

3.3.7 Recombinant cloning of rHA2

The PCR conditions were optimised where 100 ng of DNA with 200 ng of Forward and Reverse primers respectively yielded maximum product without contaminating bands (Figure 3.15). The water control substituting for DNA in Lane 6 ensured there was no contaminating DNA in the buffers and enzymes used. The genomic DNA preparation (lanes 7 and 8) was shown not to be degraded (Figure 3.15). The expression vector was constructed as described (3.2.9.1) and the insert ligated into the vector. The cloning junctions were sequenced and found to be in frame and alignment of the deduced amino acid sequence of *rHA2* clone C was shown to be 100% compatible with the *RgpA* proteinase of *P. gingivalis* [(Pavloff *et al.*, 1995) ; accession number U15282] (Figure 3.16). When the ligation mix was transformed into NM522 competent cells, 65 colonies grew on the overnight LB plates (Appendix A2). The colonies selected were then grown in broth and the purified plasmids digested to check for inserts. Clones A, B, C and D were found to have inserts when digested.

Clones A-C were chosen to screen for expression of the rHA2 protein. This was performed under denaturing conditions to isolate any tagged proteins independent of their solubility within the cell. The 6× His tag will also be completely exposed under denaturing conditions, which increases binding and therefore yields, when compared to purification under native conditions. Expression levels for all clones tested were comparable; Clone C was arbitrarily chosen as the working recombinant clone.

Clone C was initially purified under native conditions using the Ni-NTA resin (3.2.9.4). SDS-PAGE analysis (2.4.2) of the eluted fractions on 16% resolving gels showed that the recombinant clone was able to be easily isolated from contaminating proteins from *E. coli* (lane 10, Figure 3.17). Since the HA2 protein had been reported to have the functional capacity to bind haemoglobin (Nakayama *et al.*, 1998), the native configuration of the rHA2 was tested by utilising haemoglobin-Agarose beads according to the conditions described in that paper. However, it was

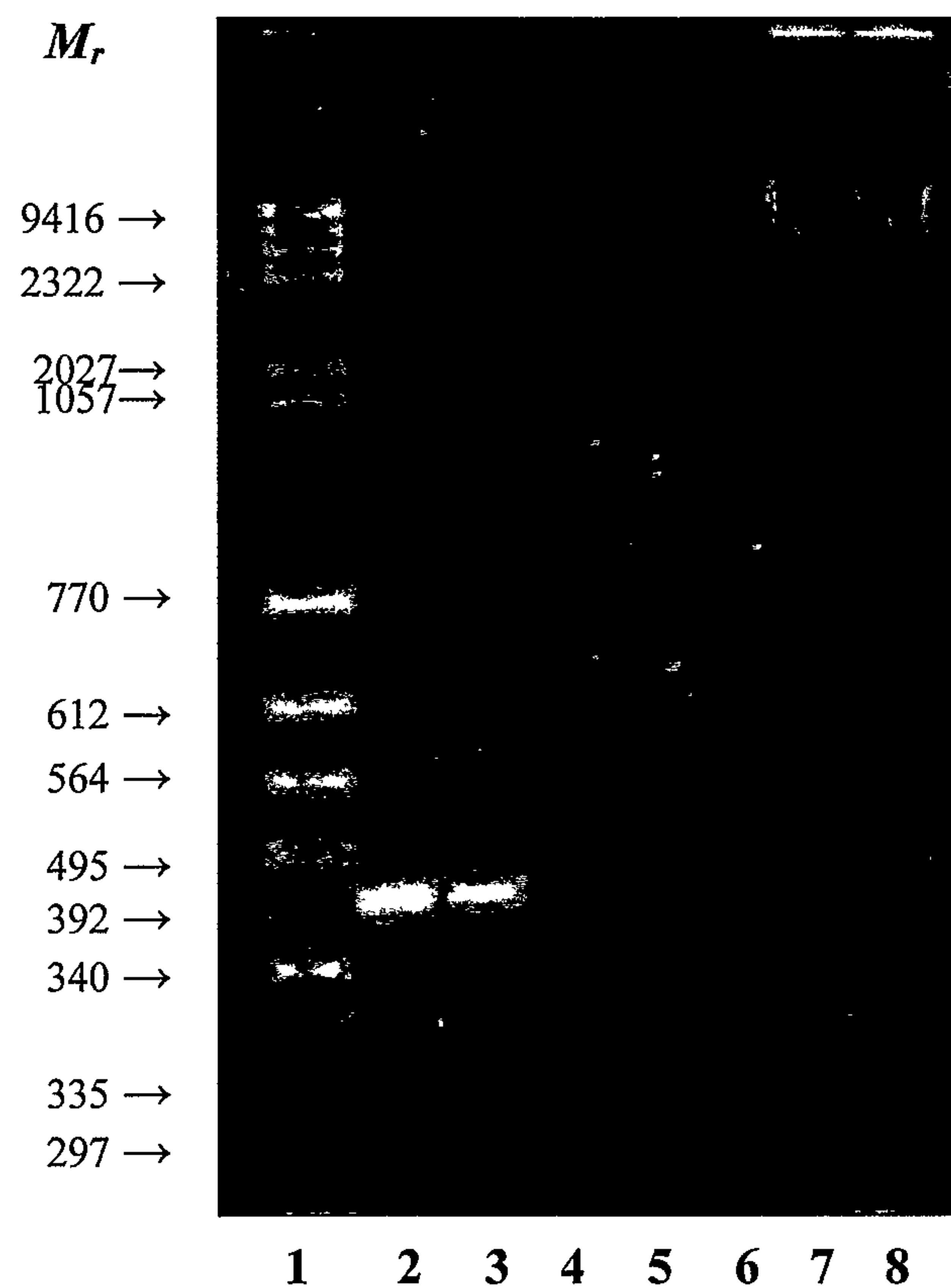


Fig. 3.15 Agarose gel (1%) showing PCR amplification of *rHA2* clone. Lane 1, λ DNA-*Hind*III / ϕ X-174-*Hinc*II M_r markers; Lane 2, PCR 1 (100ng DNA/ 200ng Primer 1+2); Lane 3, PCR 2 (50ng DNA/ 100ng Primer 1+2); Lane 4, PCR 3 (25ng DNA/ 50 ng Primer 1+2); Lane 5, PCR 4 (12.5ng DNA/ 25 ng Primer 1+2); Lane 6; H₂O control; Lane 7, 1.08 μ g genomic DNA; Lane 8, 2.70 μ g genomic DNA.

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21  .RADFTETFESSTHGEAPAEWTTIDADGDGQGWLCLSSGQLDWLTAHGGT 69
      ||||||||||||||||||||||||||||||||||||||||||||||||
1451 KRADFTETFESSTHGEAPAEWTTIDADGDGQGWLCLSSGQLDWLTAHGGT 1500
      . . . . .

70  NVVASFSWNGMALNPDNYLISKDVTGATKVKYYYAVNDGFPGDHYAVMIS 119
      ||||||||||||||||||||||||||||||||||||||||||||||||
1501 NVVASFSWNGMALNPDNYLISKDVTGATKVKYYYAVNDGFPGDHYAVMIS 1550
      . . . . .

120 KTGTNAGDFTVVFEETPNGINKGGARFGLSTEANGAKRSHHHHHH*A*LA 169
      ||||||||||||||||||||||||||||||||||||. .: . |:
1551 KTGTNAGDFTVVFEETPNGINKGGARFGLSTEANGAKPQSVWIERTVDLP 1600
      . . . . .

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Figure 3.16 Alignment of deduced amino acid sequence of *rHA2* Clone C (yellow) with the matching sequence in the RgpA proteinase of *P. gingivalis* [(Pavloff *et al.*, 1995) ; (accession number U15282)].

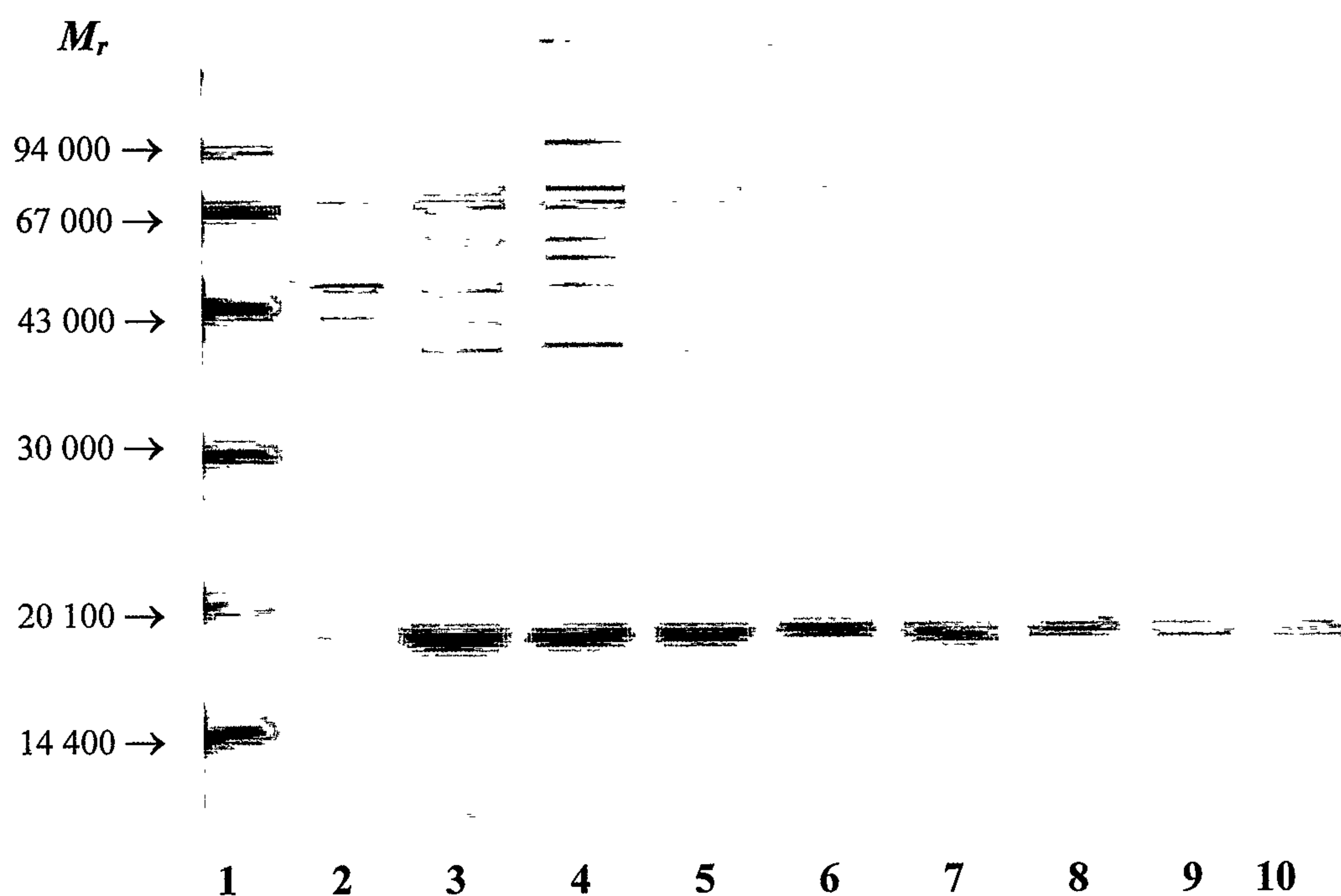


Figure 3.17 Coomassie stained SDS-PAGE showing the batch purification fractions (#1-9) of Clone C. Lane 1 contains low range M_r markers; Lane 2, fraction #1; Lane 3, fraction #2; Lane 4, fraction #3; Lane 5, fraction #4; Lane 6, fraction #5; Lane 7, fraction #6; Lane 8, fraction #7; Lane 9, fraction #8; Lane 10, fraction #9.

found that the protein sample did not bind well to the haemoglobin-Agarose. This implied that the recombinant protein was not expressed in a functionally folded form. A commonly used method to refold proteins is to denature the protein completely using a chaotropic agent (eg. guanidine or urea) then refold the protein while one end of the protein is fixed (3.2.9.5).

The crude lysate (147 mg) from Clone C was prepared under denaturing conditions and loaded onto a Ni-NTA column. While attached to the Ni-NTA column, the rHA2 from Clone C was denatured and refolded according to the method described in Section 3.2.9.5. The eluted protein was then prepared for the haemoglobin column and the sample found to bind well and elute in Tris-HCl (pH 9.0) as expected (Figure 3.18). The different stages of the purification process are demonstrated in Figure 3.19 where the lysate, flow-through, wash and eluate fractions were run on 16% SDS-PAGE gels (2.4.2). The yield of purified rHA2 using the denaturing and refolding technique was 300 µg/L of soluble protein. A 30 kDa protein was demonstrated to co-elute with the 19 kDa protein (See arrow in lane 4, Figure 3.18). Since both protein bands seemed to be equally intense, it was postulated that the higher band might be a dimer of the rHA2 protein which was possible if the reducing conditions used were not stringent enough for the dimer to be reduced. The eluate was run on a 16% SDS-PAGE gel (2.4.2) with and without the reducing agent β-mercaptoethanol. The proteins from the gel were transblotted onto a PVDF membrane (2.4.3) and probed (3.2.10), using the monoclonal antibody VAI which recognises an epitope on the HA2 domain (DeCarlo *et al.*, 1999). Under non-reducing conditions this band was demonstrated to be a dimeric form of the rHA2 (lane 1, Figure 3.19). Using different reducing conditions to the standard protocol referred to in 2.4.2, the sample was then heated at 95°C for at least 15 min with fresh β-mercaptanol and immediately loaded onto the gel where the rHA2 appeared as a monomer (lane 3, Figure 3.19). This confirmed that rHA2 behaved as a dimer under non-reducing conditions. However, when the rHA2 was tested in the *in-vitro* vascular network disruption assay, it was found over multiple experiments to be inactive in concentrations up to 6.6 µM of protein.

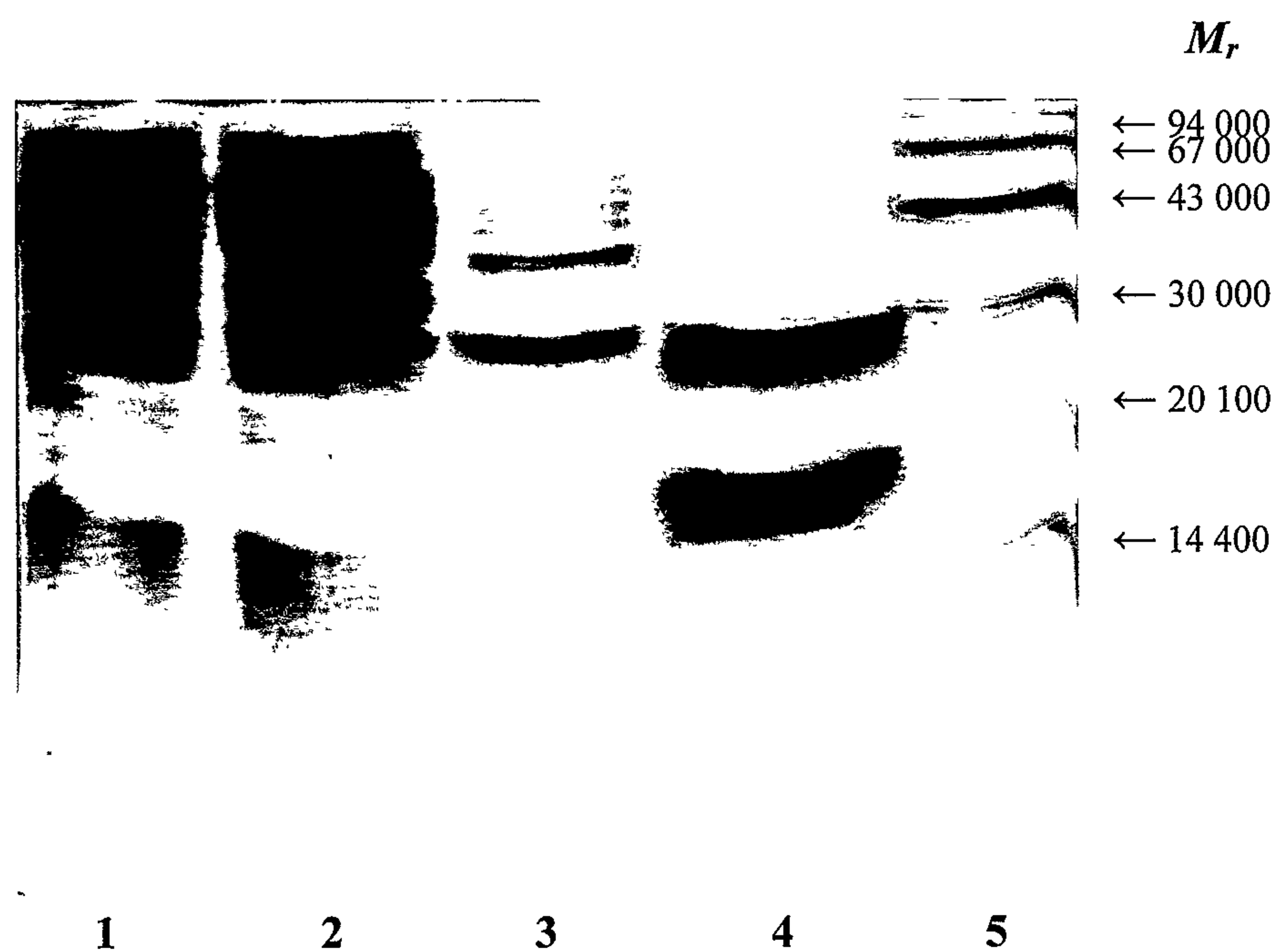


Fig. 3.18 Coomassie stained 16% SDS-PAGE gel (2.4.2) of denatured lysate containing recombinant HA2 which was refolded then eluted from Ni-NTA resin affinity columns. Lane 1, cleared lysate; Lane 2, flow through; Lane 3, wash; Lane 4, eluate showing two dominant bands at 19 kDa and 30 kDa; Lane 5, low range M_r markers.

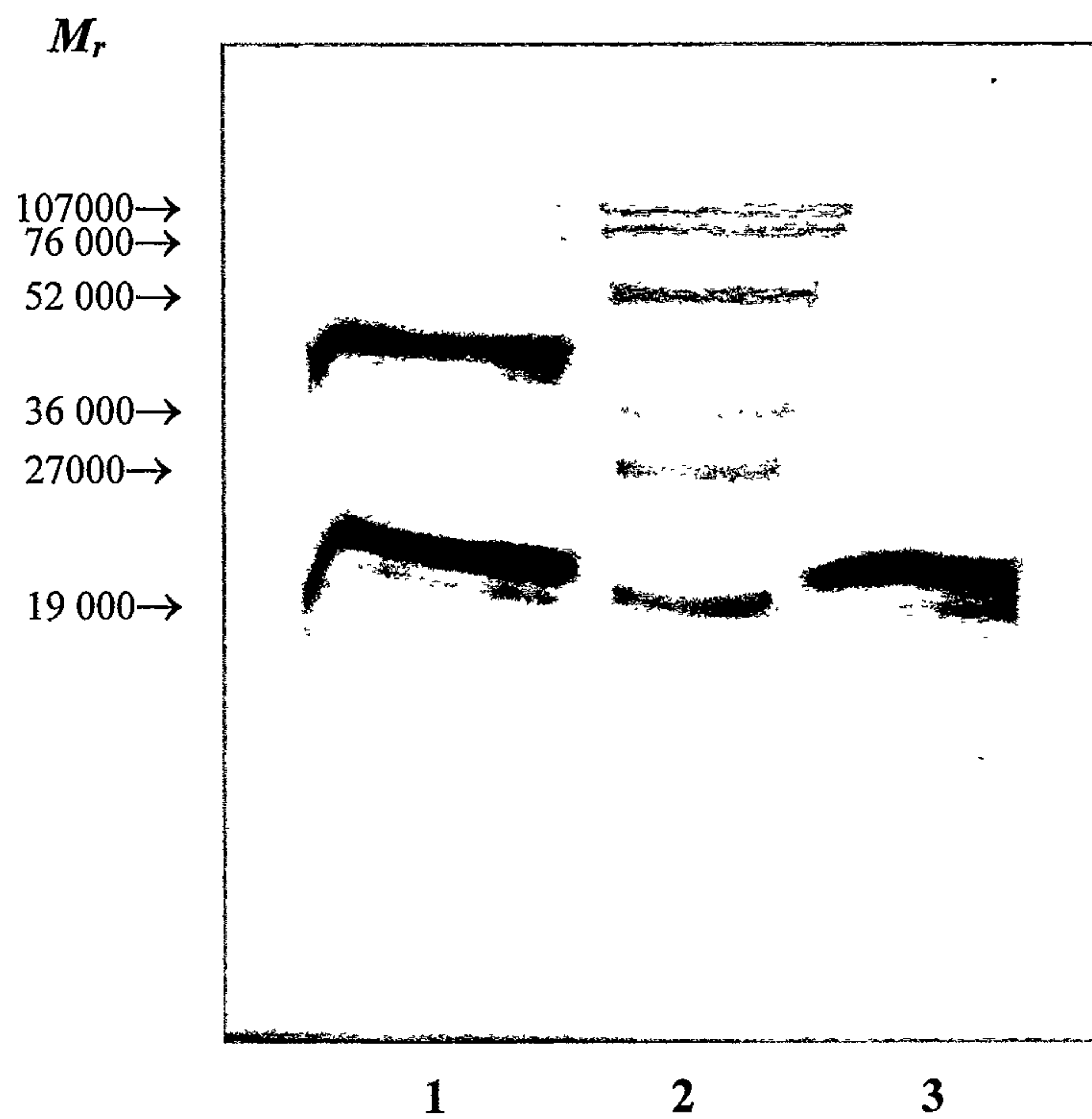


Fig. 3.19 Western blot of recombinant HA2 developed with alkaline phosphatase. Lane 1, Clone C (boiled in SDS sample buffer under non-reducing conditions (no mercaptoethanol)); Lane 2, low range M_r markers; Lane 3, Clone C (boiled in SDS sample buffer with mercaptoethanol for 15 min and loaded immediately).

3.4 DISCUSSION

The crude extract from *Porphyromonas gingivalis* was capable of causing vascular disruption of pre-formed vascular networks on Matrigel. Heat-inactivation of the sample eliminated this disruption suggesting that it was a protein effect rather than lipopolysaccharide. *Prevotella intermedius* and *F. nucleatum* extracts failed to elicit a similar reaction when used to challenge at identical protein concentrations.

It was necessary to isolate this Vascular Disruptive Factor (VDF) from *P. gingivalis* before any further study on the protein-cell-matrix interactions could be attempted.

Since the SSE was to be used in a biological system, a protease inhibitor cocktail was avoided. Despite the degradation that would have occurred, the vascular disruptive activity remained stable both at the storage temperature of 4°C indefinitely and at the 37°C needed for the bioassay. All purification stages were treated in a similar manner with the gel filtration profiles being reproducible with each batch of extract despite not using protease inhibitors and running the column at room temperature.

The presence of high molecular weight proteins in the low molecular weight fraction could be explained by the poor resolution of the sample where the tail of the middle molecular peak trails into the low molecular weight fractions. This is quite possible as the low molecular weight fraction was a minor component only and using only the middle part of the peak (theoretically the purest part of the peak) would have resulted in a very low protein yield. Since this was only the first part of the purification protocol the entire low molecular weight peak was pooled to screen for the presence of activity in a broader molecular weight range. However, the poor solubility of the high and middle molecular weight fractions was suggestive of the presence of lipopolysaccharide (LPS). The contaminating presence of LPS might make all the proteins more “sticky” with the sample being less likely to resolve efficiently. Recently, it was reported that the haemagglutinin/adhesin domains common to the high molecular weight gingipains have the capacity to bind to phospholipids (Imamura *et al.*, 2000). This might explain the presence of the HA2 domain in the high and middle molecular weight peaks. The lack of substantial

increase in potency between the crude extract and the low molecular weight gel filtration fraction could be related to the active protein of interest being dispersed throughout the fractions, reducing the biological potency of the sample during purification.

The HA2 domain has 7 phenylalanine, 5 tyrosine and 4 tryptophan residues, that is only 12% of the protein consists of residues with aromatic side chains. Fujimura *et al.* have also reported the pI of the HA2 domain to be 4.3 with implication that the protein is very acidic (Fujimura *et al.*, 1996). This would explain the extremely hydrophilic nature of the semi-purified sample and the lack of binding observed on the Alkyl-Superose column.

Although the molecular weight of the HA2 was calculated to be 15 kDa, it was shown to migrate at either 19 kDa or 17 kDa on SDS-PAGE. This variation appeared to be based on the percentage acrylamide gel used. The HA2 protein appeared to migrate at 19 kDa with 12% and 16% gels while 20% gels used in the analytical gels for the Mini Prep cell resulted in the protein migrating at 17 kDa. Poor yield from the purification process and the difficulty of separating the closely related adhesin domains required the production of recombinant HA2. The purified rHA2 was re-tested in the bioassay at concentrations of 67 nM, 0.67 μ M, 1.3 μ M, 3.3 μ M and 6.7 μ M of protein respectively. It did not appear to be active at any of these concentrations. When the major proteins in the ion-exchange fraction (3.3.4) were transblotted and identified by N-terminal sequencing (3.3.6), the HA2 domain was chosen for cloning as it was the major protein in the sample. However, it is possible that the activity might be related to the other adhesin domains sequenced, namely HA3 (17 kDa) and HA4 (27 kDa). The effect seen could also be due to incorrect refolding of the recombinant protein expressed. Despite being passed through a haemoglobin-Agarose column to ensure that only functional protein was purified, there is no certainty that the other functions have been retained after the refolding process. One of the difficulties of over-expression is the reproduction of native structure. Under native conditions the 6 $\times$ His Tag was not well exposed and did not bind well to the Ni-NTA resin. One of the standard procedures recommended was utilised whereby the pellet was solubilised using denaturing agents and the peptide

refolded while still attached to the Ni-NTA column. This resulted in the binding and elution of putative functional peptide from the haemoglobin-Agarose column. However, the anti-gingipain mAb VA1 (DeCarlo and Harber 1997; DeCarlo *et al.*, 1999) was found to bind rHA2 but not native HA2, implying a difference in the configuration of the two forms of the protein which might explain the lack of activity of rHA2 in the vascular disruptive assay.

The rHA2 was found to exist in both a monomeric and dimeric form. This dimeric form was not evident during purification of the HA2 from *P. gingivalis* ATCC 33277. The presence of the dimer was detected although the SDS-PAGE analysis in Figure 3.20 was under SDS-PAGE reducing conditions. This implied that the standard reducing conditions used were not sufficiently stringent to reduce all dimer forms to a monomeric state (2.4.2). Therefore to reduce the sample completely, it was necessary to heat for a longer time frame and load immediately to avoid re-association of the monomers. It is not clear why the dimer migrates as a 30 kDa protein which would be closer to the true molecular weight of two HA2 domains ($15 \text{ kDa} \times 2$) while the monomer runs as a 19 kDa protein. Preliminary work in a collaborating laboratory (Langley, Menz and Collyer, Department of Biochemistry, University of Sydney) has also observed the homo-dimerisation of rHA2 in the oxidised state. A 30 kDa haemin-binding cell envelope protein has been reported (Kim *et al.*, 1996). This could have been the native form of the HA2 domain. It is not clear what role the dimeric form of rHA2 would play in the *in-vitro* assays. Dimerisation of rHA2 might inhibit potential binding to the HUVEC and prevent vascular disruption of the networks.

Since attempts to over-express the rHA2 domain in *E. coli* produced only small yields of protein, it was possible that the codon bias due to an excess of rare low tRNA codons displayed by the *P. gingivalis* gene led to translational problems and prevented high level expression in an *E. coli* host (Kane 1995). Purification of the VDF proved to be a difficult task due to poor resolution of the biological activity despite the different methods used. The lack of increase in biological potency was complicated by the non-quantitative nature of the Matrigel assay. This made

assessment of this increase less precise at the different stages of the purification procedure.

Consequent sequencing of the majority protein bands showed that these proteins belonged to an important family of cysteine proteinases referred to as the “gingipains”. Interestingly, the RgpA and RgpB (Arg-gingipains) and Kgp (Lys-gingipains) complex has been reported to interact with the vasculature. Rgp-1 was reported to be the major vascular permeability enhancement (VPE) factor of *P. gingivalis* by inducing this activity through prekallikrein activation and subsequent bradykinin release (Imamura *et al.*, 1995). However, Scott *et al.* showed a lysine-specific proteinase from *P. gingivalis* to be a potent factor in the release of bradykinin from human high-molecular-weight kininogen *in-vitro* (Scott *et al.*, 1993). Imamura later showed that the organism interferes with plasma clot formation by digesting fibrinogen with either vesicle-bound or secreted cysteine proteinases, demonstrating a pathogenic correlation for fibrinogen digestion with a tendency to bleed at periodontitis sites (Imamura *et al.*, 1995).

Imamura *et al.* also reported that Rgp associated with vesicles was responsible for most of the VPE activity generation via plasma prekallikrein activation and subsequent bradykinin production while a secondary pathway for VPE activity production was dependent on the direct release of bradykinin from high molecular weight kininogen by the concerted action of Rgp and a Lys-specific cysteine proteinase (Kgp), also associated with vesicles (Imamura *et al.*, 1995). The 95-kDa Rgp-1 is capable of activating factor X with a 5-fold greater efficiency than the 50-kDa Rgp-2 which essentially contains only the catalytic domain (Imamura *et al.*, 1997). This profile has recently been shown to be similar for the activation of protein C which is a negative regulator of blood coagulation and where Rgp-1 is 18-fold more efficient than Rgp-2. It is clear from the literature that the cysteine proteinase complex plays an important role in vascular interaction. Although the rHA2 domain does not appear to play an obvious role in the vascular disruption seen in the Matrigel model, it is possible that the whole complex might play a role. It was necessary then to purify the Rgp/Kgp complex and re-test it in the *in-vitro* Matrigel model. The nature of this bioassay highlighted a need for quantitation which would allow

graphical and statistical presentation of the level of disruption. This aspect is addressed in Chapter 4.

CHAPTER FOUR

THE EFFECT OF THE RgpA COMPLEX FROM *PORPHYROMONAS GINGIVALIS* ON VASCULAR ENDOTHELIAL NETWORKS

4.1 INTRODUCTION

The need for a quantitative bioassay to more accurately analyse the observed vascular disruption was highlighted in Chapter 3. In order to obtain useful information, the model chosen needed to fulfil certain criteria. The critical aspect of the model was to be able to challenge the networks with a known concentration of bacterial product and easily observe and quantify any changes. Since the vascular pathology represented remnants of basement membrane proteins, a requirement was for the model to represent the late stage of angiogenesis.

The Matrigel assay fulfilled most of these criteria but there did not exist a known method for quantifying the surface area covered by these networks. In studies described in Chapter 3, the difficulty in monitoring an increase in biological potency could have been related to the inefficiency of the present model. Perusal of the literature did not reveal any *in-vitro* model systems which were capable of fulfilling all these criteria. To provide comparative data with statistical analysis of any in vascular disruption over time, a method to quantify the networks was developed for the existing Matrigel assay (4.2.5/4.2.6).

4.2 METHODS AND MATERIALS

4.2.1 Purification protocol for RgpA/Kgp

The use of vesicle-bound proteinases would have been an appropriate form of the Rgp/Kgp complex to test as it represents the biological carrier of the *P. gingivalis* proteinases. However, since both RgpA and Kgp would be indistinguishable in this

form, it would be necessary to specifically inhibit either one of the catalytic domains to evaluate the role of each. Leupeptin is a specific inhibitor for the RgpA complex but is reversible and therefore must be maintained within the assay. In the context of a biological assay, this could prove toxic to the cells. TLCK is also used to inhibit the complex and is irreversible but inhibits both enzymes (Table 4.1). For the purposes of this study it was necessary to be able to purify RgpA and Kgp separately for informative evaluation of their role in the vascular disruptive activity.

The method used was a modified protocol (Yun *et al.*, 1999) adapted from two separate methods by Ciborowski *et al.* (Ciborowski *et al.*, 1994) and Chen *et al.* (Chen, Z *et al.*, 1992). *Porphyromonas gingivalis* (ATCC 33277) cells were grown in CDC adjusted broth (Appendix B1) under anaerobic conditions for 48 h (3.2.1.1). The bacterial pellet was then extracted in Buffer A (0.05 M Tris, 1.0 mM. CaCl₂ (pH 7.5)) with 0.75% CHAPS (a non-denaturing zwitterionic detergent) by gentle mixing for 2.0 h. Insoluble material was separated from the detergent extract by centrifugation (8,000 × g, 15 min, 4°C), and the supernatant fractionated over a Resource-Q FPLC column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with Buffer A containing 1% CHAPS at a flow rate of 60 ml/h. Bound proteins were eluted from the column with the same buffer containing 1.0 M NaCl. The Resource-Q eluate was dialysed against Buffer A. Final purification was achieved by affinity chromatography over an arginine-Sepharose column (XK 26; Pharmacia Biotech, Sweden) previously equilibrated with Buffer A. The dialysed sample was applied at a flow rate of 60 ml/h, and the column was washed with 0.5 M NaCl in the same buffer. Kgp was eluted with 0.75 M L-lysine (pH 7.4) in Tris buffer. After re-equilibration, RgpA was eluted with 1 M L-arginine (pH 7.4) in Tris buffer. The protein concentrations of the RgpA and Kgp eluates were determined by Coomassie dye binding (2.4.1).

4.2.2 Enzyme Inhibition and activity assay

The amidolytic activities of the RgpA and Kgp eluates were measured with the substrates *N*-*p*-tosyl-Gly-Pro-Arg *p*-nitroanilide (GPR-pNA) (1.0 mM) and *N*-*p*-tosyl-Gly-Pro-Lys *p*-nitroanilide (GPK-pNA) (1.0 mM). RgpA and Kgp eluates were initially activated with 5.0 mM L-cysteine at 37°C for 5.0 min. TLCK (tosyl-L-lysine chloromethyl-ketone) was added to 4.0 mM final concentration and the samples incubated at 37°C for 30 min. The samples were then dialysed into 5.0 L of PBS (pH 7.4) (3.2.1.2) overnight to remove excess TLCK. To test for residual enzyme activity, 1.0 µg of RgpA or Kgp was pre-incubated in PBS (pH 7.4) containing 5.0 mM cysteine for 5.0 min at 37°C. The enzymes were combined with the substrate GPR-pNA (1.0 mM) and GPK-pNA (1.0 mM) respectively and the rates of hydrolysis measured at 37°C on the basis of the increase in absorbance at 414nm (A_{414}) as measured with a Titertek Twinreader PLUS photometer (ICN/Flow Labs, Sydney, Australia). For the measurement of the effect of stimulating agents or inhibitors on the activated gingipains, the compounds were pre-incubated with enzyme at room temperature for up to 30 min in assay buffer prior to assay for residual amidolytic activities.

The RgpA and Kgp isolated from the cellular fraction of *P. gingivalis* had activity and inhibition profiles characteristic of the gingipains previously published by Yun *et al.* (Yun *et al.*, 1999) (Table 4.1). Based on the activity profiles, the RgpA preparation was ≈99% pure with 1% Kgp, whereas the Kgp preparation was ≈85% pure with 15% Rgp contamination. NH₂-terminal sequencing confirmed the identity of the peptide fragments as RgpA and Kgp domains. Due to the high contamination of RgpA (15%) in the Kgp preparation and its requirement to be used in a biological system, the Kgp preparation was not amenable to testing (4.2.1). Therefore only RgpA was used to challenge endothelial networks established on Matrigel.

4.2.3 Cell culture

Refer to 3.2.2 for methodology.

Table 4.1. Inhibition profile of RgpA and Kgp on the hyrolysis of GPR-pNA and GPK-pNA. Data are the mean average of three separate experiments.

Inhibitor	Conc.	RgpA	Kgp
	mM	% Residual activity	
Leupeptin	0.1	0	100
TPCK	1	0	0
PMSF	10	100	85
Iodoacetamide	10	0	0
	1	50	50
TLCK	2	0	0
HgCl ₂	2	0	0
EDTA	5	10	100
Pepstatin A	0.1	92	100
SDS	10	30	50
N-Ethylmaleimide	5	0	0
α-1 antitrypsin	0.01	90	82

Table 4.2. Activity profiles (μM/μg/min) of RgpA and Kgp on the substrate GPR-pNA amd GPK-pNA in the absence or presence of 5mM L-cysteine.

Cys : L-cysteine. Data are the mean average of three separate experiments.

	RgpA (cys+ve)	RgpA (cys-ve)	Kgp (cys+ve	Kgp (cys-ve)
GPR-pNA assay	9.77	0.1	0.84	0
GPK-pNA assa	0.1	0.14	5.4	0.14

4.2.4 Matrigel tube assay

A range of concentrations of RgpA (40-250 nM) were initially screened in the Matrigel assay and found to be active in the presence of 5.0 mM L-cysteine at all concentrations tested. However the main difference noted was the time taken to reach complete disruption. There was a dose response to the levels of RgpA tested with the lowest levels (40 nM) resulting in 100% vascular disruption by 24 h. However, by this stage the controls started to deteriorate and interpretation of the results became more ambiguous. As the levels of RgpA increased, this window narrowed with 250 nM resulting in 70% disruption within 18 h. Since the quality of the controls was found to be optimal within this 18 h time frame, it was decided to use this concentration of RgpA for quantitative analysis of the vascular disruption observed.

Human umbilical vein endothelial cells (HUVEC) were prepared for the Matrigel assay as described in 3.2.3. Culture media was then aspirated from the wells and replaced with fresh HUVEC growth media with 20% foetal calf serum (Appendix B2) containing purified RgpA or RgpA inactivated with TLCK (40-250 nM). All RgpA samples were activated with 5.0 mM L-cysteine just prior to addition to the test wells. Negative controls received an equivalent volume of PBS (pH 7.4) (3.2.1.2). All testing was carried out in triplicate and the treatments were randomly assigned to coded plate wells.

4.2.5 Photographic recording

Coded capillary networks were photographed using a Zeiss camera mounted on an inverted phase-contrast microscope (Axiovert 10, Zeiss Germany). Photographs were taken of the networks at either $\times 5$ or $\times 20$ magnification immediately after challenge and then between 6-18 h later. A vernier scale was used to record the microscope stage co-ordinates in order to relocate the identical field of the networks at the different times chosen.

