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A STUDY OF

THE CHEMICAL DISCOLOURATION OF AUSTRALIAN ABALONE

This is the **Final Report** on the project

THE CHEMICAL CAUSES OF DISCOLOURATION IN AUSTRALIAN ABALONE FLESH

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I am indebted for much enthusiasm and out-of-hours work by Mr Richard Lim and since December 1988 by Miss Loraine Tam, who collaborated with me in developing the project.

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THE CHEMICAL DISCOLOURATION OF AUSTRALIAN ABALONE

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1.0 INTRODUCTION

1.1 The Australian abalone industry

Abalone are marine molluscs of the genus Haliotis. A number of species are found around the southern areas of Australia, from Perth in W.A to Sydney in N.S.W.. Victoria, Tasmania and South Australia are the major zones for capture of abalone. In the past 18 years or so, the Australian abalone industry has grown to become the largest in the world.

The United States and South East Asia are large export markets for Australian abalone. High quality abalone are greatly sought after and command high prices overseas. As a consequence, abalone have become a very important and lucrative commodity to the Australian Fishing Industry.

1.2 The blue discolourations of abalone flesh

Abalone are processed in a variety of ways. They are frozen, canned and sliced to make steaks and off cuts may be used to make soup. The highest value appears to be in canned abalone which is exported predominantly to the South East Asian market. For several years now, the industry has been faced with the occurrence of an unsightly discolouration of canned abalone. The abalone appears to form a blue discolouration or blue 'spot'μ the cause and nature of which is unknown. The discolouration can manifest itself in the following ways but epipodium blueing (c) is by-far the most common form:

a) Blue spot - Apparently on the outer sections of the flesh alone.

b) Internal Blueing - The blue discolouration spreads all throughout the abalone tissue.

c) Epipodium blueing - A bluish tinge around the frill where blood transport occurs.

This unsightly blueing is detrimental to the industry for two reasons. Firstly, the blued abalone fetch a lower price since they are regarded as lower grade/quality products. Secondly, at present, the addition of metabisulfite(MBS) to canned abalone is used to prevent discolouration of the canned abalone. The approximate concentration of metabisulfite in canned Australian abalone has been known to reach 150 ppm. The Japanese fishing industry presently only allow <30 ppm of metabisulfite in its own market. Due to an increased consciousness of public health, similar maximum levels of MBS are likely to be enforced in the US and SE asian markets, which could probably result in the overnight collapse of the Australian abalone industry if a commercially acceptable alternate treatment is not available.

1.3 Possible causes of the blue discolouration of abalone flesh

The blueing of abalone has been reported in other areas and tentatively linked with the copper content of the abalone flesh (53). Since hemocyanin is a copper containing protein, its involvement in the blueing problem has been postulated. Thrower (65) investigated the occurrence of blue spot in abalone and proposed that excessive localised hemocyanin caused by environmental and handling related factors contributed to the blueing phenomena. Such discolourations were shown by James (41) to be easily reduced by chemical agents such as ascorbic acid. However, after canning the blueing could not be reverted by such treatment (41). The addition of citric acid has also been shown to reduce or eliminate discolouration (65) but was detrimental to the delicate flavour of the meat.

1.4 Studies on the blueing of crabmeat

Apart from the work of Olley, Thrower and co-workers (CSIRO Hobart), very little other literature regarding the blueing of abalone is available. However, extensive studies of the blueing of crab flesh, mainly by Japanese workers, have been reported and a number of observations are pertinent to the abalone blueing problem. The work of Inoue et al. (31) points to hemocyanin or a modified form thereof as the cause of the 'blueing' in crab meat. It was shown that blued crab meat contained a higher percentage of copper when compared with normal meat. In addition, a hemocyanin specific stain produced positive results on blued meats. Normal meat showed no reactivity which inferred a relatively lower concentration of hemocyanin as compared with discoloured flesh. Furthermore, Inoue et al. (31) showed that hemocyanin from the crab, reacting with hydrogen sulfide produced a stable bluish green complex (Hc/S). They further demonstrated that the blue crab meat had a similar UV/Vis reflectance spectra to that of the Hc/S complex.

Elliot and Harvey (16) investigated the effectiveness of blood removal in the reduction of discolourations. A definite correlation was established with bled crabs showing a decrease in both copper content and blueing.

Phenolases are enzymes that catalyse the oxidation of phenols into dark melanin compounds or polyphenolics. Babbitt et al. (3) postulated that the blueing found in processed Dungeness crabs may be due to these phenolic reactions. It was shown that the hemolymph of C. magister exhibits enzymic activity and several phenols were also tentatively identified in the blood.

1.5 Project aims

The initial aim of this project had been to determine an economically and industrially viable method of preventing the discolouration of abalone. Unfortunately, the problem extends

further and the effects of the treatment to meat quality and taste must also be considered. Although citric acid treatment of blued abalone has been shown by van der Merwe to be effective in reducing discolourations, the acid flavour of the final product was totally unacceptable. With a better understanding of the nature and formation conditions of the blueing agent(s), it is hoped that a more suitable method may evolve. This study therefore , has tended to emphasize this approach as one way through the complexity of the problem. Very little literature was available which related specifically to the blueing of abalone, but it is suspected that the oxygen carrying protein, hemocyanin (Hc) is involved in the 'blueing' reactions for the following reasons:

i) Abalone have been shown to retain extractable quantities of Hc in the epipodium (or frill) - the most commonly observed area of blueing.

ii) Variations in the concentration of hemocyanin between individuals from the one area is quite common (1). This may be related to the extreme variations in discolourations observed from abalone of the same catch and to the spasmodic appearance of blueing even under strictly controlled laboratory conditions.

iii) Studies by many overseas workers produced results that roughly fall into two categories that implicate either (1) hemocyanin or (2) a modified form of Hc. as being involved with the blueing phenomenon found in untreated canned crab meats.

The initial work of this investigation was on the physical and chemical properties of abalone hemocyanin. Such data had not appeared in the literature. This study is reported as Part I of this Report. An analytical examination of likely mechanisms for the production of blueing agent(s) mentioned previously follows in Part II and III. This data base is used to interpret results from further extraction and

analysis of the actual blueing compound(s) found in Tasmanian and canned blued abalone. This work is Part IV of the Report. Part V summarizes our conclusions.

PART I. ISOLATION, PURIFICATION AND CHARACTERISATION OF ABALONE HEMOCYANIN

THE CHEMICAL CHARACTERISATION OF ABALONE HEMOCYANIN

2.1 INTRODUCTION

The blueing discolouration in crab meat has been studied for over 50 years but the causes of such still remain unknown. Evidence from various investigations into crab meat has indicated a relationship between blueing and some constituents in the blood (probably hemocyanin). Unfortunately, the complexities of this type of biochemical study are compounded by the presence of different types of blueing (see Introduction).

Elliot and Harvey (13) studied the effect of blood removal on the reduction in discolouration. It was found that bled crabs produced less pronounced discolouration and contained a lower overall copper content. The great variations in hemocyanin content between various animals has been reported by many investigators and which are echoed by this report (Section 3). These earlier findings suggest a possible relationship between hemocyanin in the blood withthe spasmodic occurrence of blueing. Even under strictly controlled laboratory conditions blueing cannot be reliably reproduced. This is illustrated by the occurrence of a discoloured animal and one with normal meat in the same can. Since the treatment for both have been identical, the blueing must have been caused by an individual dependent factor such as hemocyanin.

Very little is known about abalone blueing as most studies from European and Japanese sources investigate canned crab meat. Here in Australia abalone produce blue discolourations predominantly around the epipodium - a porous muscular region of greatest hemocyanin content. The copper content has been reported by Olley (52), Inoue and Motohiro (31), and Elliot and Harvey (16) to be significantly higher in blued meats of crabs

and abalone. The most likely source of this copper is hemocyanin and in fact some investigators (55, 57) have used the copper content in the blood as a measure of hemocyanin concentration.

To our knowledge, little data is available about the chemical characteristics of abalone hemocyanin but due to the probable involvement of hemocyanin in the blueing phenomena, it became essential to understand the physical and chemical characteristics of abalone hemocyanin. This chapter attempts to isolate and characterise the hemocyanin of N. ruber so as to establish a 'base-line' for further comparative work in later chapters.

2.2.0 METHODS

2.2.1 Purification of abalone hemocyanin

Abalone were collected and immediately washed with water. The animals were shucked and the flesh portion rinsed with distilled water and transferred into a beaker. The temperature was kept low (approx. $4^{o}C$) to reduce spoilage and bacterial action. Blood was drained from the abalone overnight and collected in a beaker. [Up to 40% of the body weight can be due to blood (53).]

The isolation of hemocyanin involves a two step procedure. The first is the precipitation of crude protein in a modification of the procedure used by Koning et al. (42). The final purification involves gel chromatography of the protein. A detailed flow chart of the procedure is shown on Figure 2.1.

2.2.2 Precipitation of hemocyanin

The hemolymph was centrifuged (8 000 rpm, 45 min., $4^{o}C$) to remove cells and other debris. The clear supernatent was diluted

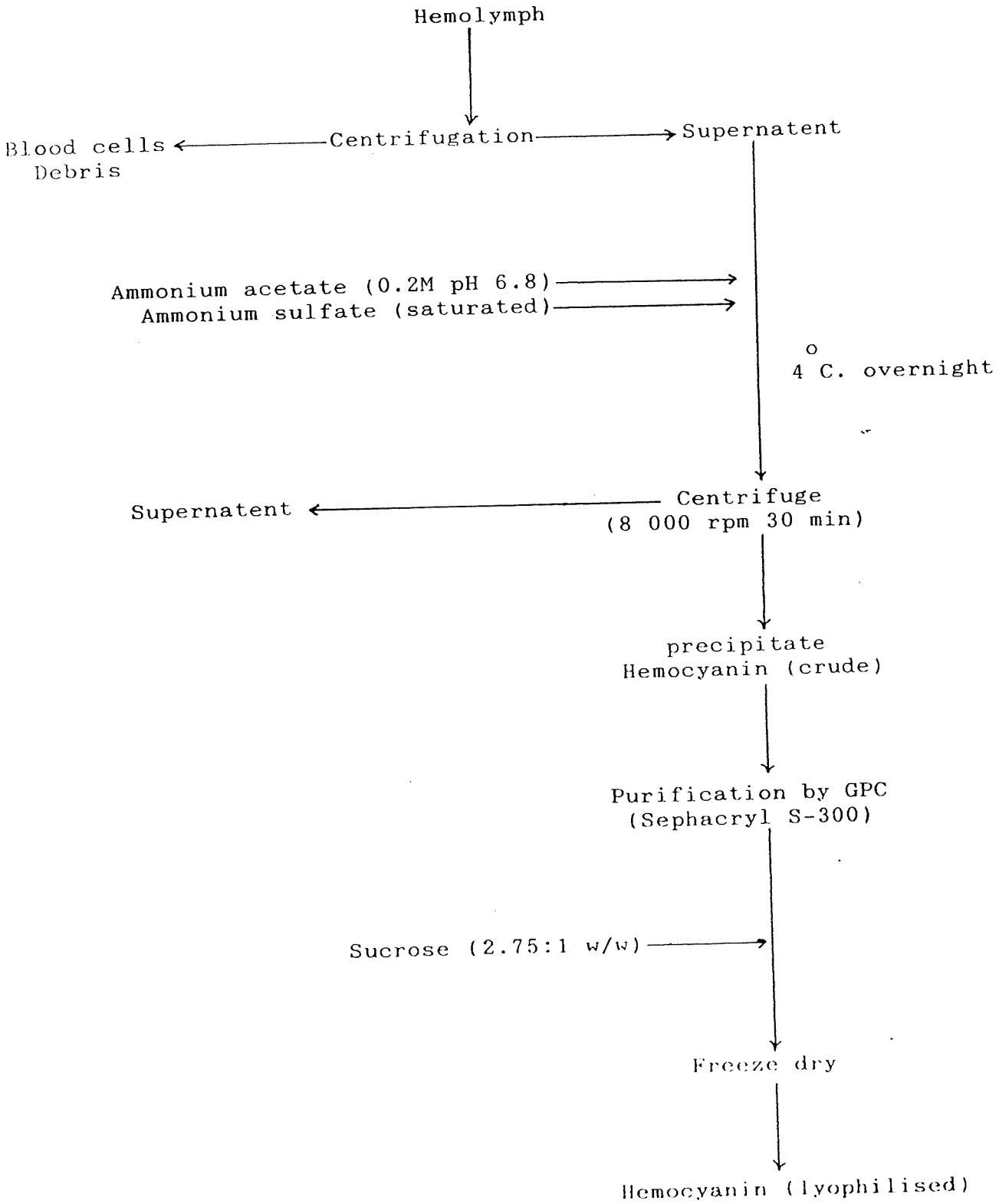


FIGURE 2.1 FLOW CHART FOR THE ISOLATION AND PURIFICATION OF ABALONE HEMOCYANIN.

with an equal volume of ammonium acetate/acetic acid buffer (0.5M, pH 6.2). The hemocyanin was then precipitated with an equal volume of $[(NH_4)_2SO_2]$. The precipitated protein was stored overnight and the next morning centrifuged (8 000 rpm, 45 min). The precipitate was a crude protein containing 61% water. Hemocyanin in this form may be stored for extended periods at 4^oC.

2.2.3 Gel filtration chromatography

Crude protein (500mg) was dissolved in 0.5M Trizma buffer (pH=7.0, I=1.0, 1.5mL) overnight. The solution was separated through Sephadex S-300 producing a major peak and a smaller minor peak with a positive UV absorbance at 280 nm. The initial high molecular weight hemocyanin fraction was dialysed against distilled water and lyophilised with sucrose in a ratio of 2.75:1. The final purified protein was used for most of the subsequent experiments.

2.2.4 Ion exchange chromatography

Abalone hemocyanin was isolated as previously described. The sample (500 mg) was dissolved in starting buffer (1.5 mLs) and left to equilibrate overnight at 4^oC. DEAE-Sepharose CL-6B (Pharmacia) is an anion exchange gel suitable for macro molecular proteins. The gel was prepared by removing initial contaminants with a concentrated salt solution (1.0M NaCl) and re-equilibrating with starting buffer (Tris-HCl, 0.05M, pH 8.2). This was then packed under flow as recommended by Pharmacia (38). A linear salt gradient of 0 to 0.5M NaCl was chosen and fractions were collected every 5.0 mLs. The UV absorbance was individually determined and plotted at 280 nm and 340 nm.

2.2.5 Ultraviolet/visible absorption

Abalone hemocyanin was prepared as previously described. The sample (10 mg) was dissolved in Tris-HCl buffer (pH 6.8 I=0.01) and the UV/Vis absorbance spectrum recorded on a Varian Superscan 3. This simulated natural hemocyanin concentrations. A much greater amount was dissolved (500 mg) in order to monitor the presence of the 540 nm absorption band. The same buffer solution was used as a reference in each case.

2.2.6 Agarose gel high voltage isoelectric focussing of hemocyanin

The pharmacia modular flat bed electrophoresis system was used for the determination of isoelectric points. This comprises of a power supply (ECPS 3000/150), a flat bed apparatus incorporating a cooling plate (FBE 3000) and a volthour integrator (VH-1). Agarose gel, cast on to hydrophobic plastic sheets, was chosen as the most suitable and convenient medium. Agarose (Pharmacia) is a suitable medium for the focussing of high molecular weight proteins due to its relatively high porosity. A linear pH gradient of 3-10 was used in each case. Conditions of focussing were within the limits set by Pharmacia (39). Isoelectric gradient calibration was performed using marker proteins (Pharmacia). The final gel was stained using coumassie blue and finally de-stained with a water:ethanol:acetic acid (55:35:10) mixture.

2.2.7 High performance liquid chromatography (HPLC)

Bio-Rad's TSK-60 column and complementary guard column were used in Gel filtration HPLC to determine molecular weights. The column is suitable for the separation of macro polymers up to 20 million Daltons. UV absorbance at 280 nm was used in detecting the protein. All buffers were made using tris(hydroxymethyl)methyl amine (BDH, AR grade) dissolved in

MilliQ water. Concentrated hydrochloric acid (33%, AR grade) was used to adjust the buffer to the desired pH. Calibration proteins (Pharmacia) were dissolved in buffer overnight before being applied to the column. The calibration graph obtained is shown on Figure 2.2. Extrapolation past the highest molecular weight marker (thyroglobulin- mwt.699,000) was necessary but work by Brouwer and Kuiper (9) produced comparable molecular weight estimates using gel filtration to those obtained by other methods.

2.2.8 Infrared analysis of abalone hemocyanin

Abalone hemocyanin was isolated and purified as described previously. However, the sample for IR analysis was dialysed against distilled water and freeze dried without the addition of sucrose. The dried protein (1.0 mg) was mixed with Potassium Bromide (400 mg, oven dried, 110^{o}C, 48 hours). The sample was mechanically homogenised (2 X 30 sec) on a modified dental amalgamator (PROGRESS - TAC DENTAL 135/A). KBr disks were produced under vacuum and pressure (8 tons). The spectrum was obtained on a Perkin-Elmer 983G previously calibrated with a polystyrene film. For a more elaborate spectral output, a Hitachi 270-30 infrared spectrophotometer with a 270-30 data processing unit was used. This instrument was capable of storing up to 4 spectra and providing difference, first and second order derivative plots.

2.2.9 Amino acid analysis of <u>N. ruber</u> hemocyanin

Hemocyanin was previously isolated (Section 2.2.1 -2.2.3) from abalone hemolymph and lyophylised before analysis. An accurate mass recorded and the proteins hydrolysed with hydrochloric acid (6M, 2 mLs) in an evacuated sealed ampule (110^{o}C, 24 hours). The hydrolysate was then dried in a dessicator (NaOH) and eluted on a Beckman system 630 High Performance Analyser. Amino acids were identified by comparisons of relative retention times (TR's) with

eluted standards.

2.2.10 Preparation of apo-hemocyanin

Hemocyanin was prepared from the hemolymph of H. ruber as previously described. A solution of hemocyanin (40mg/mL) was dialysed with several changes against Tris-HCl buffer (0.05M, pH 9.0, I=0.1). This was further dialysed against the buffer A (48 hours at 6°C). The apo-hemocyanin was then dialysed against buffer B to remove excess cyanide. The pH was reduced by further dialysis with Tris-HCl (pH 7.0, I=0.1) before analysis.

Buffer A: Tris-HCl (pH 9.0, I=0.1)
KCN (50 mM)
KCl (200 mM)
$(NH_4)_2SO_4$ (70 mM)
EDTA (20 mM)

Buffer B: Tris-HCl (pH 9.0, I=0.1)
KCl (200 mM)
$(NH_4)_2SO_4$ (70 mM)
EDTA (20 mM)

2.3.0 RESULTS AND DISCUSSION

2.3.1 Isolation of abalone hemocyanin

Abalone blood or hemolymph has a steely grey-blue colour in the oxidised form and a yellow colour in the reduced form. The protein in the blood is almost exclusively hemocyanin (1, 56). The protein was isolated by precipitation at pH 6.2 (the isoelectric point determined by isoelectric focussing). Hemocyanin was a light blue colour and is readily soluble in water. The addition of metabisulfite to a solution of the oxygenated blue protein caused it to become colourless within seconds.

The wet isolated protein consisted of 61% moisture. The protein was further purified by gel chrmatography and freeze-dried in the presence of sucrose added in a ratio of 2.75:1. If sucrose is not added, the protein obtained will not redissolve, presumably due to some conformational modification during the drying process.

The coagulation temperature of purified hemocyanin was determined to be 77^{o}C in distilled water. First sign of precipitation was noted at 67^{o}C although this may possibly be due to uneven heating of the test tube surfaces.

2.3.2 Ultraviolet/visible absorption

Abalone hemocyanin shares similar spectral characteristics with that of other molluscs and arthropods (see Fig.2.3 - 2.5). Strong ultraviolet and visible absorption peaks were present at 280 nm and 340 nm. The appearance of the first absorption band (280 nm) is mainly due to the tryptophan and tyrosine residues and can be used as an estimate of dilute protein concentrations. The second peak (at 340 nm.) is thought to arise from the oxidation valency state of the copper (ie Cu (II)) and is specific for the hemocyanins. The band at 560 nm. reported by Boon (9) for Octopus valgaris hemocyanin is extremely weak in the spectrum obtained for abalone hemocyanin (Fig.2.6) and could only be observed in a concentrated solution. At natural concentrations (10 to 20 mg/mL), the absorption band is almost absent and below the detection limits of our instrument (Varian SuperScan 3).

2.3.3 Gel filtration and ion exchange chromatography

Gel filtration of abalone hemocyanin produced only one major peak at fractions 14 to 20 (Fig. 2.7) within the exclusion limit of the gel. Minor low molecular weight proteins are present at fraction 30 and 49, but these are in relatively low concentrations and assumed to be other components of hemolymph.

Ainslie (2) has also reported the appearance of the minor constituents of abalone hemolymph with UV. maxima absorption at <260 nm and 320 nm. These compounds were not identified by Ainslie but later experiments suggests that they may be phenolic in nature.

Ion exchange at pH 8.2 produces a major peak at fraction 103 and some unretained material at fraction 18 (Fig.2.8). The major peak showed positive UV absorption at 340 nm (with a 340/280 nm ratio of 0.195) indicating that the sample was hemocyanin.

2.3.4 Agarose gel high voltage isoelectric focussing of hemocyanin

High voltage isoelectric focussing is a useful technique for analytical and semi-preparative separations of proteins. The technique is based on separating charged proteins in a porous gel which contains a pH gradient. The protein under the influence of an electric field, travels in the gel and stops at the pH equal to its isoelectric point. At the isoelectric point, the protein has a net zero charge and does not migrate further into the gel. Using this procedure, proteins differing in isoelectric points by as little as 0.05 units can be separated.

For the separation and analysis of abalone hemocyanin, we have used agarose gels which have a relatively high molecular weight exclusion limit of 2 million daltons. We employed pH gradients of 3-10 and 4-6.5. Estimation of isoelectric point was performed by specially purified marker proteins.

Abalone hemocyanin in phosphate buffer (pH 6.8) produces a single band with an isoelectric point of 6.2 (Fig.2.9). Although native hemocyanin has an estimated molecular weight of 2.5 million daltons (ie. greater than the exclusion limit of the gel) it appeared to travel quite freely from the point of application. Hemocyanins are well known to exhibit polymorphism (or different electrophoretic patterns for different animals of the same

species) (7). One of the earlier specimens examined produced a heterogeneous mixture of protein bands (Fig.2.9). This may have been caused by abalone hemocyanin polymorphism or be the result of proteolysis by bacterial degradation of the sample. Later samples showed little or no other protein contamination in the isolated hemocyanin.

Brouwer and Kuiper (9) focussed Helix pomatia a-hemocyanin which had been denatured by 6M guanidine-HCl (+ 1% mercapto-ethanol) and determined that the main component had a pI of 6.35 with a shoulder peak at pI=6.19. Comparison of this with abalone hemocyanin, however, is difficult because pI's are not absolute values and are dependent upon variables such as buffer composition.

2.3.5 Infrared analysis of N. ruber hemocyanin

The infrared spectra of abalone hemocyanin has many absorption peaks common to most proteins. The amide (I) and amide (II) absorption bands at 1538 cm-1 and 1660 cm-1 were clearly visible from the plot (Fig.2.10) The sample produced a broad phenolic -OH absorption band approx 3320 cm-1 with associated and out of plane bending of bonded -OH at the lower wavelengths (769-650 cm-1). Methyl absorption bands (2) are clearly visible at 3000 - 2850 cm-1.

The first derivative plot (Fig.2.11) was recalculated from the hemocyanin spectrum and provides data on additional peaks that are usually hidden by topographical features of the major absorption peaks. Many additional absorption bands are revealed but the complexities of functional group interactions within the protein matrix make accurate assignments difficult.

2.3.6 Amino acid analysis of N. ruber hemocyanin

The complexity and size of the hemocyanins make amino acid sequencing extremely difficult. It is only recently that the sequence for fragmented subunits of the hemocyanins from Euryplma californian and Panulinus interruptus (72) have been determined. However, the amino acid content of the hemocyanins from many arthropods and molluscs are summarised by van Holde and Van Bruggen (68).

Table 2. Amino acid analysis of a sample of hemocyanin isolated from the hemolymph of an abalone specimen (N. ruber)

Amino acid	Mole (%)*		
LYS	4.9	(±)	0.13
HIS	4.0		0.10
ARG	4.4		0.12
ASP	11.9		0.44
THR	6.2		0.43
SER	5.8		0.55
GLU	10.4		0.12
PRO	6.0		0.15
GLY	7.4		0.33
ALA	7.5		0.56
CYS	1.4		0.42
VAL	5.5		0.33
MET	1.3		0.20
ILEU	4.5		0.78
LEU	8.6		0.40
TYR	4.5		0.29
PHE	5.6		0.27

Total : 100.0

* Calculated as the average of duplicate determinations

The amino acid content of N. ruber Hc as shown in Table 2 was calculated from duplicate determinations and expressed as

mole percentage of total amino acid content. These results are characterised by high proportions of the acidic groups (up to 22%). The non-polar amino acids such as leucine, aspartic and glutamic acid is also present in larger quantities showing strong similarities when compared with the hemocyanins of other molluscs. Very little of the sulfur containing amino acids are present although acid hydrolyses are non quantitative for tryptophan residues.

2.3.7 High performance liquid chromatography (HPLC)

HPLC gel chromatography is a convenient method of determining the molecular weight of a protein. The technique may also be used as a method of separating and characterising proteins according to relative retention times (TR's). Unfortunately, no commercial protein standard could be obtained beyond 670,000 daltons (thyroglobulin) and extrapolation of the graph was necessary. Brouwer and Kuiper (9) investigated various methods of m.wt. determination and produced similar results for gel filtration (also extrapolated past the last standard protein) when compared with other methods.

At pH 7.0, N. ruber hemocyanin produced a single absorption peak with an estimated molecular weight of 2,300,000 (±) 200,000 daltons (Fig.2.12). The value was calculated from an average of 8 determinations derived from individual abalones collected over the course of the study. On some samples, a minor component of high molecular weight (>5,000,000) could be observed. It was assumed that this artifact may be the result of two or more aggregated whole hemocyanin molecules as the peak was too broad to have been produced from a single homogeneous component. The whole molecule is known to be stable at pH 7.0 (I=0.1) but undergoes dissociation into smaller subunits with higher pH conditions (Section 9.0)

2.3.8 Preparation of apo-hemocyanin

The removal of the copper from the active sites of mollusc and arthropod hemocyanins has been reported by several investigators (5, 42). For Octopus valgaris hemocyanin, cyanide treatment removes 98% of total copper compared to only 80 to 90% for mollusc Hc.. Beltramini et al. (5) showed that the effects of cyanide treatment on Octopus valgaris hemocyanin was completely reversible. The addition of copper (I) cations to apo-Hc produced a reconstituted hemocyanin with similar spectral properties and oxygen capacity when compared with the native protein. Similarities in the copper content of both hemocyanins suggested specific re-absorption at the active sites.

Apo-hemocyanin from N. ruber was produced by treatment with cyanide buffer in a method similar to that used by Beltramini et al. (5). It was assumed from spectral evidence (loss of characteristic 340 nm absorption) that a significant percentage of the active copper had been removed from the native protein (Fig.2.13). This apo-Hc. was stored and used in further experiments in later chapters.

2.3.9 Comparison of bay and ocean black lip abalone hemolymph

A visual comparison was made between bay and ocean hemolymph. Bay hemolymph was a deep blue colour and had a milky appearance. When NH_4Ac and $(NH_4)_2SO_4$ were added to precipitate the hemocyanin a deep blue flocculant precipitated out. On the other hand, ocean hemolymph was a clear pale green/blue colour and less viscous. Upon the addition of NH_4Ac and $(NH_4)_2SO_4$ a dense white coagulant precipitated. Bay hemocyanin could easily be redissolved with Tris-HCl (0.5M) overnight, whilst ocean hemocyanin didn't fully redissolve.

Ultraviolet/visible scans of bay and ocean hemolymph (Fig.2.14) were made on a Varian Superscan 3 spectrophotometer over the wavelength range, 200-600nm. A shift of 25nm (340nm to

365 nm) was observed between ocean and bay hemolymph traces. Both traces were of similar shape but a lower absorbance value at 340nm for ocean hemolymph was observed (1.9 compared to 3.5).

Absorbance at 340nm-350nm has been used to allow appoximations to be made of the % hemocyanin in an oxygenated state. The lower absorbance measured at 340nm for ocean hemolymph, 1.9, compared to 3.28 at 365nm for bay hemolymph, may suggest less hemocyanin in an oxygenated state. This could be a possible reason for the visual difference observed between ocean and bay hemolymph.

Abalone were bled overnight and the hemolymph treated to yield purified and stable hemocyanin samples. It was noted that ocean abalone yield less hemolymph compared to bay, 18.36/100g of shucked weight compared to 25.42g/100g shucked weight respectively. A difference in volume could be due to greater fluid loss on transport. It is known that abalone will lose fluid once removed from water and when under stress. Differences in salinity may also be reflected here.

Hemocyanin/water solutions (1mg/mL) were prepared and absorbance scanned between 200-600nm. Traces for bay and ocean hemocyanin were of similar shape except ocean hemocyanin recorded lower absorbances over this wavelength range. A peak recorded at 360nm for bay hemocyanin was absent for ocean hemocyanin.

Differences in diet may be responsible for the visual differences in hemolymph and precipitated hemocyanin, as well as minor differences in ultraviolet/visible scans for stored, freeze dried hemocyanin.

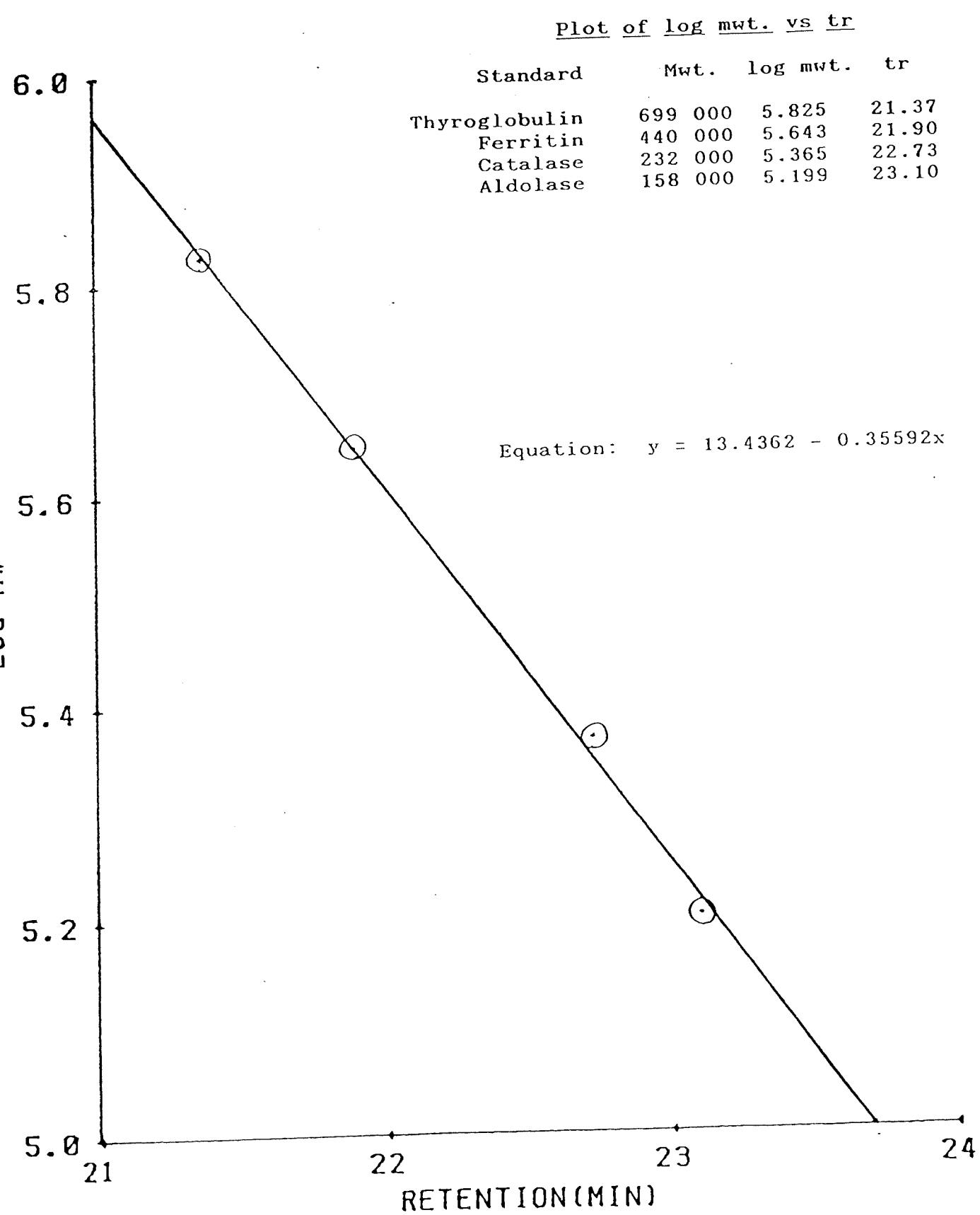
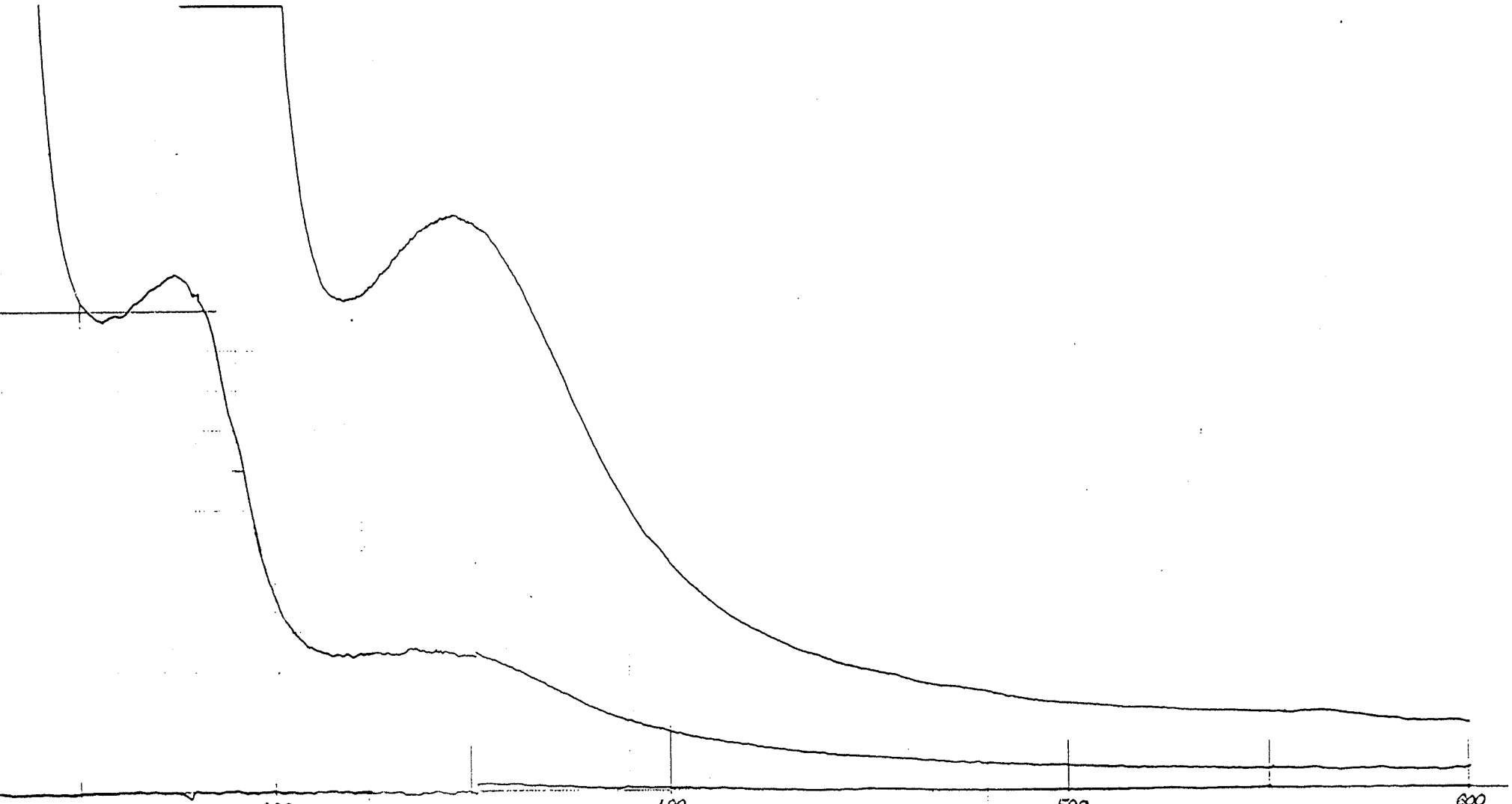


FIGURE 2.2 CALIBRATION CURVE FOR MOLECUALR WEIGHT DETERMINATIONS.

