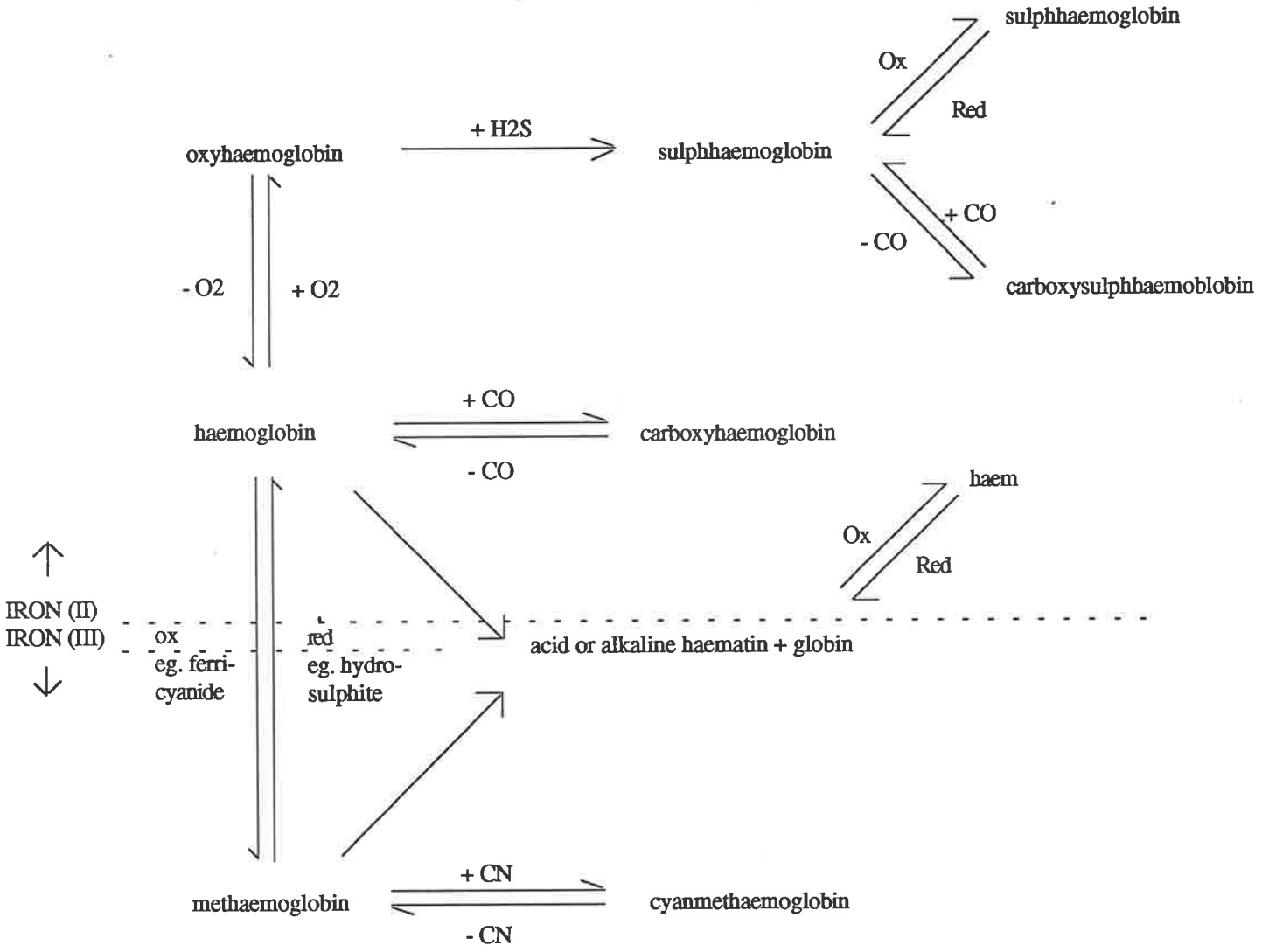



Fig 10 The interrelationships of haemoglobin and its derivatives.
(Adapted from Henry et al, 1975)

TABLE 5

Nomenclature of haemoglobin and its derivatives

| Name in old system | Lemberg & Legge and others | Pauling and Coryell | Constitution |
|---|---|---|---|
| Haemoglobin | Haemoglobin | Ferrohaemoglobin | Complex of ferrohaem and native globin |
| Oxyhaemoglobin | Oxyhaemoglobin | Oxyferrohaemoglobin | Complex of ferrohaemo-globin and $O_2$ |
| Methaemoglobin | Haemiglobin | Ferrihaemoglobin | Complex of ferrihaem and native globin |
| Haem | Haem | Ferrohaem | Ferrous porphyrin complex |
| Acid hematin | Acid hematin | Ferrihaem | Ferric porphyrin complex |
| Haemochromogen | Haemochrome | Ferrohaemochromogen | Complex of ferrohaem and nitrogenous substance |
| Parahematin | Haemichrome | Ferrihaemochromogen | Complex of ferrihaem and nitrogenous substance |
| Alkaline hematin | Alkaline hematin | Ferrihaem hydroxide | Ferrihaem plus hydroxyl ions |
| Carboxyhaemo-globin | Carboxyhaemo-globin | Carbonmonoxo-haemoglobin | Complex of ferrohaemo-globin and CO |
| Cyanmethaemo-globin | Haemoglobin-cyanide | Ferrihaemoglobin cyanide | Complex of ferrihaemo-globin and cyanide |

(Adapted from Henry et al, 1975)

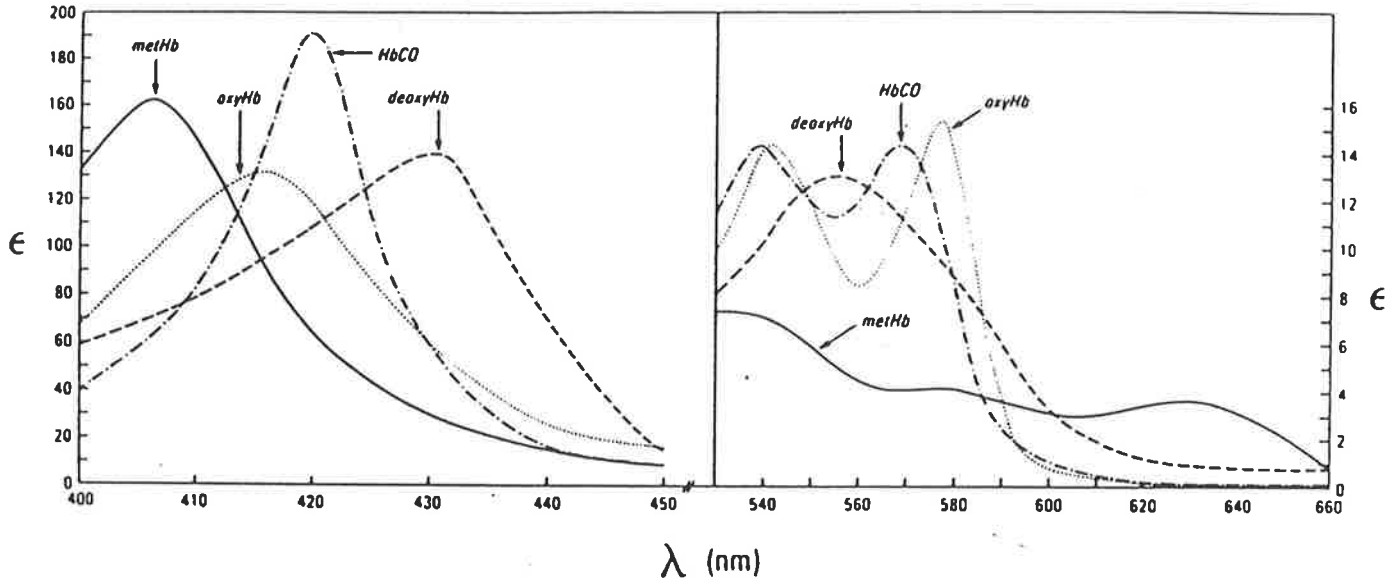



Fig 11 Absorption spectra of haemoglobin and its derivatives.
(Adapted from Ranney and Sharma, 1990)

TABLE 6

Some spectroscopic properties of haemoglobin derivatives.

Soret bands associated with the haem group are those with a Millimolar Extinction Coefficient (εmM) > 100 (see Section 7.3).

| | Wavelength (nm) | εmM | Weak Bands (nm) |
|---|---|---|---|
| Haemoglobin | 555 | 12.5 | 590 |
| | 430 | 133 | |
| | | 29.2 | |
| Oxyhaemoglobin | 541 | 13.8 | 920 |
| | 577 | 14.6 | |
| | 415 | 125 | |
| | 344 | 27 | |
| | 276 | 34.4 | |
| Carboxyhaemoglobin | 540 | 13.4 | |
| | 569 | 13.4 | |
| | 419 | 191 | |
| | 344 | 28 | |
| Methaemoglobin | 1000 | 0.8 | 581 |
| pH 6.4 | 629 | 4.4 | 540 |
| | 500 | 10 | |
| | 405 | 179 | |
| Haemichromes | 565 | 12.5 | |
| | 534 | 14.7 | |
| | 411 | 105 | |

( Adapted from Weisbluth, 1974 and Telen, 1990)

4.2.2 Oxyhaemoglobin ($HbO_2$)

Oxyhaemoglobin is bright red in colour with two sharp absorption bands in the green. On extreme dilution it gives a yellowish green colour. The spectrum is insensitive to pH between 5.5 and 10 (Datta and Ottaway, 1972).

4.2.3 Carboxyhaemoglobin (HbCO)

Carboxyhaemoglobin results from the binding of carbon monoxide to the haem iron.

The carboxyhaemoglobin content of normal blood varies considerably depending on the degree of exposure to carbon monoxide. Individuals living in a city or in rural communities have blood levels of 0.1 - 0.4 gm/100 ml whereas taxi drivers have levels 1.1 - 3.2 gm/100 ml (Lukens, 1993). The affinity of haemoglobin for carbon monoxide is 200 times that for oxygen. Thus if the partial pressure of carbon monoxide is 0.5% that of oxygen, the concentration of carboxyhaemoglobin at equilibirium will approximately equal that of haemoglobin.

The absorption spectra of carboxyhaemoglobin is very similar to that of oxyhaemoglobin. It is cherry red in colour and remains so even in dilute solution (Data and Ottaway, 1972).

4.2.4 Methaemoglobin

Oxidising agents react with haemoglobin to change the Fe(II) to Fe(III), producing methaemoglobin. This oxidation occurs stepwise ie. one Fe at a time, and thus intermediate forms exist between haemoglobin and methaemoglobin (Ranney and Sharma, 1990). Oxygen itself slowly oxidises haemoglobin. Other oxidants act more quickly (eg. ferricyanide, ozone, permanganate). Fig 12 shows the changes in the absorption spectrum as oxyhaemoglobin changes to methaemoglobin.

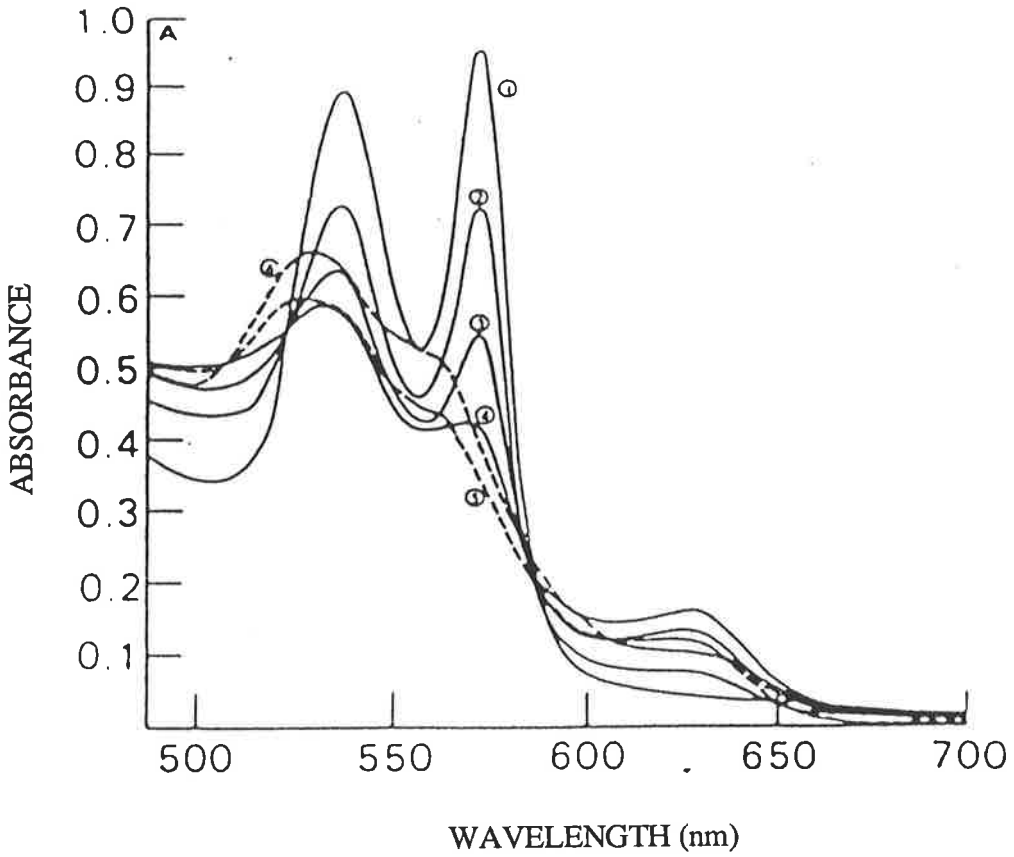



Fig 12 Changes in haemoglobin spectrum as oxyhaemoglobin changes to methaemoglobin. The numbers 1 through 6 represent curves taken in sequence as oxidation proceeds. Note in particular the appearance of the band at 631 nm and the disappearance of the band at 575 nm.
(Adapted from Ranney and Sharma, 1990)

A solution of methaemoglobin is reddish brown. Methaemoglobin may be reduced to haemoglobin by sodium dithionate (Datta and Ottaway, 1972).

In the normal red cell the methaemoglobin level is maintained at less than 7% of the total haemoglobin. This reflects a balance between its slow formation and its reduction by enzymatic pathways involving NADPH.

Methaemoglobin reacts with acids and bases. These split it into globin and either acid or alkaline haematin, with the anion of the splitting reagent occupying the third valence of the iron. Reducing agents can reverse these valency changes.

The absorption spectrum of methaemoglobin is strongly pH-dependent: At low pH a water molecule is bound to the iron and occupies the space between the ferric iron and the distal histidine. At alkaline pH, a hydroxyl ion is bound to the ferric ion (Ranney and Sharma, 1990).

### 4.2.5 Sulphhaemoglobin (sulphmethaemoglobin)

On exposure to some toxic agents, sulphhaemoglobin is formed. The iron is in the ferrous state but the oxygen affinity is about 100 times lower than that of normal haemoglobin (Lukens, 1993).

The exact structure of sulphhaemoglobin is still unknown. The sulphur is not liganded to the iron but is found in the porphyrin ring. Recent work suggests that divalent sulphur breaks the carbon-carbon double bond of the pyrrole ring and forms an episulphate bridge between the two-ring carbons (Fig 13).

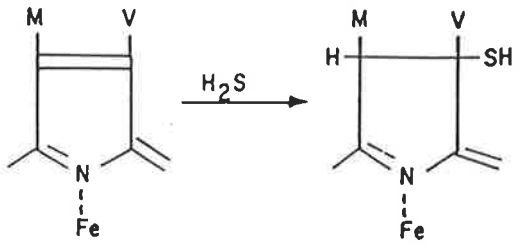



Fig 13 The probable structure of sulfhaemoglobin. One of the 4 pyrrole rings in haem is illustrated at the left. Sulphur added at the β carbon forms a thioclorin, accounting for the spectral changes characteristic of sulfhaemoglobin. (Adapted from Telen, 1990)

## 4.3 Dissociation of haemoglobin

Oxyhaemoglobin dissociates into dimers over a wide range of conditions, such as low and high pH or in strong salt solution even at neutral pH (Weissbluth, 1974).The $\alpha 1 \alpha 2 \beta 1 \beta 2$ reverts to $\alpha\beta$ dimers. The dissociation is reversible and functional haemoglobin can be reformed. Deoxyhaemoglobin (reduced haemoglobin) however rarely dissociates.

## 4.4 Oxidative denaturation of haemoglobin

Oxyhaemoglobin in solution undergoes autoxidation (Fig 14), becoming methaemoglobin ($HbFe^{3+}$). The rate of oxidation is enhanced by conditions such as increased temperature, decreased pH, presence of organic phosphate and of metal ions, and partial oxygenation of haemoglobin.

The oxidation of haemoglobin occurs in a stepwise fashion from fully reduced haemoglobin to fully oxidised haemoglobin (Fig 15). Intermediate forms are called valence hybrids. In deoxyhaemoglobin, the haem iron is in the "high spin" ferrous state, in which 6 electrons are in the outer shell, four being unpaired.

Methaemoglobin may occur *in vivo* as the result of exposure to superoxide anions:

$$2HbFe^{2+}O_2 + 2O_2^- + 4H^+ \rightarrow 2HbFe^{3+} + 3O_2 + 2H_2O$$

The formation of methaemoglobin may also result from direct reaction of reduced haemoglobin with peroxide:

$$2HbFe^{2+} + H_2O_2 \rightarrow 2HbFe^{3+}\ H_2O + O_2$$

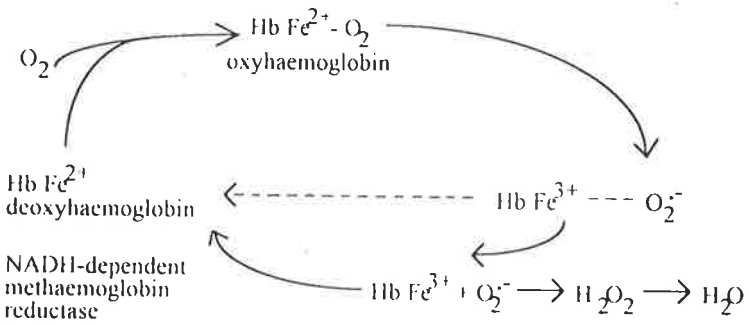



Fig 14 Haemoglobin autoxidation.

(Adapted from Rice Evans and Burdon, 1994)

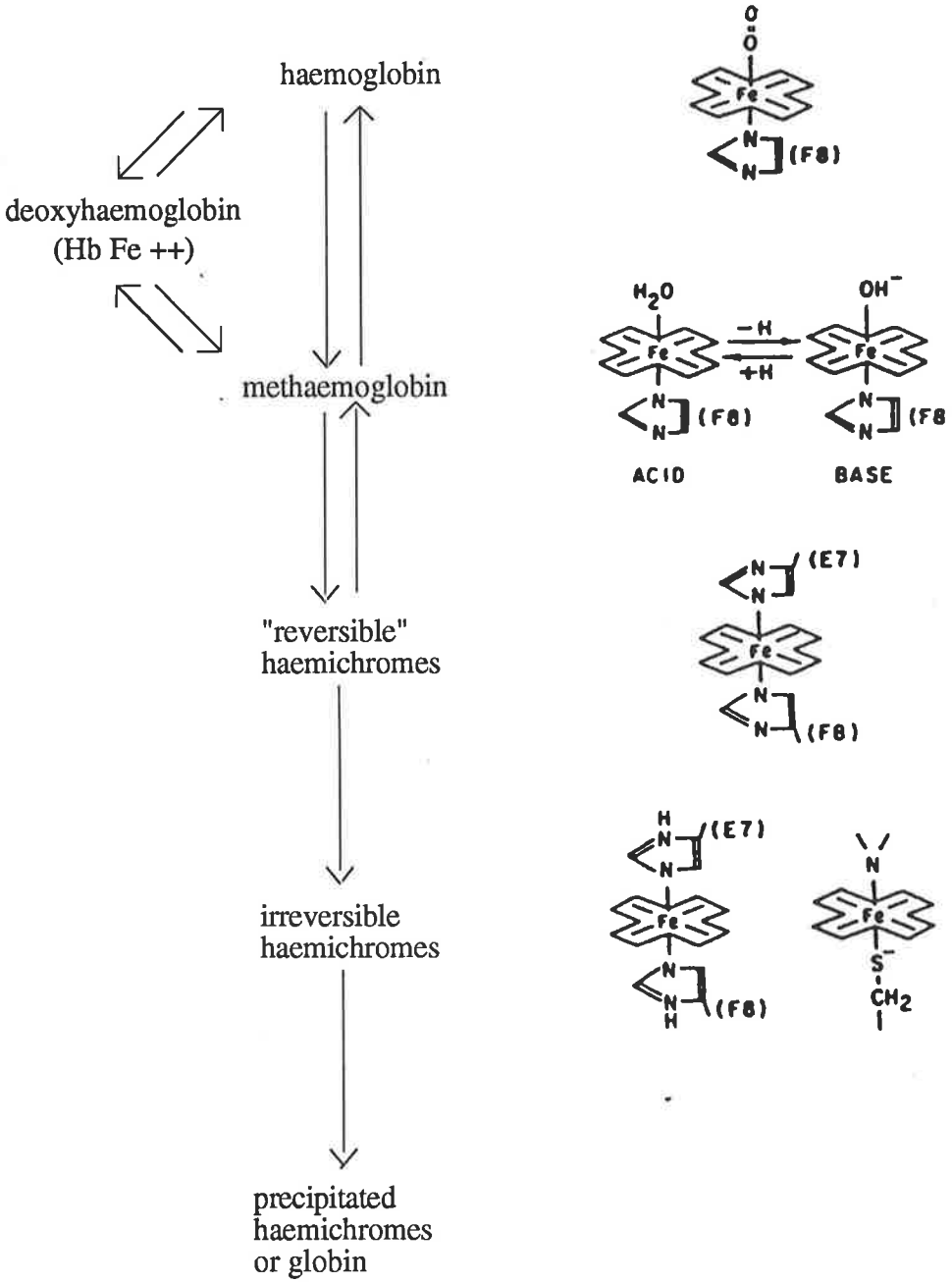



Fig 15 Stages in the oxidative denaturation of haemoglobin. Names of the stages are given on the left, proceeding from the most reduced form at the top to the most oxidised at the bottom. Partial structures are illustrated on the right. The haem group is denoted by a planar cross.
(Adapted from Telen, 1990)

As a result of these processes methaemoglobin is formed in normal cells at the rate of 0.5 to 3% per day. When methaemoglobin appears *in vivo* at concentrations greater than 15 to 20 mg/ml patients appear visibly cyanotic (Fairbanks and Beutler, 1990).

As oxidative denaturation continues, methaemoglobin is converted to derivatives known as hemichromes. The hemichromes are low spin ferric compounds with a greenish hue and characteristic spectrum. The 'irreversible' hemichromes cannot be converted back to normal haemoglobin *in vivo* or *in vitro*. These changes occur in the vicinity of the haem group. Changes are also occurring in other parts of the haemoglobin molecule. Such changes facilitate the dissociation of polypeptide chains first into $\alpha\beta$ dimers and finally into monomers. The end products of these changes are precipitated hemichromes and precipitated haem-free globin (Telen, 1990).

## 4.5 Denaturation of haemoglobin by hydrogen peroxide

Hydrogen peroxide has often been shown to induce oxidative denaturation of haemoglobin *in vitro*, whether it does so directly or by giving rise to other products, such as the hydroxyl radical is not known. The hydroxyl radical is one of the most potent redox agents known. It may be generated from the reaction of superoxide and peroxide and from peroxide directly in the presence of certain metals (Fig 16) :

$$Fe^{2+} + H_2O \rightarrow Fe^{3+} + OH^- + OH\cdot$$

The reaction of haemoglobin in the ferric, oxy- or deoxy-forms with hydrogen peroxide may also produce as intermediate products hypervalent iron species such

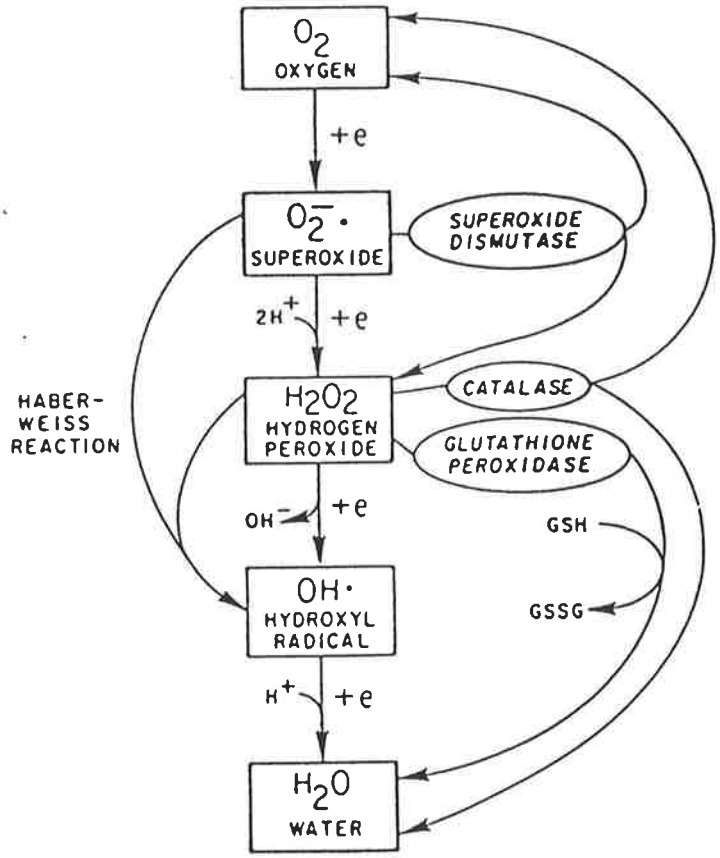



Fig 16 Steps in the univalent reduction of oxygen and enzymatic pathways affecting the intermediates. The enzymatic pathways shown on the right, provide the means for processing these intermediates without the formation of the highly reactive hydroxyl radical . This potent oxidant can be formed by the reaction shown on the left if superoxide and peroxide concentrations are sufficient and if catalytic quantities of transition metals are present. (Adapted from Telen, 1990)

as "perferryl" $(Fe=O)^{2+}$ or "ferryl" $(Fe=O^{2+})$. This reaction may be reversed with the reformation of ferric haem (Giulivi and Davies, 1990).

The prolonged incubation of haemoglobin with hydrogen peroxide however leads to the irreversible formation of low spin haemichromes, where the iron is directly ligated by both the proximal and distal histadines (Tomodo *et al*, 1978) (see Fig 15).

## 4.6 The degradation of haemoglobin *in vivo*

*Role of the monocyte macrophage system.*

Destruction of aged erythrocytes and degradation of haemoglobin occur within macrophages, chiefly in the liver and spleen. Lysosomes release their lytic enzymes into the primary phagosome of the macrophage and, within 60 minutes, digestion of the engulfed red cell is virtually complete. The globin is hydrolysed to its constituent amino acids which mix with the general amino acid pool. The haem moiety is degraded to biliverdin and carbon monoxide, and the biliverdin is reduced to bilirubin which is excreted into the bile (Robinson, 1990).

Increased rates of haemoglobin degradation occur where there is haemolysis or the resorption of haematomas. In the extravascular form of haemolysis the red cells are destroyed primarily within phagocytic cells where the haem of haemoglobin is converted directly to bilirubin (Fig 17a); there is also some liberation of free haemoglobin into the plasma. Haemoglobin released into the plasma is bound to the plasma protein haptoglobin and is carried to to the parenchymal cells of the liver where it is converted to bilirubin (Robinson, 1990) (Fig 17b).

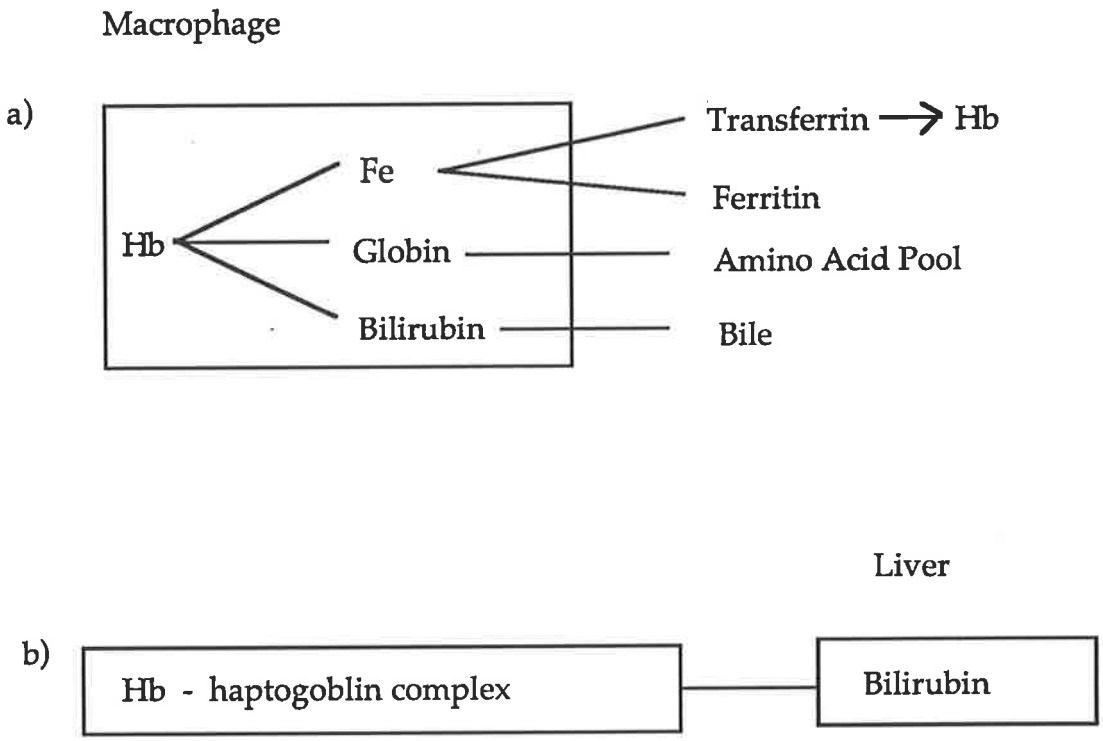



Fig 17 Schematic summary of extravascular haemoglobin degradation.
(Adapted from Robinson, 1990)

*Iron*

Haemoglobin contains 0.34 per cent of iron by weight. This means that 1 ml of packed red blood cells contains approximately 1mg of iron. In living tissues, iron does not exist except transiently as a free cation, instead it is bound by or incorporated into various proteins.

Digestion of red cells proceeds at a rate sufficient to release approximately 20% of haemoglobin iron within a few hours (Fairbanks and Beutler, 1990). The plasma iron derived by the action of macrophages is bound to transferrin and is transported to early erythroid cells in the marrow, being rapidly re-incorporated into haemoglobin (Fig 18). The remainder of the iron derived from haemoglobin enters the storage pool as ferritin or hemosiderin, and normally turns over very slowly.

Ferritin is a 440-kDa protein the central cavity of which may store 4500 iron atoms in the form of electron dense particles of about 6 nm. The uptake and release of iron from ferritin is very rapid. Ferritin occurs in virtually all cells of the body and also in tissue fluids. In blood, plasma ferritin is present in minute concentration (Hoffbrand and Pettit, 1993).

Haemosiderin is the intracellular, yellowish, iron-containing pigment visible under light microscopy in iron-loaded tissues. Under electron microscopy it is composed of dense clusters of ferritin. Ferritin is converted into haemosiderin upon partial degradation of its protein shell by lysosomal enzymes. Haemosiderin is found predominantly in cells of the monocyte macrophage system (marrow, Kupffer cells of liver, spleen). Under pathological conditions it may accumulate in large quantities in almost every tissue of the body.

The mobilisation of storage iron involves the release of intracellular ferritin iron in the divalent state (Hoffbrand and Pettit, 1993).