4.2.6 Data analysis

The percentage of vascular disruption was calculated by scanning the photographs of the networks and measuring the total network line distance within the

photographic frame at 0, 6, 12 and 18 h after challenge using Scion *Image* (NIH-Image Analysis Software). All the photographs were randomly assigned a code number to avoid bias in measurement. The Scion Image program allowed a selected line distance to be measured and the length of the selected line was automatically added to the results table. This length was then filled and remained locked on the screen to indicate that the area had already been measured and this process continued until all line distances in the field were measured. Triplicates of each time frame were imported into Excel. The total distance measured for the control was assigned a value of 100% and the total distances measured for the Rgp challenged wells were expressed as a % relative to the control. This ensured that the natural loss in distance in the controls was taken into account. Histograms of the results were created based on these percentages. The significance of the change in network complexity as measured by line distance over time was calculated using one-way analysis of variance (ANOVA) where the change between time frames was calculated.

4.2.7 Labelling for confocal analysis

HUVEC cells were seeded into Matrigel coated 4-chamber slides (Nunc, Medos Co. Australia). Cells in coded cultures were challenged with RgpA, RgpA-TLCK and PBS (pH 7.4) (3.2.1.2) controls. The networks were then gently washed 3 $\times$ with warmed PBS (pH 7.4), the buffer was removed, and then the cells fixed with 100% ice-cold methanol for 3.0 min. The slides were rinsed with cold PBS (pH 7.4). The networks were then blocked overnight in 20% horse serum (made up in PBS, pH 7.4). Probes consisted of the primary antibody IIB2 (provided by Dr. A.A. DeCarlo), a monoclonal antibody recognising an epitope on the haemagglutinin domains of the RgpA complex at 40 $\mu\text{g/ml}$ (DeCarlo and Harber 1997). The secondary antibody used was rabbit-anti mouse-FITC diluted in 0.1% bovine serum albumin (BSA)/ NaN_3 for 45 min at 23°C. All wash steps were in PBS (pH 7.4). FITC-Phalloidin for the detection of F-actin, was used at 10 $\mu\text{g/ml}$ diluted in 0.1% BSA/ NaN_3 and applied for 1.0 h at room temperature.

4.2.8 Confocal image analysis

Fluorescence staining of coded cultures was visualised using an Olympus BX60 microscope equipped with a xenon arc lamp, appropriate dichroic filters and a $\times 40$, NA 0.8 water-immersion epi-fluorescence objective. Confocal laser scanning was performed with the Optiscan F900e personal confocal system (Optiscan, Melbourne, Australia). Accumulated digital images were acquired for analysis and three-dimensional (3-D) reconstruction using Voxblast software, version 1.3.1 (Vay Tek, Inc., Fairfield, IA, USA).

4.3 RESULTS

4.3.1 Quantification of changes of network complexity over time

Figure 4.1 A-D represents the control networks remodelled over time as previously described in 3.2.1. To quantify any changes in the networks over time, the complexity was measured and expressed as a percentage relative to 0 hours of challenge (6.0 h of network formation) which represented 100% (4.2.6). The control networks lost some 20% of line length over the 18 h experimental period (Figure 4.1 M).

4.3.2 Vascular disruption

Figure 4.1 E-H clearly demonstrates the disruption of the networks within an eighteen hour study period when challenged with 250 nM Rgp. By 6.0 h there was a 30% loss of network complexity and at 12 h, there was already a 45% reduction in the line distance of the networks. By 18 h, the networks had lost 70% of the initial line distance ($p < 0.001$) (Figure 4.1 N). Vascular disruptive activity was not detected following challenge with RgpA treated with the protease inhibitor TLCK (Figure 4.1 I-L) as determined by a non-significant attenuation over 18 h ($p > 0.05$) where only a 20% loss in line length was noted (Figure 4.1 O).

Figure 4.1 Disruption of endothelial networks on Matrigel by active proteinase complex.

(A)-(D) HUVEC networks of the control well at 0, 6, 12 and 18 hours after challenge, showing remodelling of networks to form fine branches.

(E)-(H) Showing HUVEC networks challenged with RgpA. Network disruption is detectable at 6 hours after challenge and progresses over the experimental period.

(I)-(L) RgpA (TLCK treated) cells at 0, 6, 12 and 18 hours after challenge which essentially appear as the controls with little change in appearance over 18 hours. All photographs were taken at $\times 5$ magnification.

(M)-(O) Showing histograms calculated from linear distance over time and expressed as a percentage of the initial value using data pooled from three separate experiments.

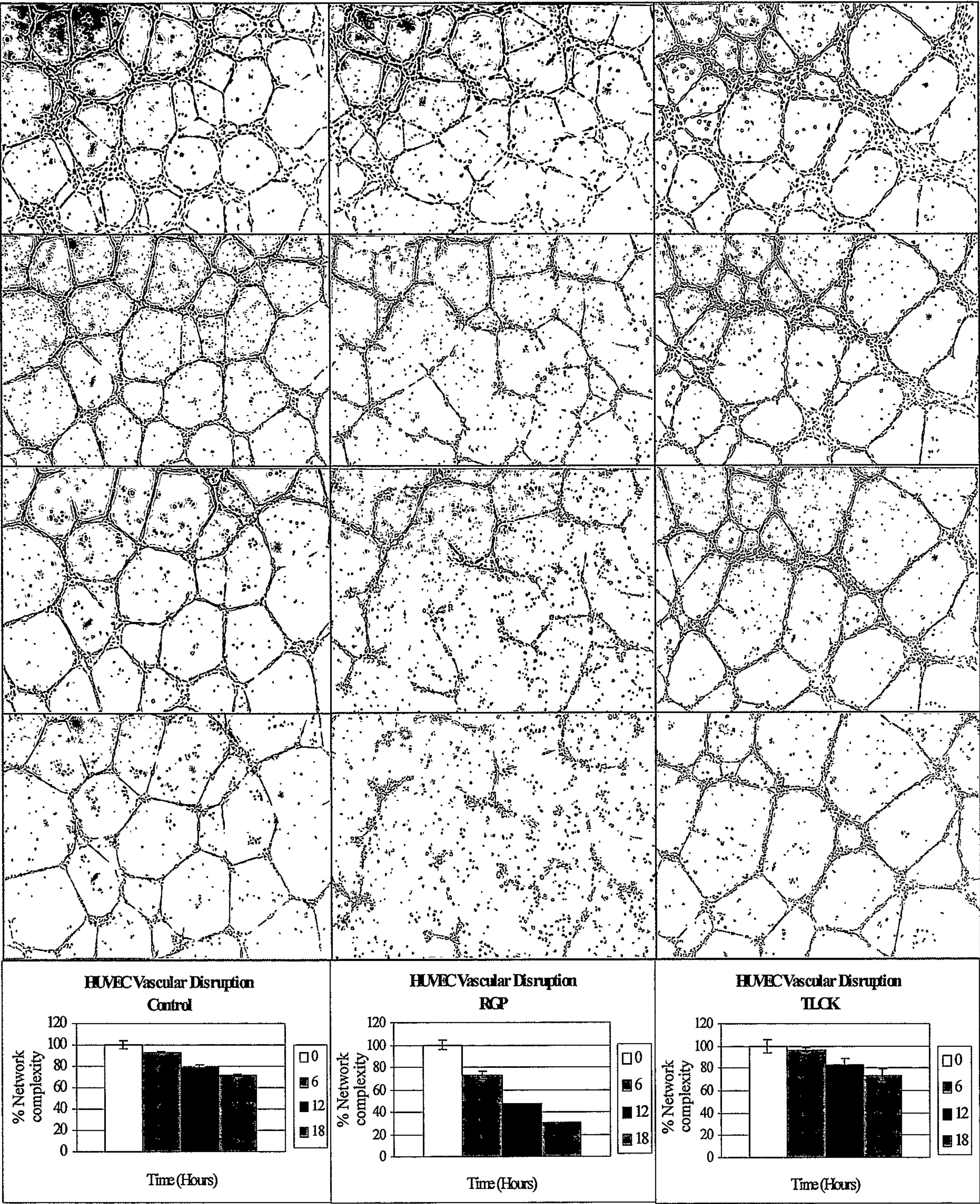


Figure 4.1

4.3.3 Confocal analysis

TLCK-inhibited RgpA was localised to endothelial networks as discrete aggregates bound to the cell surface (Figure 4.2a and b). In contrast, the active proteinase was observed to bind in a more diffuse pattern on the cell surface (Figure 4.2c). There was no evidence for internalisation of the active RgpA or TLCK-inhibited RgpA over the study period. By 6 hours after challenge, rupture of the networks was associated with intense labelling of the affected sites (Figure 4.2d). High resolution, 3-D reconstruction revealed that the surface localisation of the proteinase at foci of disruption was characterised by extensive membrane degradation (Figure 4.2e and f). In control cultures and in cultures challenged with TLCK-inhibited proteinase, network formation was clearly related to the orientation of F-actin filaments by 6.0 h (Figure 4.3a and b), whereas clumping of F-actin preceded network disruption in cultures treated with active proteinase (Figure 4.3c).

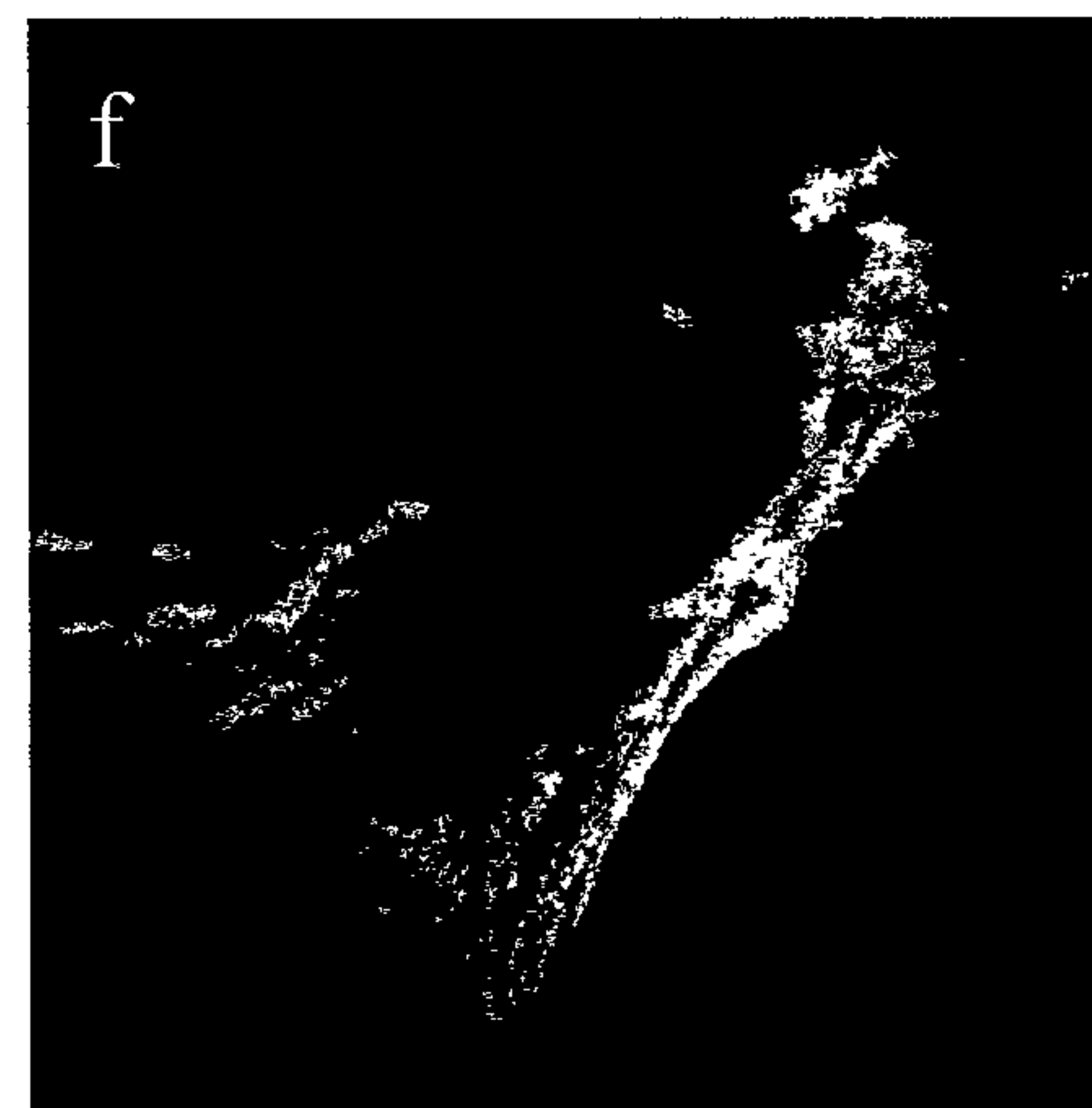
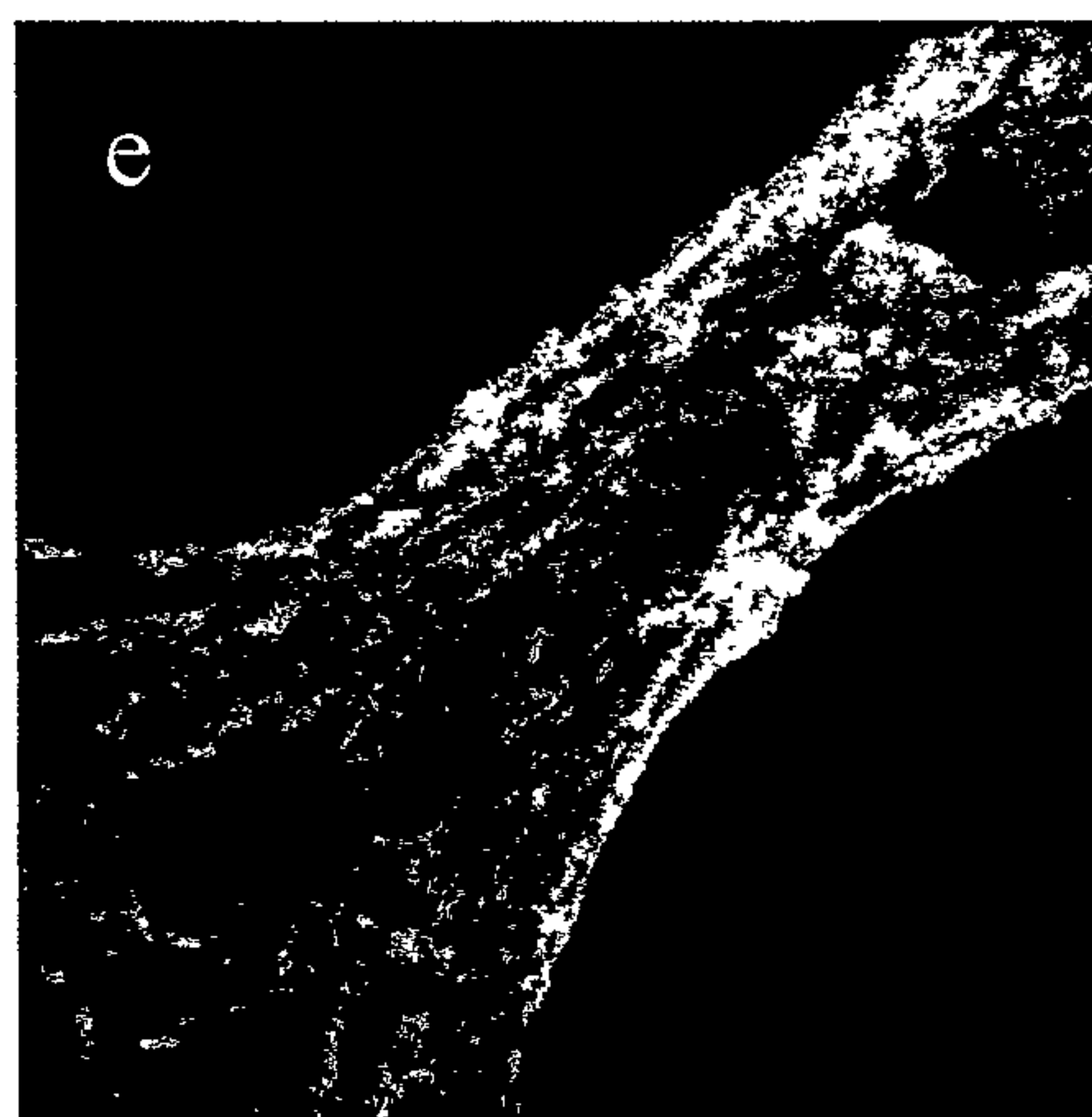
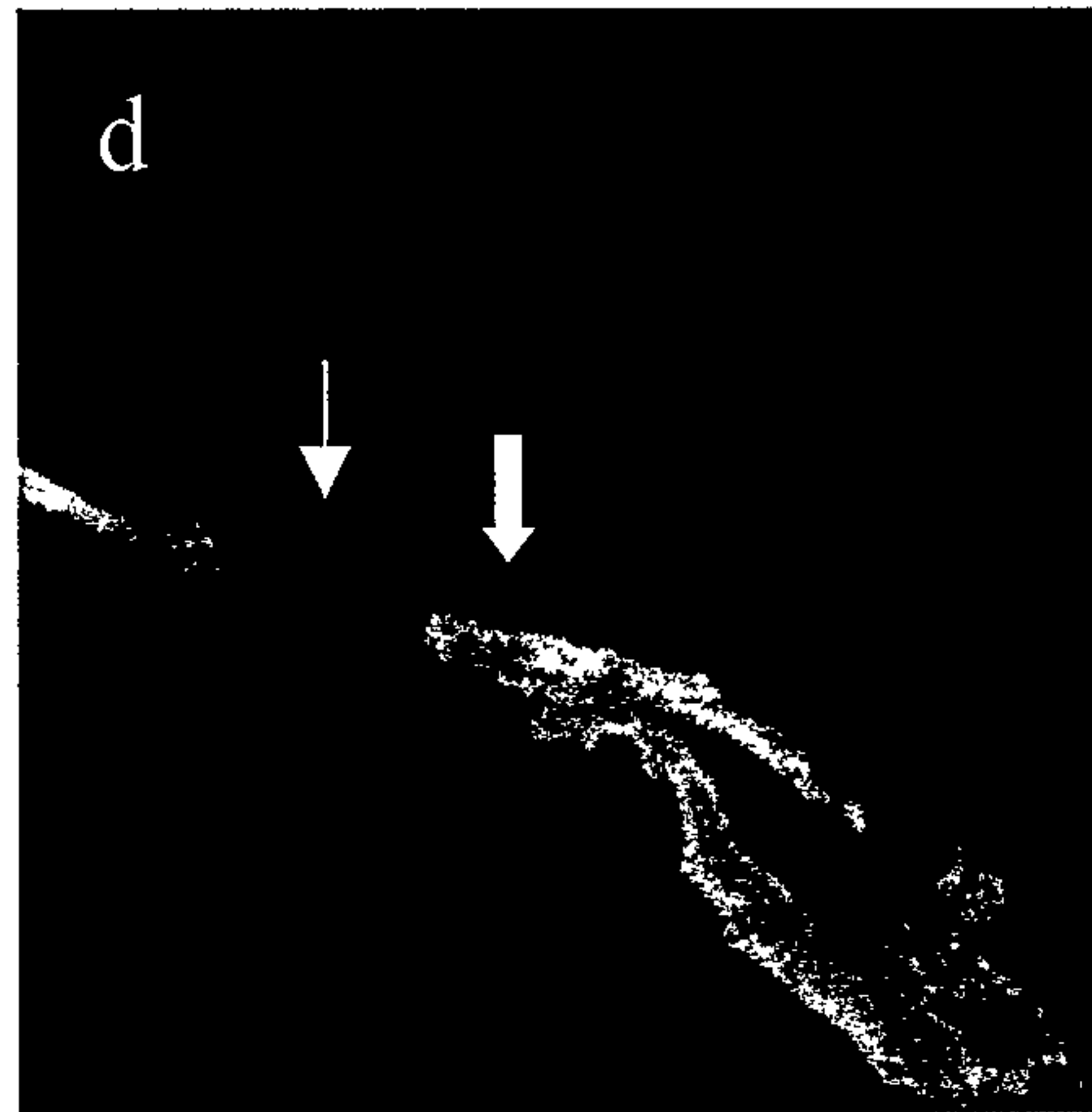
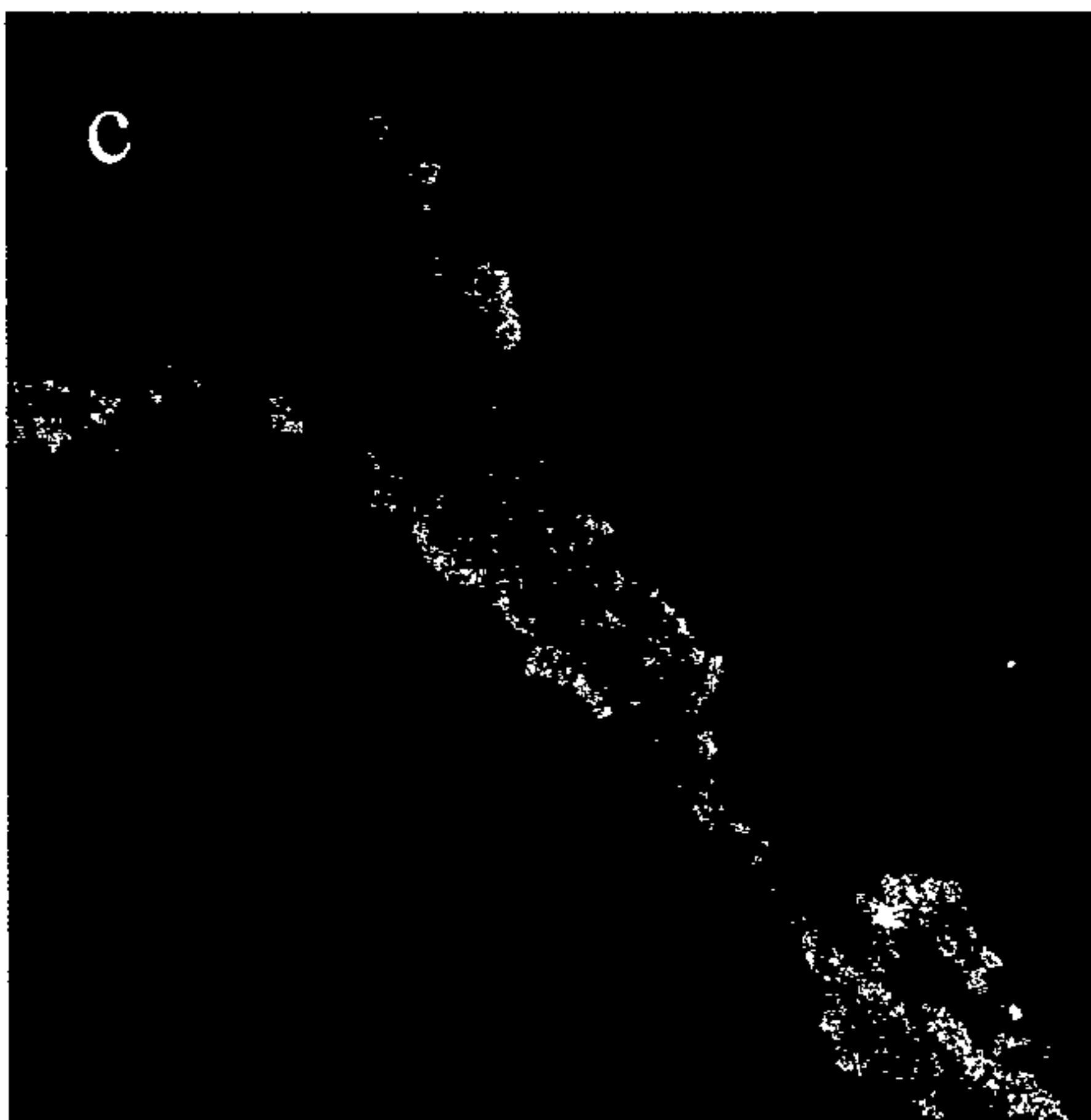
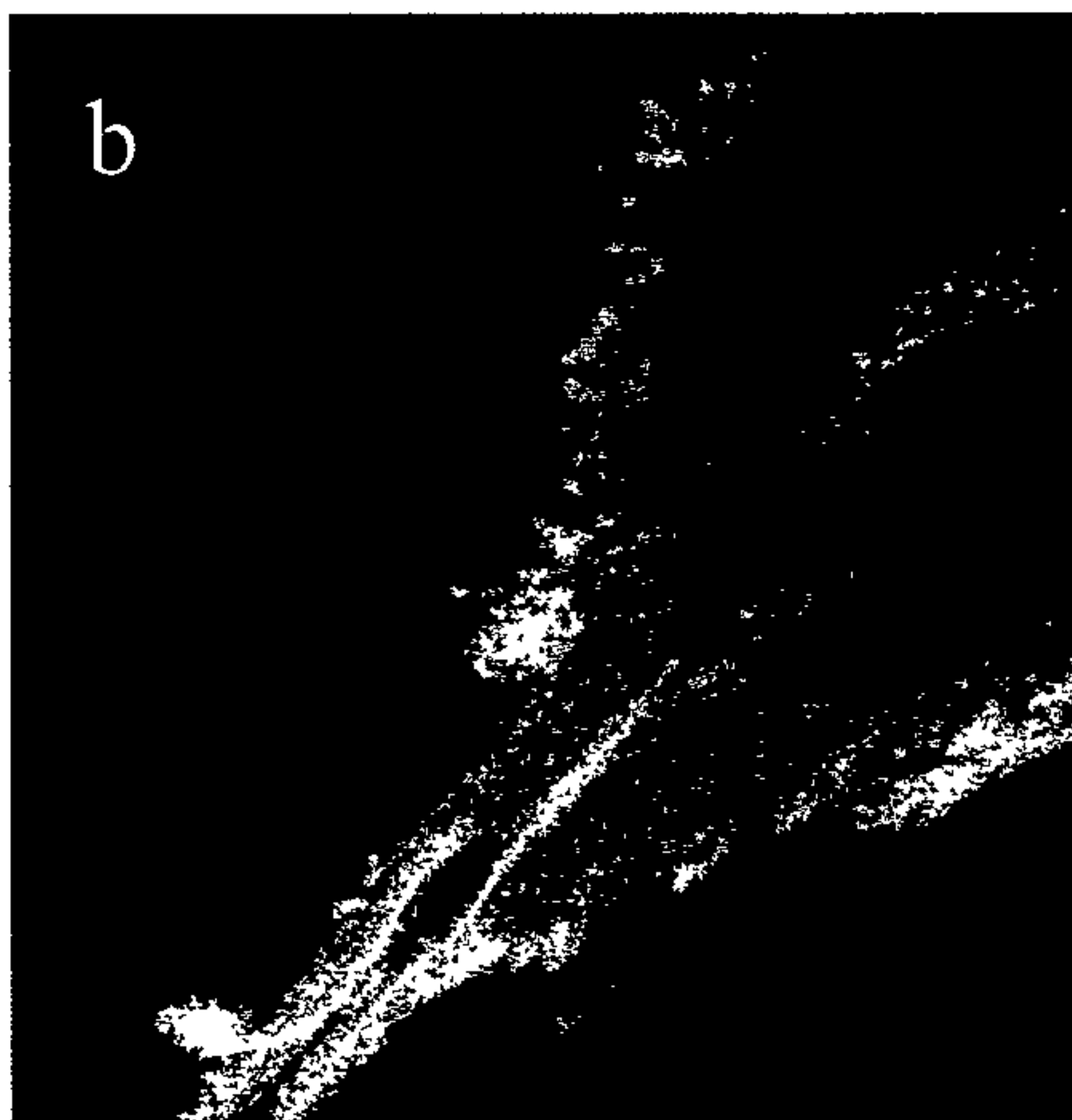
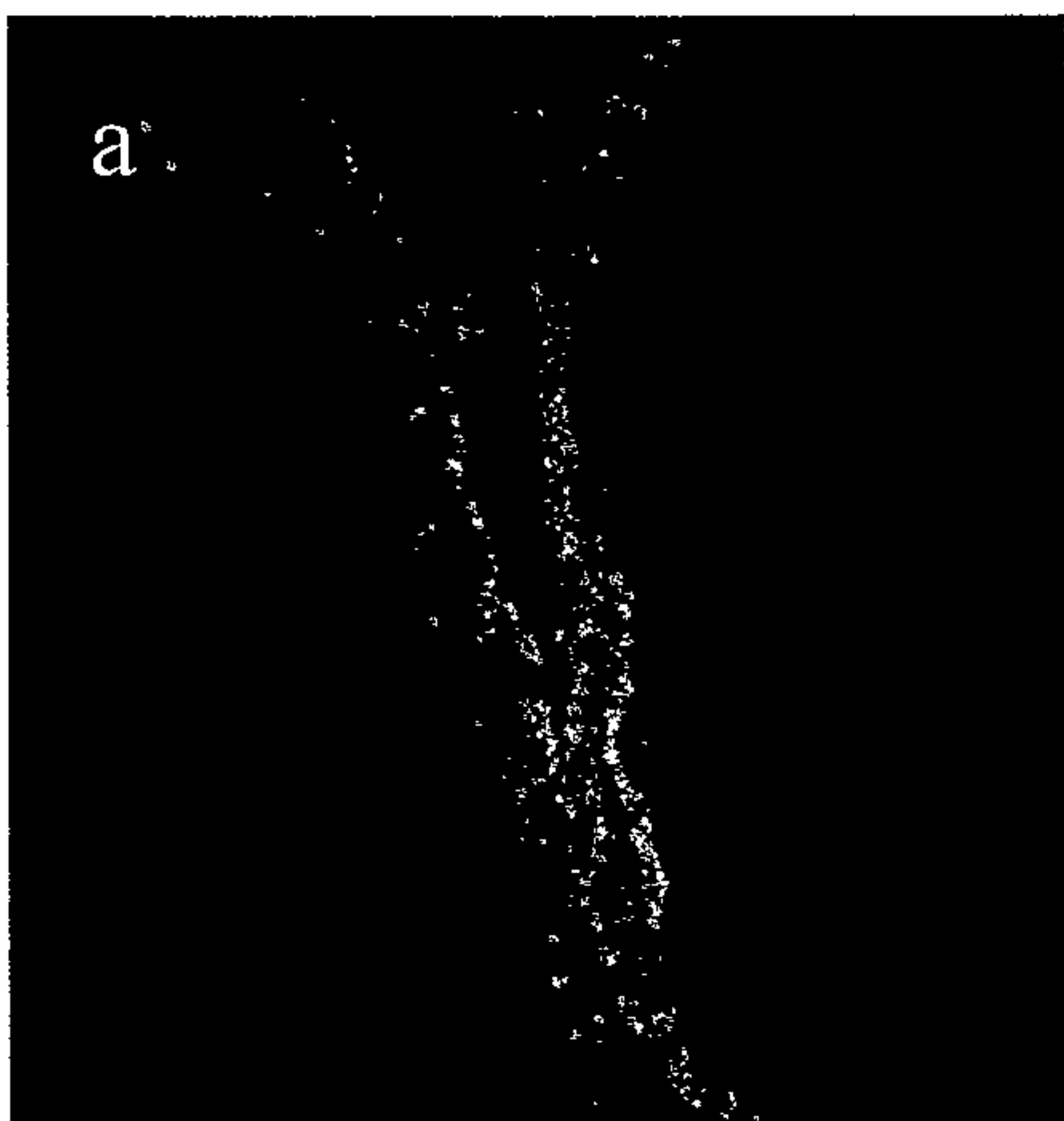
4.4 DISCUSSION

Under culture conditions, *P. gingivalis* is found to either release the Rgp/Kgp complex into the medium or localise the enzymes either in vesicles or on the outer membrane, depending on the strain. *Porphyromonas gingivalis* H66 produces very few vesicles unlike the strain used, *P. gingivalis* ATCC 33277, which both releases the complex into the medium and produces vesicles. Although it is probable that the vesicles would represent the biological carriers of these proteinases, both the RgpA and the Kgp complex are co-purified with the vesicles. Whilst it would be possible to purify the vesicle fraction and test it in the assay, the difficulty of discriminating between the two different enzyme activities would prevent interpretable results.

While there has been extensive analysis of the catalytic action of the gingipains on extracellular proteins, there have been fewer investigations of the capacity of these enzymes to attack cellular components. Recently, it was reported that the haemagglutinin/adhesin domains common to the high molecular weight gingipains have the capacity to bind to phospholipids (Imamura *et al.*, 2000). This is compatible with the membrane binding observed in the present study for the inhibited proteinase/adhesin complex. Our findings indicated that binding of the RgpA to the

Figure 4.2. Confocal images of endothelial networks 6 hours after challenge showing localisation of the proteinase to the endothelial surface. All images were manipulated from data acquired at $\times 40$ magnification.

- (a) An optical section of a culture challenged with TLCK-inhibited RgpA showing localisation in a fine granular pattern to an interconnecting process.
- (b) As for (a) but showing a more intense staining pattern.
- (c) An optical section of a culture challenged with Active RgpA, demonstrating a more diffuse pattern of staining.
- (d) As for (c) but showing intense staining (coarse arrow) in a region of network disruption (fine arrow).
- (e) 3-D reconstruction of a series of optical sections through an interconnecting cell process. Demonstrating extensive degradation of the plasma membrane in association with localisation of RgpA.
- (f) An optical section mid-way through the cell process shown in (e) demonstrating the surface only localisation of RgpA.



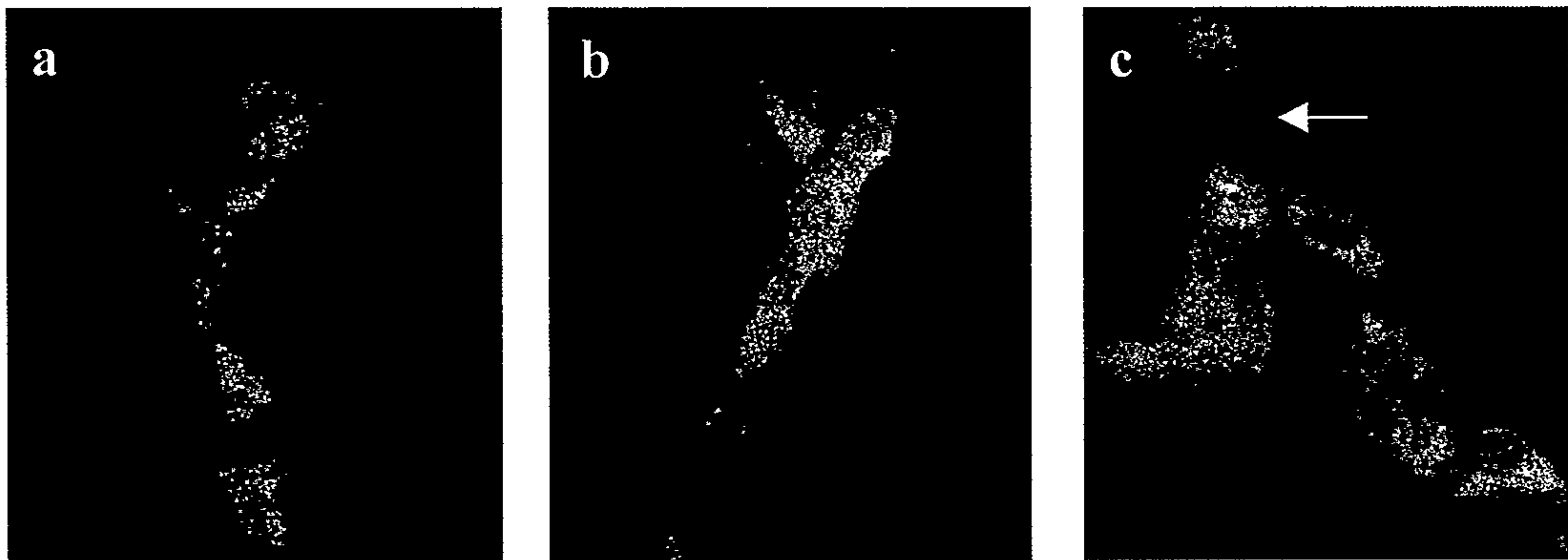


Figure 4.3 Confocal images of F-actin distribution related to network formation and disruption at 6 hours after challenge with proteinase. All images were manipulated from optical sections acquired at $\times 40$ magnification.

- (a) An optical section from a control culture showing arrangement of F-actin in an interconnecting region.
- (b) RgpA-TLCK treated culture demonstrating the abundance of F-actin in the fine interconnecting cell process.
- (c) Culture challenged with active RgpA illustrating retraction of F-actin from extended cell processes of an intact network region (arrow) prior to disruption.

endothelial surface was modified by catalytic activity. The inhibited enzyme bound in a more discrete, aggregated pattern, whereas active enzyme was more diffusely spread but also in a similar pattern that was confined to the cell surface. The mechanism of network disruption was related to apparent attack and permeation of the cell membrane. There was no evidence from confocal analysis to indicate internalisation of the RgpA. Since preferred binding of RgpA to the cytoplasmic membrane of endothelial cells occurred in an environment supplemented with 20% foetal calf serum and in the presence of a substantial amount of basement membrane proteins, this may indicate that some type of homing mechanism was involved.

Re-arrangement of F-actin filaments occurred before physical disruption of the networks. Proteolytic attack on cadherins mediating intercellular adherens junctions has the potential to induce this response through linkage via α -catenin to F-actin (Yap *et al.*, 1997). An alternative mechanism is the attack, either on basement membrane proteins or cellular integrins that link to the matrix, thereby causing disruption of F-actin filaments through a system of connecting proteins including talin, vinculin and α -actinin (Sastry and Horwitz 1993). Targeted disruption of fibronectin-integrin interactions by the RgpA complex in fibroblasts has been reported to cause loss of the cell-associated fibronectin network in human gingival fibroblasts under conditions in which $\alpha_5\beta_1$ integrin receptor loss was also observed (Scragg *et al.*, 1999). It was also shown that the pattern of staining of the RgpA complex coincides with that of fibronectin and its cellular receptors. This effect was dependent on catalytic activity. Other studies have implicated hydrolysis of the extracellular matrix components in detachment of cultured epithelial cells and fibroblasts (Shah *et al.*, 1992; Johansson and Kalfas 1998). In contrast, the findings of the present study indicated that the RgpA complex produced an extensive and direct attack on endothelial cell membrane proteins. Degradation of extracellular matrix proteins and endothelial membrane proteins could contribute to the observed effect on vascular networks.