SuperScan

Part No. 71-100033-00



SENTATION

nm
m (1 nm/cm)
" (2 ")
" (5 ")
" (10 ")
" (20 ")
50. 100. 200

PHOTOMETRIC PRESENTATION

MODE	%T, Abs, Der.
FULL SCALE	0·05 Abs
Y AXIS	0·10 "
	0·50 "
	1·0 "
	2·0 "
	4·0 "
	100% T
OFFSE	bs.

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE	20
X AXIS	40
	100
	200
	400

Sample & Formula		
Concentration 15	Date 16/5/87	Ref. No.
Reference		
Path Length mm	Operator R1	
th		

FIGURE 2.3 UV-VISIBLE SPECTRUM OF ISOLATED ABALONE HEMOCYANIN AT TWO DIFFERENT CONCENTRATIONS TO EMPHASIZE THE MAXIMUM AT 340 nm.

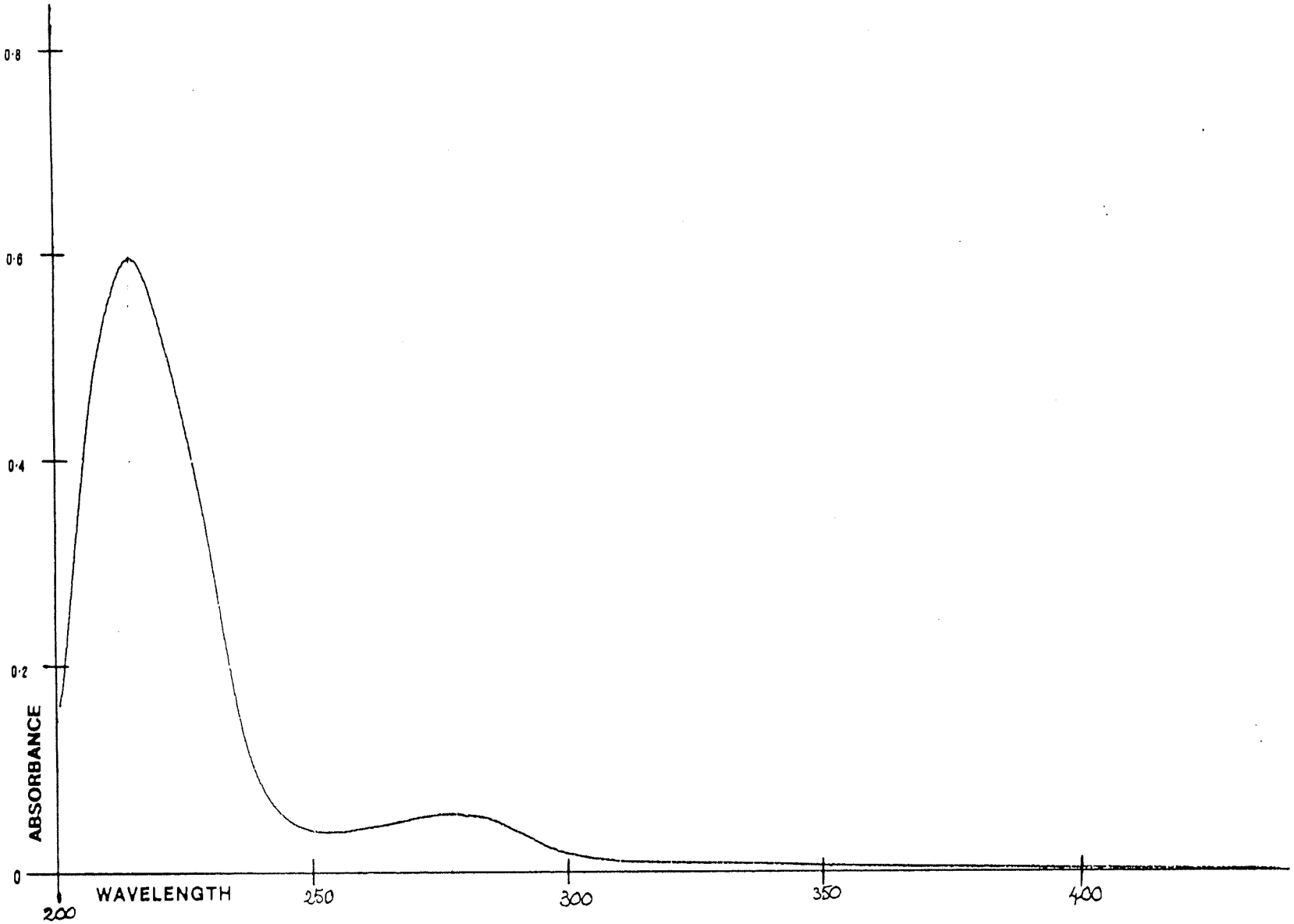


FIGURE 2.4 UV SPECTRUM OF ABALONE HEMOCYANIN.

WAVELENGTH PRESENTATION

SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm	(1 nm/cm)
	80 "	(2 "
	200 "	(5 "
	400 "	(10 "
	800 "	(20 "
SCAN SPEED	10, 20, 40, 100, 200 nm/min	

PHOTOMETRIC PRESENTATION

MODE	%T, Abs, Der.	
FULL SCALE Y AXIS	0·05	Abs
	0·10	"
	0·50	"
	1·0	"
	2·0	"
	4·0	"
	100%	T
REC. OFFSET		Abs.

TIME PRESENTA

UNITS	Sec.
FULL SCALE X AXIS	20
	40
	100
	200
	400

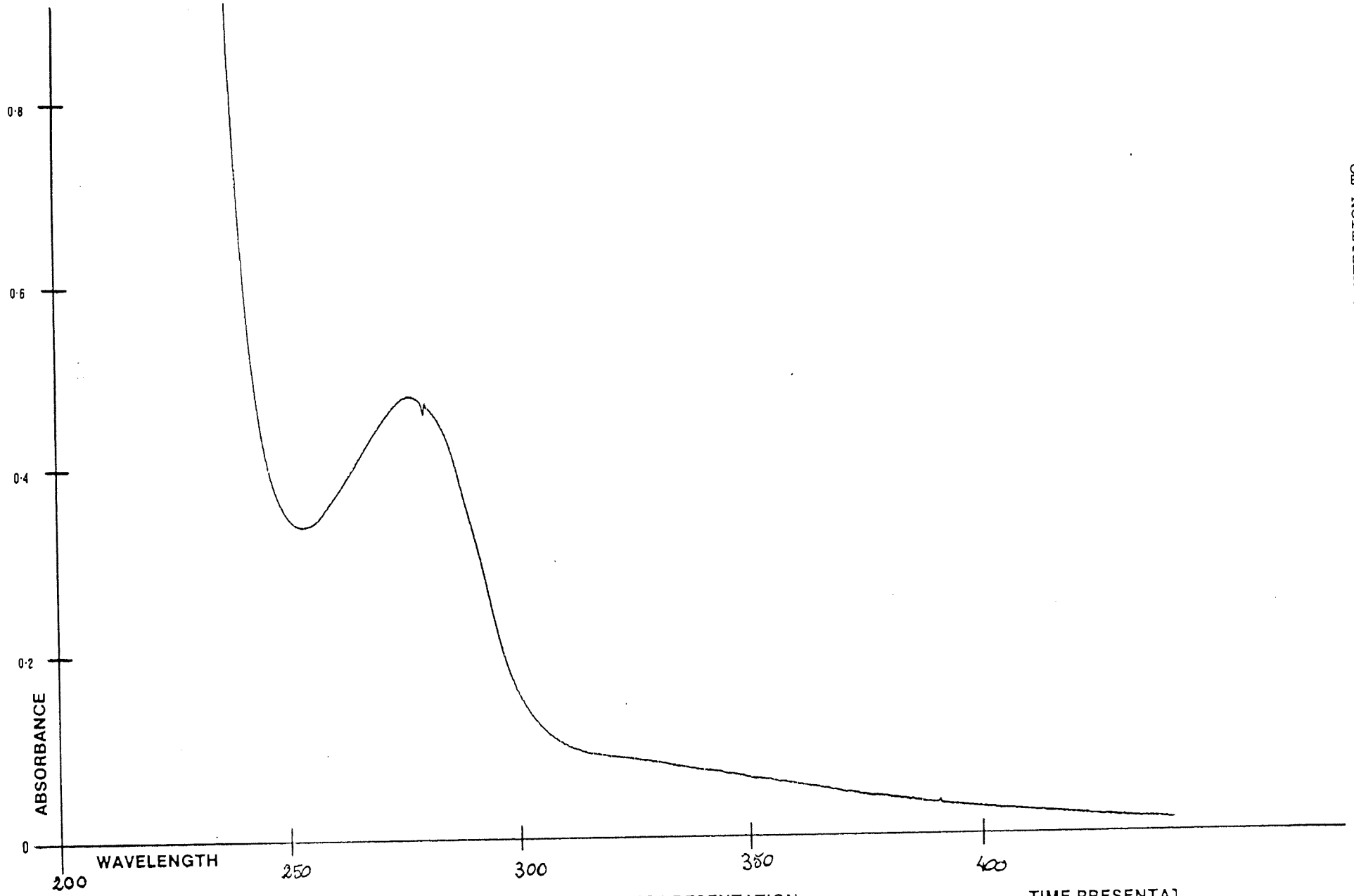


FIGURE 2.5 UV SPECTRUM OF HEMOCYANIN AT A CONCENTRATION TO EMPHASIZE THE MAXIMUM AT 280 nm.

WAVELENGTH PRESENTATION

SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm (1 nm/cm)	
	80 " (2 ")	
	200 " (5 ")	
	400 " (10 ")	
	800 " (20 ")	
SCAN SPEED	10. 20. 50. 100. 200 /min	

PHOTOMETRIC PRESENTATION

MODE	%T, Abs, Der,	
FULL SCALE Y AXIS	0·05	Abs
	0·10	"
	0·50	"
	1·0	"
	2·0	"
	4·0	"
	100%	T

TIME PRESENTAT

UNITS	Sec,
FULL SCALE X AXIS	20
	40
	100
	200
	400

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△

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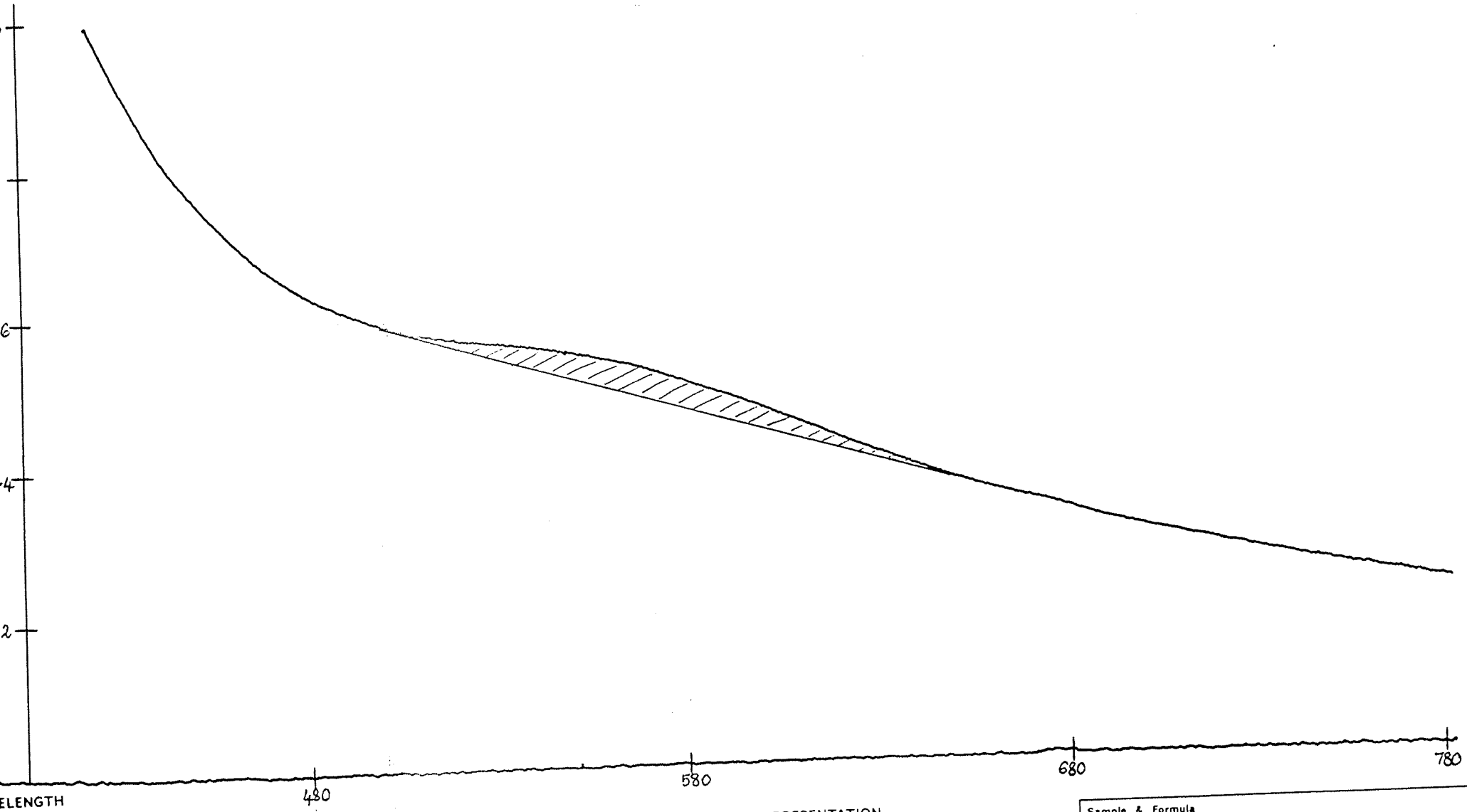


FIGURE 2.6 ABALONE HEMOCYANIN IN WATER AT A HIGH CONCENTRATION SHOWING THE WEAK BROAD MAXIMUM

ELENGTH

PRESENTATION

	nm
40 nm	(1 nm/cm)
80 "	(2 ")
200 "	(5 ")
400 "	(10 ")
800 "	(20 ")

10 , 20 (50), 100 , 200

PHOTOMETRIC PRESENTATION

MODE	%T, (Abs), Der,
FULL SCALE	0·05 Abs
Y AXIS	0·10 "
	1·0 "
	2·0 "
	4·0 "
	100 % T

RE ET

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE	20
X AXIS	40
	100
	200
	400

Sample & Formula: HEMOCYANIN · H_2O		
Concentration	Date 12/9/86.	Ref. No.
Reference H_2O		
Path Length mm	Operator RL	
Slit Width nm		

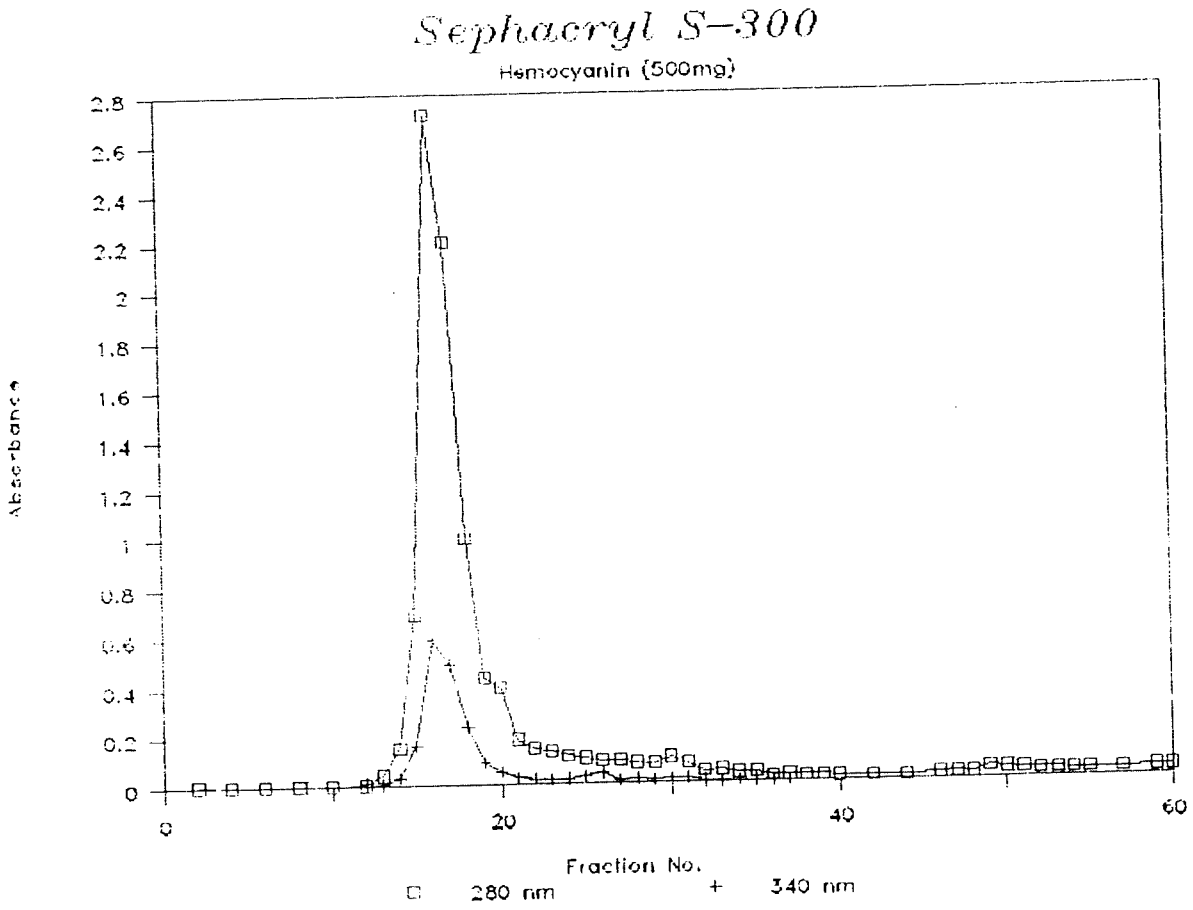


FIGURE 2.7 GEL CHROMATOGRAPHY OF PRECIPITATED HEMOCYANIN ON SEPHACRYL S-300.

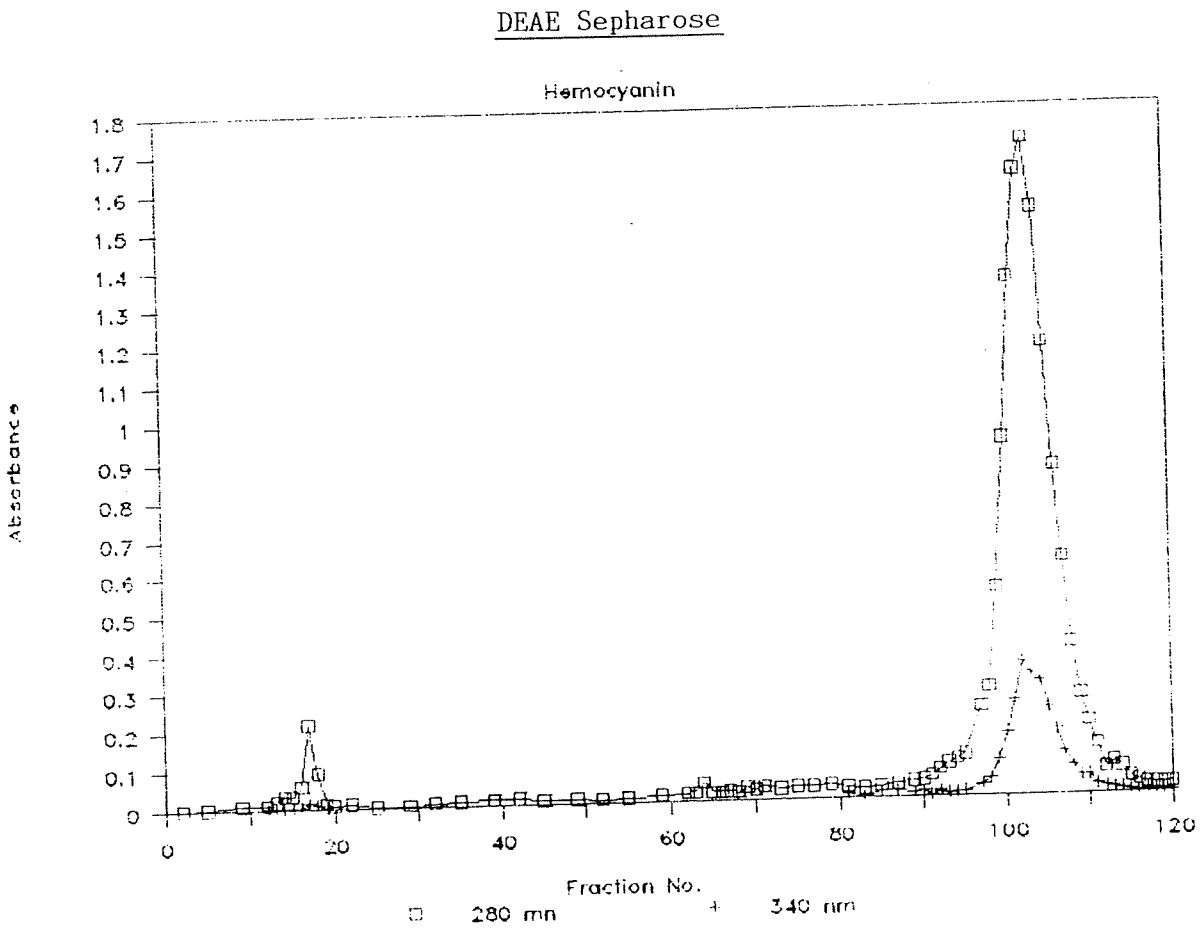


FIGURE 2.8 ION EXCHANGE CHROMATOGRAPHY OF A HEMOCYANIN ISOLATE.

FIGURE 2.9 ISOELECTRIC FOCUSSING OF N. RUBER HEMOCYANIN AT pH 6.8.

pI indicators

Medium: Agarose gel / pharmalyte 3-10

Final Conditions: ~ 1650 Volt/hour
1500 Volt
9.8 ma
15.0 Watt

SAMPLE :HEMOCYANIN
REMARKS :S-300 TB

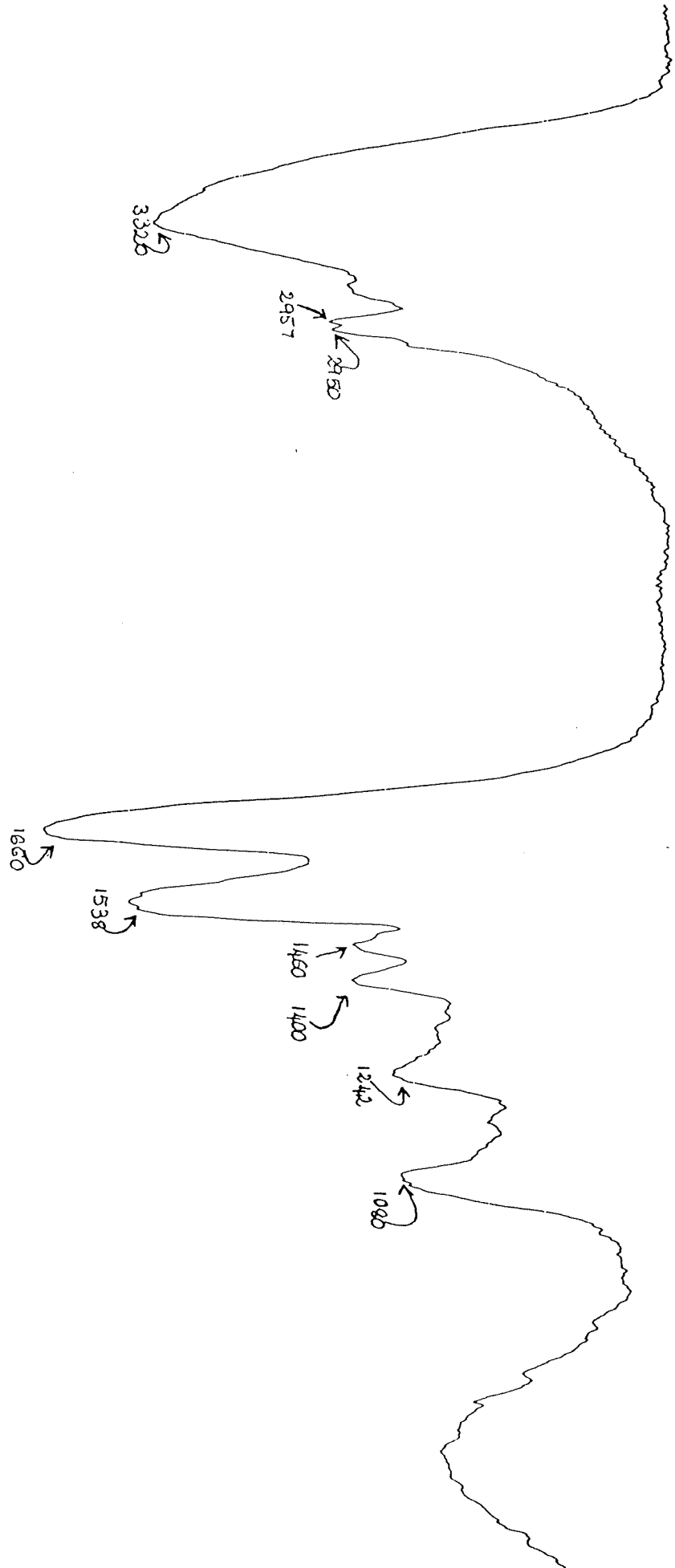


FIGURE 2.10 TRANSMISSION IR SPECTRUM OF N. RUBER HEMOCYANIN

SAMPLE :HEMOCYANIN
REMARKS :S-300 TB

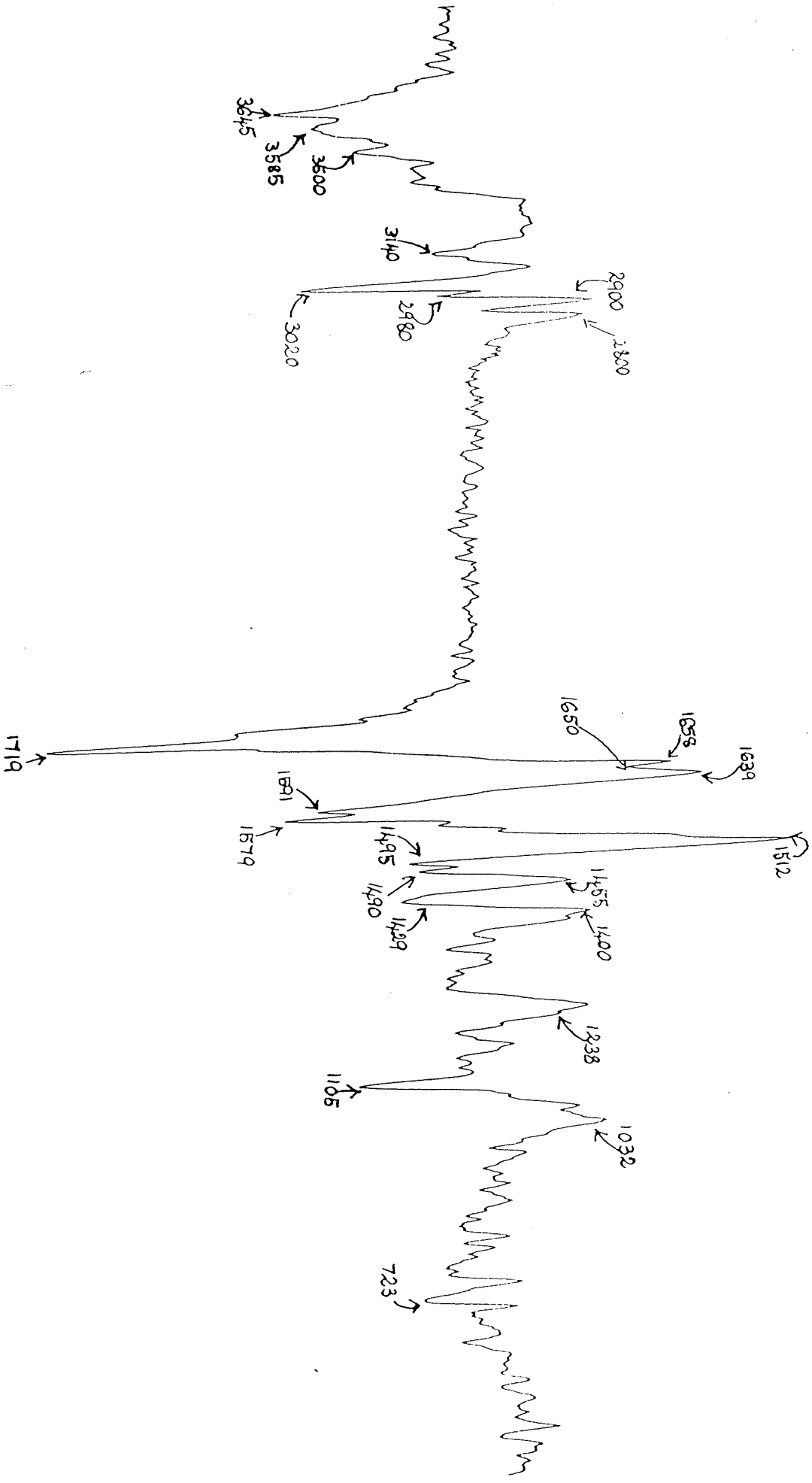
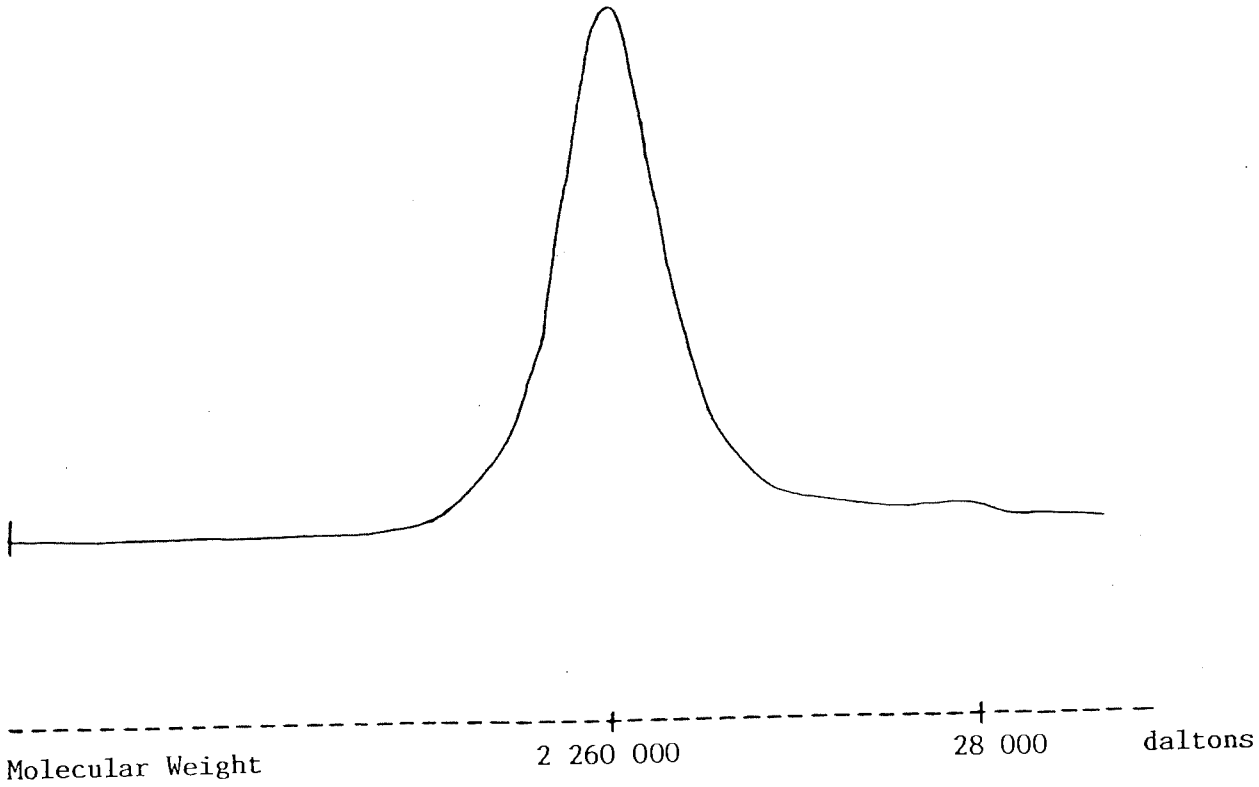


FIGURE 2.11 FIRST DERIVATIVE IR SPECTRUM OF HEMOCYANIN ISOLATED FROM N. RUBER HEMOLYMPH.