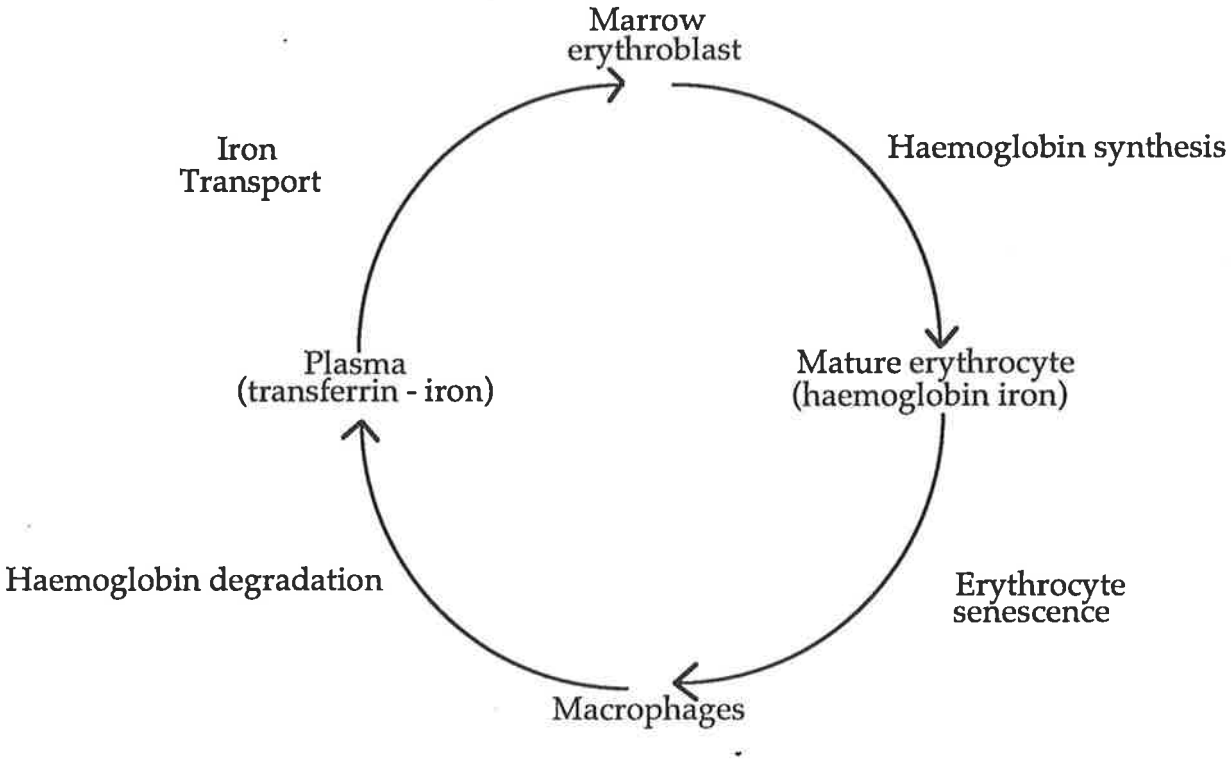



Fig 18 Internal iron cycle. Once an atom of iron enters the body, it is in a virtually closed system where it cycles repeatedly. A small quantity of iron atoms escapes each day, to other iron proteins or to the exterior of the body, and an equal number enter the system.
(Adapted from Fairbanks and Beutler, 1990).

# 5 BLEACHING

*Definition*

Bleach : To make white or whiter

## 5.1 Mechanism of bleaching

The splitting of the chromophore structure in organic molecules results in the loss of colour. The loss of colour makes the substance appear white.

Bleaches are chemical substances that remove unwanted colours by oxidation or reduction.

### 5.1.1 Oxidation bleaching

When chlorine gas, hypochlorite laundry bleach or hydrogen peroxide are used to bleach fabrics, hair, paper pulp and other such substances, the mechanism involved is the breaking of the π-component of a double bond, Equation 1.

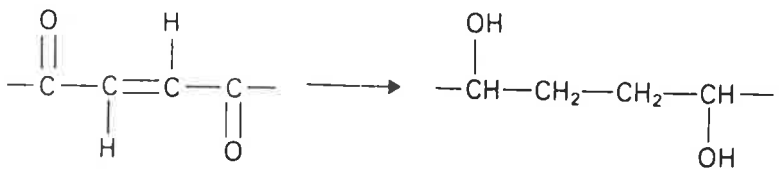
*Equation 1*

(Fremantle, 1987)

### 5.1.2 Reductive bleaching

Reducing bleaches convert these double bonds to single bonds, Equation 2 . Reducing bleaches include gaseous sulphur dioxide and sodium sulphite, $Na_2SO_3$.

*Equation 2*



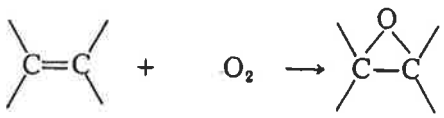
(Fremantle, 1987)

Whichever process occurs the resulting break up of the conjugated double bond system will produce a hypsochromic blue shift (see Chapter 7) into the ultraviolet and the loss of colour. These processes constitute *chemical bleaching*.

### 5.1.3 Photochemical bleaching

The energy derived from the absorption of ultraviolet radiation can produce the formation of excited π bonds; these are then able to react with atmospheric oxygen Equation 3. In the past linen was bleached in this way, by exposure to the sun (Nassau, 1983).

*Equation 3*




### 5.1.4 Physical bleaching

Physical bleaching is employed to enhance the whiteness of natural textiles and yarns which still retain a faint creamy colour even after chemical bleaching. The addition of a small amount of blue dye, the 'blueing' during laundering is an example of physical bleaching. The addition of the complementary colour blue (yellow absorbing) balances out the yellow.

A more sophisticated approach to physical bleaching of textiles uses a colourless dye that absorbs the small amount of ultraviolet present in daylight and produces a blue fluorescence. This adds extra blue light to balance the blue-absorbing yellow. These optical bleaches or fluorescent whitening agents are often incorporated into synthetic fibres before they are spun into yarn or added to detergents (Fremantle, 1987).

## 5.2 Oxidising bleaching agents

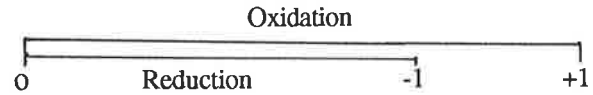
For a review of the chemistry of oxidation and reduction see Appendix 2.

### 5.2.1 The halogens

All halogens are oxidising agents (Table A2). The oxidising strength of the halogens decreases down group VII, hence the strongest is fluorine and the weakest is iodine.

The halides have a strong tendency to complete their outer shell octet by forming the halide ion $X^-$ by reacting with metals, or a single covalent bond with another non-metal.

Except for fluorine, the other halogens are only slightly soluble in water.




$$Cl_2\,(g) + 2H_2O \rightarrow H_3O^+ + Cl^-\,(aq) + HOCl\,(aq)$$

Hypochlorous acid

(g = gas)

(aq = aqueous)

#### a. Oxo-acids of chlorine

Table 7 shows the new and traditional names of the four oxo-acids of chlorine and their salts. As the oxidation number increases, both the thermal stability and the acid strength of the oxo-acids increase:

TABLE 7

Oxo-acids of Chlorine and their salts

| Oxidation state | Acid | | | Anion | | |
|---|---|---|---|---|---|---|
| | Formula | Systematic name | Traditional name | Formula | Systematic name | Traditional name |
| +1 | $HOCl$ | chloric(I) | hypochlorous | $OCl^-$ | chlorate(I) | hypochlorite |
| +3 | $HOCl_2$ | chloric(III) | chlorous | $ClO_2^-$ | chlorate(III) | chlorite |
| +5 | $HClO_3$ | chloric(V) | chloric | $ClO_3^-$ | chlorate(V) | hypochlorate |
| +7 | $HClO_4$ | chloric(VII) | perchloric | $ClO_4^-$ | chlorate(VII) | perchlorate |

(Adapted from Fremantle, 1987)

$$HOCl < HClO_2 < HClO_3 < HClO_4$$

$HClO_3$ and $HClO_4$ are both strong acids. The oxidising strength of the acids decreases with increasing oxidation number:

$$HOCl > HClO_2 > HClO_3 > HClO_4$$

HOCl and $HClO_2$ are particularly good oxidising agents. For example, in acid solution, HOCl oxidises iron (II) ions to iron (III) ions:

$$2Fe_{2+}\,(aq) + H_3O+\,(aq) + HOCl\,(aq) \rightarrow 2Fe_3+\,(aq) + 2H_2O\,(l) + Cl-\,(aq)$$

In the presence of sunlight HOCl decomposes to form oxygen.

$$2OCl-\,(aq) \rightarrow 2Cl-(aq) + O_2\,(g)$$

b. Salts of oxo-acids

In solution the oxidising strength of oxo-acid salts increases with oxidation number. However, they are not as good an oxidising agent as the acids. Most domestic liquid bleaches contain sodium hypochlorite, NaOCl, whereas bleaching powders more often contain calcium hypochlorite.

Calcium hypochlorite $Ca(OCl)_2$ is made from a reaction between $Cl_2$ and $Ca(OH)_2$. It is much more stable than bleaching powder, CaCl(OCl), and contains twice as much chlorine. It is used for bleaching, disinfecting swimming pools and for water purification. These uses are based on the strongly oxidising nature of $OCl^-$ and HOCl.

### 5.2.2 Hydrogen peroxide (hydrogen dioxide, hydroperoxide)

Discovered by Louis-Jacques Thenard in 1818, hydrogen peroxide is a weakly acidic, colourless liquid which is miscible with water. It is formed as an intermediate in the reaction of oxygen with a wide variety of substances including hydrogen, metals, and various organic compounds. Molecules of hydrogen peroxide have the structure shown in Fig 19.

Like water molecules hydrogen peroxide molecules are hydrogen bonded, however the -O-O- bond is weak and thus hydrogen peroxide is unstable. It decomposes spontaneously into water and oxygen at room temperature at a rate of 0.5% per year (Fremantle, 1987).

$$2H_2O_2\,(l) \rightarrow 2H_2O\,(l) + O_2\,(g)$$

Hydrogen peroxide can act as either an oxidising agent or a reducing agent depending on conditions. It oxidises iodide ions for example:

$$H_2O_2\,(aq) + 2H^+\,(aq) + 2I^-\,(aq) \rightarrow 2H_2O\,(l) + I_2\,(s) \qquad (s = \text{solid})$$

In the presence of another strong oxidising agent, hydrogen peroxide acts as a reducing agent:

$$H_2O_2\,(aq) \rightarrow O_2\,(g) + 2H^+\,(aq) + 2e^-$$

Hydrogen peroxide is manufactured and marketed (eg "Perhydrol" and "Hioxyl") as an aqueous solution in concentrations of 3 to 90 weight % or higher. Superoxol is a 30% concentration of hydrogen peroxide and contains approximately 100 vols of oxygen.

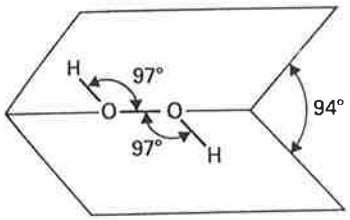



Fig 19 The structure of hydrogen peroxide.
(Adapted from Fremantle, 1987)

Solutions of hydrogen peroxide, if free from contaminants, are very stable. A small amount of stabilising agent such as acetanilide or sodium stannate is usually added to help counteract the catalytic effect of traces of impurities such as iron, copper, and other heavy metals (which may exist in processing equipment or packaging materials).

Hydrogen peroxide is catalytically decomposed by a wide variety of substances, particularly, the oxides and hydroxides of metals of relatively high molecular weight such as manganese, iron and silver. It is rapidly decomposed by alkalis and the presence of mineral acid renders it more stable. Rapid decomposition with the generation of large quantities of oxygen poses the risk of explosion. Hydrogen peroxide is a dangerous fire risk in contact with organic materials and is corrosive to skin and mucous membranes.

About 70% of U.S. production is used for bleaching of textiles, wool and cotton. Other uses of hydrogen peroxide include bleaching of ground wood pulp, straw, flour, gelatine, epoxidation of unsaturated compounds, oxidation of vat and other dyed yarns, depolymerisation of starch, adhesives etc, as rocket fuel and as an analytical reagent.

### 5.2.3 Urea peroxide (urea hydrogen peroxide, hydrogen peroxide carbamide, percarbamide, carbamide peroxide)

$CO(NH_2)_2.H_2O_2$

Urea peroxide is available ("Perhydrit", "Perhydrol-Urea," "Hyperol") as white crystals or a crystalline powder. It decomposes above 60° C to urea, oxygen and

water. It is soluble in water, alcohol, and ethylene glycol and is partly decomposed by alcohol, acetone and ether into hydrogen peroxide and urea.

Urea peroxide contains about 35 % $H_2O_2$ or a minimum of 16% active oxygen. It is sometimes compounded with waxes into pellet form.

Urea peroxide is a strong oxidising agent, an irritant and a dangerous fire risk in contact with organic materials. It is used as a source of water-free (ie dry) hydrogen peroxide, as a bleaching disinfectant, in the manufacture of cosmetics, pharmaceuticals, as a blueprint developer and for the modification of starches.

5.2.4 Perborates such as sodium perborate (perborax, sodium peroxyborate, "Dexol")

Sodium perborate is obtained by the action of hydrogen peroxide or sodium peroxide on sodium metaborate ($NaBO_2$) or pyroborate ($Na_2B_4O_7$, borax) (Fig 20). It is probably not a true perborate, $NaBO_3.4H_2O$ but more likely a borate containing hydrogen peroxide of crystallisation, $[NaBO_2.(OH)_2]_2$ which can exist anhydrous or as monohydrate tri- and tetra-hydrates (see Table 8).

The compound is stable in the dry state and only sparingly soluble in water. The solution is alkaline and has bleaching and antiseptic properties due to the liberation of hydrogen peroxide and then oxygen. Sodium perborate tetrahydrate contains about 9-10% available oxygen.

Perborates are used extensively in washing powders because they afford hydrogen peroxide in solution i.e. they act as a convenient dry source of hydrogen peroxide.

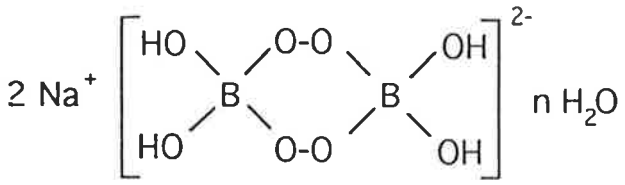



Fig. 20 Sodium perborate ring with water of crystallisation

n = 0 monohydrate
n = 4 trihydtrate
n = 6 tetrahydrate

(Adapted from Weiger *et al*, 1994

TABLE 8

Comparison of sodium perborate-monohydrate, trihydrate, and tetrahydrate

| Sodium perborate | Formula | Content of active oxygen (%)* | Solubility in water |
|---|---|---|---|
| Monohydrate | 2 x $NaBO_2(OH)_2$ | 16.0 | 15g/l |
| Trihydrate | 2 x [$NaBO_2(OH)_2$]. $4H_2O$ | 11.8 | - |
| Tetrahydrate | 2 x [$NaBO_2(OH)_2$]. $6H_2O$ | 10.4 | 23g/l |

* The content of active oxygen is a measure for the potential bleaching afficacy

(Adapted from Weiger *et al*, 1994)

## 5.3 Reducing bleaching agents

### 5.3.1 Sulphur dioxide ( sulphurous oxide, $SO_2$)

Sulphur dioxide is a colourless gas (or liquid at -10°C or less) with a sharp pungent odour which is highly irritant to the eyes and respiratory tract. It is soluble in water and forms sulphurous acid ($H_2SO_3$). It is also soluble in alcohol, methanol, chloroform and ether. It can act as an oxidising agent and a reducing agent depending on conditions.

Sulphur dioxide is used as a disinfectant, a food preservative (eg. prevention of browning of fruits) as a reducing agent, anti-oxidant and as a bleach for textiles.

### 5.3.2 Sulphurous acid

A solution of (usually around 6%) sulphur dioxide in water. Sulphurous acid is a colourless liquid, with a suffocating sulphur dioxide odour. The theoretical formula $H_2SO_3$ is used but the acid is known only by its salts. Sulphurous acid is toxic by ingestion and inhalation, and a strong irritant to tissues. It gradually oxidises in air to sulphuric acid.

Sulphurous acid has many uses including organic synthesis, bleaching straw and textiles, paper manufacture, wine manufacture, medicine (antiseptic), reagent in analytical chemistry, as a preservative for fruits, nuts, foods and beverages.

### 5.3.3 Hydrogen sulphide (sulphureted hydrogen sulphide, $H_2S$)

Hydrogen sulphide is a colourless and highly toxic gas with a characteristic smell of rotten eggs. It can from an explosive mixture with air. It is usually prepared in the laboratory by adding dilute hydrochloric acid to iron (II) sulphide:

$$FeS(s) + 2HCl\ (aq) \rightarrow FeCl_2\ (aq) + H_2S(g)$$

Hydrogen sulphide molecules do not form hydrogen bonds. This accounts for its low solubility in water. It is soluble in alcohol and glycerol. Aqeous solutions are unstable due to absorbed oxygen precipitating sulphur. Low concentrations in air are irritable to the eyes and mucous membranes.

In solution, hydrogen sulphide is a weak dibasic acid:

$H_2S(g) + H_2O(l) \quad H_3O^+ (aq) + HS^- (aq)$

hydrogen sulphide ion

$HS^-(aq) + H_2O(l) \quad H_3O^+ + S_2^- (aq)$

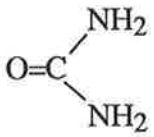
sulphide ion

Hydrogen sulphide is a reducing agent. For example it reduces iron (III) ions to iron (II) ions:

$2Fe_3^+(aq) + H_2S (g) \rightarrow 2Fe_2^+(aq) + 2H^+(aq) + S(s)$

# 6 THE UREAS

## 6.1 Urea (Carbamide

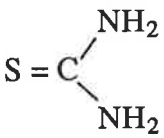


Urea occurs in urine and other body fluids as a product of protein metabolism. It was the first organic compound to be synthesised (Wohler 1894) and is the 12th highest volume chemical produced in the USA (Hampel and Harvey, 1973).

Urea consists of white crystals or powder (Mwt = 60.06), almost odourless and with a saline taste, it can develop the odour of ammonia when wet or on storage. It is very soluble in water (1 gm dissolves in 1 ml water), alcohol, benzene and concentrated HCl and is slightly soluble in ether and almost insoluble in chloroform. The pH of 10% water solution is 7.2.

Urea has numerous uses in industry and manufacture as well as a fertiliser, animal feed, resins and plastics, adhesives, pharmaceuticals, cosmetics, dentrifices, antiseptic and as a diuretic.

## 6.2 Thiourea (thiocarbamide; sulphocarbamide)



Thiourea is a crystalline solid, (MWt 76.12), soluble in 11 parts water, soluble in alcohol, and sparingly soluble in ether. It forms addition products with metallic salts and can dissolve insoluble sulphides (Hampel and Harvey, 1973.)

It has a toxicity (oral $LD_{50}$) of 0.125-0.640 gm/kg in the rat, (Dieke, George and Curt, 1947). The nature of the toxic reaction is not described. Repeated exposure may induce agranulocytosis and thrombocytopenia. It is also a skin irritant and allergenic (Hampel and Harvey, 1973). Thiourea is excreted unchanged in the urine (Schulman and Keating, 1950).

Fed chronically to rats, thiourea induced hepatic tumours, bone marrow depression and goitres (Newcomb and Deane, 1944). Fautz *et al* (1991) investigating DNA damage in rat hepatocytes reported that thiourea does not have genotoxic activity. Giri, Hollinger and Rice (1991) reported that intraperitoneal administration of thiourea in mature male rats produced pulmonary oedema similar to that produced by ammonium salts. Young sexually immature rats however were resistant to this effect.

Commercial uses of thiourea are in animal glue liquefiers and silver tarnish removers. In silver polishes it is usually acidified with citric or hydrochloric acid. It is also used in the production of flame retardant resins and as a vulcaniser and rust removers. Thiourea has been used as a fixer in photography and for photocopying papers, organic synthesis (dyes, drugs, hair preparations), rubber vulcanising accelerator, analytical reagent, amino resins, mould inhibitor (Hampel and Harvey, 1973)

Exposure to thiourea is not significant for the public except in tarnish remover dips, which dissolve silver sulphide tarnish, through skin contact or by ingestion if

newly cleaned utensils are not thoroughly washed. The FDA prohibits the use of thiourea in food for humans. OSHA lists thiourea as a carcinogen.

Thiourea compounds have been used in medicine as antithyroid agents. Thiourea compounds block the production of thyroid hormones through inhibition of thyroid peroxidase. Thiourea and dimethylthiourea (DMT) have also been investigated as scavengers of oxygen free radicals to decrease reperfusion injury in lung transplants (Detterbeck, Keagy, Paull and Wilcox, 1990). The use of thiourea as an antidote to anticancer agents such as asplatin (CDDP) has been reported (Shibiya , Hirosawa and Niitani, 1990).

### 6.2.1 Thiourea as a scavenger of radicals

Thiourea is a highly cell permeable scavenger of $H_2O_2$ and hydroxyl radicals (Stahl, Pan, and Longhurst (1993). Iron can be released from ferritin by superoxide but not hydroxyl radicals (Thomas and Aust, 1986). Kelner, Bagnell and Welch (1990) demonstrated that thiourea and DMT prevented the release of iron from ferritin and thus confirmed that thiourea is a scavenger of superoxide. The reaction of superoxide with thiourea produced a sulphydryl product distinct from the products formed by hydrogen peroxide or hydroxyl radicals which was identified as formamidine sulphide. This decomposed to a cyanamide (R–C≡N). Urea was also examined as a superoxide scavenger but was not effective (Kelner *et al*, 1990)

The rate constant for the reduction of thiourea by superoxide is comparable to the reaction of superoxide with methaemoglobin. Hence Kelner *et al* concluded that it is plausible that the reaction of thiourea and superoxide occurs *in vivo* and is biologically important.

It has been postulated that thioureas also inhibit production of iron-oxygen complexes commonly referred to as "tetravalent iron" or 'per-oxo iron" complexes (Winterbourn, 1987).

Thiourea reacts with hydroxyl radicals to form the sulphydryl enol form. The rate of this reaction is several orders of magnitude above that of the superoxide radical reaction. The relative biological significance of these reactions would depend on the relative *in vivo* concentrations of superoxide and hydroxyl radicals (Kelner, 1993).

### 6.2.2 The reaction of thiourea and hydrogen peroxide

Thiourea reacts with hydrogen peroxide to produce thiourea dioxide (sulfinic acid, formamidinesulfinic acid, aminoiminomethanesulfinic acid). In the presence of excess hydrogen peroxide thiourea is converted to urea (Kelner *et al*, 1990).

### 6.2.3 Decomposition of thiourea in acid medium

Dilruba (1975) has reported that thiourea hydrolysis in acid medium produces hydrogen sulphide and cyanamide as an intermediary products. The cyanamide ($H_2NC{\equiv}N$) is then hydrolysed to urea.

### 6.2.4 Thiourea as a reducing bleach for textiles

Conventional bleaching processes commonly employed for wool and other textiles utilise the oxidising potential of hydrogen peroxide. Recently, however the sequential oxidative reductive bleaching of wool using hydrogen peroxide and thiourea has been reported to produce greatly superior bleaching of pigmented and stained wool with less physical damage when compared to conventional bleaching (Arifoglu, Marmer and Carr, 1990).

This process involves an oxidative hydrogen peroxide bleaching (Step 1) then in-situ formation of thiourea dioxide from the reaction of thiourea with unspent peroxide (Step 2). Subsequent hydrolysis of thiourea dioxide forms the reducing agent, sulfinate ion, with urea as a byproduct, and reductive bleaching ensues. Alternatively reductive bleaching is achieved by the complete decomposition of unspent hydrogen peroxide and then the addition of the reductive substances thiourea dioxide or sodium hydroxymethane-sulfinate with pH adjustment to 7 - 8 (Gacen *et al*, 1993; Arifoglu and Marmer, 1992).

The use of reducing agents for bleaching ground wood pulp has also been investigated. Tchakeva, Ivanova, Zuikov and Trefilova (1981) reported that the brightness of groundwood pulp was increased by treating it with thiourea dioxide solution at pH 9.5 - 11.5. Kanada, Jinnouchi, Shimpo, Koshitsuka and Kimura (1994) patented a process for the production of thiourea dioxide in situ by mixing thiourea with a peroxide, a reaction catalyst and/or a chelating agent. A process was patented by Devic (1994), which uses the reducing agents sodium sulphite and sodium borohydride and then an alkaline hydrogen peroxide bleach to produce pulps with improved whiteness.

### 6.2.5 The chemical reaction of thiourea and hydrogen peroxide in the bleaching of wool

Arifoglu, Marmer and Dudley (1992) investigated the reaction of thiourea with hydrogen peroxide in solution under bleaching conditions using $^{13}C$ NMR spectroscopy. They found that different intermediate products are formed during the reactions, depending on the pH of the medium and the molar ratio of the reactants.

The reaction goes through a thiourea dioxide intermediate; this then hydrolyses under heat and neutral or alkaline conditions, to yield sulfinate anion and urea. There is a rapid change in redox potential from a positive value to a high negative value. The species causing the negative redox potential, and hence the species responsible for reductive bleaching, is believed to be the sulfinate anion.

Cardome, Marmer and Arifoglu (1992) compared conventional bleaching of wool with alkaline hydrogen peroxide to variations of the single-bath sequential oxidation/reduction bleaching process. Mechanistic studies for this reaction, using $^{13}C$ NMR in the absence of wool, showed the appearance of urea as a marker for the conversion of thiourea dioxide to sulfinate. The conversion of thiourea dioxide was immediate and complete. Only under the mildest conditions (50°C, 8 g/L peroxide, pH 7.8 for the reductive bleaching step) did a stable thiourea dioxide signal appear, and this disappeared upon the addition of wool. The whiteness achieved was equal to or surpassed conventional alkaline bleaching carried out within the same time and temperature limits.

## 6.3 The effects of the ureas on human haemoglobin

Elbaum and Herskovits (1974) reported that the urea and thiourea are effective subunit dissociating agents of human haemoglobin at concentrations of 1M and below. Nearly complete dissociation into half-molecules can be achieved before pronounced changes in the physical properties of haemoglobin are produced, indicating unfolding of the intact $\alpha\beta$ subunits. At higher concentrations (above 5M) urea is an effective denaturing agent producing unfolding of the haemoglobin chains and dissociation into single chains. The effectiveness of ureas and amides as denaturants of haemoglobin increases with increasing hydrocarbon content requiring correspondingly lower concentrations to produce unfolding of native haemoglobin (Elbaum, Pandolfelli and Herskovits, 1974).

## 6.4 Decolourisation of blood

The decolourisation of blood has been extensively reported in the literature. The purpose of such research appears to be the desire to use otherwise wasted by-products from the meat industry, in particular blood, as a versatile source of protein in human diets.

Nakamura, Yamamoto and Yaguchi (1994) decolourised red blood cells obtained from pig's blood using hydrochloric acid and sodium chlorite. The decolourised haemoglobin suspension was dehydrated and freeze-dried to give a white powder. Hydrogen peroxide was used by Ermolenko, Gulko, Lyubliner, Sudakov, Rusakova and Ivanov (1986) as the bleaching agent. Oluski, Tomkov, Oluski and Dzinic (1984), separated the haem from the globin of bovine haemoglobin with hydrochloric acid (0.1 or 0.2N). The acid haemolysate thus obtained was mixed with concentrated NaCl and decolourised by sodium peroxide (0.25-1%). Finally the proteins were separated from the solution by ultrafiltration.

Piot, Guillochon, Charet and Thomas (1994) used hydrochloric acid (pH 2) to denature bovine blood. This was then incubated with pepsin, centrifuged and passed through a column containing basic alumina to leave a colourless eluate which was lyophilised to produce a white powder. An effective decolourisation of bovine blood by the enzyme, alkalase, was reported by Clark, Cutler, O'Meara and Munro (1987).

Wismer-Pederson (1980) has reviewed the decolourisation of haemoglobin by bleaching agents and enzymes and reported on the effects of these processes on the functional properties of the protein in food.

In order to achieve a colourless product, it appears necessary to first liberate the haem from the globin component of haemoglobin. Hydrochloric acid is commonly used for this purpose. The haem is then acted upon by one of the oxidising bleaching agents and decolourised. Alternatively enzymes may be used to denature the haemoglobin and to decolourise it. The final product is a white protein powder.

# 7 SPECTROPHOTOMETRY

Spectrophotometry is the measurement of the absorption of radiation energy by the use of a spectrophotometer.

## 7.1 Principles of spectrophotometry

When a light beam passes through a layer of absorbing material, the transmitted radiation will have less intensity than the incident light. This loss is accounted for by the absorption of the radiation energy by the material.

When colour is being measured, it is understood that the apparent colour of a solution is the complement of the colour which is absorbed, for example, a solution that absorbs yellow light appears blue (see Table 9).

The quantitative treatment of absorption is based on the combined Beer's and Lambert's laws (Wallwark and Grant, 1977) :

$$dI/dn = -kI$$

dI is the fraction of light intensity absorbed by an increment dn in the number of absorbing molecules in the light path, and K is a constant characteristic of the absorbing material.

By rearranging and integrating the above equation, one gets:

$$In\ I/Io = -kN$$

where

*Io* is the intensity of incident light

TABLE 9

Colours of visible radiation

| Wavelength range (nm) | Absorbed colour | Apparent colour |
|---|---|---|
| 400-450 | Violet | Yellow green |
| 450-480 | Blue | Yellow |
| 480-490 | Green -blue | Orange |
| 490-500 | Blue-green | Red |
| 500-560 | Green | Purple |
| 560-575 | Yellow-green | Violet |
| 575-590 | Yellow | Blue |
| 590-625 | Orange | Green-blue |
| 625-750 | Red | Blue-green |

*I* is the intensity of transmitted light

*N* is the amount of absorbing molecules in the light path of a defined cross sectional area (1cm by convention)

The number N can be specified as a product of the concentration, c, and the length of light path, b:

$$In\ I/Io = -kbc$$

For convenience the natural logarithm is converted into a decimal one (k multiplied by 0.43 becomes a) and the ration I/Io is reversed to remove the negative sign. The above equation then becomes:

$$logI/Io = abc = A$$

where A is the ***absorbance***

Absorbance is the measure of the absorption of radiation energy by the material. The absorbance profile over a range of wavelengths is termed an absorption spectrum and is characteristic of individual chemical compounds.

## 7.2 Quantitative spectrophotometric parameters

Spectrophotometers are so arranged that one can obtain a comparison of the intensity of transmitted radiation with that of the pure solvent (blank) which corresponds to the intensity of the incident radiation, Io.

The light path b is usually 1cm for a standard quartz cuvette.

The constant, a, in the above equation is characteristic of a particular combination of solute and solvent for a particular wavelength and is called **absorptivity**.

The optimum accuracy of absorbance reading is achieved when A = 0.43. The error remains small for readings of 0.2 and 0.8 and increases for lower and higher readings.

The **molar absorptivity** (or extinction coefficient, ε) is defined as follows:

$$\varepsilon = A/bM$$

where M equals the moles per litre and b is the light path in cm.

The **millimolar absorptivity** ($\varepsilon_{mM}$) is also commonly used as a more practical unit.

**Specific absorbance** ($E_{1cm}1\%$) is the absorbance of a 1% w/v solution.

**Peak absorbance** ($\lambda_{max}$) indicates that the given value applies to the wavelength at which the absorbance is at its maximum. Thus the degree of absorbance of a solution of a substance is a measure of the concentration of that substance in solution.

## 7.3 Optical spectra

All proteins absorb strongly in the ultraviolet region of the spectrum. At wavelengths of 190-200 nm, the absorption is associated with the peptide bond while at 260-280 nm, the absorption is due to the aromatic group associated with

the three amino acids tyrosine, tryptophan and phenylalanine. Haem proteins have additional absorption bands associated with the haem group (Weisbluth, 1974).

Metal porphyrins like haemoglobin have spectra which consist of a strong band in the near ultraviolet (Soret band, see Table 6) and two other bands in the visible; the latter two are responsible for the characteristic colour of most haem proteins. The Soret band is located at 400-420 nm and has a millimolar extinction coefficient ($\varepsilon_{mM}$) of about 100. The visible bands generally designated $\alpha$ (longer wavelength) and $\beta$ (shorter wavelength) are typically found in the region of 650 and 550 nm respectively with $\varepsilon_{mM}$ approximately 10.

Although these gross features are recognisable in the haemoglobin spectra, there are significant differences in detail amongst the spectra of various derivatives (Table 6 Fig 11).

The spectrum is obtained by recording the light absorbed as a function of the wavelength. The ultraviolet region lies to the left of the spectrum and the visible region lies to the right.

The vertical scale of an absorption spectrum gives an indication of the amount of monochromatic radiation absorbed by the test substance measured relative to the reference cell in the spectrophotometer.

The human eye is so sensitive to small changes in transition energies that distinctly different colours may in fact arise from a shift in an absorption spectrum band of only a few nanometres. This makes acceptable colour matching a difficulty in many areas of manufacturing and of course, dentistry.

The following terms are used in connection with electronic absorption spectra (Fig 21)

Bathochromic shift. The displacement of an absorption band towards longer wavelengths.

Hypsochromic shift. The displacement of a band to shorter wavelengths.

Red shift. Synonymous with the term bathochromic shift, implying a movement towards the red end of the spectrum.

Blue shift. Synonymous with hypsochromic shift ie a movement towards the blue end of the spectrum.