In order for a similar pathogenic action to occur *in vivo*, the proteinase complex must diffuse through the connective tissue to reach the underlying vascular networks. Reported marked alteration in filamentous actin expression and a reduction

in staining for E-cadherin, involucrin and connexins 26 and 43 in the epithelium of the periodontal pocket lining indicate profound perturbation of the epithelial structure, supporting the concept that the ability of the lining epithelium to function as an effective barrier against the ingress of microbial products is severely compromised (Ye *et al.*, 2000). Analysis by indirect immunofluorescence demonstrated that products derived from *Bacteroides melaninogenicus* could be detected in human gingival sub-epithelial connective tissue (Courant and Bader 1966). It has also been reported that bacteria can be detected penetrating between the enlarged intercellular spaces of the pocket epithelial surface (Saglie 1988).

Of direct relevance, *P. gingivalis* has been found to adhere to and invade endothelial cells (Deshpande *et al.*, 1998). Although there have been few reports of a direct effect of pathogenic bacteria on the vasculature, Quinn *et al.* reported findings, similar to those noted in the present study, in which the presence of the Brazilian purpuric fever-associated *Haemophilus influenzae* biogroup *aegyptius* caused the disruption of the endothelial networks (Quinn *et al.*, 1994).

Interpretation of findings in the model system indicates the capacity of the gingipains to induce disruption of vascular networks as a key aspect of the action of these virulence factors in perturbation of the neovascularisation critical for the formation of reparative granulation tissue.

CHAPTER FIVE

EVIDENCE THAT *PORPHYROMONAS GINGIVALIS* IS A PORPHYRIN AUXOTROPH : THE CRITICAL ROLE OF HA2 AS A PORPHYRIN RECEPTOR

5.1 INTRODUCTION

Porphyrin precursors are essential for several cell components of bacteria such as cytochromes, catalases, peroxidases, sirohaem and vitamin B₁₂. These cellular components are not ubiquitous but are instead found scattered throughout the domain bacteria. The requirement for porphyrin can be met by an organism by either scavenging from the environment or by *de novo* synthesis. Although *P. gingivalis* has a critical need for haem for growth, its requirement for porphyrin has not been resolved.

Recently, studies at this Institute showed that the capacity of the rHA2 domain to bind haemin or haemoglobin was inhibited by protoporphyrin IX, thereby demonstrating an essential interaction with the porphyrin moiety (DeCarlo *et al.*, 1999). This binding interaction implied that *P. gingivalis* was not capable of synthesising porphyrin and required an external source of protoporphyrin IX for growth.

The aim of the studies described in this Chapter was to investigate the postulate that *P. gingivalis* is a porphyrin auxotroph by examining the genomic sequence of strain W83 for evidence of enzymes used for *de novo* synthesis of a tetrapyrrole ring and for the synthesis of haem. Following confirmation of the auxotrophic status, the ability of porphyrin to substitute for haem as a growth supplement was investigated. Neutralising ELISA assays established the importance of the porphyrin-mediated binding of HA2 to haemoglobin in relation to total haemoglobin binding of *P. gingivalis* and while it has been demonstrated that the recognition of the porphyrin moiety was through the propionic face which is exposed in haemoglobin (DeCarlo, Paramaesvaran *et al.*, 1999), the structural importance of the vinyl-face was further investigated using similar binding and growth assays.

5.2 METHODS AND MATERIALS

Note: Chemicals, reagents and enzymes are listed in Chapter 2

5.2.1 Genomic data search

All entries for proteins used in the porphyrin biosynthesis pathway that are listed in the Swiss-Prot database (Release 37.0) were searched with TBlastN (Altschul *et al.*, 1990) against the available genomic data for the *P. gingivalis* genome. Preliminary sequence data for *P. gingivalis* was obtained from The Institute for Genomic Research through the website at <http://www.tigr.org>.

This search comprised 257 eukaryotic, archaeal and prokaryotic protein sequences used in different pathways related to porphyrin biosynthesis. These include the standard enzymes in porphyrin biosynthesis, alternative enzymes used by subsets of organisms, the enzymes of the C₄ and C₅ pathways for 5-aminolevulinic acid synthesis, and enzymes used in vitamin B₁₂ synthesis.

Open reading frames (ORF) with more than 10% identity to enzymes required for haem synthesis were further examined by comparison with enzymes from 4 reference organisms; *Escherichia coli*, *Bacillus subtilis*, *Synechocystis*, and *Aquifex aeolicus*, and the enzyme 5-aminolevulinic acid synthase from *Bradyrhizobium japonicum*. Open reading frame's (ORF's) were identified using the program Map and translated with Translate (Genetics Computer Group 1995). Sequence identity between protein sequences was calculated using the program GAP (Genetics Computer Group 1995) and multiple alignments performed with PileUp (Genetics Computer Group 1995).

Enzymes used solely in the pathway for vitamin B₁₂ synthesis were reported as significant if there was an ORF which had a Blast score greater than 100. Enzymes and proteins involved in cytochrome synthesis were also blast searched against the genome. Proteins were aligned and identity calculated using GAP (Genetics Computer Group 1995).

5.2.2 Growth curve assays

5.2.2.1 Stock cultures and medium preparation

Porphyromonas gingivalis ATCC 33277 was used in the growth curve assays. A 4-5 day growth of *P. gingivalis* was inoculated into CDC adjusted broth (Appendix A1). All cultures were grown in an anaerobic chamber at 37°C in a 5% CO₂-10% H₂- 85% N₂ atmosphere (3.2.1.1). Growth rates of *P. gingivalis* were determined by inoculation of a 10% (v/v) inoculum of a late-logarithmic-phase culture ($A_{660} = 1.0$) into anaerobically equilibrated CDC modified broth. Optical density was determined at 660 nm (A_{660}). Culture purity was assessed by Gram stain and anaerobic subculture on CDC modified blood agar plates. A 1% inoculum was transferred to fresh CDC modified media without haemin and horse serum or without haemin, horse serum and menadione. Horse serum had been routinely used in the altered CDC media (Appendix A2) but was eliminated from this study as the complexity of proteins involved might result in difficulty in interpreting the results seen. Menadione has been reported to act as a growth supplement for *P. gingivalis* and has been thought to play a role in electron transport (Gibbons and Macdonald 1960). The importance of this growth supplement was tested during the various stages of growth. Endogenous stores of haemin in *P. gingivalis* whole cells were exhausted by serial passage of the culture at least five times into haemin-free medium after each culture achieved stationary phase. At this point, the porphyrins to be tested were added to the haemin-depleted media with or without the presence of menadione and the subsequent growth monitored by measuring the OD₆₆₀.

5.2.2.2 Porphyrin stocks

The porphyrins to be tested in the growth curve assays were deuteroporphyrin IX 2,4 bis-ethylene glycol (DBEG), deuteroporphyrin IX dihydrochloride (DDH) and deuteroporphyrin IX disulfonic acid (DDS) representing modified porphyrins with varying substitutions on the vinyl aspect but with an intact propionate face. For the binding and competition assay (5.2.7), DBEG, DDH and DDS were used as well as three dipyrroles which were structurally analogous “half” porphyrins containing no vinyl

aspect (designated #1, #2 and #3). All products were obtained from Porphyrin Products Ltd, Utah, USA. Stock solutions of the porphyrins were prepared in 0.1 N NaOH. These were added to the haemin-depleted CDC medium at 5.0 µg/ml with the control medium including haemin at the same concentration.

5.2.3 Extraction and biotinylation of surface proteins from *Porphyromonas gingivalis*

5.2.3.1 Preparation of biotinylated CHAPS extracted surface proteins (BCE)

Porphyromonas gingivalis (ATCC 33277) cells were grown in modified CDC broth (Appendix A1) under anaerobic conditions for 48 h (3.2.1.1). The cell pellet was washed 3 × in ice-cold PBS (pH 7.4) (3.2.1.2) to remove any contaminating proteins and resuspended at a concentration of approximately 25×10^6 cells/ml in bicarbonate buffer (A = 8.4 g NaHCO₃/100ml (1.0 M), B = 10.6 g Na₂CO₃/100ml H₂O (1.0 M) where 45.3 ml A was added to 18.2 ml B, made up to 1.0 L in H₂O and adjusted to pH 9.6). Sulfosuccinimidyl-6-(biotinamido)hexanoate-Biotin (Sulfo-NHS-LC-Biotin) was added at 0.5 mg/ml of reaction volume. The mixture was rotated gently at 23°C for 30 min and washed 3 × in ice-cold PBS (pH 7.4) to remove any remaining biotinylation reagent. The cell pellet was then resuspended in 10 ml of Tris buffer with 0.25% CHAPS and 2.0 mM TLCK to inhibit proteolytic degradation and rotated gently overnight. The suspension was centrifuged at $3,000 \times g$ for 15 min and the protein in the supernatant calculated to be 2.0 mg/ml using Coomassie® Plus Protein Assay Reagent.

5.2.3.2 ELISA conditions for CHAPS Assay (2.4.6)

Bovine haemoglobin (used in all experiments requiring haemoglobin) was used to coat the surfaces of the wells in PBS (pH 7.4) (3.2.1.2) with 10 mM NaN₃ (PBS/N₃). Dilutions of biotinylated CHAPS extract (BCE) made in PBS (pH 7.4) with 0.1% Tween 20 (PBS/Tween) were incubated for 4.0 h at 23°C on haemoglobin-coated plates before washing in PBS/Tween. Streptavidin-alkaline phosphatase (Strep-AP) was applied in PBS/Tween at a concentration of 0.5 µg/ml for 1.0 h at 37°C and then AP activity was monitored at A₄₁₄ (2.4.6).

5.2.4 Expression and purification of recombinant HA2 (rHA2).

Refer to 3.2.7

5.2.5 Neutralisation of haemoglobin binding

Initially rabbit polyclonal antibodies to HA2 had been prepared by immunisation of New Zealand White rabbits with native HA2 isolated by native gel electrophoresis and emulsified in Freund's Complete Adjuvant for the first challenge and in Freund's Incomplete Adjuvant for subsequent booster challenges. The polyclonal antibodies produced were tested for the capacity to bind the haemoglobin binding site on the HA2 protein in the CHAPS extract of *P. gingivalis* (method not shown). If polyclonal antibodies to this site had been produced, it would have been possible to test for the capacity of the extract to bind haemoglobin after the HA2 haemoglobin-binding sites had been blocked. Unfortunately, the polyclonal antibodies produced were not capable of blocking the haemoglobin binding sites on the HA2 molecule and a different approach had to be used. Patient sera which had been collected for the purpose of the clinical study described in Chapter 6 had been found to have a range in capacity to neutralise haemoglobin binding. It was decided to select a patient with a strong neutralising capacity for haemoglobin binding and one with weak neutralising capacity for haemoglobin binding to be used for the purposes of this study.

5.2.5.1 Serum IgG isolation

Venous blood was collected in plastic tubes and serum stored frozen in aliquots at -70°C until use. Once thawed for use, NaN₃ was added to a final concentration of 10 mM and the serum samples were kept at 4°C. The IgG fraction was isolated from patient sera by protein-G affinity chromatography. Protein-G columns (Pharmacia Biotech, Sweden) were equilibrated with 50 mM Tris, 25 mM NaCl, 1 mM CaCl₂, 10 mM NaN₃ (pH 7.4) then loaded with a 1/10 dilution of patient sera in the same buffer. Columns were washed with 8 column volumes of equilibration buffer then bound IgG was eluted with 0.1 M glycine (pH 2.7). IgG fractions were adjusted to pH 8.4 with 1/20 volume of 2.0 M Tris buffer (pH 8.4). IgG concentrations were determined by absorbance at A₅₉₅.

5.2.5.2 Neutralisation assay conditions

Patient sera for the assay were selected by the capacity to neutralise haemoglobin binding. Of the 38 purified IgG fractions tested, the patients with the strongest (IgG #125) and the weakest (IgG #65) neutralising capacity were selected. By using the standard ligand binding assay described herein, BCE at a concentration which produced 50% saturation binding to a haemoglobin-coated plate was pre-incubated for 1.0 h in PBS/Tween (5.2.3.2) with dilutions of patient IgG #125 and the control patient IgG #65 and then allowed to bind to haemoglobin-coated plates overnight. Although haemoglobin, protoporphyrin IX and haemin were initially used in the preliminary experiments, haemoglobin was selected for the final experiments as the protein was found to evenly coat the ELISA plates resulting in reproducible data.

5.2.5.3 Preparation of rHA2-bound-resin

Recombinant HA2 was dialysed overnight into 0.2 M NaHCO₃-0.5 M NaCl. NHS-activated Sepharose 4 fast flow beads (Pharmacia Biotech, Melbourne) were washed 6× with ice-cold 0.1 M HCl. The resin was pelleted, the rHA2 added and the suspension rotated gently at 23°C overnight. The beads were washed 5 × with buffer to remove unbound protein. The resin was resuspended in 50 mM Tris (pH 8)–10 mM NaN₃ and rotated overnight to block unbound sites. The rHA2-bound-resin was pelleted and resuspended in PBS/N₃ (5.2.3.2) at 716 µg/ml.

5.2.5.4 Inhibition of neutralisation assay

Increasing amounts of rHA2-bound resin suspension were pre-incubated with patient IgG #125 chosen at the highest level of neutralisation of haemoglobin binding and patient IgG #65 as the IgG control, and rotated gently at 23°C overnight. This was done as a single absorption procedure. The optimum amount of rHA2-bound resin suspension to produce the greatest inhibition was 200 µl of resin. As a resin control, the same amount of beads (prepared using the same method as rHA2) was pre-incubated with the IgG samples. The resin was pelleted and the supernatant added to a pre-blocked haemoglobin plate. BCE at a concentration which produced 50% saturation binding to a haemoglobin-coated plate, was added and incubated at 23°C for 4.0 h then detected with

Streptavidin-Alkaline Phosphatase (Strep-AP). The midpoint of the linear portion of the inhibition curve and the standard neutralisation curve was calculated to the nearest 2 fold dilution. This difference was then expressed as a percentage of total binding inhibited by the rHA2-bound resin. The percentage range was the mean average of 6 separate experiments.

5.2.6 Development of Mabs VA1 and 11B2.

Anti-gingipain mAbs VA1 and 11B2 were prepared in mice and characterised as previously described (DeCarlo and Harber 1997; DeCarlo *et al.*, 1999).

5.2.7 Binding and competition assays

5.2.7.1 ELISA conditions (2.4.6)

Porphyrins and haemoglobin were used to coat the well surfaces in either 0.1 N NaOH or 1 M bicarbonate buffer (pH 9.0) (5.2.3.1) to determine optimal coating concentrations for saturation binding of rHA2. Dilutions of rHA2 in 50 mM acetate buffer containing 137 mM NaCl, 0.1% Tween 20 and 10 mM NaN₃ (pH 5.5) (Acetate/Tween) were incubated overnight before washing in PBS/Tween (5.2.3.2). Primary murine mAb (VA1) was applied in PBS/Tween at a concentration of 0.5 µg/ml for 1.0 h at 37°C. Secondary goat anti-mouse antibodies conjugated with AP were applied at a concentration of 1.1 µg/ml for 1.0 h at 37°C, and then AP activity was monitored at 414 nm (A_{414}) by hydrolysis of the substrate 4-nitrophenylphosphate (Boehringer, Mannheim, Germany) in 5.0 mM Tris (pH 9.5) by using a Titertek Twinreader PLUS photometer (absorbance maximum of 3.0 ELISA units) (2.4.6). Mean apparent dissociation constants (K_{ds}) were derived by solid-phase ELISA as previously described (DeCarlo *et al.*, 1999).

5.2.7.2 Ligand binding assay

The ligand-binding assay was a variant of the ELISA in which the ligand (ie., haemoglobin, DDH, DBEG, DDS and the dipyrroles 1, 2 and 3) (5.2.2.2) that had been used to coat the wells in bicarbonate buffer (5.2.3.1) or 0.1 N NaOH was subsequently allowed to bind to a second ligand-binding protein (ie., rHA2) in Acetate/Tween (5.2.6.1)

or PBS/Tween (5.2.3.2). The ligand-binding protein was then detected with mAb VAI, followed by a goat anti-mouse AP conjugate, and developed as described for ELISA (2.4.6).

5.2.7.3 Competition assay

The IC_{50} s for ligand binding in solution phase competition assays were determined. By using the standard ligand binding assay described herein, rHA2 at a concentration which produced 50% saturation binding to a haemoglobin-coated plate was pre-incubated for 1.0 h in Acetate/Tween (5.2.7.1) with dilutions of the porphyrins and then allowed to bind to haemoglobin-coated plates overnight. In preliminary studies, the loading capacity of porphyrin onto rHA2 was tested with both Acetate/Tween and PBS/Tween (5.2.3.2). It was found that rHA2 bound to haemoglobin more efficiently in the presence of Acetate/Tween. However PBS/Tween was maintained for the antibody steps as the low pH of acetate was not ideal for antibody interactions. Haemoglobin was used as it was considered to represent the dominant form of porphyrin presentation in the pathological environment of chronic periodontitis.

5.3 RESULTS

5.3.1 Genomic database searches

5.3.1.1 Proteins potentially involved in porphyrin biosynthesis

The genome of *P. gingivalis* was searched for evidence of proteins involved in the biosynthesis of protoporphyrin IX and haem. A flow diagram of this pathway is presented in Figure 5.1. The proteins commonly required for porphyrin biosynthesis (Table 5.1) were Blast searched against the available sequence data for the *P. gingivalis* genome.

Three proteins in the haem synthesis pathway have notable identity to open reading frames in the *P. gingivalis* genome. These are the enzymes for the final three steps in the pathway to convert coproporphyrinogen III to protohaem and have the

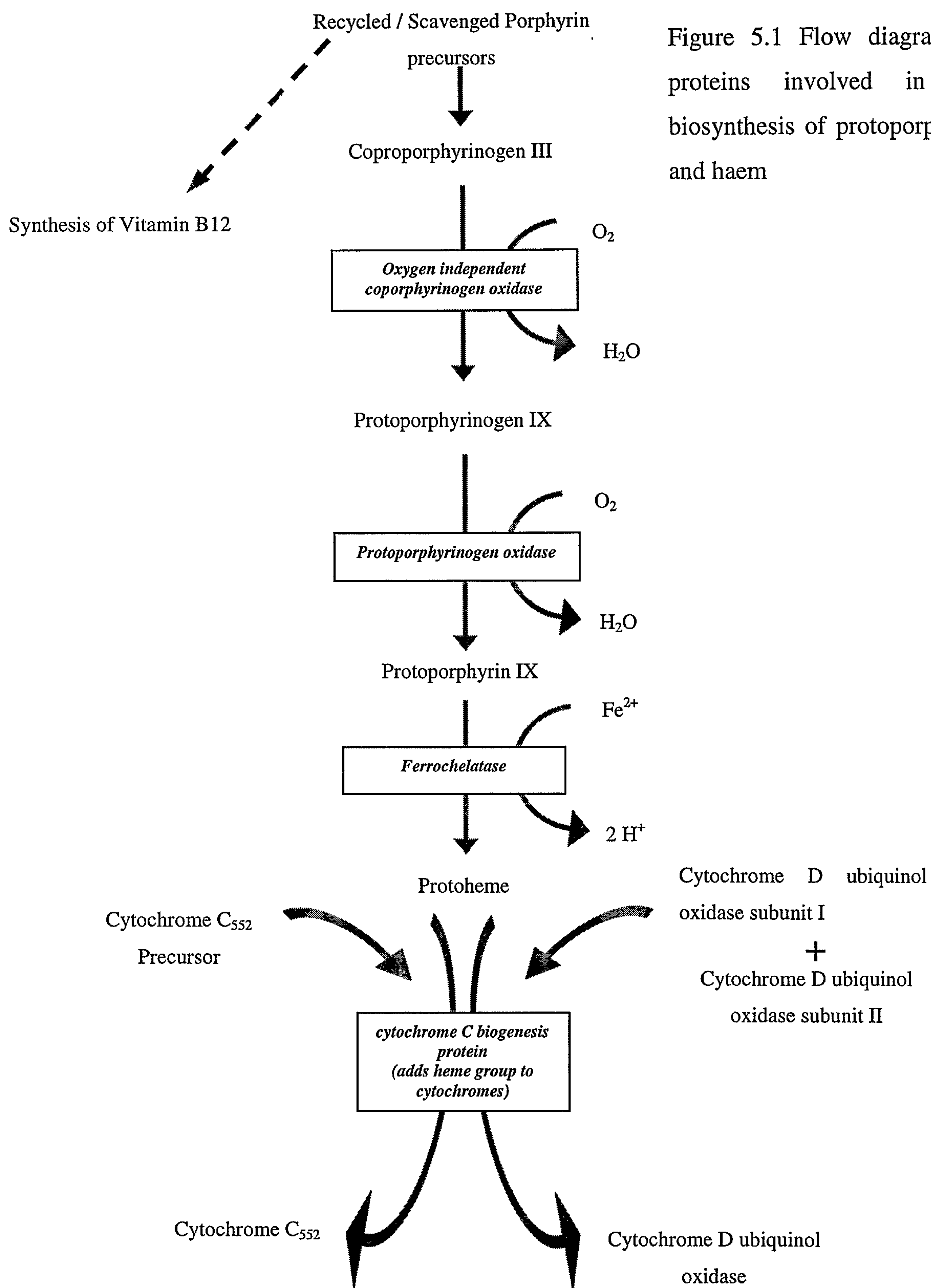


Table 5 1 – Enzymes Used in Haem Biosynthesis

Enzyme	Function
Hem1/ Hem0 / HemA	5-aminolevulinic acid synthase
HemA/ Hem1	Glutamyl-tRNA reductase
HemB/ Hem2	Porphobilinogen synthase
HemC / Hem3	Porphobilinogen deaminase
HemD / CysG / NasF	Uroporphyrinogen III cosynthase
HemE / DcuP	Uroporphyrinogen decarboxylase
HemF / Hem6	Coproporphyrinogen III oxidase
HemY / HemG / HemK	Protoporphyrinogen oxidase
HemH / HemZ	Ferrochelatase
HemL / HemK	Glutamate -1- semialdehyde 2,1 aminotransferase
HemN	Oxygen-independent coproporphyrinogen III oxidase
HemX / CysG / CobA	Uroporphyrinogen III methylase
GltX	Glutamyl-tRNA synthetase

Table 5.2 – Enzymes for Haem Synthesis with Significant Identity to ORFs of *P. gingivalis* (get refs from Liz)

Putative Enzyme / Protein	% Identity			
	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Synechocystis</i>	<i>Aquifex aeolicus</i>
HemG	-	26.11	20.55	33.56
Protoporphyrinogen oxidase				
HemN	30.09	-	-	26.61
Oxygen independent coporphyrinogen III oxidase				
HemH	37.30	24.40	32.79	29.70
Ferrochelatase				

capacity to modify the vinyl face of the porphyrin molecule. These proteins and their identities to other proteins are shown in Table 5.2.

No significant matches were detected for the proteins used in the initial stages of porphyrin biosynthesis for synthesis of the tetrapyrrole ring. These are the proteins glutamyl-tRNA reductase, porphobilinogen synthase, porphobilinogen deaminase, uroporphyrinogen III cosynthase, uroporphyrinogen decarboxylase and uroporphyrinogen III methylase.

One ORF of *P. gingivalis* has a slight identity to glutamate semi-aldehyde 2,1 aminomutase (HemL) used in the C₅ pathway for synthesis of 5 - aminolevulinic acid, a precursor of the porphyrin ring. HemL is a member of the pyridoxal phosphate-dependent aminotransferase class of proteins. The translated open reading frame resembling this protein was Blast searched against the NR Protein Database (ANGIS compiled Database) to identify similar proteins. This ORF has much higher identity to another protein of this class, ornithine aminotransferase. For example, the identity of the ORF to HemL from *Bacillus subtilis* (Accession Number P30949) is 28.4% whereas the identity to ornithine aminotransferase from *B. subtilis* (Accession Number P38021) is 51.1%. This open reading frame is much more likely to perform the function of ornithine aminotransferase in *P. gingivalis*. Ornithine aminotransferase is involved in arginine and proline metabolism and appears to be unrelated to porphyrin biosynthesis.

Another two open reading frames have some identity to the enzyme 5-aminolevulinic acid synthase of the C₄ pathway for 5-aminolevulinic acid synthesis. Similarly, these open reading frames encode proteins that are more likely to perform other functions based on higher identity to proteins other than 5-aminolevulinic acid synthase. One open reading frame appears more likely to be 2-amino-3-ketobutyrate coenzyme A ligase (58.6% identity to *E. coli* Kbl) rather than 5-aminolevulinic acid synthase (31.5% identity to *Bradyrhizobium japonicum* 5-aminolevulinic acid synthase; Accession Number P08262). The other open reading frame also has higher identity to 2-amino-3-ketobutyrate coenzyme A ligase (41.8% identity to *B. subtilis* 2-amino-3-ketobutyrate coenzyme A ligase; Accession Number O31777, compared to 31.9% identity to *Bradyrhizobium japonicum* 5-aminolevulinic acid synthase; Accession

Number P08262). The enzyme 2-amino-3-ketobutyrate coenzyme A ligase is used in glycine, serine and threonine metabolism.

5.3.1.2 The requirement for porphyrin by *P. gingivalis*

It is possible that the absence of these critical enzymes is an indication that the organism has lost the requirement for porphyrin. Accordingly, the genome was searched for evidence to support or refute this theory. Several genes associated with synthesis of tetrapyrrole rings can be identified within ORFs in *P. gingivalis*. For example, there appear to be several ORFs in the genome of *P. gingivalis* with significant identity to the genes that encode the enzymes used in the synthesis of cobalamin or vitamin B₁₂. The structure of this vitamin is basically a tetrapyrrole ring complexed with a cobalt ion. The synthesis of vitamin B₁₂ usually occurs in a pathway that uses up to 20 different enzymes. In the genome of *P. gingivalis* there are 16 ORFs that have significant identity to enzymes used in vitamin B₁₂ synthesis (Table 5.3).

Also present are several ORFs that have significant identity to proteins and enzymes that are used in the electron transport chain. These include flavodoxin, ferredoxin, thioredoxin, cytochrome components and biogenesis proteins. Of these proteins, the cytochrome component and biogenesis proteins are established as haem-binding proteins. These haem-binding proteins are shown in Table 5.4 and are described below.

5.3.1.2.1 Cytochrome C552 Precursor Protein

An ORF in the *P. gingivalis* genome has a high identity to the NrfA protein of *E. coli* at 43.19% (Figure 5.2). It also has conserved within its sequence several residues that have been identified as essential for haem binding. The five haem-binding motifs of the NrfA protein are also conserved in this open reading frame. Four of these are conventional CXXCH motifs that each covalently attach a haem group to the two cysteine residues (Eaves *et al.*, 1998). Also partially conserved is an unusual haem binding motif CWSCK (Eaves *et al.*, 1998). This unusual motif is believed to be the site for nitrite reduction in the formate-dependent nitrite reduction pathway of *E. coli*.

Table 5.3 – Vitamin B₁₂ Synthesis Enzymes with Significant Blast Scores¹
Against the Genome of *P. gingivalis*

Protein	Organism	Enzyme
CbiA	<i>Salmonella typhimurium</i>	Cobyrinic acid A,C – diamide synthase
CobB	<i>Pseudomonas denitrificans</i>	Cobyrinic acid A,C – diamide synthase
CobC	<i>Pseudomonas denitrificans</i>	CobC Protein
CobL	<i>Pseudomonas denitrificans</i>	Precorrin-6Y C5,15-methyltransferase
CobD	<i>Pseudomonas denitrificans</i>	CobD Protein
CobH	<i>Pseudomonas denitrificans</i>	Precorrin-8X methylmutase
CobI	<i>Pseudomonas denitrificans</i>	Cob(I)alamin adenosyltransferase
CobJ	<i>Pseudomonas denitrificans</i>	Precorrin-3B C17-methyltransferase
CobM	<i>Pseudomonas denitrificans</i>	Precorrin-4 C11-methyltransferase
CobN	<i>Pseudomonas denitrificans</i>	Cobalt insertion protein
CobP	<i>Pseudomonas denitrificans</i>	Cobinamide kinase
CobQ	<i>Pseudomonas denitrificans</i>	Cobyrinic acid synthase
CobS	<i>Escherichia coli</i>	Cobalamin [5'-phosphate] synthase
CobT	<i>Salmonella typhimurium</i>	Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase
CobU	<i>Salmonella typhimurium</i>	Cobinamide phosphate guanylyltransferase
CobV	<i>Pseudomonas denitrificans</i>	Cobalamin [5'-phosphate] synthase

¹ A significant Blast score is greater than 100.

Table 5.4 – Cytochrome-Associated Proteins with Significant Identity to ORFs of *P. gingivalis*

Putative Enzyme / Protein	% Identity			
	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Synechocystis</i>	<i>Aquifex aeolicus</i>
NrfA				
Cytochrome C 552 precursor (terminal reductase of the formate- dependent pathway for nitrite reduction to ammonia)	43.19	-	-	-
CydA	40.56	40.67	34.90	38.48
Cytochrome D ubiquinol oxidase subunit I				
CydB	29.67	25.67	22.19	26.71
Cytochrome D ubiquinol oxidase subunit II				
CcsA / CcmC	21.39	31.28	33.17	27.27
Cytochrome C biogenesis protein	(CcmC)		(CcsA)	

ORF1	1	MKNKERKLKSWQGWLIFSSSMVVVFCLGLLAASVTERRAEIQSIYANKKD	50
		. : : : . . . : . : . . .	
NRFA	1	..MTRIKINARRIF....SLLIPIFFFFFTSVHAEQTAAPA.....	33
ORF1	51	KIAPFEARNEMYRGNYPREYETWYTYTADTSFRSEFNGSQAIDVLEQRPNM	100
		. . . : : . . . : . . . : : . : . :	
NRFA	34	KPVTVEAKNETFAPQHPDQYLSWKATSEQSER.....VDALAEDPRL	75
ORF1	101	VIFWAGYAFSRDYTSRGRHMHAIQDMQRTLRGTGNPGIDGAGDMQPAT	150
		: : . : : : .	
NRFA	76	VILWAGYPFSRDYNKPRGHAFVTDVRETLRGTGAPKNAEDGPL.PMA	124
ORF1	151	SPDVPRMMQAIGVDEFYKNKWSSLGSDIVNPIGADNDP.....E	193
		: : : : : : : . :	
NRFA	125	SPDVARLIQKDGEDGYFHGKWARGGPEIVNNLGADNTASPEFAKG	174
ORF1	194	TMDLHISRPALIEAFQRRGLDITKASHQEMRSLVQAQGEVEYYFKGEGKY	243
		. : : : : . . . : : . : : . . .	
NRFA	175	KPELTLSRPYAARAMEAIGKPFKAGRFDQQSMVGGQGEVEYYFDGKNKA	224
ORF1	244	LTFPWDKGMTMEDAERYYYDEAEYYDYIHTLSRAPILKAQHPDFEISQHGI	293
		: . . : : : . . : : . . : : . .	
NRFA	225	VKFPWDDGMKVENMEQYYDKIAFSDWNTSLSKTPMLKAQHPEYETWTAGI	274
ORF1	294	HAQRGVSADMPYISEGGVK.FSDHHITSPLAHMDRTQOTORESEEE	342
		: : : : . : . . : : . . . :	
NRFA	275	HGKNNVTIDMPKVQNAEGKLYTDHKIGNPFDNFAQTANSTQDKAA	324
ORF1	343	LRKNVYERQRKANEVRNQLLENELAKAHLEAQFAWDKGATEKEMTPILKYI	392
		. . . : : . : : : . : . : .	
NRFA	325	LQKVVAERKQSINDLKIKVEDQLVHAHFEAKAALDAGATEAEMKPIQDDI	374
ORF1	393	RQSQRWDYGVASHGASFHAPQEITRILSNGLERAMQARIEIARVLARHG	442
		: . . : : : : : : : : : . : : .	
NRFA	375	RHAQWRWDLAIAASHGIHMHAPEEGLRMLGTAMDKAADARTKLARLLATKG	424
ORF1	443	YTDEVPLPDVSTKEKAQKYIGLDMGDLHKNKEKFLETVPKWKVKAKGKG	492
		. . : : : . : : . . : : . : . : . . . : .	
NRFA	425	ITHEIQIPDISTKEKAQQAIGLNMEQIKAQKQDFIKTVIPQWEEQARKNG	474
ORF1	493	LLIAAK	498
		.	
NRFA	475	LLSQ..	478

Figure 5.2 - A putative protein (ORF1) from *P.gingivalis* has significant identity to the NrfA protein of *Escherichia coli*. The protein sequences were aligned with the program GAP (Genetics Computer Group, 1995) and have an identity of 43.19%. The residues needed for haem binding in NrfA are highlighted in blue and the corresponding conserved residues in the putative protein are also highlighted in blue.

5.3.1.2.2 The cytochrome D ubiquinol oxidase complex

In *E. coli* the CydAB complex is one of two terminal oxidases in the aerobic respiratory chain (Spinner *et al.*, 1995). This complex catalyses the oxidation of ubiquinol-8 and the reduction of oxygen to water. Levels of this complex are elevated under anaerobic conditions. This is because it has a high oxygen affinity and it can scavenge oxygen molecules for electron transport-linked energy generation. The oxidase CydAB contains 3 haem moieties.

In the genome of *P. gingivalis* there is an ORF that has 40.56% identity to CydA of *E. coli* and an adjacent ORF that has 29.67% identity to the CydB protein of *E. coli* (Figure 5.3). Also conserved in the ORFs are two histidines and a methionine which are needed for ligation to haem b558 in the *E. coli* complex (Spinner *et al.*, 1995).

5.3.1.2.3 Cytochrome C biogenesis protein

CcSA or CcmC is needed for haem attachment to C type cytochromes in *E. coli* and other bacteria. An ORF in *P. gingivalis* has 33.17% identity to an ORF of *Synechocystis sp.* PCC 6803 that has been putatively identified as CcsA (Figure 5.4). The ORF of *P. gingivalis* has a conserved tryptophan rich motif that is used in haem binding in the CcmC protein of *E. coli*, and conserved histidine residues that may serve as ligands to the haem iron (Xie and Merchant 1998).

The genomic data base search found *P. gingivalis* to be lacking the critical enzymes for porphyrin synthesis. However, the presence of cytochromes which contain haem as a prosthetic group, implied the organism still had a requirement for porphyrin. This need apparently had to be met from external sources of porphyrin. Since the organism possessed the last three enzymes in the haem synthesis pathway that are involved in modification of the vinyl-face of the porphyrin molecule, it should be able to scavenge a wide range of porphyrins. The ability of *P. gingivalis* to utilise a range of porphyrins as a substitute for haem required investigation.

5.3.2 Ability of porphyrins to substitute for haem as a growth supplement

The ability of the porphyrins DBEG, DDH and DDS to substitute for haem as a growth supplement was tested under various culture conditions. *Porphyromonas gingivalis* cells grown for 48 h were transferred at a 1% inoculum into supplement-free media to deplete the cells of internal stores of haemin by continuous passage until growth stagnated. The growth rate was stagnant at the first passage after 24 h and the “haemin depleted” *P. gingivalis* cells were then transferred at a 1% inoculum into supplement free CDC media (5.2.2.1). The addition of the test porphyrins into the culture had different effects as can be seen in Figure 5.5a. It took at least 40 h for DDH and haemin (control) to re-establish growth. The growth profiles were found to be very similar but the final biomass reached was only up to $OD_{660} = 0.5$ despite allowing the cultures to grow for 100 h. Under standard growth conditions (5.2.2.1), the cells are normally able to reach an $OD_{660} = 1.0$. DBEG and DDS were unable to re-establish growth under these conditions and paralleled the growth rate of the negative control (no supplements with the 1% inoculum). Due to the unusually short time required to deplete the cells of haemin, it was decided to include menadione at all stages of the experiment and observe any differences to the growth rates with the test porphyrins.

The haemin-depleted cells were then prepared by inoculating from a 48 h haemin-supplemented culture into haemin-free (supplemented with menadione) medium. The first difference noted was the number of passages required to reach growth stagnation. As compared to the first experiment described, it now took 5 passages for this to occur. When the growth rate had stagnated, the haemin-depleted cells were transferred into haemin-free media supplemented with menadione with the addition of the respective test porphyrins. When the test porphyrins were added to this medium under these conditions, they were all able to substitute for haemin (although with different responses) (Figure 5.5b). Cultures supplemented with both DDH and haemin grew at identical rates again. The growth profiles in the presence of DBEG and DDS were quite divergent when compared to the first experiment (Figure 5.5a). In order to calculate the efficiency of the test porphyrins under these conditions, the shortest time for the haemin control to reach

Figure 5.5. Growth curves of *P. gingivalis* demonstrating the effect of substituting iron-free porphyrin for haem as a growth supplement

a. *Porphyromonas gingivalis* cells grown for 48 h were transferred at a 1% inoculum into supplement-free media to deplete the cells of internal stores of haemin by continuous passage until growth stagnated. The effect of DBEG, DDH and DDS on the growth rate of *P. gingivalis* in haemin-depleted media without the presence of menadione is shown. DDH(--) is demonstrated to support growth at the same rate as the positive control haemin(—) while DBEG (—) and DDS (—) were unable to re-establish growth and paralleled the growth rate of the negative control (—).

b. The haemin-depleted cells were prepared under these conditions by inoculating from a 48 h haemin-supplemented culture into haemin-free (supplemented with menadione) media until growth stagnated. The effect of DBEG, DDH and DDS on the growth rate of *P. gingivalis* in haemin-depleted media supplemented with menadione is shown with DDH(---), DBEG (—) and DDS (—) shown to be able to substitute for haemin (positive control) (—) with different growth rates

c. These conditions involved preparing the haemin-depleted cells by inoculating from a 48 h haemin-supplemented culture into haemin-free media supplemented with menadione until growth stagnated. The effect of DBEG, DDH and DDS on the growth rate of *P. gingivalis* in menadione-free recovery media. The growth profiles for DBEG (—) and DDS (—) supplementation were shown to have altered slightly under these conditions while DDH(--) and haemin (positive control) (—) supported identical growth rates.