INSTRUMENT : Waters HPLC
COLUMN : Bio Rad TSK - 60
DETECTOR : UV at 280 nm
BUFFER : Tris-HCl (0.025M) pH 7.0
FLOW RATE : 0.5 mL/min

FIGURE 2.12 HPLC OF HEMOCYANIN FROM N. RUBER AGAINST A MOLECULAR WEIGHT SCALE AT pH 7.

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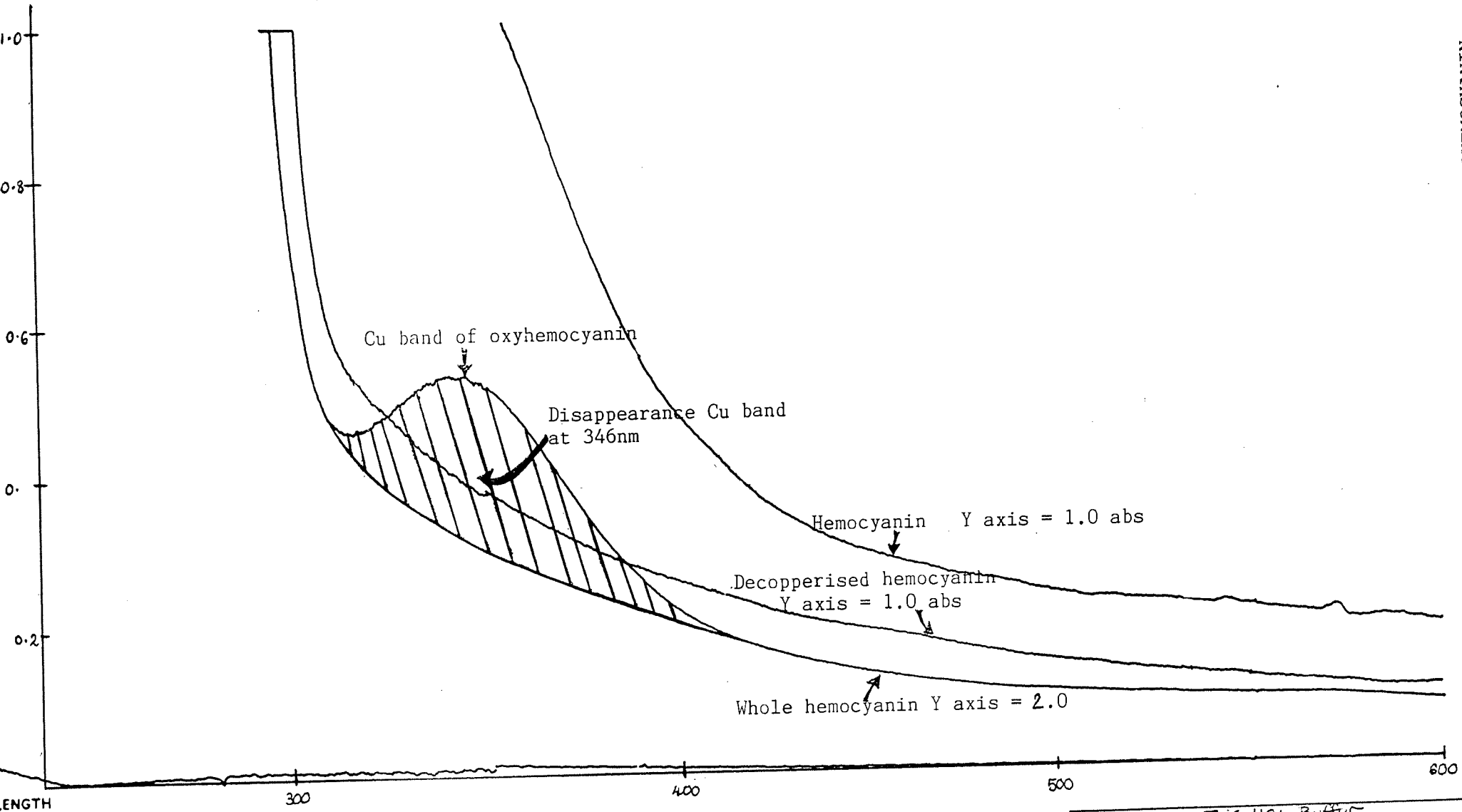


FIGURE 2.13 UV-VISIBLE SPECTRA OF HEMOCYANIN AND APOHEMOCYANIN.

LENGTH

PRESENTATION

600 nm

nm (1 nm/cm)
" (2 ")
" (5 ")
" (10 ")
" (20 ")

20 50 100 200
nm/min

PHOTOMETRIC PRESENTATION

MODE	%T, Abs, Der,
FULL SCALE Y AXIS	0·05 Abs 0·10 " 0·50 " 1·0 " 2·0 " 4·0 " 100 % T
REC. OFFSET	Abs.

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE X AXIS	20 40 100 200 400

Sample & Formula Tris HCl Buffer

Concentration	Date 2/4/66.	Ref. No.
Reference		
Path Length mm	Operator R. Lim	
Slit Width nm		

varian

SuperScan

Part No.

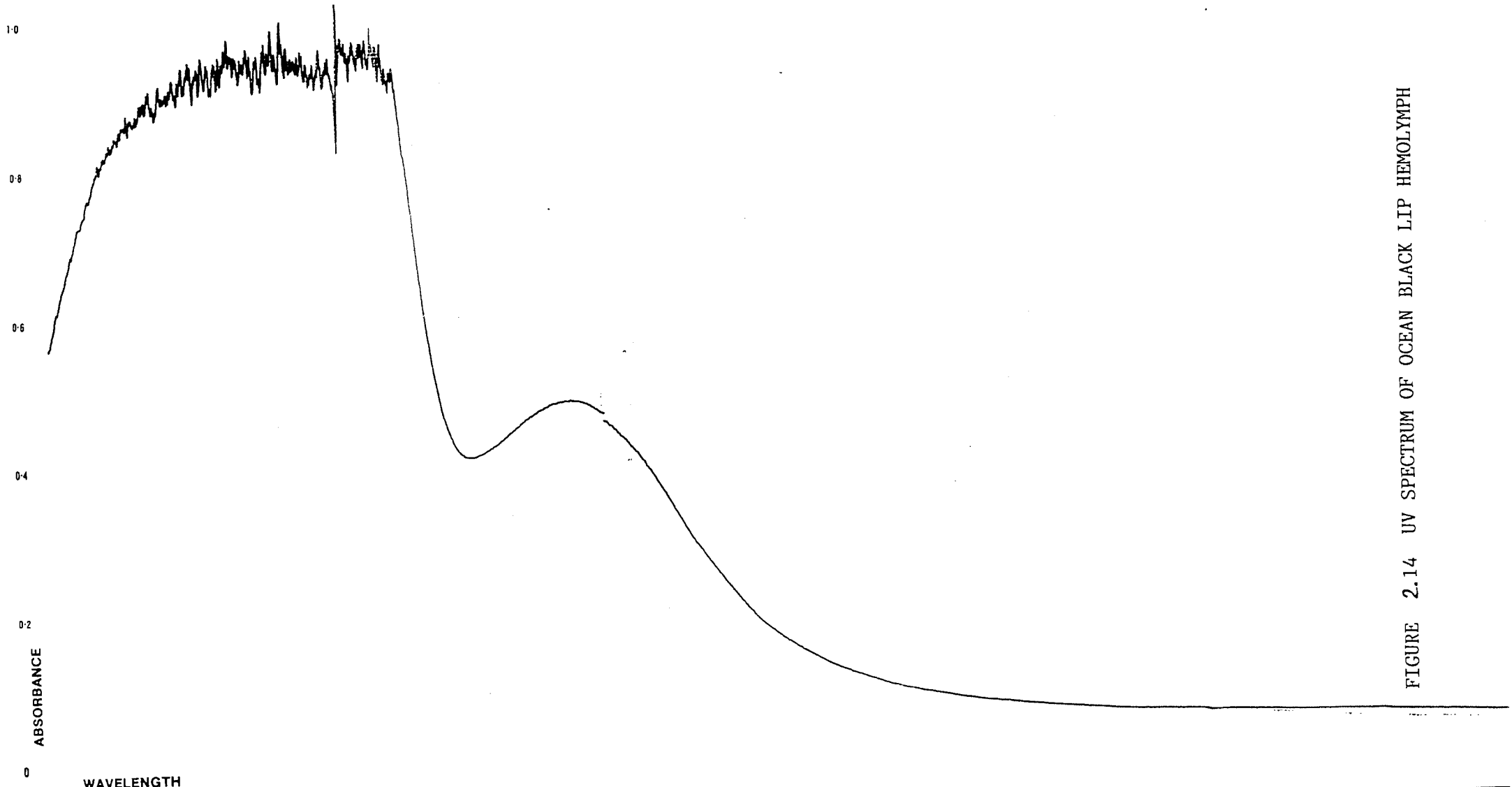


FIGURE 2.14 UV SPECTRUM OF OCEAN BLACK LIP HEMOLYMPH

WAVELENGTH PRESENTATION

SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm	(1 nm/cm)
	80 "	(2 ")
	200 "	(5 ")
	400 "	(10 ")
	20 "	

PHOTOMETRIC PRESENTATION

MODE	%T, Abs, Der,
FULL SCALE Y AXIS	0·05 Abs
	0·10 "
	0·50 "
	1·0 "
	2·0
	4·0

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE X AXIS	20
	40
	100
	3

Sample & Formula: OCEAN BLACK LIP HEMOLYMPH

Concentration

Date 23/3/87

Reference H_2O

Path Length 10 mm

Operator

PART II. ANALYSIS OF ABALONE FLESH CONSTITUENTS RELATED TO THE BLUEING PHENOMENUM

3.0 HEMOCYANIN CONCENTRATIONS IN TASMANIAN AND VICTORIAN ABALONE HEMOLYMPH

3.1 INTRODUCTION

Variations in hemocyanin (Hc) concentration in abalone hemolymph have been reported. Pilson (55) monitored the Hc levels of four species of Californian Haliotis and found variations of up to 900 fold for members of H. currugata (ave. 3.8 mg/mL). Other species studied included H. fulgens (ave. 5.4 mg/mL) and H. cracherodii (ave. 1.5 mg/mL). The changes recorded could not be correlated with weight, sex, reproductive stage, depth of water from which the animals were collected, or the time of year.

Ainslie (1) published results of a similar study on three species of South Australian abalone. Large differences in concentrations were observed that could not be correlated with any biological pattern. The species studied were H. laevigata (ave. 5.9 mg/mL) and N. ruber (ave. 4.1 mg/mL).

Betzer and Pilson (6) however, suggested that abalone have lower overall levels of Hc during the winter months when the animals are less active. In summer when the animals require more respiratory oxygen, up to a 4 fold increase in Hc concentration was observed.

This chapter investigates the seasonal variation in Hc concentrations of Victorian N. ruber. Four collections of abalone specimens were collected from February 1987 to June 1988 (representing 2 summer and 2 winter samples). The results are also compared with the Hc concentration of Tasmanian N. ruber collected in March 1987.

3.2 MATERIALS AND METHODS

The Hc concentrations of N. ruber were recorded at capture during various times of the year. Blood samples were collected by the insertion of a syringe into the median pedal vein and withdrawing approximately 1 mL of blood from the animal. Samples were stored in ice and kept at 6°C until analysis.

Each blood sample was centrifuged to remove cells and other debris. The remaining plasma was quantitatively diluted with a saline solution (0.1M NaCl) and oxygenated by shaking before measurement. Hemocyanin content was estimated with UV absorbance at 340 nm assuming E1%=2.23 (58)

Table 3. Variations of hemocyanin concentrations (mg/mL) in the hemolymph of N. ruber species

Specimens collected at Flinders, Victoria

Date	Ave.Conc.	High	Low
13 Feb. 87	18.1	27.4	12.4
14 May 87	8.9	12.0	6.9
20 Jan. 88	17.2	25.5	8.4
24 June 88	10.2	19.1	3.7

Specimens collected off Dover, Tasmania

March 87	Specimen No.	Conc.	Specimen No.	Conc.
	1	16.5	6	45.0
	2	22.9	7	21.6
	3	21.1	8	28.5
	4	16.4	9	19.8
	5	20.9	10	15.7

3.3 RESULTS AND DISCUSSION

The average Hc concentrations of N. ruber were found to be 14.6mg/mL (range 6.9 - 27.4) for Victorian samples and 24.4 mg/mL (range 16.8 - 48.0) for Tasmanian samples as set out in Table 3. The average concentration (14.6 mg/mL, from 35 animals) was higher than the 4.1 mg/mL reported by Ainslie (1) for South Australian N. ruber. Large variations in Hc concentration have been recorded even for specimens of the same genetic pool [Pilson (55)]. This may produce the differences observed for Victorian, and Tasmanian N. ruber, and South Australian N. ruber.

The specimens of N. ruber were collected from Flinders in Victoria at various intervals during the course of the study and the seasonal variations in Hc content are shown in Figure 3. All samples were taken at low tide from the same location (many off the same rock) in order to provide a better comparison between the samples which presumably have had similar environmental and dietary input. Seasonal data indicates lower overall Hc levels in the winter months. The lowest Hc content for an individual animal was also found during May/June.
Betzer and Pilson (6) suggested that abalone have higher levels of Hc during the summer months possibly due to an increase in metabolic activity.

A comparison of the Hc content of Victorian and Tasmanian specimens suggests that Tasmanian abalone have a naturally higher level of Hc when compared with either Victorian or South Australian samples (see Tables 3.1-3.5). The maximum of 48.0 mg/mL being significantly higher than any figure reported in the literature for any species of abalone (Pilson - 20.3mg/mL, Ainslie - 15.1 mg/mL). This particular animal (specimen No.6) produced the most intense blueing after brining and also after canning (see attached photograph (Figure 11.3 in Section 11).

Abalone are known to undergo dramatic changes in Hc levels (1). Ainslie sampled, tagged, and released animals to determine the changes in the animals over a period of time. Recaptured

animals produced all three variations (fall, stable, rise) that could not be adequately explained. However this does indicate that the abalone metabolism may produce or remove Hc from the circulatory system in a relatively short time. There are suggestions that Hc may be catabolised in order to maintain energy supplies for the organism (2). Since copper is highly toxic to most marine organisms (including abalone) its storage must be in the form of either another metal/protein complex or as granules completely engulfed by cellular amebocytes. It is likely that this stored copper plays an active role in the blueing phenomena.

3.4 CONCLUSIONS

The data reported in this Section represent a monitoring over two summers and two winters. The specimens analysed at any one sampling are numerous enough, and selected carefully enough to allow us to have confidence in the trend which clearly shows that hemocyanin concentrations in the hemolymph are higher during warmer summer months.

Of special interest is the observation that Tasmanian abalone have a significantly higher average concentration of Hc than samples from Victorian waters and in turn these show a higher concentration than South Australian abalone. Our understanding is that Tasmanian and Victorian abalone show blueing on canning but the problem is not so pressing in South Australia.

Table 3.1

Sample Area : Flinders (Vic.)
Date : 13th Feb 1987

Specimen	Conc.(mg/mL)	
1	20.8	
2	14.7	
3	12.4	min.
4	27.4	max.
5	17.2	
6	21.5	
7	16.6	
8	14.3	
Average	18.1 mg/mL	

Table 3.2
Sample Area : Flinders (Vic.)
Date : 14th May 1987

Specimen	Conc.(mg/mL)	
1	6.0	min.
2	10.5	
3	10.4	
4	7.2	
5	7.8	
6	11.2	
7	10.0	
8	6.9	
9	9.0	
10	7.8	
11	7.9	
12	12.0	max.
Average	8.9 mg/mL	

Table 3.3

Sample Area : Flinders (Vic.)

Date : 20th Jan 1988

Specimen	Conc.(mg/mL)	
1	12.5	
2	10.6	
3	13.8	
4	20.5	
5	11.6	
6	25.4	
7	20.0	
8	24.6	
9	8.4	min.
10	18.7	
11	19.0	
12	14.5	
13	13.9	
14	25.5	max.
15	19.7	
Average	17.2 mg/mL	

Table 3.4

Sample Area : Flinders (Vic.)

Date : 24th June 88

Specimen	Conc.(mg/mL)	
1	19.1	max.
2	12.9	
3	3.7	min.
4	19.1	
5	9.1	
6	7.6	
7	11.3	
8	4.2	
Average	10.2	

Table 3.5

Sample Area : Dover (Tas.)

Date : 6th March 1987

Specimen	Conc.(mg/mL)	
1	30.2	
2	32.4	
3	17.7	
4	24.5	
5	22.5	
6	17.5	
7	22.2	
8	48.0	max.
9	23.0	
10	30.4	
11	21.2	
12	16.8	min.
Average	24.4 mg/mL	

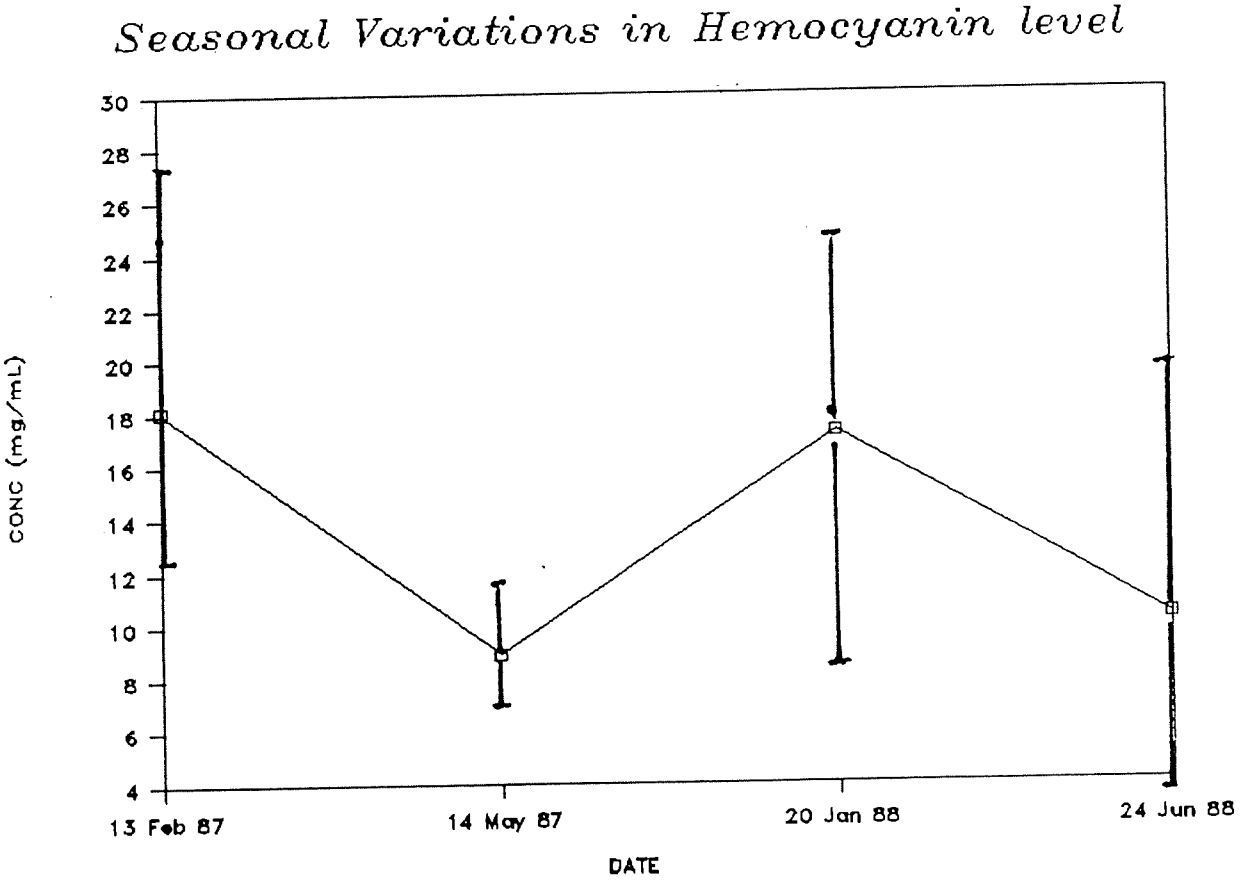


FIGURE 3. HEMOCYANIN VARIATIONS AS FOUND IN N. RUBER SPECIMENS COLLECTED FROM FLINDERS, VICTORIA.

4.0 THE INDUCED BLUEING OF N.RUBER

4.1.0 MATERIALS AND METHODS

4.1.1 Abalone

Abalone were supplied by Australian Abalone Exports (Laverton, Vic.). The abalone were kept alive in an oxygenated tank until collection. Just prior to transportation to the University of Melbourne the abalone were packed in a foam esky lined with hesshen that had been soaked in sea water. Transport was by van (ambient temperature) and undertaken within 45 minutes.

4.1.2 Induced blueing

Black lip abalone, shucked/unshucked, were left at room temperature (24h) and in saturated salt/ice solution (2L/450g) shucked weight) for four days in an attempt to induce blue discolouration. Abalone were observed everyday for signs of blueing.

4.2 Results and discussion

Abalone were left at room temperature and in a saturated salt/ice solution in a shucked and unshucked state. The following results were observed-

Table 4a The effect of storage at room temperature and in saturated salt ice solution on the colour of unshucked abalone

	Ambient temp. 25°C, 24h	Saturated salt/ice solution (4days) 30 min.,room temp, exposed to air
Shucked	No visible change	No visible change
Unshucked	Blueing of frill	No visible change

	Deep blue/grey frill area.When cut in half the exposed flesh took on a darker colour

The process of shucking and the removal of viscera resulted in the immediate loss of hemolymph and therefore hemocyanin. The rapid revoval of hemolymph and therefore substrate/enzyme needed for the formation of hemocyanin sulfide complex and catalyst for phenolase-produced melanin compounds was also removed. This would support the observed results of no visual change in shucked abalone samples.

The saturated salt/ice solution concentrated the hemocyanin in the flesh by osmosis, especially in the frill region which is used for oxygen transfer. The transport of abalone out of water, handling, confinement and rapid temperature change would result in stress (Wagner, 1987). In a stressed state utilization of carbohydrate, protein and lipid stores would increase as well as energy demands. It is possible that unshucked abalone in saturated salt/ice solution showed no visual signs of blueing due to the hemocyanin in the flesh becoming deoxygenated due to near total utilization of dissolved oxygen in the salt/ice solution. Upon exposure of wet surfaces to the atmosphere, hemocyanin would

oxygenate and because of being in high concentration, give a deep blue/grey colour to the frill and flesh.

4.3 CONCLUSIONS

Table 4 leaves one in no doubt that if the abalone is bled of hemolymph no blueing is induced in the frill or the flesh. This points strongly to the involvement of Hc in the blueing reactions. It also suggests a method of prevention.

5.0 AMINO ACID CONTENT OF HEMOCYANIN AND ITS USE IN THE ANALYSIS OF ABALONE FLESH EXTRACTS

5.1 INTRODUCTION

The amino acid content of various extracts and protein fractions were determined on a Beckman auto analyser. Selected samples were extracted by a modification of a method used by Pyeuns et al. (56). Some of the samples produced characteristic amino acid compositions which were also exhibited by the hemocyanin standard.

A statistical method developed by Teller and used by Van Holde and Van Bruggen (68) allows the use of amino acid composition data to test the relatedness of proteins. The general equation is shown below:

$$D=1/N \; E \; (X_i - Y_i)^2$$

D=measure of relatedness in proteins
N=number of amino acids
X_i=mole fraction of an amino acid from sample X
Y_i=mole fraction of an amino acid from sample Y

Teller was able to demonstrate that related hemocyanins will produce low values of 'D'. Obviously from the equation, identical proteins with identical amino acid compositions will have D = 0. This same calculation can also be used to numerically compare the similarities of protein fractions. It can also be used to estimate the influence of the hemocyanin amino acids upon the composition of the overall extracts of the flesh.

5.2 MATERIALS AND METHODS

Hemocyanin was previously isolated (see Section 2)

from abalone hemolymph by the method of Koning et al. (42) Extraction of salt and base soluble proteins is described in Section 11. All samples were freeze dried overnight and an accurate mass recorded. The proteins were hydrolysed with hydrochloric acid (6M, 2 mLs) in an evacuated sealed ampoule at 110^{o}C for 24 hours. In later determinations, phenol (0.2%) was added during hydrolysis in order to obtain quantitative recoveries of tyrosine. The hydrolysate was then dried in a desiccator (NaOH) under reduced pressure (48 hours). The samples were quantitatively diluted and eluted on a Beckman system 630 High Performance Analyser.

5.3 RESULTS

Samples analysed included the following in Table 5:

Table 5. Samples used for chemical extractions

(1)	Canned treated abalone	(white flesh)
(2)	Canned untreated abalone	(bluish frill)
(3)	Unprocessed abalone	(bluish frill)
(4)	Unprocessed abalone	(blue spotted)
(5)	Hemocyanin	
(6)	Coagulated Hemocyanin	
(7)	Hemocyanin Sulfide complex (after H_2S treatment)	

Samples (1) to (4) were extracted using salt (1.0M KCl) and basic (0.1M NaOH) solutions. Hence, KCl (1) refers to the salt soluble extract of sample (1) and NaOH (2) is the alkali soluble fraction of sample (2). Untreated flesh samples of each flesh type were also hydrolysed. The results are expressed as a percentage of total amino acid (moles) and are summarised in Table 5.1. Further calculations of 'D' are shown (Table 5.2) for comparisons between the various samples.

Table 5.1 **Amino acid content expressed as a percentage of the total**

Sample	H'cyanin	KCl 1	KCl 2	KCl 3	KCL 4
Lys	4.75	2.24	1.37	2.32	8.16
His	4.12	0.00	1.19	2.78	1.33
Arg	4.32	5.76	5.88	5.91	5.23
Asp	12.32	9.17	8.67	8.78	11.14
Thr	5.80	2.93	2.81	2.96	5.31
Ser	5.29	5.39	5.71	5.61	4.88
Glu	10.32	12.00	11.62	11.83	16.02
Pro	5.88	8.22	8.99	7.90	5.15
Gly	7.04	30.01	32.89	29.92	7.16
Ala	6.95	9.39	9.41	9.28	8.63
Cys	0.97	0.00	0.00	0.00	Trace
Val	5.79	2.86	2.56	2.84	6.17
Met	1.52	1.58	1.14	1.20	2.24
Ileu	5.27	1.69	1.40	1.60	4.53
Leu	9.01	4.11	3.57	3.97	7.75
Tyr	4.81	1.98	1.17	1.34	2.79
Phe	5.85	2.68	1.62	1.75	3.51

Sample	H'cyanin	NaOH 1	NaOH 2	NaOH 3	NaOH 4
Lys	4.75	5.27	3.48	5.74	5.85
His	4.12	1.80	0.99	1.49	2.19
Arg	4.32	5.48	5.05	5.32	6.24
Asp	12.32	10.78	10.09	10.97	10.84
Thr	5.80	5.44	4.58	5.78	5.03
Ser	5.29	5.20	5.63	6.58	5.89
Glu	10.32	12.02	11.83	13.24	14.32
Pro	5.88	6.16	6.79	5.20	3.83
Gly	7.04	13.26	18.33	9.38	7.27
Ala	6.95	7.89	7.89	8.02	8.39
Cys	0.97	Trace	0.00	Trace	Trace
Val	5.79	6.03	4.81	5.97	5.55
Met	1.52	2.23	2.03	2.41	2.58
Ileu	5.27	4.61	3.54	4.72	4.82
Leu	9.01	6.99	6.03	7.78	8.99
Tyr	4.81	3.03	3.71	3.34	3.53
Phe	5.85	3.82	5.22	4.07	4.69

Table 5.2 Calculated 'D' values of the extracts from samples listed in Table 5.

	KCl1	KCl2	KCl3	KCl4	NaOH1	NaOH2	NaOH3	NaOH4
Hc	38.2	48.1	37.9	*4.2	3.8	9.7	*2.2	*2.3
KCl1	0.0	*0.8	*0.6	37.1	19.9	9.9	29.5	36.3
KCl2		0.0	*0.8	47.3	27.5	15.5	38.7	46.1
KCl3			0.0	37.2	20.0	10.4	29.7	36.6
KCl4				0.0	*3.8	10.6	*1.3	*1.0
NaOH1					0.0	*2.2	*1.2	*3.2
NaOH2						0.0	5.9	9.3
NaOH3							0.0	*0.7
NaOH4								0.0

* Refers to those samples that have D<5

5.4 DISCUSSION

Hydrolysed abalone flesh contained very similar amino acids between blued and white specimens as shown by the relatively low 'D' values. This is not unexpected as the blueing compound is expected to make a minor contribution to the overall amino acid proportions. However, differences become more evident between the various extracted protein fractions.

The extraction procedure produced two different groups of

proteins. The salt soluble fraction was characterised by high levels of glycine and glutamic acid while in the base soluble proteins, glycine, glutamic acid and aspartic acid predominated. The amino acid composition of N. ruber hemocyanin compared well with other molluscs reported in the literature and contained a high proportion of aspartic acid, glutamic acid and leucine. No tryptophan was found in our samples although acid hydrolysis does not yield quantitative recoveries.

Cysteine was determined to be present in hemocyanin but absent in KCl (1), (2), and (3) extracts. Trace amounts were found in KCl (4), NaOH (1), (3), and (4) which also show a remarkably similar amino acid content to hemocyanin. Since cysteine was not found in abalone flesh, it is assumed its presence relates directly to hemocyanin, which is in relatively small amounts in unextracted flesh samples. It is likely that the trace amounts of cysteine found in the latter group is due to the presence of hemocyanin in the flesh samples. Perhaps, cysteine content may be used as an estimate of hemocyanin levels in abalone samples including flesh.

The calculated 'D' values separated the extracted samples into two distinct groups. Statistical comparisons (D values) between KCl (1), (2), and (3) produced values below 5 indicating similarities in amino acid composition. The other similar group of proteins consisted of KCl (4), NaOH (1), (3), and (4) although the latter two (which represented alkali soluble extracts of blued unprocessed abalone) had the lowest 'D' value when compared to hemocyanin. Intergroup comparisons produced 'D' values of 35 to 50 indicating dissimilar amino acid composition.