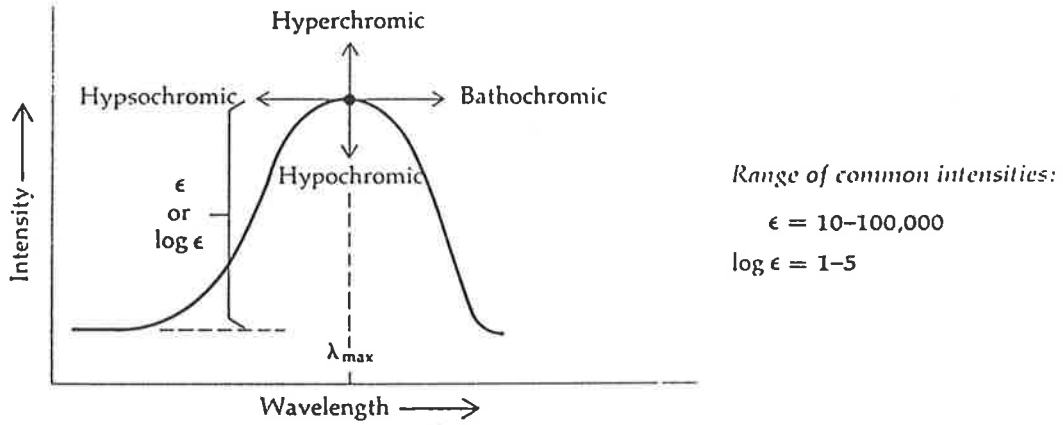



Fig 21 Shifts in absorption position.
(Adapted from Hendrickson *et al*, 1970).

# 8 MATERIALS AND METHODS

## 8.1 Spectrophotometric studies

A "Lambda - 5" UV/VIS spectrophotometer (Perkin Elmer) and matched quartz cuvettes were used for all spectrophotometric recordings.

### 8.1.1 Preparation of haemoglobin and its derivatives

Packed red blood cells[2] obtained from the Red Cross Blood Bank, Adelaide, were diluted[3] 1:40 with demineralised water to provoke haemolysis. These were then placed in ultracentrifuge tubes and spun using an L8-80 Ultracentrifuge (Beckman) at 35, 000 rpm for 1 hour (RCF = 100,000 g) to sediment the red blood cell ghosts from the haemoglobin solution. The supernatant was carefully drawn off with a pipette providing a clear red solution of haemoglobin .

*Haemoglobin*

Haemoglobin in a dilution 1:40 is too strongly coloured to allow sensitive spectrophotometric analysis. Samples were therefore carefully diluted with distilled water 1:400 and the absorption spectrum, in the visible range, was recorded.

A dilution of 1:400 haemoglobin was also used in the spectrophotometric study of the effects of the various test bleaching solutions. This is equivalent to $4.97 \times 10^{-6}$ M haemoglobin.

---

2 Blood type O Rh+ve
Tested negative for HIVAb, HCVAb, HTLV-I Ab, HBSAg and Syphilis
Concentration of Hb = 13.5 gm/dl

3 Universal precautions were used at all times when handling blood and blood products.

*Methaemoglobin*

A stock solution of methaemoglobin was prepared by dissolving 1.35 gm of methaemoglobin powder (human haemoglobin dessicate) in 10 ml of distilled water. The resultant dark brown solution was diluted 1:400 as with haemoglobin above and the absorption spectrum recorded.

8.1.2 Preparation of test bleaching solutions

The chemicals used in the preparation of bleaching solutions were analytical grade. The sources of the chemicals are detailed in Appendix 3. All chemicals were stored as recommended by the suppliers. The preparation of the test bleach solutions is detailed in Appendix 4. The pH of the various bleaching solutions was measured using a Townson digital pH meter.

8.1.3 The absorption spectra of the test bleaching solutions

The test bleach solutions were colourless. The degradation of the bleaching solutions with time was studied by recording the absorption spectra in the UV range immediately after preparation and at 10, 20 and 60 days. As thiourea has a very high absorbance (Log$\varepsilon$ of thiourea = 4.1) dilute solutions of thiourea at 1:50,000 were used for spectrophotometric analysis. The thiourea dioxide was used in a dilution of 1:1,000.

8.1.4 The effect of test bleach solutions on haemoglobin and methaemoglobin

To 8 test tubes each containing 2 ml of haemoglobin solution at 1:400 dilution was added 2ml of one of the following solutions :

0.1M aqueous thiourea

0.1M acidified thiourea

0.1M acidified thiourea

0.1M thiourea dioxide

0.01M hydrochloric acid

distilled water

30% w/v hydrogen peroxide

The addition of 2ml of either 0.01M HCl (pH= 2.02) or distilled water formed the negative controls for this phase of the project and the 2ml of 30% hydrogen peroxide was the positive control.

Similarly, 2 ml of each test bleach solution and control was added to test tubes containing 2 ml of 1:400 dilution methaemoglobin solution. The test tubes were left for 24 hours at room temperature. The solution in each test tube was transferred in turn to one of a pair of matched quartz cuvettes and the solution scanned in the range $\lambda$= 330-760 nm.

## 8.2 The effect of various agents on blood stained dentin

### 8.2.1 Preparation of stained dentin samples

Single rooted premolar teeth, extracted for orthodontic purposes, were collected from consenting patients. The teeth were stored in phoshpate-buffered isotonic saline (pH 7.4) at 4°C until they were used.

An endodontic access cavity was prepared through the occlusal surface of the teeth with a tungsten carbide bur at high speed using water spray. Pulp extirpation was then carried out using endodontic files. Irrigation with 1-2 ml of 1% sodium

hypochlorite (NaOCl) was used to enhance removal of intracanal organic debris. This was followed by copious rinsing with sterile saline.

The teeth were discoloured with blood using an adaptation by Marin (1992) of the technique described by Freccia and Peters (1982).

The prepared teeth, with access cavities left open, were immersed in a suspension of packed red blood cells in centrifuge tubes. The packed red blood cells were those from the stock suspensions used for the spectrophotometry. The teeth were centrifuged using a IEC Centra-MP4R High speed bench centrifuge (Appendix 3) at 5,500 rpm twice daily for 30 minutes over three consecutive days. In the interim period, they were stored in the centrifuge tubes in packed red blood cell suspensions in an incubator room at 37°C.

The relative centrifugal force (RCF) exerted on the teeth:

$$RCF = 11182 \times 10^{-8} \times R \times (rpm)^2$$

where R = radius of centrifuge arm = 13 (cm)

and rpm = 5,500

$$RCF = 11182 \times 10^{-8} \times 13 \times (5,500)^2$$

$$= 43,973 \text{ g}$$

Following centrifugation the teeth were sectioned transversely at 1 mm thickness using a Van Moppes diamond cutting disc (Appendix 3) at 12,000 rpm with water spray cooling. The dentin samples were stored in the centrifuge tubes in the packed red blood cell suspensions.

8.2.3 The measurement of the decolourisation of blood-stained dentin

Light absorbance by the dentin samples was measured, prior to them being subjected to the various bleaching protocols, with a 502M Photometer (Photovolt) and light reflectance with a "Speedmaster" R75-CP Reflection Densitometer (Electronic Systems Engineering Co.).

The R75-CP Reflection Densitometer measures the light that is reflected back from a sample. As seen in Fig 4 the total light is the sum of the light reflected at the surface and that which enters the sample for a short distance and is reflected back out again. The densitometer records the reflectance density of a sample on a linear scale from zero to 2.5. It was calibrated as recommended by the manufacturer so that the ANSI Standard White on the calibration plaque registered 0.80 and ANSI Standard Black registered 1.86. Therefore as a dentin sample became lighter or 'bleached', the Reflection Densitometer reading became lower and approached the ANSI Standard White.

The 502M Photometer measures the amount of incident light that is transmitted through a sample, i.e. the optical density of the sample, directly in absorbance (OD) units. The more darkly stained the dentin samples the lower the OD value recorded. As the samples bleached or lightened they allowed more light to be transmitted through. Hence a higher OD value after a bleaching regimen indicates superior bleaching efficacy.

The instruments were turned on and allowed to reach thermal stability prior to use. The warm up time allowed was 1 hour.

The various test solutions were placed into the wells of a culture plate as per the bleaching regimens in Table 10.

Table 10 Bleaching regimens for dentin discoloured by blood

| Bleaching regimens | Stage 1 | Stage 2 | Stage 3 |
|---|---|---|---|
| A1 | acidified thiourea | 5% NaOCl | hydrogen peroxide |
| A2 | acidified thiourea | NaOH* | hydrogen peroxide |
| A3 | acidified thiourea | distilled water | hydrogen peroxide |
| A4 | acidified thiourea | acidified thiourea | hydrogen peroxide |
| A5 | acidified thiourea | acidified thiourea | acidified thiourea |
| B1 | aqueous thiourea | 5% NaOCl | hydrogen peroxide |
| B2 | aqueous thiourea | NaOH* | hydrogen peroxide |
| B3 | aqueous thiourea | distilled water | hydrogen peroxide |
| B4 | aqueous thiourea | aqueous thiourea | hydrogen peroxide |
| B5 | aqueous thiourea | aqueous thiourea | aqueous thiourea |
| C1 | 0.1M HCl | 5% NaOCl | hydrogen peroxide |
| C2 | 0.1M HCl | NaOH* | hydrogen peroxide |
| C3 | 0.1M HCl | 0.1M HCl | hydrogen peroxide |
| C4 | 0.1M HCl | 0.1M HCl | 0.1M HCl |
| D1 | 5% NaOCl | 5% NaOCl | hydrogen peroxide |
| D2 | NaOH* | NaOH* | hydrogen peroxide |
| D3 | hydrogen peroxide | hydrogen peroxide | hydrogen peroxide |
| E1 | hydrogen peroxide | acidified thiourea | acidified thiourea |
| E2 | hydrogen peroxide | aqueous thiourea | aqueous thiourea |
| E3 | thiourea dioxide | thiourea dioxide | thiourea dioxide |
| F1 | 5% NaOCl | 5% NaOCl | 5% NaOCl |
| F2 | NaOH* | NaOH* | NaOH* |
| F3 | distilled water | distilled water | distilled water |

* The pH of NaOH was adjusted to 11.7 (i.e. the same pH as determined for 5% NaOCl)

A centrifuge tube was selected at random, a dentin sample was removed from the centrifuge tube and was blotted on damp filter paper to remove excess blood (but left moist). The sample was placed on the standard white calibration plaque, the probe of the R75-CP Reflection Densitometer was depressed over the sample and the reflectance density was recorded. The sample was then immediately transferred to the 502M Photometer. The sample was centred in the path of the light beam and the optical density (absorbance) recorded at zero time (T0).

The dentin sample was then immersed in the test bleaching agent (Stage 1) for 5 minutes, blotted as before and the reflectance density and absorbance measured as above.

It was then immersed in the second agent (Stage 2) for 60 seconds, blotted but left moist and the reflectance density and absorbance measured as before.

The sample was then immersed in the third agent. The reflectance density and absorbance of the sample was measured at 1 hour, Stage 3 (i) and then at 24 hours Stage 3 (ii). A flow diagram of the experimental design appears as Fig 22.

This was repeated giving 4 samples for each of the 23 different bleaching treatments.

### 8.3 Statistical method

The statistical analyses were performed on the original data. A log transformation of the Reflection Densitometer data was considered but not carried out as it was felt that it would not change the basic conclusions. Comparisons of the different bleaching regimens were made using a method of Least Significant Difference (LSD) and/or Analysis of Variance. These methods were applied separately to the

data obtained using the 502M Photometer and to that obtained using the R75-CP Reflection Densitometer. Both these methods involve comparisons of the means of the treatments. One sample was lost and in this case the mean of the remaining values in that group was taken.

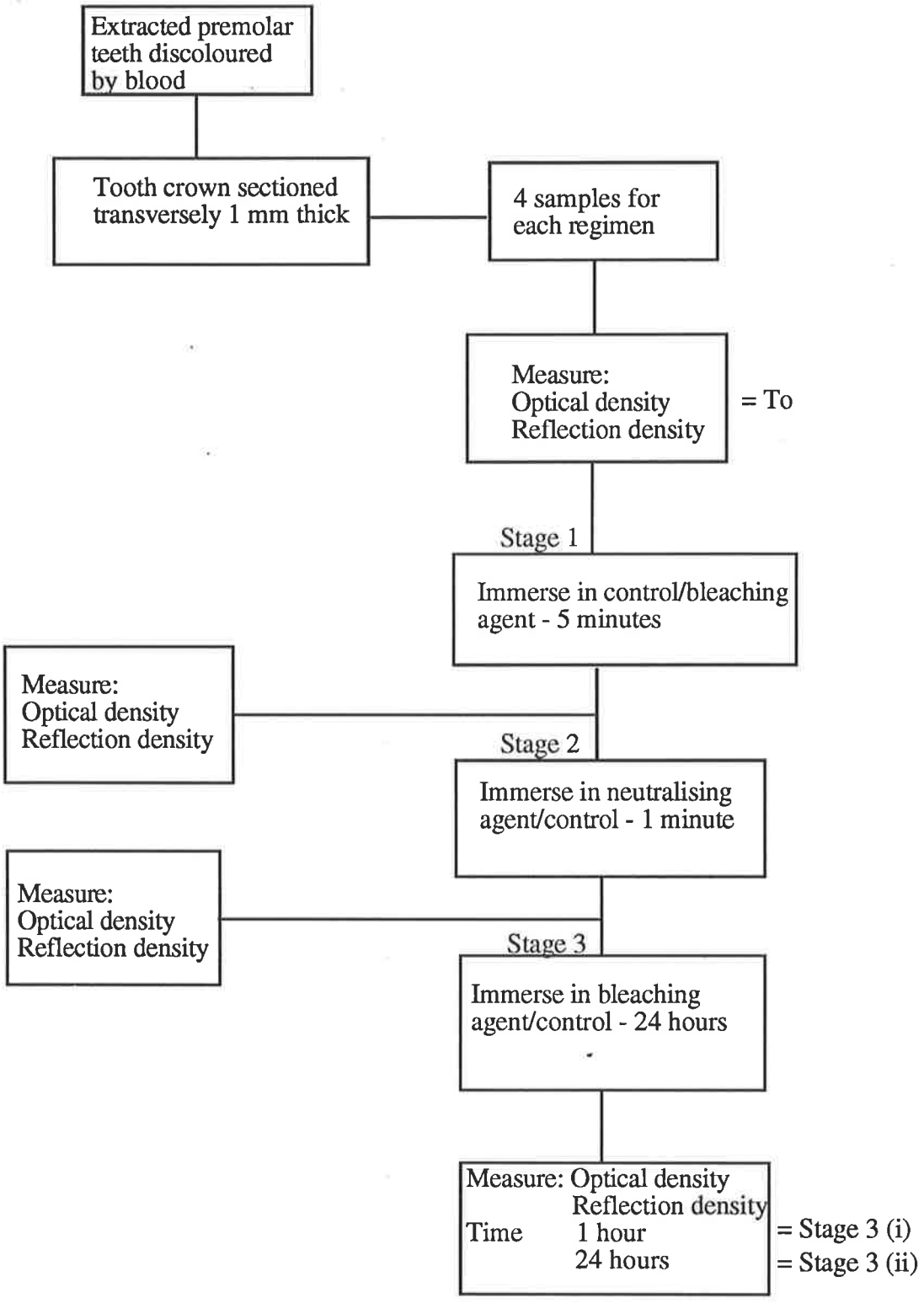



Fig 22 Method used to determine the bleaching efficacy of different regimens on dentin discoloured by blood.

# 9. RESULTS

## 9.1 Spectrophotometric studies

### 9.1.1 Absorption spectra of haemoglobin and methaemoglobin

The absorption spectrum of the haemoglobin derived from the packed red blood cells (Fig 23) shows a peak at 415 nm (the Soret band) and absorption peaks at $\lambda$ = 540 nm and $\lambda$ = 580 nm, the $\alpha$ and $\beta$ bands, as well as absorption peaks at $\lambda$ = 344 nm and 276 nm and is consistent with the absorption spectrum for oxyhaemoglobin reported by Weisbluth (1974). The absorption spectrum of the methaemoglobin (Fig 24) shows the Soret band at $\lambda$ = 405 nm and two minor peaks at $\lambda$ = 500 nm and $\lambda$ = 630 nm. This too is in accordance with Weisbluth (see Table 6).

### 9.1.2 Absorption spectra of the bleaching solutions

Sample absorption spectra of the experimental bleaching solutions in the UV range are presented. The bleaching solutions containing thiourea demonstrated absorption peaks at $\lambda$ = 238 nm and at $\lambda$ = 198 nm (Fig 25 and 26). This is consistent with the presence of urea as a product of the hydrolysis of thiourea. A sample absorption spectrum of 0.1M urea is also presented. It may be seen that the urea solution showed a single characteristic peak at $\lambda$ < 200 nm (Fig 27). Thiourea dioxide showed a maximum absorption at $\lambda$ = 210 nm and a weaker band at $\lambda$ = 270 nm (Fig 28). The absorption spectra of hydrochloric acid and hydrogen peroxide was also recorded (Figs 29-30). 0.1 M hydrochloric acid ($\lambda$ max 237 nm) did not record an absorption spectrum until a dilution of 1: 5 was used and hence it can be concluded that this substance could not interfere with the spectra recorded in the study. Hydrogen peroxide 30% w/v in a 1: 200 dilution showed a single absorption band at $\lambda$ < 200 nm which is a characteristic of simple molecules containing double bonds. The absorbance of the solutions remained constant over 60 days under the conditions in this project.

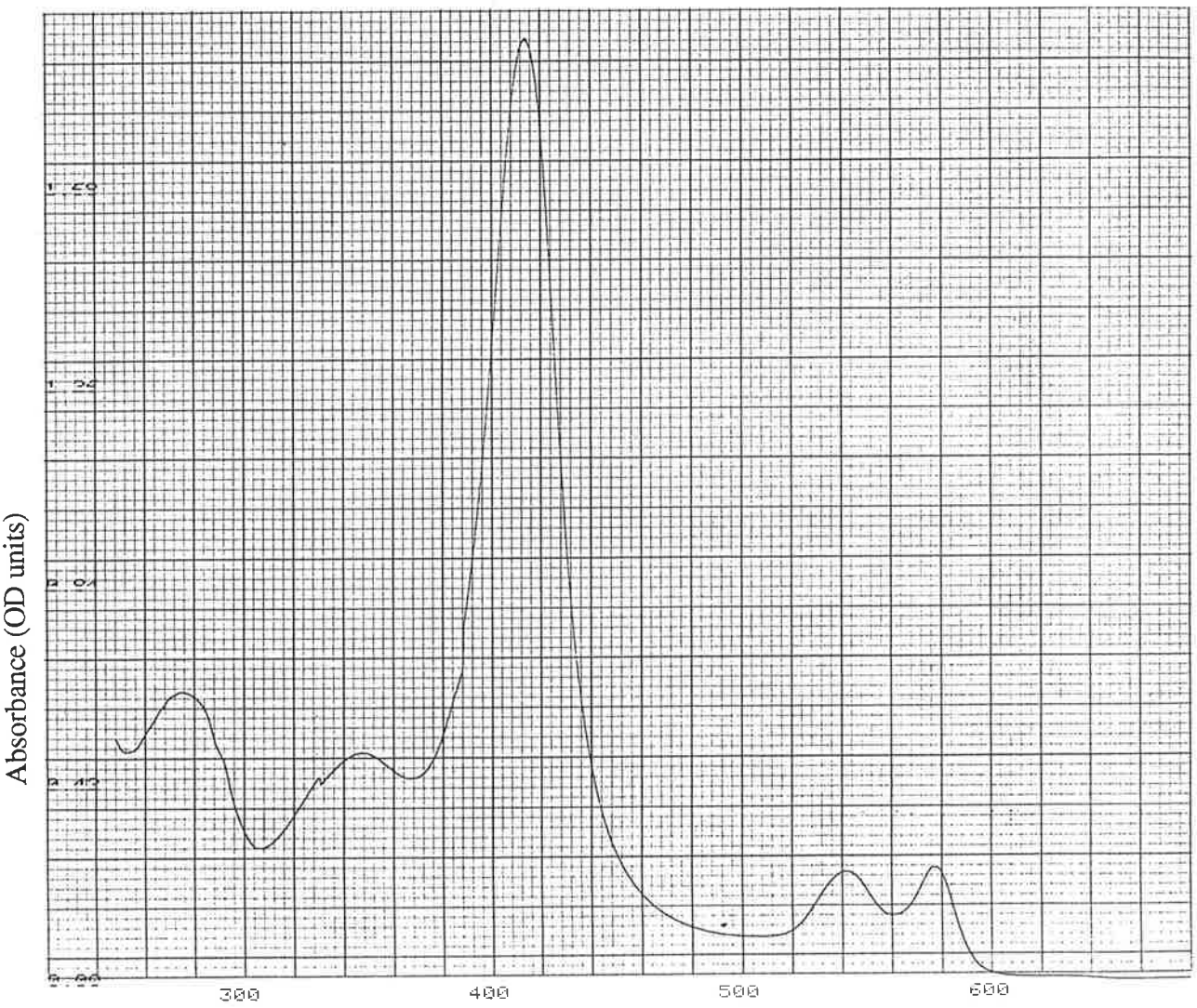



Fig 23 Absorption spectrum of haemoglobin from packed red blood cells.

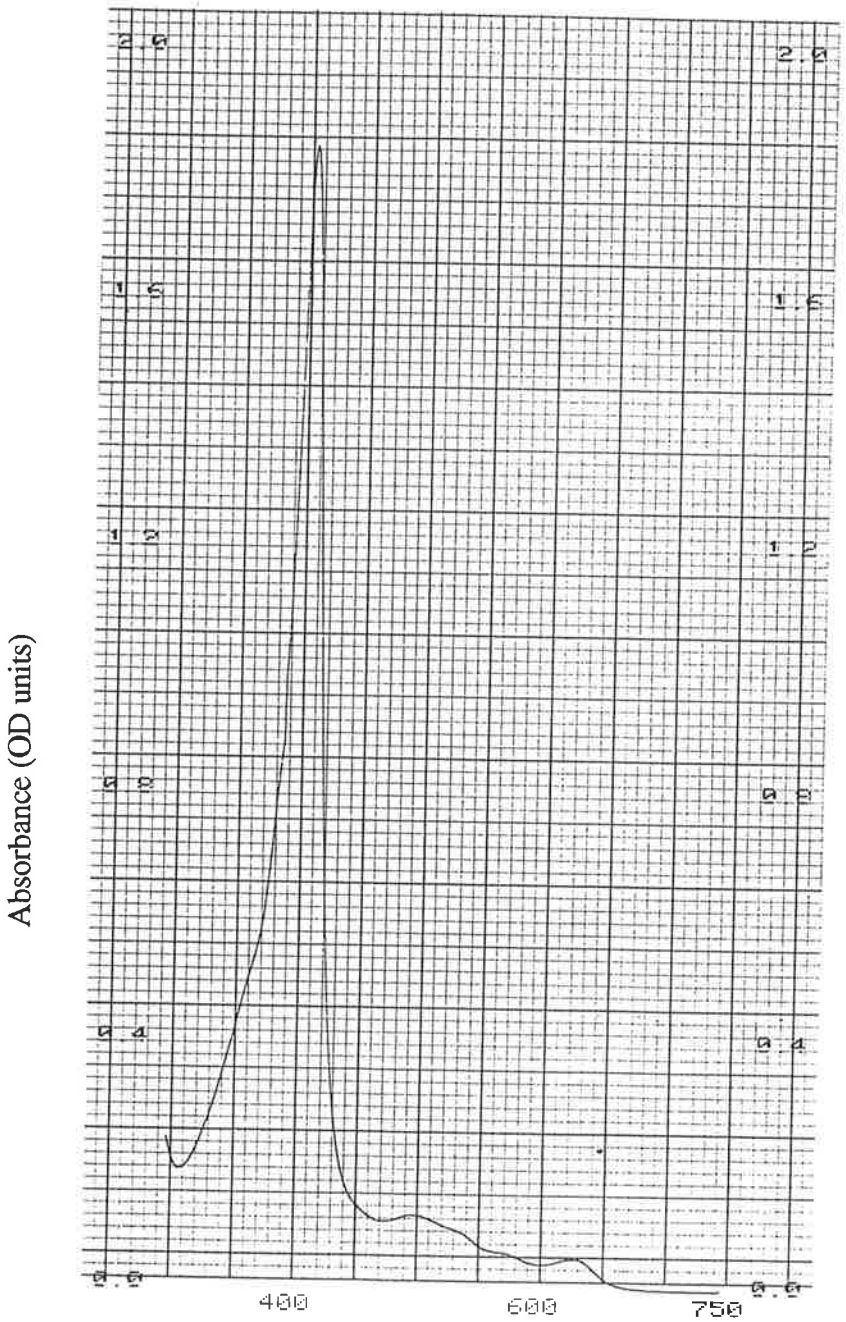



Fig 24 Absorption spectrum of methaemoglobin.

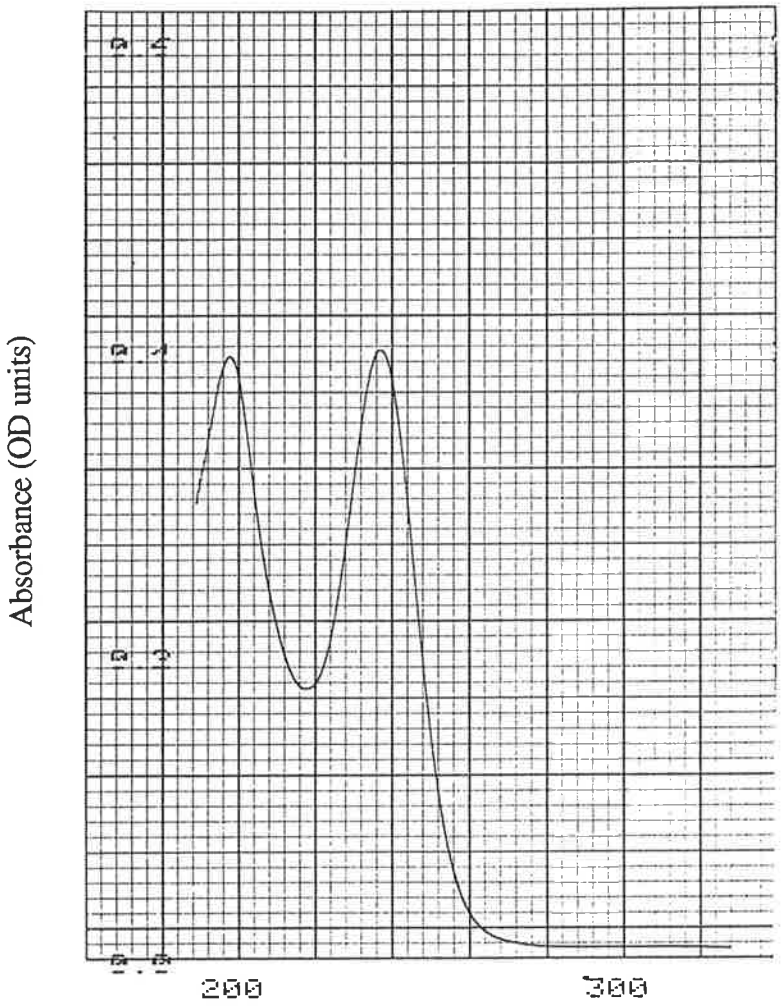



Fig 25 Absorption spectrum of aqueous thiourea solution 1:50,000 dilution.

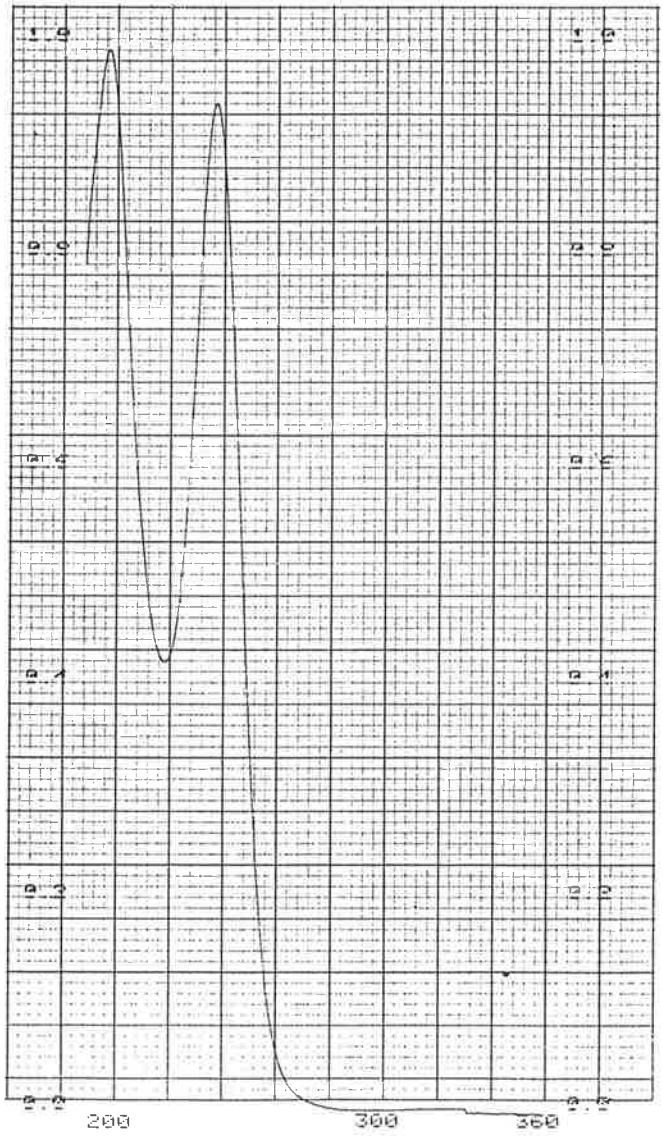



Fig 26 Absorption spectrum of 0.1 M acidified thiourea solution 1:20,000 dilution.

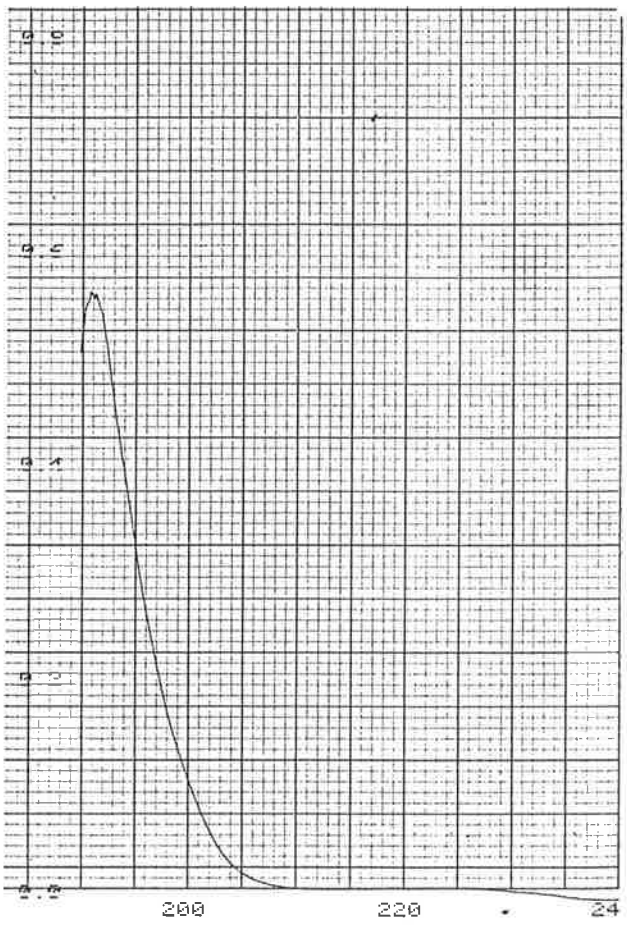



Fig 27 Absorption spectrum of 0.1 M urea solution 1:4,000 dilution.

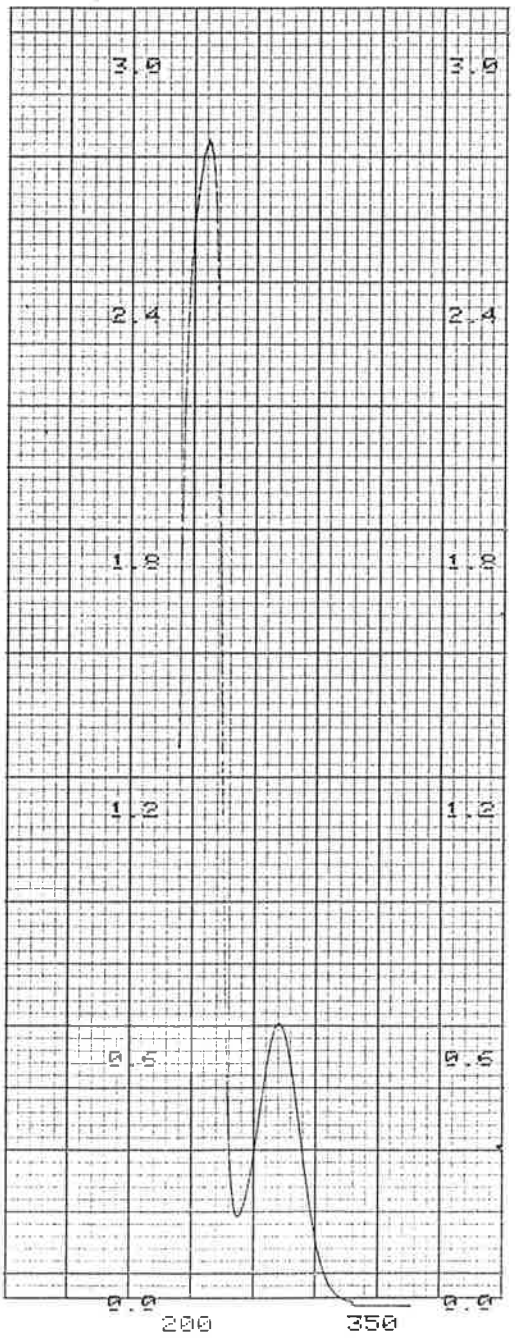



Fig 28 Absorption spectrum of thiourea dioxide.