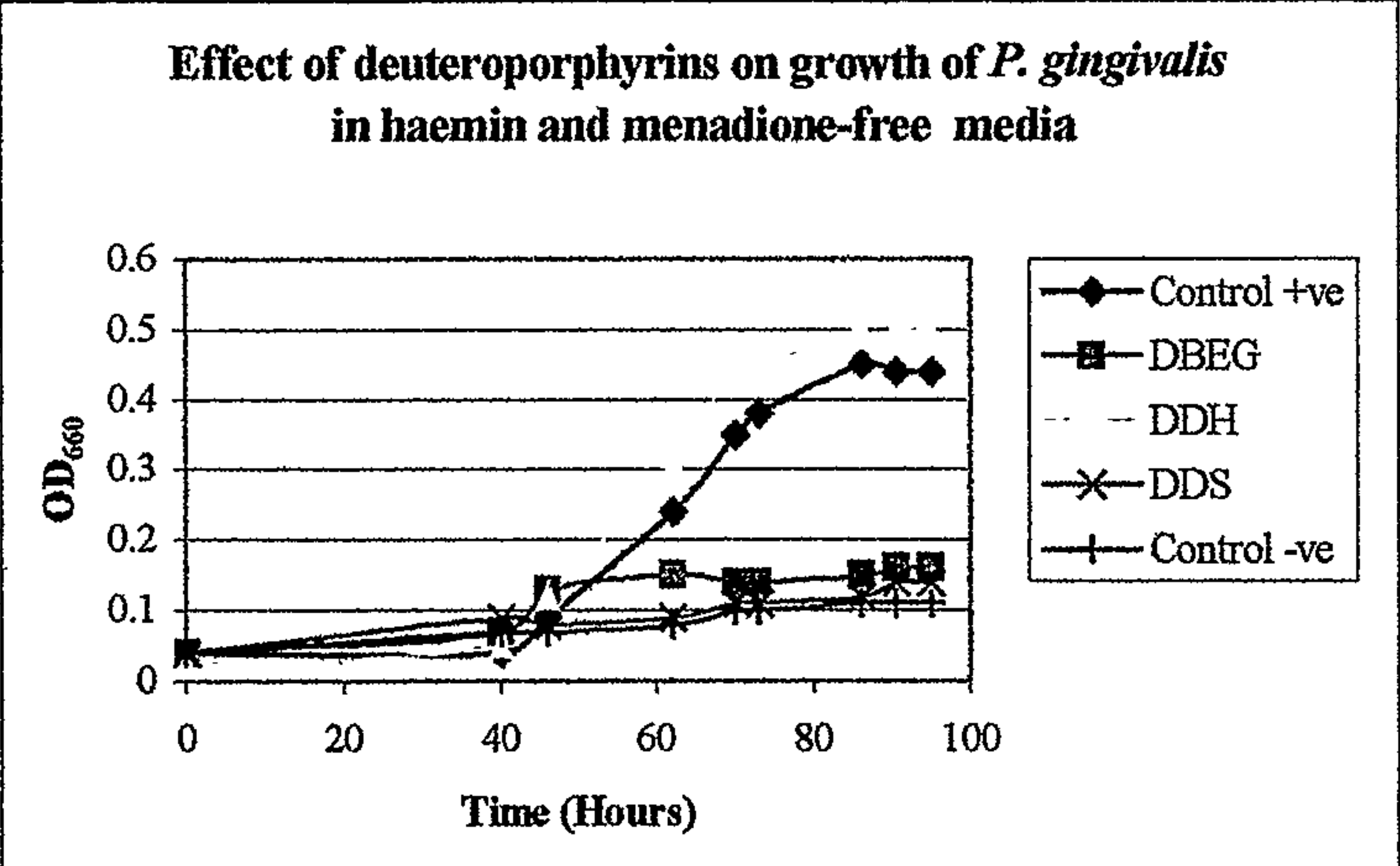


Figure 5.5a

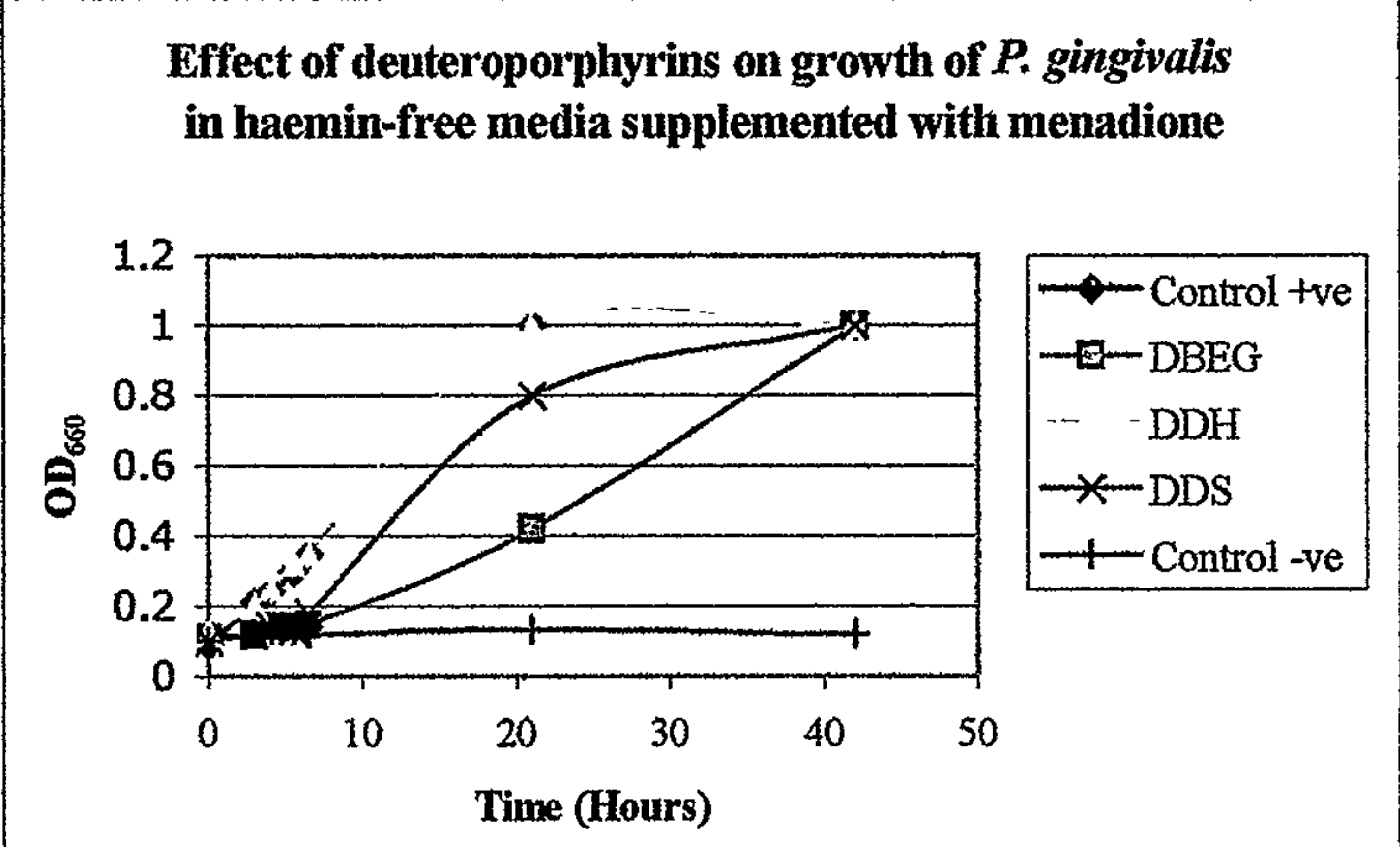


Figure 5.5b

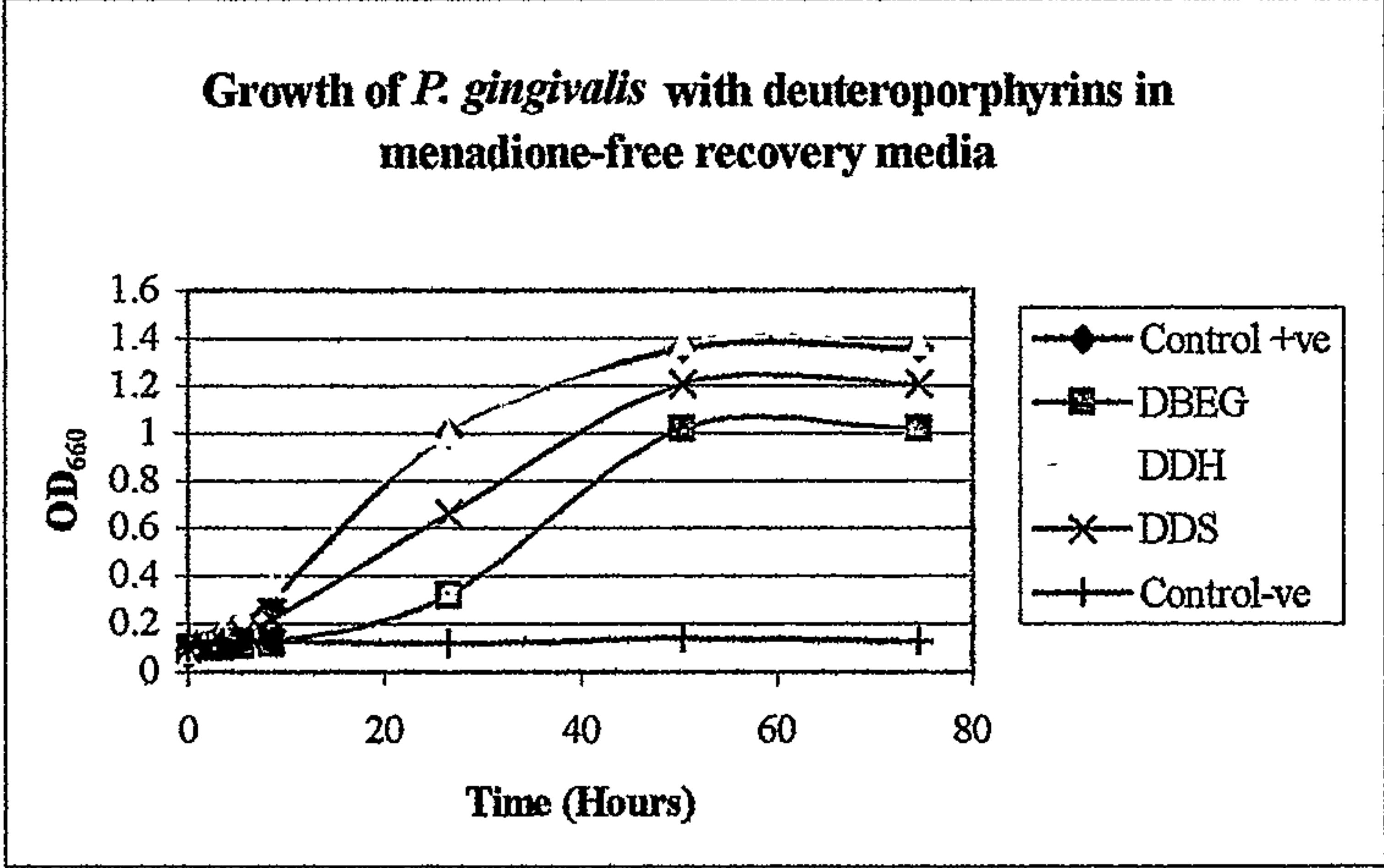


Figure 5.5c

an $OD_{660} = 1.0$ was designated 100 %. In this experiment, the time taken to reach this OD was approximately 20 h. The efficiency of the test porphyrins was calculated as a percentage of the OD reached in this time frame. DBEG was able to act as a porphyrin supplement at approximately 40% the efficiency of DDH while DDS was closer to DDH at approximately 80% efficiency. The efficiency of the porphyrins was calculated as a percentage of the maximum OD reached. Despite the differences in the initial growth rate, all the cultures were able to reach a final biomass of $OD_{660} = 1.0$. As it was evident that the presence of menadione affected both the number passages required to deplete the cells of haemin and the recovery growth curves of the substituted porphyrin, a final set of conditions with no menadione was investigated.

These conditions involved preparing the haemin-depleted cells by inoculating from a 48 h haemin-supplemented culture into haemin-free media supplemented with menadione. When the growth rate had stagnated again after 5 passages, the haemin depleted cells were then transferred into supplement-free media with the addition of the respective test porphyrins. Under these conditions, the growth profiles of DBEG and DDS were again found to have changed slightly from previous observations (Figure 5.5c). DDH had an identical growth pattern to haemin. Despite the difference in the test growth conditions for Figure 5.5b and 5.5c, DBEG was shown in both instances to consistently support a final OD_{660} of 1.0. In order to calculate the efficiency of the test porphyrins under these conditions, the shortest time for the haemin control in this experiment to reach to reach an $OD_{660} = 1.0$ was approximately 25 h.. This OD was designated 100 % with the efficiency of the test porphyrins calculated as a percentage of the OD reached in this time frame. DBEG was able to act as a porphyrin supplement at approximately 30% the efficiency of DDH while DDS had approximately 65% efficiency.

The CDC medium is a complex mixture with a variety of undefined ingredients. It would have been preferable to have used a chemically defined medium for the growth curve experiments. This was attempted by using a chemically defined and minimal media for *P. gingivalis* containing 4 amino acids, 5 mineral salts, cysteine hydrochloride as a reducing agent and the growth factors haemin and menadione (Seddon *et al.*, 1988). However attempts to adapt the organism from the CDC media into this defined medium

were not successful. Since the importance of porphyrin to the organism had now been established, the role of HA2 as a porphyrin-mediated haemoglobin binding protein required further investigation.

5.3.3 Neutralisation of haemoglobin-binding

The importance of HA2 as a porphyrin-mediated haemoglobin binding peptide in *P. gingivalis* was investigated by using immunoassays involving neutralising patient sera (5.2.5.2). Haemoglobin was used in the assay rather than protoporphyrin IX or haemin because haemoglobin was found to coat the plates more efficiently and produced more consistent results. A standard curve of the binding of biotinylated CHAPS extract to haemoglobin is shown in Figure 5.6a. This curve established the ability of surface proteins from *P. gingivalis* to successfully bind haemoglobin. The 50% saturation point of the binding curve of biotinylated CHAPS extract to haemoglobin was calculated and used as a fixed reference point for the pre-incubation mix in the neutralisation assay of haemoglobin binding.

The ability of human IgG sera #125 to neutralise haemoglobin binding where OD reached close to 0 is shown in Figure 5.6b. In contrast, the control IgG #65 sera was only weakly neutralising. This established the ability of human IgG sera #125 to successfully bind the haemoglobin-binding proteins in the CHAPS extract of *P. gingivalis* and eliminate haemoglobin binding. However, since the IgG used was not specific to HA2, a further step was required to relate this neutralising capacity specifically to HA2.

To demonstrate the importance of HA2 in haemoglobin binding, the rHA2-bound resin prepared (5.2.5.3) was used to remove HA2-related IgG from the human sera #125 with HA2-IgG depleted sera added back into the assay resulting in a shift in the profile of the curve. The midpoint of the linear portion of the inhibition curve and the standard neutralisation curve was calculated to the nearest 2 fold dilution. This difference was then expressed as a percentage of total binding inhibited by the rHA2-bound resin. A 70-85% reduction was demonstrated in ability of the sera to neutralise haemoglobin binding (Figure 5.6c). Although only a single absorption procedure was used for this assay due to the low expression levels of the rHA2 clone (3.3.7), the percentage range was the mean average 6 experiments.

Figure 5.6. Binding curves demonstrating the importance of HA2 as the major porphyrin-mediated haemoglobin binding protein (representative of 6 experiments with triplicates).

- a. Shows the standard binding curve of biotinylated CHAPS extract (BCE) from *P. gingivalis* to haemoglobin. Dilutions of BCE were incubated on haemoglobin-coated plates. Strep-AP was applied then AP activity monitored at A_{414} . The 50% saturation point of the binding curve of biotinylated CHAPS extract to haemoglobin was calculated and used as a fixed reference point for the pre-incubation mix in the neutralisation assay of haemoglobin binding.
- b. Shows the binding curve of biotinylated CHAPS extract to haemoglobin after neutralisation of haemoglobin binding. The patients with the strongest (IgG #125) and the weakest (IgG #65) neutralising capacity were selected. Using the standard ligand binding assay (5.2.5.2), BCE at a concentration which produced 50% saturation binding to a haemoglobin-coated plate was pre-incubated with dilutions of patient IgG #125 (—) and the control patient IgG #65 (—) then allowed to bind to haemoglobin-coated plates overnight.
- c. Shows two sets of binding curves of biotinylated CHAPS extract to haemoglobin. rHA2-bound resin suspension and a resin control was pre-incubated with patient IgG #125 (highest level of neutralisation of haemoglobin binding) and patient IgG #65 as the IgG control in a single absorption procedure. BCE at a concentration which produced 50% saturation binding to a haemoglobin-coated plate was added, incubated and detected with Strep-AP. The midpoint of the linear portion of the inhibition curve and the standard neutralisation curve was calculated to the nearest 2 fold dilution. This difference was expressed as a percentage of total binding inhibited by the rHA2-bound resin. 125/CE and 65/CE represent the positive control where IgG #125 and #65 had been used to neutralise haemoglobin binding of the biotinylated CE. 125/HA2 and 65/HA2 represent the same assay after rHA2 bound resin had been used to remove rHA2-related IgG from the human sera #125 and added back to the sera resulting in a shift in the profile of the curve.

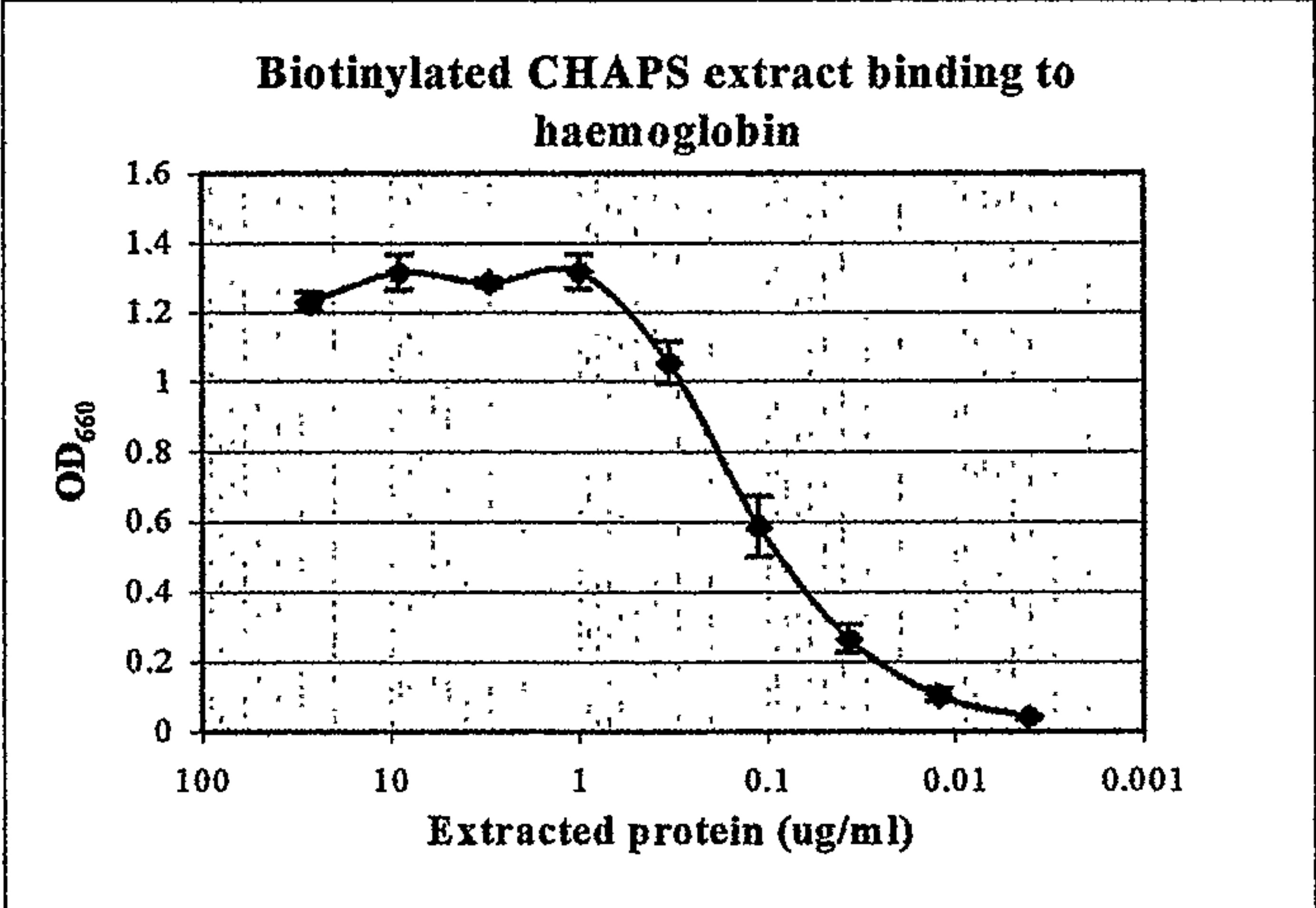


Figure 5.6a

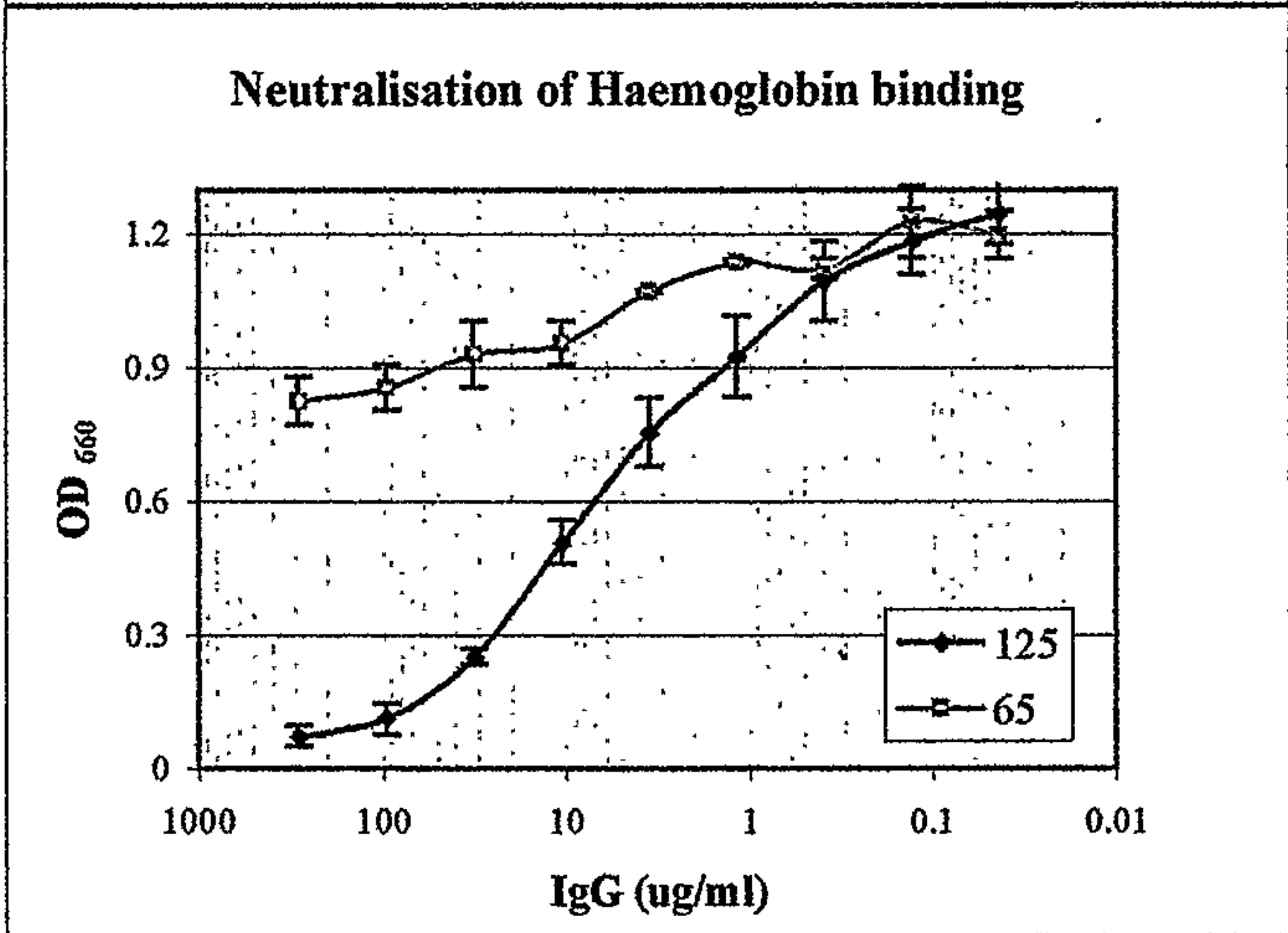


Figure 5.6b

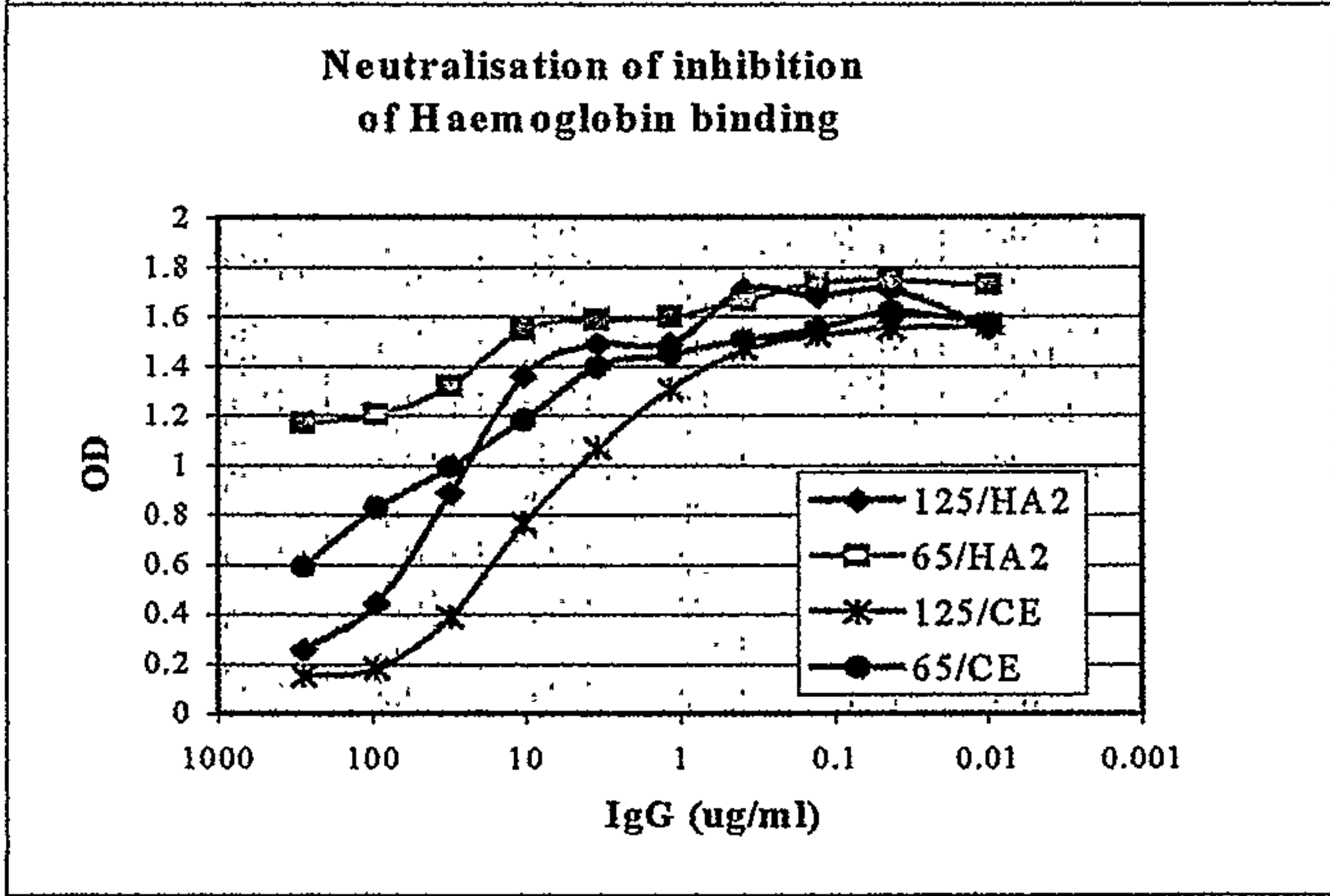


Figure 5.6c

separate experiments. These results demonstrated the importance of HA2 as the main porphyrin-mediated haemoglobin binding protein in *P. gingivalis*. On this basis, it was of interest to further clarify the nature of this binding interaction between the HA2 molecule and protoporphyrin IX.

5.3.4 Solid-phase ligand binding assay of deuteroporphyrins to HA2

Solid-phase ligand binding assays were set up to test the capacity of a range of porphyrins to bind HA2. Since it has been found that HA2 appears to bind protoporphyrin IX (PPIX) via the propionic face (DeCarlo *et al.*, 1999), porphyrins were chosen which resembled PPIX with differing levels of modifications on the vinyl face to further support this finding. To optimise binding conditions, each porphyrin was tested using either a pre-coat of ethylene diamine, a coat in PBS/Tween (5.2.3.2) or bicarbonate buffer (5.2.3.1) and incubating the rHA2 in PBS/Tween or Acetate/Tween (5.2.7.1). It was found that DBEG and DDS required to be coated in 0.1 N NaOH and bound best to rHA2 in PBS/Tween. C1 coated the plate in 0.1 N NaOH but interacted more strongly with rHA2 in Acetate/Tween. C2 coated best in 0.1 N NaOH and bound to rHA2 in PBS/Tween. Finally C3 required a pre-coat in ethylene diamine, before being coated in 0.1 N NaOH then bound to rHA2 in Acetate/Tween. Despite the different binding conditions required to optimise binding in each case, in the solid-phase ligand-binding assay, rHA2 bound the porphyrins with similar affinities (Figures 5.7a-e). Deuteroporphyrin IX dihydrochloride was not able to attach to the plate under the conditions tested, but was shown in the competition assay to compete with haemoglobin for rHA2. This confirms earlier data which showed that rHA2 was capable of binding to the propionic acid chains despite modification on the vinyl aspect (DeCarlo *et al.*, 1999). All the porphyrin derivatives tested were modified at the 2,4 vinyl aspect and the dipyrroles tested were structurally analogous “half” porphyrins containing no vinyl aspect. Despite these differences the ligands tested demonstrated comparable interactions with rHA2, all with apparent K_d s of between 15-20 nM (Figure 5.7a-e). Since the deuteroporphyrins can bind HA2 with similar binding affinities, it was of interest to investigate the ability of these porphyrins to compete with haemoglobin for HA2.

Deuteroporphyrin IX 2,4
Disulfonic acid

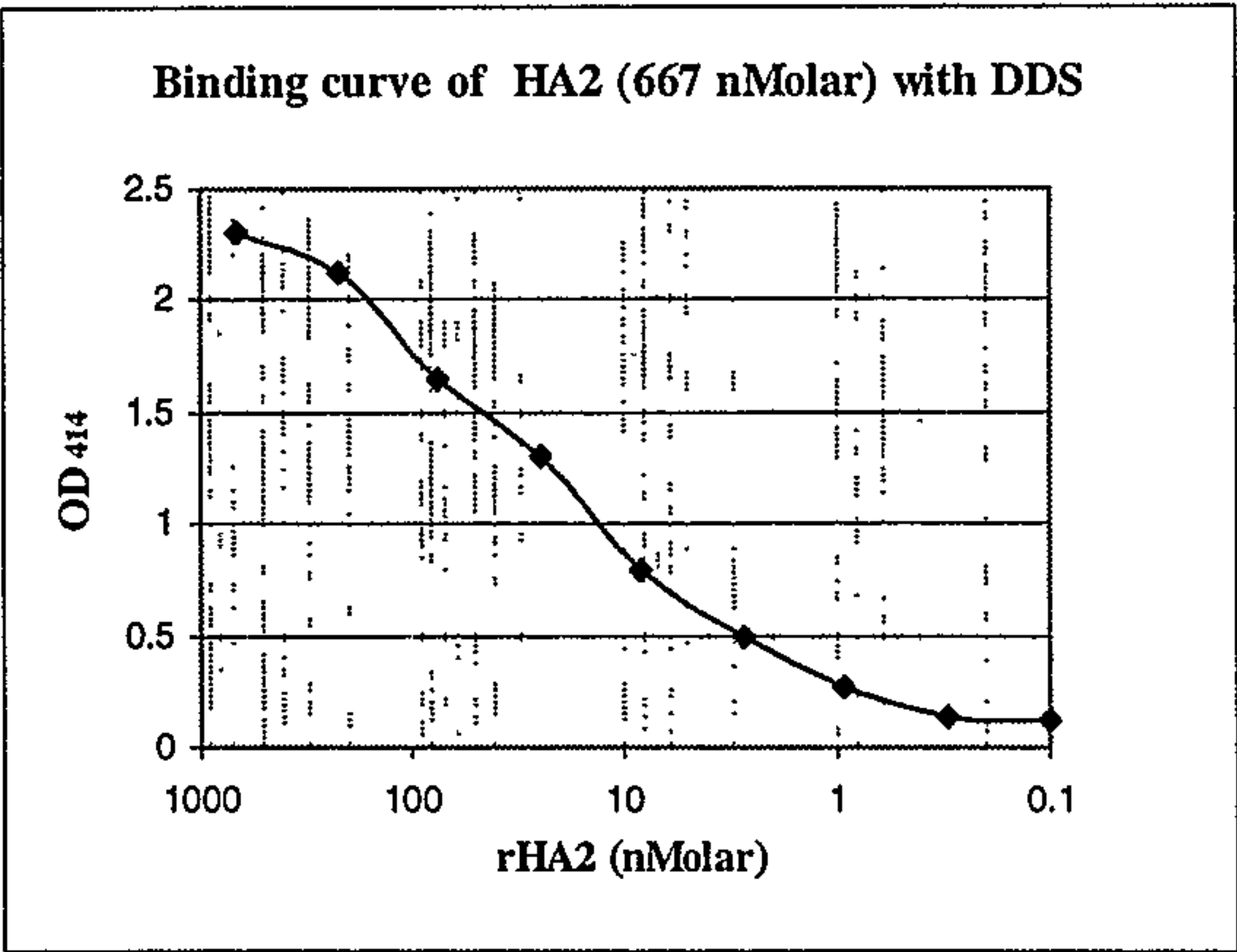
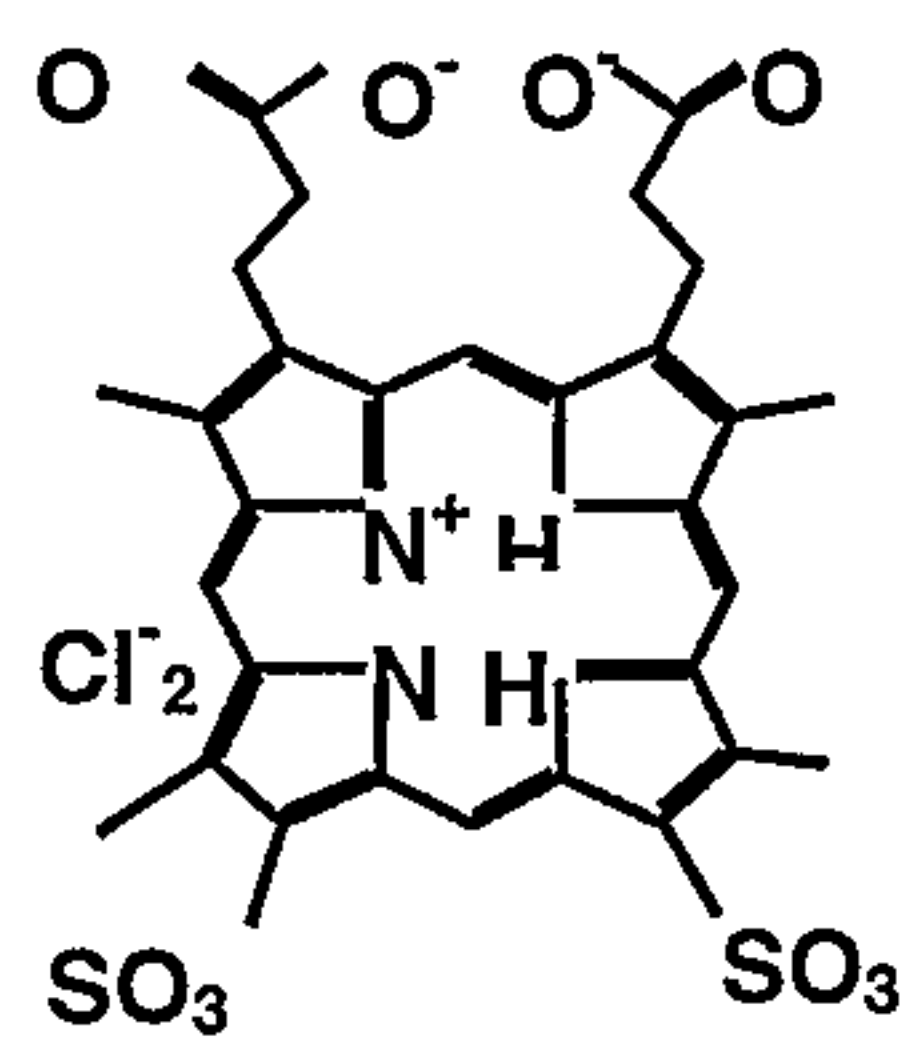