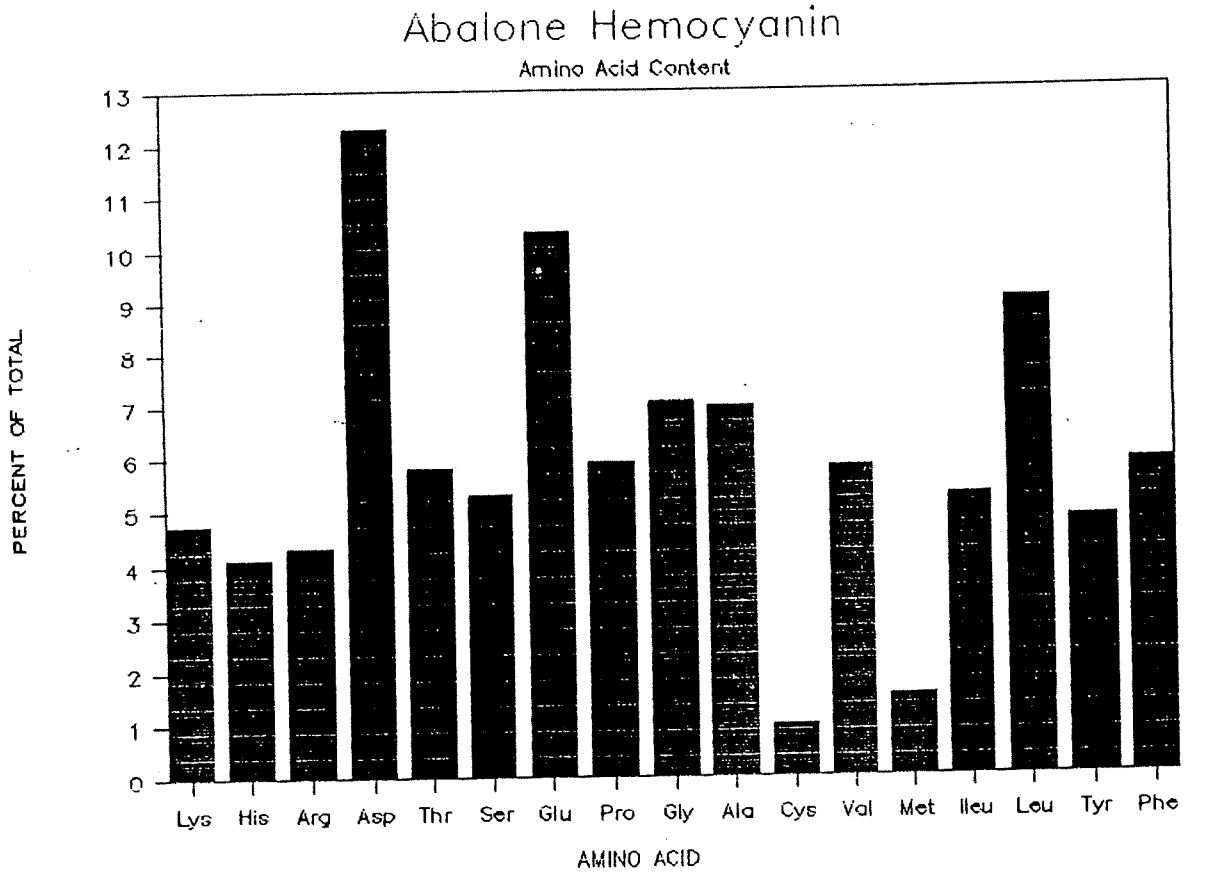
Our experiments (Section 2) have shown that heat coagulated hemocyanin is insoluble in saline solution but is soluble in aqueous alkaline solvents. Thus the results suggest that hemocyanin was extracted into the base soluble extracts. KCl (4) contained the salt soluble proteins extracted from a blue spotted unprocessed abalone (Figures 5 and 11.3). The amino acid content of KCl (4) and in particular, the presence of trace levels of cysteine suggests unusually high levels of hemocyanin in this fraction.

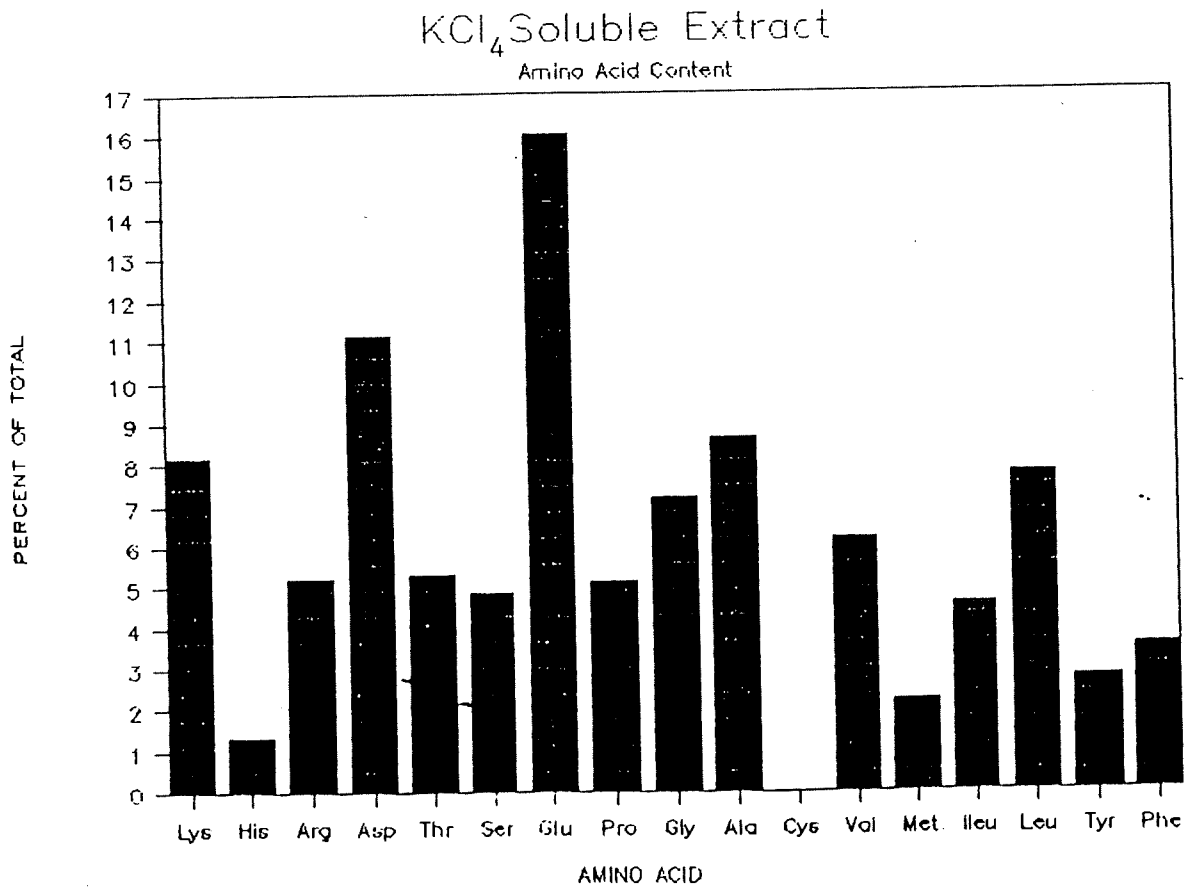
5.5 CONCLUSIONS

Amino acid content of the flesh extracts has been successfully used to demonstrate relatedness of the various extracts. The salt (KCl) extracts are differentiated from the base extracts. In relatedness one would conclude that base extracts bring out a protein component which is clearly very similar to Hc itself. In section it was shown that heat denatured Hc is soluble in base but insoluble in saline solution. The data in Table 5.2 suggests that base is extracting Hc from the abalone flesh. The KCl extracts in general did not extract from the flesh except in the case of sample (4) (Unprocessed abalone showing blue spots). The relatively low value for D in the KCl 4 extract does suggest that the base spot area is very high in hemocyanin to overcome the other extractables which would otherwise lead to high D values. Figure 5 confirms this relatedness as the histograms show a close resemblance overall.

It is interesting to note that even after processing, Hc can still be released from the flesh by base extraction.

FIGURE 5. HISTOGRAMS OF AMINO ACID CONTENT OF ABALONE HEMOCYANIN AND KCL EXTRACT.





6.0 THE COPPER CONTENT OF AUSTRALIAN N.RUBER FLESH

6.1 Introduction

Many investigators agree that copper is associated with the discolouring found in processed crab meat. From the various proposed blueing reactions, several are attributed directly or indirectly to the presence of copper. These include inorganic copper precipitates, copper catalysed oxidation of phenols (melanin formation), biuret complexes and hemocyanin compounds. A reasonable correlation was found for copper concentration and blueing intensity for Dungeness crab by Elliot and Harvey (16), Babbit et al. (3) and in King crab by Inoue and Motohiro (31). Unpublished data by Olley (52) indicated that the copper content is significantly higher in blued abalone samples. Reducing agents have also been shown to reduce discolourations which suggests that the oxidation state of copper plays an important role in the reactions.

Copper content of flesh samples were determined by Atomic Absorption Spectrophotometry (AAS) as described in Section 6.2. The results show dramatic increases from white to blued flesh (see Table 6). However, comparison between investigators is difficult because many calculate percentage copper from a wet mass of flesh which is both unreliable and unreproducable.

The extremely blued sample (c) contained a relatively high content when compared with white flesh (a) or even abalone hemolymph (d). This would suggest that the extremely blued flesh was either saturated with hemocyanin or contained copper in another form possibly another metalloprotein or as granules. The mildly discoloured sample (b) contains a proportionately lower concentration of copper. The results obtained by Inoue and Motohiro (31) and by Olley (52) also show similar increases in copper content of blued flesh.

6.2. MATERIALS AND METHODS

Copper content was determined by AAS (Atomic Absorption Spectrophotometry). Samples of flesh were freeze dried and crushed to a fine powder. An accurate mass was taken (approx. 0.1g) and the samples dissolved in Aqua Regia with heating ($3HNO_3$:1HCl, 100^oC). Heating was continued until a clear solution was obtained. The acid level was also maintained with further additions of conc. nitric acid when necessary. Copper standards of 1 to 20 ppm were prepared from copper metal (BDH) and a fresh calibration curve prepared for each determination. The copper content of the flesh samples were determined from a standard curve and calculated as a percentage of total mass.

6.3 Results

Table 6. The % copper found in a range of abalone samples

Sample	Origin	[Copper%]
(a) White	Tasmania	0.0007 to 0.001
(b) Blue	Tasmania	0.007
(c) Extremely blue	Tasmania	0.013
(d) Abalone Hemolymph	Victoria	0.026
(e) Hemocyanin	Victoria	0.035 to 0.044

6.4 CONCLUSIONS

The data in the Table clearly relate blued flesh with increased copper content. Indeed, the concentrations in blued flesh exceed the controls by x10 to x20. The more probable source of copper will be the Hc.

7.0 SULFIDE DETERMINATIONS

7.1 MATERIALS AND METHODS

The sulfide content in abalone was determined by a modification of a method by Ohashi. In this technique, the labile sulfur (predominantly organic sulfides) in a sample was quantitatively reduced to hydrogen sulfide by heating the sample to high temperatures in the presence of tin(II)-strong phosphoric acid. The evolved hydrogen sulfide gas is carried in a stream of helium gas from the reaction vessel to the absorption vessel. The hydrogen sulfide is then absorbed into a zinc acetate solution where a zinc sulfide complex is formed. The amount of sulfide extracted was determined directly by using the methylene blue method of Fogo (18) and form part of AOAC standard method of analysis for gaseous hydrogen sulfide.

7.2 RESULTS AND DISCUSSION

Inoue and Motohiro (31) suggested that the blueing found in king crab is due to a hemocyanin sulfide complex. Their results indicated that greater levels of sulfide was found in the flesh of discoloured crab meats. Examination of our results indicates that coagulated hemocyanin exhibits the greatest percentage mass of labile sulfide. The increase in sulfide content is caused by a denaturation process which opens the polypeptide chains and exposes more labile sulfide groups. Generally, a higher sulfide content was given by blued flesh when compared to white flesh but this may be due to the presence of coagulated hemocyanin rather than hemocyanin sulfide. Since sulfide is thought to coordinate through the copper active sites, its contribution to the hemocyanin sulfide complex is relatively small compared with the natural organic sulfhydryl present. This is shown by a comparison between heat coagulated hemocyanin and hemocyanin sulfide complex (also heated) which shows no significant increase in sulfide levels.

Table 7. Analyses for sulfide content of abalone flesh and various extracts of the frill.

	Sulfide content (ppm)
Flesh:	
White Frill	1.84
Blue Frill	2.46
White Foot	1.34
Blue Foot	1.62
Hemocyanin	0.63
Hemocyanin/sulfide*	3.09
Coagulated Hc.	3.46
Extracts:	
Water Sol.	
Blue Frill	2.00
White Frill	1.66
KCl Sol.	
Blue Frill	0.27
White Frill	0.15
NaOH Sol.	
Blue Frill	0.77
White Frill	2.00
Residue.	
Blue Frill	1.55
White Frill	1.72

* Obtained by H_2S treatment of a hemocyanin solution

7.3 CONCLUSIONS

The data obtained show a trend only which suggests that blued samples have a higher sulfide content. This is best illustrated by the analyses of the flesh samples themselves. In such cases it would be reasonable to expect that we are dealing with coagulated Hc. Interestingly it is coagulated (denatured) Hc which gives a higher sulfide value than is found for Hc itself. The water soluble extract is not necessarily associated with Hc (the latter, as coagulated Hc is likely to be insoluble) and hence the results from the extracts are not helpful to an interpretation.

8.0 SCANNING ELECTRON MICROSCOPY

A scanning electron microscope (SEM) is a useful tool that has the capacity for a detailed investigation of microstructure within a sample. When a specimen is illuminated with an electron beam, backscattered and secondary electrons are given off and collected to provided a 3-dimensional visual image of the sample. Electrons share similar characteristics with light but have wavelengths hundreds of times shorter. Since resolution is inversely proportional to wavelength, an electron microscope has resolving power and depth of image that is far greater than can theoretically be achieved with an optical light microscope. Apart from the obvious advantages in increased resolution, a SEM may also provide a basic semi-quantitative elemental analysis from the characteristic x-ray emission patterns.

This technique was applied to the investigation of the microstructural features of white and blued abalone flesh and also to provide an elemental compositional comparison between the samples.

8.1 MATERIALS AND METHODS

Abalone flesh were obtained from a freshly opened can purchased from Dover Fisheries. These specimens were deliberately processed without the addition of any reducing agent (MBS) to allow any natural discolourations to occur. Blued and white samples were selected from the same can which suggests that the abalone experienced identical handling and processing and were also likely to be collected from the same general area.

Transverse sections of flesh (approximately 0.1 cm thick) were cut to fit on to a 1.5cm diameter carbon stud. Each specimen included adjacent connected sections of epipodium and foot muscle rather than discrete pieces (from various positions on the animal) so that differences associated with the foot and frill muscle could

also be directly compared.

The samples were prepared by initial removal of water in a dessicator (P_2O_5, 24 hours) followed by vacuum drying (24 hours) to ensure complete dryness. The samples were secured on to the carbon stud with general purpose glue (bosh-stick) and allowed to dry (70°C, 60 min). A conductive layer of carbon was applied before analysis.

8.2 Results and discussion

The spectral output showed peaks that had positions characteristic of the elements in the sample area. Although each element can produce several peaks, the position and intensity observed allow confident assignments of the elements found. In theory, the intensity of a given peak is proportional to the concentration of a particular element in a sample. This intensity is influenced by matrix effects so that calculated corrections must usually be applied to obtain quantitative values. However, with the determinations on abalone flesh, it is assumed a constant flesh matrix exists between the samples so that the results are directly comparable.

Pilson (55) determined the proportions of ions in the abalone H. fulgens and found that chlorine concentrations were almost identical with the surroundings sea water suggesting a passive uptake. Sodium and magnesium also remained constant while potassium, calcium, and phosphate appeared to be regulated by the abalone. The results presented in Table 8 are the x-ray elemental output from the SEM which have been corrected (relative to the chlorine concentration) for direct comparisons between the samples.

The spectra show strong K shell emissions for sodium, magnesium, phosphorus, sulfur, chlorine, potassium and calcium with smaller peaks corresponding to silicon and iron detected in a few samples.

PROPORTIONS OF ELEMENTS STANDARDISED TO CHLORINE

Sample	Na	P	S	Cl	K	Mg	low	high
1:b foot overall	0.16	0.6	0.79	1	0.32	0	Na S K	
2:b foot vascularised	0.33	0.83	1.02	1	0.4	0		
3:b frill overall	0.36	1.82	1.27	1	0.67	0.39		S K
4:b frill particles	0.4	0.72	1	1	0.4	0		
5:b frill overall	0.38	2.03	1.06	1	0.5	0.53		
6:b frill fibres	0.57	2.39	1.29	1	0.64	0.68		Na P S K
7:b fril' particles	0.28	0.78	1.02	1	0.45	0		
8:b foot overall	0.15	0.98	1	1	0.44	0	Na	
9:w frill overall	0.4	0.53	0.74	1	0.29	0	K	
10:w frill fibres	0.25	0.83	1.07	1	0.43	0		
11:w frill flat region	0.5	3.45	1.1	1	0.65	0.9		Na P K
12:w frill particles	0.31	0.89	1.09	1	0.53	0		
13:w frill flat region	0.55	3.05	1.14	1	0.64	1.18		Na P S K
14:w frill particle from 13	0.23	0.58	0.8	1	0.33	0	S K	
15:w frill fibres	0.44	1.37	0.68	1	0.41	0.68	S	Na
16:w foot particles	0.21	0.67	0.77	1	0.33	0	S K	
17:w foot fibres/overall	0.26	1.26	0.96	1	0.44	0.26		
18:w foot overall 80x	0.25	0.97	0.98	1	0.42	0		
19:w frill/foot overall 80x	0.21	0.95	0.98	1	0.44	0		
20:w frill fibres 80x	0.31	0.79	1	1	0.43	0		
21:b foot overall 80x	0.4	1.4	0.98	1	0.42	0.33		
22:b frill overall 80x	0.22	0.75	1	1	0.52	0		
23:b foot/frill overall 80x	0.22	0.73	0.89	1	0.35	0		
24:b foot overall/fibres								
Average	0.321	1.233	0.984	1	0.454			
Std. Dev	0.116	0.781	0.15	0	0.107			

Table 8

PART III. STUDIES OF BLUED ABALONE FLESH AND SIMULATION EXPERIMENTS

9.0 THE HEMOCYANIN SULFIDE COMPLEX

9.1 Introduction

As discussed in Section 1.4 extensive studies on the blueing of crab meat have shown that a reaction between hemocyanin and sulfide produces a stable 'blueish' protein complex. Inoue and Motohiro (31 - 36) published a series of papers that investigated the blueing discolouration of canned crabmeat and concluded that the hemocyanin sulfide complex was responsible for the following reasons:

(i) There was an observable increase in both copper and sulfur content of blued crabmeat.

(ii) Hemocyanin was detected in blued meat but was absent in white meat.

(iii) Reflectance UV/Vis spectra of the hemocyanin sulfide complex was similar to the blue meat.

(iv) Isolation of the blueing agent by proteolysis produced similar crystals to the hemocyanin sulfide complex.

(v) The hemocyanin sulfide complex forms under similar conditions to those encountered during the canning of crab meat.

It is not known whether the blueing discolouration of Australian abalone is a hemocyanin sulfide complex, but some evidence suggests that this is possible. Johnson and Vickery (42) measured the evolution of hydrogen sulfide during the

heating of meat as a function of time and pH. Up to 50 µg of hydrogen sulfide per minute (per 100 g of meat) was released in the first 4 minutes of heating. Hydrogen sulfide production was also shown to increase with pH reaching a maximum at pH 11.0. Gruenwedel and Patnaik (27) measured the release of hydrogen sulfide from sulfur containing amino acids and found that the reaction was catalysed by the presence of such cations as Al(III), Fe(III), and Fe(II) (in order of effectiveness). The presence of Fe (from anodic dissolution of defective cans) and sulfides was thought to be responsible for the darkening of the dead space found in some cans and may also cause the observed discolourations in abalone.

This chapter investigates the formation of the abalone hemocyanin sulfide complex in an attempt to determine its involvement in the blueing problem.

9.2.0 METHODS

9.2.1 Preparation of hemocyanin sulfide

Abalone hemocyanin was isolated as described in Section 2. 3 mLs of hemocyanin solution (0.5g/5mL) were prepared and separated into three tubes. Hydrogen sulfide was bubbled for 30s through the first tube. To the second test tube, a basic solution of sodium sulfide was added. The third test tube contained only hemocyanin and represented the 'control'. All test tubes were heated to 100^{o}C for 15 minutes and cooled. The 'control' was then treated with hydrogen sulfide (similar to test tube (1)) and heated to 100^{o}C for a further 15 minutes.

9.2.2 Solubility and stability of hemocyanin sulfide

The solubility and stability of the hemocyanin sulfide complex was examined with the following solvents:

i) Sodium hydroxide (0.1M)

ii) Urea (8.0M)

iii) Sodium dodecyl sulfate (1.0%)

9.2.3 Comparison of a model copper sulfide system to hemocyanin sulfide complex

Aqueous solutions of copper (II) nitrate were reacted with a hydrogen sulfide solution. The test was performed with and without the presence of a reducing agent such as metabisulfite (MBS). Hemocyanin solutions as prepared in 9.2.1 were also reacted instead of the copper (II) nitrate and the results compared.

9.2.4 Sulfide induced blueing in Victorian abalone

Abalone specimens were collected from Flinders and washed thoroughly in distilled water. The animals were left overnight in the cold room (4^{o}C) and processed the following day. The abalones was shucked and the viscera removed by manual scrubbing. The cleaned meat was used for the following experiments. One specimen was divided into two portions, one of which was subjected to the standard industrial canning procedure. The second sample was placed into a solution containing a high concentration of hydrogen sulfide. This sample was removed the following day and processed identically to the previous half. Both samples were stored (4^{o}C) for extended observations.

9.2.5 Ultraviolet/visible spectrophotometry

Hemocyanin sulfide was prepared by the reaction of hydrogen sulfide and hemocyanin (Section 9.2.2). The product was centrifuged and washed with several changes of distilled water to remove excess sulfides and other soluble contaminants. The isolated hemocyanin sulfide was treated with basic solution (0.10M NaOH) and

centrifuged after 30 mins.. The residue was further extracted with base and centrifuged after 15 hours of storage. Both alkali extracts were extensively dialysed against Tris-HCl buffer (0.05M, pH 8.2) and the UV/Visible spectra determined on a Varian SuperScan 3. Hemocyanin was heat coagulated by heating in solution (distilled water) to 100^{o}C. The white precipitate was centrifuged and washed with several changes of distilled water. The protein was dissolved in basic solution (0.10M NaOH), dialysed against Tris-HCl buffer and the UV/Visible spectrum recorded for comparison.

9.3.0 RESULTS AND DISCUSSION

9.3.1 Preparation of hemocyanin sulfide

The formation of the hemocyanin sulfide complex has been suggested as the cause of blueing discolourations in canned crab meat (33). A similar coloured complex was produced when a solution of abalone hemocyanin and sulfide is heated to 100^{o}C.

Very little literature is available concerning the reaction mechanism but evidence suggests that it proceeds in two distinct steps during the heating process. The first is the initial coagulation of hemocyanin at 77^{o}C (a white powdery precipitate) followed by reaction/complexation with sulfide at 100^{o}C to form the green product. The formation of the blue/green precipitate occurs even for denatured hemocyanin (see results in Table 9.1) which suggest that the reaction proceeds via the coagulated intermediate. This observation is relevant to abalone canning as it indicates that even denatured hemocyanin may react with sulfides during the canning process.

A basic solution of sulfide was used in a second test in place of hydrogen sulfide, to provide sulfide anions. The test produced a clear solution when heated to 100^{o}C but the coloured complex formed on neutralization. Similar results were obtained by Inoue and Motohiro (31) for arthropod hemocyanin.

Table 9.1. Solubility characteristics of hemocyanin samples

		Solvent Systems		
Sample		**Urea**	**SDS**	**NaOH**
Hemocyanin	(Wet)	vs	vs	vs
Hemocyanin sulfide	(F.D)	i	i	vs
Coagulated Hc.	(F.D)	i	i	vs

SDS - Sodium Dodecyl Sulfate (0.1% soln.)
NaOH - Sodium Hydroxide (0.1M) with overnight storage
Urea - 9M solution
F.D - Samples lyophilised (freeze dried)

9.3.2 Solubility and stability of hemocyanin sulfide

The solubility characteristics for hemocyanin sulfide has not been reported in the literature. For chromatographic analysis, the solubility of the hemocyanin sulfide complex was determined in some common protein solvent systems (see Table 9.1). Urea and sodium dodecyl sulfate (SDS) are popular solvents for proteins but showed little solvency effects towards hemocyanin sulfide. Inoue and Motohiro reported the presence of hemocyanin in basic extracts of crab meat. The sulfide complex was also found to be soluble in 0.10M sodium hydroxide after overnight storage. Stronger solutions of NaOH (up to 1.0M) solubilised the sulfide complex in a much shorter time but a slower process would minimise any modification to the protein structure. In contrast heat coagulated hemocyanin was shown to be soluble in sodium hydroxide (0.10M) after only 30 minutes. The blue/green protein complex retained its colour in solution indicating that the chromophore is still intact under these basic conditions.

9.3.3 Comparison of a model copper sulfide system to hemocyanin complex

Inoue and Motohiro (33,36) suggested that the complex formed between hemocyanin and sulfide had the general formula Cu-S-Cu-R or R-Cu-S^-, where R represented a protein molecule. This formula suggests that the chemistry of copper and sulfur are intimately involved in the reaction. Evidence from the reaction of copperless apo-hemocyanin (prepared in Section 2.3.8) with sulfide produced no blue precipitate suggesting the involvement of the copper site in the formation of the protein/chromophore.

The solubility product for copper (I) and (II) sulfides are 1.28×10^{-36}, 2.24×10^{-48} (SI Data, 1984) respectively and can be considered very high. However, the addition of MBS before the reaction with sulfide for both copper and hemocyanin prevents the formation of the coloured precipitate (see Table 9.2) but does not reverse the reaction of either (CuS or HcS) once the product has formed.

Table 9.2 Comparision of Hc and Cu^{2+} reactions with sulfide ions.

$Cu + S^{2-}$	CuS (s)
$Cu + S^{2-}$ + MBS	No precipitate
$Hc + S^{2-}$	Hc/S precipitate
$Hc + S^{2-}$ + MBS	No precipitate

The addition of MBS to canned abalone would prevent/inhibit the formation of the hemocyanin sulfide complex by removal of sulfides through a series of chemical reactions. This observation suggests that the observed blueing in canned abalone may be due to sulfides since MBS has been commercially proven to be effective in preventing such blueing discolourations.

9.3.4 Sulfide induced blueing in Victorian abalone

The abalone was cleaned and discolouration was induced in the flesh. Due to a high individual dependence reported for blueing, the abalone was divided into two portions so that the results of the test may be directly compared with the control sample.

The results of the experiments, lead to the conclusion that discolourations in the frill of abalone specimens occur in the presence of hydrogen sulfide when treated under canning conditions and in the absence of MBS. However, the colour was more intense than that generally found in untreated canned abalone. Factors that may have contributed to the colour intensity found in this specimen may be due to the following reasons:

1. A higher concentration of hydrogen sulfide was used than is encountered in production-line specimens.

2. The discolouration of canned abalone has previously been shown to be individual dependent.

The frill represents the area of respiratory exchange of gases and hence may be expected to contain the highest concentration of hemocyanin in surface membranes. The reaction with hydrogen sulfide has been observed to occur only in the frill and seems to be quite specific since other areas of flesh remained unaffected. It seems reasonable to conclude that the colouration involved hemocyanin in the frill membranes.

9.3.5 Ultraviolet/Visible spectrophotometry

The solubility of denatured hemocyanin was shown to be different to those of the sulfide complex. In basic solution, coagulated hemocyanin dissolves in a relatively short time (approx. 30 min.) while the sulfide complex requires overnight

storage. This difference in solubility was exploited to obtain a 'pure' fraction of hemocyanin sulfide by a two stage separation. Initial treatment of hemocyanin sulfide with basic solution removed unreacted coagulated hemocyanin and a second extraction produced the green protein solution of hemocyanin sulfide.

The UV/Visible spectra of coagulated and sulfide treated hemocyanin is illustrated in Figure 9.1. The spectra were originally plotted in absorbance units but have been replotted as percent transmittance to provide a better comparison with the diffused reflectance spectra obtained by Inoue and Motohiro (34). The maximum transmittance was at 600 - 610 nm. compared with 530 - 580 nm. reported for crab hemocyanin sulfide. However, examination of the overall appearance of both hemocyanin sulfide spectra indicate similarities that may imply comparable chromophoric sites (Fig. 9.2).

Treatment of blued flesh with 0.10 M sodium hydroxide and subsequent plotting of the UV/Vis absorbance did not produce any observable maximum at 600 - 610 nm. (Fig.9.1). The spectra produced resembled that of coagulated hemocyanin which suggests a greater influence from this form of the protein. However, the solution was also likely to contain other proteins (solubilised during the extraction) that may interfere or alter the overall characteristics of the spectrum.

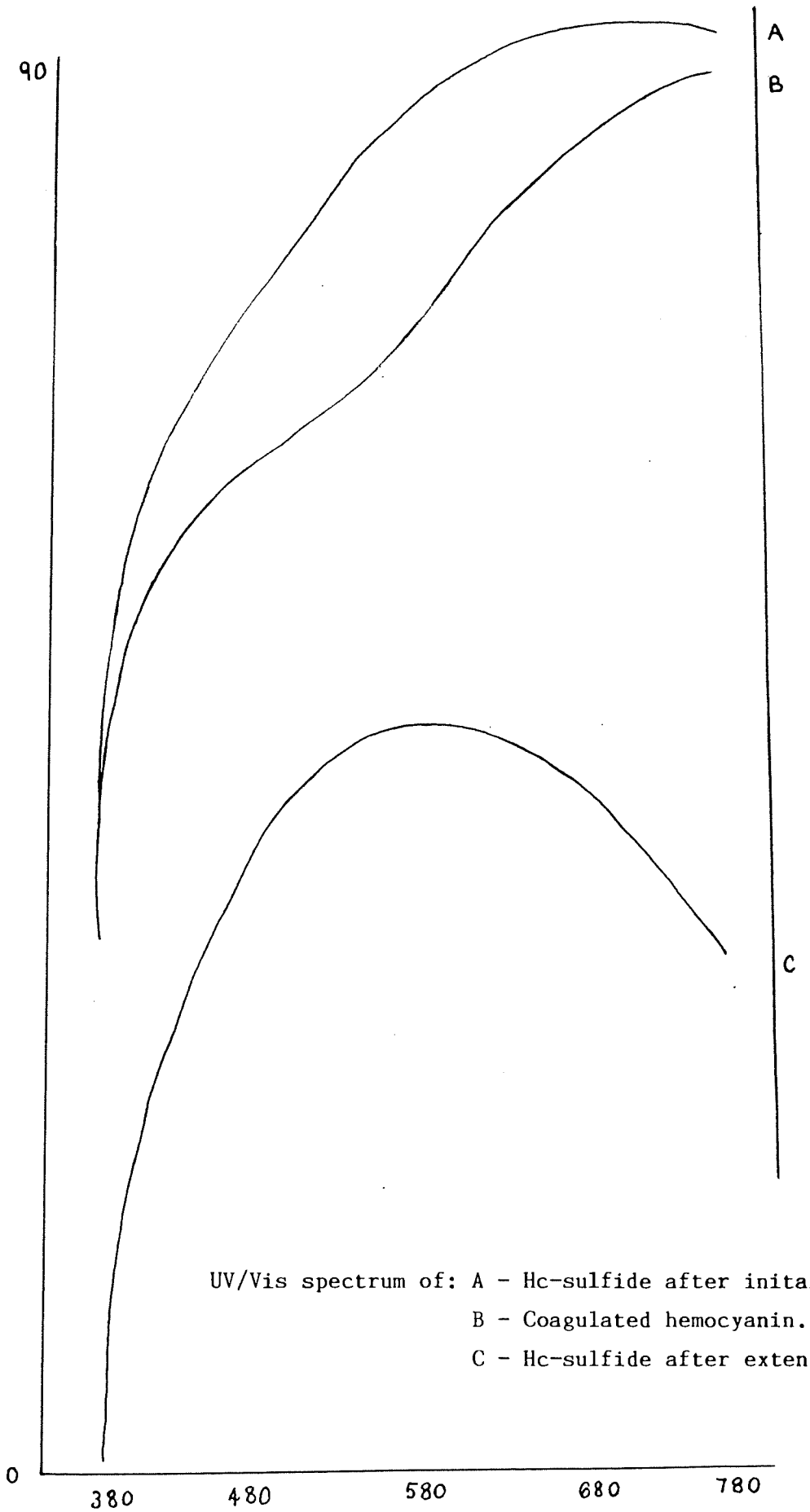
9.4 CONCLUSIONS

The results recorded in this Section allow us to put into perspective much of the inferences which can be drawn from earlier Sections.