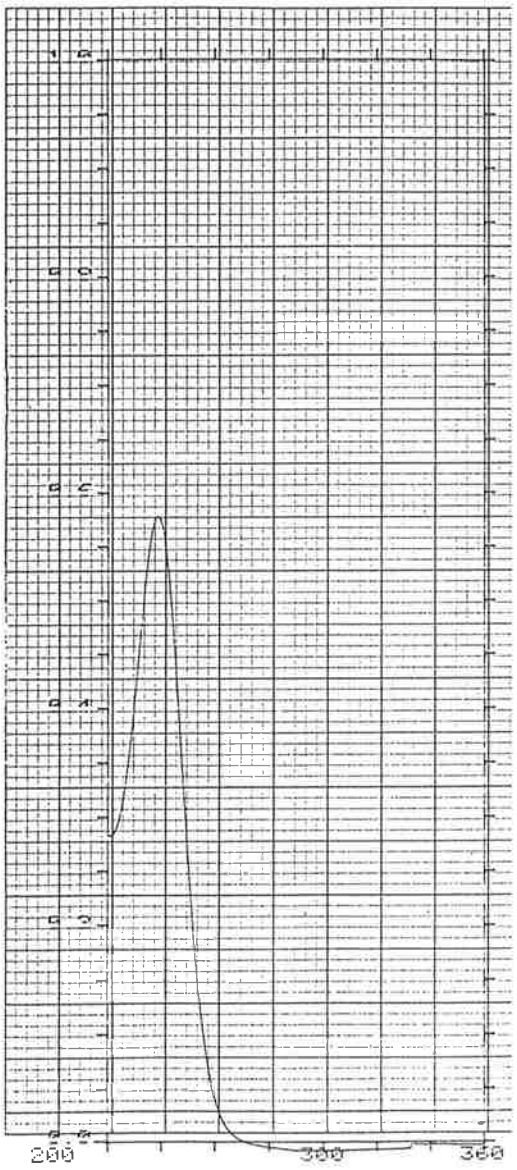



Fig 29 Absorption spectrum of 0.1 M hydrochloric acid in a dilution of 1:5.

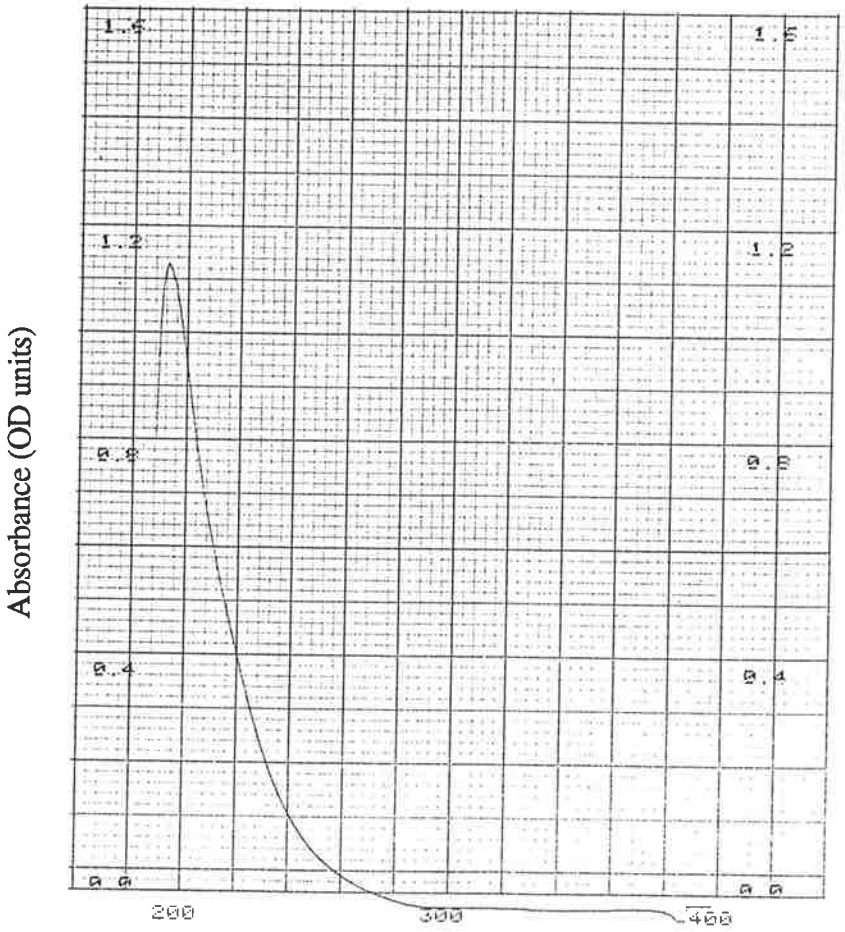



Fig 30 Absorption spectrum of 30 w/v hydrogen peroxide in a dilution of 1:200.

9.1.3 The effect of various bleaching solutions on the absorption spectra of haemoglobin and methaemoglobin

*Haemoglobin*

The aqueous thiourea did not produce any changes in the absorption spectra of the solutions of haemoglobin as compared with the control (Fig 31 cf Fig 23).

The 0.01M hydrochloric acid and acidified thiourea solutions produced very similar changes to the absorption spectra of haemoglobin (Figs 32, 33, 34). They eliminated the $\alpha$ and $\beta$ bands of the haemoglobin (responsible for the characteristic red colour) and significantly reduced absorption in the near ultraviolet region, i.e. the Soret band. Fig 34 shows an overlay of the absorption spectrum of haemoglobin bleached with 0.1M acidified thiourea and 0.01 M hydrochloric acid. Fig 35 illustrates the changes in the absorption spectrum of haemoglobin that is produced by 0.1M thiourea dioxide. It also eliminates the $\alpha$ and $\beta$ bands and produces a broad band at the near ultraviolet region ($\lambda$ max 396 nm). The 30% w/v hydrogen peroxide rendered the haemoglobin colourless and no absorbance could be recorded in the wavelength 330-760 nm (Fig 36). This effect was repeated when a weaker solution of 3% hydrogen peroxide was used.

*Methaemoglobin*

The aqueous thiourea did not affect the absorption spectrum of methaemoglobin compared with the control. A sample absorption spectrum is presented as Fig. 37. The absorption spectrum of methaemoglobin ($\lambda$ = 360 - 750 nm) produced by 0.1 M acidified thiourea, by 0.01M hydrochloric acid and 0.1M thiourea dioxide appear as Figs 38 - 40. The acidified thiourea and hydrochloric acid produced similar changes in the absorption spectrum of methaemoglobin, greatly reducing the absorption at the near ultra-violet region ($\lambda$ = 400 nm) and almost eliminating

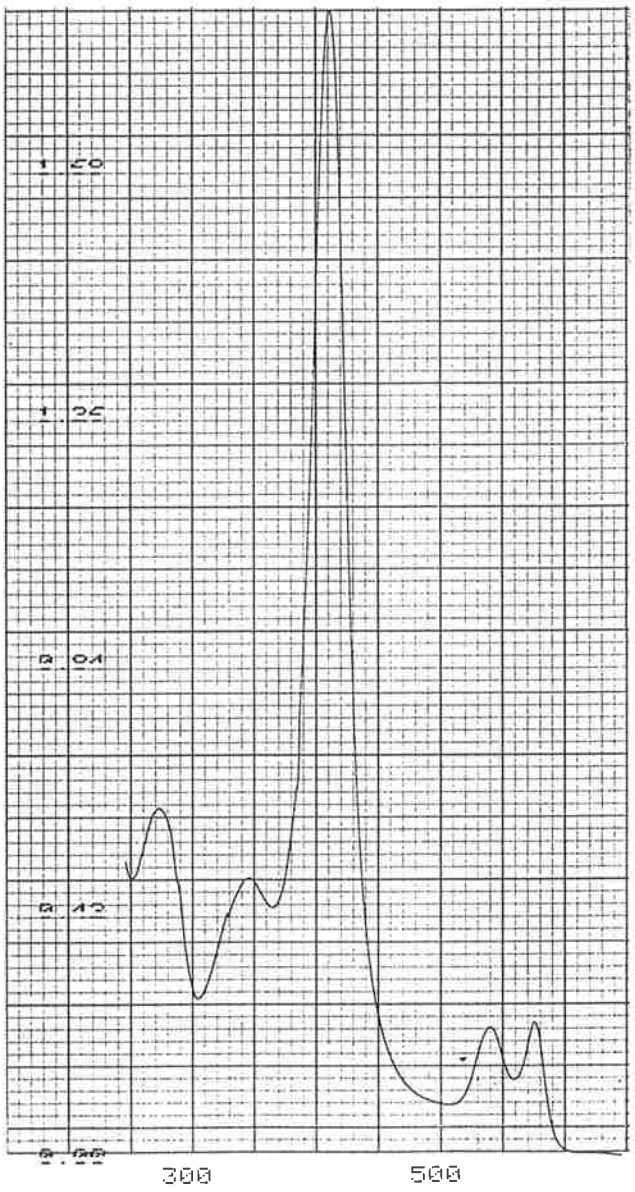



Fig 31 The absorption spectrum of haemoglobin after the addition of 0.1M aqueous thiourea.

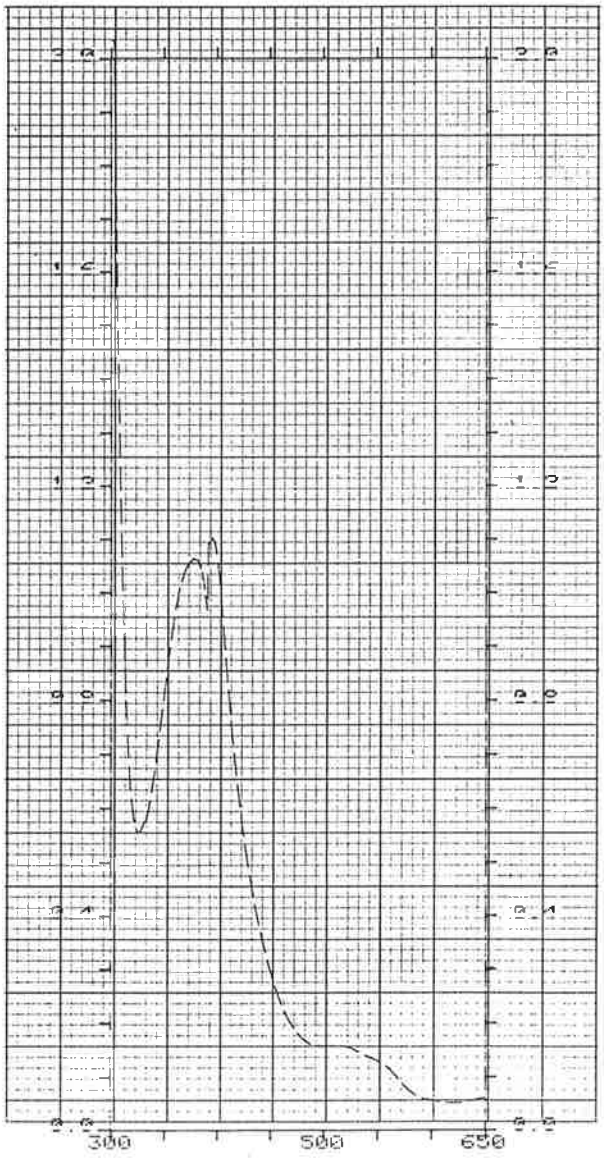
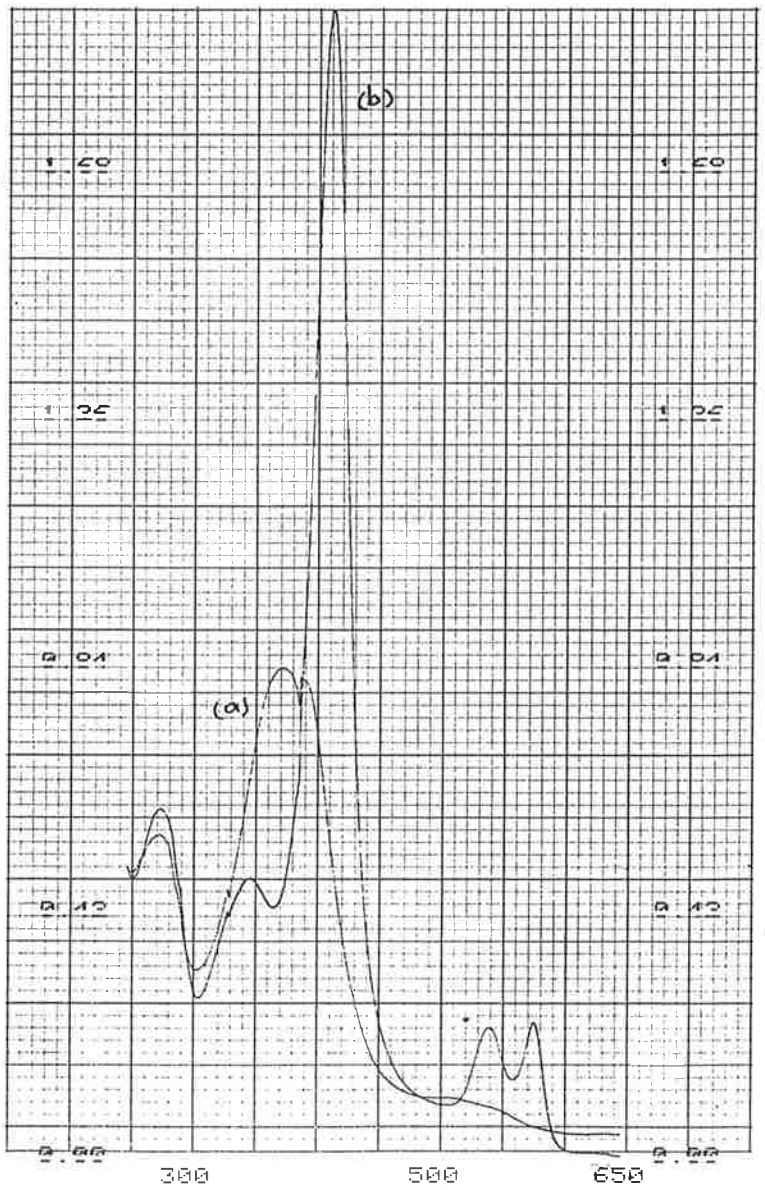



Fig 32 The absorption spectrum of haemoglobin after the addition of 0.1 M acidified thiourea.




Absorbance (OD units)

Wavelength (nm)

Fig 33 An overlay of the changes in the absorption spectrum of haemoglobin after the addition of 0.1 M acidified thiourea (a) vs haemoglobin control (b).

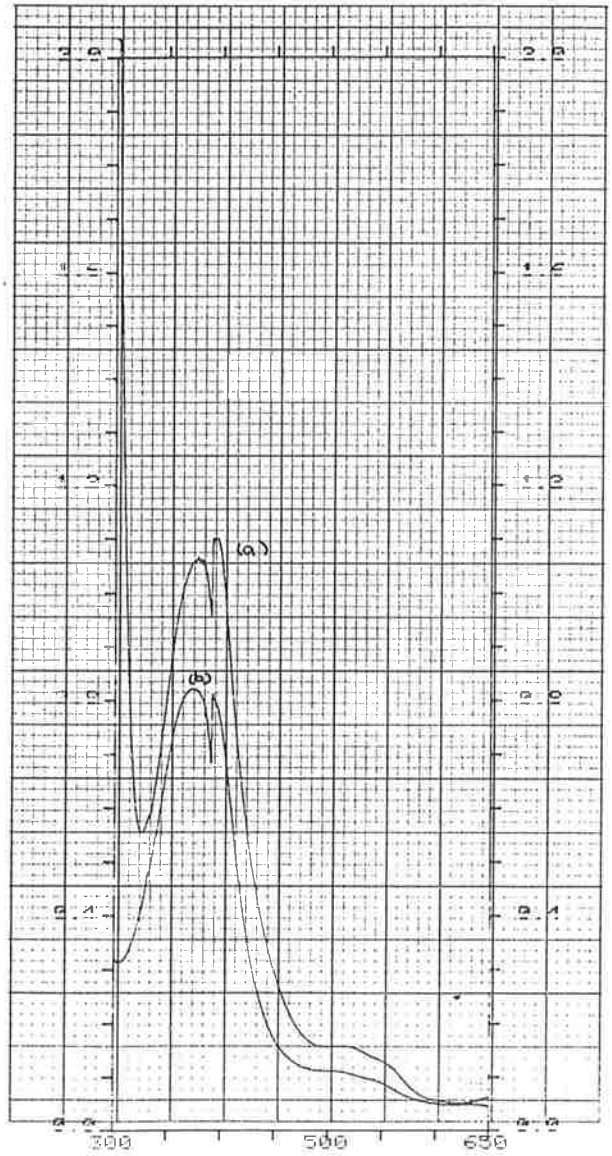



Fig 34 An overlay of the changes in the absorption spectrum of haemoglobin produced by 0.01 M hydrochloric acid (a) and 0.1 M acidified thiourea solution (b).

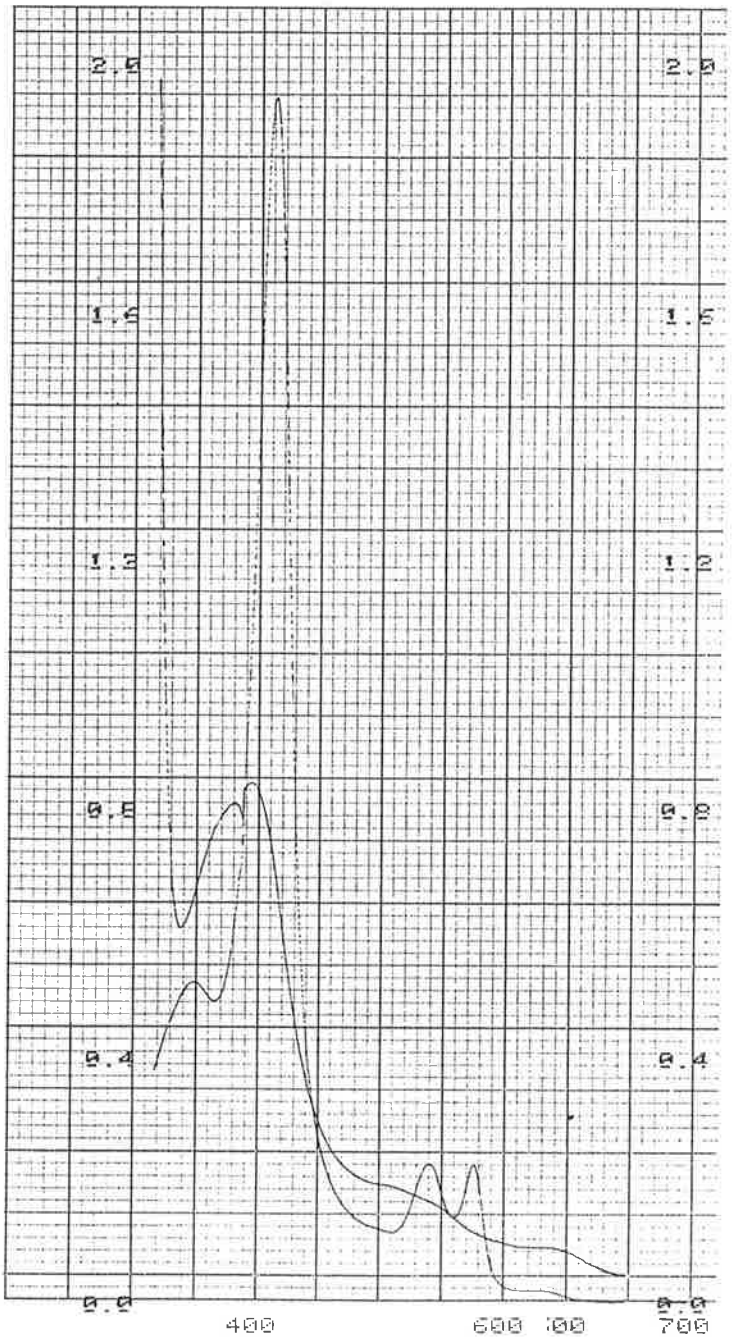



Fig 35 The absorption spectrum of haemoglobin after the addition of 0.1M thiourea dioxide solution vs water.

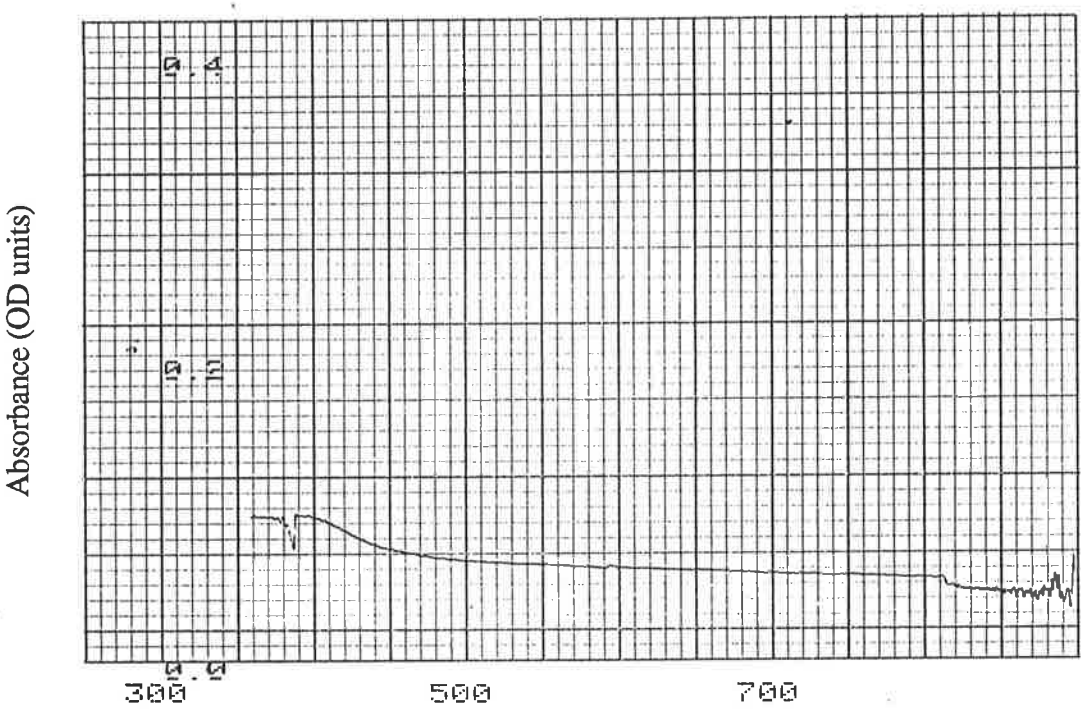



Fig 36 The absorption spectrum of haemoglobin after the addition of 30% w/v hydrogen peroxide.

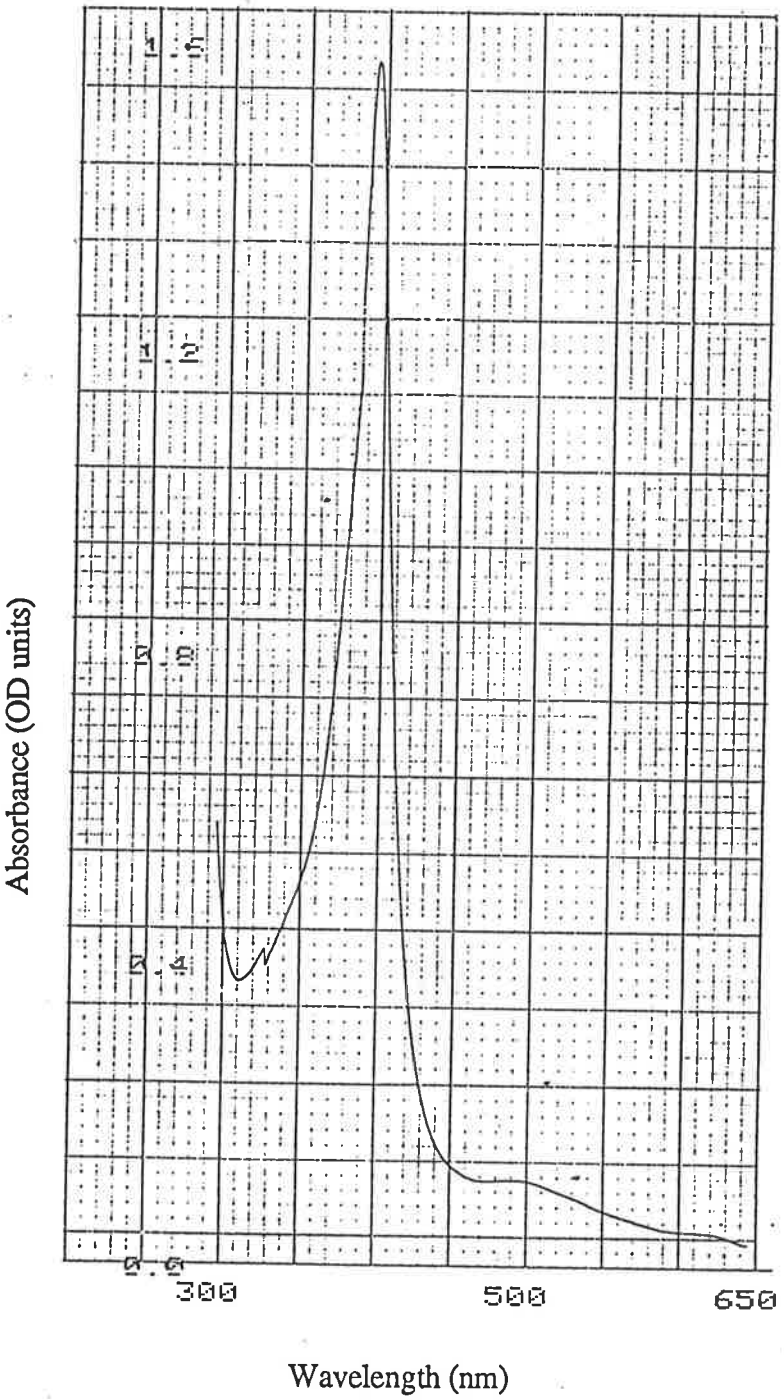



Fig 37 The absorption spectrum of methaemoglobin after the addition of 0.1M aqueous thiourea.

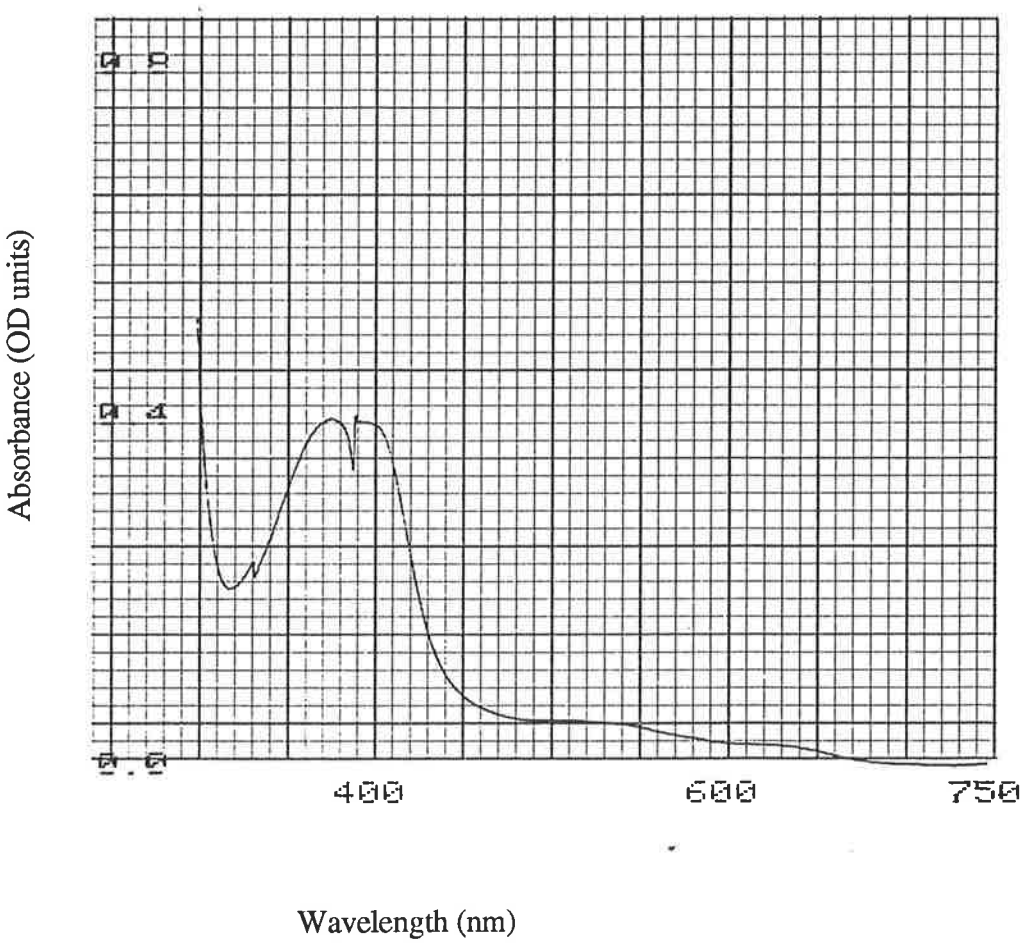



Fig 38 The absorption spectrum of methaemoglobin after the addition of 0.1M acidified thiourea solution.

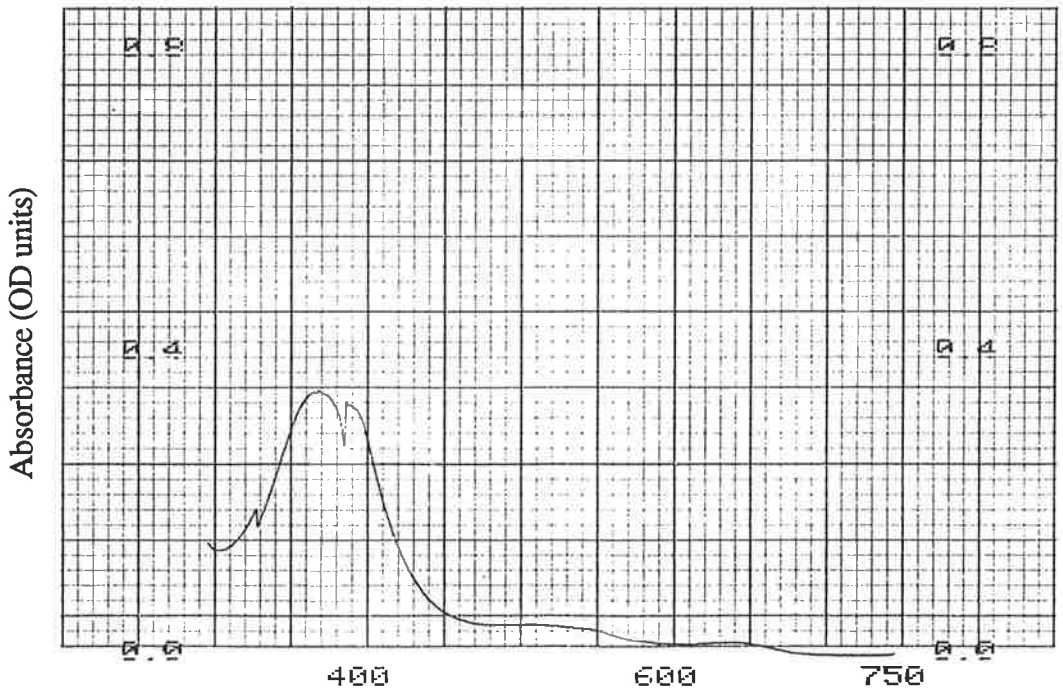



Fig 39 The absorption spectrum of methaemoglobin after the addition of 0.01M hydrochloric acid.