Figure 5.7a $K_d=20\text{nM}$

Deuteroporphyrin IX 2,4
BisEthylene Glycol

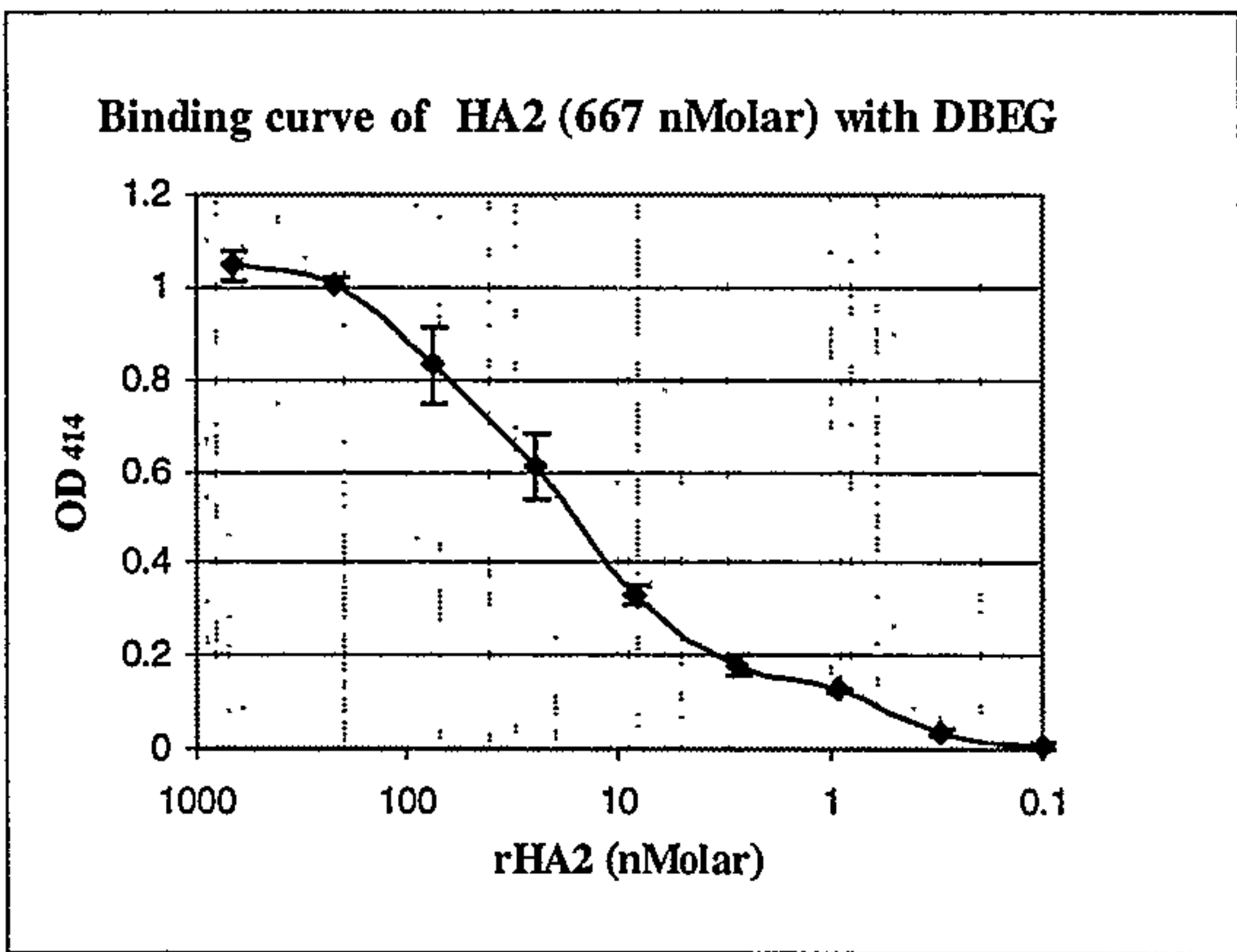
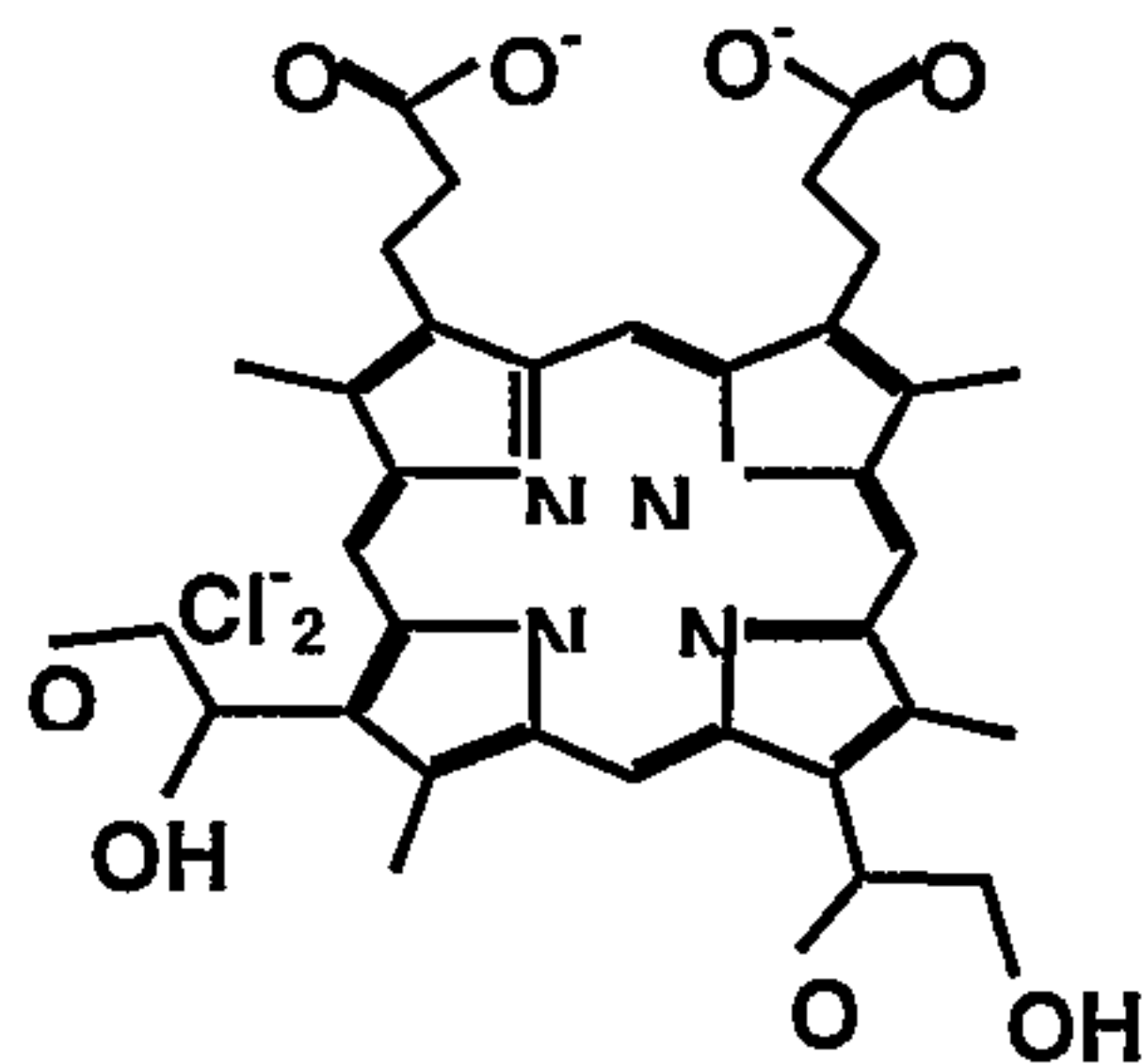


Figure 5.7b $K_d=20\text{nM}$

Dipyrrole No. 1

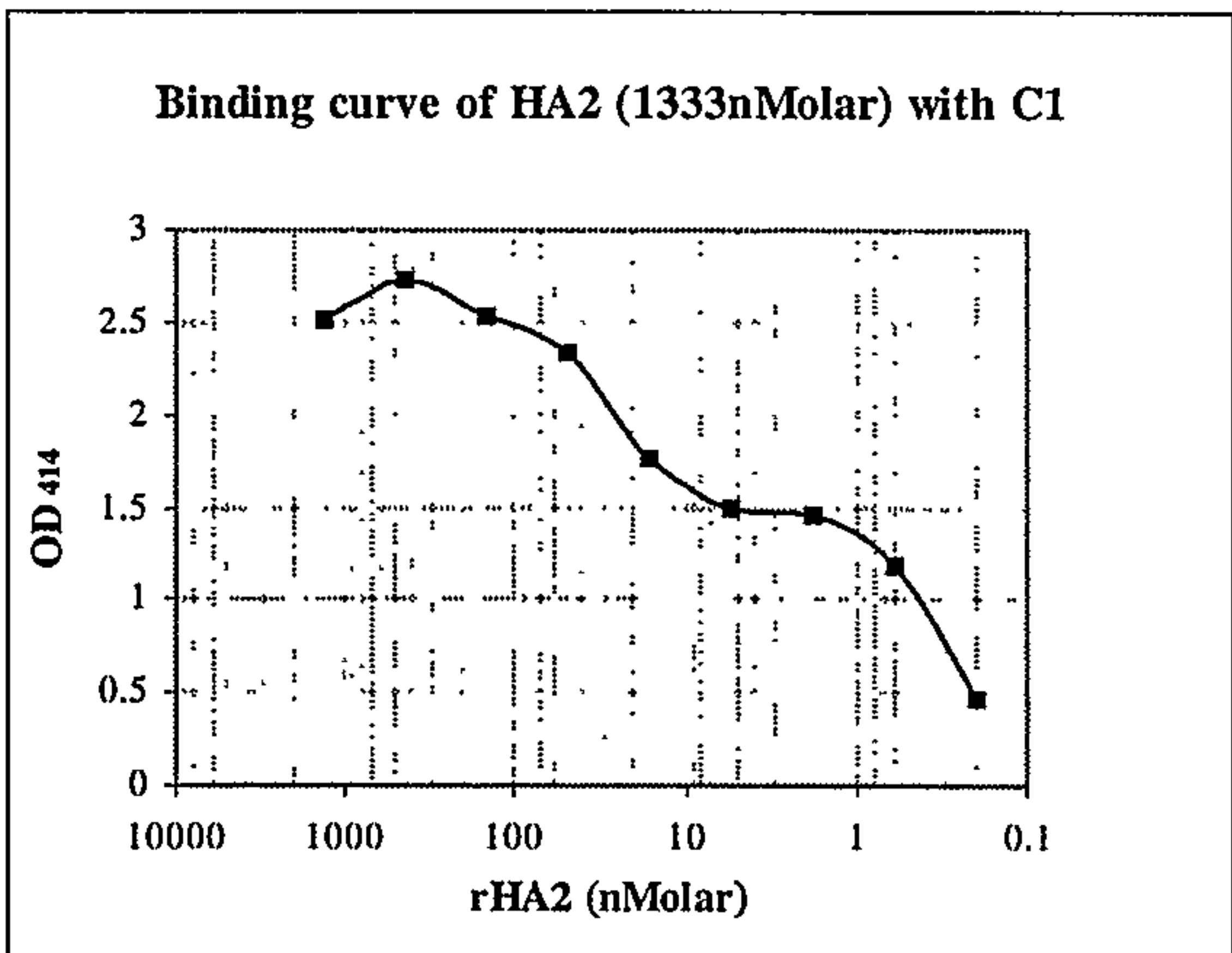
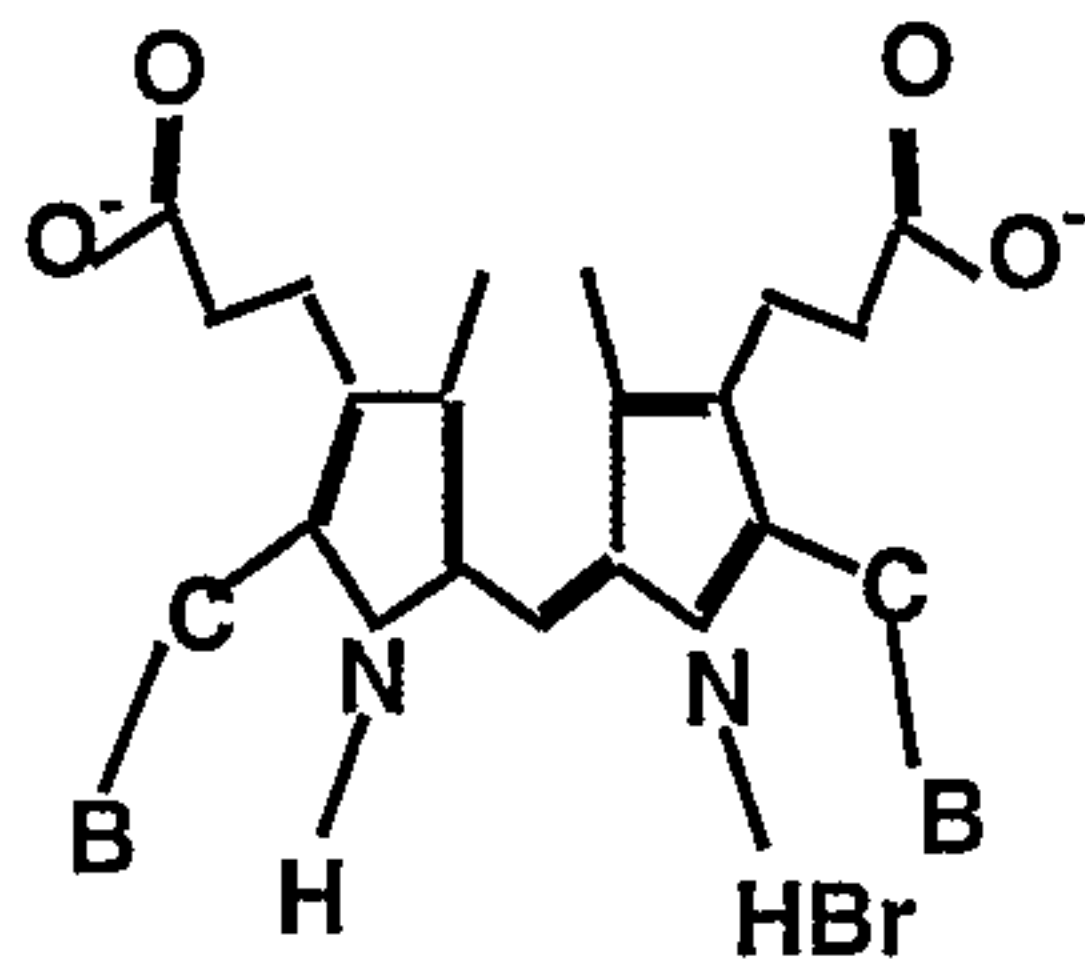


Figure 5.7c $K_d=15\text{nM}$

Dipyrrole No. 2

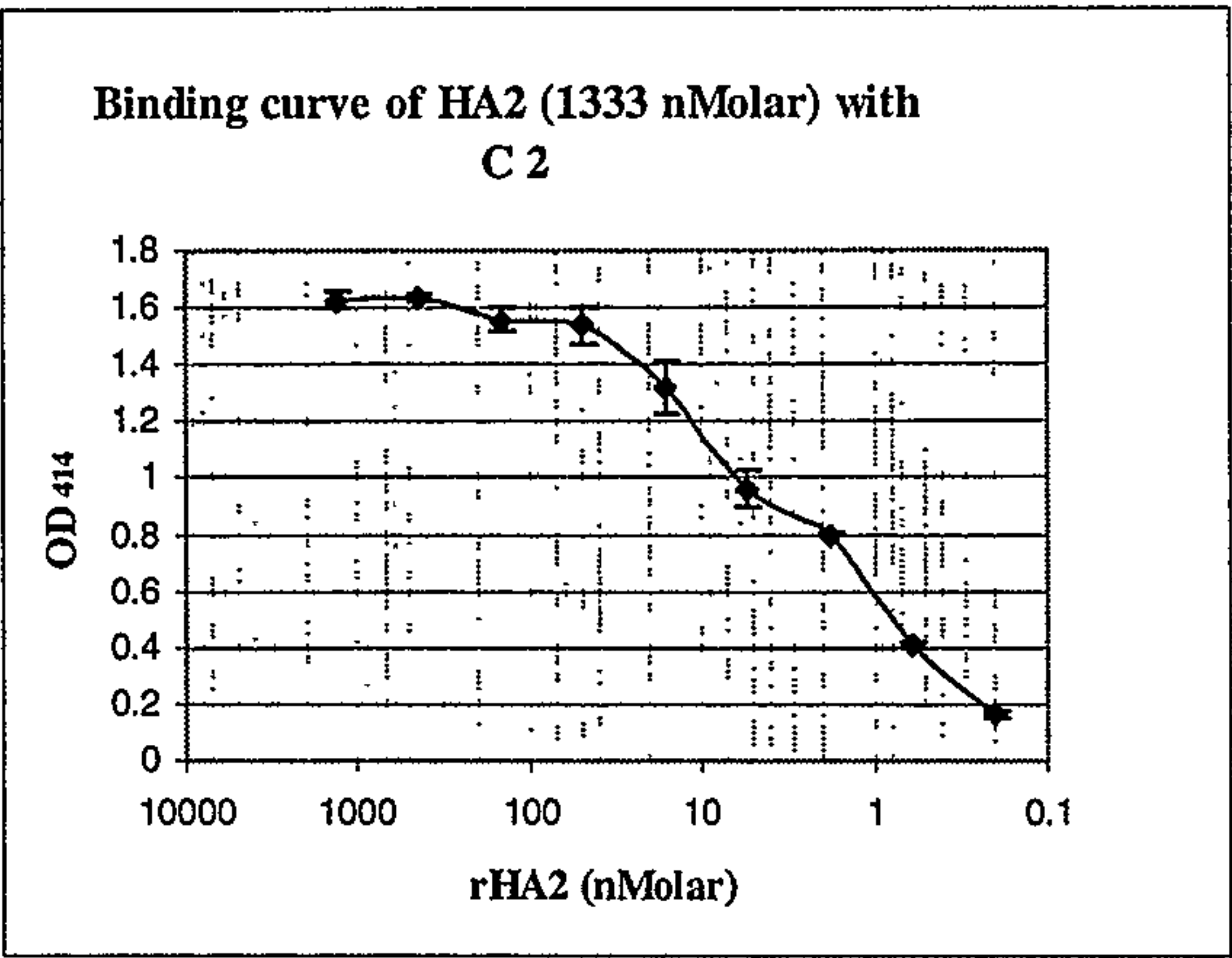
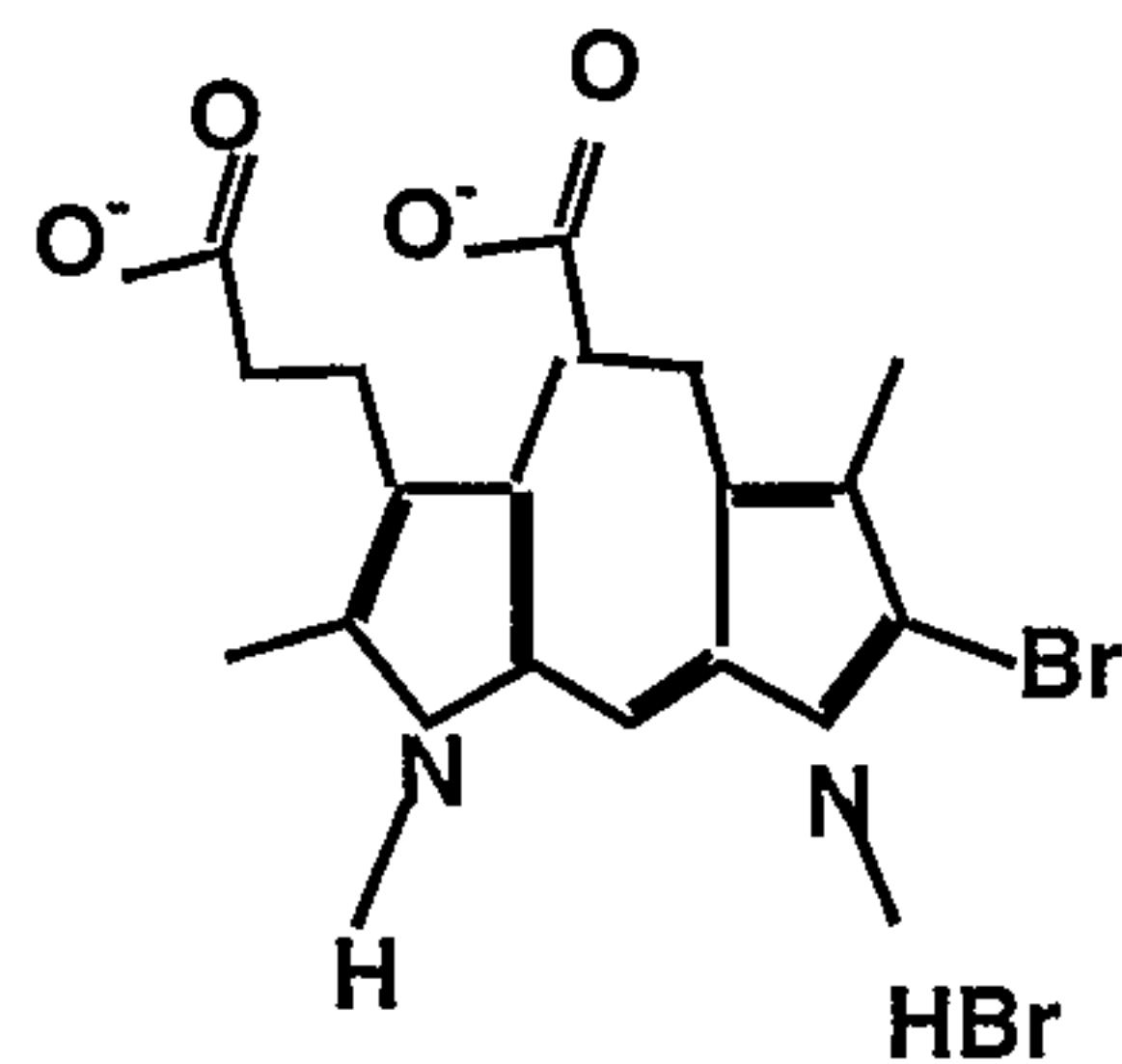


Figure 5.7d $K_d=20\text{nM}$

Dipyrrole No.3

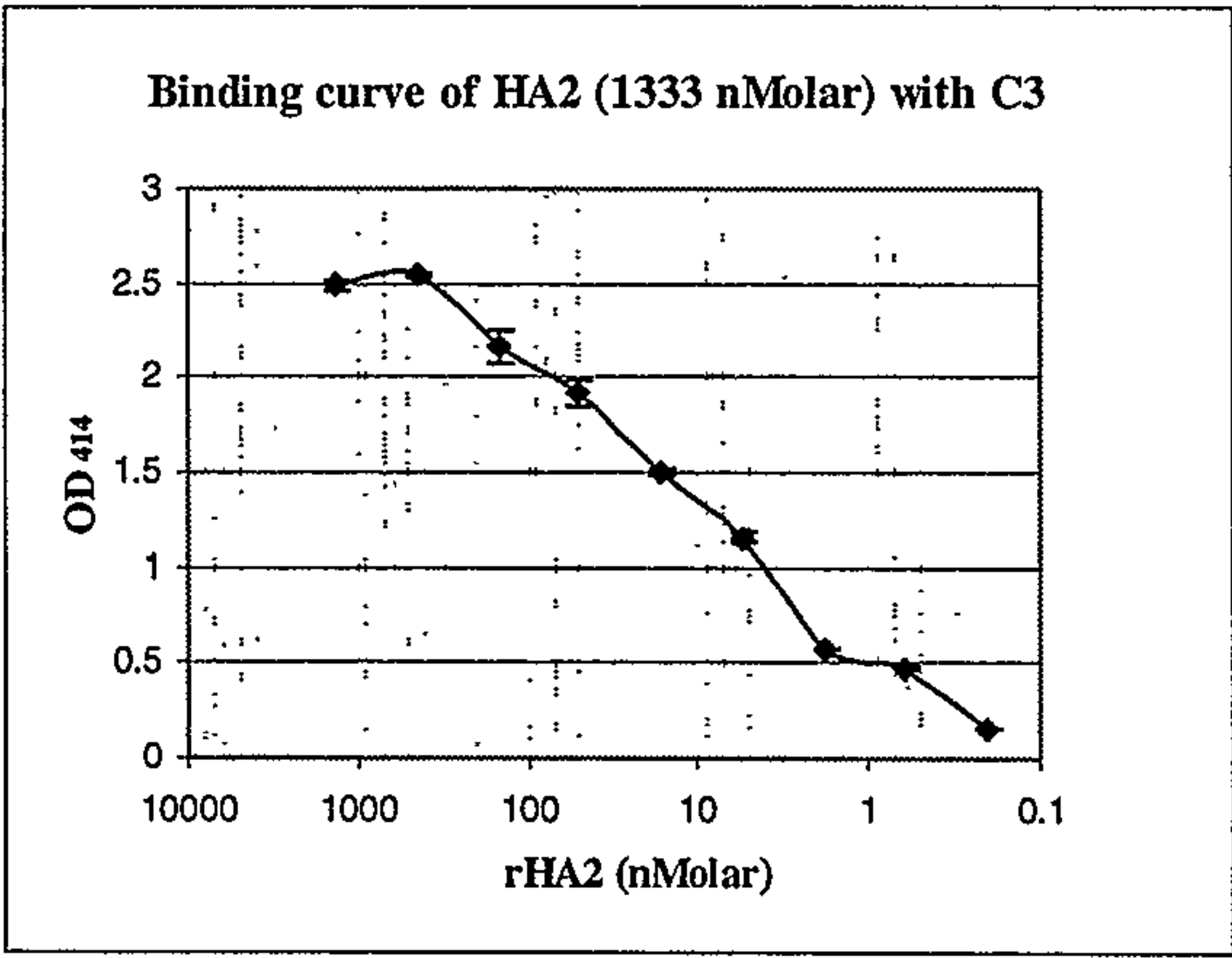
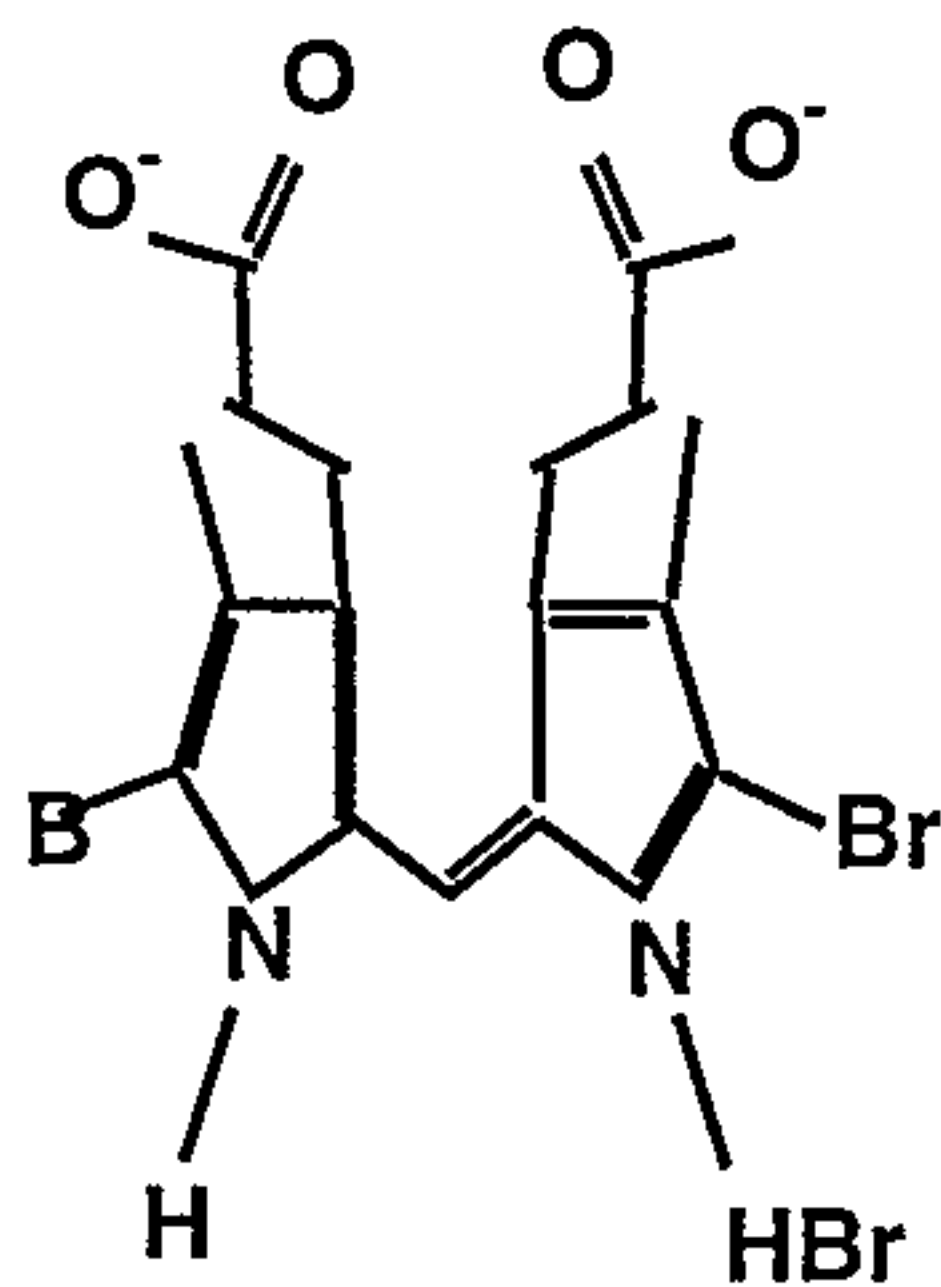


Figure 5.7e $K_d=20\text{nM}$

Figure 5.7a-e. Solid-phase ligand binding curves showing the binding affinities of DDS, DBEG, Dipyrrole no.1, 2 and 3 to rHA2. Despite the different binding conditions used all the porphyrins tested bound to rHA2 with similar affinities ($K_d=20\text{nM}$).

5.3.5 Competition ELISA between deuteroporphyrins and haemoglobin for HA2.

Competition ELISA immunoassays were set up to investigate the ability of deuteroporphyrins with varying degrees of modification on the vinyl-face to compete with haemoglobin for HA2. Haemoglobin was utilised as a source of protoporphyrin IX in this study as this represents the most likely presentation of the molecule *in-vivo*. The apparent IC₅₀s for the deuteroporphyrins were similar in value ranging from 3-8 µM (Figure 5.8a-h). Of interest, tested at similar concentrations, the dipyrroles were incapable of competing with haemoglobin for rHA2 even though they were demonstrated to bind to rHA2 in the solid-phase assays.

5.4 DISCUSSION

Analysis of the genomic database of *P. gingivalis* W83 indicates that the organism is probably unable to undergo *de novo* synthesis of porphyrin with the early and essential steps for synthesis of the tetrapyrrole ring absent from the genome. A recent study by Roper *et al.* has supported this finding with the presence of 5-aminolevulinic acid dehydratase (ALA) and phorphobilinogen deaminase (PBG), two key enzymes involved in the synthesis of uroporphyrinogen III (the first macrocyclic intermediate in tetrapyrrole synthesis) targeted using two different methods (Roper *et al.*, 2000). Extracts of bacteria were tested for ALA and PBG activity supported by genetic investigation for evidence of these early enzymes using degenerate polymerase chain reaction (Roper *et al.*, 2000). Both methods were not able to detect the presence of these enzymes.

The genomic data search in this study suggested that the bacterium has lost most of its haem biosynthetic genes since there is no *hemD*, *hemE* or *hemG* although *hemN* and *hemH* genes are present. Roper *et al.* also support this finding using the GCG software package (Genetics Computer Group, Inc., Madison, WI) with searches of the Unfinished Bacterial Genomic Data Base performed using the BLAST suite (Roper *et al.*, 2000). While the functions of the recognised gene *hemN* is not yet fully understood, the gene *hemH* encodes an enzyme which has significant identity to ferrochelatase, an

The chemical structure of chlorophyll *a* is shown. It features a central magnesium atom (Mg) coordinated by four nitrogen atoms (N) in a porphyrin-like ring. The structure includes a long phytol chain (CH₂)₂₀ and a vinyl group (CH=CH₂).



The diagram shows a complex chemical structure, likely a chlorophyll derivative. It features a central magnesium atom (Mg) coordinated by four nitrogen atoms (N) in a porphyrin-like ring. A chlorine atom (Cl) is attached to one of the ring carbons. Two long side chains are attached to the ring, each ending in a carboxylate group (COO-). The structure is labeled with 'C', 'O', 'N', 'H', and 'Cl'.



Deuteroporphyrin IX 2,4
BisEthylene Glycol

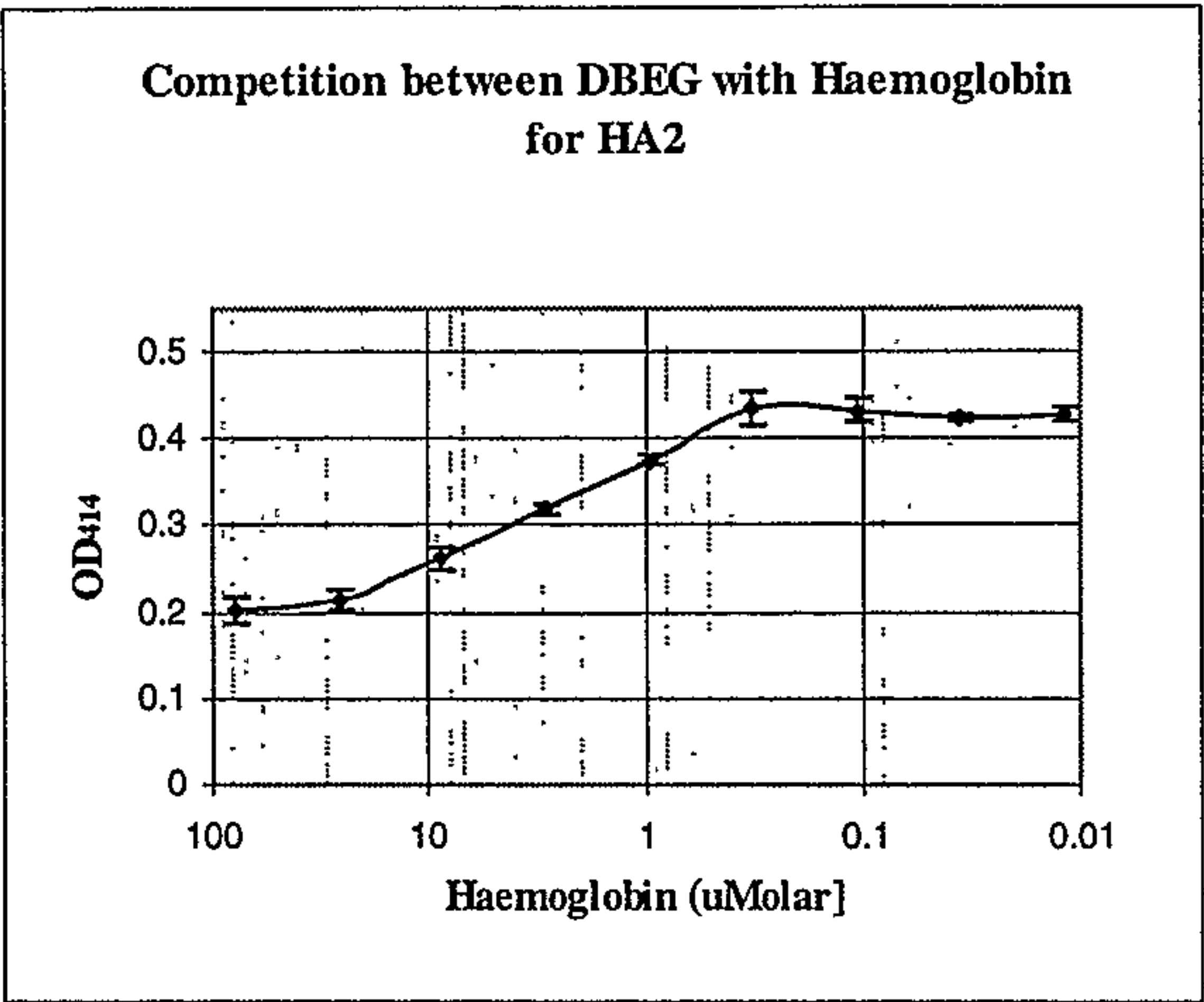
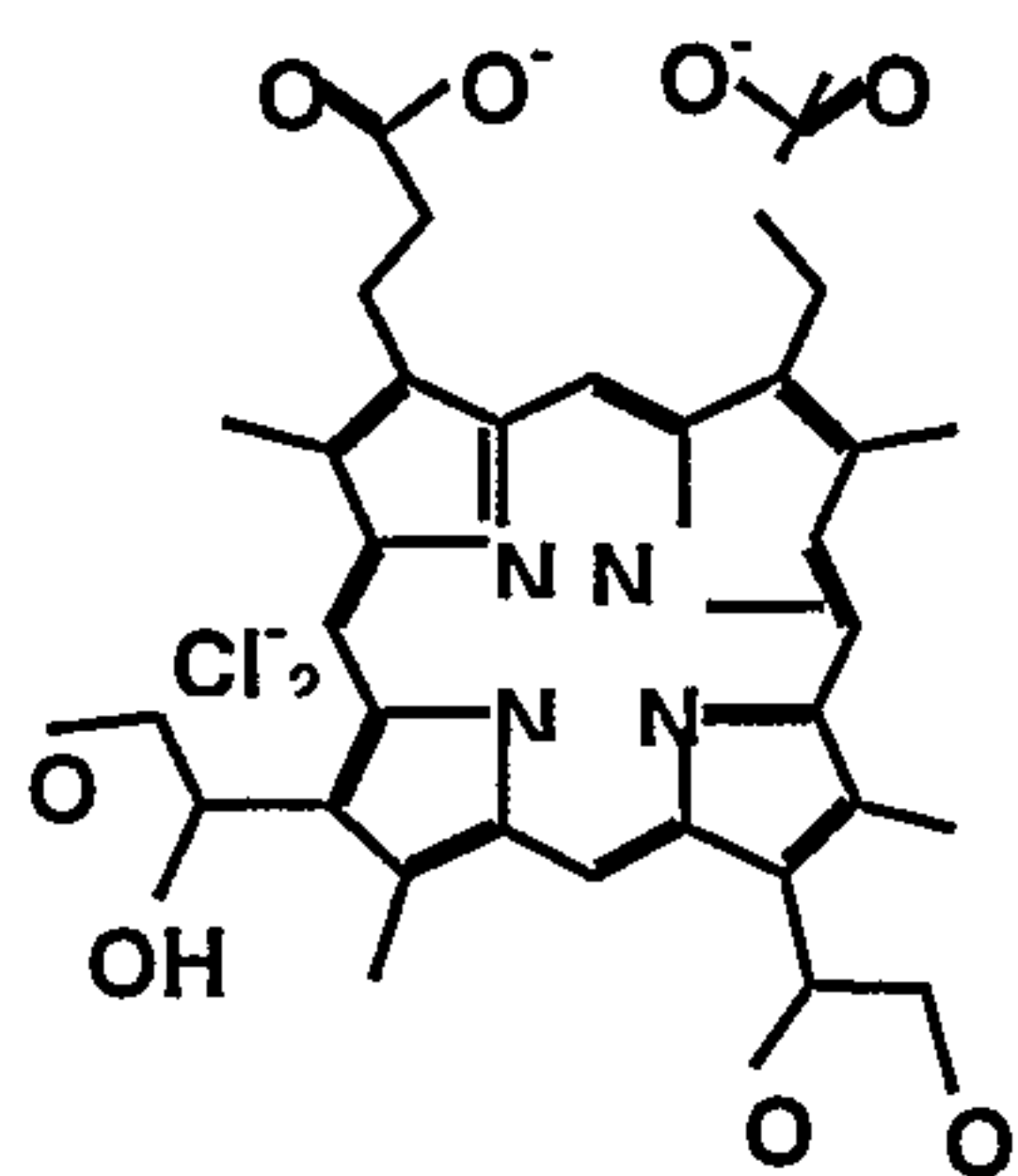