In Part II it became clear that Hc and Cu were involved in all cases of blueing. One potential cause is reaction with released sulfide anion or sulfhydryl groups on the protein of the flesh. To model this reaction and particularly to test solubilities and stabilities to heat and reducing agents such as

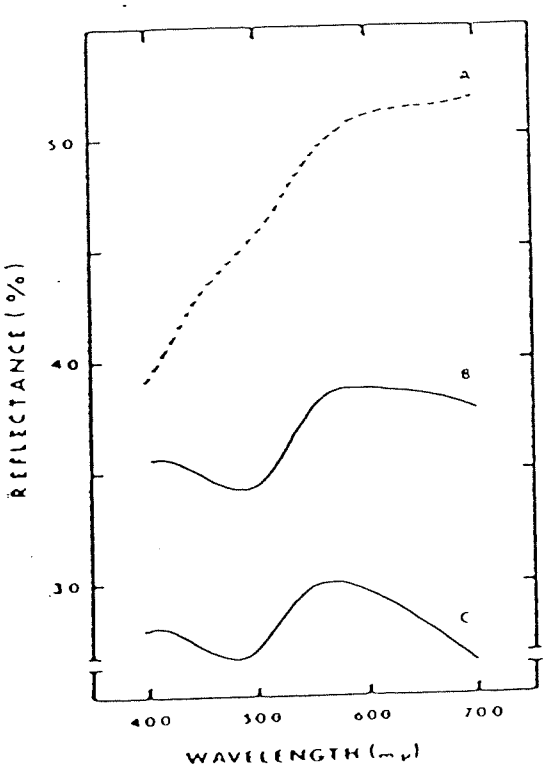
MBS, Hc was reacted with a solution of H_2S. A blue/green complex precipitated out at acidic pH. This precipitate was soluble in base and stable in basic solution (overnight was the most extended test). Apo-Hc produced no colouration or precipitate when treated with sulfide. This conclusively involves the Hc copper in the formation of the complex. More interestingly, if the reaction is carried out in the presence of MBS no precipitate forms. Once the complex has been formed, however, MBS does not reverse its formation. On this basis, MBS during canning is likely to be effective only if the blueing has not already occurred. The complex is soluble in basic solution but not in reagents which conventually solubilise proteins. Basic extraction of the blued flesh did not extract out the coloured complex. The UV/Vis spectrum of the extract resembled coagulated Hc most closely but other components were likely to be present. The important point to take is that the blueing agent was not extractable from the flesh. Given that Hc has been strongly implicated in the formation of the complex this result suggests that the Hc-complex is strongly held by the proteins in the flesh.

Related to the last of these inferences is the observation that a sulfide wash blues the frill of the abalone **but not the flesh in the foot.** Whilst this result is understandable in terms of Hc being readily accessible through the membraneous tissues of the frill, it does not explain other observations we have made that the blueing can occur throughout the flesh of the epipodium. Some additional interactions must be occurring.

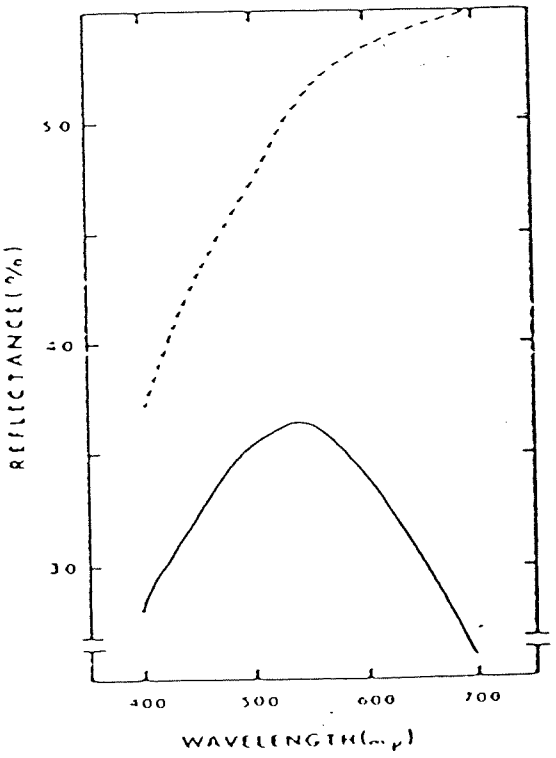


UV/Vis spectrum of: A - Hc-sulfide after inital extraction.
B - Coagulated hemocyanin.
C - Hc-sulfide after extended extraction.

FIGURE 9.1 A COMPARISON OF THE VISIBLE SPECTRA FROM RELATED HEMOCYANIN SAMPLES



Reflectance spectra of the normal meat(A), slightly coloured blue meat(B) and stronghly coloured blue meat(C) in canned king crab.



Reflectance spectra of the heat coagulated hemocyanin and hemocyanin sulfide complex.
----- Heat coagulated hemocyanin
——— Hemocyanin sulfide complex

FIGURE 9.2 A COMPARISON OF REFLECTANCE VISIBLE SPECTRA OF CRAB MEAT AND ABALONE HEMOCYANIN SAMPLES

10.0 PHENOLASE ACTIVITY IN N.RUBER

10.1 Introduction

Tyrosinases are copper containing enzymes that catalyse the o-hydroxylation of monophenols and oxidation of diphenols to melanins and other polyphenolic compounds. They are widely distributed in both plants and animals and are responsible for the dark surface pigments found in abalone viscera. They are physically and chemically similar to the hemocyanins exhibiting reversible oxygen binding characteristics from a dimeric copper pair. Huber and Leach (30) compared two tyrosinases with several hemocyanins and noted spectral and physical similarities. The amino acid ligands around the copper binding sites of S. glaucescens tyrosinase showed remarkable similarities to those of E. californicum hemocyanin. It is suspected that many hemocyanins may also possess phenolase activity which may be related to the blueing problem.

Babbit et al. (3) postulated that the blueing of Cancer magister is due to the oxidation of phenols to melanins. It was shown that the phenol oxidation reaction is catalysed by the presence of ionic copper and is optimal at pH 8.0. Phenols such as dopa and tyrosine were tentatively identified by TLC (3) and the blood shown to exhibit phenolase activity. However, Groninger and Dassow (26) studied blueing in the king crab, P. camtschatica, and decided that polyphenols were not the cause of the discolouration because of the absence of both phenols and phenolase activity in the blood.

It is not known whether the discolourations found in abalone are caused by oxidation of phenols but some basic observations indicates that this is possible. Abalone already contain dark surface pigments that are formed from naturally occurring 'melanin-like' oxidation reactions. The copper catalysed (non-enzymic) oxidation of dopa has an optimum pH of 8.0 and

is inhibited by acidic conditions. Similarly, abalone are canned in a phosphate 'soup' that buffers the pH at around 8.9. The addition of reducing agents prevents the formation of polyphenols (3) which is also consistent with observations on the blueing of abalone.

10.2.0 MATERIALS AND METHODS

10.2.1 Qualitative determination of phenolase activity in *N. ruber* hemocyanin

Abalone blood was previously isolated and stored as lyophilised protein, 20mg was dissolved in NaCl (5%, 1mL) solution. Tyrosine (5mg) was added and dissolved with gentle agitation and heated to 55^{o}C for 30 mins. The temperature was then raised quickly and maintained at 100^{o}C for a further 30 mins. This procedure was repeated with the addition of DL-dopa (10mg) instead of tyrosine. A metabisulfite (MBS) solution (5%) was added to the dopa/hemolymph reaction to determine the effect of a reducing agent on melanin formation. A 'blank' was also performed without abalone hemolymph to observe the effects and contribution of atmospheric oxidation and also to provide a better basis for comparison.

10.2.2 Gel permeation chromatography (GPC)

Hemolymph (500mg) was reacted with dopa (100mg) at 100^{o}C in a procedure similar to that outlined above. The resultant solution was isolated by centrifuging and the residue retained for further analysis. The solution was separated on Sephacryl S-300 (Pharmacia) which had been previously equilibrated with 0.05M Tris-HCl (pH 7.0 I=0.01). 5ml fractions were collected and the elution profile determined by UV absorption at 280nm.

10.2.3 Extraction of phenols from abalone

Blued abalone frill was removed from a sample of canned abalone. Trichloroacetic acid (TCA) solution (5%, 100mL) was added to abalone flesh (20g) and macerated. The solution was left overnight at 4^{o}C and centrifuged the following day (8,000 rpm, 45min) to remove any solid material.

10.2.4 Isolation of phenols

Phenols extracted by TCA (outlined above) were separated on activated alumina following the method used by Drell (14). This involves the absorption of phenols on activated aluminim hydroxide (BDH). The various classes of phenols were then selectively desorbed by buffers of decreasing pH. Alumina (2g) was packed into a suitable column and equilibrated by acetone (20mL) followed by distilled water (20mL) and buffer A (20mL). TCA extract (10mL) was diluted with buffer B (8mL) and applied to the column followed by buffer B (20mL) wash solution. Distilled water (30mL) and buffer C (10mL) were passed through the column and catechol amines collected. The remaining phenolic compounds were eluted with solution D (0.1M, 40mL). The composition of buffers A, B, C and solution D are shown below:

Buffer A - 0.2M NH_4Ac pH 6.1
Buffer B - 0.5M NH_4Ac pH 6.1
Buffer C - 0.01M NH_4Ac pH 4.0
Solution D - 0.1M HCl

All buffers were made from analar reagent (AR) grade chemical and adjusted to the required pH with glacial acetic acid.

Initial determinations of dopa by the above procedure were used to calculate percentage recoveries. Dopa concentrations were estimated by UV absorption at 280 and 218nm and determined by reference to a freshly prepared standard curve.

10.2.5 Supply of abalone for the quantitation of phenolase activity in hemolymph and flesh

Ocean and black lip abalone were supplied by Australian Abalone Exports (Laverton, Vic.). The abalone were kept alive in an oxygenated tank until collection. Just prior to transportation to the University of Melbourne the abalone were packed foam esky lined with hesshen that had been soaked in sea water Transport was by van at ambient temperature and undertaken within 45 minutes.

10.2.6 Phenolase quantitation of abalone hemolymph and flesh

L-proline (10mM) and catechol (10mM) solutions were prepared daily in phosphate buffer (67mM, pH=6.4). 25.00mL of each solution was mixed in a 50.00mL standard flask. Abalone hemolymph/flesh extract (1mL) was immediately added to the L-proline/catechol solution and mixed thoroughly. This mixture (3mL) was transferred to a 1 cm light path cuvette. Absorbance values were determined using a Varian Superscan 3 spectrophotometer at 520nm against a reagent blank every five minutes over a period of forty minutes.

A standard curve was constructed to allow the quantitation of phenolase activity. Abalone blood was isolated as lyophylised protein and stored in a freeze dried form. Hemocyanin (20mg) was mixed with L-proline/catechol solution (0.0005-0.0025M, 5.00mL), allowed to react for one hour then analysed as above against corresponding blanks.

The phenolase activities of bay and ocean black lip abalone hemolymph were determined using a spectrophotometric method in which catechol was used as a substate for determining changes in enzymic activity. o-Benzoquinone formed from catechol reacted with proline to form 1,2-benzoquinone-4-pyrrolidine-carboxylic acid with an absorption maximum at 520nm.

It was determined that 8.97×10^{-6} and 2.99×10^{-6} umoles of

catechol reacted over the fifteen minute interval for bay and ocean black lip hemolymph respectively. The change in absorbance during the 15-minute interval, between 15 and 30 minutes after mixing was multiplied by 0.0013 to determine the micromoles of catechol oxidised during this time interval. The plots for these measurements are shown in Figure 10.1 (ocean samples of N.ruber) and Figure 10.2 (bay samples of N. ruber). Figure 10.3 is a calibration curve.

Tris-HCl and KCl extracts of minced white and blue flesh were prepared as outlined in Figure 11.1. When analysed neither Tris-HCl nor KCl extracts showed an observable change in absorbance at 520nm. These results would suggest that the hemocyanin was tightly bound to the flesh and was not extracted.

The Tris-HCl extract of freshly shucked, unbled abalone flesh showed minimal phenolase activity. Absorbance readings at 520 nm were of the order of six times less than that observed for bay black lip hemolymph.

10.3.0 RESULTS AND DISCUSSION

10.3.1 Qualitative determination of phenolase activity in N. ruber hemocyanin

The discolouration of dungeness crab (Cancer magister) was thought to be caused by the oxidation of phenolic compounds to dark melanin-like pigments (3). This reaction may be initiated by phenolase present in the hemolymph but at higher temperatures experienced during canning, the oxidation may proceed non-enzymically in the presence of copper and ferric cations and at alkaline pH.

Preliminary testing on abalone hemolymph indicated similar reactions to those observed for C. magister by Babbitt et al. However, no mono-phenolase (tyrosinase) activity was apparent

under the conditions tested (see Table 10). Perhaps the monophenolases if present are at low concentrations as reported by Summers (64) for hemolymph of the fiddler crab, U. pugnax. Since tyrosine is also present in the blood (see amino acid analysis - Section 5.0) the coexistence of both enzyme and substrate can only occur if the concentration of tyrosinase is extremely low and reducing substances are present in the blood .

Dopa (3,4-dihydroxyphenyl alanine), on the other hand produced severe discolourations in the presence of abalone hemolymph when heated to 50°C for 1 hour. Further heating to 100°C produced a light greyish/blue precipitate on cooling. The solution exhibited enhanced blueing after storage overnight at 4°C indicating that further oxidation reactions are occurring. When the sample was heated again to 100°C, loss of dark bluish colour occurred leaving a light greyish/blue suspension. This suspension was centrifuged producing a green solution and a greyish/black precipitate. These results are summarized in Table 10.

Table 10. Colour changes observed in phenolaes activity of abalone hemolymph using Dopa as substrate.

	Sample		
Treatment	Blood NaCl(10%) DL-Dopa	Blood NaCl(10%) DL-Dopa MBS(1%)	Control NaCl(10%) DL-Dopa
50°C. 60min.	Slightly darker	No Change	No Change
2.5mLs left for observations	Blue/black tint	Colorless	Colorless

Sequential Treatments

100°C 60min.	Bluish/ grey color	Colorless	Colorless Slight cloudiness
Cooled 1 hour	Grey color	Colorless	Clear Dark tint
24 Hours later	Very dark blue/grey	Colorless slight cloudiness	Clear Dark tint

10.3.2 Chromatography

Gel permeation chromatography (GPC) of the blue/green solution produced a major peak at the exclusion limit of Sephacryl S-300 (Pharmacia). Some low molecular weight material was present with a similar retention time to those previously found in abalone blood. Dark melanin-like pigments were still produced from the hemocyanin fraction after 2 days storage indicating that dopa is present and may have been weakly coordinated to the hemocyanin and further oxidation reactions are occurring. In contrast, natural hemocyanin heated above 77°C denatured to produce a white precipitate that could not be chromatographed.

10.3.3 The Hemocyanin/dopa complex

In the presence of dopa, some hemocyanin heated to 100°C remains soluble producing a blue/green complex. This complex (Dopa/Hc) exhibits unique oxidative/reductive properties towards reducing agents such as MBS. Exposure to a solution of MBS results in a lessening of colour similar to the effects of heat treatment at 100°C (Table 10).

10.3.4 Determination of phenols in abalone

Phenols were extracted from abalone flesh with Trichloro-acetic acid (TCA) and were isolated by a method of Drell (14). This involves the absorption of catechol derivatives on active aluminium hydroxide and selective desorption by various buffers of decreasing pH. The phenols are tightly bound to the alumina in their ionic form but the binding is totally reversed by acid. Drell (14) reports that phosphates are potential competitors for binding sites but this problem was overcome by the use of larger columns that had a sufficient capacity to bind both phenols and any residual phosphate anions from solution. Initial determinations using known amounts of dopa indicated a recovery of 82% . A sample of flesh extract was processed following the above procedure and repeated with the addition of a known amount of dopa followed by passing the solution immediately through the same column. Results indicated that the alumina media was not saturated as 79% of dopa was recovered from the second determination.

10.3.5 Ultraviolet spectrophotometry

The UV spectra of the extracts indicated successful separation of the phenolic compounds. The catechol amine fraction of canned abalone extracts showed broad absorption bands at 258 and 355 nm (Figs.10.4 - 10.7) while the brined blue sample (Fig.10.5) produced a lower but sharper peak at 247 nm with a skewed profile caused by absorption at 258 nm. There seems to be a difference in the types of catechol amines found in extracts of brined and canned abalone flesh (Figs.10.5 and 10.7) which may be attributed to the canning process (e.g. differences in E values). In contrast the second set of extracts from the flesh samples (Figs.10.9, 10.10) produced similar absorption profiles for all samples and are 'typical' of phenolic compounds. Bands at 207 and 260 nm are clearly defined (eg. Fig.10.5 and 10.9) but comparison with the spectrum of dopa (Fig.10.4)indicates clear

differences. Further attempts to characterise and identify these phenolic compounds have failed. Adaptations of GC and HPLC methods for the identification of phenolic biomolecules in plants were tried (54) but produced no data possibly due to the complexities and size of these compounds.

10.4 CONCLUSIONS

There are few reports in the literature which have shown that hemocyanins can have phenolase activity. Probabily the most directly related work was the blueing of Cancer magister. There, it was shown that phenol oxidation was catalysed by the presence of ionic copper, was optimal at a pH 8.0 and was inhibited by acidic conditions. In the canning of abalone, the phosphate buffer 'soup' is about pH 8.0. In our test experiments not one showed any development of colour when abalone hemolymph and Dopa were reacted together in the presence of metabisulfite (MBS). These observations accord with experience in the canning process.

Abalone hemolymph was found to have no tyrosinase activity which is in accord with the fact that it is unlikely that a monohydroxy phenolase would co exist with the tyrosine substrate present in the Hc. When we tested for dihydroxy phenolase activity using Dopa, a strong colour developed in the solution. In quantitative measurements, hemolymph from bay abalone was more reactive by a factor of 3 than hemolymph from ocean abalone samples.

Flesh taken from shucked, but unbled abalone also gave a positive test for dihydroxy phenolase activity. This is in keeping with our data that : (1) Hc can be extracted from uncanned abalone flesh, particularly when the abalone has **not** been bled, (2) Hc is associated with blueing.

Experiments using Dopa and abalone Hc with heating to 100^{o}C (similar to canning treatments) led to the formation of a light grey/blue suspension. This colour is similar to that

observed throughout the epipodium of blued samples of abalone. The data in Table 10 suggest that colour production can continue after the initial reaction with Dopa despite 100°C treatment.

Extracts of precanned abalone flesh from frozen samples and other treatments showed UV absorptions samples which can be associated with 'phenolic' components. This was found not to be the case for samples after canning. Neither of these sets of UV spectra resembled that for Dopa and we must conclude that if it is present is a minor component. Colour development, however, would require only a minimal concentration level to give the deep colourations observed.

The dihydroxy phenolase activity of unbled, shucked abalone flesh was measured. Attempts to extract the blued abalone flesh by methods which were successful (Section 2 and 5) earlier did not succeed. We must conclude that the molecular complex causing the blue colouration is tightly held to the flesh.

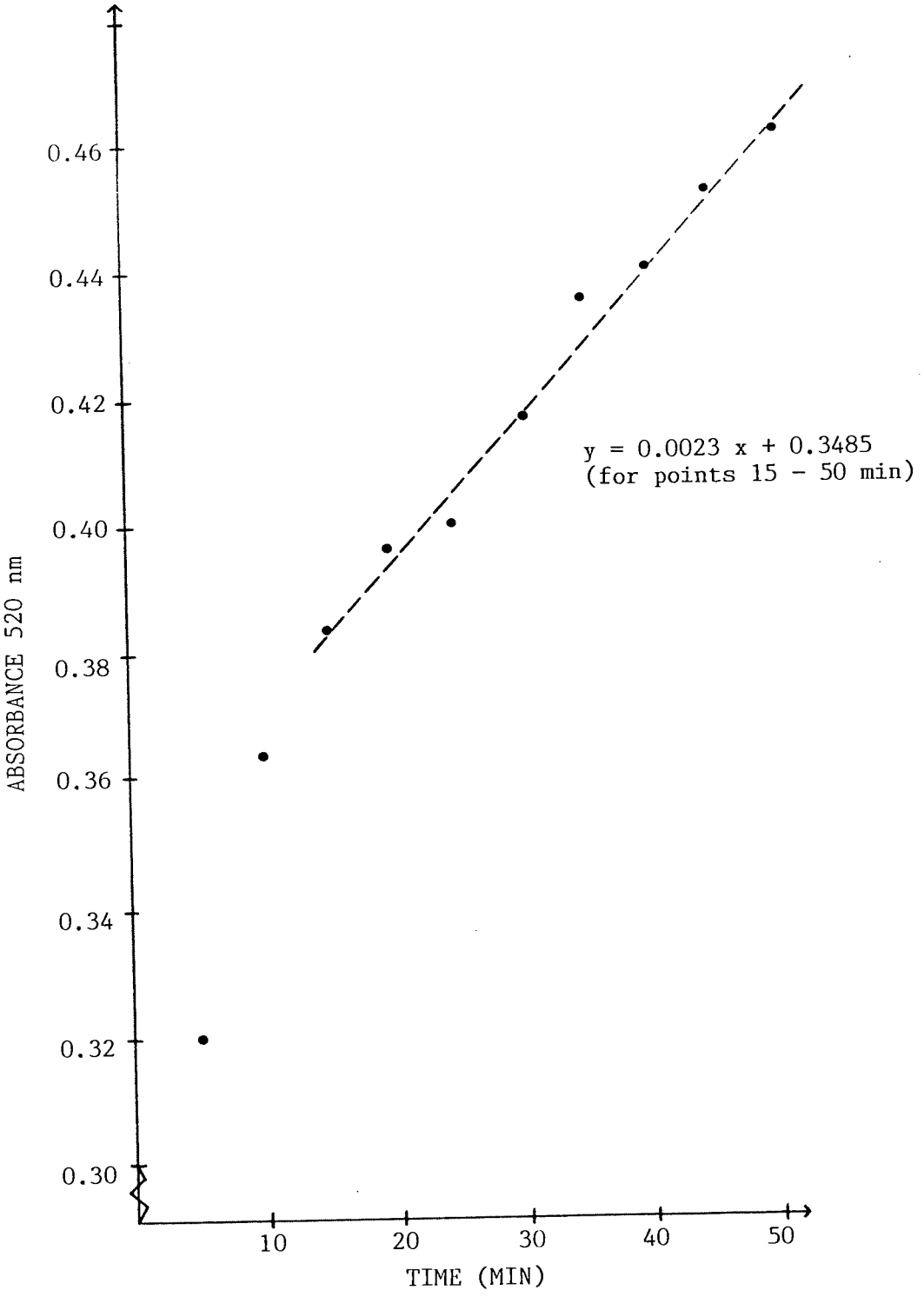


FIGURE 10.1 CATECHOL PROLINE ASSAY FOR HEMOCYANIN PHENOLASE ACTIVITY IN OCEAN BLACK LIP ABALONE BLOOD

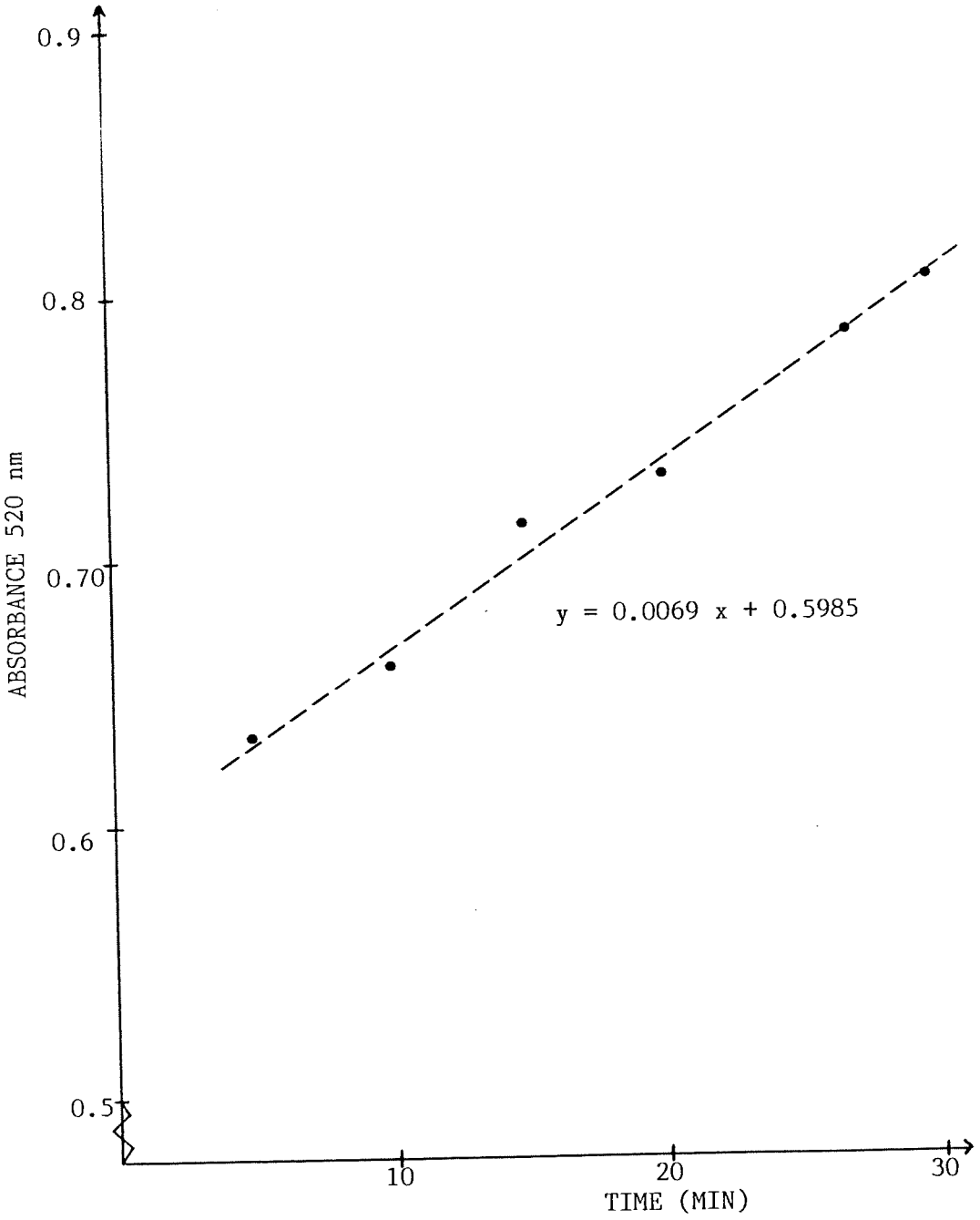


FIGURE 10.2 CATECHOL PROLINE ASSAY FOR HEMOCYANIN PHENOLASE ACTIVITY IN BAY BLACK LIP ABALONE BLOOD

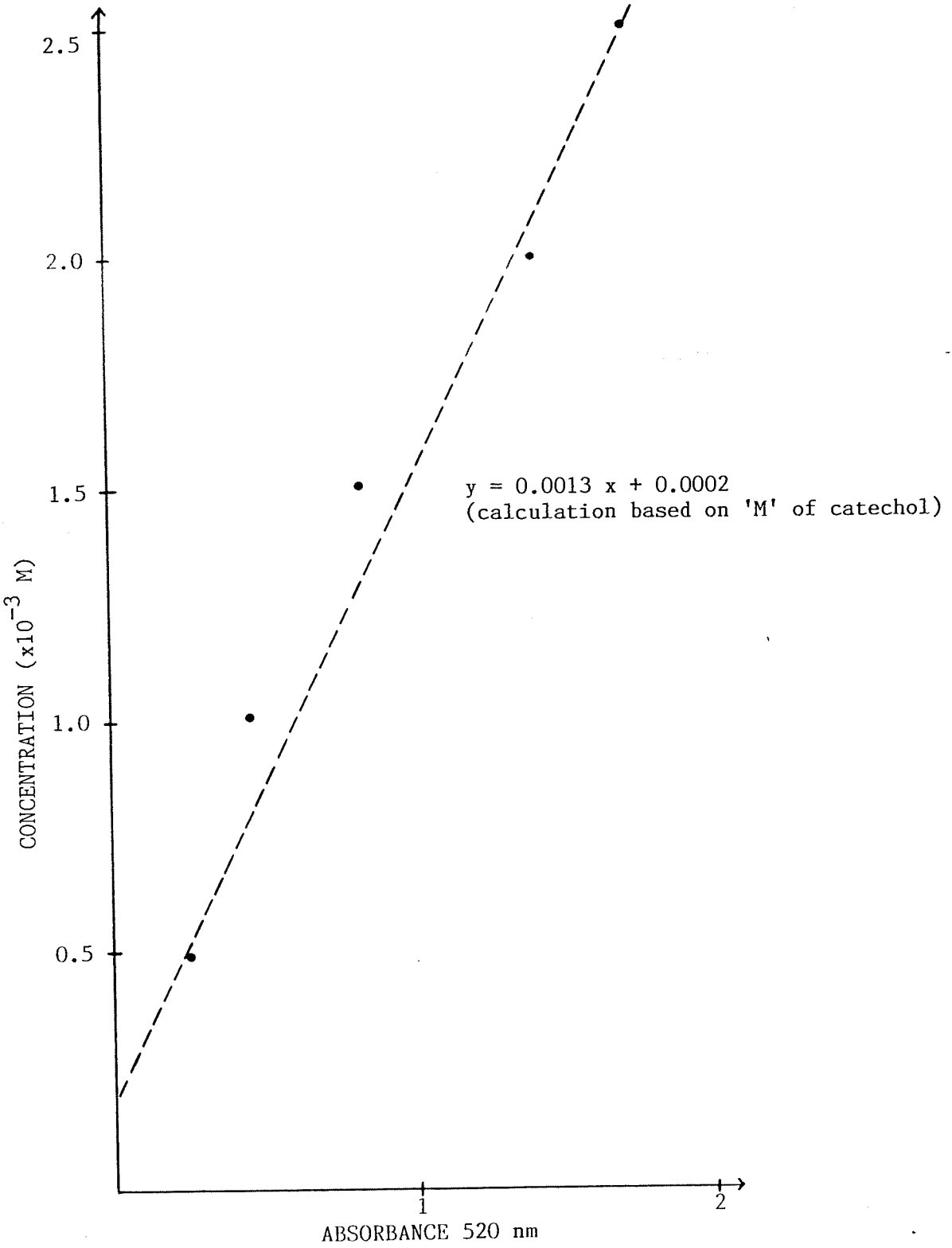


FIGURE 10.3 STANDARD CURVE FOR CATECHOL PROLINE ASSAY OF PHENOLASE ACTIVITY

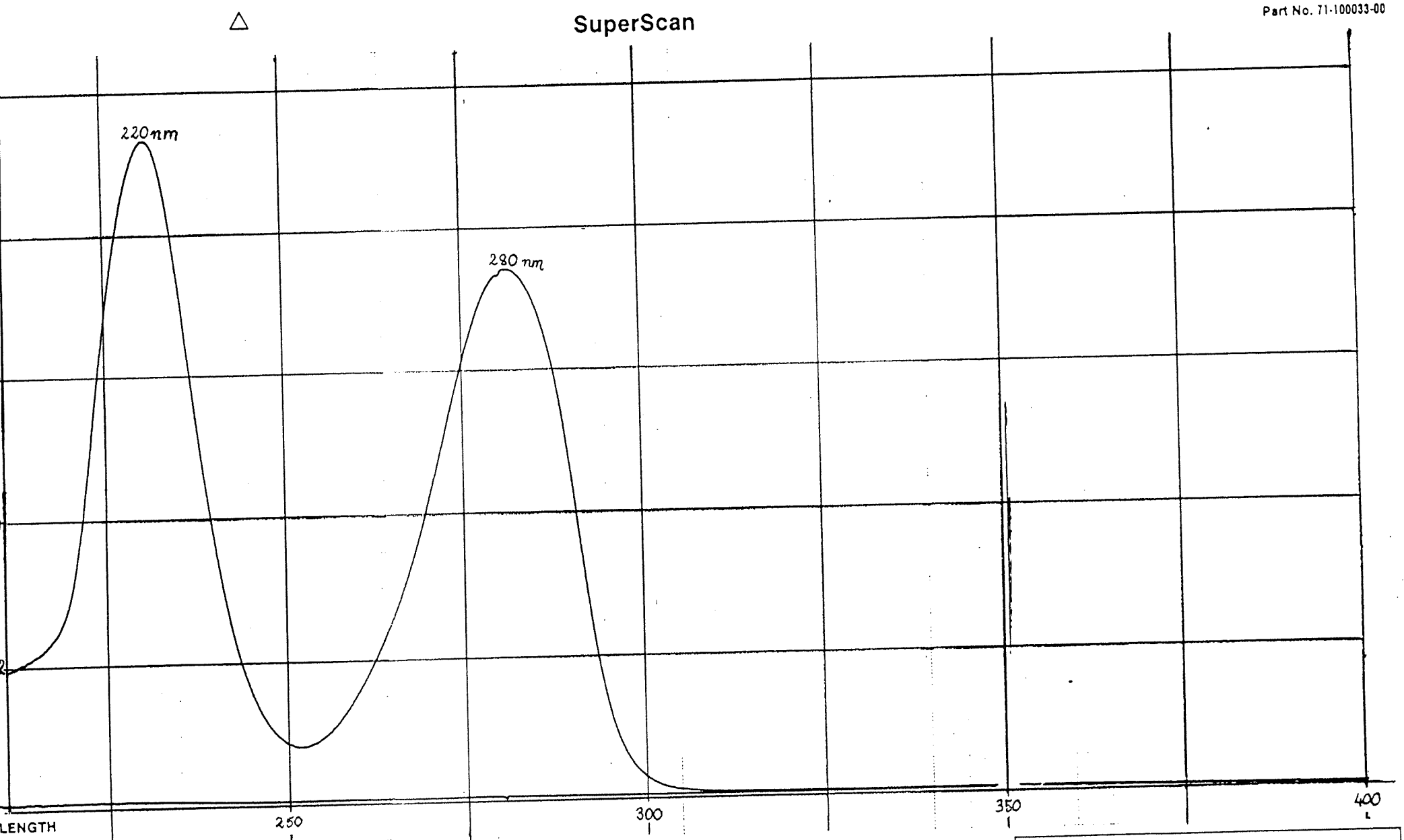


FIGURE 10.4 UV SPECTRUM OF DOPA

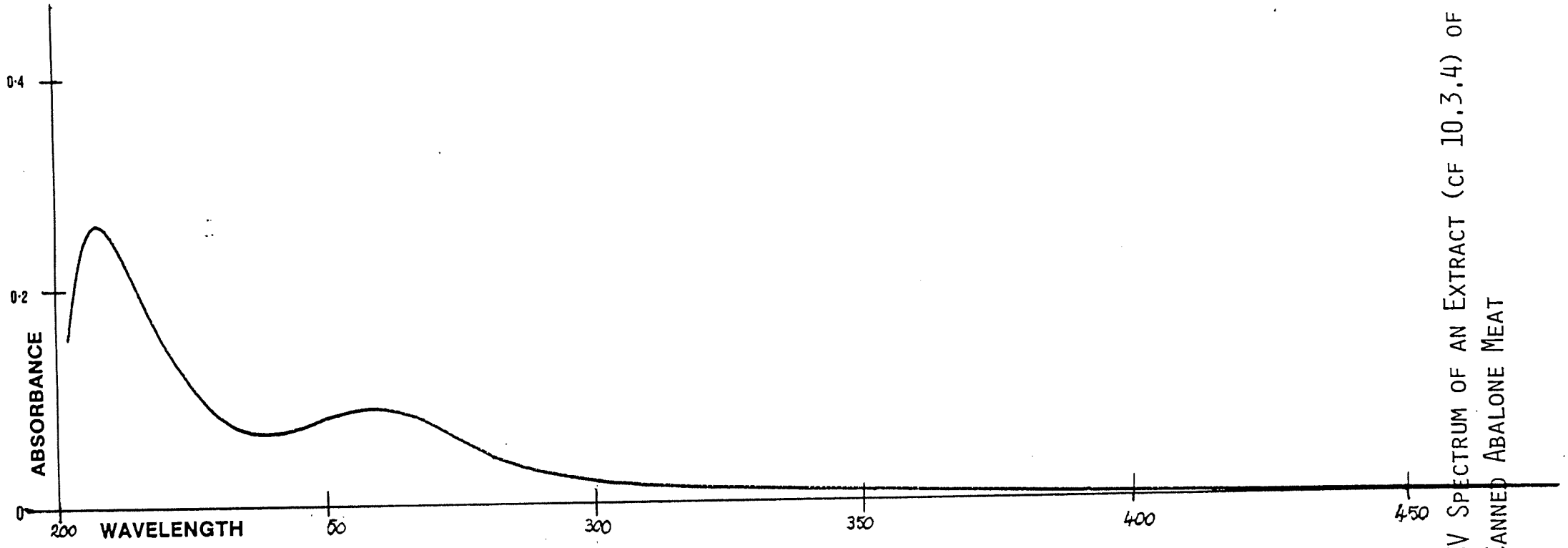


FIGURE 10.5 UV SPECTRUM OF AN EXTRACT (CF 10.3.4) OF CANNED ABALONE MEAT

WAVELENGTH PRESENTATION

SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm	(1 nm/cm)
	80 "	(2 ")
	200 "	(5 ")
	(400) "	(10 ")
	800 "	(20 ")
SCAN SPEED	10, 20, (50), 100, 200	nm/min

PHOTOMETRIC PRESENTATION

MODE	%T, (Abs) Der,	
FULL SCALE Y AXIS	0·05	Abs
	0·10	"
	0·50	"
	1·0	"
	2·0	"
	(4·0)	"
	100%	T
REC. OFFSET		Abs.