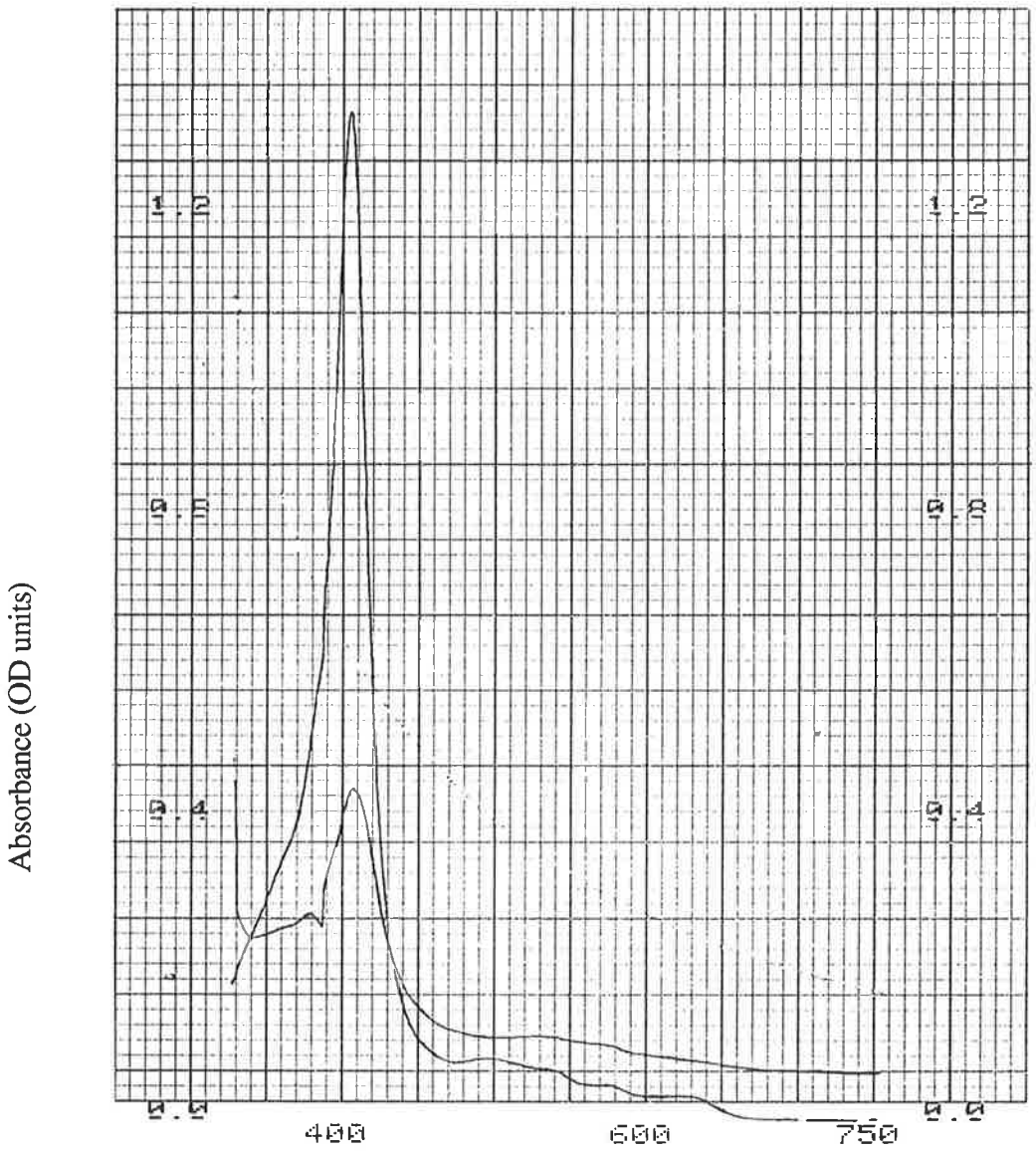



Fig 40 The absorption spectrum of methaemoglobin after the addition of 0.1M thiourea dioxide solution vs water.

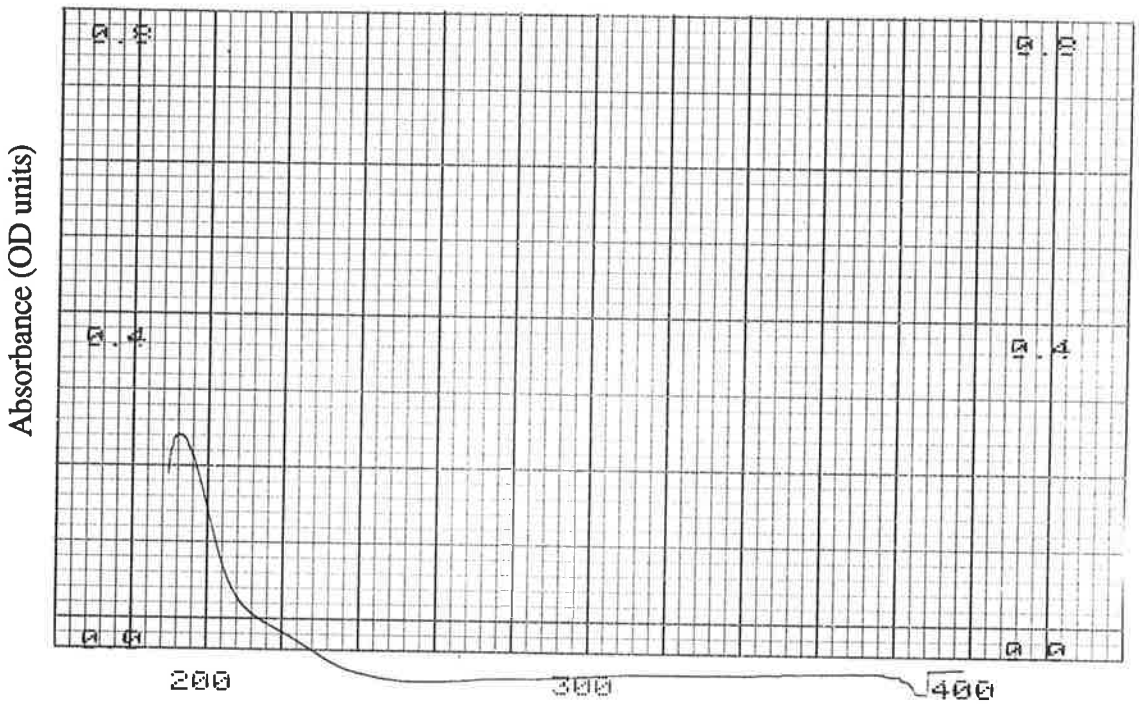



Fig 41 The absorption spectrum of methaemoglobin after the addition of 30% w/v hydrogen peroxide.

absorption at the wavelength 450 - 750 nm. Thiourea dioxide also produced an equivalent reduction in absorption of methaemoglobin at the near ultra-violet region ($\lambda = 410$) nm and minimal absorption at the wavelength 450 - 750 nm. The effect of 30% w/v hydrogen peroxide on the absorption spectrum of methaemoglobin is presented in Fig 41. It may be seen that no absorption peaks were recorded. The methaemoglobin was rendered colourless.

## 9.2 The efficacy of various bleaching protocols on blood stained dentin

The raw data obtained using the Photometer and the Reflection Densitometer for the 23 different bleaching protocols tested are presented in Appendix 5. Table 11 shows the raw data at the end of Stage 3 (24 hours). In summary the highest mean value for the Photometer data, ie the lightest dentin samples, was achieved by the bleaching regimen, acidified thiourea/NaOH/hydrogen peroxide (A2). The lowest mean value, ie the darkest or most stained dentin samples at 24 hours, was recorded for the bleaching regimen NaOH (F2). The greatest bleaching effect ie lowest mean score for the Reflection Densitometer was achieved by the bleaching regimen HCl/hydrogen peroxide (C3) and the highest mean (darkest samples) was obtained with NaOH (F2).

### 9.2.1 Statistical analysis

The complete statistical analysis of the results from this study is presented in Appendix 5. The preliminary analysis was made using the final measurements (Stage 3ii) made for each bleaching regimen, namely those at 24 hours after the bleaching process. The appropriateness of the statistical analysis was confirmed after which an Analysis of Variance of the complete data set for the Photometer and for the Reflection Densitometer was carried out. This indicated that a significant

difference in the results from the different bleaching regimens existed and allowed the more successful treatments to be identified.

### 9.2.2 Results of the preliminary analysis of the Photometer data at 24 hours (Stage 3ii)

From the Photometer data (Table 11) it was possible to separate the results from the bleaching regimens into 4 groups which were statistically significantly different from each other: They are listed in increasing order of effectiveness of 'bleaching'.

- The first group comprising aqueous thiourea (B5), hydrochloric acid (C4) and sodium hydroxide (F2) were similar with means approximately 10 OD units.
- The second group comprised acid thiourea alone (A5), 30% hydrogen peroxide followed by acid thiourea (E1) and 30% hydrogen peroxide followed by aqueous thiourea (E2) had means of around 16 OD units.
- The third group comprised 5% sodium hypochlorite alone (F1) and distilled water (F2) were also similar with means approximately 23 OD units.
- The fourth group comprised thiourea dioxide and all those bleaching regimens which included 30% w/v hydrogen peroxide at the third stage of the bleaching process ie A1-A4, B1-B4, C1-C3, D1-D3. This fourth group of bleaching regimens were all similar with means approximately 30-40 OD units. The results of this group equate in clinical terms to successful bleaching of the blood-stained dentin.

### 9.2.3 Results of the preliminary analysis of the Densitometer data at 24 hours (Stage 3ii)

A similar pattern emerged from the Densitometer data, at 24 hours. The bleaching regimens separated into the following four groups listed in order of decreasing means which equates in clinical terms with increased effectiveness of bleaching.

- Aqueous thiourea alone (B5) and hydrochloric acid (C4) comprised the first group, mean approximately 0.96.
- Acidified thiourea alone (A5), 30% hydrogen peroxide followed by acidified thiourea (E1), 30% hydrogen peroxide followed by aqueous thiourea (E2), as well as sodium hydroxide (F2), and distilled water (F3) comprised the second group with means approximately 0.90.
- The third group consisted only of thiourea dioxide (E3) which had a mean of 0.86.
- The fourth group comprised of all those bleaching regimens with 30% w/v hydrogen peroxide at the third stage of the bleaching process namely, A1-A4, B1-B4, C1-C3, D1-D3, as well as 5% sodium hypochlorite. This group had means approximately 0.82 and most closely approximated the ANSI Standard White, hence this group was the most effective at bleaching the blood-stained dentin.

In summary the most effective bleaching regimens consisted of thiourea dioxide, 5% sodium hypochlorite and all the bleaching regimens which employed 30% hydrogen peroxide at the final stage of the bleaching process. Table 12 shows the means of the bleaching regimens, the standard deviation and the standard error of the mean (s.e.m.) for the Photometer data and for the Reflection Densitometer. The means of the different bleaching regimens were compared using an Analysis of Variance or the LSD value where appropriate (see Appendix 6 for the detailed analyses). The LSD values are shown in the last row of Table 12. These values provide a simple method of pairwise comparisons of the means of the bleaching regimens. They were used to test whether the observed difference between two means was significant, at a 5% level of significance. If the difference between the means of two bleaching regimens was greater than the appropriate LSD, then we concluded that there was a significant difference between the underlying means.

Table 11 Raw data at 24 hours.

| | Mean ± sem (n=4) | |
|---|---|---|
| | Photometer (OD units) | Reflection densitometer (units) / 0 - 2.5 |
| A1 | 28.5 ± 2.2 | 0.823 ± 0.006 |
| A2 | 39.8 ± 3.1 | 0.838 ± 0.002 |
| A3 | 32.5 ± 2.8 | 0.823 ± 0.008 |
| A4 | 30.8 ± 2.0 | 0.820 ± 0.008 |
| A5 | 16.5 ± 3.2 | 0.893 ± 0.016 |
| B1 | 25.0 ± 4.3 | 0.813 ± 0.002 |
| B2 | 36.8 ± 2.9 | 0.815 ± 0.008 |
| B3 | 33.8 ± 0.8 | 0.820 ± 0.004 |
| B4 | 30.3 ± 3.5 | 0.835 ± 0.009 |
| B5 | 9.1 ± 0.4 | 0.953 ± 0.014 |
| C1 | 36.8 ± 2.5 | 0.815 ± 0.010 |
| C2 | 35.8 ± 4.1 | 0.803 ± 0.010 |
| C3 | 37.0 ± 2.0 | 0.798 ± 0.002 |
| C4 | 10.3 ± 2.4 | 0.975 ± 0.003 |
| D1 | 33.5 ± 2.7 | 0.810 ± 0.007 |
| D2 | 32.0 ± 5.0 | 0.838 ± 0.006 |
| D3 | 34.8 ± 6.8 | 0.833 ± 0.005 |
| E1 | 14.5 ± 2.2 | 0.903 ± 0.013 |
| E2 | 18.6 ± 3.0 | 0.898 ± 0.012 |
| E3 | 33.5 ± 4.4 | 0.863 ± 0.010 |
| F1 | 22.8 ± 2.0 | 0.835 ± 0.012 |
| F2 | 9.0 ± 1.3 | 0.917 ± 0.024 |
| F3 | 23.3 ± 0.9 | 0.903 ± 0.020 |

Table 12 Results at 24 hours

Summary of Means and Standard Deviations/s.e.m.(n = 4)

| | Photometer Data | | | Densitometer Data | | |
|---|---|---|---|---|---|---|
| Regimen | Mean | St Dev | s.e.m. | Mean | St Dev | s.e.m. |
| A1 | 28.50 | 4.435 | 2.212 | 0.823 | 0.013 | 0.006 |
| A2 | 39.75 | 6.185 | 3.062 | 0.838 | 0.005 | 0.002 |
| A3 | 32.50 | 5.568 | 2.784 | 0.823 | 0.017 | 0.008 |
| A4 | 30.75 | 3.594 | 1.797 | 0.820 | 0.008 | 0.008 |
| A5 | 16.50 | 6.351 | 3.176 | 0.893 | 0.032 | 0.016 |
| B1 | 25.00 | 8.679 | 4.340 | 0.813 | 0.005 | 0.002 |
| B2 | 36.75 | 5.737 | 2.869 | 0.815 | 0.017 | 0.008 |
| B3 | 33.75 | 1.500 | 0.750 | 0.820 | 0.008 | 0.004 |
| B4 | 30.25 | 6.946 | 3.473 | 0.835 | 0.019 | 0.009 |
| B5 | 9.13 | 0.854 | 0.427 | 0.953 | 0.028 | 0.014 |
| C1 | 36.75 | 4.924 | 2.462 | 0.815 | 0.020 | 0.010 |
| C2 | 35.75 | 8.180 | 4.090 | 0.803 | 0.021 | 0.010 |
| C3 | 37.00 | 3.916 | 1.958 | 0.798 | 0.005 | 0.002 |
| C4 | 10.25 | 4.856 | 2.428 | 0.975 | 0.064 | 0.003 |
| D1 | 33.50 | 5.431 | 2.716 | 0.810 | 0.014 | 0.007 |
| D2 | 32.00 | 9.933 | 4.967 | 0.838 | 0.013 | 0.006 |
| D3 | 34.75 | 13.574 | 6.787 | 0.833 | 0.010 | 0.005 |
| E1 | 14.50 | 4.466 | 2.233 | 0.903 | 0.026 | 0.013 |
| E2 | 18.63 | 5.963 | 2.982 | 0.898 | 0.024 | 0.012 |
| E3 | 33.50 | 8.813 | 4.407 | 0.863 | 0.021 | 0.010 |
| F1 | 22.75 | 4.113 | 2.057 | 0.835 | 0.024 | 0.012 |
| F2 | 9.00 | 3.606 | 1.303 | 0.917 | 0.047 | 0.024 |
| F3 | 23.25 | 1.708 | 0.854 | 0.903 | 0.040 | 0.020 |
| LSD | 8.949 | | | 0.0350 | | |

Table 13

Comparison of the means of Bleaching sets at 24 hours.

| | Photometer | Reflection densitometer |
|---|---|---|
| Comparison | Result | Result |
| A1-A4 | Not Significant | Not Significant |
| B1-B4 | Not Significant | Not Significant |
| C1-C3 | Not Significant | Not Significant |
| (A1-A4) vs A5 | P-value < 0.001 | P-value < 0.001 |
| (B1-B4) vs B5 | P-value < 0.001 | P-value < 0.001 |
| (C1-C3) vs C4 | P-value < 0.001 | P-value < 0.001 |
| (A1-A4) vs (B1-B4) vs (C1-C3) | Not Significant | Not Significant |
| D1, D2, vs A1, A2 vs B1, B2 vs C1, C2 | Not Significant | Not Significant |
| E1 vs E2 vs D3 | P-value < 0.001 | P-value < 0.001 |
| D3 vs E3 | Not Significant (LSD) | Not Significant (LSD) |
| F1 vs D1 | P-value < 0.05 (LSD) | Not Significant (LSD)* |
| F2 vs D2 | P-value < 0.05 (LSD) | P-value < 0.05 (LSD) |
| F1 vs F2 vs D3 | P-value < 0.001 | P-value < 0.001 |
| F3 vs D3 | P-value < 0.05 (LSD) | P-value < 0.05 (LSD) |

* The comparison of the bleaching sets F1 and D1 (NaOCl vs NaOCl/HP) was the only one not consistent between the Photometer and Reflection densitometer.

Table 14 Means of the Photometer data at each stage of bleaching (n = 4). An increase in value indicates a loss of colour.

| Regimen | To | Stage 1 | Stage 2 | Stage 3 (i) | Stage 3 (ii) |
|---|---|---|---|---|---|
| A1 | 13.5 | 14.75 | 13.25 | 25.75 | 28.5 |
| A2 | 18.875 | 20.25 | 21.75 | 33.5 | 39.75 |
| A3 | 17 | 16.825 | 16.25 | 31 | 32.5 |
| A4 | 12.125 | 14.5 | 14.5 | 23.125 | 30.75 |
| A5 | 13.75 | 14.25 | 14.25 | 17 | 16.5 |
| B1 | 6.75 | 7.25 | 9.75 | 22.5 | 25 |
| B2 | 15.625 | 15 | 14.5 | 30.25 | 36.75 |
| B3 | 14 | 14 | 13.25 | 30 | 33.75 |
| B4 | 12.25 | 11 | 11 | 26.25 | 30.25 |
| B5 | 5.75 | 6.25 | 6.25 | 8.625 | 9.125 |
| C1 | 12.75 | 14.75 | 19.125 | 33.25 | 36.75 |
| C2 | 14.375 | 15.75 | 18 | 32 | 35.75 |
| C3 | 13.75 | 15.5 | 15.5 | 33.25 | 37 |
| C4 | 4.12 | 5.12 | 5.125 | 6.75 | 10.25 |
| D1 | 13.875 | 17.25 | 17.25 | 29.75 | 33.5 |
| D2 | 12.375 | 11.375 | 11.375 | 22.5 | 32 |
| D3 | 13.375 | 19.625 | 19.625 | 26 | 34.75 |
| E1 | 4.75 | 10.75 | 10.75 | 15.625 | 15.375 |
| E2 | 5.875 | 9.125 | 9.125 | 14.75 | 18.625 |
| E3 | 6.125 | 6.75 | 6.75 | 13 | 33.5 |
| F1 | 10 | 13.25 | 13.25 | 14.5 | 22.75 |
| F2 | 4.25 | 5 | 5 | 5.3 | 6.75 |
| F3 | 16 | 15.75 | 15.75 | 18 | 23.25 |

| sig diff $p < 0.5$ | Stage 1 - To | Stage 2 - Stage 1 | Stage 3 (1) - Stage 2 | Stage 3(ii) - Stage 3(i) | Stage 3(ii) - To |
|---|---|---|---|---|---|
| LSD value | 3.123 | 1.574 | 6.160 | 6.640 | 8.248 |

Table 13 shows the results of the comparison of the bleaching regimens that were made for the Photometer and the Reflection Densitometer. It may be seen that the comparisons were consistent for the Photometer and the Reflection Densitometer data except for that of F1 (NaOCl control) vs D1 (NaOCl /hydrogen peroxide).

9.2.4 Results of the Photometer at each stage of the bleaching process

The mean results of the 23 bleaching regimens at each stage of the bleaching process are presented in Table 14. The LSD values for the change in the mean between subsequent bleaching stages is also shown. Histograms of the mean change of the 23 bleaching regimens for Stage 1 - To, Stage 2 - Stage 1, Stage 3 (1 hour) - Stage 2, Stage 3 (24 hours) - Stage 3 (1 hour) and Stage 3 (24 hours) - To including the appropriate LSD values are shown in Figs 42 - 46. These plots allowed a simple comparison of the change in the mean at various stages of the bleaching process. Again the LSD values were used to test whether the observed difference between the different stages of the bleaching process was statistically significant at a 5% level of significance. If the difference between the means of two stages of a bleaching regimen is greater than the appropriate LSD, we concluded that there was a significant difference between the bleaching stages.

*Stage 1-versus To (Fig 42)*

The bleaching regimens which showed a statistically significant improvement in colour after Stage 1 were those which employed NaOCl namely D1 and F1 or 30% w/v hydrogen peroxide (D3, E1, E2) as the initial solution.

*Stage 2 versus Stage 1 (Fig 43)*

Most of the samples remained unbleached. The bleaching regimens which showed a statistically significant change (ie loss of colour) were C1 and B1 which had 5%

NaOCl as the second stage, and C2 (HCl followed by NaOH). Three bleaching regimens had a lower OD value at stage 2 which equates to a deepening in colour but this was not statistically significant.

*Stage 3 (1 hour) versus Stage 2 (Fig 44)*

All the bleaching regimens which had 30% hydrogen peroxide as the third stage of the bleaching process showed a statistically significant reduction in colour at this time (ie after immersion in the hydrogen peroxide for 1 hour) namely A1-A4, B1-B4, C1-C3, D1-D3. The thiourea dioxide (E3) also registered a statistically significant bleaching at this time.

*Stage 3 (24 hours) versus Stage 3 (1 hour) (Fig 45)*

The bleaching effect with some of the regimens which incorporated hydrogen peroxide in the final stage of the bleaching process did not continue at the same rate and did not show a statistically significant difference between Stage 3 (1 hour) and Stage 3 (1 day). The bleaching regimens which were statistically significantly different were acidified thiourea/hydrogen peroxide (A4), NaOH/hydrogen peroxide (D2), hydrogen peroxide alone (D3), NaOCl alone (F1) and thiourea dioxide (E3). The thiourea dioxide was significantly better at 24 hours than 1 hour compared with all other bleaching regimens.

*Stage 3 (24 hours) versus To (Fig 46)*

All the bleaching regimens which employed hydrogen peroxide or 5 % NaOCl as a part of the bleaching process showed a statistically significant improvement compared with To ie were successfully bleached. However thiourea dioxide showed the greatest mean change observed between Stage 3 (24 hours) and To .

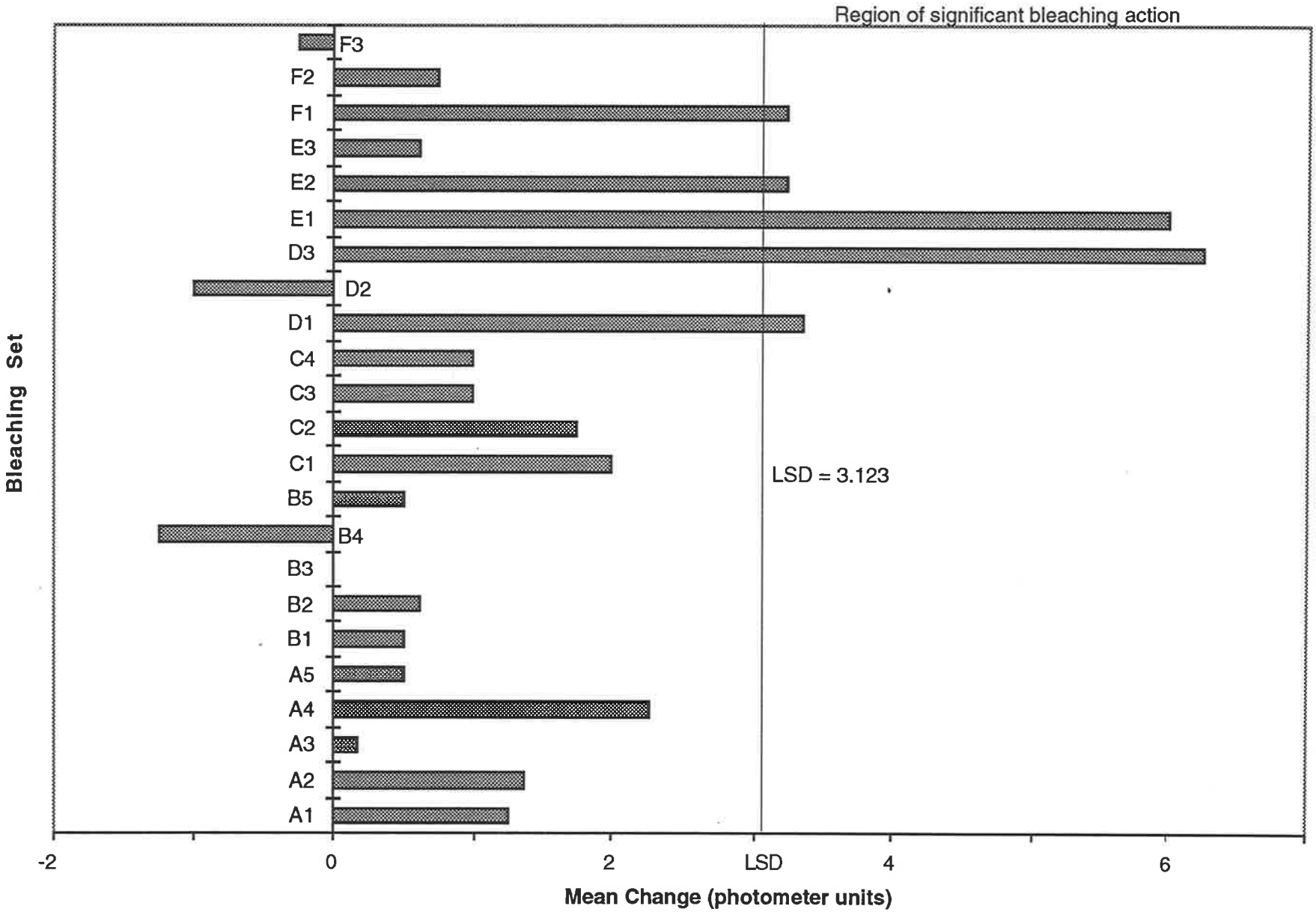



Fig 42 Mean change for the Photometer data Stage 1 - To.

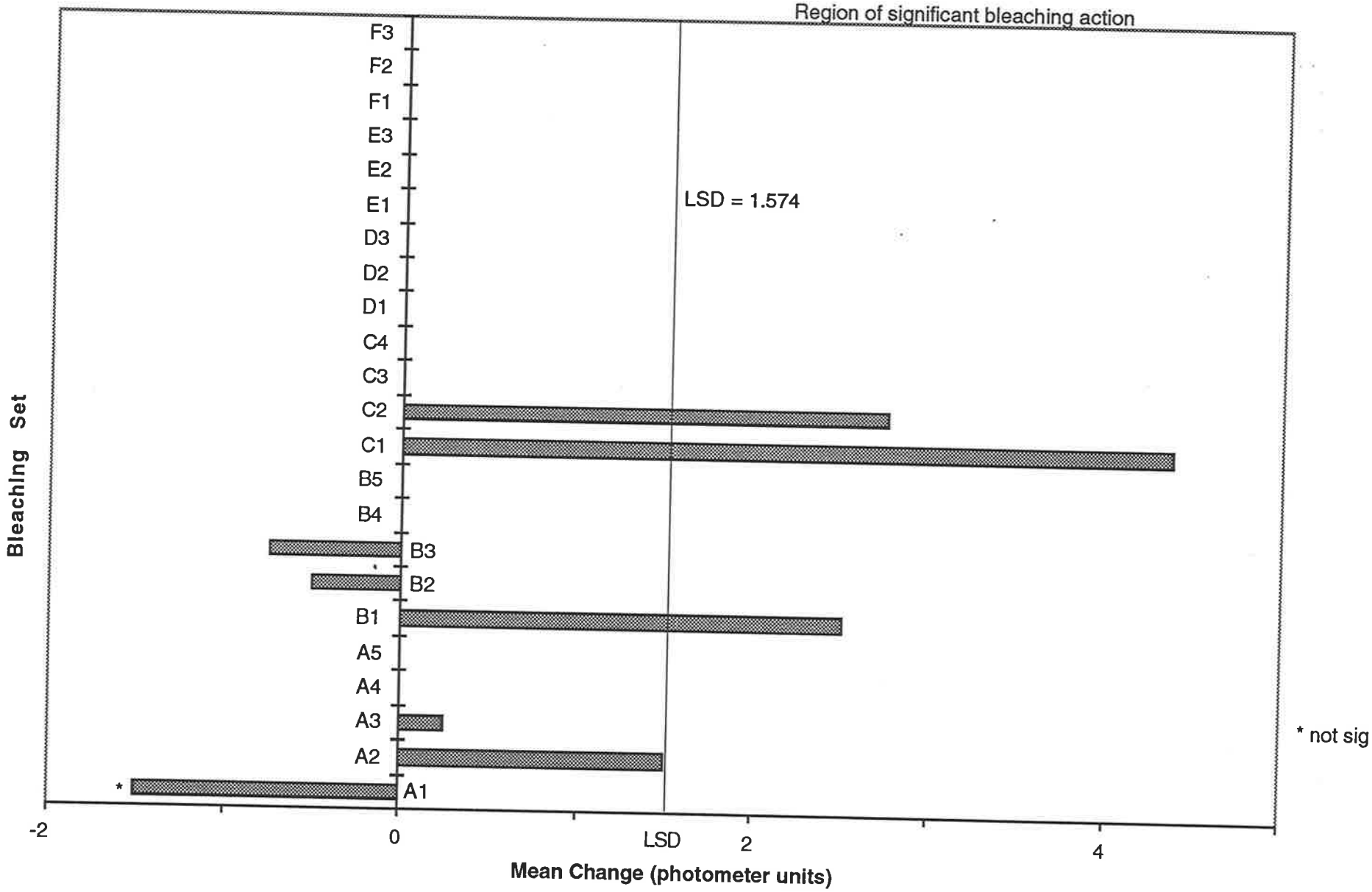



Fig 43 Mean change for the Photometer data Stage 2 - Stage 1.

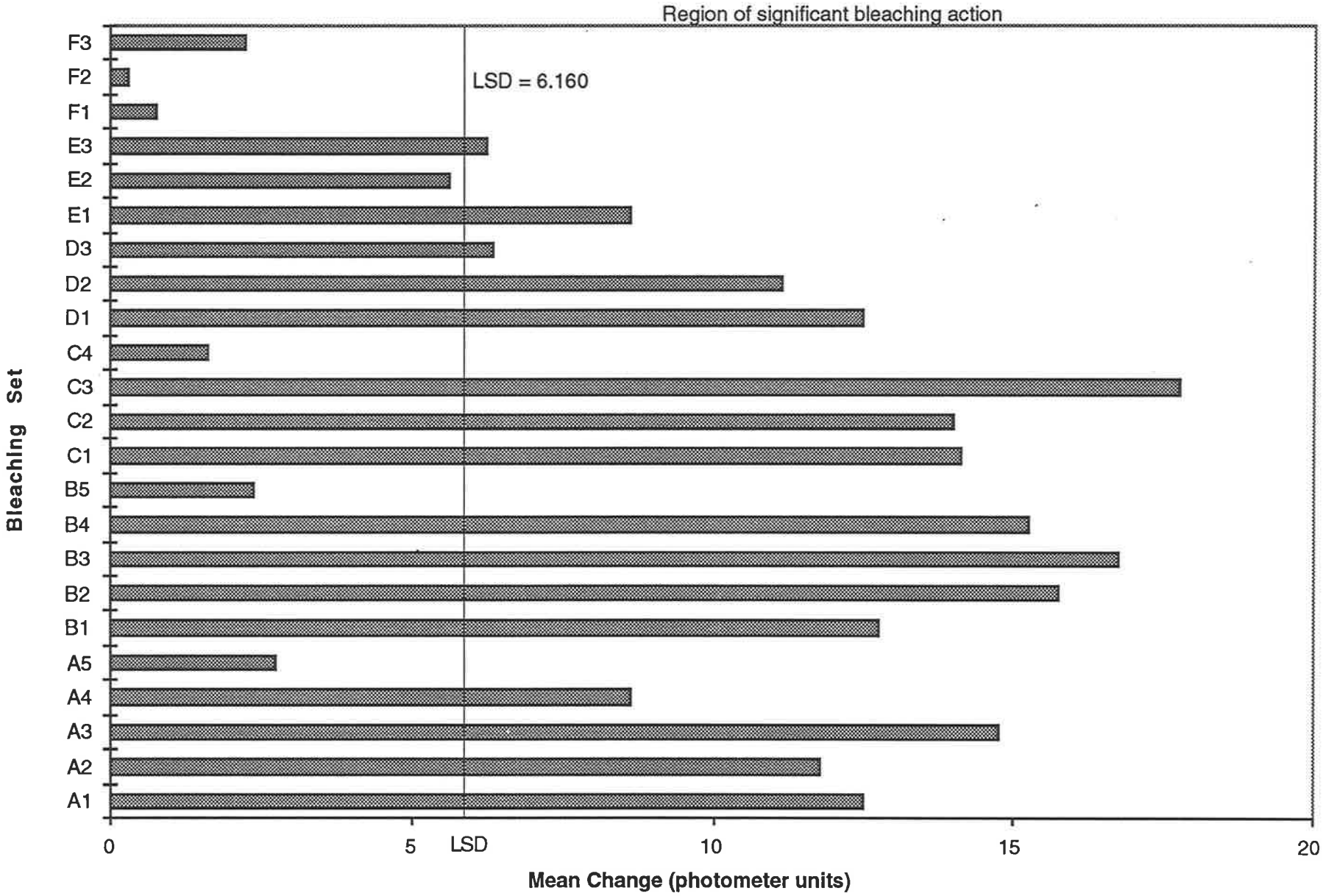



Fig 44 Mean change for the Photometer Stage 3(1 hour) - Stage 2.