Figure 5.8d $IC_{50}=3\mu M$

Dipyrrole No. 1

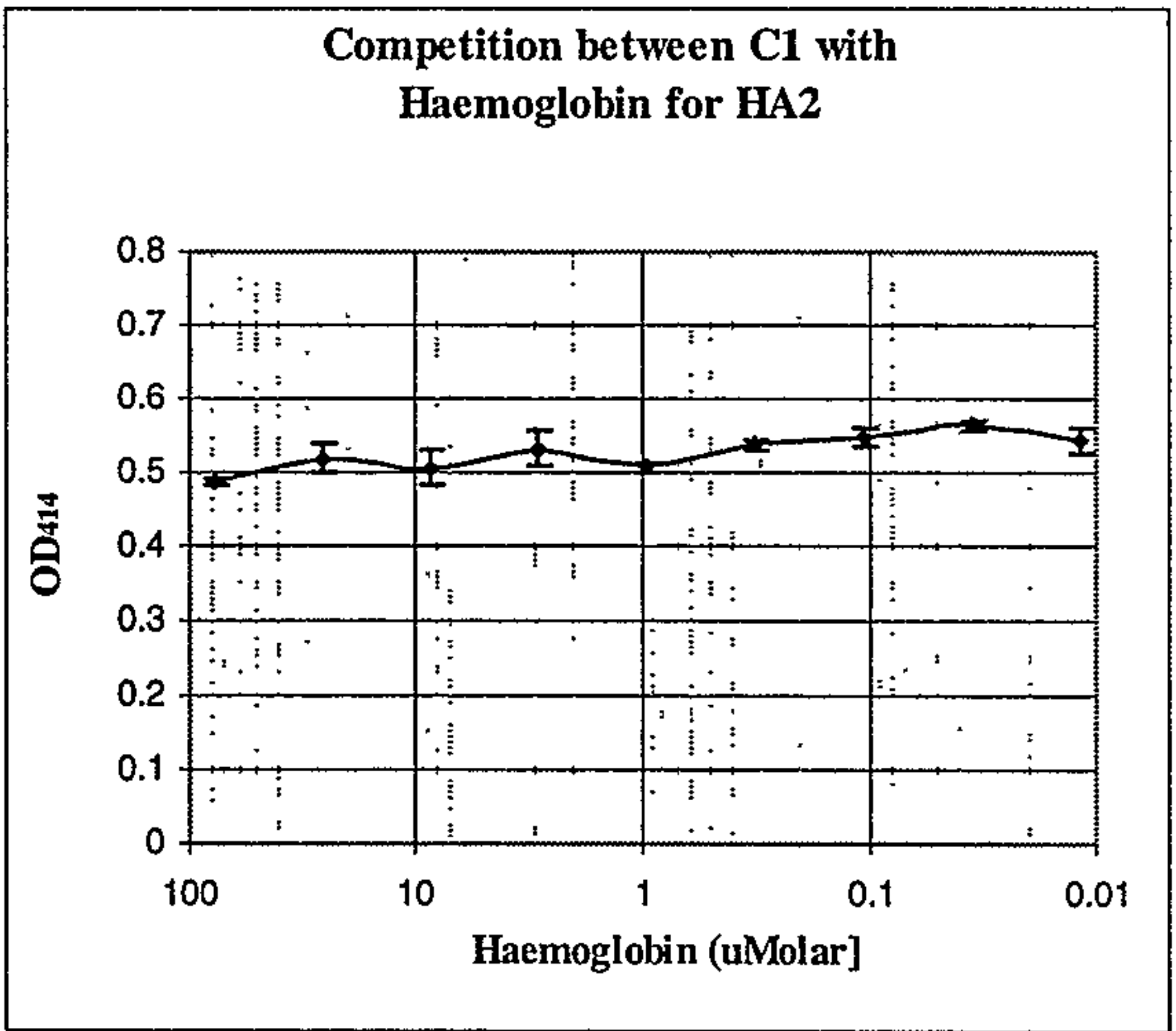
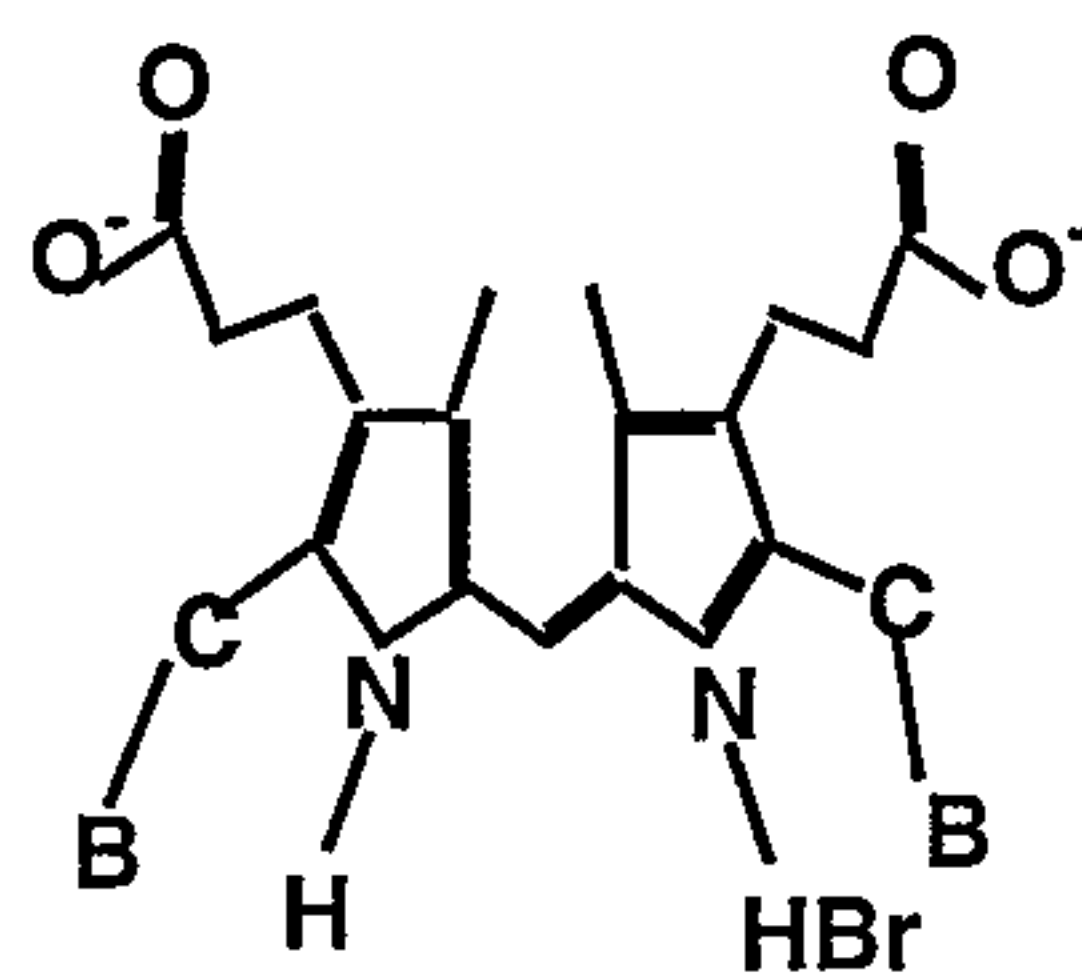


Figure 5.8e

Dipyrrole No. 2

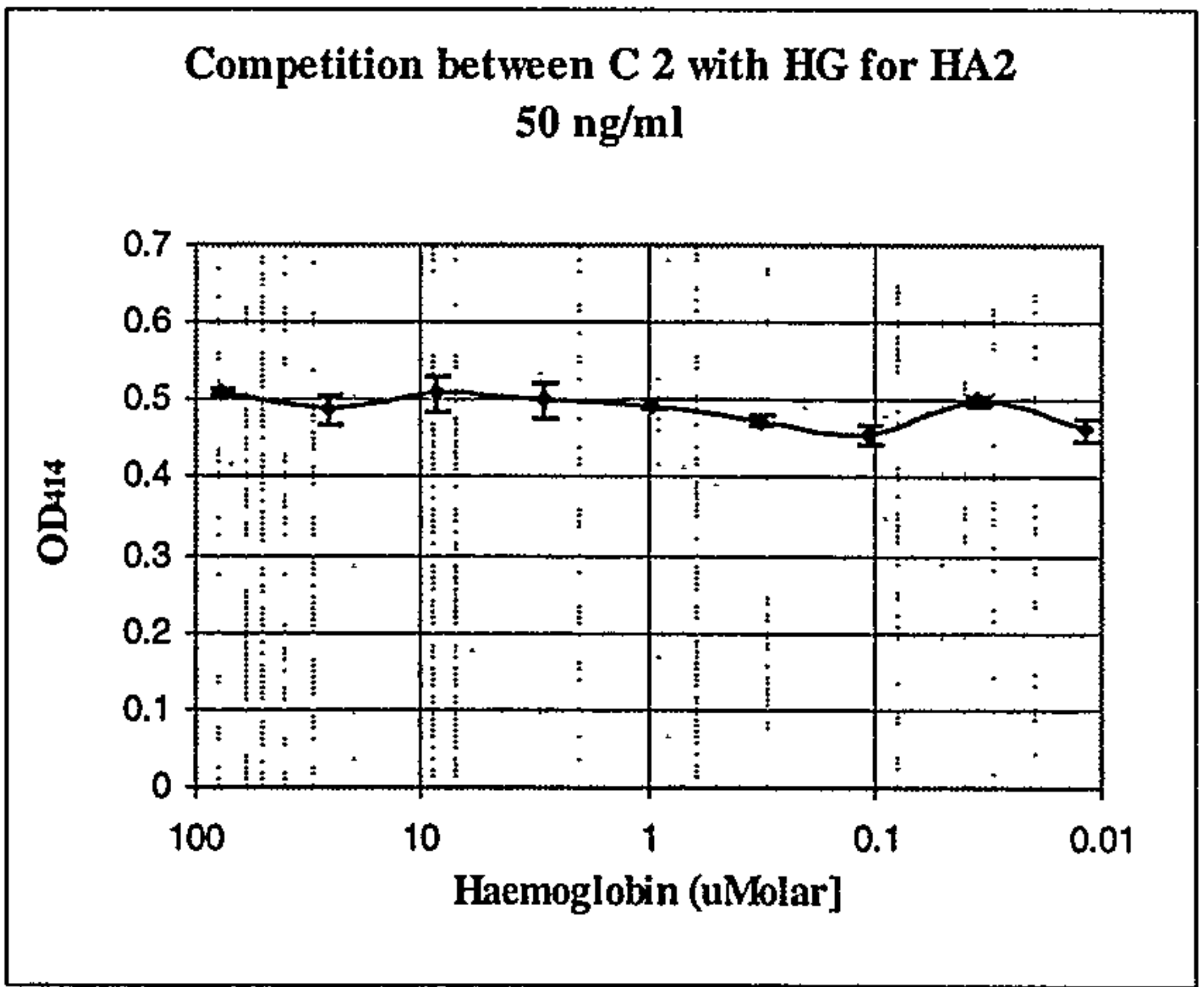
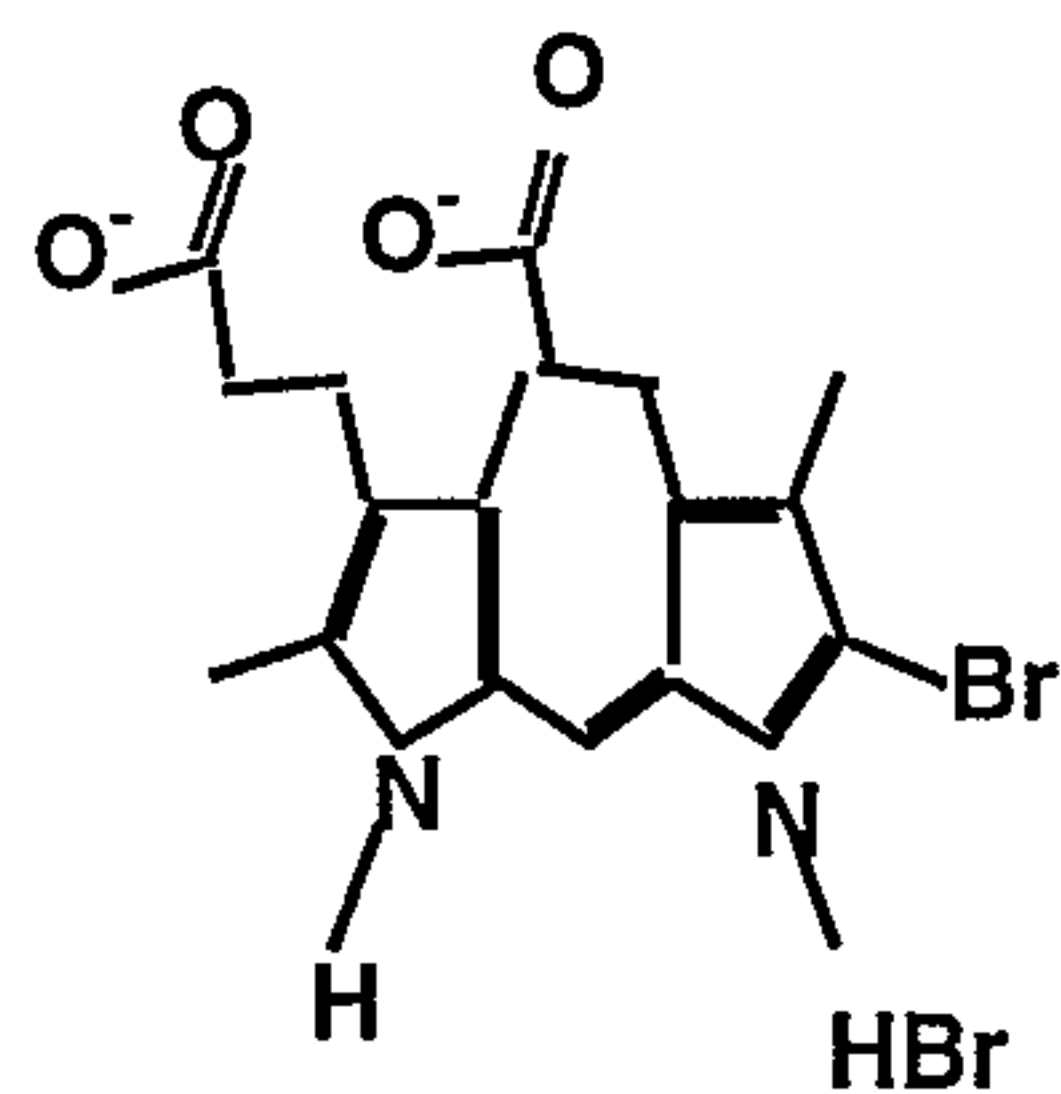


Figure 5.8f
65

Dipyrrole No.3

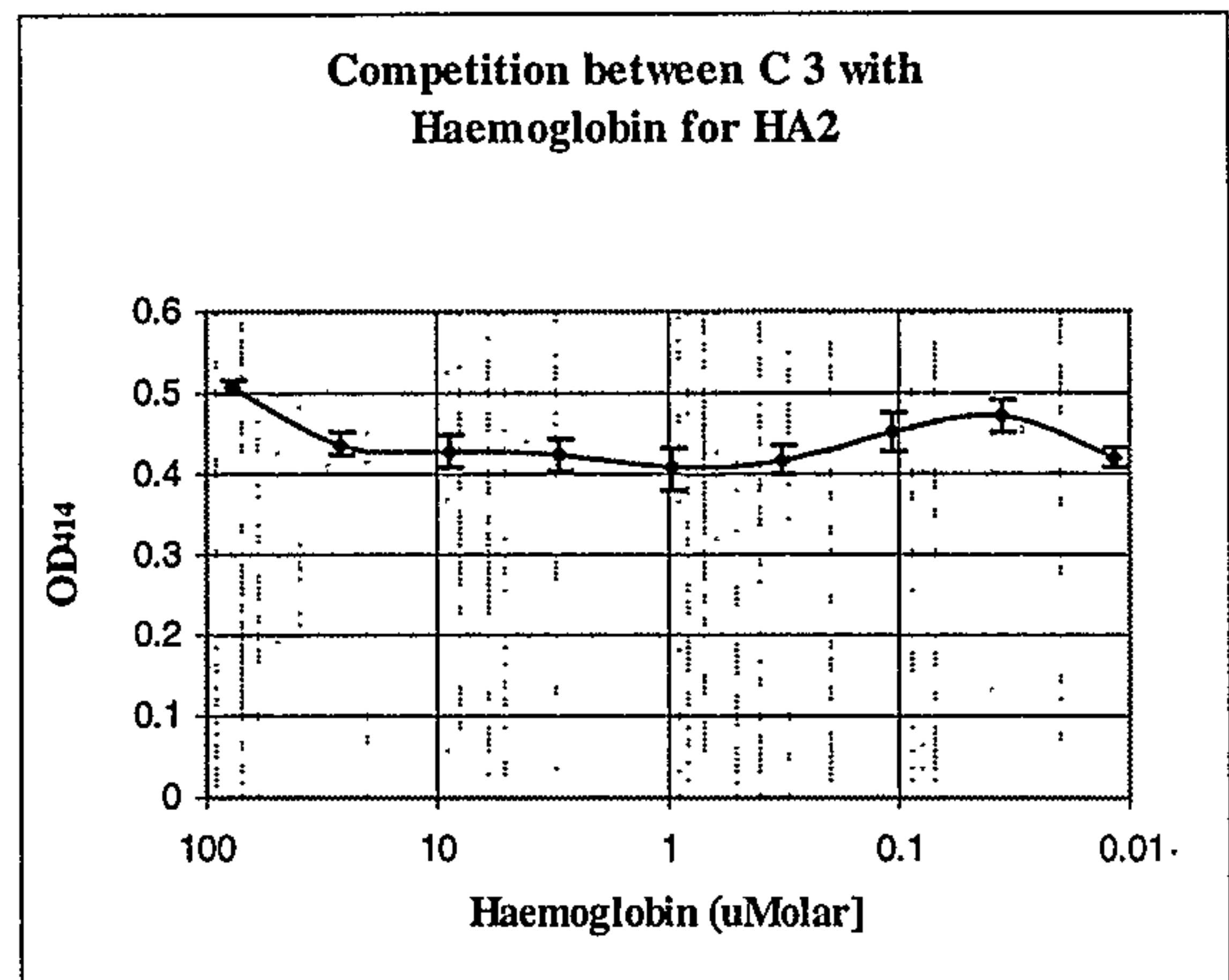
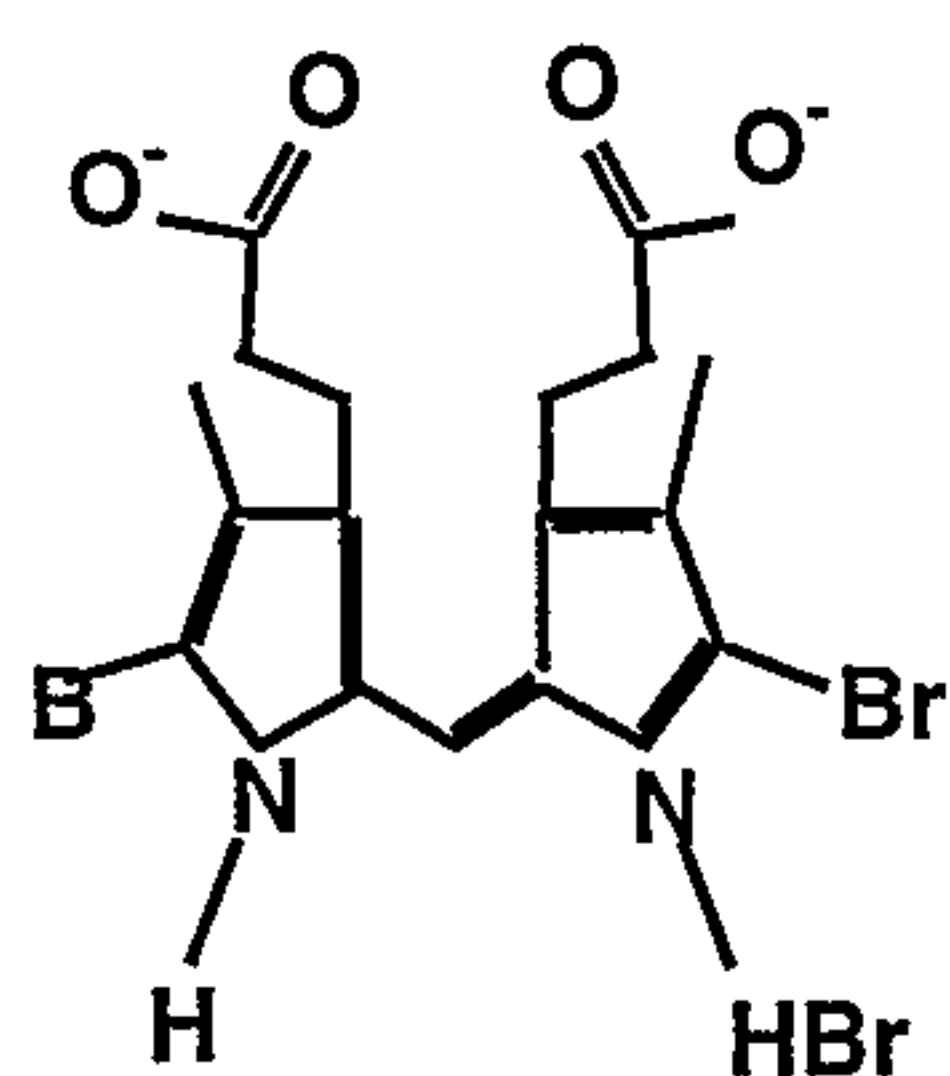


Figure 5.8g

Figure 5.8a-g showing the binding curves of the competition assays between PPIX, DDH, DDS, DBEG, Dipyrrole no.1, 2, and 3 with haemoglobin for rHA2. These figures all demonstrate the similar apparent IC_{50} s which ranged from 3-8 μ M.

enzyme used in haem synthesis. Hence it is possible for the bacterium to insert free iron in the medium into the porphyrin ring to meet its haem requirements.

The presence of cytochromes in the genome indicates that although the organism lacks the capacity for porphyrin synthesis, it has a requirement for porphyrin. It was of interest that the three critical enzymes needed for modification of the vinyl end of porphyrin molecules were present. The ability of porphyrin molecules with modifications of the vinyl-face (DBEG/DDS) to support growth was evidence for this as well, emphasising the scavenging role of the organism and its capacity to acquire exogenous sources of porphyrin and modify them for use by the organism. According to the collaborators on the *P. gingivalis* genome project, the strain W83 was chosen because it is virulent in the mouse abscess model (Neiders *et al.*, 1989; Genco *et al.*, 1991), has been extensively studied by the *P. gingivalis* scientific community, including sequencing; and it can be manipulated genetically. For the present experiments, a different strain (ATCC 33277) was used ; however, a recent study by Roper showed that genetic analyses of the haem biosynthesis pathway did not differ in the 6 different strains of *P. gingivalis* tested (W83, W50, PgM3, PgM4, Hg405, Hg1241 and Hg189) (Roper *et al.*, 2000). There has been no clear evidence for limitation of bacterial properties (eg. virulence) to particular clonal types or genetic lineages in the genome of *P. gingivalis*. Evidence that most strains of the organism possess the well-studied virulence factors, the gingipains (gingipains-R and K), (Mikolajczyk-Pawlinska *et al.*, 1998) which contain the HA2 adhesin domain, supports the potential role of the multi-domain proteinases in porphyrin acquisition.

Although the contigs of the genome have not been finally ordered, nor the sequence annotated (implying that short sections of sequence may be missing or short sections of sequence may be duplicated in the available data), it seems unlikely that all of the four or six enzymes necessary for *de novo* synthesis of porphyrin would be encoded in those regions of the genome that have not been sequenced satisfactorily. However, as these genes occur in an operon in some bacteria it may be that an entire section containing an operon of these genes is not included in the sequencing to date. For example, the genes for hemL, hemB, hemC, hemD, hemX and hemA occur within a region of 6250 base pairs in the genome of *B. subtilis* (Kunst *et al.*, 1997). However, in

some other bacteria, such as *Chlamydia trachomatis*, these genes are not associated on the genome (Stephens *et al.*, 1998).

The ability of *P. gingivalis* to grow with an iron-free source of porphyrin supports the findings of the genomic data above. It was necessary to establish the capacity of the porphyrins to promote growth in a culture assay using haemin-depleted cells. DDH was capable of promoting growth and since it was the least modified on the vinyl end, it fitted the predicted behaviour of the porphyrin molecules. DBEG and DDS were also shown to support similar rates of growth. Although there was an initial lag phase observed, the cells were eventually able to reach the desired OD. This suggested that the enzymes protoporphyrinogen oxidase, oxygen-independent coporphyrinogen III oxidase and ferrochelatase were capable of modifying the sulfonated and glycol groups on the vinyl-face as predicted, allowing the porphyrin molecule to be used in the assembly of cytochromes. If not, the sulfonated groups on these modified porphyrins would not fit into the hydrophobic cleft for haem of the cytochromes and hence could not be utilised. The slower rate of growth in comparison to DDH (least modified) and haemin might be related to a reduction in efficiency of these enzymes due to the bulkier than normal modifications on this face.

An unexpected finding was the need for both menadione and porphyrin in the culture. Preliminary experiments of the haemin depletion assays without menadione resulted in premature retardation of growth. During the depletion stage, the cells were inoculated into porphyrin-free media at a 5% inoculum. At this early point, where the biomass of cells is low, the need for menadione appears to be critical, probably as a source of energy currency to allow the cells to still grow and utilise stored supplies of porphyrin in the absence of an exogenous source of porphyrin. The addition of the porphyrins to these cultures resulted in resumption of growth for only DDH and haemin although the cells only reached 50% of the maximum biomass expected. The more heavily modified porphyrins were unable to support the resumption of growth. The combination of compromising the system at this critical point and the addition of a porphyrin source in the recovery culture which is less efficiently utilised by the organism, could explain this last observation. When haemin depletion was carried out under menadione supplemented conditions, as expected, it took at least 5 passages to reach

stationary growth and the presence of menadione in the recovery culture does not appear to be as critical.

The relation between menadione and porphyrin is not well understood. Menadione has been shown to function in anaerobic respiration as an electron acceptor (Lee *et al.*, 1997) and the distal haem centre in *B. subtilis* succinate : quinone reductase appears to be crucial for electron transfer to menaquinone (Matsson *et al.*, 2000). In *P. gingivalis*, vitamin K (1,4-naphthoquinone) is used to synthesise a nine unit polyprenyl side chain. This electron carrier forms part of an electron transport chain in which fumarate acts as an electron sink for reducing equivalents from various electron donors resulting in the possible synthesis of at least 1 mole of ATP (Shah and Gharbia 1995). Perhaps there exists a fine equilibrium between the roles of porphyrin and menadione in energy transport for the organism. Under porphyrin excess conditions, the role of menadione diminishes but under porphyrin limited conditions menadione is apparently critical for energy transfer.

The role of PPIX as an essential growth factor for *P. gingivalis* remains equivocal. Studies using chemically defined media as opposed to complex media have not as yet addressed this issue. Approaches have been either to use haemin as a growth factor or to attempt substitution of haemin with PPIX in the presence of free iron (Seddon *et al.*, 1988; Wyss 1992; Minhas *et al.*, 1993). One study did report an attempt to examine the effect of PPIX limitation on *P. gingivalis* and found it to replace haemin as a growth factor (Schifferle *et al.*, 1996). However, trace amounts of iron were present in the media. The authors noted that PPIX did not readily bind iron under conditions of growth, a finding that appears to be contrary to other work where PPIX could replace haemin as a growth factor in the presence of free iron (Barua *et al.*, 1990; Bramanti and Holt 1991; Minhas *et al.*, 1993). *Porphyromonas gingivalis* is also capable of using non-porphyrin sources of iron for growth (Inoshita *et al.*, 1991; Bramanti and Holt 1991).

Bramanti and Holt (1991) performed haemin depletion studies to investigate the role of PPIX in the growth of *P. gingivalis*. After haemin depletion, which required 8 passages into haemin-free media, PPIX, Zn-saturated PPIX and ZnCl₂ plus PPIX at 7.7 µM were found to be unable to support the growth of *P. gingivalis*. This finding was in complete contrast to our findings where all the deuteroporphyrins tested were found to be

capable of supporting *P. gingivalis* growth, especially DDH which resembles PPIX most closely.

Barua *et al.* (1990) found that *P. gingivalis* could use PPIX as a substitute for haemin. The growth rate was the same as in the presence of haemin. It is not clear how depleted the medium was of free iron. Schifferle *et al.* again confirmed their earlier finding that PPIX could replace haemin as a growth supplement (Schifferle *et al.*, 1996). Wyss found that protoporphyrin IX could mimic the growth-promoting effects of haemin but only in the presence of at least 10^{-7} M ferrous sulfate (Wyss 1992). Minhas *et al.* demonstrated, using continuous culture, that protoporphyrin IX at 2 mg/L could substitute for haemin as well (Minhas *et al.*, 1993).

The ability of the HA2-bound resin to absorb nearly 80% of the total haemoglobin binding capacity of the organism suggests that the HA2 domain is the major porphyrin receptor for *P. gingivalis*. Although haemoglobin is the major source of porphyrin *in vivo*, it was not possible to use protoporphyrin IX or haemin in the assay as the CHAPS extract did not interact consistently with these compounds when they were used to coat the plates. If it were possible to have generated a higher concentration of functionally folded rHA2, a multiple absorption approach could have been utilised, where the IgG fractions would be re-absorbed with fresh batches of rHA2-bound resin suspension until all ability of the sera to neutralise haemoglobin binding was removed. Hence the other 20% unabsorbed could be either due to the absorption technique used or the presence of another haemoglobin-binding protein.

Amano and Smalley found monophasic, negatively sloped Scatchard plots for haemoglobin binding in *P. gingivalis*, indicating the involvement of a single "receptor" in cell surface binding (Amano *et al.*, 1995; Smalley *et al.*, 1998a) and suggesting that the HA2 domain is the only receptor for haemoglobin binding. There has also been some evidence of previous reports suggesting the existence of this protein. A 14-15 kDa protein was identified when *P. gingivalis* W50 was grown under haemin-starved conditions and appeared to be lost from the cell surface upon transfer to a porphyrin-replete environment (Bramanti and Holt 1992). Grenier previously reported the haemin-binding property of the lipid A region of lipopolysaccharides (Grenier 1991). As HA2

has been found to be a lipid-A associated protein of *Porphyromonas gingivalis*, binding of lipid A to haemin could be explained (Sharp *et al.*, 1998).

Preliminary work in a collaborating laboratory (Langley, Menz and Collyer, Department of Biochemistry, University of Sydney) has indicated the homo-dimerisation of rHA2 in the oxidised state. This oligomerisation is stabilised by disulfide bond formation. This would increase the molecular weight of the protein to approximately 30 kDa. A 30 kDa (unheated) haemin-binding cell envelope protein was reported (Kim *et al.*, 1996). This could possibly have been the native form of the HA2 domain. Since the commencement of this work, Shi *et al.* have reported the construction of *rgpA rgpB kgp* and *rgpA kgp hagA* triple mutants by homologous recombination with cloned *rgp* and *kgp* DNA interrupted by drug resistance gene markers (Shi *et al.*, 1999). By using immunoblot analyses with anti-HA2 antiserum on cell lysates and intact cells, they demonstrated that both mutants were deficient in haemoglobin receptor (HbR) protein. These results suggested that all three of the genes *rgpA*, *kgp* and *hagA* contributed to the HbR expression of *P. gingivalis*. Both mutants also failed to bind haemoglobin which was tested using a solid-phase haemoglobin binding assay, supporting findings from the present study that HA2 is the major haemoglobin binding protein. The *tla* gene was also reported to have a HbR domain region; however, there has been no evidence to date that this gene expresses protein (Aduse-Opoku *et al.*, 1997).

The ability of human lactoferrin to bind and remove HA2 protein was recently reported (Shi *et al.*, 2000). This study showed that lactoferrin as well as haemoglobin bound HA2. The results suggest that the cationic lactoferricin region of lactoferrin may interact with the N-terminal anion-rich region of HA2 in an electrostatic action, loosen the non-covalent bond between HA2 and other components of the complex, hence releasing HA2 from the cell. It was also capable of suppressing the growth of *P. gingivalis* in medium containing haemoglobin as the sole iron source but not in medium containing haemin (Shi *et al.*, 2000). This indicates again that HA2 is probably the main haemoglobin binding protein for *P. gingivalis*. The lack of effect in haemin-containing medium might be due to a separate protein-receptor interaction or passive diffusion of haemin into the cell.

In previous studies at the Institute of Dental Research it was reported that in the solid phase ligand binding assay, rHA2, RgpA and Kgp each bound to haemoglobin with similar binding affinities ($K_d=2.1 \pm 0.6$ nM) and the binding curves of neither rHA2 nor the gingipains were indicative of multi-site binding (DeCarlo *et al.*, 1999). Haemoglobin binding of the HA2 domain was found to be mediated through the haem moiety where rHA2 bound to wells coated with haemin and proteolytically degraded haemoglobin. Binding of the rHA2 domain to haemin-coated wells was approximately eightfold weaker than binding to haemoglobin in solid-phase assays ($K_d=16 \pm 1$ nM). Using solution phase competition assays, binding of the gingipains or rHA2 to haemin was inhibited by the addition of protoporphyrin IX ($IC_{50}=2.5 \pm 0.3$ μ M) indicating that binding of rHA2 or the gingipains was specific for some aspect of the protoporphyrin ring. Binding of rHA2 or the gingipains to haemoglobin was also inhibited by protoporphyrin IX ($IC_{50}=10 \pm 2$ μ M). Binding of rHA2 with either haemin, protoporphyrin IX, or haematoporphyrin was abolished by establishing covalent linkage of the protoporphyrin propionic acid side chains to fixed amines, demonstrating specific and directed binding of the rHA2 to these protoporphyrins (DeCarlo *et al.*, 1999).

Results from the present study showed that HA2 bound to DBEG, DDS, C1, C2 and C3. However, DDH did not produce a binding curve as the substrate was not capable of coating the plates under any of the conditions tested. However, it was shown to play an active role in the competition assay, confirming its ability to bind to HA2. Hence DDH was only found to be functional in a solution phase assay. The K_d 's for all the derivatives tested were comparable despite the degree of modification on the vinyl aspect of these molecules. This confirmed the minimal contribution of the vinyl face to the binding interaction. In competition assays there was little difference in the capacity of DBEG, DDH and DDS to inhibit the binding of HA2 to haemoglobin-coated plates. However, although C1, C2 and C3 produced solid phase binding curves which were comparable to those of DBEG and DDS, they were not able to inhibit HA2 from binding to haemoglobin in solution phase. This could be explained by the solution structure of the dipyrroles which may not include a planar conformation. However, when bound to the well, the dipyrroles could adopt a conformation which is closely analogous to the porphyrin planar structure and hence present the propionate face in a more aligned plane.

This implies that the planar position of the propionic side-chains is important for competitive binding and strongly supports a specific binding interaction between the haemoglobin-binding receptor of RgpA /Kgp, HA2 and the porphyrin ring. Although many pathogenic bacteria have mechanisms for the recognition of iron within the context of the haem moiety (Létoffé *et al.*, 1994), the pattern of recognition of haem by HA2 is distinctive. *Haemophilus influenzae* also lacks the enzymes for *de novo* synthesis of porphyrin (Tatusov *et al.*, 1996) but there has been no reported porphyrin-binding protein as yet. Further work to test the ability of porphyrin derivatives modified at the propionic acid face to support growth will be required to confirm the proposed mediation by HA2, of porphyrin acquisition.

This study has highlighted the important role the HA2 domain would contribute as part of this organism's well equipped array of mechanisms to fulfil its iron and porphyrin needs. Data reported in Chapter 4 also supported an already well-established role for the gingipains in the acquisition of blood. The clinical relevance of these functional properties is investigated in Chapter 6.

THE BIOLOGY OF THE HA2 DOMAIN

The experimental results reported in Chapter 3 did not support a role for the HA2 domain in vascular disruption using the *in-vitro* Matrigel assay. In contrast, challenge of endothelial networks with the RgpA cysteine proteinase complex resulted in vascular disruption, with the activity mainly being a feature of the catalytic activity. These results implied that the RgpA complex has a capacity to actively disrupt pre-formed blood vessels to obtain haem, a critical growth supplement needed for the organism's electron transport system.

At this time, the HA2 domain was reported by Nakayama *et al.* to be a haemoglobin-binding receptor (HbR) (Nakayama *et al.*, 1998). As noted previously, haem is central to metabolism in nearly all organisms, especially as a prosthetic group involved in electron transport. Its synthesis occurs via a branched biosynthetic pathway, which is also responsible for the synthesis of other modified tetrapyrroles. *Porphyromonas gingivalis* has a critical need for haem as a growth supplement making the interaction between the HA2 domain and haemoglobin/haem of considerable interest. In collaborative studies, a visiting fellow, Dr. A.A. DeCarlo, used the rHA2 prepared in 3.2.7 to further investigate the nature of the binding interaction of HA2 to haemoglobin. It was found that while rHA2 demonstrated tight binding to haemin ($K_d=16$ nM); this binding was inhibited by iron-free protoporphyrin IX ($IC_{50}=2.5$ μ M). Haemoglobin binding to the gingipains and rHA2 ($K_d=2.1$ nM) was also inhibited by protoporphyrin IX ($IC_{50}=10$ μ M), demonstrating an essential interaction between the HA2 domain and the haem moiety in haemoglobin binding. Binding of rHA2 with either haemin, protoporphyrin IX or haemotoporphyrin was abolished by establishing covalent linkage of the protoporphyrin propionic acid side chains to fixed amines, demonstrating specific and directed binding of rHA2 to these protoporphyrins [DeCarlo *et al.*, 1999].