TIME PRESENTATION

UNITS	Sec,	Min,
FULL SCALE X AXIS	20	
	40	
	100	
	200	
	400	

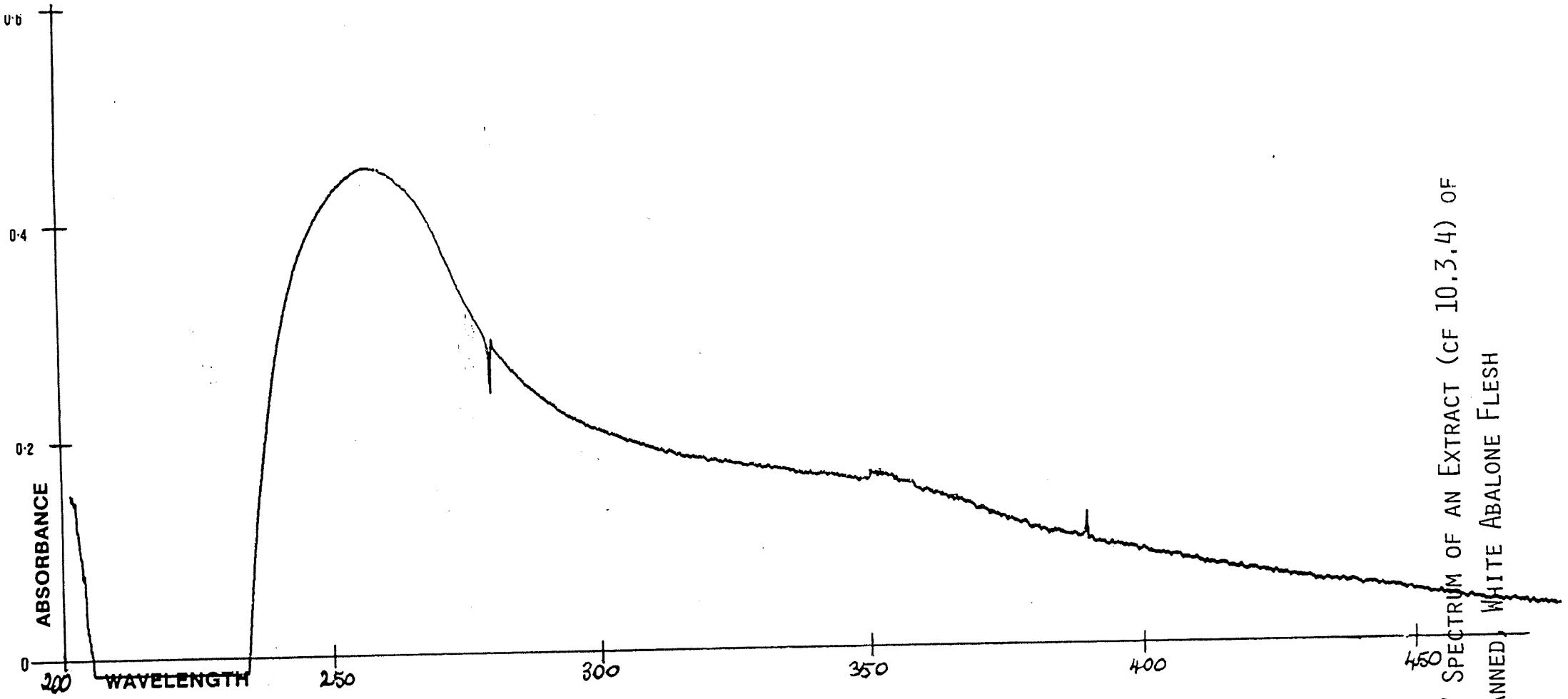


FIGURE 10.6 UV SPECTRUM OF AN EXTRACT (CF 10.3.4) OF CANNED WHITE ABALONE FLESH

WAVELENGTH PRESENTATION

SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm (1 nm/cm)	
	80 " (2 ")	
	200 " (5 ")	
	(400) " (10 ")	
	800 " (20 ")	
SCAN SPEED	10, 20, (50), 100, 200	nm/min

PHOTOMETRIC PRESENTATION

MODE	%T, (Abs), Der,	
FULL SCALE Y AXIS	(0·05)	Abs
	(0·10)	"
	0·50	"
	1·0	"
	2·0	"
	4·0	"
	100%	T
REC. OFFSET		Abs.

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE	20
X AXIS	40
	100
	200
	400

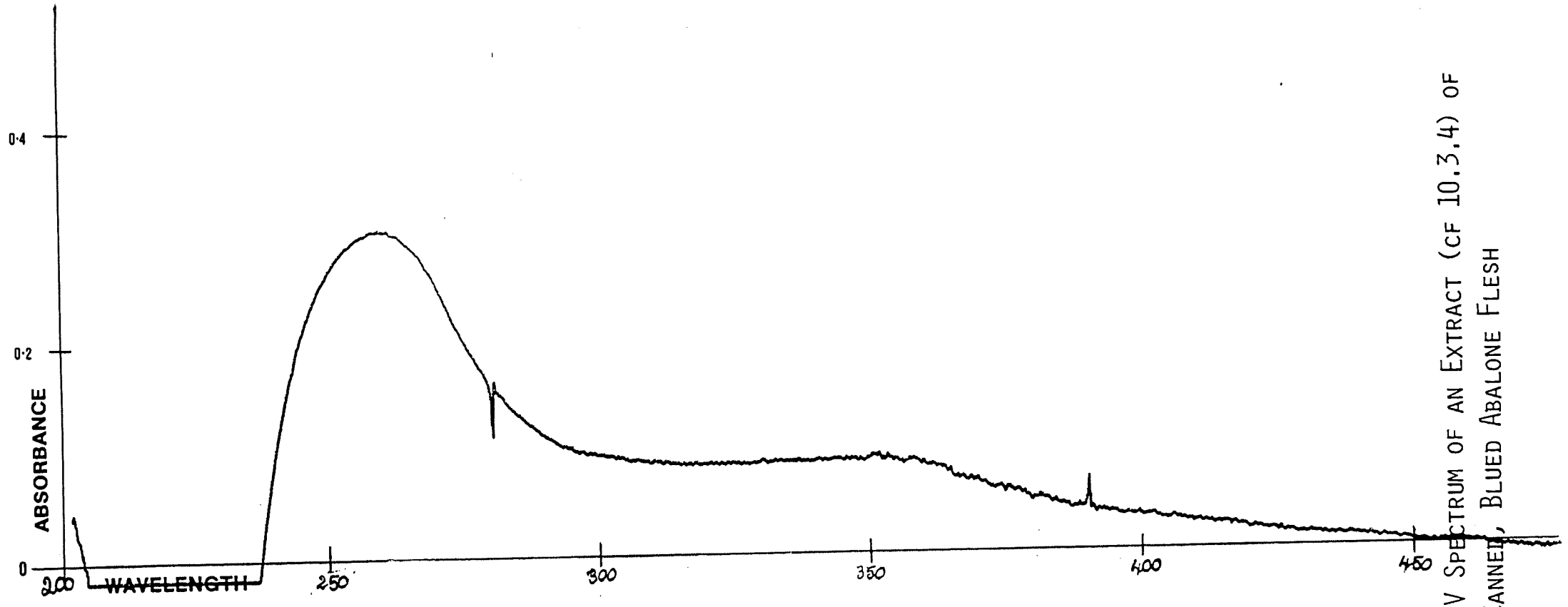


FIGURE 10.7 UV SPECTRUM OF AN EXTRACT (CF 10.3,4) OF CANNED, BLUED ABALONE FLESH

WAVELENGTH PRESENTATION

SET UPPER λ	(200)	nm
SCAN RANGE X AXIS	40 nm	(1 nm/cm)
	80 "	(2 ")
	200 "	(5 ")
	(400) "	(10 ")
	800 "	(20 ")
SCAN SPEED	10, 20, (50) 100, 200 nm/min	

PHOTOMETRIC PRESENTATION

MODE	%T, (Abs), Der,	
FULL SCALE Y AXIS	0·05	Abs
	(0·10)	"
	0·50	"
	1·0	"
	2·0	"
	4·0	"
	100%	T
REC. OFFSET		Abs.

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE X AXIS	20
	40
	100
	200
	400

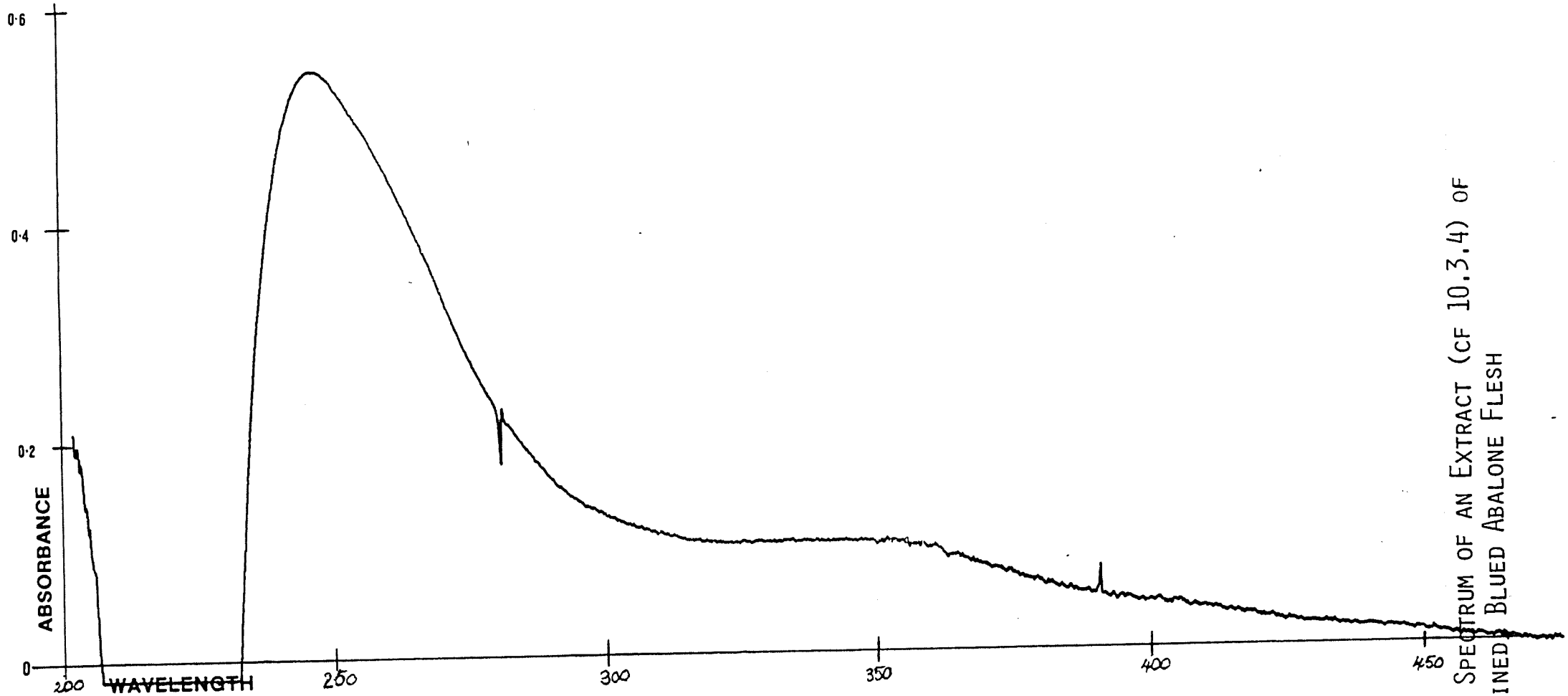


FIGURE 10.8 UV SPECTRUM OF AN EXTRACT (CF 10.3.4) OF BRINED BLUED ABALONE FLESH

WAVELENGTH PRESENTATION

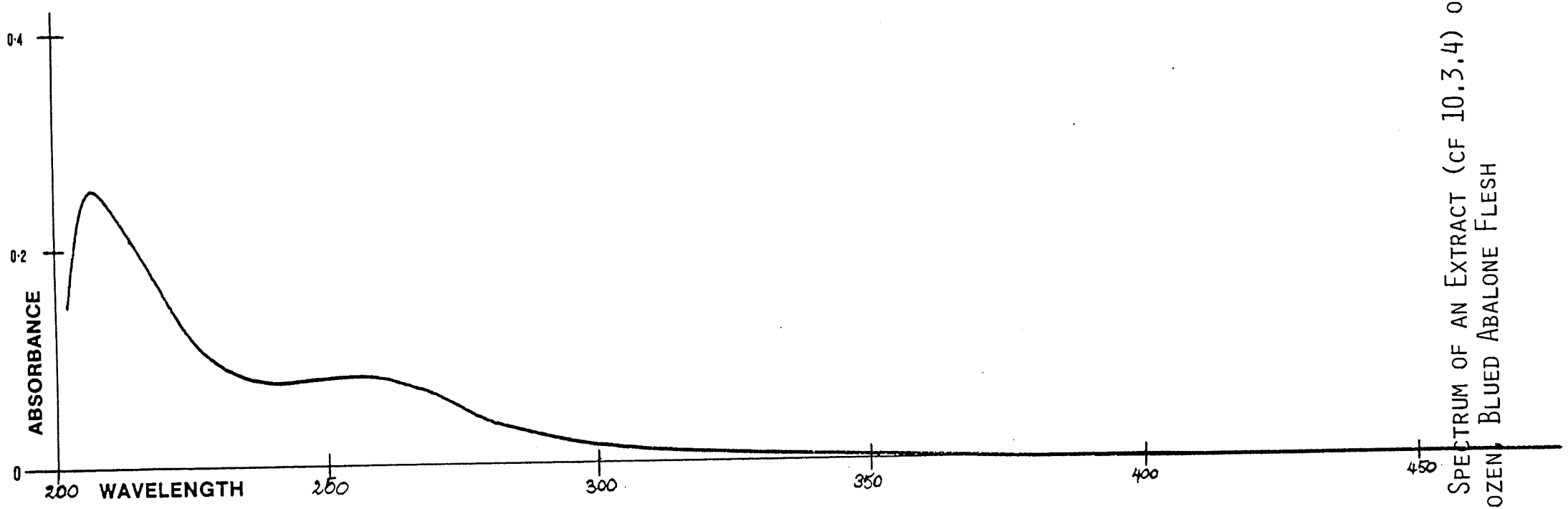
SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm	(1 nm/cm)
	80 "	(2 ")
	200 "	(5 ")
	(400) "	(10 ")
	800 "	(20 ")
SCAN SPEED	10 , 20 , (50) , 100 , 200	nm/min

PHOTOMETRIC PRESENTATION

MODE	%T, (Abs) Der,	
FULL SCALE Y AXIS	0·05	Abs
	(0·10)	"
	0·50	"
	1·0	"
	2·0	"
	4·0	"
	100%	T
REC. OFFSET		Abs.

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE	20
X AXIS	40
	100
	200
	400



WAVELENGTH PRESENTATION

SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm (1 nm/cm)	
	80 " (2 ")	
	200 " (5 ")	
	(400) " (10 ")	
	800 " (20 ")	
SCAN SPEED	10 , 20 , 50 , 100 , 200	nm/min

PHOTOMETRIC PRESENTATION

MODE	%T, (Abs), Der,	
FULL SCALE Y AXIS	0·05	Abs
	0·10	"
	0·50	"
	1·0	"
	2·0	"
	(4·0)	"
	100%	T
REC. OFFSET		Abs.

TIME PRESENTATION

UNITS	Sec,	Min,
FULL SCALE X AXIS	20	
	40	
	100	
	200	
	400	

FIGURE 10.9 UV SPECTRUM OF AN EXTRACT (CF 10.3.4) OF FROZEN, BLUED ABALONE FLESH

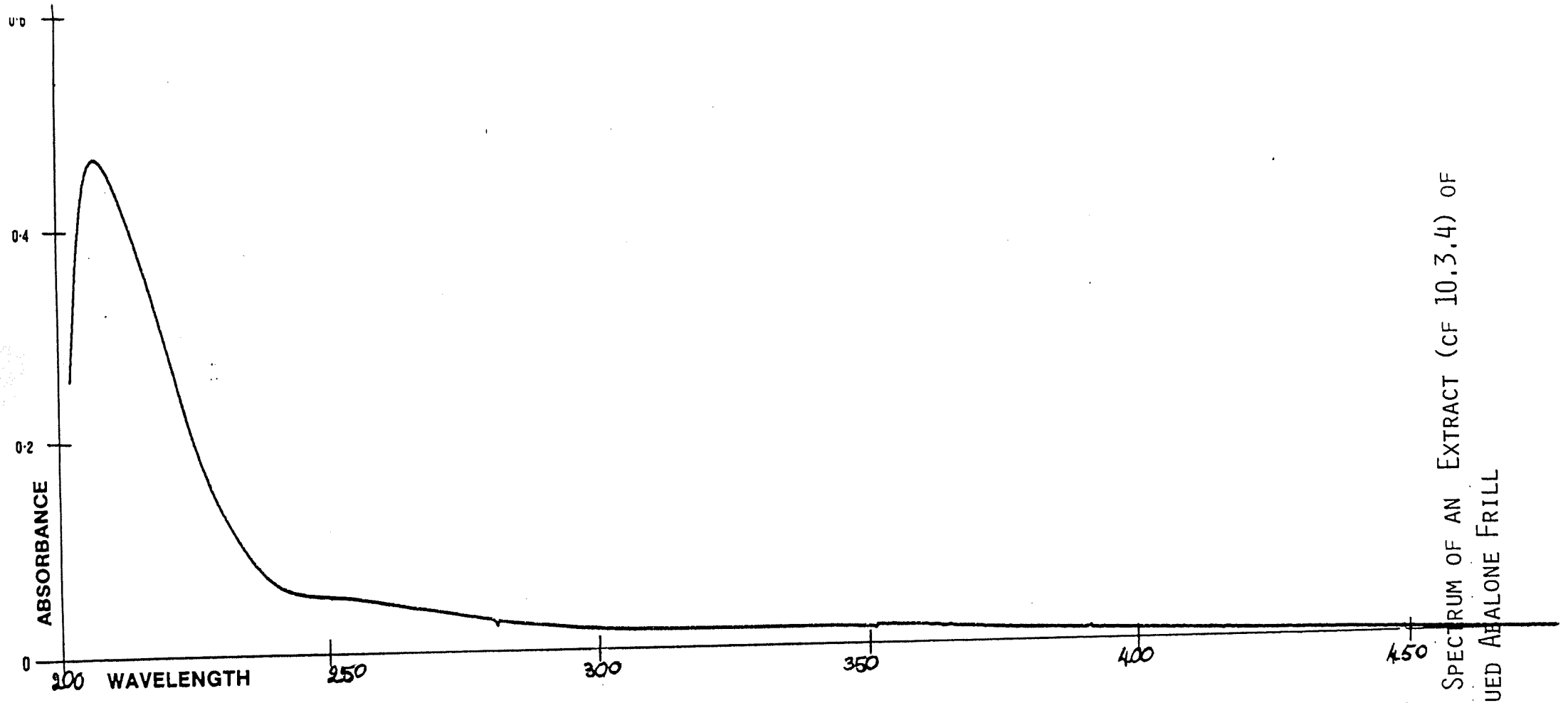


FIGURE 10.10 UV SPECTRUM OF AN EXTRACT (CF 10.3.4) OF BLUED ABALONE FRILL

WAVELENGTH PRESENTATION

SET UPPER λ	600	nm
SCAN RANGE X AXIS	40 nm	(1 nm/cm)
	80 "	(2 ")
	200 "	(5 ")
	(400) "	(10 ")
	800 "	(20 ")
SCAN SPEED	10, 20, (50) 100, 200	nm/min

PHOTOMETRIC PRESENTATION

MODE	%T, (Abs,) Der,	
FULL SCALE Y AXIS	0·05	Abs
	0·10	"
	0·50	"
	(1·0)	"
	2·0	"
	4·0	"
	100%	T
REC. OFFSET		Abs.

TIME PRESENTATION

UNITS	Sec, Min,
FULL SCALE	20
X AXIS	40
	100
	200
	400

PART IV. ANALYSES OF BLUED COMMERCIAL ABALONE

11.0 ANALYSIS OF BLUED ABALONE SAMPLES FROM TASMANIAN WATERS

11.1.0 The post-harvest handling of abalone

Abalone are caught from their natural habitat by commercial divers. The transport of the catch is governed by regulations that vary between different states. Tasmanian law require that the catch is delivered to the processing plant 'live' which serves to reduce the number of undersize specimens captured and also to improve the quality of the catch. Unfortunately, no specific regulations exist for the handling of live abalones which are left to the individual divers. This leads to varied holding procedures which are likely to affect the quality of the final canned product.

Small runabouts used to harvest abalone, stay out at sea for periods of up to 10 hours and keep their catch live in nets or bags. These day trips avoid many of the problems associated with transport and storage as the catch is taken from the jetty and delivered to the factory that same day. Larger vessels that operate in the heavy seas around the south-west coast of Tasmania may collect several tonnes of abalones over a period of several days. Here the catch is usually stored in steel baskets and kept live by circulating sea water. Several problems arise from such handling practice. The animals are usually kept in crowded environments to maximise available space thus reducing water circulation and oxygen supply. Abalone stored under such restricted conditions are likely to revert to anaerobic respiration with accompanying changes in physiology. The blueing problem has been commonly reported to occur to animals that are held in the center of the holding tanks which suggests that stress and overcrowding are factors to be considered in this investigation.

Commercial canned abalone (without the addition of MBS) provided initial samples to establish techniques for extraction and analysis. However, experience from local and overseas workers have shown that handling plays an important role in the formation of the discolorations found in both crabs and abalone. Environmental factors such as water temperature, storage temperature, holding time, availability of oxygen etc, leads to related changes in the animals. CSIRO investigators in Tasmania have received reports of blue spot in abalones that often coincides with summer months and abnormally high water temperatures (65). Based on feedback from experienced fishermen, it seems that handling practices such as water temperature and circulation affects the quality of the final product. The animals in the center of the holding wells are likely to experience warmer temperatures and are most prone to blue spot. The death of an abalone is also difficult to determine. The lack of activity from a specimen does not automatically imply death and although the catch is required to be delivered 'live', many of the unfortunate animals in the center and bottom of the holding vessels may have already been dead many days before arrival. Dead abalone undergo slow break down of glycogen (glycolysis) within the muscle along with many unknown chemical reactions that also affect muscle quality.

Abalone held in air have been shown to undergo physiological changes that are not well understood. Olley (52) monitored the loss of liquid from abalones held in air and determined that the initial liquor had very little protein content. However, after a period, the liquid lost becomes a blue color and is composed almost entirely of hemolymph. The reasons for this bleeding is still unknown but the phenomenum relates to the initial handling and almost certainly affects the final product.

In order to understand the handling problems and to better follow the treatment phases in the cannery, a detailed study of the handling and processing of abalones from Tasmanian fishery was undertaken in March 1987. With help from local divers, we were

able to monitor the treatment of individual abalones from capture through to the final canned product. This provided us with the precise knowledge of environmental factors of our samples such as temperature, hemocyanin concentrations, blood pH and first hand experience of the various stages up to the final product. The samples were then analysed in our laboratory and the results evaluated with consideration to the history of each animal.

11.2.0 PRECANNING BLUEING

11.2.1 MATERIALS AND METHODS

11.2.1.1 Collection of experimental abalone

Abalone (Haliotis ruber) were collected by hookah divers around the south coast of Tasmania in March 1987. Ten specimens were chosen to be monitored from the point of capture through to the initial canning stages. The animals were carefully handled to ensure minimum stress that may lead to related changes in their physiology.

Blood samples were taken by the insertion of a syringe into the median pedal vein running the length of the foot. Approximately one mL of hemolymph from each abalone was collected. The blood was sampled shortly after capture and within 24 hours (before the brining process). No blood could be obtained after the brining.

The animals were transported to the factory and stored in a refrigerated room. A salt/ice bath kept the abalone at below $-10^{o}C$ overnight. They were shucked, brined, cleaned manually, and canned approximately 18 to 24 hours after capture.

11.2.1.2 Qualitative testing

Three blued portions of abalone epipodium were subjected to initial testing. (i) A sample was suspended in a solution of 0.25% sodium metabisulfite (standard pre-canning, reducing, dip) overnight at 4°C, (ii) the control was suspended in distilled water and treated under similar conditions, (iii) the third sample was heated to a boil for 10 minutes.

11.2.1.3 Hemocyanin concentration

Samples of blood had been refrigerated at between 3-5°C and transported back to the University of Melbourne for analysis. In each case, the blood was centrifuged to remove cells and other debris. A dilution by a factor of 15 was also necessary to obtain an absorbance within the linear range of the instrument. A vigorous shaking ensured oxygention of the hemocyanin. Estimates of hemocyanin concentration were made spectrophotometrically at 340 nm where E was taken to be 2.23 abs. units (13).

11.2.1.4 Column chromatography

Blood/protein samples were obtained from brined blued abalone that were frozen then thawed. Olley (52) reports that a contraction of the flesh occurs during this slow freezing process. This leads to a loss of body weight through the expulsion of the blood and other body fluids. The deep blued sample obtained after thawing was fractionated by gel permeation chromatography over Sephacryl S-300 (Pharmacia.). The portion eluting in the exclusion region (greater than 1,500,000 Daltons) was further separated on Sepharose CL-4B (Pharmacia, m.wt. range $60,000-20x10^6$). Samples were dialysed overnight with two changes of Tris-HCl buffer (0.025M, pH 7.0) before application to the column. Buffers were made up in distilled water and adjusted with a calibrated pH meter. GPC fractions obtained were further analysed by both HPLC/GPC and isoelectric focussing. The

centricon microconcentrator (Amicon Corp.) was used as a convenient method of concentrating small volumes of dilute fractions for further analysis.

11.2.1.5 Extraction of abalone flesh

Strongly blued frill was removed from three brined abalones and subjected to extraction by aqueous buffers. A modification of the method of Pyeun et al.(56) was used to separate the proteins into four main groups. A detailed flow chart of the separation scheme is given in Figure 11.1. The temperature was kept below 6^{o}C at all times to minimise possible denaturation of the proteins especially during maceration/blending of the flesh.

11.2.1.6 High performance liquid chromatography

Bio-Rad's TSK-60 was used in gel filtration HPLC to determine molecular weights. The column is suitable for the separation of macro polymers up to 20 million Daltons. The complementary guard column (Bio-Rad TSK-Guard) was also used. UV absorbance at 280 nm was used in detecting the protein. All buffers were made using AR grade tris(hydroxylmethyl)methyl amine (BDH) dissolved in Milli Q water. AR grade hydrochloric acid was used to adjust the buffer to the desired pH. Calibration proteins (Pharmacia) were dissolved in buffer overnight before being applied to the column. The calibration graph obtained is shown on Figure 11.2. Extrapolation past the highest molecular weight marker (thyroglobulin - mwt. 699,000) was required but work by Brouwer and Kuiper (9) produced comparable molecular weight estimates to those obtained by other methods.

11.2.1.7 Isoelectric focussing

Isoelectric focussing of the proteins were performed as previously described in Section 2.3.4 for abalone hemocyanin.

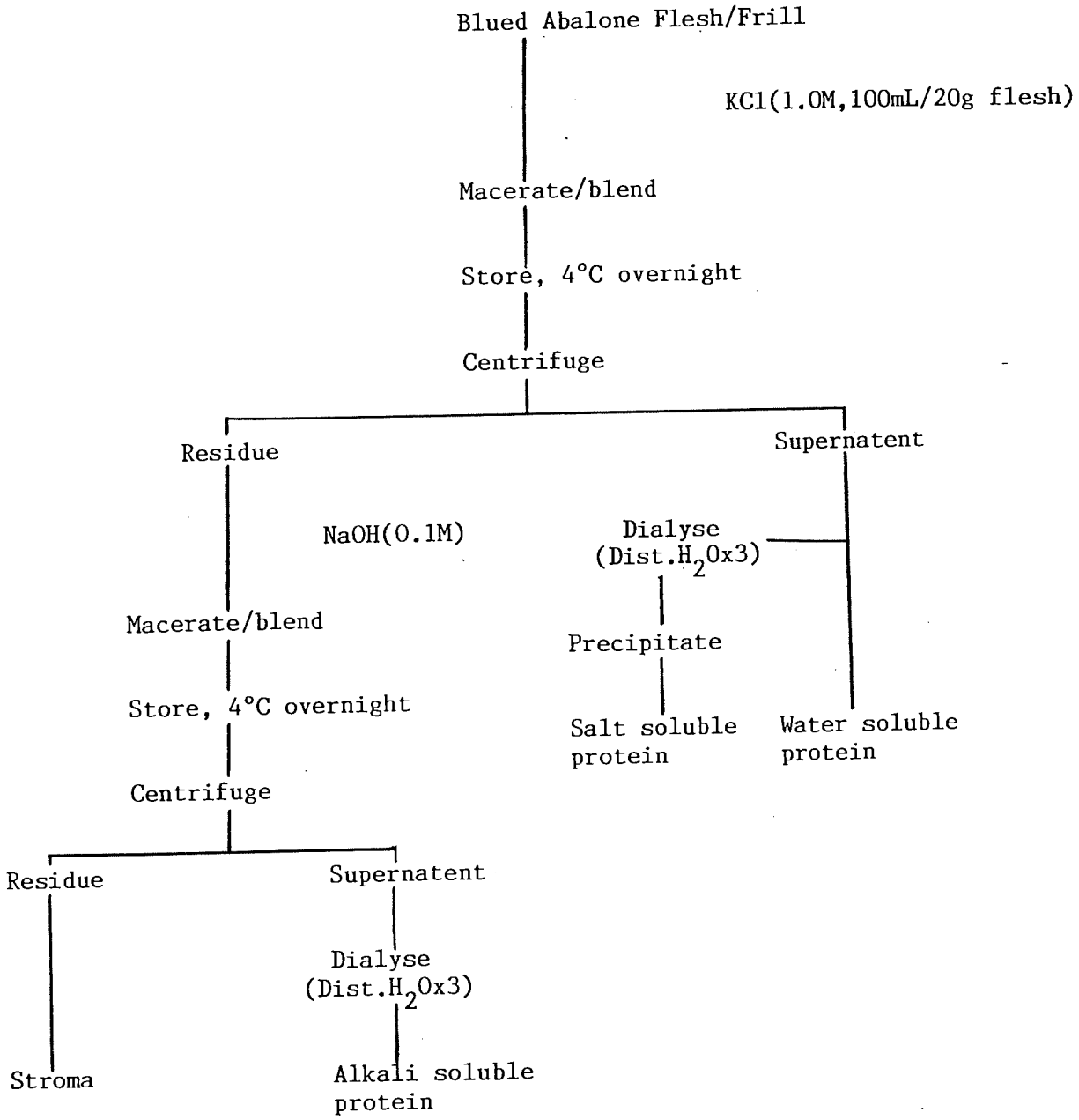


FIGURE 11.1 FLOW CHART SHOWING THE EXTRACTION OF ALKALI, SALT AND WATER SOLUBLE PROTEINS FROM ABALONE FLESH.