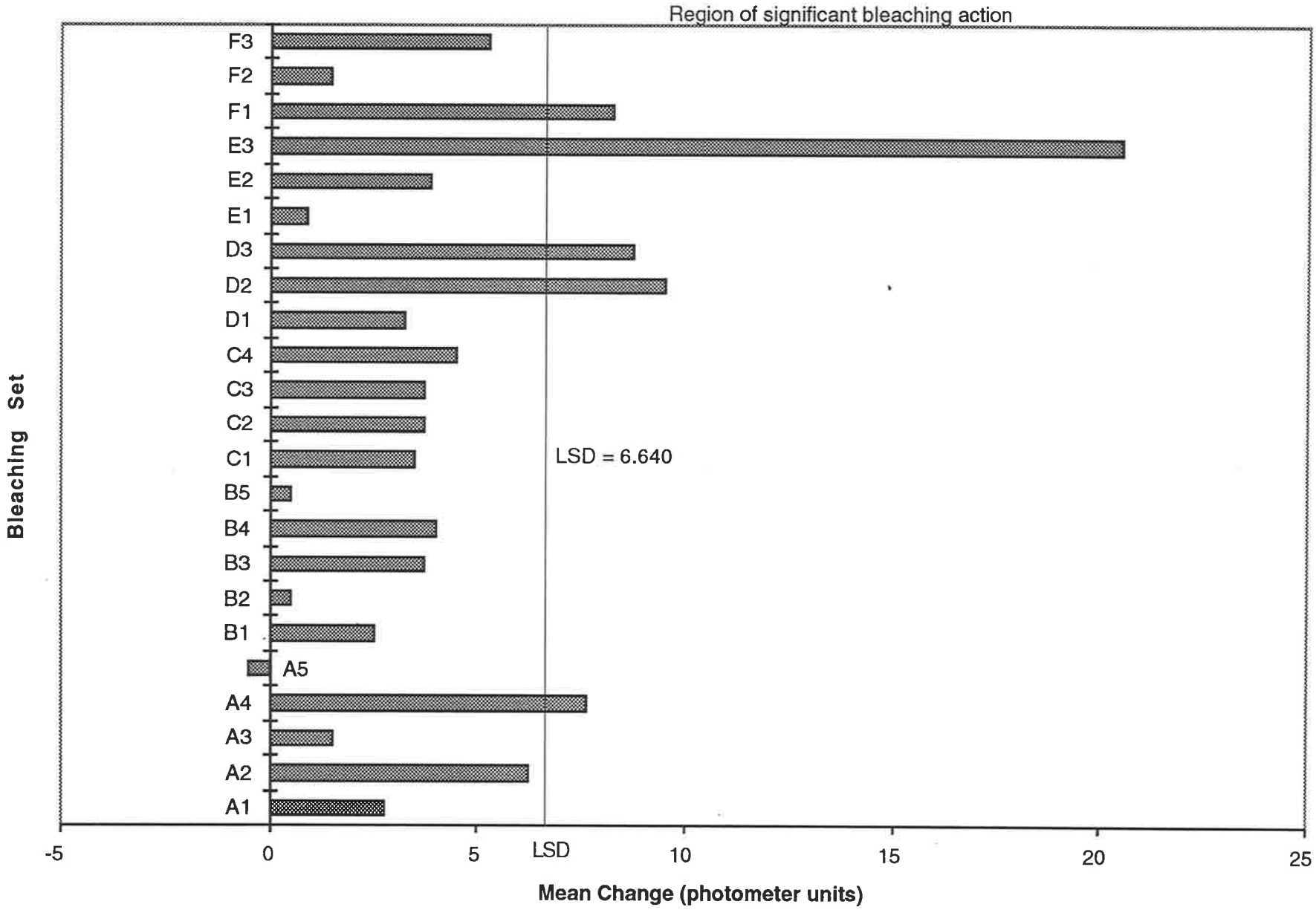



Fig 45 Mean change for the Photometer data Stage 3 (24 hours) - Stage 3 (1 hour).

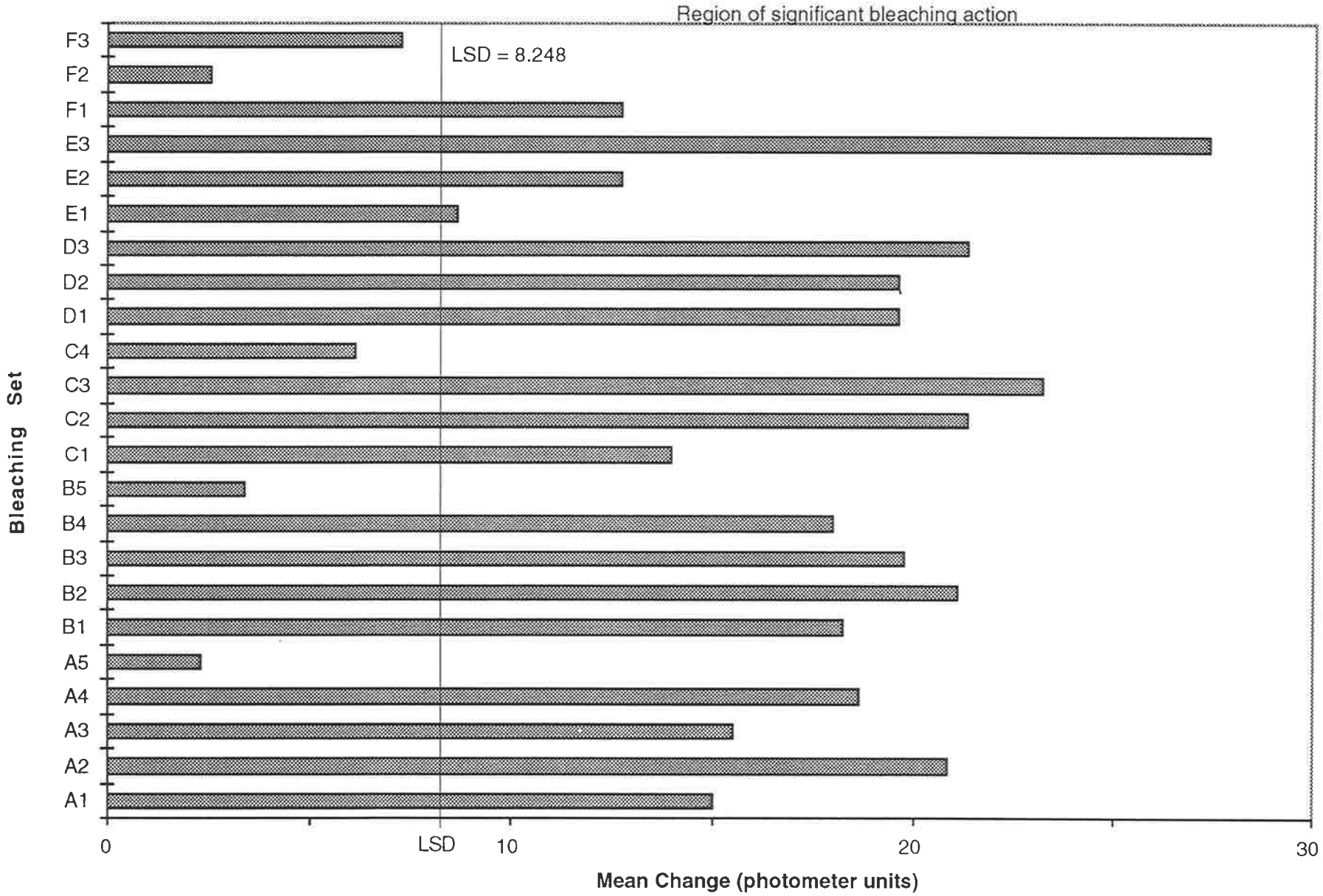



Fig 46 Mean change for the Photometer Stage 3 (24 hours) - T0.

9.2.4 Results of the Reflection Densitometer at each stage of the bleaching process

The means of the 23 bleaching regimens at each stage of the bleaching process for the Reflection Densitometer are presented in Table 15. The LSD values for the change in the mean between subsequent bleaching stages is also shown. Histograms of the mean change of the 23 bleaching regimens for Stage 1 - To, Stage 2 - Stage 1, Stage 3 (1 hour) - Stage 2, Stage 3 (24 hours) - Stage 3 (1 hour) and Stage 3 (24 hours) - To including the appropriate LSD values are shown in Figs 47 - 51.

*Stage 1 versus To (Fig 47)*

The results for the Reflection densitometer were similar with those obtained for the Photometer. The same bleaching regimens showed a statistically significant improvement however D1 was statistically significant at a probability of 1%.

*Stage 2 versus Stage 1 (Fig 48)*

Unlike the Photometer data only aqueous thiourea dioxide/NaOH (B2) showed a statistically significant change between these stages.

*Stage 3 (1 hour) versus Stage 2 (Fig 49)*

All the bleaching regimens which showed a statistically significant difference for the Photometer data were also significantly better with the Reflection Densitometer with the exception of thiourea dioxide (E3) which had a mean change equal to 0.0375 (LSD = 0.383).

*Stage 3 (24 hours) versus Stage 3 (1 hour) (Fig 50)*

The greatest variation in the results between the Photometer and Reflection Densitometer occurred at this stage. The bleaching regimens which were statistically significantly different were acidified thiourea/NaOCl/hydrogen peroxide (A1),

aqueous thiourea/NaOCl/hydrogen peroxide (B1), aqueous thiourea/NaOH/ hydrogen peroxide (B2), aqueous thiourea (B5), HCl/NaOCl/hydrogen peroxide (C2), NaOCl/hydrogen peroxide (D1), thiourea dioxide (E3), NaOCl (F1) , the latter two were also statistically significant with the Photometer, and NaOH (F2). C3 (HCl/hydrogen peroxide) was almost statistically significant (c.f. C3 mean change = 0.025, LSD = 0.0252).

*Stage 3 (24 hours) versus To (Fig 51)*

There was good correlation between the results of the Photometer and Reflection Densitometer at this stage. All the bleaching regimens which were statistically significantly better with the Photometer also showed a significant improvement with the Reflection Densitometer. NaOH (F2) was also significantly different however it must be remembered that one sample of F2 was lost at stage 3.

*Summary of the results*

The results of this study indicate that in general 30% w/v hydrogen peroxide bleached the blood-stained dentin quickly and reached its maximum effectiveness by 1 hour. Bleaching by the reducing agent thiourea dioxide however showed the greatest effect at the end of the 24 hours. Although slower thiourea dioxide was equally effective at 24 hours at bleaching blood-stained dentin as 30% w/v hydrogen peroxide. Acidified thiourea, aqueous thiourea and HCl were ineffectual as bleaching agents for blood-stained dentin. At 24 hours there was no statistical significance between the bleaching regimens that employed aqueous thiourea or acidified thiourea at Stage 1 and their controls. 5% NaOCl also bleached the dentin samples but the result obtained was not consistent for the Photometer and Reflection Densitometer. The hydrogen peroxide was better than the 5% NaOCl as measured by the Photometer but the Reflection Densitometer showed no difference between them.

Table 15 Means of the Reflection densitometer data at each stage of bleaching (n = 4). A decrease in value indicates a loss of colour.

| Regimen | To | Stage 1 | Stage 2 | Stage 3 (i) | Stage 3 (ii) |
|---|---|---|---|---|---|
| A1 | 0.965 | 0.9225 | 0.9225 | 0.86 | 0.8225 |
| A2 | 0.98 | 0.94 | 0.9375 | 0.825 | 0.8375 |
| A3 | 0.925 | 0.925 | 0.92 | 0.84 | 0.8225 |
| A4 | 0.9375 | 0.915 | 0.915 | 0.8425 | 0.82 |
| A5 | 0.93 | 0.9075 | 0.9075 | 0.9 | 0.8925 |
| B1 | 0.9475 | 0.935 | 0.91 | 0.845 | 0.8125 |
| B2 | 1.015 | 1.005 | 0.9325 | 0.85 | 0.815 |
| B3 | 0.9475 | 0.93 | 0.915 | 0.8225 | 0.82 |
| B4 | 0.9425 | 0.93 | 0.93 | 0.845 | 0.835 |
| B5 | 0.99 | 0.995 | 0.995 | 0.9875 | 0.9525 |
| C1 | 0.9355 | 0.9025 | 0.9175 | 0.8275 | 0.815 |
| C2 | 0.925 | 0.9125 | 0.9025 | 0.8375 | 0.8025 |
| C3 | 0.9275 | 0.9125 | 0.9125 | 0.8225 | 0.7975 |
| C4 | 1.035 | 1.0225 | 1.0225 | 1.015 | 1 |
| D1 | 0.94 | 0.8925 | 0.8925 | 0.845 | 0.81 |
| D2 | 0.97 | 0.955 | 0.955 | 0.8475 | 0.8375 |
| D3 | 0.95 | 0.8975 | 0.8975 | 0.8475 | 0.8325 |
| E1 | 0.995 | 0.935 | 0.935 | 0.8975 | 0.9025 |
| E2 | 0.9725 | 0.92 | 0.92 | 0.89 | 0.8975 |
| E3 | 0.975 | 0.9625 | 0.9625 | 0.925 | 0.8625 |
| F1 | 0.9975 | 0.8975 | 0.8975 | 0.88 | 0.835 |
| F2 | 0.9925 | 0.9925 | 0.9925 | 0.9575 | 0.916 |
| F3 | 0.995 | 0.935 | 0.935 | 0.925 | 0.9026 |

| Sig diff p< 0.05 | Stage 1 - To | Stage2 - Stage1 | Stage 3 - Stage 2 | Stage 3 (ii)- Stage 3 (i) | Stage 3 (ii)- To |
|---|---|---|---|---|---|
| LSD value | -0.0503 | -0.0332 | -0.0382 | -0.0252 | -0.0659 |

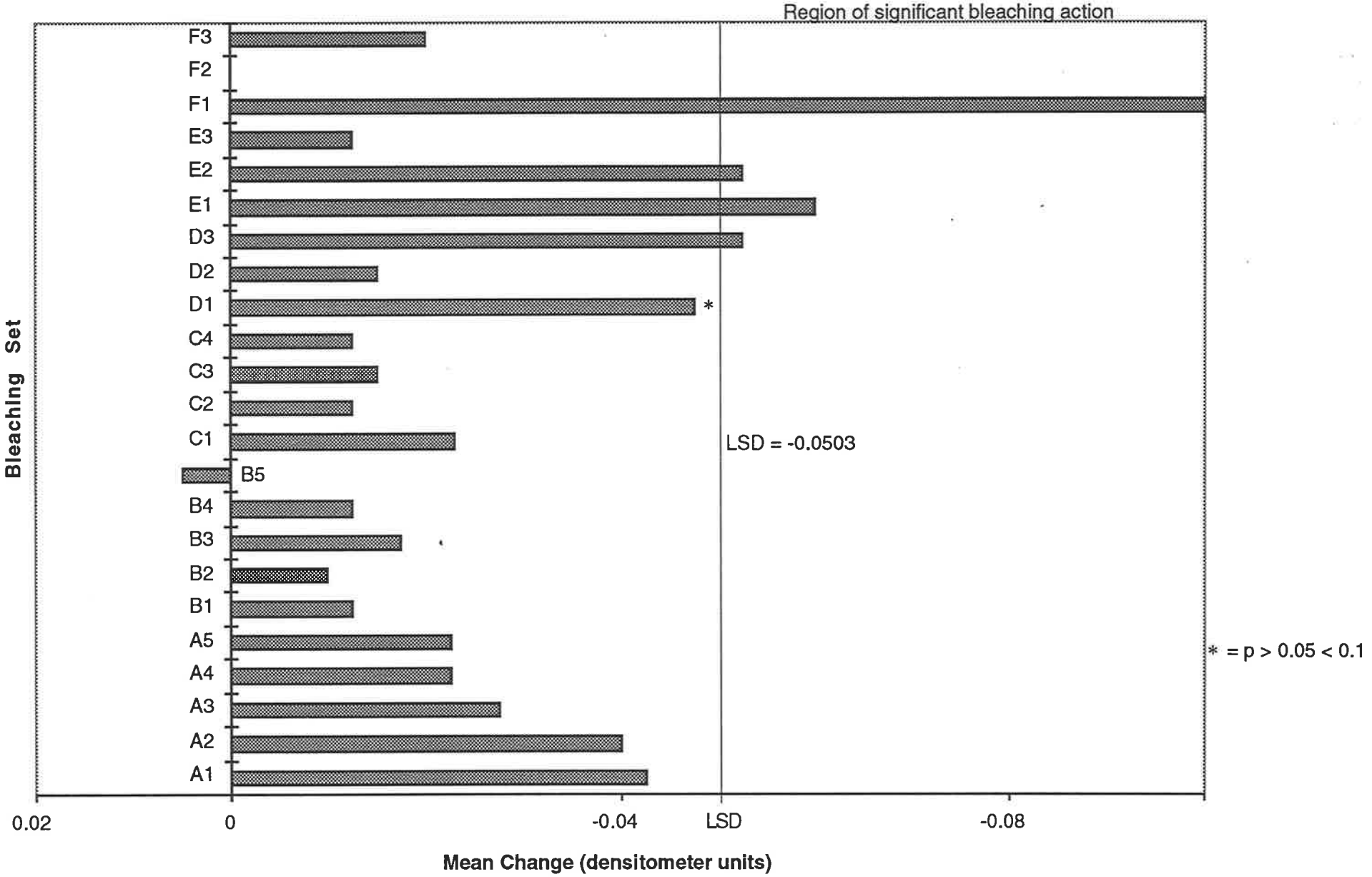



Fig 47 Mean change for the Densitometer data Stage 1 versus To.

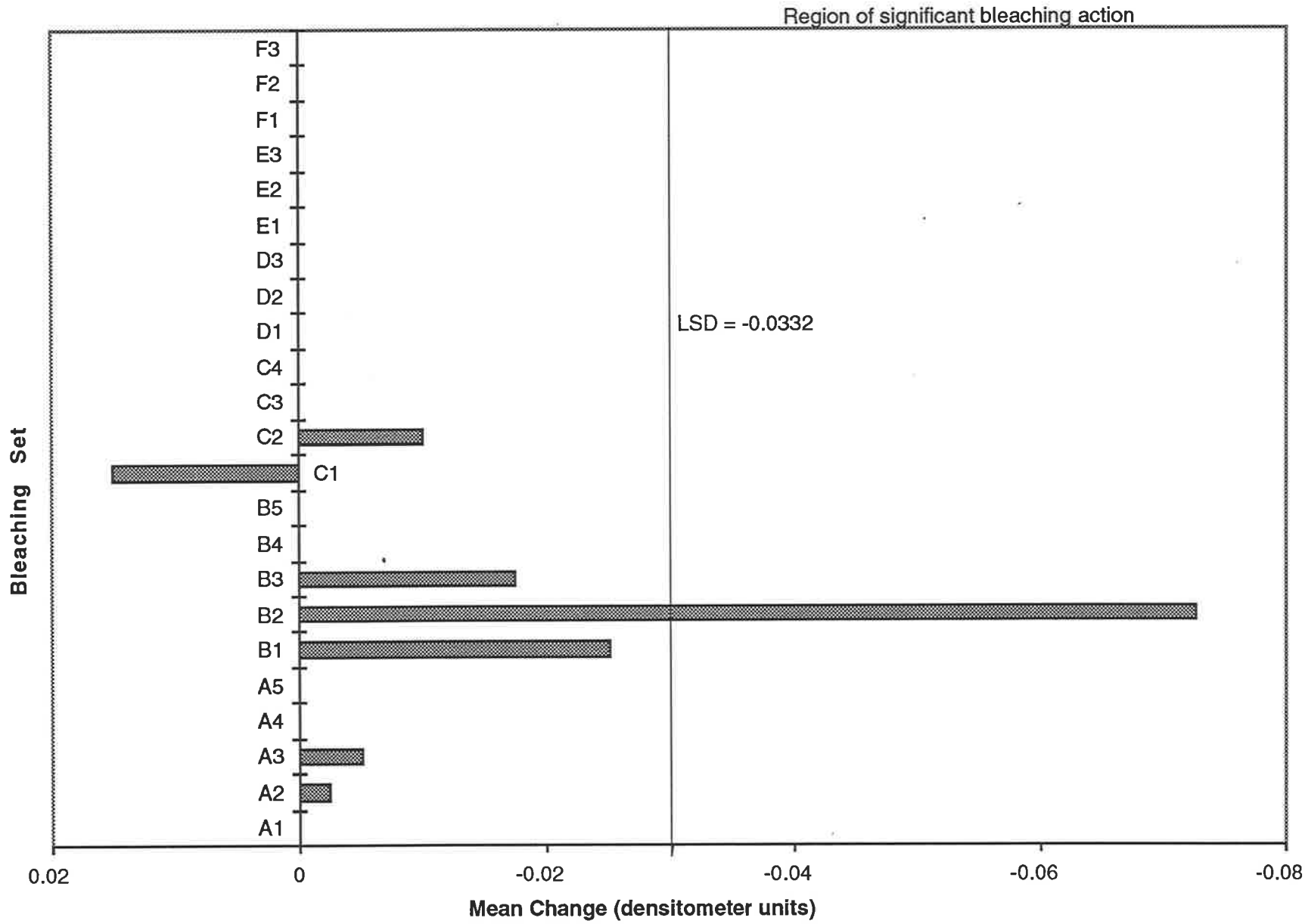



Fig 48 Mean Change for the Reflection densitometer Stage 2 versus Stage 1.

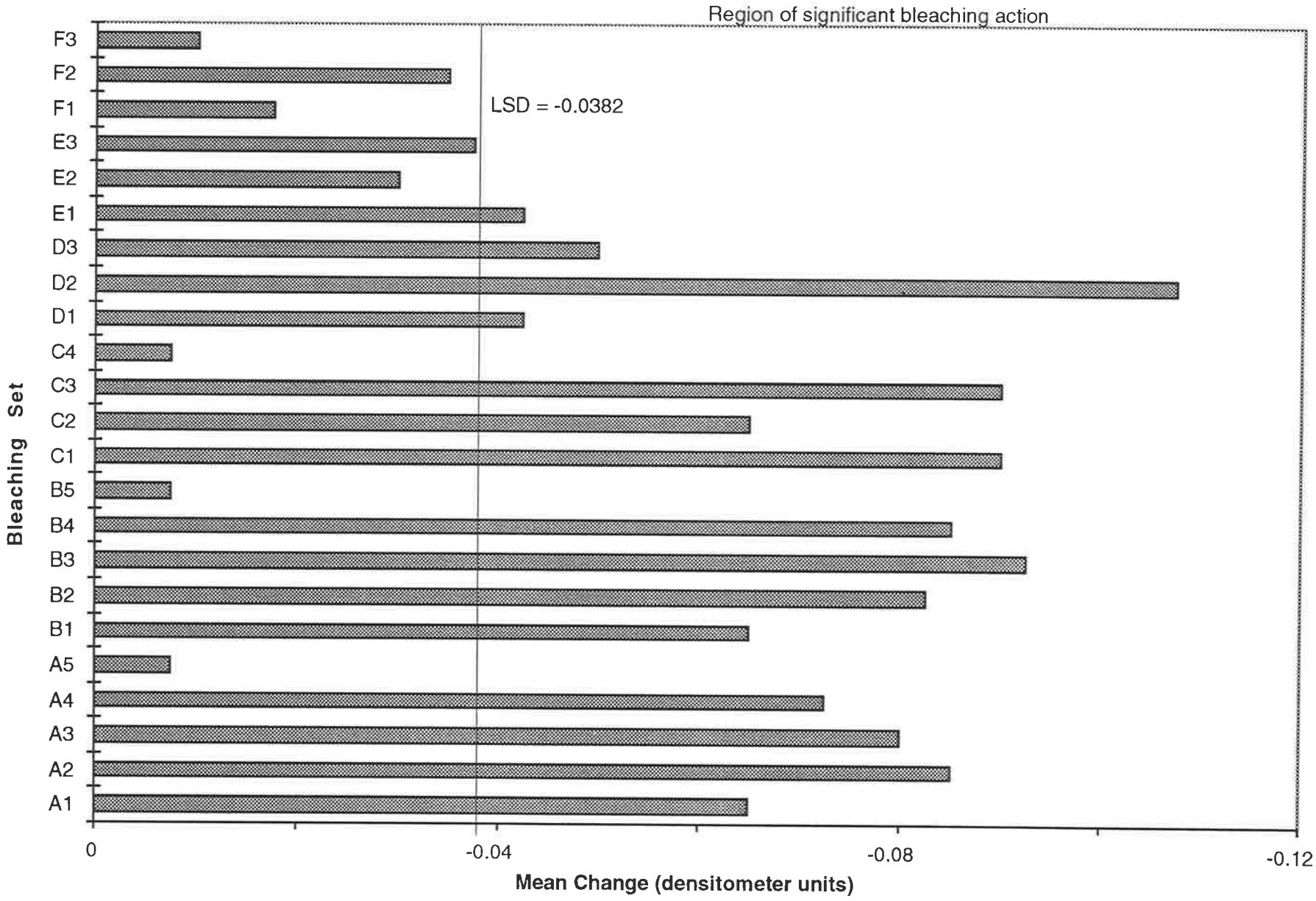



Fig 49 Mean change for the Reflection densitometer Stage 3 (1 hour) versus Stage 2.

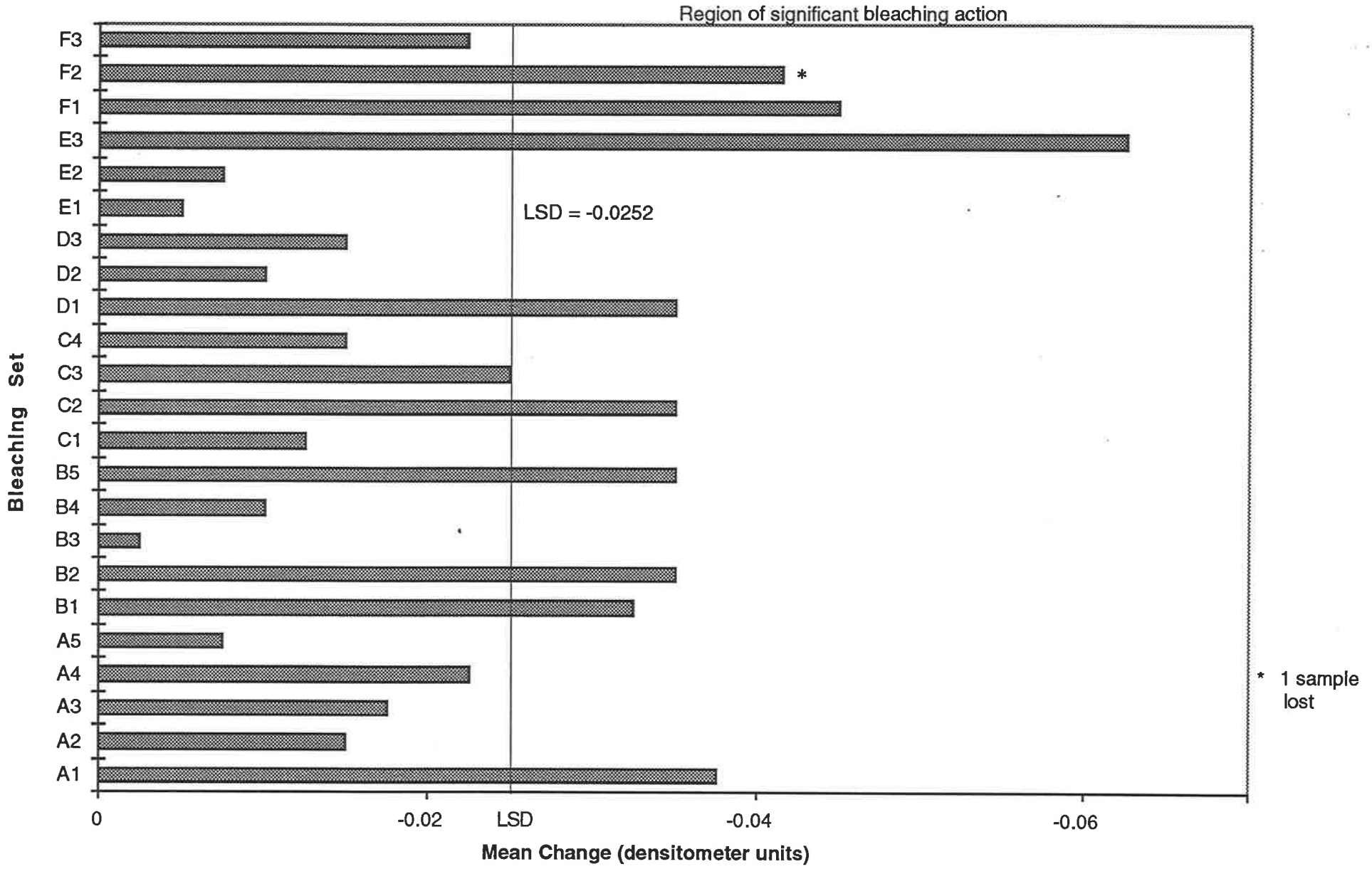



Fig 50 Mean change for the Reflection densitometer Stage 3 (24 hours) versus Stage 3 (1 hour).

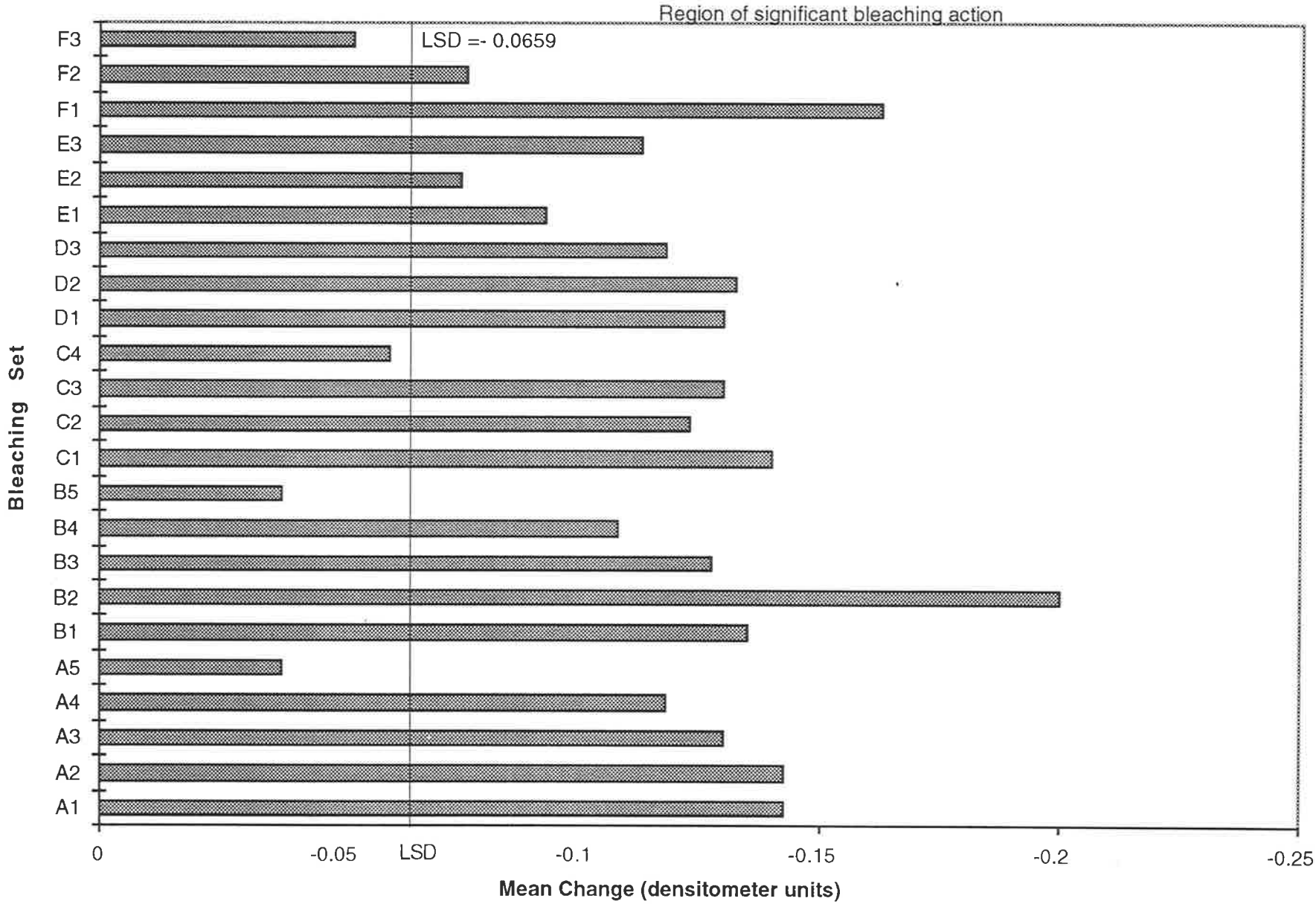



Fig 51 Mean change for the Reflection densitometer Stage 3 (24 hours) versus To.