Although the critical need for haem by *P. gingivalis* has not been disputed, it is not known whether this exogenous haem is used to complement an inability of the organism to synthesise this product or whether the exogenous haem is used as a

source of iron. It has been generally believed that interaction between *P. gingivalis* and haemoglobin would be targeted towards the acquisition of the required iron while the role of the porphyrin molecule has been unclear. The ability of the HA2 domain to bind to haemoglobin via the porphyrin ring suggested a possible need for the organism to obtain its porphyrin requirements via an external source, highlighting the importance of further investigation regarding the importance of porphyrin for *P. gingivalis*.

CHAPTER SIX

CLINICAL RELEVANCE OF *PORPHYROMONAS GINGIVALIS* PROTEINASES (GINGIPAINS) AND HA2 DOMAIN IN ADULT PERIODONTITIS PATIENTS

6.1 INTRODUCTION

The host response of adult periodontitis patients to the putative pathogen *Porphyromonas gingivalis* remains unclear. Previous investigations have resulted in a confusing plethora of literature. Earlier studies focused on finding immunodominant antigens from *P. gingivalis* with major bands of peptides of apparent molecular weights of 140, 130, 107.6, 92, 80, 67.5, 63, 53, 51, 43, 40.5, 41, 37, 32, 28 and 15.5 kDa and lipopolysaccharide reported (Kurihara *et al.*, 1991; Polak *et al.*, 1995; Bouts *et al.*, 1996; Pietrzak *et al.*, 1998; Fan *et al.*, 2000). Iron repressible membrane proteins (IRMPs) of 46, 43, 37.5 and 22 kDa. were also found to be immunodominant indicating that *P. gingivalis* IRMPs are immunogenic and expressed *in-vivo* (Chen *et al.*, 1991). The huge variation in molecular weight is probably a reflection of proteolytic degradation of the proteins studied, by the proteinases of *P. gingivalis*.

It became apparent that reporting a host response to antigens from *P. gingivalis* did not provide adequate information about the type of antibody response that was involved and the avidity of the antibodies became of major interest. Analysis of the titre and avidity of IgG antibodies to *P. gingivalis* whole cells and a 47-kDa cell surface protein found half the patient pool with adult periodontitis to have low antibody titres and avidities that were similar to healthy control values. This indicated either susceptibility due to poor host response or that disease was not associated with this particular pathogen (Benjamin *et al.*, 1997). The effect of periodontal therapy on specific serum antibody concentration was measured where patients who were initially sero-positive showed a significant increase in antibody avidity and had demonstrable treatment outcomes in terms of reduced number of deep pockets and sites which bled on probing, suggesting

that periodontal therapy affects the magnitude and quality of the humoral response to the suspected periodontopathogen (Mooney *et al.*, 1995). While it was interesting to note differences in avidities of antibodies implying that antibodies with high avidities would be more protective, the function of these protective antibodies was not well characterised (Takahashi *et al.*, 1998).

The mouse abscess model was used to demonstrate protection after challenge with periodontopathic organisms. An outer membrane preparation was used to immunise BALB/c mice and at the highest dose level of 100 µg/immunisation, was able to induce high levels of specific antibody and subsequent protective immunity from challenge with live *P. gingivalis* organisms (Bird *et al.*, 1995). Attempts to characterise the antigen(s) recognised by sera from periodontitis patients have been reported by Curtis *et al.* using monoclonal antibodies specific for *P. gingivalis* whole cells (Curtis *et al.*, 1996). In particular, Mab 1A1 was found to be reactive with protein determinants within the beta subunit, a haemagglutinin and/or adhesin component of the Arg1 dimer. This antibody strongly inhibited the agglutination of human erythrocytes by *P. gingivalis* W50 culture supernatant, suggesting that the binding site contains residues that are critical for the interaction with the erythrocyte surface (Curtis *et al.*, 1996). An oligonucleotide probe corresponding to the coding sequence for the region of the Arg1 beta component containing the Mab 1A1 binding site hybridised to multiple bands on genomic digests of *P. gingivalis* DNA. The Mab 1A1 epitope may be a component of a binding domain common to multiple gene products of this organism and may thus represent a functionally important target of the host's specific immune response (Curtis *et al.*, 1996). No studies to date have clearly defined the interactions between *P. gingivalis* and human peripheral blood polymorphonuclear leukocytes (PMN), nor has a protective role for antibodies to *P. gingivalis* been defined. Sera from a non-immune rabbit and healthy subject were not opsonic for virulent *P. gingivalis* strains A7346, W83 and HG405. Phagocytosis of these strains (but not of ATCC 33277) required opsonisation with hyperimmune antiserum (RaPg). Diluting RaPg with a constant complement source decreased proportionally the number of *P. gingivalis* A7436 cells phagocytosed per phagocyte (PMN). Enriching for the immunoglobulin G fraction of RaPg enhanced for opsonic activity toward A7436. An opsonic evaluation of 18 serum samples from adult periodontitis patients revealed that only 3 adult periodontitis sera of 17 with elevated immunoglobulin G to *P. gingivalis*

A7436 were opsonic for A7436 (Cutler *et al.*, 1991). The outer membrane proteins of the bacteria are potentially important targets for interaction with host defense systems. A 40-kDa outer membrane protein is conserved among many strains of *P. gingivalis*. Antibody against a recombinant 40-kDa OMP has opsonic activity for human neutrophil phagocytosis of *P. gingivalis* (Saito *et al.*, 1999). The literature to this point had presented a confusing array; antiserum responses without a clear definition of the antigen, protective roles of non-specific antibodies, and protective roles with no specific function.

The importance of the cysteine proteinase Rgp-Kgp complex in virulence has been the focus of intense investigation and the relative role this family of molecules plays in clinical presentation is of interest. The Rgp-Kgp complex, when used as an immunogen has been reported to have a protective role against challenge with *P. gingivalis* (Booth *et al.*, 1996; Moritz *et al.*, 1998; Katz *et al.*, 1999; O' Brien-Simpson *et al.*, 2000). The host immune responses to the HA2 domain and the gingipains may play a critical role in the incidence and severity of adult periodontitis.

In this Chapter, the titres of serum antibody specific for the gingipains and HA2, are studied relative to neutralisation capacity. In turn, characteristics of the antibody responses are related to levels of the antigens in subgingival plaque and to clinical status in a group of patients with adult periodontitis. Perusal of the literature at the time did not show evidence of an antibody response to gingipains in patients with no clinical signs of periodontitis. Therefore, the value of an aged/sex matched periodontally healthy group was questionable. It was decided that it would be appropriate to use each patient as an internal control. Plaque was sampled from both the healthiest (mild gingivitis) and most diseased (advanced periodontitis) site in each patient. In addition sera were collected pre- and post-treatment. This approach would counteract the diverse cultural and social backgrounds of the patient pool which might otherwise complicate any differences observed between patients.

6.2 MATERIALS AND METHODS

6.2.1 Collection of patient data

The collection of patient sera, plaque and clinical indices of disease was necessary prior to the laboratory work. Of the 25 participants selected from patients presenting to

the United Dental Hospital in Sydney, Australia, 4 were Asian, 1 was of African descent and the rest Caucasian. There were 9 females and 16 males in the study with an age distribution of 36-69 years respectively. Full medical and dental histories were obtained for each subject. The criteria for acceptance into the study were; 1) no professional periodontal treatment within the prior three years, and 2) no use of antibiotics within the prior six months. Subjects had no history of systemic diseases affecting the periodontium directly or indirectly and no apparent impairment to the ability to perform adequate oral hygiene. Dr. A.A. DeCarlo, a specialist periodontist, was the designated operator for the collection of all samples to standardise the procedures. With informed consent a dental curette was used to collect 5-15 µl of subgingival dental plaque in 300 µl PBS (pH 7.4) (3.2.1.2) containing 10 mM NaN₃. Two sites were chosen ranging from the greatest level of attachment loss (diseased sites), to one with a minimal level of periodontal disease (healthy sites) as determined by visual clinical and radiographic inspection.

6.2.2 RgpA and Kgp isolation.

Gingipains were isolated and characterised from *P. gingivalis* ATCC 33277 as previously described (4.2.1)

6.2.3 Expression and purification of recombinant HA2.

The HA2 domain was cloned, expressed, purified and characterised as previously described (3.2.7)

6.2.4 Monoclonal antibodies VA1 and IIB2.

Monoclonal antibodies (mAb) VA1 and IIB2 were prepared in mice against gingipains as described (DeCarlo and Harber 1997). In denatured samples, mAb VA1 recognises the HA2 as well as the HA1 gingipain domain (DeCarlo *et al.*, 1999), so antigen recognised by mAb VA1 is termed gingipain/HA2 in experiments using denatured plaque samples. MAb IIB2 recognises the co-linear HA3 and HA1 domains of the gingipains in non-denatured samples (unpublished data). Cross-reactivities of mAb VA1 and mAb IIB2 with extracts of two other plaque bacteria suspected to have a role in periodontal diseases, namely *F. nucleatum* and *P. intermedia* were not detected by ELISA (data not shown).

6.2.5 ELISA.

Standardised ELISA conditions were used (2.4.6). Coating was in 2.7 mM KCl, 1.5 mM KH_2PO_4 , 137 mM NaCl, 8.1 mM Na_2HPO_4 (PBS) with 10 mM sodium azide (PBS/ N_3). All wells were blocked and washed in PBS/ N_3 with 0.1% Tween (PBS/Tween). To detect human immunoglobulin, alkaline-phosphatase conjugated goat anti-human IgG, IgM, or IgA antibody preparations were used (Sigma Chemical Co.). Primary murine mAbs VA1 or IIB2 were detected with a 1 h incubation of 0.5 $\mu\text{g}/\text{ml}$ alkaline-phosphatase conjugated rabbit anti-mouse IgG (Dako Corp., USA). Alkaline phosphatase activity was monitored at 414nm (A_{414}) by hydrolysis of the substrate 4-nitrophenylphosphate (Boehringer, Mannheim, Germany) in 5mM Tris (pH 9.5) by using a Titertek Twinreader PLUS photometer (absorbance maximum of 3.0 ELISA units). The experimental conditions only used OD's between 0.1-2.5 where the Beer-Lambert law was obeyed for product and instrument.

6.2.6 Haemoglobin binding assay.

The haemoglobin binding assay (5.2.7.2), was a variant of the ELISA in which haemoglobin was coated onto the wells in PBS/ N_3 then subsequently allowed to bind plaque proteins or the gingipains in PBS/Tween (5.2.3.2). Non-denatured plaque samples were diluted 1/3 in PBS/Tween. Haemoglobin-binding protein in plaque was then detected with mAb IIB2 followed by a rabbit anti-mouse AP conjugate and developed as described for ELISA (2.4.6). Haemoglobin binding levels were calculated from parallel control binding measurements of mAb IIB2 to known gingipain amounts. Human haemoglobin from a 1 mg/ml stock solution in PBS/ N_3 was used in these experiments. Haemoglobin binding activity was detected in non-denatured plaque with mAb IIB2.

6.2.7 Serum IgG Isolation.

Patient sera were collected and the IgG fractions purified as in 5.2.5.1. IgG concentrations were determined by absorbance at A_{280} (extinction coefficient = 1.4) and eluants were each diluted to a final IgG concentration of 460 $\mu\text{g}/\text{ml}$ with elution buffer, pH 8.4 for use in neutralisation assays.

6.2.8 Haemoglobin-binding inhibition assays.

IgG fractions of 460 µg/ml (6.2.1) were pre-incubated with 1/3 volume 1 nM RgpA, or Kgp in PBS/Tween (5.2.3.2) at room temperature in microtiter plates that had been blocked in PBS/Tween. Separate microtiter plates were coated with 5 µg/ml haemoglobin then blocked. The pre-incubated mixtures were added to the haemoglobin-coated plates and incubated 1 h at room temperature. Levels of gingipains binding to haemoglobin were measured with mAb IIB2 as described above. Percent inhibition of gingipain/HA2 haemoglobin-binding relative to the highest levels of binding for either RgpA or Kgp in a constant IgG concentration was calculated.

6.2.9 Gingipain proteinase neutralisation assays.

In microtiter wells that had been blocked with PBS/Tween (5.2.3.2), IgG fractions were pre-incubated with an equal volume of 1 nM Kgp or trypsin in 50mM Tris, 1mM CaCl₂, 10 mM NaN₃, pH 7.4 (Tris buffer). N-tertiary-butoxycarbonyl-Glu-Lys-Lys-7-amido-4-methylcoumarin (80 µM) in Tris buffer with 5 mM L-cysteine were added to equal volumes of pre-incubated Kgp or trypsin then hydrolysis of substrates was monitored by absorption at 460 nm (A₄₆₀) using a 380nm excitation beam on a Perkin Elmer LS 50B luminescence spectrophotometer. Kgp activity was normalised to the trypsin controls then percent inhibition relative to the highest levels of activity for Kgp in IgG was calculated.

6.2.10 Statistics

Differences between categories were established by independent Student's 2-tailed t-test or Pearson's correlation using 95% confidence levels using the statistical program SPSS. The relationships of values between categories were established by linear regression, R² values were determined and significance (F value) with a 95% confidence level calculated using SPSS. For regression analysis, levels of periodontal disease severity were assigned the following values: 0, none; 1, localised mild; 2, generalised mild; 3, localised moderate; 4, generalised moderate; 5, localised advanced; 6, generalised advanced (Table 6.1)

6.3 RESULTS

6.3.1 Patient data

Clinical indices of disease for each patient were noted and periodontal diagnosis scores ranging from 0-6 were assigned to participants (Table 6.1). Clinical data for the participants in the study group are displayed in Table 6.2 and Figure 6.1. The age of the participant was not significantly associated with any of the data presented in this report (data not shown).

6.3.2 Analysis of plaque samples

Plaque samples collected from healthy and diseased sites in each patient were analysed for the presence of gingipains and HA2 in ELISA assays detected with mAbs VA1 and IIB2 (Figure 6.2a-b). Samples from healthy and diseased sites in 23 patients were detected with IIB2 for the presence of gingipains (Figure 6.2a). Levels of gingipains were detected in 4 healthy sites where apart from an OD₄₁₄ of 1.842 in one site, the level of detection ranged from 0.01-0.119 in the three other sites. In the diseased sites, however, 17 patients were shown to have detectable levels of gingipains ranging from 0.059-1.146. If gingipains were not detected in a diseased site, the healthy site in the same patient also failed to demonstrate the presence of gingipains. Figure 6.2b presents the data from healthy and diseased sites probed with the monoclonal antibody VA1 for the presence of HA2. The general levels of HA2 in the plaque samples appeared to have lower OD₄₁₄ as compared to the gingipains. Only 6 healthy sites had detectable levels of HA2 with OD₄₁₄ values ranging from 0.012-0.107. In contrast 16 of the diseased sites were shown to contain the HA2 protein with OD₄₁₄ values ranging from 0.013-0.516. As shown with the gingipains, if a diseased site did not have detectable levels of HA2, this was mirrored in the healthy site of the same patient.

The HA2 domain is recognised specifically by mAb VA1 but only in denatured plaque samples. The epitope of HA2 is essentially cryptic in native gingipains although it is apparently fully available in functional recombinant HA2 (DeCarlo *et al.*, 1999). Detection of gingipains with mAb IIB2 also correlated significantly and positively with immunochemical detection of the HA2 epitope recognised specifically by the anti-HA2

TABLE 6.1. Derivation of diagnosis scores based on American Academy of Periodontology (AAP) case-type description.

AAP Case Type	1	2	3	4
Periodontal Disease Level	gingivitis	slight	moderate	advanced
Localised (L) or Generalised (G)		L G	L G	L G
Diagnosis Score	0	1 2	3 4	5 6

Table 6.2: Study group clinical data.

N	25	
mean age	49 ± 9 years	
diagnosis score	4.7 ± 1.5	
pocket depth	max 6.7 ± 2.2 mm	min 2.3 ± 0.5 mm
attachment loss	max 8.4 ± 2.0 mm	min 2.8 ± 0.9 mm

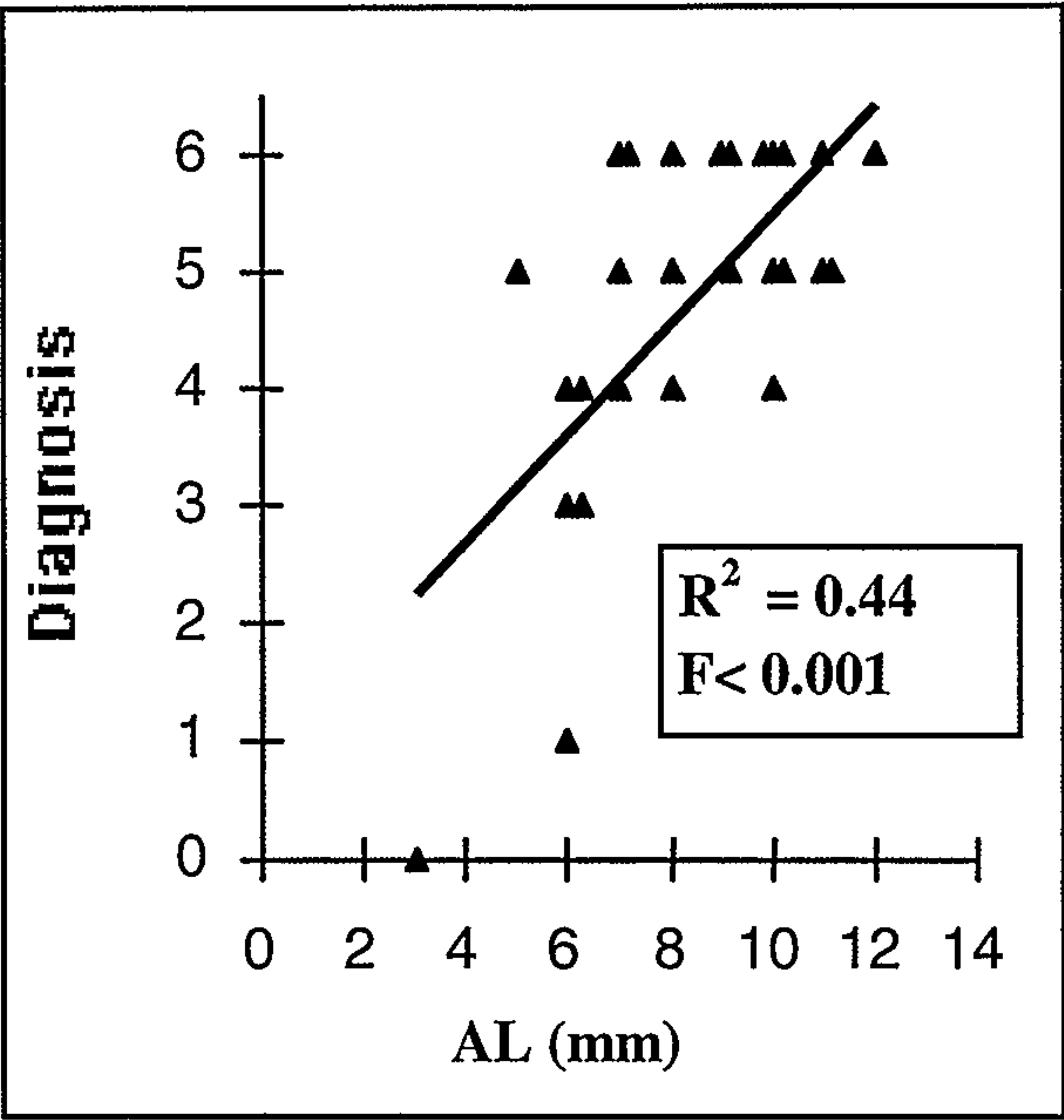
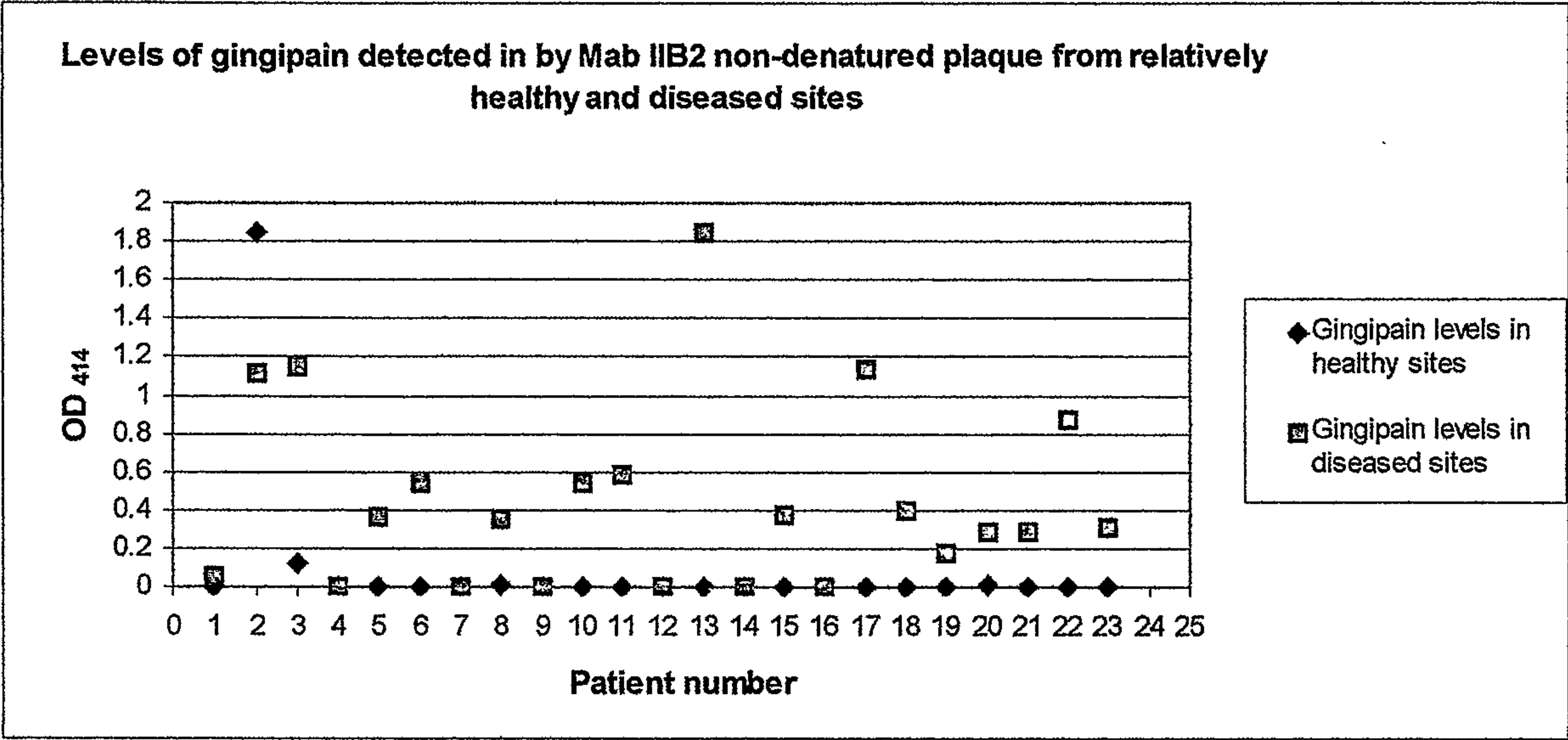
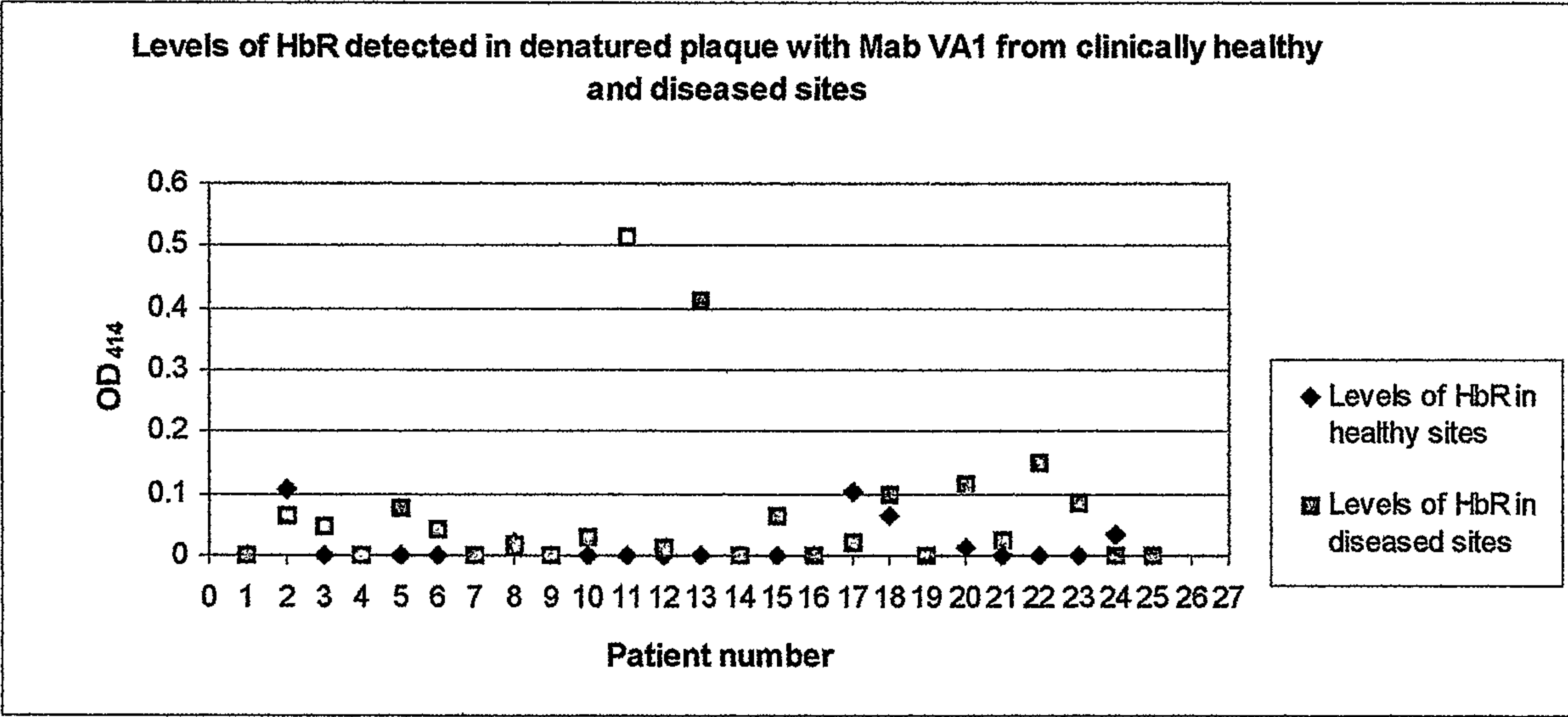


Figure 6.1 Periodontal attachment loss and independent correlation with participant diagnosis scores. Linear regression and best fit plot of diagnosis scores vs. maximal attachment loss (AL) within participants.



(a)



(b)

Figure 6.2 Shows scatter graphs of plaque samples analysed for the presence of gingipains and HbR in ELISA immunoassays

(a) Non-denatured plaque samples from healthy and diseased sites in 23 patients detected with IIB2 for the presence of gingipains

(b) Denatured plaque samples from healthy and diseased sites in 23 patients detected with VA1 for the presence of HbR (HA2)

mAb VA1 (Pearson's correlation, $p=0.008$) suggesting that the HA2 had a significant association with the gingipains *in vivo*.

6.3.3 Inflammation is associated with the presence of gingipains and the HA2 domain

The presence of gingipains and HA2 was co-related to important markers of disease to look for potential relationships between these virulence factors and disease activity. Using the epitope for mAb VA1 as a marker for the *P. gingivalis* HA2 and gingipains in plaque, a significant positive association was found between the immunochemical detection of HA2 in plaque and gingival inflammation at the corresponding sites (2-tailed independent Student's t-test, $p=0.025$) (Figure 6.3) and was more frequent in plaque samples from the diseased donor sites than in those from healthier sites (Student's t = test, $p=0.05$; data not shown). The monoclonal antibody IIB2 was also capable of demonstrating a similar co-relation between the presence of gingipains and gingival inflammation (2-tailed independent Student's t-test, $p<0.0001$) again being more frequent in samples from diseased donor sites (Student's t-test, $p=0.05$; data not shown). These data associate the HA2 of *P. gingivalis* with gingival inflammation and periodontal disease severity.

6.3.4 Detection of haemoglobin binding activity in plaque

Haemoglobin binding activity was measured in plaque samples from 50 healthy and diseased sites. Where haemoglobin binding activity was detectable in non-denatured plaque samples (7 of 50 healthy and diseased sites), significantly higher levels of HA2 in the plaque were found in the matched, denatured plaque samples (2-tailed independent Student's t-test, $p=0.029$) (Figure 6.4) using parallel solid phase assays (6.2.8). These results support the ability of HA2 to function as a haemoglobin binding moiety in dental plaque.

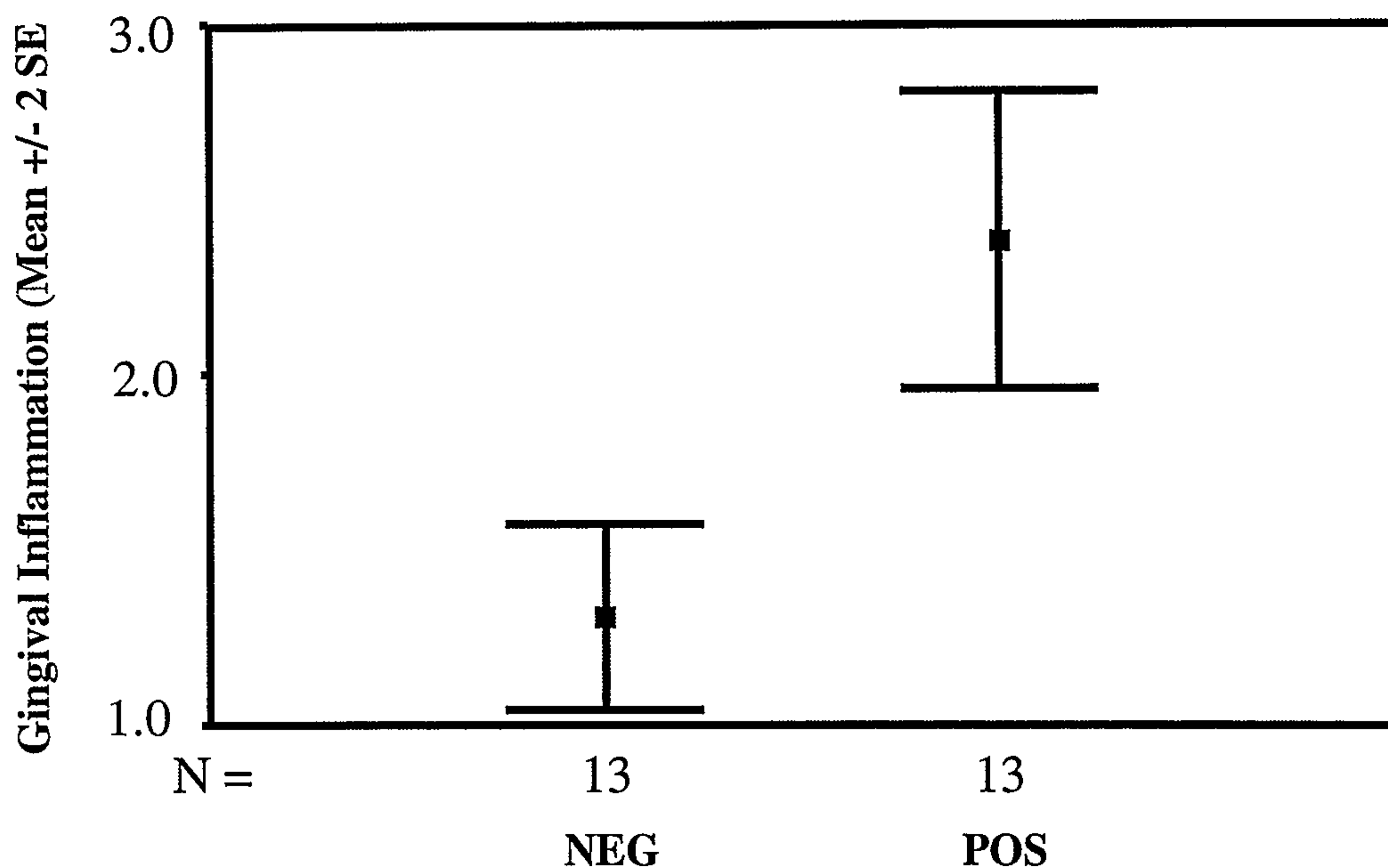


Figure 6.3 Detection of HA2 is associated with amount of clinical inflammation in the gingiva. Means (square) $\pm$ 2 standard errors (2SE) of gingival inflammation score as defined by the American Academy of Periodontology (1, mild inflammation; 2 moderate inflammation; 3, severe inflammation with spontaneous bleeding) measured in denatured plaque samples by ELISA that either did (pos) or did not (neg) demonstrate the presence of HA2 by ELISA with mAb VA1.

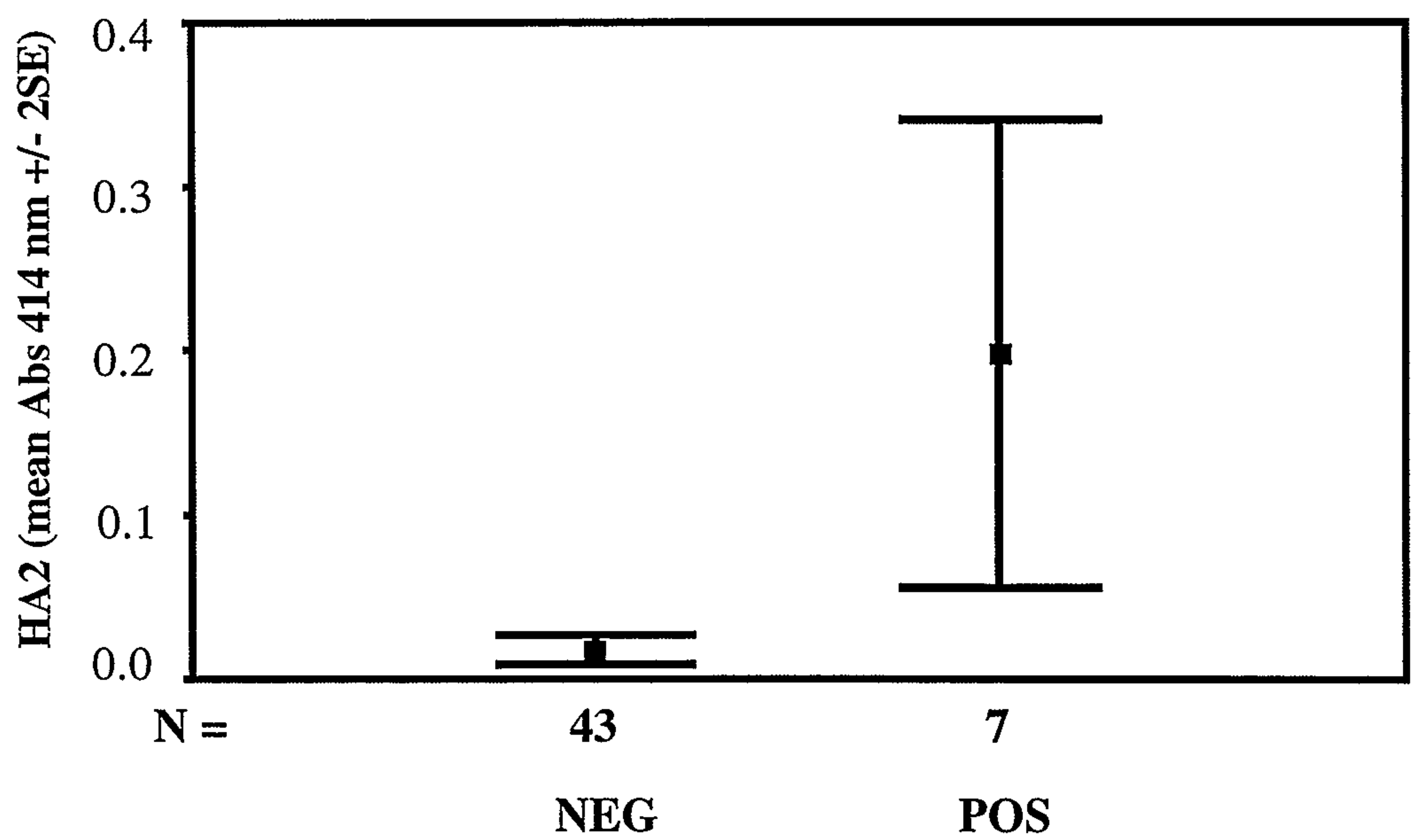


Figure 6.4. Haemoglobin binding activity in dental plaque associated with detection of HA2. Means (square) $\pm$ 2 standard errors (2SE) of receptor levels measured in denatured plaque samples by ELISA that either did (pos) or did not (neg) demonstrate detectable receptor-associated haemoglobin binding activity.

6.3.5 The HA2 domain is antigenic and elicits protective antibodies capable of neutralising haemoglobin binding

Pre- and post-therapy sera were used to measure the antigenic and protective properties of the HA2 protein in ELISA immunoassays (6.2.3). Pre-therapy serum was collected from 23 of the 25 participants but only 15 patients donated post-therapy sera as a result of difficulties encountered in patient attendance. Although all 23 of the participants donating sera had pre-therapy sera recognising HA2, only the 15 patients with paired pre- and post-therapy sera for HA2 are depicted graphically in Figure 6.5, demonstrating the change in serum titre pre- and post-treatment.