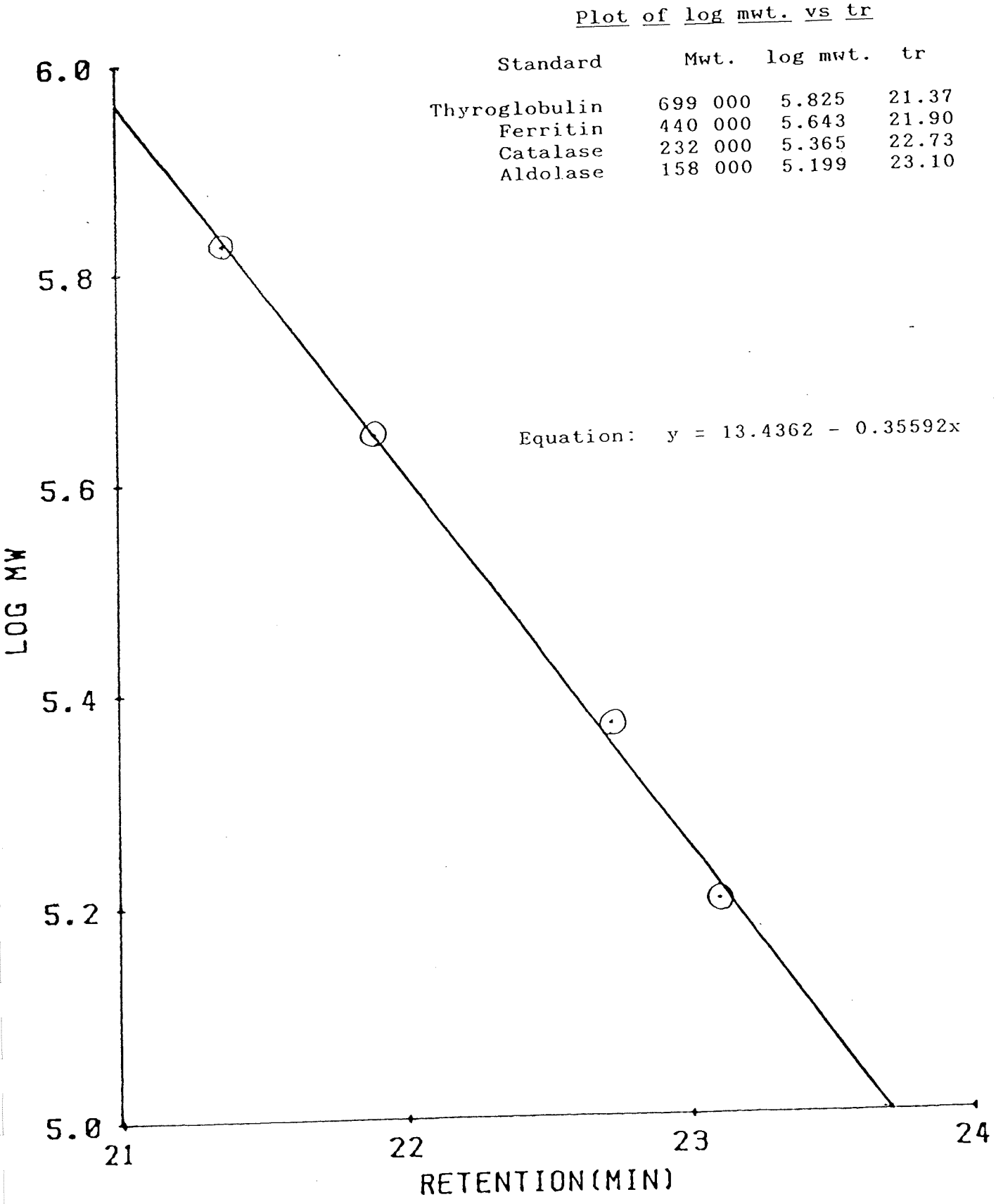


FIGURE 11.2 STANDARD CURVE FOR CALIBRATION AND MOLECULAR WEIGHT DETERMINATION OF HEMOCYANIN

11.3.0 RESULTS AND DISCUSSION

11.3.1 Hemocyanin concentration

Concentrations of hemocyanin in the blood of Tasmanian abalone Haliotis ruber (mean conc. 22.8mg/L. Table 11) were compared with samples of Victorian N.ruber (determined from 43 specimens captured at Flinders. Mean conc. 13.9 mg/L). These results indicate a naturally higher concentration of hemocyanin in the blood of Tasmanian abalone. There was also a significant increase in the effective hemocyanin concentration through the the processing stages for all specimens (Table 11).

11.3.2 Qualitative testing

The results of qualitative testing indicated that with sulfite dip and heat the blueing agent is easily reduced to a colourless form. Heat treatment without the sulphite dip also resulted in the loss of colour, suggesting that the blue material is heat labile and possibly proteinaceous in nature.

11.3.3 Extraction of abalone flesh

Abalone specimens showing the most intense discolourations were collected from the production line (these are shown in Fig.11.3). The extraction of the abalone frill with aqueous buffers gave a blue solution in the KCl/water soluble fraction. Spectrophotometric analysis of this sample gave positive UV. absorbances at 280 and 340 nm (Fig.11.4). These resemble the characteristic spectrum of oxygenated abalone hemocyanin. The appearance of the first absorption peak (280 nm) is mainly due to the tryptophan and tyrosine residues and can be used as an estimate of dilute protein concentrations. The second peak (approx. 340nm) is thought to be due to the oxygenated state of copper and is characteristic of mollusc hemocyanin. Its

isoelectrophoretic pattern produced two bands, with pI's of 6.2 and 5.3 corresponding to undissociated hemocyanin (major component) and an unidentified hemolymph protein (relatively minor component) respectively. HPLC/GPC molecular weight estimations gave values of approximately 2,300,000 Daltons for the extracted blued protein. This compares well with the molecular weight of hemocyanin (Fig.11.5). The minor blood component with an apparent molecular weight of 29,000 Daltons remains unidentified. Similar low mol. wt. compounds have also been reported by Ainslie (1) to be present in the blood of different abalone species, but these were not adequately characterised.

11.4 Analysis of thawed blood

Sephacryl S-300 gel permeation chromatography gave one major peak in the exclusion region (mol. wt. 1,500,000). Minor peaks were observed but these are within the limits of uncertainty. The fraction was further separated using Sepharose CL-4B (Fig. 11.6). This produced a single major peak with two early minor shoulder peaks. Isoelectric focussing of fraction 19 (major component) produced a strong band with a pI of approx. 6.2. No visible protein band appeared in the focussing of fractions 9 and 14 (high mol. wt. minor components). Spectrophotometric determinations show characteristic oxy-hemocyanin absorptions at 340 nm and were similar to the spectrum of the KCl soluble extract of blued frill. The concentration determined at 340 nm was 92.5 mg/mL, almost 5 times higher than the average natural concentration measured (Table 11).

11.5.0.0 POST CANNING BLUEING

11.5.1.0 MATERIALS AND METHODS

11.5.1.1 Sample history

Abalone specimens were collected during our March visit to a Tasmanian seafood cannery. These were monitored from the time of capture through to the processing stages. Most samples had discolourations to varying degrees.

Samples 1 to 10 were canned without the sulfite reducing dip so as to stimulate a high level of 'blueing' (cf Fig.11.3). A heavily blued specimen (No.6, Table 11) was chosen for chemical analysis. Abalone No.1 showing least colouration was used as the control for all extraction procedures.

11.5.1.2 Preliminary testing

Discoloured abalone flesh was subjected to various qualitative testing. Samples were removed in wedge shaped pieces which were refitted after treatment. This allowed better comparisons of the treated sections with that of the original coloured flesh.

Blued abalone flesh samples were treated in a similar method to that used for the preliminary testing of brined blue abalone (refer to Section 11.1.2.1). Heat stability of the blueing agent was tested by heating the sample to 100^{o}C in distilled water. The section was then refitted and left overnight (6^{o}C). Another section was subjected to a 1% solution of sodium MBS for 30 min at 20^{o}C. The dependency on oxygen for the discolouration was investigated. Two samples were immersed in Milli Q water and helium was bubbled through for 120 mins. Another sample was exposed to a reduced pressure of 0.5 Torr for two mins. All

Table 11. Hemocyanin concentrations measured in Tasmanian abalone hemolymph 'at capture' and after storing overnight.

Specimen	Hc. concentration (mg/mL) at capture	pre-brining	*Blueness
1	16.5	29.3	1
2	22.9	-	-
3	21.1	37.8	3
4	16.4	30.9	1
5	20.9	43.0	2
6	45.0	59.9	5
7	21.6	39.0	1
8	28.5	50.2	-
9	19.8	52.1	2
10	15.7	38.0	3
Average	22.8 mg/mL	42.2 mg/mL	

* Blueness was a subjective comparison with the highest discolouration obtaining a rating of 5.

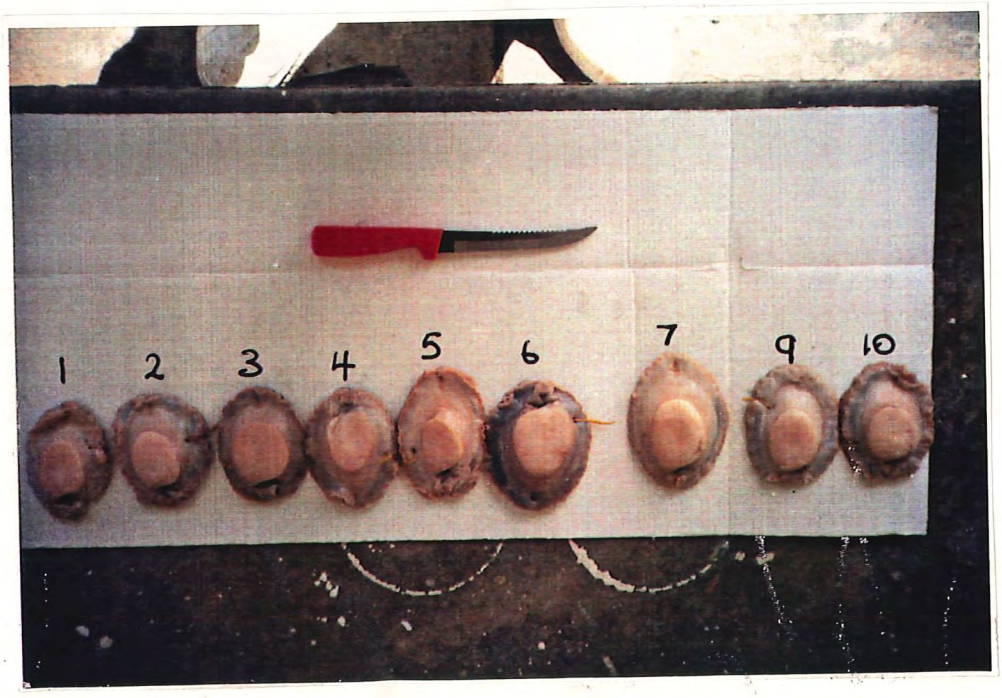


Figure 11.3

samples showed little apparent reversion of colour under these conditions.

11.5.1.3 Extraction of blued flesh

The blued frill of specimen No.6 was subjected to extraction with various buffer solutions. This method was used for the extraction of brined blue samples (Section 10.2.6.Fig. 11.1) produced water soluble proteins, salt soluble proteins, base soluble proteins and an insoluble residue. Extraction was carried out with a simultaneous control sample (mentioned earlier). The colour of the residues after each extraction provided a basis for comparison to indicate the solublising medium for the blueing agent.

11.5.2.0 Results and discussion

11.5.2.1 Initial examination

The canned abalone showed very similar blueing when compared to the photographs of the 'brined' blue samples collected at the same time in March (Section 5). The discoloured areas after canning remained blue/grey with the same approximate intensities as those before canning (see attached photographs). This 'before and after' observation strongly suggests that the blueing observed after brining is related to that occurring after canning.

Heating of the sample (100^{o}C, 10min.) resulted in loss of most colour. The loss of colour was observed to begin at approx. 60^{o}C with the most striking colour change at 80^{o}C. The sample of flesh had lost most of its colour with only a slight pale grey remaining. Reversion of colour in the heat treated flesh occurred on storage overnight at -15^{o}C. Treatment of another blued section with MBS resulted in loss of colour similar to those observed for the heat treatment. No reversion of colour could be induced under the reduced oxygen conditions used.

The initial testing indicates that the compound exhibits oxidative/reductive properties as well as other reversible reactions when subjected to heat and chemical treatment.

11.5.2.2 Extraction of blued flesh

Specimen (6) was extracted with buffered solutions in an identical method as used for brined blued abalone (11.5.1.3). A control sample was also extracted to provide a more accurate comparison between the specimens. Results suggest that the blueing agent was not extracted from the flesh by either the potassium chloride or sodium hydroxide solutions. The insoluble residue remained coloured by comparison with the control. The extraction produced pale white solutions indicating that the blueing agent was not successfully extracted.

11.6 CONCLUSIONS

In this particular Section we report on a study which encompassed the harvesting of abalone on board ship and a follow through to the canning stage. This situation allowed us to take blued abalone samples at various stages in the 'catch-to-can' process.

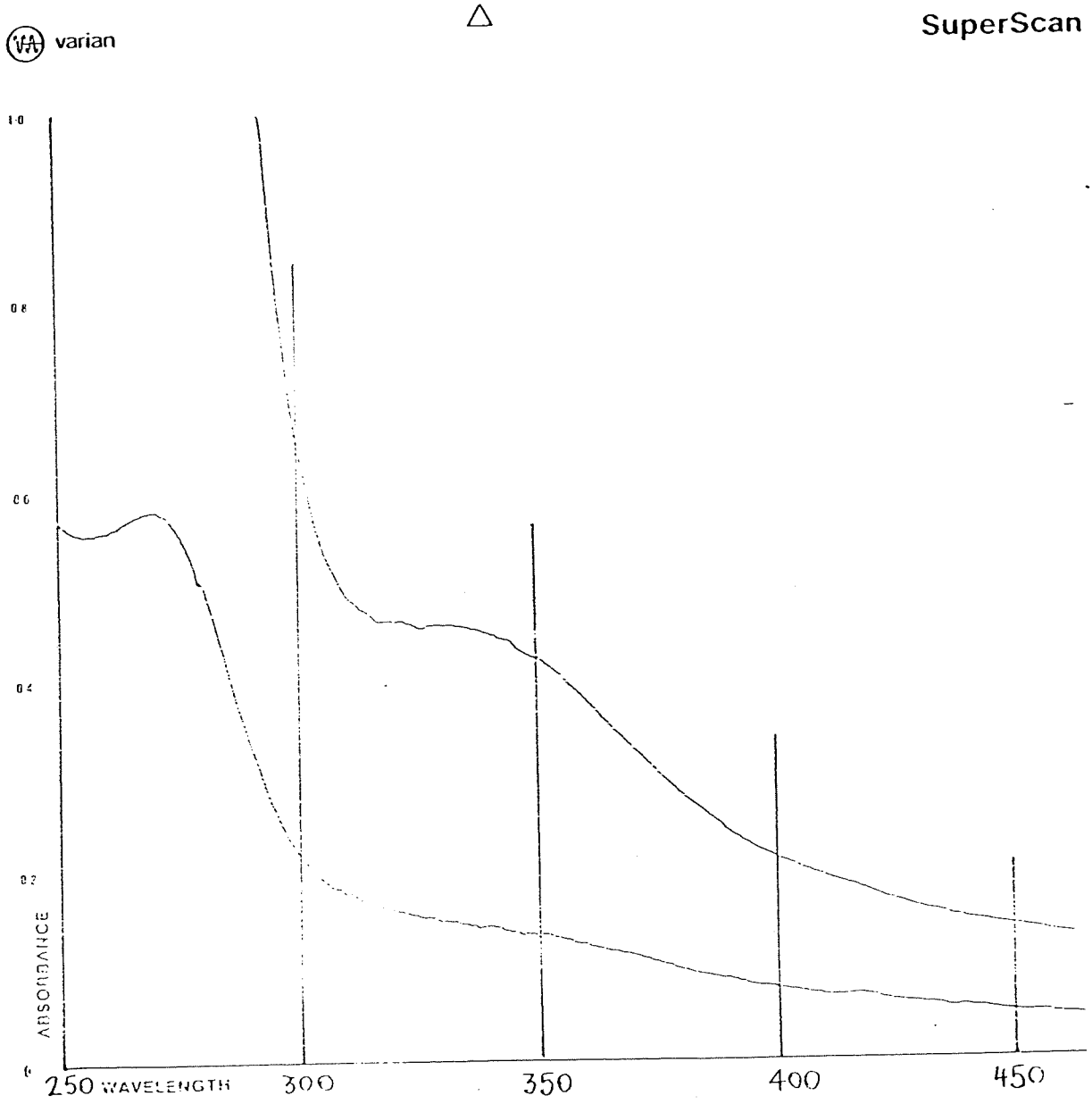
We were able to observe in the factory that as the abalone were thawed for brine washing, blue fluoresence showed in the draining water. This presumably relates to the gradual loss of Hc/hemolymph from the abalone. Table 11 reports the very high concentration of Hc in the hemolymph taken after storing at $-10^{o}C$ in an ice-salt bath. These values are significantly greater than found at capture. One likely reason is that the ice/salt mix removes water from the abalone hemolymph thereby increasing the concentration of the Hc. What is of interest is that after brining in the factory ($49^{o}C$ treatment), no hemolymph from the animals bodies was yielded by the abalone samples. A surprising number of abalone were blued before factory brining was commenced.

At this point in the precanning process, the blued frill of the abalone could be made colourless by dipping in a solution of sodium metabisulfite (MBS) and also by boiling at 100°C for 10 minutes. These results are what one would expect of Hc in the oxygenated form. A KCl aqueous extract of blue abalone frill gave a solute which had a UV spectrum which agreed with that for Hc. HPLC/GPC chromatography gave a fraction with a protein of M.wt 2.3×10^6 Daltons and Isoelectric Focussing further confirmed the presence of Hc. Figure 11.5 illustrates well the relationships between the various samples analysed. These data appear to link Hc with this form of blueing.

Samples examined after canning, but without the MBS dip gave distinctly different results from those described in the above paragraphs. The abalone were part of the catch used in the prebrining tests. The blueing after canning was comparable in colour intensity to that before canning. The canned and blued flesh was heated to 100°C for 10 minutes when it lost most of its colour. Most loss of colour appeared to occur at approximately 80°C which also corresponds to the temperature when Hc is denatured by heating. When the flesh was washed and stored overnight at -15°C it reverted to its original colouration. When the blued flesh was 'deoxygenated' it did not lose any colour. MBS dip at 1% concentration at room temperature (20°C) caused the flesh to lose its blue colouration. It did not revert to the original colour on storage overnight. Finally, aqueous KCl and NaOH solutions did not extract any of the blueing agent.

These observations leave one in no doubt that the heat treatment during canning 'fixes' the blueing agent in the flesh since it cannot be extracted by chemical procedures which do succeed at the precanning stage. Our observations also suggest that once the abalone is blued, either at the frill or through the flesh of the epipodium, the blueing will continue through and become 'fixed' during the canning stage. It would seem that a MBS dip can reduce the colouration. The action of the dip is not to remove oxygen as physical procedures to acheive this end had no effect. Rather, it must be a chemical breaking in part of the

complex with copper in the Hc. Once the sample has been heated during canning, however, the Hc appears to bind strongly to the protein of the flesh. Two distinct forms of blueing are thus recognisable. In the early pre-canning steps the blueing is almost certainly due to the presence of Hc in the oxygenated form. This can be relatively easily removed. However, as Hc is invariably left throughout the circulation system of the abalone flesh the 'fixing' during canning is due to a further reaction of the Hc with reactive centres in the flesh proteins or even in the hemolymph itself. The reactions which impart the permanent blue to the abalone are accentuated when or if the Hc concentration in the residual hemolymph is raised.



Instrument : Varian Superscan 3

Scan speed : 50nm/min

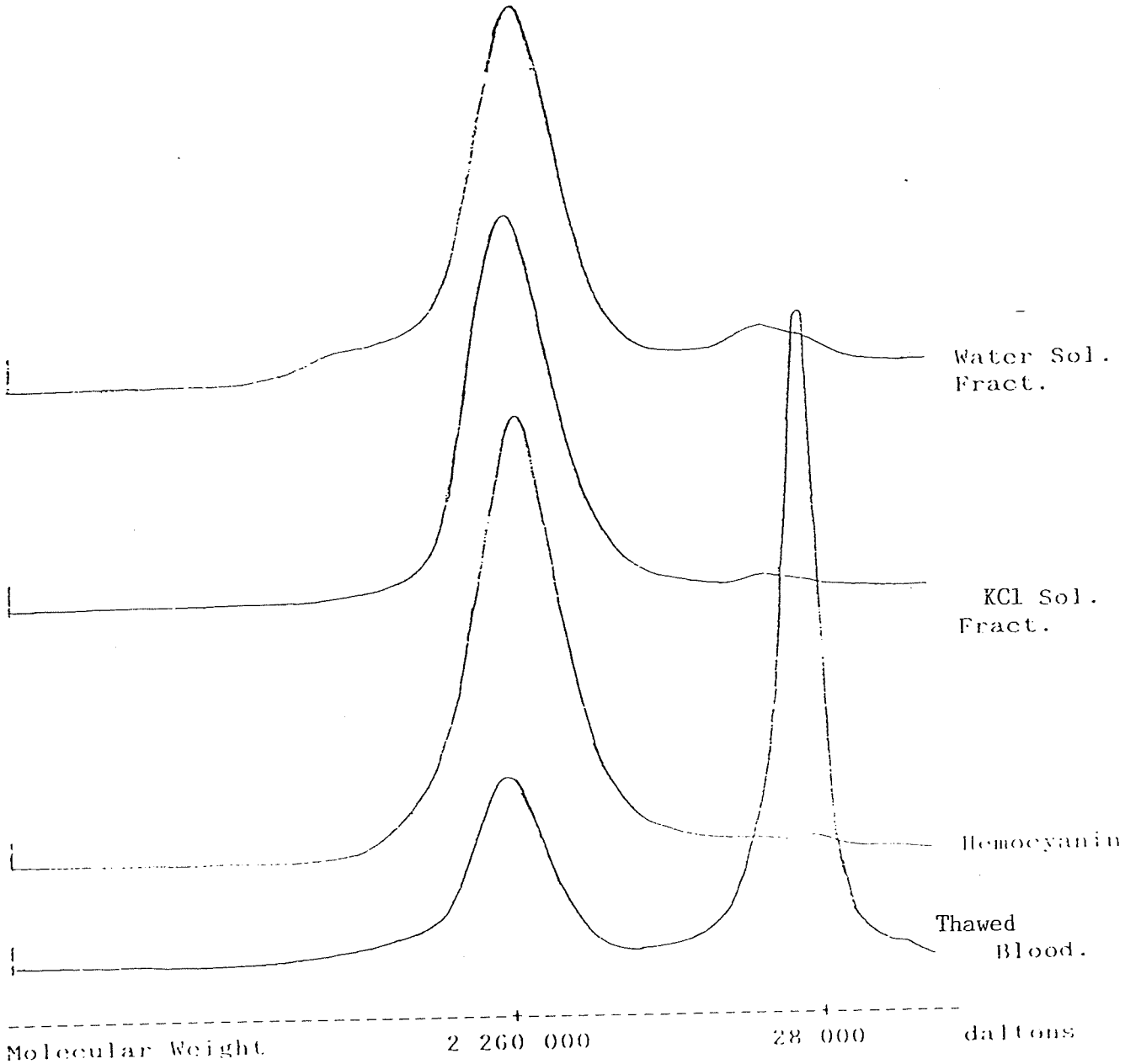
Path length : 10mm

Sample : KCl soluble extract of bled abalone frill

Concentration : Upper 2mg/mL (undiluted)
Lower diluted 1/5 with distilled water

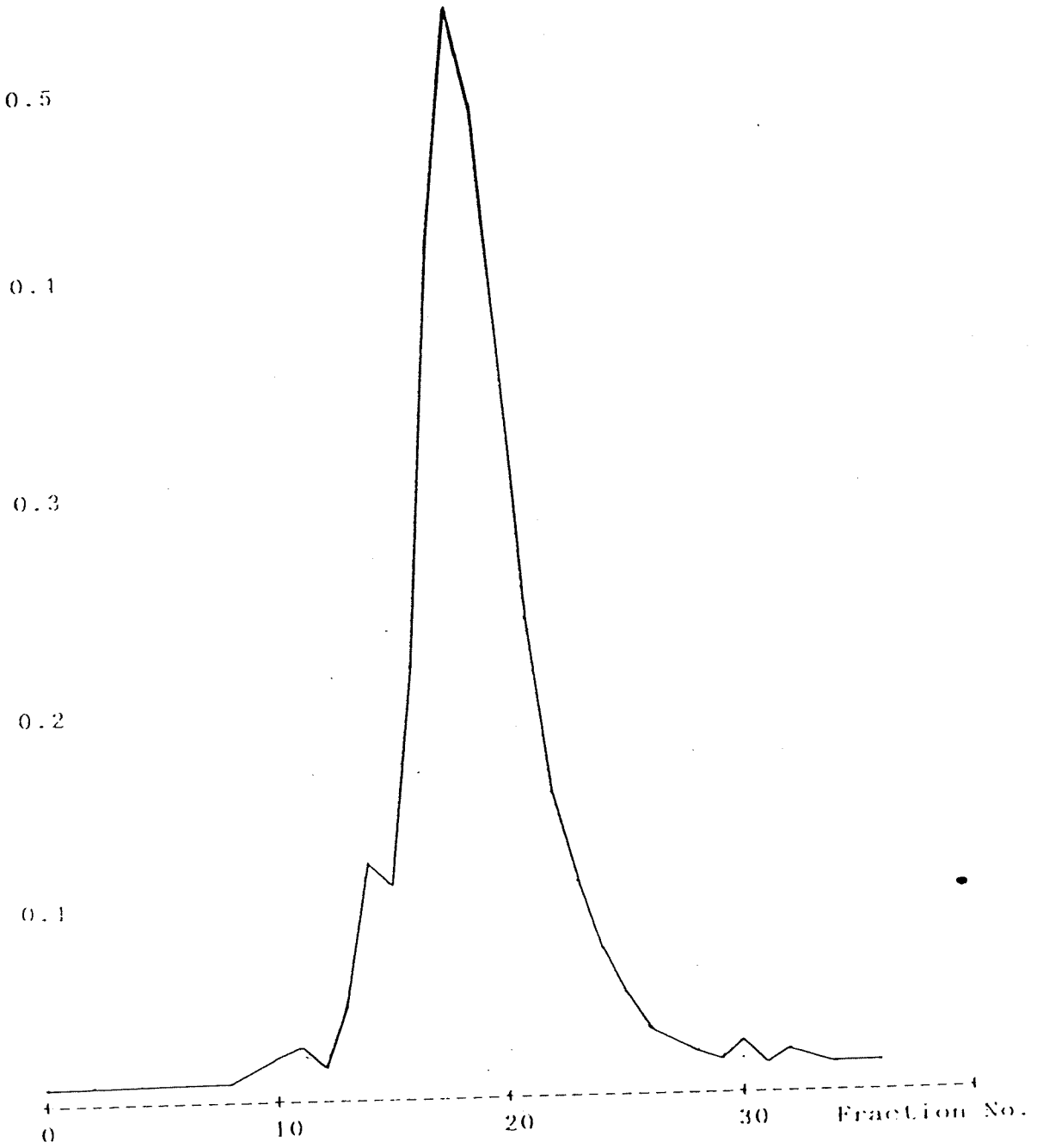
Reference : Distilled water

FIGURE 11.4 UV SPECTRUM OF KCL SOLUBLE EXTRACT OF A BLUED TASMANIAN ABALONE FRILL



Instrument	: Waters HPLC
Column	: Bio Rad TSK - 60
Detector	: UV at 280 nm
Buffer	: Tris-HCl (0.025M) pH 7.0
Flow Rate	: 0.5 ml/min.

FIGURE 11.5 HPLC-GPC PLOT OF MOLECULAR WEIGHT DETERMINATIONS OF SAMPLES FROM TASMANIAN ABALONE FRILL



GEL PERMEATION CHROMATOGRAPHY

Column : Pharmacia C 26/40

Medium : Pharmacia Sepharose CL-4B

Buffer : Tris-HCl (0.025M) pH 7.0

Flow : 10 mL/hr (5mL/fraction)

Detection : UV at 280 nm

FIGURE 11.6 HPLC-GPC CHROMATOGRAPHY PLOT OF THAWED ABALONE BLOOD STORED AT 3 - 5 C

PART V. SUMMARY OF RESULTS

12.0 CONCLUSIONS AND RECOMMMENDATIONS

Although it is generally agreed that blueing of abalone meat is caused by some constituent of the hemolymph, there is little agreement as to the actual cause. The major reported mechanisms of blueing relate to crab meat and may be separated into five broad categories which are not necessarily mutually exclusive. These are the formation of (i) iron compounds, (ii) copper compounds, (iii) melanin, (iv) copper proteins and biuret complexes, and (v) hemocyanin compounds. Very little previous work has been reported specifically for abalone as the five classes mentioned above are essentially related to research on different crab species. These reported mechanisms for discolouration in crabmeat were taken as our starting point for a specific study of abalone, and the results indicate that several different types of blue discolouration are possible. We have been able to identify the following specific types of blueing in abalone -

(i) heavy blueing within major veins of the hemolymph system,
(ii) pale blueing to heavy blueing of the frill,
(iii) blueing throughout the flesh of the foot.
(iv) blueing as a concentration of oxygenated hemocyanin

The involvement of hemocyanin

Fifty years of investigations into the blueing of canned crabmeat have provided little agreement as to the cause(s). However, it is often postulated that hemocyanin plays an active role as either a reactant or a source of inorganic copper. In our study of abalone, we naturally put a high priority on attempts to find sound chemical grounds for implicating hemocyanin in causing the blue colouration.

The copper content of abalone flesh samples were determined and the results indicated that heavily blued frill may contain up to 100 times the concentration found in white flesh. Olley (52) monitored both copper and iron content of normal and discoloured flesh and

found that iron content varied between the samples but copper content was clearly much higher (up to ten fold) in blued sections. Inoue and Motohiro (31) also reported a ten fold increase in copper content between white and blued canned crabmeat and suggested that blueing may be noticeable whenever the copper levels reach 2 mg%. The most likely source of this copper is hemocyanin and in fact some investigators have used copper content in the blood as an estimate of hemocyanin concentration.

The results of amino acid determinations on the various abalone flesh extracts have shown that blue areas of flesh contain a relatively high proportion of the hemocyanin amino acids. The salt soluble protein extract of blued frill flesh was pale blue in colour and remarkably similar to hemocyanin when statistically compared. These observations suggest strongly that hemocyanin concentration plays a key role in the formation of this form of blueing. The reported spasmodic occurrence of blueing is probably the result of the great variation in the hemocyanin concentration of a particular abalone population. We put effort into comparative analyses of Tasmanian and Victorian abalones in order to obtain some 'hard' data on this variation. It is not known why different abalone living in the same environment may have such a wide variation in hemocyanin levels. This study has noted a four fold difference in hemocyanin concentrations of some abalone caught within 10 meters of each other. [This compares with a nine hundred fold difference reported by Pilson(55)]. Blueing is often observed to occur in only one of two abalone in the same can, suggesting that the phenomenon must be due to an individual-dependent factor such as hemocyanin levels. This is a particularly important observation in understanding blueing as a whole.

During a visit to a Tasmanian seafood processing factory, the hemocyanin concentration of ten animals was monitored at various stages up to canning. Heavily blued specimens were collected from the production line and analysed at the University of Melbourne. The results suggested that the animals monitored had higher overall hemocyanin levels when compared with Victorian specimens. One Tasmanian specimen recorded the highest level of hemocyanin and also

exhibited the most pronounced blueing. From the samples analysed, it was clear that discoloured animals contained an extraordinarily high level of hemocyanin when compared with normal specimens.

Monitoring local (Victorian) populations of abalone over a two year period suggested that average hemocyanin levels are higher in the summer months. Coincidently, most reports of blueing and fatalities in abalone populations occur during the warmer summer months or during periods of abnormally high water temperature. During this time the abalone may suffer heat stress and be prone to blueing through a combination of higher levels of natural hemocyanin and accelerated deterioration which may ultimately lead to the formation of sulfide associated problems.

Our data has led us to conclude that Victorian bay abalone differ from ocean abalone in the concentration of hemocyanin in the hemolymph. In turn,Victorian abalone have on the average much less hemocyanin/ml of hemolymph than the Tasmanian abalone population. It is not within our area of expertise to suggest why this can be the case, but rather to note that since we conclude that the degree of blueing is related to hemocyanin concentration,it is likely that Tasmanian abalone will be more prone to blueing than are Victorian abalone.