# 10 DISCUSSION

Haemorrhage into the pulp cavity after trauma is an important aetiological factor for tooth discolouration (Ingle and Beveridge, 1976). The early pink discolouration is due to haemoglobin released from red blood cells penetrating into the dentinal tubules (Cohen and Burns, 1980). Subsequently darkly coloured derivatives produced by the decompositon of haemoglobin shift the colour from pink to grey (Goldstein, 1976). It is a commonly held belief that the degradation of haemoglobin proceeds to release the Fe from the haem moiety and that this combines with sulphides produced by bacteria to form FeS. As FeS is black it is suggested that this is the cause of darkly stained teeth. In a recent *in vitro* study of teeth discoloured by blood products, Marin (1993) concluded that in the absence of bacteria Fe was not released from the haem structure and that discolouration was due to haemoglobin or one of the haematin molecules.

Current techniques for intra-coronal bleaching of root-filled teeth employ oxidative bleaching with hydrogen peroxide. However, concern over the potential for external cervical resorption in teeth that have been treated by intra-coronal bleaching particularly with 30% hydrogen peroxide has often been expressed in the dental literature and recommendations have been made to limit the use of this agent (Friedman et al, 1988; Madison and Walton, 1990). Recommendations have also been made on the placement of an intermediate base over the root filling, prior to bleaching, to limit the diffusion of hydrogen peroxide down the root dentin (Hansen-Bayless, and Davis, 1992; Smith, Cunningham and Montgomery, 1992). The use of catalase, an enzyme that breaks down hydrogen peroxide following intracoronal bleaching has been suggested (Rotstein, 1993). Dahlstrom, Bridges and Heithersay (1993) demonstrated that hydroxyl radicals are produced when teeth stained with blood products are bleached with hydrogen peroxide. Hydroxyl

radicals are extremely reactive and can destroy connective tissue products and may be a causative factor in bleaching related invasive cervical resorption (Dahlstrom, Bridges and Heithersay, 1993). Thiourea is a highly cell permeable scavenger of hydrogen peroxide and hydroxyl radicals (Stahl, Pan, and Longhurtst, 1993). It has been used in the textile industry in combination with 30% hydrogen peroxide to bleach wool and (ground) wood pulp with results superior to 30% hydrogen peroxide alone. Bleaching with this dual process is believed to occur by the interaction of hydrogen peroxide and thiourea to produce the reducing agent thiourea dioxide. Chemical bleaching with reducing agents has been discussed in Chapter 2.

The development of a combined oxidative-reductive bleaching protocol for root-filled teeth using hydrogen peroxide and thiourea offers the possibility of producing superior clinical bleaching and potentially may have the added benefit of removing toxic hydroxyl radicals.

The aim of the present study was to investigate the effectiveness of combining thiourea and hydrogen peroxide in bleaching root-filled teeth discoloured by blood products.

In the first part of the study the effectiveness of various amine bleaching agents was compared with 30% hydrogen peroxide by quantitative and qualitative analysis of the absorption spectra of haemoglobin and methaemoglobin. The amine bleaching agents studied were 0.1 M aqueous thiourea, 0.1 M acidified thiourea and 0.1 M thiourea dioxide. The negative and positive controls for the spectrophotometric studies were 0.01 M hydrochloric acid and 30% w/v hydrogen peroxide respectively. Since haemoglobin undergoes auto-oxidation fresh haemoglobin was prepared by the haemolysis and ultracentrifugation of packed red blood cells. The

absorption spectra obtained using the "Lambda - 5" UV/VIS spectrophotometer were consistent with those reported in the literature. Methaemoglobin was obtained from commercial sources.

The spectrophotometric study showed that aqueous thiourea alone had no effect on the absorption spectra of haemoglobin, or of methaemoglobin and hence no effect on the colour. It was therefore concluded that aqueous thiourea alone is ineffective as a bleaching agent for haemoglobin and methaemoglobin. This preliminary finding was confirmed by the latter study of the decolourisation of blood-stained dentin.

Acidified thiourea and 0.1M hydrochloric acid produced similar changes to the absorption spectra of haemoglobin and methaemoglobin. The magnitude of the absorbance in the visible range was reduced but not completely eliminated. Oxidative denaturation of hemoglobin is increased as the pH is lowered (Rice Evans and Burdon, 1994). Hydrochloric acid in particular has been used to denature haemoglobin prior to decolourisation with oxidative agents (Nakamura et al, 1994). It seems reasonable to conclude that the changes noted in the absorption spectra of haemoglobin and methaemoglobin in the present study is most likely due to denaturation of haemoglobin and methaemoglobin in response to the low pH of these agents. This effect of low pH on haemoglobin has not previously been considered in bleaching blood-stained teeth. Acid etching of dentin with 50% phosphoric acid prior to intra-coronal bleaching has been recommended in order to open up the dentinal tubules and enhance penetration by the bleaching agent (Walton, O'Dell and Lake, 1983). The benefit of dentinal etching has been disputed by Casey, Schnilder, Murata and Burgess (1989) who found no significant difference in bleaching with and without dentinal etching with phosphoric acid. The authors used visual colour determination by matching to a shade tab. They reported

that the individual investigators were only able to reproduce their own findings about 50% of the time and that at no time during the study were the investigators able to reproduce the findings of another. Lack of consistency among inidvidual dentists in visual colour determination has been reported previously (van der Bergt, Ten Bosch, Borsboom, Palsschaaert, 1985).

The present study demonstrated that the reducing agent thiourea dioxide produced changes to the absorption spectrum of haemoglobin and methaemoglobin which were similar to those with hydrochloric acid and acidified thiourea, with a partial decolourisation of these blood products. The methaemoglobin was not reduced to haemoglobin. The products formed by the reaction of thiourea dioxide and haemoglobin and methaemoglobin were unknown and it was beyond the scope of this project to identify them.

Since thiourea dioxide, acidified thiourea, and the hydrochloric acid produced a partial decolourisation of haemoglobin and methaemoglobin in the spectrophotometric studies some bleaching effect in the tests on blood-stained dentin was anticipated.

As discussed in Chapter 4, hydrogen peroxide induces oxidative denaturation of haemoglobin *in vitro* (Telen, 1990). In this study 30% hydrogen peroxide oxidised haemoglobin and methaemoglobin to a colourless product. 3% hydrogen peroxide also rendered the haemoglobin and methaemoglobin colourless. For this to occur the 18-member conjugated $\pi$ system of haem which is responsible for the red colour would have to be disrupted. This indicates that an irreversible change has occurred, i.e. the bleaching is permanent and neither haemoglobin nor methaemoglobin can be expected to re-form.

Some colour return or 'relapse' following successful bleaching of root-filled teeth has been reported (Brown, 1965; Howell, 1981). It has been suggested that this is due to a return or re-formation of the original pigment. Several authors dispute this claim and maintain that fresh stain has been taken up by the tooth (Hayashi, Takamizu, Momoi, Furuya, Kusunoki and Komo, 1980; Chandra and Chawla, 1972). The present spectrophotometric study has shown that hydrogen peroxide is a very effective bleaching agent for haemoglobin and methaemoglobin and supports the previous reports of successful bleaching of blood-stained teeth by this agent (Casey *et al*, 1989; Marin, 1993; Warren, Wong and Ingram, 1990; Ho and Goerig, 1989). The findings also support the view that if bleaching progresses to the full extent regression should not occur, at least in the case of blood-stained teeth.

The aim of the second part of the present study was to determine the efficacy of various protocols which combined thiourea and hydrogen peroxide in bleaching blood-stained dentin. Extracted premolar teeth were discoloured by blood using an adaptation of the technique described by Freccia and Peters (1982). This method of staining teeth for bleaching studies has been used by several authors (Freccia, Peters, Lorton and Bernier, 1982; Ho and Goerig, 1989; Warren, Wong and Ingram, 1990; Marin, 1992; Dahlstrom, Bridges and Heithersay, 1993). Because of the large number of bleaching regimens which were to be tested transverse sections of blood-stained coronal dentin were used rather than whole teeth for this experiment. The degree of bleaching or decolourisation of the blood-stained dentin samples was determined with optical instruments. This method was chosen to provide an objective measure of the colour change and to avoid the difficulties which arise from visual colour determination as illustrated by the example of Casey et al (1989) cited earlier.

The colour of natural teeth is due in the most part to the light absorbed by the dentin but unlike artificial crowns natural teeth also have the property of translucence i.e. they can transmit light, and the appearance is enhanced in the oral cavity by the background of the tissues. Two intruments were therefore employed to measure the colour changes with bleaching. The reflection densitometer measures the light reflected back from the sample and the photometer measures the light transmitted through it. As the blood-stained dentin sample bleaches or lightens it becomes more translucent and more light is transmitted. The photometer reading increases. The reflection densitometer measures the light that is reflected back from the sample. As seen in Fig 4 the total of the reflected light is the sum of the light reflected at the surface and that which enters the sample for a short distance and is reflected back out again. The quality of the surface will affect the light that is reflected back. As the sample bleaches more light is reflected back so the value of the reflection densitometer decreases and approaches the ANSI Standard White. A rougher surface will reveal the colour of the sample more than a smooth surface. The changes in the surface characteristic of the sample (such as acid etching of the surface) will have a more significant effect on the readings with the reflection densitometer than the photometer.

In the second part of the study a total of 23 bleaching regimens including negative and positive controls were tested. A code was used to identify the bleaching sets and an assistant other than the author read the instruments. This assistant did not know the identity of the bleaching sets .

A comparison of the means of the results of the bleaching sets at 24 hours (Table 13) was consistent between the photometer and reflection densitometer. This result indicates that both instruments were measuring the same quality i.e. bleaching. It also indicates that the use of either instrument would have been sufficient for the

purpose of comparing the bleaching regimens. The photometer however is less affected by surface characterisitics, it showed a lower and more uniform variation and in the opinion of the author is the preferred instrument.

From the spectrophotometric study some bleaching effect was anticipated with the use of acidified thiourea, hydrochloric acid and thiourea dioxide. As shown by the preliminary analysis of the mean results of the Photometer data and Reflection Densitometer data at 24 hours (Stage 3 ii) however the acidified thiourea and hydrochloric acid were ineffective as bleaching agents for the blood-stained dentin. The reason for this may lie in the fact that in blood-stained teeth the stain resides in the dentinal tubules (Marin, 1993). In the spectrophotometric study the hydrochloric acid was able to interact with the haemoglobin and methaemoglobin in solution whereas in the blood-stained dentin the hydrochloric acid had to diffuse into the dentin to reach the stain. Acids dissolve the hydroxyapatite mineral crystallites from both intertubular and peritubular dentin and increase the porosity of the dentin (Pashley, 1992). Strong acids also denature proteins (Pashley, 1992). As denatured proteins change their dimensions, the dentin becomes less porous and so strong acids have a self-limiting effect in penetrating into dentin. This may be a cause of the lack of bleaching found with the hydrochloric acid and the acidified thiourea on the blood-stained dentin samples. Dentin can also act as a buffer to acid solutions (Wang and Hume, 1988).

Some bleaching effect was expected with thiourea dioxide, however the preliminary analysis showed that thiourea dioxide alone was as successful in bleaching blood-stained dentin as 30% hydrogen peroxide as measured by the photometer. Thiourea dioxide has a higher pH than the hydrochloric acid and the acidified thiourea and is unlikely to denature the protein matrix of dentin. It must therefore have been able to diffuse into the dentinal tubules and produce reducing bleaching of the haemoglobin

derivatives present. Bleaching of teeth with this reducing agent has not previously been reported.

A 5 % solution of sodium hypochlorite was also shown to be as effective at bleaching the samples as 30% hydrogen peroxide as determined with a reflection densitometer. As discussed previously, the reflection densitometer is sensitive to surface changes. Since 5% sodium hypochlorite is an alkaline oxidising agent it could have bleached the surface layer of the dentin samples enough to allow sufficient reflection of light to register equivalently low readings as the other treatments without actually being able to penetrate the matrix to bleach internal staining.

All the bleaching regimens which included 30% w/v hydrogen peroxide at the third stage of the bleaching process successfully bleached the stained dentin samples and were not statistically significantly different to 30% hydrogen peroxide alone. The conclusion that can be drawn from this result is that bleaching of blood-stained dentin by 30% hydrogen peroxide although not improved by pre-treatment with the agents tested was also not impaired. If thiourea acts as a hydroxyl scavenger under clinical bleaching conditions, a dual bleaching regimen of thiourea and hydrogen peroxide offers the advantage of reducing oxidative damage to the periodontal ligament cells without diminishing the bleaching power of the hydrogen peroxide.

The preliminary analysis (0 vs 24 hours) identified the more successful bleaching regimens but it did not indicate the amount of bleaching that had occured between zero time and 24 hours. For example distilled water showed a mean result at 24 hours (Stage 3ii) of 23 OD units similar to 5% NaOCl. Nevertheless an analysis of the change between zero time and 24 hours (see Fig 49 ) shows that there was no bleaching of the dentin samples immersed in distilled water for 24 hours but the

samples treated with 5% NaOCl showed a significant difference in the colour i.e. were bleached. This seemingly incongruent finding is explained when one considers that the dentin samples that had been used for the distilled water were less stained initially than those that had been treated with the 5% NaOCl.

In order to clarify the amount of decolourisation produced by the various bleaching regimens and to determine the degree of bleaching produced by the various components an analysis of the mean change between each stage of the bleaching process was performed. The results of this analysis including the appropriate LSD values were presented, for simplicity, as a series of histograms.

The pattern that emerged indicated that hydrogen peroxide is an effective and rapid bleaching agent for blood-stained dentin. Although slower to act thiourea dioxide proved to be equally effective at 24 hours as hydrogen peroxide. The reducing agent sulphur dioxide was used early this century to bleach root-filled teeth. The results of the present study suggest that the reducing agent thiourea dioxide may be an alternative bleaching agent to hydrogen peroxide. Alternatively a dual bleaching system using thiourea and hydrogen peroxide may be clinically an effective bleaching system whilst reducing the risk of oxidative damage from hydroxyl radicals.

## 10.1 Summary of discussion

This *in vitro* study has shown that the reducing agent, thiourea dioxide, not previously used as a bleaching agent in dentistry can successfully bleach blood-stained dentin. Under the conditions in this study bleaching with thiourea dioxide alone was as effective as 30% hydrogen peroxide. The bleaching effect of thiourea

dioxide however was slower than hydrogen peroxide and may have required more time to reach its maximum effect.

This study also showed that the bleaching regimens which employed the sequential use of acidified thiourea or aqueous thiourea and 30% hydrogen peroxide, although not superior, were as effective at bleaching blood-stained dentin as 30% hydrogen peroxide alone.

The results suggest that the use of a bleaching technique that employs the hydroxyl scavenger, thiourea in combination with 30% hydrogen peroxide may eliminate toxic hydroxyl radicals and hence potentially reduce the risk of external cervical resorption without reducing the bleaching effectiveness of hydrogen peroxide.

In view of the concerns regarding the association of bleaching root-filled teeth with hydrogen peroxide and the condition of invasive cervical resorption, further research into the use of thiourea and thiourea dioxide to bleach blood-stained teeth is warranted.

In particular an investigation (similar to the study of Dahlstrom, Bridges and Heithersay; 1993) of the ability of thiourea to act as a hydroxyl scavenger under these bleaching conditions is recommended. Tests of the ability of thiourea and thiourea dioxide to diffuse through radicular dentin and on the potential toxicity of these agents on fibroblasts should also be undertaken.

# 11 CONCLUSIONS

The principal findings of this study are:

30% w/v hydrogen peroxide renders solutions of haemoglobin and methaemoglobin colourless. The products formed were not identified.

30% hydrogen peroxide is an effective bleaching agent for blood-stained dentin.

Bleaching using 30% hydrogen peroxide is not improved or impaired by pre-treatment of blood stained dentin by other agents, as determined by a reflection densitometer and a photometer. In view of this finding it is suggested that further study be conducted to test the ability of thiourea to act as a hydroxyl scavenger under bleaching conditions.

Thiourea dioxide, a reducing agent, was equally effective as 30% hydrogen peroxide in bleaching blood-stained dentin. It is suggested that a further study be conducted to investigate the efficacy of thiourea dioxide and a combined thiourea/ hydrogen peroxide technique in removing dentin discolourations from other causes.

The recognition that bleaching discoloured teeth is a chemical process which can be achieved by both reducing agents and oxidising agents offers the possibility of developing new clinical bleaching protocols.

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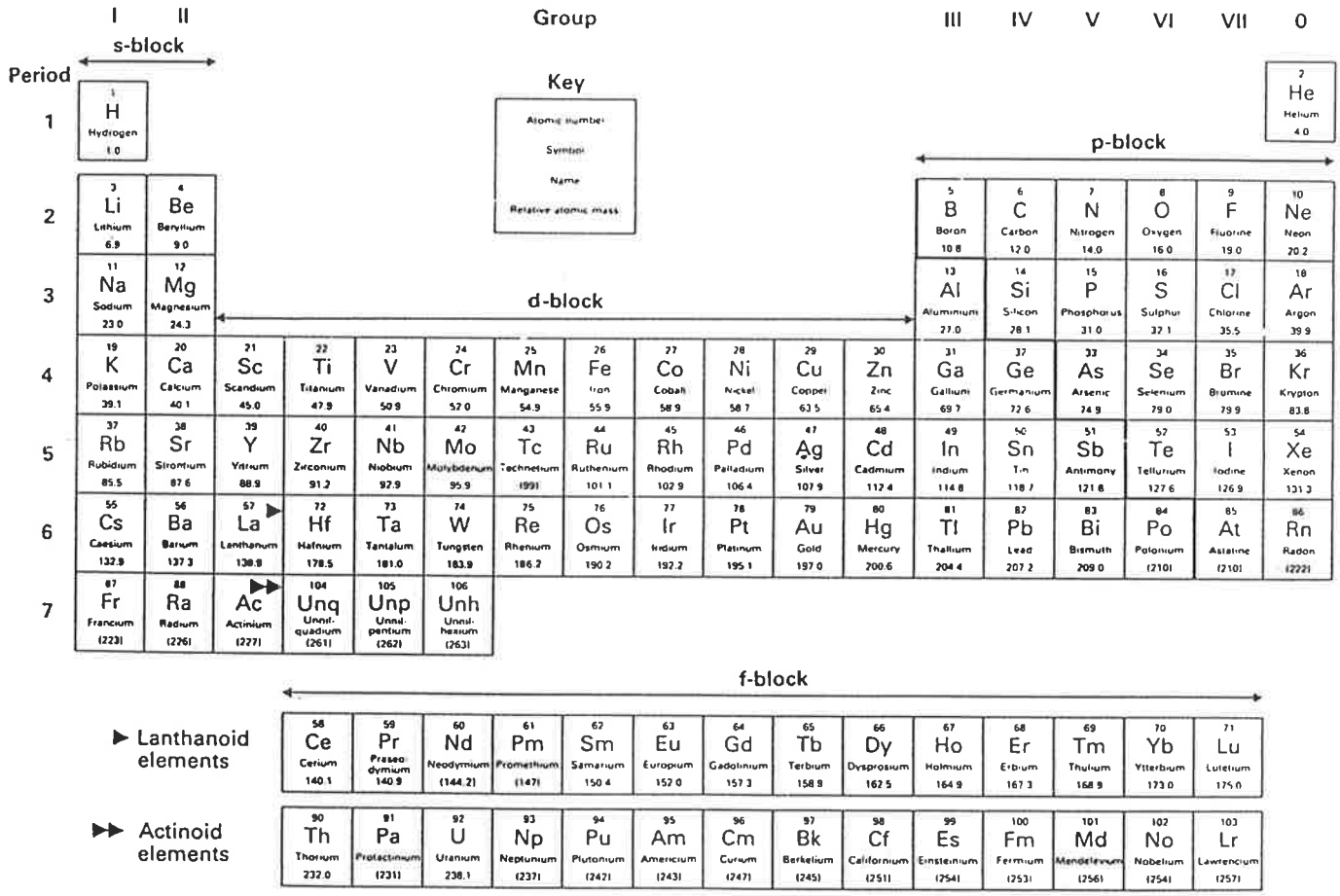
# APPENDIX I

## THE TRANSITION METAL COMPOUNDS

The transition metal compounds comprise 10 groups of elements found between the alkaline earth elements of group IIA and the elements of group IIIB in the periodic table (Table A1). All the elements are metals with typical metallic properties.

TABLE 1A

The Periodic Table



## APPENDIX 2

## OXIDATION AND REDUCTION

Oxygen combines with most metals and non-metals to form oxides. It is not surprising that such well-known reactions came to be called oxidation reactions early in the history of modern chemistry. Similarly reduction was removal of oxygen from an oxygen compound.These simple ideas developed as chemical knowledge increased.

Oxidation and reduction were seen to occur simultaneously and the term 'redox' was coined to indicate this. Later, electron transfer reactions were included as a special class of redox reaction.

In such reactions

(1) oxidation is a process in which electrons are lost,

(2) reduction is a process in which electrons are gained,

(3) an oxidising agent removes electrons from another substance,

(4) a reducing agent surrenders electrons to another substance.

The development of the concept of oxidation numbers provided a unified approach to redox reactions. The oxidation number is the charge on an atom whether real or imaginary and is a directed number. It possesses both sign and magnitude.

In a redox reaction

(1) an increase in the oxidation number of an element indicates oxidation,

(2) a decrease in the oxidation number indicates reduction .

It is convenient to classify certain substances as oxidising agents, and others as reducing agents. In redox reactions, an oxidising or reducing agent almost always yields one particular product, eg acidified hydrogen peroxide is reduced to water. When sulphite ion $SO_3^=$ acts as a reducer, sulphate ion $SO_4^=$ is formed.

Hydrogen peroxide acts as an oxidising agent with sulphite ions.

$$SO_3^= + H_2O_2 \rightarrow SO_4^= + H_2O$$

In the presence of certain oxidisers, hydrogen peroxide can act as a reducer. Its most common example as a reducer is with permanganate ions.

$$2MnO_4^- + 6H^+ + 5H_2O_2 \rightarrow 2Mn^{++} + 8H_2O + 5O_2$$

Table A4 lists common redox pairs.

TABLE A2

Examples of redox pairs

| Oxidising agent | | Usual product | Reducing agent | | Usual product |
|---|---|---|---|---|---|
| oxygen | $\overset{0}{O_2}$ | $O^{2-}$ | alkali metal | $\overset{0}{M}$ | $M^+$ |
| hydrogen peroxide | $\overset{-1}{H_2O_2}$ | $\overset{-2}{H_2O}$ | sulphur dioxide | $\overset{+4}{SO_2}$ | $\overset{+6}{SO_4^=}$ |
| hypochlorite ion | $\overset{+1}{ClO^-}$ | $Cl^-$ | sulphite | $\overset{+4}{SO_3^=}$ | $\overset{+6}{SO_4^=}$ |
| permanganate ion | $\overset{+7}{MnO_4^-}$ | $Mn^{2+}$ | hydrogen sulphide | $\overset{-2}{H_2S}$ | $\overset{0}{S}$ |
| halogen | $\overset{0}{X}$ | $X^{-1}$ | hydrogen peroxide | $\overset{-1}{H_2O_2}$ | $\overset{0}{O_2}$ |
| ferric ions | $Fe^{3+}$ | $Fe^{2+}$ | ferrous ion | $Fe^{2+}$ | $Fe^{3+}$ |
| dichromate ion | $\overset{+6}{Cr_2O_7^=}$ | $Cr^{3+}$ | iodide ion | $I^-$ | $\overset{0}{I_2}$ |

## APPENDIX 3

## SOURCES OF EQUIPMENT AND CHEMICALS

| | |
|---|---|
| Beckman L8-80 Ultracentrifuge | Beckman Instrument Co, USA |
| IEC Centra - MP4R High speed centrifuge | International Equipment Co, USA |
| Lambda 5UV/VIS Spectrophotometer | Perkin -Elmer Inc, USA |
| 502 M Photometer | Photovolt Co, USA |
| Speedmaster R-75 CP Reflection Densitometer | Electronic Systems Engineering, USA |
| Townson digital pH meter | TPS Pty Ltd, Australia |
| Van Moppes diamond cutting discs | IDP Pty Ltd., UK |
| Formamidinesulfinic acid (Thiourea dioxide) | Sigma Chemical Company, USA |
| Hydrogen peroxide 30% w/v | BDR AnalaR Chemical Ltd Victoria, Australia |
| Hemoglobin Human dessicate (Methaemoglobin) | Sigma Chemical Company, USA |
| Thiourea (Analar) | BDH Lab Supplies, England |
| Urea (Analar) | Merck Pty Ltd, Victoria |

## APPENDIX 4

## PREPARATION OF TEST BLEACH SOLUTIONS

1M aqueous thiourea

MW thiourea = 76.12

Distilled water was added to 76.12 gm of thiourea powder to make 1 litre of stock solution .

1M thiourea acidified with HCl

Distilled water acidified with 0.1M hydrochloric acid (pH 0.2 ) was added to 76.12 gm of thiourea powder to make 1 litre of stock solution.

1M aqueous urea

MW urea = 60.06 gm

Distilled water was added to 60.06 gm of thiourea powder to make 1 litre of stock solution.

0.1 M thiourea dioxide

FW = 108.1

It was not possible to produce a 1M solution of thiourea dioxide consistent with the molarity of the other test bleach solutions. A saturated solution of approximate molarity 0.1 M was used.

The pH of the bleaching solutions was measured using a Townson Digital pH meter (Table A4).

TABLE A4

The pH of the bleaching solutions

| Bleaching solution | pH |
|---|---|
| 1M acidified thiourea | 2.0 |
| 1M aqueous thiourea | 4.9 |
| 1M aqueous urea | 7.3 |
| 0.1 M thiourea dioxide | 4.5 |
| 30% w/v hydrogen peroxide | 3.0 |
| 0.1M HCl | 2.1 |
| 5% NaOCl | 11.7 |
| NaOH* | 11.7 |

* adjusted to match the pH of NaOCl solution

## APPENDIX 5

## RAW DATA

Table A5-1 Raw Data for the Photometer

| Bleaching Set | Sample | Time o | Stage 1 | Stage 2 | Stage 3 (i) | Stage 3 (ii) |
|---|---|---|---|---|---|---|
| A1 | 1 | 9 | 13 | 11 | 20 | 24 |
| | 2 | 15 | 14 | 14 | 27 | 26 |
| | 3 | 14 | 15 | 15 | 27 | 30 |
| | 4 | 16 | 13 | 13 | 29 | 34 |
| | | | | | | |
| A2 | 1 | 27 | 31 | 31 | 32 | 37 |
| | 2 | 11.5 | 12.5 | 12 | 32 | 36 |
| | 3 | 24 | 25 | 25 | 33 | 49 |
| | 4 | 13 | 12.5 | 19 | 37 | 37 |
| | | | | | | |
| A3 | 1 | 14 | 15 | 14 | 25 | 25 |
| | 2 | 19 | 19 | 18 | 39 | 38 |
| | 3 | 22 | 21 | 24 | 32 | 35 |
| | 4 | 6 | 9 | 9 | 28 | 32 |
| | | | | | | |
| A4 | 1 | 8 | 11 | 11 | 16 | 28 |
| | 2 | 17.5 | 19 | 19 | 31.5 | 36 |
| | 3 | 11 | 17 | 17 | 25 | 30 |
| | 4 | 12 | 11 | 11 | 20 | 29 |
| | | | | | | |
| A5 | 1 | 12 | 11 | 11 | 13 | 13 |
| | 2 | 7 | 9 | 9 | 14 | 14 |
| | 3 | 24 | 24 | 24 | 26 | 26 |
| | 4 | 12 | 13 | 13 | 15 | 13 |
| | | | | | | |
| B1 | 1 | 6 | 5 | 7 | 21 | 23 |
| | 2 | 3 | 4 | 9 | 12 | 15 |
| | 3 | 6 | 8 | 12 | 26 | 26 |
| | 4 | 12 | 12 | 11 | 31 | 36 |
| | | | | | | |
| B2 | 1 | 14 | 12 | 12 | 26 | 34 |
| | 2 | 14 | 15 | 14 | 28 | 36 |
| | 3 | 19 | 17 | 17 | 39 | 45 |
| | 4 | 15.5 | 16 | 15 | 28 | 32 |
| | | | | | | |
| B3 | 1 | 14 | 12 | 12 | 35 | 33 |
| | 2 | 17 | 19 | 19 | 30 | 35 |
| | 3 | 12 | 13 | 11 | 25 | 32 |
| | 4 | 13 | 12 | 11 | 30 | 35 |
| | | | | | | |
| B4 | 1 | 11 | 9 | 9 | 22 | 35 |
| | 2 | 11 | 9 | 9 | 28 | 32 |
| | 3 | 10 | 9 | 9 | 21 | 20 |
| | 4 | 17 | 17 | 17 | 34 | 34 |
| | | | | | | |
| B5 | 1 | 8.5 | 9 | 9 | 13 | 9 |
| | 2 | 6 | 7 | 7 | 7 | 10 |
| | 3 | 4.5 | 5 | 5 | 8 | 8 |
| | 4 | 4 | 4 | 4 | 6.5 | 9.5 |

Table A5-1 Raw Data for the Photometer (ctn)

| Bleaching Set | Sample | Time o | Stage 1 | Stage 2 | Stage 3 (i) | Stage 3 (ii) |
|---|---|---|---|---|---|---|
| C1 | 1 | 15.5 | 16 | 21 | 37 | 36 |
| | 2 | 12.5 | 13 | 15.5 | 29 | 37 |
| | 3 | 14 | 18 | 24 | 43 | 43 |
| | 4 | 9 | 11 | 16 | 24 | 31 |
| | | | | | | |
| C2 | 1 | 17 | 16 | 19 | 40 | 42 |
| | 2 | 11 | 13 | 13 | 24 | 26 |
| | 3 | 17 | 19 | 22 | 38 | 43 |
| | 4 | 12.5 | 15 | 18 | 26 | 32 |
| | | | | | | |
| C3 | 1 | 15 | 19 | 19 | 35 | 35 |
| | 2 | 15 | 15 | 15 | 33 | 33 |
| | 3 | 11 | 13 | 13 | 28 | 38 |
| | 4 | 14 | 15 | 15 | 37 | 42 |
| | | | | | | |
| C4 | 1 | 1 | 2 | 2 | 6 | 13 |
| | 2 | 6.5 | 6.5 | 6.5 | 8 | 15 |
| | 3 | 2 | 7 | 7 | 9 | 9 |
| | 4 | 7 | 5 | 5 | 4 | 4 |
| | | | | | | |
| D1 | 1 | 16.5 | 23 | 23 | 37 | 40 |
| | 2 | 13 | 13 | 13 | 27 | 31 |
| | 3 | 16 | 19 | 19 | 34 | 35.5 |
| | 4 | 10 | 14 | 14 | 21 | 27.5 |
| | | | | | | |
| D2 | 1 | 13 | 13 | 13 | 29 | 36 |
| | 2 | 12 | 12.5 | 12.5 | 19 | 22 |
| | 3 | 9.5 | 9 | 9 | 17 | 26 |
| | 4 | 15 | 11 | 11 | 25 | 44 |
| | | | | | | |
| D3 | 1 | 6.5 | 6.5 | 6.5 | 14 | 39 |
| | 2 | 7 | 13 | 13 | 33 | 39 |
| | 3 | 11 | 13 | 13 | 15 | 15 |
| | 4 | 29 | 33 | 33 | 42 | 46 |
| | | | | | | |
| E1 | 1 | 1 | 13 | 13 | 17 | 21 |
| | 2 | 6 | 11 | 11 | 14 | 14 |
| | 3 | 3 | 6.5 | 6.5 | 9 | 10 |
| | 4 | 9 | 11 | 11 | 14.5 | 13 |
| | | | | | | |
| E2 | 1 | 12 | 14.5 | 14.5 | 25 | 24.5 |
| | 2 | 2 | 3 | 3 | 7 | 14 |
| | 3 | 4 | 13 | 13 | 18 | 23 |
| | 4 | 5.5 | 6 | 6 | 9 | 13 |
| | | | | | | |
| E3 | 1 | 6.5 | 6 | 6 | 12 | 29 |
| | 2 | 6 | 5 | 5 | 8 | 24 |
| | 3 | 7 | 8 | 8 | 15 | 44 |
| | 4 | 5 | 8 | 8 | 17 | 37 |

Table A5-1 Raw Data for the Photometer (ctn)

| Bleaching Set | Sample | Time o | Stage 1 | Stage 2 | Stage 3 (i) | Stage 3 (ii) |
|---|---|---|---|---|---|---|
| F1 | 1 | 6 | 9 | 9 | 12 | 28 |
| | 2 | 7 | 8 | 8 | 9 | 19 |
| | 3 | 13 | 16 | 16 | 17 | 24 |
| | 4 | 14 | 20 | 20 | 20 | 20 |
| | | | | | | |
| F2 | 1 | 3 | 4 | 4 | 4 | 13 |
| | 2 | 2 | 3 | 3 | 6 | 6 |
| | 3 | 3 | 3 | 3 | 6 | 8 |
| | 4 | 9 | 10 | 10 | sample lost | |
| | | | | | | |
| F3 | 1 | 14 | 14 | 14 | 17 | 23 |
| | 2 | 16 | 15 | 15 | 17 | 24 |
| | 3 | 20 | 20 | 20 | 21 | 25 |
| | 4 | 14 | 14 | 14 | 17 | 21 |

Table A5-2 Raw Data for the Densitometer

| Bleaching set | Sample | Time o | Stage 1 | Stage 2 | Stage 3(i) | Stage 3(ii) |
|---|---|---|---|---|---|---|
| A1 | 1 | 1.00 | 0.92 | 0.92 | 0.88 | 0.82 |
| | 2 | 0.98 | 0.96 | 0.92 | 0.83 | 0.82 |
| | 3 | 0.90 | 0.89 | 0.92 | 0.88 | 0.84 |
| | 4 | 0.98 | 0.92 | 0.93 | 0.85 | 0.81 |
| | | | | | | |
| A2 | 1 | 0.95 | 0.92 | 0.89 | 0.85 | 0.84 |
| | 2 | 1.00 | 0.94 | 0.94 | 0.84 | 0.83 |
| | 3 | 0.99 | 0.93 | 0.94 | 0.86 | 0.84 |
| | 4 | 0.98 | 0.97 | 0.98 | 0.86 | 0.84 |
| | | | | | | |
| A3 | 1 | 0.95 | 0.90 | 0.92 | 0.84 | 0.84 |
| | 2 | 0.99 | 0.99 | 0.96 | 0.82 | 0.80 |
| | 3 | 0.88 | 0.88 | 0.88 | 0.84 | 0.82 |
| | 4 | 0.99 | 0.93 | 0.92 | 0.86 | 0.83 |
| | | | | | | |
| A4 | 1 | 0.95 | 0.92 | 0.92 | 0.83 | 0.83 |
| | 2 | 0.92 | 0.90 | 0.90 | 0.84 | 0.81 |
| | 3 | 0.94 | 0.92 | 0.92 | 0.84 | 0.82 |
| | 4 | 0.94 | 0.92 | 0.92 | 0.86 | 0.82 |
| | | | | | | |
| A5 | 1 | 0.90 | 0.91 | 0.91 | 0.93 | 0.92 |
| | 2 | 0.98 | 0.94 | 0.94 | 0.94 | 0.92 |
| | 3 | 0.89 | 0.88 | 0.88 | 0.86 | 0.86 |
| | 4 | 0.95 | 0.90 | 0.90 | 0.87 | 0.87 |
| | | | | | | |
| B1 | 1 | 0.94 | 0.94 | 0.94 | 0.85 | 0.82 |
| | 2 | 1.00 | 0.94 | 0.90 | 0.86 | 0.81 |
| | 3 | 0.95 | 0.96 | 0.90 | 0.83 | 0.81 |
| | 4 | 0.90 | 0.90 | 0.90 | 0.84 | 0.81 |
| | | | | | | |
| B2 | 1 | 1.20 | 1.20 | 1.00 | 0.87 | 0.84 |
| | 2 | 0.93 | 0.96 | 0.89 | 0.84 | 0.80 |
| | 3 | 0.98 | 0.91 | 0.90 | 0.84 | 0.81 |
| | 4 | 0.95 | 0.95 | 0.94 | 0.85 | 0.81 |
| | | | | | | |
| B3 | 1 | 0.98 | 0.95 | 0.92 | 0.82 | 0.81 |
| | 2 | 0.90 | 0.92 | 0.92 | 0.83 | 0.82 |
| | 3 | 0.97 | 0.94 | 0.91 | 0.83 | 0.83 |
| | 4 | 0.94 | 0.91 | 0.91 | 0.81 | 0.82 |
| | | | | | | |
| B4 | 1 | 0.94 | 0.92 | 0.92 | 0.86 | 0.84 |
| | 2 | 0.91 | 0.92 | 0.92 | 0.82 | 0.82 |
| | 3 | 0.98 | 0.96 | 0.96 | 0.86 | 0.86 |
| | 4 | 0.94 | 0.92 | 0.92 | 0.84 | 0.82 |
| | | | | | | |
| B5 | 1 | 0.98 | 1.00 | 1.00 | 0.99 | 0.97 |
| | 2 | 0.99 | 0.98 | 0.98 | 0.98 | 0.92 |
| | 3 | 0.99 | 0.98 | 0.98 | 0.98 | 0.94 |
| | 4 | 1.00 | 1.02 | 1.02 | 1.00 | 0.98 |

Table A5-2 Raw Data for the Densitometer (ctn)

| Bleaching set | Sample | Time o | Stage 1 | Stage 2 | Stage 3(i) | Stage 3(ii) |
|---|---|---|---|---|---|---|
| C1 | 1 | 0.96 | 0.88 | 0.90 | 0.81 | 0.82 |
| | 2 | 1.00 | 0.92 | 0.98 | 0.85 | 0.84 |
| | 3 | 0.91 | 0.87 | 0.85 | 0.79 | 0.79 |
| | 4 | 0.95 | 0.94 | 0.94 | 0.86 | 0.81 |
| | | | | | | |
| C2 | 1 | 0.93 | 0.90 | 0.88 | 0.83 | 0.78 |
| | 2 | 0.96 | 0.91 | 0.92 | 0.85 | 0.83 |
| | 3 | 0.90 | 0.90 | 0.91 | 0.81 | 0.80 |
| | 4 | 0.91 | 0.94 | 0.90 | 0.86 | 0.80 |
| | | | | | | |
| C3 | 1 | 0.95 | 0.89 | 0.89 | 0.82 | 0.80 |
| | 2 | 0.93 | 0.92 | 0.92 | 0.82 | 0.80 |
| | 3 | 0.89 | 0.91 | 0.91 | 0.82 | 0.79 |
| | 4 | 0.94 | 0.93 | 0.93 | 0.83 | 0.80 |
| | | | | | | |
| C4 | 1 | 1.05 | 1.03 | 1.03 | 1.00 | 1.00 |
| | 2 | 0.99 | 1.00 | 1.00 | 0.98 | 0.88 |
| | 3 | 1.02 | 1.04 | 1.04 | 1.04 | 1.00 |
| | 4 | 1.08 | 1.02 | 1.02 | 1.05 | 1.02 |
| | | | | | | |
| D1 | 1 | 0.92 | 0.90 | 0.90 | 0.84 | 0.80 |
| | 2 | 0.96 | 0.91 | 0.91 | 0.85 | 0.80 |
| | 3 | 0.94 | 0.86 | 0.86 | 0.84 | 0.81 |
| | 4 | 0.94 | 0.90 | 0.90 | 0.85 | 0.83 |
| | | | | | | |
| D2 | 1 | 0.95 | 0.95 | 0.95 | 0.82 | 0.82 |
| | 2 | 0.98 | 0.97 | 0.97 | 0.87 | 0.85 |
| | 3 | 1 | 0.98 | 0.98 | 0.86 | 0.84 |
| | 4 | 0.95 | 0.92 | 0.92 | 0.84 | 0.84 |
| | | | | | | |
| D3 | 1 | 0.99 | 0.98 | 0.98 | 0.86 | 0.83 |
| | 2 | 0.93 | 0.85 | 0.85 | 0.82 | 0.82 |
| | 3 | 0.96 | 0.88 | 0.88 | 0.86 | 0.84 |
| | 4 | 0.92 | 0.88 | 0.88 | 0.85 | 0.84 |
| | | | | | | |
| E1 | 1 | 1.08 | 1.02 | 1.02 | 0.94 | 0.94 |
| | 2 | 0.98 | 0.90 | 0.90 | 0.90 | 0.90 |
| | 3 | 0.98 | 0.94 | 0.94 | 0.86 | 0.88 |
| | 4 | 0.94 | 0.88 | 0.88 | 0.89 | 0.89 |
| | | | | | | |
| E2 | 1 | 0.95 | 0.88 | 0.88 | 0.87 | 0.88 |
| | 2 | 0.97 | 0.97 | 0.97 | 0.93 | 0.93 |
| | 3 | 0.98 | 0.91 | 0.91 | 0.87 | 0.88 |
| | 4 | 0.99 | 0.92 | 0.92 | 0.89 | 0.90 |
| | | | | | | |
| E3 | 1 | 0.93 | 0.93 | 0.93 | 0.88 | 0.84 |
| | 2 | 1.02 | 1.00 | 1.00 | 0.95 | 0.88 |
| | 3 | 1.00 | 0.99 | 0.99 | 0.94 | 0.88 |
| | 4 | 0.95 | 0.93 | 0.93 | 0.93 | 0.85 |

Table A5-2 Raw Data for the Densitometer (ctn)

| Bleaching set | Sample | Time o | Stage 1 | Stage 2 | Stage 3(i) | Stage 3(ii) |
|---|---|---|---|---|---|---|
| F1 | 1 | 0.97 | 0.93 | 0.93 | 0.88 | 0.83 |
| | 2 | 1.20 | 0.95 | 0.95 | 0.92 | 0.87 |
| | 3 | 0.92 | 0.87 | 0.87 | 0.87 | 0.82 |
| | 4 | 0.90 | 0.84 | 0.84 | 0.85 | 0.82 |
| | | | | | | |
| F2 | 1 | 1.05 | 1.05 | 1.05 | 1.00 | 0.97 |
| | 2 | 1.00 | 1.00 | 1.00 | 1.00 | 0.90 |
| | 3 | 0.99 | 0.97 | 0.97 | 0.93 | 0.88 |
| | 4 | 0.93 | 0.95 | 0.95 | 0.90 | sample lost |
| | | | | | | |
| F3 | 1 | 0.94 | 0.94 | 0.94 | 0.93 | 0.90 |
| | 2 | 0.92 | 0.92 | 0.92 | 0.91 | 0.88 |
| | 3 | 0.86 | 0.88 | 0.88 | 0.86 | 0.87 |
| | 4 | 1.10 | 1.00 | 1.00 | 1.00 | 0.96 |

## APPENDIX 6

## STATISTICAL ANALYSIS

*Preliminary analysis*

In order to determine the appropriateness of the statistical analysis for the data the standard deviations of the bleaching sets was plotted against the means of each bleaching set for the Photometer data (Fig A6-1) and for the Reflection Densitometer data (Fig A6-2).

The plot for the Photometer data (Fig A6-1) shows that the variation is reasonably uniform as the means of the bleaching sets increase. There is more variation in the standard deviations as the means increase, which is to be expected due to the larger number of bleaching regimens present at the higher means. The plot for the Reflection Densitometer data (Fig A6-2) reveals a reasonably strong increase in standard deviations as the means of the bleaching sets increased. As a result, consideration was given to a further analysis using log transformation of the data in order to stabilise the variation. However it was felt that this would not change the basic conclusions.

The plots of Means vs Bleaching set for the Photometer data (Fig A6-3) and the Reflection Densitometer data (Fig A6-4 ) show there is considerable variation in the means of the different bleaching treatments. The vertical lines in these figures are used to separate the various bleaching regimen groupings, namely A1-A5, B1-B5, C1-C4, D1-D3, E1-E3, and F1-F3.

An Analysis of Variance of the complete Photometer data set at Stage 3 (24 hours) was made (Table A6-1). The large F Value (9.148128) confirmed that there are some significant differences between the means of the 23 bleaching regimens, as observed in the plot of Means of the bleaching sets (see Fig A6-1). The Residual

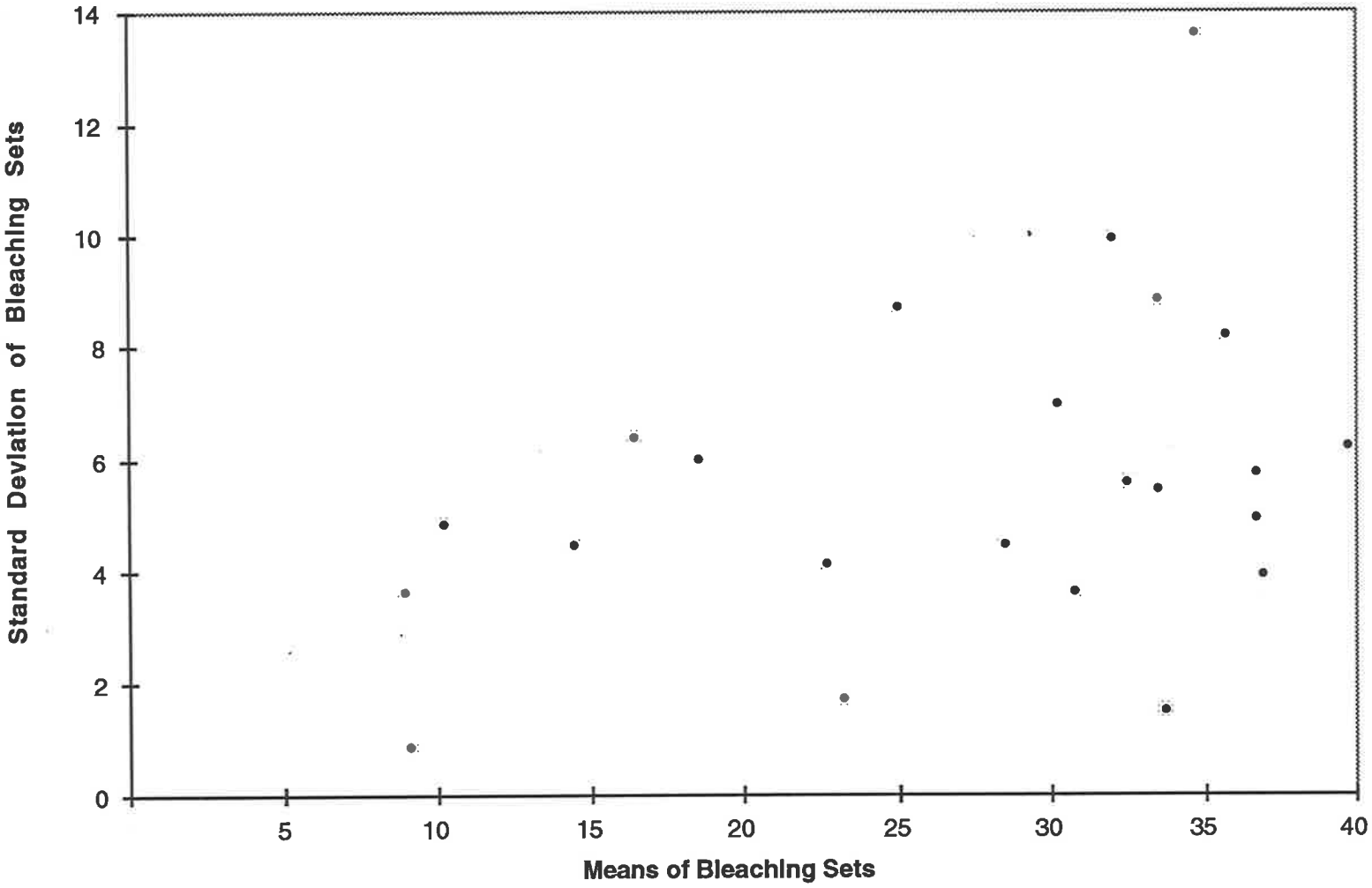



Fig A6-1 Standard deviation plotted against the means of the bleaching sets for the Photometer (n = 4).

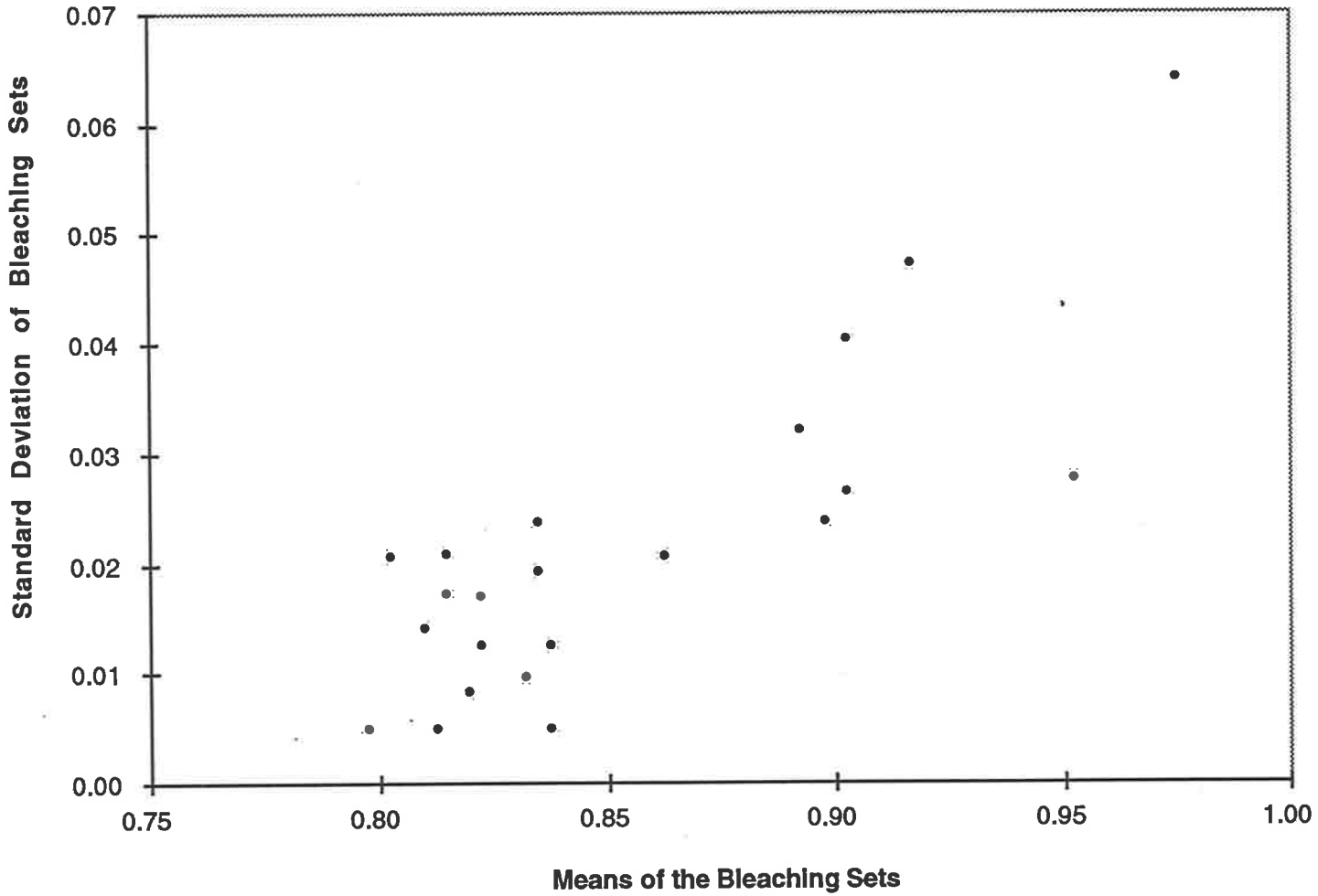



Fig A6-2 Standard deviation plotted against the means of the bleaching sets for the Reflection Densitometer (n = 4).

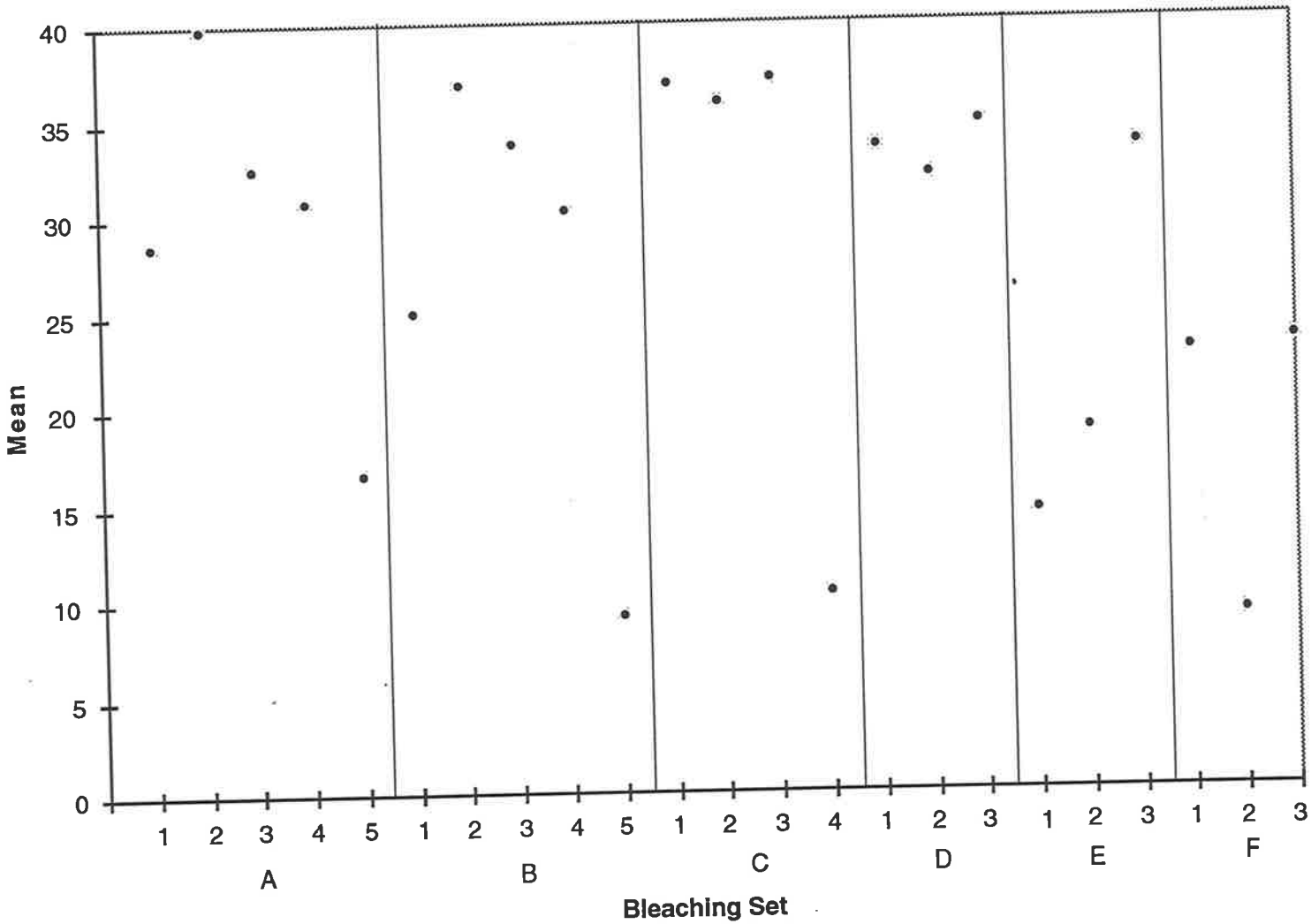



Fig A6-3 The means for the Photometer for each bleaching set (n = 4).
The vertical lines are used to separate the bleaching sets as per Table 10.

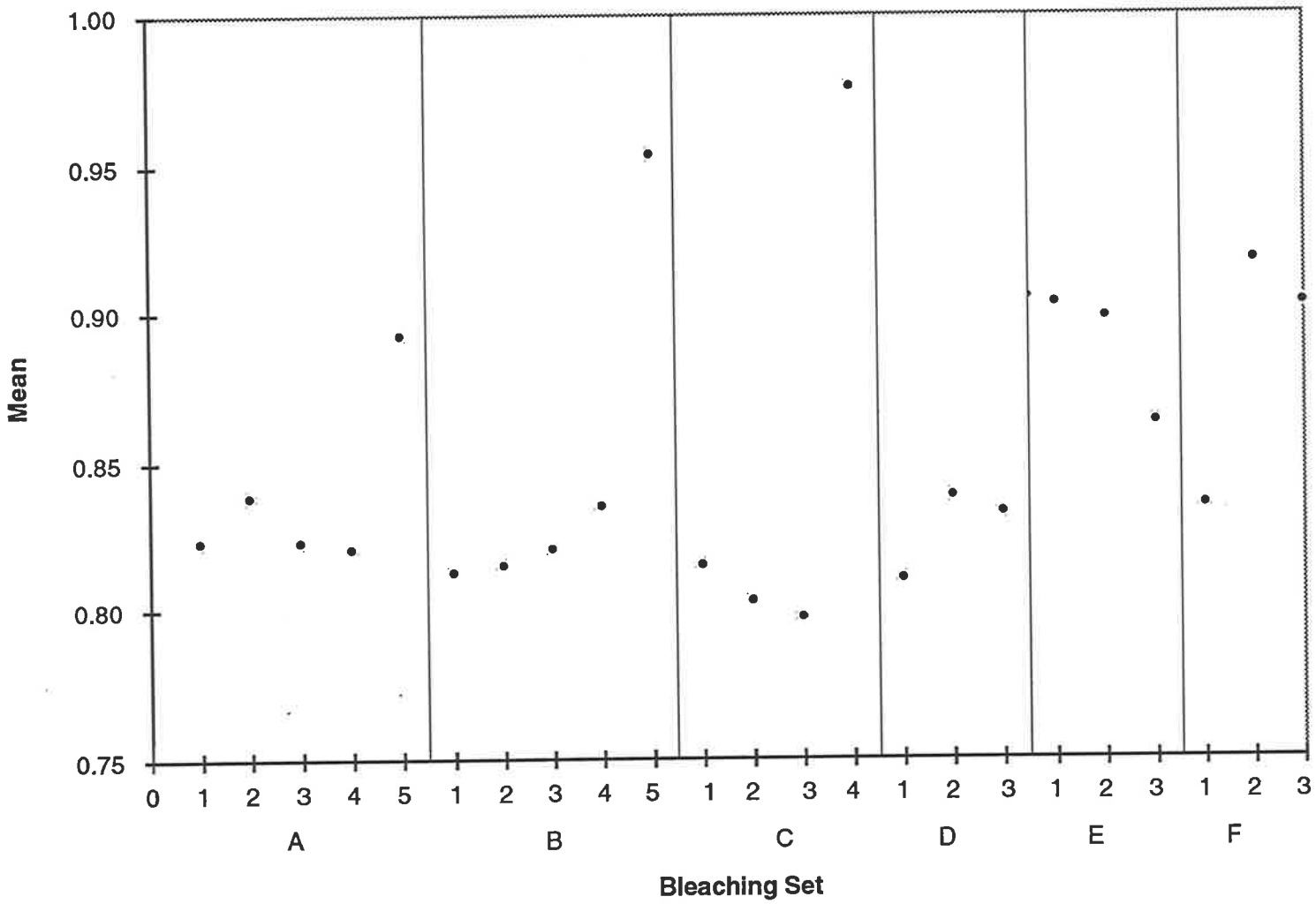



Fig A6-4 The means for the Reflection Densitometer data for each bleaching set (n =4 ). The vertical lines are used to separate the bleaching sets as per Table 10.

Mean Square is a stable, unbiased estimate of the variance of the population (Snedecor and Cochran, 1967). The square root of this is 6.3276 which estimates the standard deviation within groups. Due to the stability of this estimate, it was used in subsequent Analyses of Variance as the estimate of the standard deviation.

Similarly, an Analysis of Variance of the complete data set obtained using the Reflection Densitometer appears as Table A6-2. Again, the large F value indicates significant differences between the means of the bleaching regimens. The standard deviation within groups for the Reflection Densitometer data set is 0.02474.

*Comparisons of the progressive treatments*

The means of the various bleaching regimens were compared using Analysis of Variance or the LSD values where appropriate. The Analyses of Variance are included in this Appendix as Table A6-3. When referring to these analyses, it is important to remember that the Residual Mean Squares discussed previously for the complete data sets are based on more degrees of freedom and hence are more stable estimates of the variance than those Residual Mean Squares in a particular analysis. Hence when calculating the F Values, the Residual Mean Squares from the full analyses were used in preference to the values from the separate tables.

For example, when comparing A1-A4 for the Photometer data, F equals 3.7218 (94.750/25.458) . However to get the results in Table 13, we always took the F Ratio as the Mean Square for Groups divided by the Residual Mean Square for the full analysis. Thus, the correct F value to use is F equals (94.750/40.0386),which is 2.366 which has (3, 68) degrees of freedom rather than (3, 12). This gives a P-value of 0.078, which is not significant.

The Least Significant Differences (LSD) values provide a simple method of pairwise comparisons of treatment means. The LSD is calculated as 2x(standard error of the difference between 2 means) (Snedecor and Cochran , 1967). For the Photometer data, this is equal to SQRT(2 x 40.0386) which is 8.9486, and for the Densitometer data, SQRT(2 x 0.000612010) which equals 0.034986 . The Least Significant Differences (LSD) for the means of the Photometer data and for the Reflection Densitometer are shown in the last row of Table 12 (Chp 9), which includes a summary of the Means and the Standard Deviations for the 23 bleaching regimens.

The LSD values were used to test whether the observed difference between two means was significant, at a 5% level of significance. If the difference between the means in the same column was greater than the appropriate LSD, then we concluded that there was a significant difference between the means of the bleaching regimens.

Table A6-1

Analysis of variance of the Photometer data set at 24 hours.

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 22 | 8058.122 | 366.2783 | 9.148128 | 7.137623e-13 |
| Residuals | 68 | 27722.625 | 40.0386 | | |

Table A6-2

Analysis of variance of the Reflection Densitometer data set at 24 hours.

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 22 | 0.2157438 | 0.009806535 | 16.02349 | 0 |
| Residuals | 68 | 0.0416167 | 0.000612010 | | |

Table A6-3

Comparing A1-A4

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 3 | 284.25 | 94.75000 | 3.721768 | 0.04218588 |
| Residuals | 12 | 305.50 | 25.45833 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 3 | 0.00076875 | 0.0002562500 | 1.892308 | 0.1847359 |
| Residuals | 12 | 0.00162500 | 0.0001354167 | | |

Comparing B1-B4

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 3 | 305.6875 | 101.8958 | 2.567454 | 0.1031977 |
| Residuals | 12 | 476.2500 | 39.6875 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 3 | 0.00121875 | 0.0004062500 | 2.142857 | 0.1480382 |
| Residuals | 12 | 0.00227500 | 0.0001895833 | | |

Comparing C1- C3

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 2 | 3.5 | 1.75 | 0.04929577 | 0.9521547 |
| Residuals | 9 | 319.5 | 35.50 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 2 | 0.00065 | 0.0003250000 | 1.103774 | 0.3726436 |
| Residuals | 9 | 0.00265 | 0.0002944444 | | |

Comparison (A1-A4) vs A5

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| comp5 | 1 | 858.05 | 858.0500 | 21.73043 | 0.0001941292 |
| Residuals | 18 | 710.75 | 39.4861 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| comp5 | 1 | 0.01431125 | 0.01431125 | 47.10446 | 2.020859e-06 |
| Residuals | 18 | 0.00546875 | 0.00030382 | | |

Comparing (B1-B4) vs B5

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| comp5 | 1 | 1593.113 | 1593.113 | 36.57073 | 1.022938e-05 |
| Residuals | 18 | 784.125 | 43.562 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| comp5 | 1 | 0.05565125 | 0.05565125 | 173.6464 | 1.10259e-10 |
| Residuals | 18 | 0.00576875 | 0.00032049 | | |

Comparing (C1-C3) vs C4

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| comp5 | 1 | 2067.187 | 2067.187 | 73.5 | 6.06436e-07 |
| Residuals | 14 | 393.750 | 28.125 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| | 1 | 0.0867 | 0.08670000 | 77.80769 | 4.315475e-07 |
| Residuals | 14 | 0.0156 | 0.00111429 | | |

Comparison of (A1-A4) vs (B1-B4) vs C1-C3)

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| | 2 | 181.199 | 90.59943 | 2.191895 | 0.1246245 |
| Residuals | 41 | 1694.688 | 41.33384 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| | 2 | 0.003067045 | 0.001533523 | 6.843476 | 0.002725543 |
| Residuals | 41 | 0.009187500 | 0.000224085 | | |

Comparing D1, D2 vs A1, A2 vs B1, B2 vs C1, C2

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| | 3 | 123.25 | 41.08333 | 0.6797656 | 0.5718082 |
| Residuals | 28 | 1692.25 | 60.43750 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| | 3 | 0.002209375 | 0.0007364583 | 2.763261 | 0.06055406 |
| Residuals | 28 | 0.007462500 | 0.0002665179 | | |

Comparing E1, E2, D3

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 2 | 916.1250 | 458.0625 | 5.690708 | 0.02526601 |
| Residuals | 9 | 724.4375 | 80.4931 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 2 | 0.012200 | 0.0061000000 | 13.63975 | 0.00188632 |
| Residuals | 9 | 0.004025 | 0.000447222 | | |

Comparing F1, F2, D3

AOV Photometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 2 | 1139.045 | 569.5227 | 7.237779 | 0.01605159 |
| Residuals | 8 | 629.500 | 78.6875 | | |

AOV Densitometer data

| | Df | Sum of Sq | Mean Sq | F Value | Pr (F) |
|---|---|---|---|---|---|
| Group | 2 | 0.01501288 | 0.007506439 | 9.322357 | 0.008126728 |
| Residuals | 8 | 0.00644167 | 0.000805208 | | |