Since blueing is reported more often from periods when seawater temperatures are raised and/or when the animals have suffered stress, it was a reasonable approach to explore the reaction of sulfide ion with hemocyanin as one possible cause of the blue discolouration.

The hemocyanin sulfide complex

Extensive studies on the blueing of crabmeat have shown that a reaction between hemocyanin and sulfide produces a stable 'blueish' protein complex. Inoue and Motohiro (31-37) published a series of papers that investigated the blue discolouration of canned crabmeat and concluded that the hemocyanin sulfide complex was responsible.

Hydrogen sulfide has been shown to react with N. ruber

hemocyanin to produce a protein complex with similar chemical and uv/visible spectrophotometric properties to that reported by Inoue (33). The mechanism is thought to proceed via the coagulated hemocyanin intermediate because it does not form unless the reaction vessel is heated. This requirement implies that even denatured hemocyanin is still reactive towards free and bound organic sulfides. Once formed, the protein complex is insoluble in many common solvent systems and the colour cannot be reversed by treatment with chemical reducing agents such as metabisulfite (MBS).

The hemocyanin sulfide complexation proceeds under similar conditions to those experienced during canning and is inhibited by the presence of MBS. Reactants are readily available with many sulfides forming part of the organic matrix. In addition, bacterial degradation or putrification of the flesh can occur releasing large amounts of hydrogen sulfide. Sulfides are also released by thermal degradation of sulfur containing amino acids. Johnson and Vickery (42) reported that up to 50 μg of hydrogen sulfide may be released from 100 g of meat within the first four minutes of heating. This reaction was shown to increase with pH and be catalysed by the presence of aluminium and ferric cations which may be introduced directly from a defective can wall surface or through the canning process.

Artificial blueing of the abalone flesh was induced by the addition of hydrogen sulfide to the abalone soup before processing under standard canning conditions. The abalone portion showed pronounced discolouration concentrated around the epipodium with other areas being unaffected. However, the colour did not resemble that of 'normal' canned blued specimens and was of an intense green/blue colour more closely resembling the shade of New Zealand specimens.

Sulfide determinations on canned blued and white flesh varied but could not be correlated with the observed colour. Although Inoue (31) found higher levels of sulfides in blued meats, data from this study indicate that even purified hemocyanin sulfide complex has an organic sulfide content comparable to that of coagulated hemocyanin,

making its detection in the flesh matrix unlikely by the analytical method we have used.

It is possible that sulfide blueing may be responsible for the intense green/blue colouration found in the low grade New Zealand specimens but we conclude that this discolouration is completely different to the blueing experienced by local animals.

These experimental observations would lead to the conclusion that if a sulfide ion concentration developed in the flesh and reacted with hemocyanin a discolouration would result. The intensity of the colour, however, would be dependant upon both [S^-] and [hemocyanin] concentrations. More importantly this form of blueing requires heat to form it and therefore would be observed after canning. The reaction would involve denatured hemocyanin and could occur throughout the flesh. The amino-acid content of abalone flesh contains potential sources for the generation of sulfide ion, particularly if stress releases taurine into the hemolymph and if poor storage after capture caused the death of the animals. MBS treatment can reduce the intensity or prevent this form of blueing, but once the complex has formed it has no observable effect. The colour cannot be extracted from the flesh of the frill suggesting that the protein matrix binds strongly to the Hc-S complex. We associate this form of blueing primarily with the frill.

Phenolase activity

A form of blueing was observed which penetrated throughout the whole of the flesh and was unaffected by MBS treatment. This form of blueing did not correspond to a sulfide - hemocyanin derived discolouration since it was present already at the brining (pre-canning) stage. Furthermore, the colouration could not be extracted from the abalone flesh requiring that the complex be associated with the protein matrix. This extensive form of blueing was brought to our attention rather late in the project when we were provided with suitably discoloured samples. It also seems to be the least desirable form of blueing from the Industry's point of view.

Polymerisation of phenols has been investigated as a possible cause of blueing in canned crabmeat. Babbit et al. (3) postulated that the blueing of Cancer magister is due to the oxidation of phenols to melanins. It was shown that the phenol oxidation reaction is catalysed by the presence of ionic copper and optimizes at pH 8.0. Phenols such as dopa and tyrosine were tentatively identified by TLC (3) and the blood was shown to exhibit phenolase activity. Our study indicates that N. ruber hemocyanin catalyses the oxidation of dihydroxy phenols (dopa) but not tyrosine to any significant extent.

The data from our tests showed that a dark blue complex forms between hemocyanin and dopa when heated to 50°C, under simulated standard brining conditions. The enhanced blue complex had similar oxidative/reductive properties as observed in the body fluids obtained from heavily blued abalones. In particular the dopa-induced colouration could be prevented by an MBS dip. However, after induced blueing of the flesh by treatment with a dopa solution (in the absence of MBS), the colouration could not be extracted from the flesh (protein) matrix by procedures which were successful in removing hemocyanin. Treatment of Hc with dopa led to the formation of an intense blue/green solution. Gel permeation chromatography of this solution showed that the colour was associated with the Hc fraction and not with the low molecular weight protein fraction which is also present in abalone hemolymph. Discolouration of the hemocyanin/dopa mixture continued slowly on standing even after 100°C treatment.

A major conclusion which can be drawn from these observations is that a dihydroxyphenolase activity can be associated with abalone hemolymph. The reactant is hemocyanin. Once the discolouration forms in the abalone flesh it is bound strongly to the protein matrix suggesting that the o-dihydroxy constituent is part of or becomes part ofthe protein matrix thereby binding the hemocyanin to it in the process of forming the complex.

Analysis of blued abalone samples

Abalone specimens were collected around the southern coast of Tasmania and carefully monitored from capture through to canning and

final analysis. Extremely blued samples were also collected off the production line for extraction and analysis. The results suggest that current commercial processing procedures concentrate the hemocyanin at two separate stages.

Olley and Thrower(53) have shown that live abalone held in air lose body fluids which had very little amino acid content initially but eventually became a blue colour composed exclusively of hemolymph. The abalone monitored were kept overnight in a salt/ice bath and produced a two fold increase in hemocyanin levels. This can be regarded as the first step in concentrating hemocyanin in the hemolymph.

Further losses in body fluids and a concentration of the hemocyanin occurred during the cleaning brine. Brine solutions with greater than 4% salt led to loss of body fluids through osmosis, mostly in the form of water, and brine cleaning may be regarded as the second step in concentrating the hemolymph. Although no hemolymph samples could be obtained from abalone after the brine washing, body fluids extruded from the muscle during a subsequent freezing process were analysed. The hemocyanin concentration of the fluid was almost twice that of the highest recorded level in the hemolymph.

HPLC gel filtration of the body fluids produced a large low molecular weight absorption peak. This peak is usually an unidentified minor component of the hemolymph but UV/Visible spectrophotometric analysis suggests that it may be phenolic in nature. However, GPC separation of the dopa-treated hemolymph did not appear to involve this protein fraction in the blueing colouration. Our data show that the processing techniques are providing the ideal situation for phenolic induced blueing in abalone by concentrating the reactants (hemocyanin) and providing suitable conditions.

Blueing as a consequence of the presence of oxygenated hemocyanin.

This is a form of blueing which occurs as a consequence of hemocyanin binding with oxygen from the seawater and/or the air. This

form of blueing is hemocyanin-dependant and hence is observed in organs of the abalone which allow greatest exposure of hemolymph to oxygen. It is reasonable to consider precanning blueing of the frill in fresh and properly handled abalone to be due to this chemical complex. An important property of the complex is that the colour can be discharged by heating after about 60°C which is well below the canning temperature of 100°C. The colour can also be removed by dipping in a MBS solution. These observations are also reproduced by solutions of isolated hemocyanin. The deep blue colouration often observed in abalone after low temperature treatment in ice/salt mixtures is probably due to this form of blueing. The colour is accentuated because of the concentration of the hemocyanin in the hemolymph through loss of water.

A sample in which a deep blue stain was clearly apparent in one section of the circulatory system of the abalone showed on analysis to contain a high concentration of hemocyanin, and is almost certain to be another example of this form of blueing.

This particular form of precanning, oxidative blueing is normally not a problem in that the colouration will be reversed either on heat treatment or by a reducing dip. However, because hemocyanin is invariably left within the circulatory system which itself extends to quite fine veins within the flesh, the blueing can be 'fixed' by the canning procedure. In such cases further reactions which we identify as sulfide-Hc and phenolase-Hc complexes can occur through reactive centres in the protein matrix. These bind the hemocyanin and hence the colour to the flesh.

Hemocyanin concentrations and possible mechanisms for prevention

Our data, whilst pointing to three distinct mechanisms which can lead to a blue discolouration always involve hemocyanin. These reactions also appear to centre upon the presence of copper metal. It is known (from work in Dr.J.D.Smith's Laboratory, University of Melbourne) that there can be other centres of accummulation of copper in addition to hemocyanin. It is possible that the hemocyanin provides a biological mechanism for distributing the metal throughout

the animal's body which may aid concentration or dilution. From the point of view of this Report, however, the invariable involvement of hemocyanin copper points not only to a cause, but also to a mechanism of prevention.

Treatments which concentrate hemocyanin in the precanning stages are potentially deleterious to the quality of the canned product.

Removal of hemolymph and thereby hemocyanin as early after capture as convenient would appear to be advantageous in that bled animals will have a strongly reduced propensity to undergo subsequent blueing. With a reduced Hc concentration, the forms of blueing which can become 'fixed' by canning will have a much lowered intensity of colouration as a consequence.

More direct ways of preventing blueing by the use of reducing dips do not show much promise. A brief survey of possible chemicals to replace MBS usually led to the conclusion based on literature comments, that possible alternatives leave an unacceptable flavour in the abalone meat. Furthermore, such dips are predicated on the basis that the blue discolouration is chemically reversible or reducable. Whilst this is true of blueing due to oxygenated hemocyanin, it is not true of blueing formed by sulfide or phenolase action. It is this form of residual blue colour which is undesirable in the canned product, and if the abalone is placed in the can already blued, i.e., after brining, it is probable that the discolouration will persist, albiet at a lower intensity even if a dip has been used.

The answer to the blueing problem would appear to lie with its prevention. This involves careful handling and storage before arrival in the factory and up to the brine-cleaning step. From the limited information in the literature which can be taken over to the abalone case, there is little doubt that stress factors will generate chemical substrates in the plasma which will facilitate the undesirable forms of blueing and which appear to become 'fixed' during the canning treatment.

Finally, the obvious method of prevention is to bleed the animal

soon after it has been caught. Whilst this procedure would not disadvantage weight loss, because no hemolymph is extractable after brine-cleaning and before canning, it does run up against current legislation controlling the handling of abalones before they reach the factory.

13.0 BIBLIOGRAPHY INCLUDING LITERATURE CITED

1. AINSLIE, R. (1980) Hemocyanin Concentrations in Field Populations of Three Species of Southern Australian Abalone. Aust. J. Mar. Freshwater Res. 31: 627-633.

2. AINSLIE, R. (1980) The Quantative Role of Hemocyanin in the Respiration of Abalone (genus Haliotis). J. Exp. Zool. 211:87-99.

3. BABBITT, J.K., D.K. LAW, and D.L. CRAWFORD (1973) Blueing Discoloration in Canned Crab Meat (Cancer magister). J. Food Sci. 38: 1101-1103.

4. BAILEY, K. (1957) Invertebrate Tropomyosin. Biochimica et Biophysica Acta. 24: 612-619.

5. BELTRAMINI, M., F. RICCHELLI, A. PIAZZESIS, A. BAREL, and B. SALVATO (1984) Removal of copper from Octopus valgaris haemocyanin. Biochem. J 221: 911-914.

6. BETZER, S., PILSON M.E.Q (1974) The seasonal cycle of copper concentration in Busycon canaliculatum. Biol. Bull. 146, 165-75.

7. BONAVENTURA, J., C. BONAVENTURA, and B. SULLIVAN (1975) Hemoglobins and Hemocyanins. J. Exp. Zool. 194: 155-174.

8. BOON, D.D. (1975) Discoloration in Processed Crabmeat. A Review. J. Food Sci. 40: 756-761.

9. BROUWER, M., and H.A. KUIPER (1973) Molecular-Weight Analysis of Helix pomatia a-Hemocyanin in Guanidine Hydrochloride, Urea and Sodium Dodecylsulfate. Eur. J. Biochem. 35: 428-435.

10. BROUWER, M., M. WOLTERS, and E.F.J. VAN BRUGGEN (1976) Proteolytic Fragmentation of Helix pomatia a-Hemocyanin: Structural Domains in the Polypeptide Chains. Biochem. 15(12): 2618-2623.

11. COOMBS, T.L., and S.G. GEORGE (1977) Mechanisms of Immobilization and Detoxification of Metals in Marine Organisms. In (ed): European Symposium on Marine Biology. pp. 179-187.

12. C.S.I.R.O.
Division of food research report. 1971-1972, pp.22.

13. DIJK, J., M. BROUWER, A. COERT, and M. GRUBER (1970) Structure and Function of Hemocyanins VII. The Smallest Subunit of a- and b-Hemocyanin of Helix pomatia: Size, Composition, N- and C-Terminal Amino Acids. Biochim. Biophys. Acta. 221: 467-479.

14. DRELL, W. (1970) Separation of catecholamines from catechol acids by alumina. Anal. Biochem. 84, 142-151.

15. ELLIOT, F.G., R. WITTERS, H. BROGINON, and R. LONTIE () The

Dissociation Studies by Ultracentrifugation. Comparison with the a-Hemocyanin of Helix pomatia. : 649-653.

16. ELLIOTT, H.H., and E.W. HARVEY (1951) Biological Methods of Blood Removal and Their Effectiveness in Reducing Discolouration in Canned Dungeness Crabmeat. Food Tech. APRIL:| 163-166.

17. FAULKNER, M.B., and B.M. WATTS (1955) Deteriorative Changes in Frozen Shrimp and Their Inhibition. Food Tech. Dec:| 632-635.

18. FOGO, J.K. and POPOWSKY, M. (1949) Spectrophotometric Determinations of Hydrogen Sulfide. Anal. Chem. 21, pp.732.

19. GANGAL, S., and N.G. MAGAR (1976) Canning and Storage of Crabmeat. Food Tech. 21(397):| 79A-82A.

20. GANGAL, S.V., and N.G. MAGAR (1963) Freezing of Crabmeat. Food Tech. Dec.:| 101-105.

21. GEORGE, S.G. (1980) Correlation of Metal Accumulation in Mussels with the Mechanisms of Uptake, Metabolism and Detoxification:| Biochemical and Ultrastructural Studies. In (ed):| VIth Int. Conf. Chem. Medit. Rovinj, Yugoslavia:|.

22. GIELENS, C., M. LANCKRIET, and R. LONTIE (1979) Isolation of the Collar Fragment of bc-Haemocyanin of Helix pomatia after proteolysis with Enteropeptidase. In U. WESER (ed):| Metalloproteins. New York:| Georg Thieme Verlag Stuttgart, pp. 81-87.

23. GIELENS, C., G. PREAUX, and R. LONTIE (1973) Active subunits of crystalline B-haemocyanin of Helix pomatia obtained by trypsin treatment. Arch. Int. Physiol, Biochim. 81: 968.

24. GIELENS, C., G. PREAUX, and R. LONTIE (1975) Limited Trypsinolysis of B-Haemocyanin of Helix pomatia. Eur. J. Biochem. 60: 271-280.

25. GIELENS, F.S., G. PREAUX, and R. LONTIE (1974) Sodium dodecyl sulfate electrophoresis on polyacrylamide gel of functional tryptic fragments of crystalline B-haemocyanin of Helix pomatia. Arch. Int. Physiol. Biochim. 82: 787.

26. GRONINGER, H.S., and H.A. DASSOW (1964) Observations of the 'Blueing' of King Crab, Paralithodes camtschatica. Fish. Ind. Res. 2(3): 47-52.

27. GRUENWEDEL, D.W., and R.K. PATNAIK (1971) Release of Hydrogen Sulfide and Methyl Mercaptan from Sulfur-Containing Amino Acids. J. Agr. Food Chem. 19(4): 775-779.

28. GULLICK, W.J., and E.J. WOOD (1978) Physical Properties of Small Functtional Units Obtained from Gastropod Mollusc Haemocyanins by Tryptic Digestion. Biochem. Soc. Trans. 573rd Meet. 6: 386-389.

29. HENDRICKSON, W.A. (1977) The molecular architecture of oxygen-carrying proteins. TIBS. May: 108-112.

30. HUBER and LEACH (1986) Invertebrate oxygen carriers. Ed. Bernt Linzen, B.

31. INOUE, N., and T. MOTOHIRO (1970) A Cause and Mechanism of Blue Discoloration of Canned Crab Meat - I. A Chemical Analysis and Histological Observation of Blue Meat. Bull. Jap. Soc. Sci. Fish. 36(6): 588-591.

32. INOUE, N., and T. MOTOHIRO (1970) A Cause and Mechanism of Blue Discoloration of Canned Crab Meat - II. Detection of Haemocyanin in the Blue Meat. Bull. Jap. Soc. Sci. Fish. 36(7): 692-694.

33. INOUE, N., and T. MOTOHIRO (1970) A Cause and Mechanism of Blue Discoloration of Canned Crab Meat - III. Sulphide Reaction of Crab Haemocyanin. Bull. Jap. Soc. Sci. Fish. 36(7): 695-697.

34. INOUE, N., and T. MOTOHIRO (1970) A Cause and Mechanism of Blue Discoloration of Canned Crab Meat - IV. Spectrophotometric Study of Blued Meat. Bull. Jap. Soc. Sci. Fish. 36(9): 945-949.

35. INOUE, N., and T. MOTOHIRO (1970) A Cause and Mechanism of Blue Discoloration of Canned Crab Meat - V. Isolation of the Causitive Substance of Blued Meat. Bull. Jap. Soc. Sci. Fish. 36(10): 1040-1043.

36. INOUE, N., and T. MOTOHIRO (1970) A Cause and Mechanism of Blue Discoloration of Canned Crab Meat - VI. The Mechanism of Copper and Sulphide Reaction in Heat-coagulated Haemocyanin. Bull. Jap. Soc. Sci. Fish. 36(10): 1040-1043.

37. INOUE, N., and T. MOTOHIRO (1971) A Cause and Mechanism of Blue Discoloration of Canned Crab Meat - VIII. Some Observations of Clotting of Crab Haemolymph. Bull. Jap. Soc. Sci. Fish. 37(10): 1011-1014.

38. ION EXCHANGE CHROMATOGRAPHY - Principles and Methods. Pharmacia Sweden.

39. ISOELECTRIC FOCUSING - Principles and Methods. Pharmacia Sweden.

40. JAMES, D.G., and J. OLLEY (1970) Moisture and pH Changes as Criteria af Freshness in Abalone and their Relationship to Texture of the Canned Product. Food Tech. Aust. July: 350-357.

41. JAMES, D.G., and J. OLLEY (1974) The Abalone Industry in Australia. In R. Kreuzer (ed): Fishery Products. Surry, England: Fishery News (Books) Ltd., pp. 238-242.

42. JOHNSON, A.R., and J.R. VICKERY (1964) Factors Influencing the Production of Hydrogen Sulfide from Meat During Heating. J. Sci. Fd. Agric. 15: 695-701.

43. KARLIN, K.D., and Y. GULTNETH (1985) Bioinorganic Chemical Modeling of Dioxygen-Activating Copper Proteins. J. Chem. Educ. 62(11): 983-1001.

44. LESTER, R.J.G., and G.H.G. DAVIS (1981) A New Perkinsus Species (Apicomplexa, Perkinsea) from the Abalone Haliotis ruber. J. Inverterbr. Pathol. 37: 181-187.

45. LONTIE, R., M. DE LEY, H. ROBBERECHT, and R. WITTERS (1973) Isolation of Small Functional Subunits of Helix pomatia Haemocyanin after Subtilisin Treatment. Nature New Biology 242: 180-182.

46. LYTTON, D.G., A.R. EASTGATE, and N.J. ASHBOLT (1985) Digital mapping of metal-bearing amoebocytes in tissue sections of the Pacific oyster by scanning microphotometry. J. Microscopy 138: 1-14.

47. LYTTON, D.G., C.D. GARLAND, and N.T. TUYETHONG (1987) The Gill as a Site of Metal Depuration in the Oyster. Tas. Fish. Res. 28: .

48. MANWELL, C., and C.M. ANN BAKER (1963) Starch Gel Electrophoresis of Sera from some Marine Arthropods. Comp. Biochem. Physiol. 8: 193-208.

49. MONTGOMERY, W.A. (1966) Processing and canning abalone. Australian Fisheries Newsletter. June.

50. MOTOHIRO, T., and N. INOUE (1970) pH of Canned Crab Meat. Food Tech. 24,1389: 71-73.

51. NICKERSON, K.W., and K.E. VAN HOLDE (1971) A Comparison of Molluscan and Arthropod Hemocyanin - I. Circular Dichroism and Adsorption Spectra. Comp. Biochem. Physiol. 39B: 855-872.

52. OLLEY, J. (1970). Unpublished Results.

53. OLLEY, J., and S.J. THROWER (1977) Abalone - An Esoteric Food. In (ed): ADVANCES IN FOOD RESEARCH. : ACADEMIC PRESS INC., pp. 143-183.

54. PECINA, R., P. BURTSCHER, G. BOON, and O. BOBLETER (1986) GC-MS and HPLC analyses of lignin degradation products in biomass hydrolyzates. Fresenius Z Anal. Chem. 325: 461-465.

55. PILSON, M.E.Q. (1965) Variation of Hemocyanin Concentration in the Blood of Four Species of Haliotis. Biol. Bull. 128: 459-472.

56. PYEUNS, J.H., K. HASHIMOTO, and F. MATSUURA (1973) Isolation and Characterization of Abalone Paramyosin. Bull. Jap. Soc. Sci. Fish. 39(4): 395-402.

57. REDFIELD, A.C. (1934) The Haemocyanins. Biol. Rev. 9: 175-212.

58. RICCHELLI, F., G. JORI, L. TALLANDINI, P. ZATTA, M. BELTARAMINI, and B. SALVATO (1984) The Role of Copper and Quaternary Structure on the Conformational Properties of Octopus valgaris Hemocyanin. Arch. Biochem. Biophys. 235(2): 461-469.

59. RUDDELL, C.L. and RAINS, D.W. (1975) The relationship between zinc and copper and the basophils of crassostreid oysters, C. gigas and C. virginica. Com. Biochem. Phys. 51A, 585-591.

60. SAMUEL, E.L. (1985) Structure of Abalone Hemocyanin. Unpublished Results.

61. SIEZEN, R.J., and R. VAN DRIEL (1973) Structure and Properties of Hemocyanins III. Microheterogeniety of a-Hemocyanin of Helix pomatia. Biochim. Biophys. Acta. 295: 131-139.

62. SIEZEN, R.J., and R. VAN DRIEL (1974) Structure and Properties of Hemocyanins XIII. Dissociation of Helix pomatia a-Hemocyanin at Alkaline pH. J. Mol. Biol. 90: 91-102.

63. SULLIVAN, B., J. BONAVENTURA, and C. BONAVENTURA (1974) Functional Differences in the Multiple Hemocyanins of the Horseshoe Crab, Limulus polyphemus L. Proc. Nat. Acad. Sci. USA 71(6): 2558-2562.

65. THROWER, S.J. (1977) Blue spot in abalone - possible causes and solutions. Aust. Fish. 36(10): reprint.

66. THROWER, S.J., and H.A. BREMNER (1987) Orange roughy - a guide to handling, chilling and processing. Aust. Fish. Nov. : 22-28.

67. VAN BRUGGEN, E.F.J. (1980) Hemocyanin: the mystery of the blue blood. TIBS. July: 185-188.

68. VAN HOLDE and VAN BRUGGEN (1971) Subunits in biological systems. (Eds. S.N. Timasheff and G.D. Fasman). pp.1-53. Marcel Dekker N.Y.

69. WARNE, A.D., and N. BROWN (1982) The Influence of thermal processing severity on the yield and texture of canned abalone (Notohaliotis ruber). Food Tech. Aust. 34(10): 448-451.

70. WAXMAN, L. (1975) The Structure of Arthropod and Mollusc Hemocyanins. J. Biol. Chem. 250(10): 3796-3806.

71. YOUNG, F., and J. OLLEY (1974) Studies on the Processing of Abalone VI The Effect of Brine Composition on the Quality and Yields of Canned Abalone. Food Tech. Aust. March: 96-107.

72. NEWEBOOM, B., BEUKEVELD, G.J.J., BEINTEMA, J.J. (1986) Amino acid sequence studies on the c chain of Panularus interruptus hemocyanin. Invertebrate oxygen carriers. pp169-176.

73. WAGNER, E.J. (1987) Stress in cultured fish. Aquaculture Magazine Nov/Dec 1987.

74. KONINGS, W.N., R. VAN DRIEL, E.F.J. VAN BRUGGEN, and M. GRUBER (1969) Structure and Properties of Hemocyanins V. Binding of Oxygen and Copper in Helix pomatia Hemocyanin. Biochim. Biophys. Acta 194: 55-66.

75. LAMMY, J., J. LAMMY, and J. WEILL (1979) Arthropod Hemocyanin Structure: Isolation of Eight Subunits in the Scorpion. Arch. Biochem. Biophys. 193(1): 140-149.

Appendix A

A HISTOLOGICAL INVESTIGATION OF BLUED FRILL.

I. A histological investigation of blued Tasmanian abalone was undertaken by Mr Chris Russel (Department of Zoology, University of Melbourne), and is reported as Parts II and III of this Appendix. Blued section of flesh were examined under an optical microscope using a variety of different staining techniques and compared with similarly prepared white tissue sections. An in-depth investigation of hemolymph and accurate cell structural identification was difficult due to the poor quality of the samples, however, the study revealed definite differences between the white and blued flesh.

Large numbers of unidentified bodies (UBs) were found throughout the muscular tissue matrix of the foot muscle and the epipodium (see Fig. A1, A2, A3). Comparisons of the different samples indicate that the UBs are more prevalent in the New Zealand abalone (all blued), with an intermediate amount in the blued frill tissue, and almost totally absent in the white tissues - which were used as a control. Examination of canned blue and white abalone tissue sections produced a similar distribution that seems to correlate the presence of these UBs to the observed discolourations. A summary of the main features of the UBs is as follows -

(i) The UBs consisted of non-staining, spherical bodies, 6 to 10 μm in diameter in the blue (frill) tissue, and 10 to 18 μm in diameter in the "all Blue" (New Zealand) abalone tissue.

(ii) An encapsulating pellicle appeared to surround the bodies.

(iii) The contents of the bodies appear granular - the single granules being approximately 1 to 2 μm in diameter.

(iv) The UBs appear to lack a nucleus.

(v) Many of the spherical bodies appear to have lysed, releasing their contents into the surrounding tissues. Whether this is a result of the tissue processing techniques or not is uncertain.

(vi) It is probable that the UBs are trapped within the smaller blood channels. In abalone, such blood channels exists as spaces within the muscular connective tissue matrix of the foot and are difficult to recognize in the distorted tissue investigated here.

Preliminary identification attempts from local sources (within the University) were unfortunately unsuccessful. However, zinc and copper containing amoebocytes have been reported to be concentrated in the gills of green sick oysters (O. edulis) (21). It is thought that these cells serve to detoxify the metals that may accumulate by active intake through the food chain or passive osmotic diffusion from the environment. They are also thought to serve in a defensive role against bacterial infections (11).

The processes of metal ion uptake from aquatic environments are not well understood. It has been proposed that for some metals, the concentration within the organism is directly equivalent to the environment suggesting a passive uptake mechanism. Although many ions are essential for most biological processes, the metals accumulated often exceed the required amount many times over. Such accumulated excess metal must be detoxified and stored in a form that can be moved around the animal for utilisation or expelled from the animal. The poorly developed kidney in bivalves such as oysters has led to the loss of metal-containing amebocytes from the body surface as the major route of metal excretion.

Ruddell (59) found copper to be concentrated around wounded areas of oyster muscle and proposed that metal bearing amebocytes play an essential role in its inflammatory response. It was reported that 2-12 hours after making an incision in the oyster mantle, the areas directly adjacent to the wound became a pale green colour which has been attributed to the deposition of copper by cellular amebocytes. Hence, the presence of green discolourations in oysters may be associated with a disease process.

It has not been confirmed whether the UBs observed here are cellular amebocytes similar to those reported for other molluscs but the existence of such mechanisms are likely. The extremely high levels of copper in flesh found in blued abalone (see Section 6) and also noted by Olley (52) would probably involve some sort of copper transport and storage

other than hemocyanin. Little is known about the pathogenic defence system of molluscs but the lack of a blood clotting mechanism suggests the need for amebocyte-like cells. The amebocytes of C. gigas as described by Lytton et al., (47) also show remarkable similarities in size and micro-cellular features to those found in abalone. Further examinations under electron microscopy of our samples did not prove to be practical.

In yet unpublished work by Dr J. D. Smith (Department of Inorganic Chemistry, University of Melbourne) it has been found that copper tends to be distributed more evenly throughout abalone flesh unlike some other metals examined. This observation does not conflict with the likely concentration of copper in the UBs which appear to be associated with blued regions; it does help however to explain generalised blueing throughout the flesh observed in some samples where phenolase activity can be suspected.

II. Materials and Methods.

The following samples of abalone underwent histological investigation:

1. "Control" - white flesh.

2. "Blue frill" - blue discolouration of the flesh of the epipodium and extending approximately 5 to 8 mm dorsally from the ventral surface of the foot.

3. "All blue" - more intense discolouration of the flesh in the same distribution as blue frill. In addition, a slight discolouration throughout the remaining flesh of the foot and shell muscle.

4. "Canned blue" - specimens possessing discolouration approximately that of "blue frill", but which have undergone the canning process.

5. "Canned control" - canned white flesh.

Routine histology:

Pieces of tissue extending from the epipodium to the ventral surface of the foot were dissected from each of the samples and thawed at room temperature if frozen. Tissues were fixed in both Bouin's fluid with 3% NaCl and 10% formalin in sea water. Fixed tissues underwent routine paraffin embedding, were sectioned at 10 μm, and stained with Delafield's haematoxylin and eosin or Mallory's triple stain.

III. Results and Discussion.

Comparison of gross tissue morphology did not reveal any recognizable differences between the blue and non-blue samples. Investigation at this level was made difficult by the poor quality of the tissues, with a large degree of cellular distortion and patchy staining characteristics occurring. Such distortion, caused by ice crystal formation within the tissue, made conclusions regarding differences in the predominating muscular/connective tissue matrix difficult. To avoid such ice formation in the future, tissues should be either snap frozen in liquid nitrogen or placed in fixative when fresh.

Blood vessels and haemolymph:

Very little haemolymph remained in the blood vessels in the prepared sections. This may be due to large amounts of blood draining from the animals as a result of shell and viscera removal. When haemolymph was visible, no abnormalities were observed, however the resolution of the light microscope is too low for useful study of haemolymph - an electron microscopic study would provide more information in this regard.

Smear samples of haemolymph exhibited large amounts of bacteria within the plasma. The origin of the bacteria is most likely due to contamination of the sample after extraction from the animal (pers. comm. - Microbiology Department, University of Melbourne), however this could be further investigated by ensuring totally aseptic conditions from the time of sample extraction to microscopic examination.

Abnormalities in the discolured tissue:

The histologial study revealed large numbers of unidentified foreign bodies (F. Bs.) throughout the muscular/connective tissue matrix of the foot muscle and the epipodium. The F. Bs. were present in very large numbers in the "all blue" tissue, were almost totally absent in the "control" tissue, and were present in an intermediate amount in the "blue frill" tissue. The F. Bs. were present in the "canned blue" but were absent in the "canned control". Details of the F. Bs. are as follows:

- Distribution of the F. Bs. appears to correlate with the distribution of the discolouration in the raw tissue - "blue frill" tissue contained many more F. Bs. in the epipodium (frill) and in the region of the ventral surface of the foot than deeper within the foot. The F. Bs. within the "all blue" tissue was much more evenly dispersed.

- The F. Bs. consisted of non-staining, spherical bodies, 6 to 10 μm in diameter in the "blue frill" tissue, and 10 to 18 μm in diameter in the "all blue" tissue.

- An encapsulating pellicle appeared to surround the bodies.

- The contents of the bodies appeared granular - the single granules being approximately 1 to 2 μm in diameter.

- The F. Bs. appeared to lack a nucleus.

- Many of the spherical bodies appeared to have lysed, releasing their contents into the surrounding tissue. Whether this is as a result of the tissue processing techniques or not is uncertain.

- It is probable that the F. Bs. are trapped within the smaller blood channels. In abalone, such blood channels exist as spaces within the muscular/connective tissue matrix of the foot and are difficult to recognize in the distorted tissue investigated here.

- Identification attempts:

The lack of a distinct nucleus suggests that bodies are prokaryotic, however they are too large to be bacteria.

The possibility that the F. Bs. were fungal spores of some type was suggested, however this was ruled out following assistance from Dr Harry Swart (Botany Department, University of Melbourne).

The F. Bs. may represent a stage in a parasitic life cycle, with the nucleus not taking up stain. Parasites usually cause an obvious disruption to the surrounding tissue, a phenomenon which has not occurred in the tissues examined.

The possibility remains that the F. Bs. are a type of algae.

General Comments:

As the abalone apparently possessed, or were predisposed to, the blue discolouration immediately after removal from the water, it appears that some factor is affecting the abalone in their natural habitat. The discovery of the F. Bs. in a distribution and abundance approximating the degree of discolouration in the affected abalone strongly suggests that the bodies are relevant to the condition.