Suggesting a protective role, both pre-therapy and post-therapy serum IgG was found to have the capacity to specifically neutralise haemoglobin binding activity of the gingipains ($50\% \pm 18\%$, range 3% - 91% inhibition) in solid phase neutralisation assays similar to those described (DeCarlo *et al.*, 1999). Higher pre-therapy serum IgG titres specific for rHA2 were associated positively and significantly with detection of the HA2 in plaque samples (independent 2-tailed Student's t-test, $p=0.01$) (Figure 6.6). Levels of receptor-associated haemoglobin-binding activity measured in these plaque samples were also significantly associated with pre-therapy serum anti-receptor IgG titres (Pearson's correlation, $p=0.001$; data not shown). These data strengthen the link between HA2 and haemoglobin binding activity *in-vivo* and, importantly, indicate that HA2 is immunogenic in a human population, eliciting a humoral IgG response in proportion to antigenic load. Accordingly, pre-therapy and post-therapy anti-receptor IgG titres in patients were positively correlated within participants (Pearson's correlation $p<0.001$) (Fig. 6.7).

6.3.6 Gingipains are antigenic and elicit protective immunity by neutralising gingipain proteinase activity

Pre-therapy serum was collected from 23 of the 25 participants and used to measure the antigenic properties of the gingipains in ELISA immunoassays (6.2.3). Although all 23 of the participants donating sera had pre-therapy sera recognising gingipains only the 15 patients with paired pre- and post-therapy sera for Kgp have been depicted graphically in Figure 6.8 demonstrating the change in serum titre pre- and post-treatment. There does

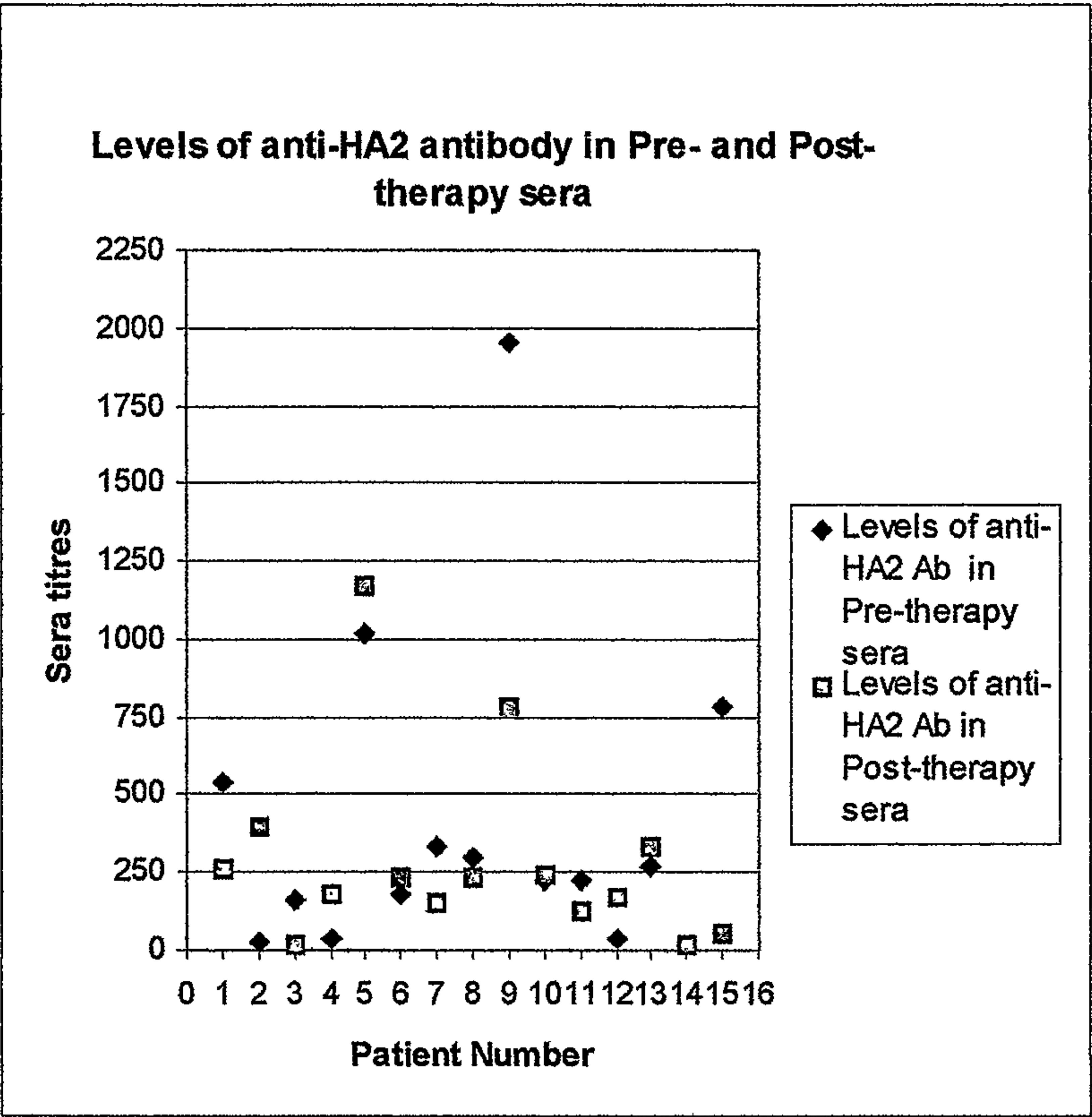


Figure 6.5 Shows a scatter-graph of matching pre- and post-therapy sera titres to the HA2 protein for 15 patients with adult periodontitis.

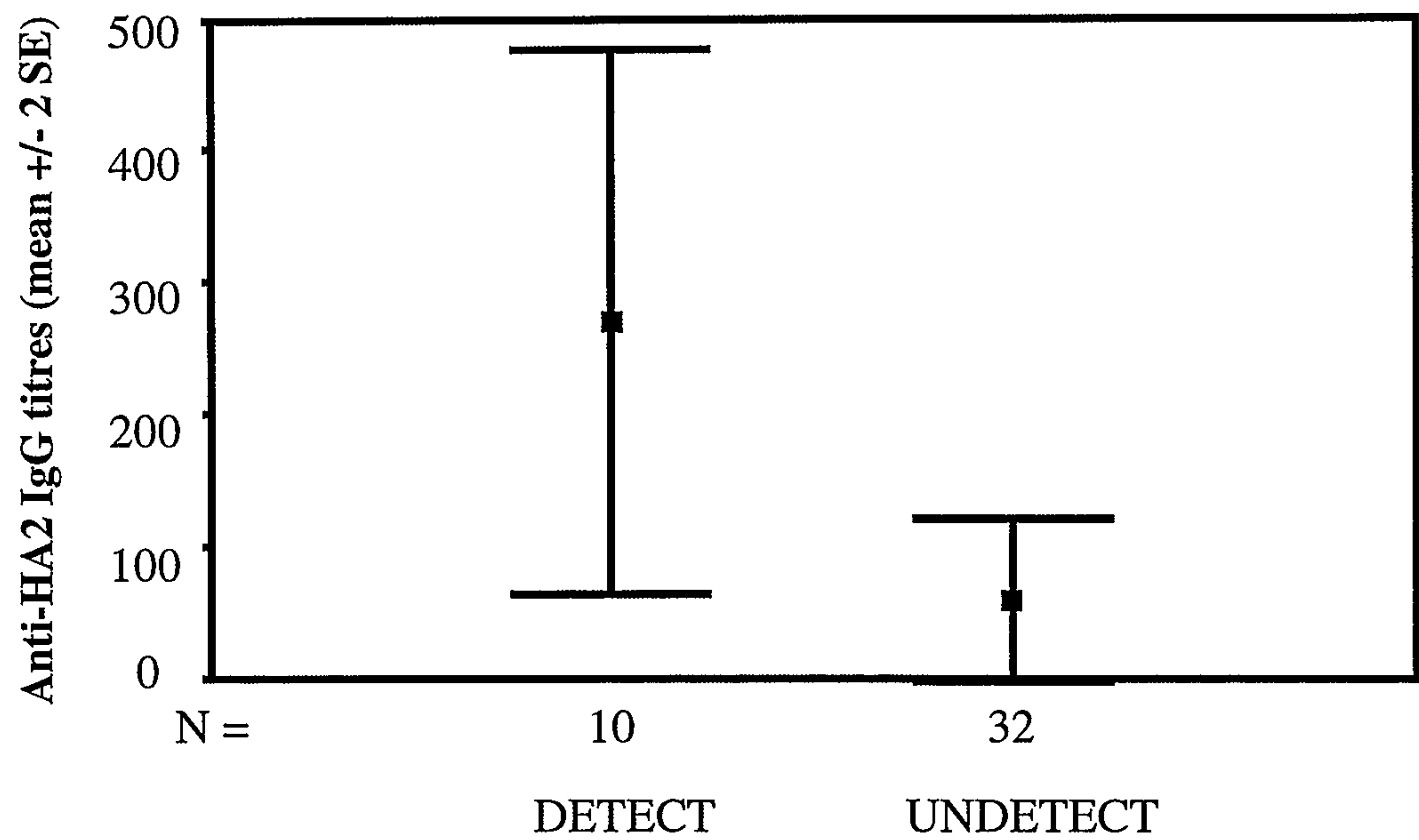


Figure 6.6. Higher IgG titres were associated with HA2 detection. Means (square) $\pm$ 2 standard errors (2SE) of IgG titres specific for HA2 in denatured plaque samples where HA2 was detectable (detect) or undetectable (undetect).

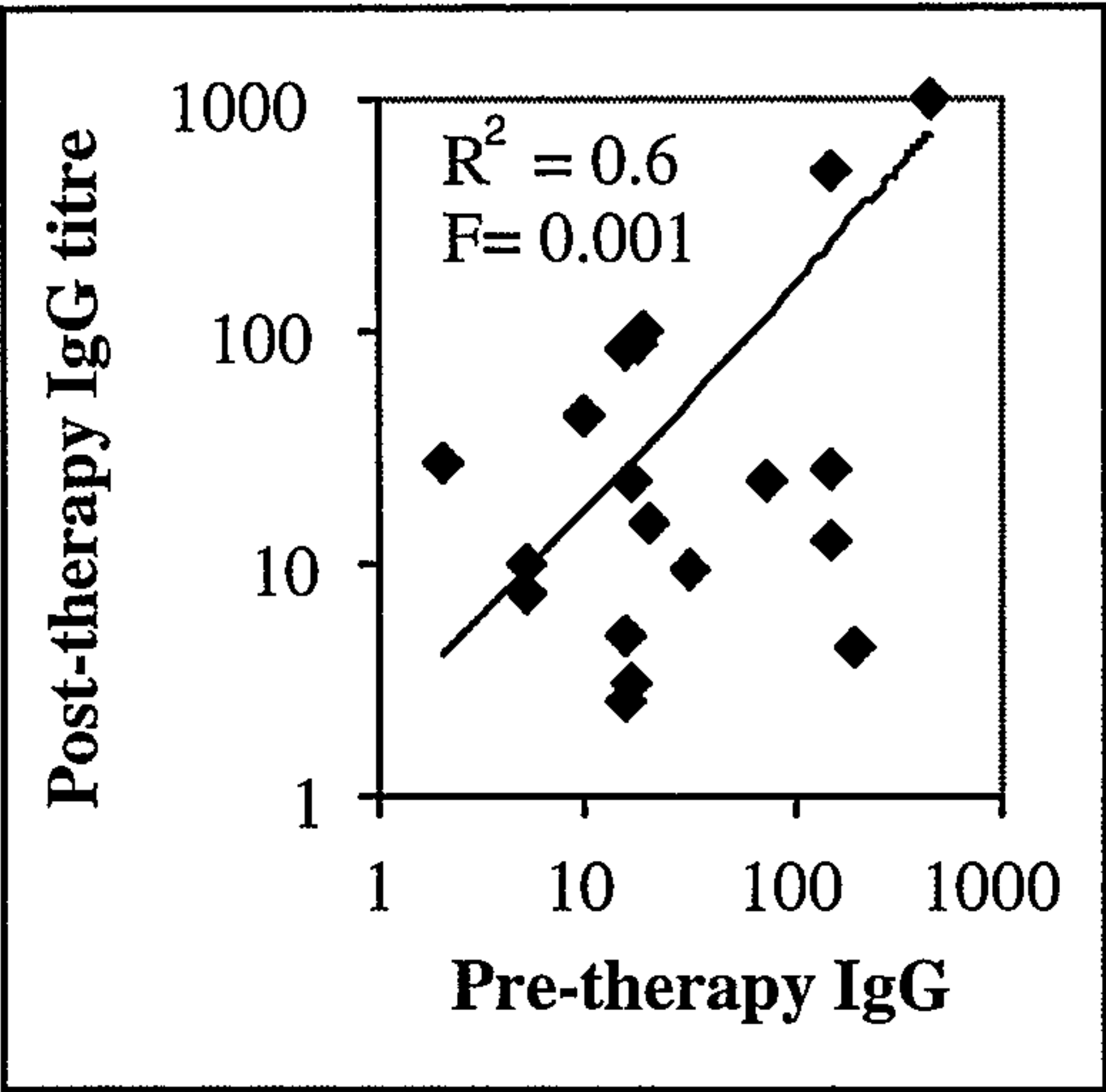


Figure 6.7 Post-therapy IgG titres correlated with pre-therapy IgG titres. Linear regression and best fit plot of IgG titers specific for the rHA2 from serum collected before the start of periodontal therapy vs. titres from serum collected some time after the start of periodontal therapy

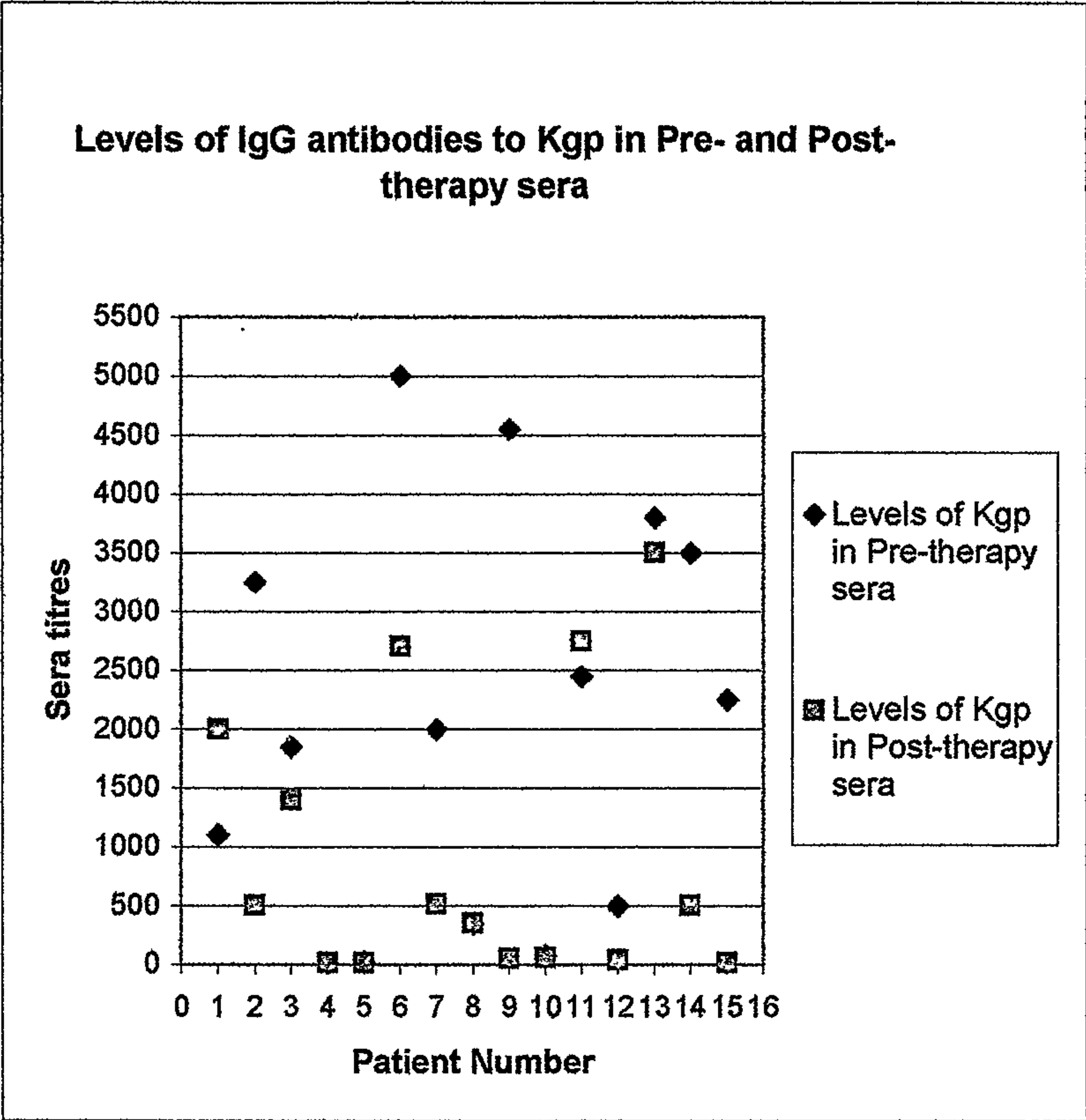


Figure 6.8 Shows a scatter-graph of matching pre- and post-therapy sera titres to gingipain Kgp combined for 15 patients with adult periodontitis.

not appear to be a definite trend in the change in titre levels between pre- and post-therapy sera in the patient pool. However, this is not a reflection of the quality of the antibodies. There was a range in the capacity of the patient sera to neutralise gingipain proteinase activity. However, a higher post-therapy capacity to neutralise gingipain protease activity was significantly and negatively correlated with severity of attachment loss in participants (Fig. 6.9), demonstrating a relation between anti-gingipain IgG and periodontal health.

6.4 DISCUSSION

In summary, these data suggest that higher titres and increased functional antibodies to the gingipains and to the associated HA2 domain may be desirable endpoints in the prevention or management of adult periodontitis.

In these experiments, the gingipains in the plaque were measured independently with two different mAbs, neither of which detected antigen in whole cell preparations of two unrelated bacteria. The epitope of mAb VA1 has been described in collaborative studies (DeCarlo *et al.*, 1999) and is uniquely specific in the databases for the HA1 and HA2 domains. Levels of detection of the gingipains with mAb IIB2 correlated with detection by mAb VA1 in participant plaque samples. This correlation supports the assumption that the gingipains, and the HA2 domain of the gingipains, were the primary target of mAb VA1 in the data presented.

This appears to be the first report specifically identifying the HA2 domain *in vivo*. Detection of the HA2 domain was directly and significantly correlated with haemoglobin-associated binding activity in the samples, demonstrating that the HA2 domain was functional *in vivo*, probably within the gingipains of *P. gingivalis*. Using ELISA, a direct statistical association of the HA2 domain with the gingipains, and with *P. gingivalis* in the dental plaque, was identified. Also identified was a direct and significant correlation of the HA2 domain with gingival inflammation, an accepted indicator of periodontitis.

Both pre-and post-therapy serum antibody titres specific for the gingipains and HA2 domain ranged widely. Higher pre-therapy antibody titres specific for the HA2 domain of the gingipains were associated with measurements of clinical health and with detection of the HA2 domain in the plaque samples, suggesting that the HA2 domain in

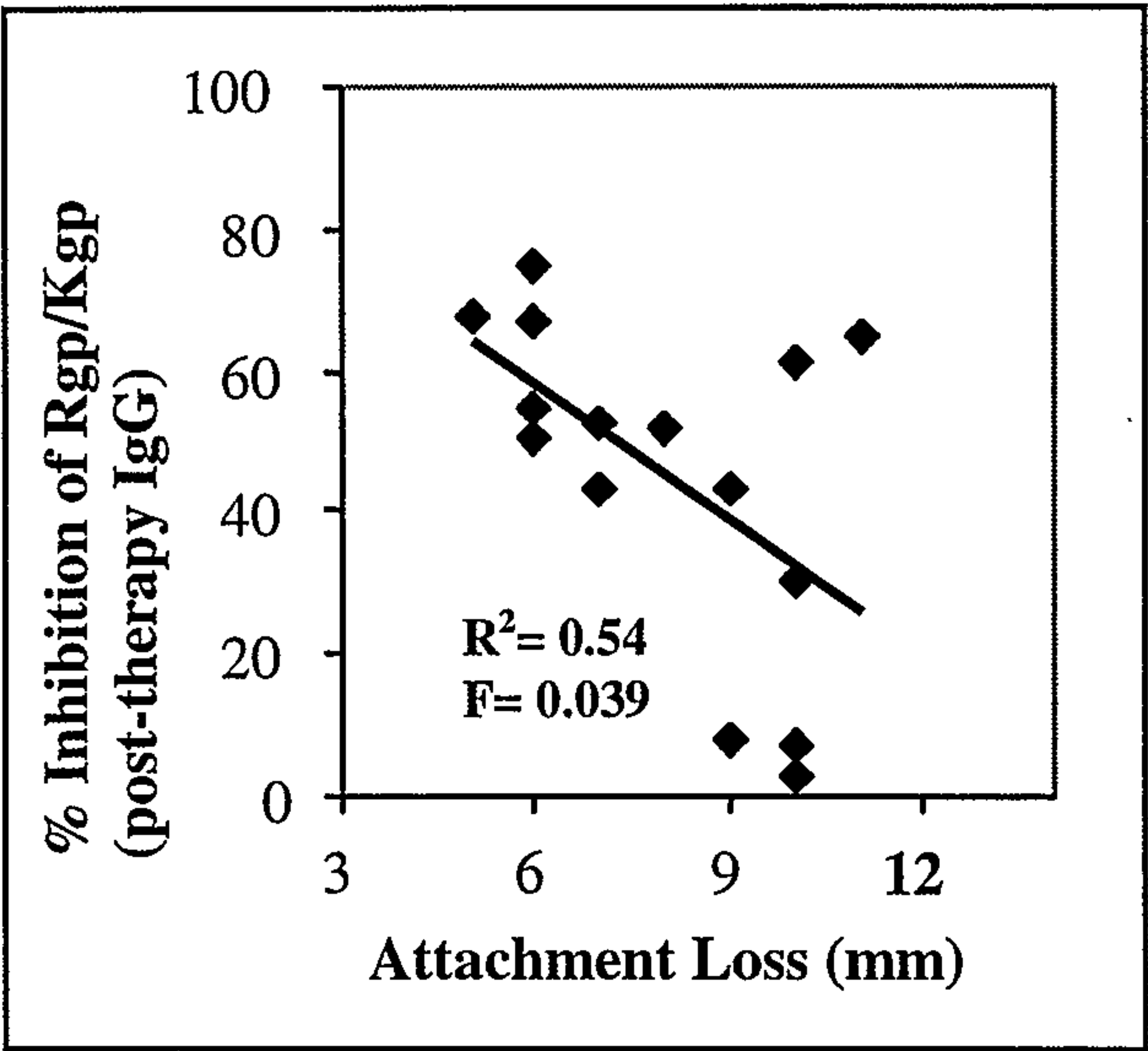


Figure 6.9 Inhibition of gingipain proteinase activity by serum IgG correlates with periodontal health. Linear regression and best fit plot of maximal dental attachment loss in participants vs. inhibition of gingipain proteolytic activity within participants (n=15). The IgG fraction was isolated from sera and gingipain activities against synthetic substrates were normalised to trypsin controls then percent inhibition relative to the highest levels of activity for either RgpA or Kgp in IgG was calculated.

the plaque was immunogenic and that higher anti-HA2 titres would be desirable.

When the work was commenced there had been no reported extensive investigation of the host response to the Rgp-Kgp proteinase-adhesin complex in periodontitis patients. Since then O'Brien-Simpson have recently reported the IgG and IgG subclass responses to the Rgp/Kgp complex (O'Brien-Simpson *et al.*, 2000). All of the subject sera showed an immunoreactive response to a 44-kDa band that corresponded to the Rgp44 and/or Kgp44 adhesins of the complex. It was not clear if the immunoreactive band corresponded to the 44-kDa adhesin or the 45-kDa catalytic domain from RgpA (O'Brien-Simpson *et al.*, 2000). The RgpA27 and Kgp39 adhesins were detected with IgG4-specific sera but there was no evidence of immunoreactivity to the RgpA15 or Kgp15 adhesin domain. It was unusual that the apparent 15-kDa adhesin domain migrated under SDS-PAGE conditions with an apparent molecular weight of 15-kDa. Nakayama *et al.* have supported findings reported herein (Figure 3.14) that demonstrated the 15-kDa adhesin domain to migrate at approximately 19-kDa (Nakayama *et al.*, 1998). It is possible that the strain differences might account for this discrepancy as their study used W50. However, another study by Curtis *et al.* using the W50 strain, identified the HA2 domain as a lipid A-associated protein with an apparent molecular weight of 17-kDa under SDS-PAGE conditions (Curtis *et al.*, 1998).

O'Brien-Simpson reported immunoblot analysis of the Rgp-Kgp complex showing that sera from healthy subjects and those with low levels of disease and with high IgG4 and low IgG2 responses, reacted with Rgp27, Kgp39, and Rgp44 adhesins while sera from diseased subjects with low IgG4 and high IgG2 responses reacted only with the Rgp44 and/or Kgp44 adhesins (O'Brien-Simpson *et al.*, 2000). This implied that the response to the adhesins may be generally protective. In contrast Genco *et al.* reported that in mice, the major IgG response was targeted to sequences within the adhesin/haemagglutinin domain of the 95-kDa gingipain R1 and gingipain K implying that the adhesins were highly immunogenic (Genco *et al.*, 1998). However, the antibodies were not found to be protective against further challenge with *P. gingivalis* in a mouse virulence assay. These findings were also supported by examination of sera from 15 patients with advanced periodontitis. Reactivity of the IgG antibodies to the haemagglutinin domains did not appear to function in a protective capacity (Genco *et al.*, 1999). Close analysis of the 95-kDa gingipain R1 utilised by Travis *et al.* revealed that

the complex consisted of a 50-kDa catalytic domain bound to two different 44-kDa proteins (since the 44-kDa and 27- and 17-kDa bands differ in their NH₂-terminal sequences), one a single chain molecule of 44-kDa and the other consisting of 27- and 17-kDa proteins, which presumably associate non-covalently to give a 44-kDa protein (Pike *et al.*, 1994). It is evident that the results from the Genco study did not involve the HbR protein (HA2) at all as the adhesin was not present in the 95-kDa gingipain R1 complex which might explain the apparent lack of protective immunity observed in the sera.

O'Brien-Simpson *et al.* found that the diseased group had a significantly higher total IgG response ($P < 0.001$) to the gingipains than the control healthy group which had almost no reactive antibody (O'Brien-Simpson *et al.*, 2000). This correlated well with our findings where pre-therapy sera from 23 of the 25 participants with periodontitis were shown to recognise the gingipains. Post-therapy sera were found to neutralise the proteolytic activity of both gingipains. In contrast, Genco *et al.* found, using Western Blot and ELISA studies, that immunisation of mice with the 95-kDa gingipain R1 or whole cells of *P. gingivalis* did not induce a high titre to the catalytic domain (Genco *et al.*, 1998). Antisera raised to gingipain R1 in rabbits or chickens also contained a strong reactivity to the haemagglutinin domain of gingipain R1 and gingipain K but with little reactivity to the catalytic domain(s) (Genco *et al.*, 1999). Since it is not clear that the neutralising capacity observed in the post-therapy sera was a function of an immunological reaction to the catalytic domain, this effect might be a result of antibodies to the adhesin domains (possibly the HA2 domain) inhibiting the proteolytic capacity of the gingipains. While there did not appear to be a distinct pattern observed in the levels of serum titre pre- and post-treatment, the capacity of post-therapy sera to neutralise proteolytic activity of the gingipains was significantly and negatively correlated with attachment loss in patients. This implied that the quality of the antibodies pre- and post-treatment was altered with the post-therapy sera developing protective qualities. The periodontal therapy received by the participants was sub-gingival root planing which usually results in a minor bacteremia, thereby indicating the inoculation of the patient with the contents of the periodontal pocket. Sub-gingival root planing has been associated with the establishment of periodontal health for the primary reason that aetiological agents are removed from the teeth, but more consideration should also be

given to the inoculating effect of root planing. Further, once antigens are identified that are relevant clinically and bacteriologically, such as the gingipains and HA2, the role of vaccination in the prevention and treatment of periodontal disease should be explored.

The gingipains and the HA2 domain have a potential role in acquiring necessary porphyrin or iron for *P. gingivalis* metabolism. Establishment of the clinical relevance for these virulence factors in periodontal disease is both valuable and timely. Data from this report provide evidence that the gingipains and the HA2 domain are clinically relevant to periodontal disease, and demonstrate that the host immune response might be successfully employed in limiting gingipain proteinase activity and haemoglobin binding activity of HA2 in the control of periodontal disease.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 SUMMARY

Immunohistological analysis of biopsied tissues from patients with chronic adult periodontitis revealed unusual evidence of remnants of the basement membrane proteins laminin and type IV collagen in the connective tissue. As these remnants were organised in a trabecular network, it was postulated that abortive angiogenesis had occurred at some stage of the host response. The resulting pathology would compromise the ability of the tissues to heal and could explain the chronicity of the disease. It was proposed that the vascular pathology observed was related to the action of products from the major putative pathogens associated with periodontal disease. To test this hypothesis, crude protein extracts from *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25611 and *Fusobacterium nucleatum* ATCC 10953 were used to challenge pre-formed vascular networks in an *in-vitro* model of angiogenesis.

Experimental results discussed in Chapter 3 demonstrated that crude protein extracts from *P. gingivalis* disrupt pre-formed vascular networks on Matrigel whereas *P. intermedius* and *F. nucleatum* were inactive at identical concentrations. The Matrigel assay presented both advantages and disadvantages. This assay represents the late stage of angiogenesis with the networks formed on the surface of the Matrigel, allowing bacterial products to interact with both endothelial cells and basement membrane. Purification of this Vascular Disruptive Factor (VDF) using standard protein chromatography techniques proved difficult as a result of the three closely related proteins in the active fraction. At this point, the inadequacy of the non-quantitative nature of the Matrigel bioassay became apparent making accurate analysis of the increase in biological potency difficult at the sequential stages of

purification. N-terminal sequencing of these proteins found them to be the haemagglutinin related adhesin domains, HA2, HA3 and HA4, from the well-established family of cysteine proteinases referred to as the “gingipains”. These gingipains (RgpA and Kgp) are multi-domain proteins containing a catalytic domain with four closely related adhesin domains (HA1, HA2, HA3 and HA4).

Since the dominant protein band in the active fractions tested in the bioassay was HA2, a recombinant clone of the HA2 protein was constructed using the genomic sequence of the *rgp1* (HG66) (Pavloff *et al.*, 1995) gene of the Arg-gingipain complex available at the time. The purified rHA2 protein was found to be inactive at all concentrations tested in the bioassay. Although recombinant cloning of proteins has become a routine approach to produce high levels of purified protein, it is not possible to ensure that the expressed recombinant protein is natively folded. Using a reported haemoglobin binding function of the HA2 protein at the time (Nakayama *et al.*, 1998), the natively purified recombinant protein was not able to bind haemoglobin. Recombinant protein with haemoglobin binding activity was produced using a denaturing and refolding technique. It was found that rHA2 recovered by elution from haemoglobin-Agarose was also inactive in the vascular network disruption assay. Demonstration of one function of the native protein does not, however, imply that all other binding functions of the protein have been maintained.

There was a growing literature which supported a role for the entire gingipain complex in an integrated strategy for obtaining haem from blood. The gingipain complex has been shown to cause vascular permeability enhancement to interfere with plasma clot formation (Imamura *et al.*, 1995; Imamura *et al.*, 1995; Imamura *et al.*, 1997), degrade fibrinogen (Scott *et al.*, 1993), to lyse erythrocytes (Shah and Gharbia 1989b), and bind and degrade haemoglobin (Nakayama *et al.*, 1998; Lewis *et al.*, 1999a).

The RgpA complex was shown to play an active role in the vascular disruption of the pre-formed vascular networks in the Matrigel assay. As a result of the difficulties encountered with the bioassay earlier, this assay was made more definitive by developing a method to quantify the integrity of vascular networks in the Matrigel bioassay. Using TLCK-inhibited preparations of the complex, this

activity was shown to be related to the catalytic activity of the complex. The mechanism of network disruption was related to apparent attack and permeation of the cell membrane as determined by confocal analysis. It was of interest that this binding of RgpA to the cytoplasmic membrane of endothelial cells occurred in an environment with 20% foetal calf serum and in the presence of a substantial amount of basement membrane protein. These findings suggest that the RgpA complex produces an extensive and direct attack on endothelial cell membrane proteins, perhaps following targeted binding.

At the time, the apparent poor increase in biological potency observed in the studies reported in Chapter 3 was thought to be related to multiple proteins being involved in the disruption. Although HA2 could not be related to the vascular disruption in the bioassay, coincidentally this protein was reported in the literature to bind haemoglobin (Nakayama *et al.*, 1998). This function was of interest as it contributed to the original hypothesis of the role of *P. gingivalis* in the initiation of haemorrhage and sequestration of haem. Collaborative studies at the IDR found the HA2 protein to bind haemoglobin specifically through the propionic acid face of the protoporphyrin IX molecule (DeCarlo *et al.*, 1999). This novel finding brought to light some important issues which needed resolution. It has always been a well-accepted fact that *P. gingivalis* has an obligatory need for haem (Gibbons and Macdonald 1960). However, the need for porphyrin had never been resolved. In findings reported in Chapter 5, analysis of the genome of *P. gingivalis* indicated that the organism was not capable of synthesising porphyrin as it lacked the enzymes needed for this process. An essential requirement for porphyrin was evident by the demonstration that iron-free deuteroporphyrin dihydrochloride could support growth in haemin-depleted medium. All the porphyrin derivatives with intact propionate faces that were tested functioned as haem substitutes despite modification of the vinyl aspect. This supported a role for HA2 as a prime porphyrin receptor. Neutralisation assays supported the function of this domain as the main haemoglobin binding receptor (Shi *et al.*, 1999) for *P. gingivalis*.

The ability of RgpA and the HA2 adhesin domain to interact with the vasculature to acquire essential porphyrin has been highlighted. It was also necessary

to investigate the potential antigenicity of these proteins in the context of the disease process as the impact of these functions on the host response related to periodontal disease were not evident. In investigations described in Chapter 6, the objective was to examine the relation between plaque levels of these virulence factors, specific serum antibody titres and clinical status. The results from this study demonstrated that site-specific detection of the HA2 and gingival inflammation were correlated with plaque measurements of gingipains and HA2. In turn these indices were directly related to levels of HA2-associated haemoglobin binding activity *in-vivo*. Higher pre-therapy anti-HA2 and higher post-therapy anti-gingipain serum antibody titres, as well as the capacity of serum IgG to neutralise gingipain proteolytic activity were inversely associated with clinical periodontitis. Although the patient pool was limited to 25 patients, the strong evidence for a role in protective immunity by the gingipain family with a particular focus on the HbR (HA2) protein, provides impetus for a further study with a larger patient population. One of the difficulties faced in designing a clinical study is that of minimising variables to allow valid interpretation of results. In this particular study, the negative control used was the healthiest gingival site within each patient with adult periodontitis to provide an internal control standard.

7.2 FURTHER WORK

It would be of interest to test the ability of RgpA to cause haemorrhage in an animal model to evaluate the actual potential *in-vivo*. However, as this work was only revisited later in the thesis after evidence for the vascular role of the gingipains became evident, further experimental work was not feasible. Probing for the presence of the gingipains and HA2 in the subgingival plaque adjacent to lesions of chronic adult periodontitis and comparing these findings with that of healthy sites using the monoclonal antibodies VA1 and IIB2 would be a useful addition to the clinical study. Increasing the patient pool and acquiring plaque and sera from patients with no clinical evidence of disease to act as a negative control are additional steps to further strengthen the findings.

7.3 FUTURE APPLICATIONS

The role of antibiotics in the treatment of periodontal disease is targeted towards eliminating all anaerobes in the environment. Metronidazole has successfully been used for this purpose in the treatment of severe cases of periodontitis (Muller *et al.*, 1977; Loesche *et al.*, 1981). This seems to be a very unsophisticated approach to the elimination of specific putative pathogens with the concern of antibiotic resistance becoming an issue in the future. It was not possible to specifically eliminate *P. gingivalis* from the microbial flora in the past which has also made it difficult to prove conclusively that this putative pathogen is truly a pathogen directly involved in the initiation and/or progression of adult periodontitis *in-vivo*. There is a need for the discovery of new bacterial vulnerabilities and for the development of new antibacterial compounds that would circumvent bacterial resistance mechanisms. A particularly attractive antibacterial drug should target the virulence mechanisms of bacterial pathogens and should not be affected by existing resistance mechanisms in these bacteria.

The findings from this work provide the basis to revisit Kochs' postulates and to address a fundamental yet unanswered question. On this basis, the binding interaction of HA2 (HbR protein) to haemoglobin becomes of interest as a focus for the development of a highly specific antibacterial agent. Firstly, the binding interaction of HA2 to the porphyrin ring via the propionate face is unusual as the vinyl aspect is a more common docking site for receptors. It is presumed that HA2 binds porphyrins via the propionate face and to present the tetrapyrrole ring to the organism via a cell surface receptor resulting in a porphyrin exchange from the vinyl end of the molecule. The importance of porphyrin to *P. gingivalis* has already been highlighted with the HA2 protein representing the major porphyrin carrier for the organism. This represents a point of vulnerability for the organism which could be exploited by the synthesis of a specific porphyrin-like inhibitor.

Heavy modification of the vinyl end aspect would mean that the inhibitor would be able to compete with environmental haemoglobin for the HA2 molecule forming the binding interaction via the propionate end but will then be unable to present it to the organism as a result of the vinyl modification. The result would be

specific elimination of *P. gingivalis* in the context of the existing microbial population. As a result it would be possible to acquire conclusive data implicating this organism as the most important pathogen associated with periodontal disease, in effect answering a key element of Kochs' postulates.

The humoral arm of the immune system provides protection from many significant pathogens. The antigenic epitopes of the pathogens which induce these responses, and the subsequent characteristics of the host response, have been extensively documented in the medical literature, and in many cases have resulted in the development and implementation of effective vaccines or diagnosis tests. The production of a successful vaccine requires identification of an antigen common to all strains of the organism, that has been shown to have virulence properties, is strongly immunogenic and has the capacity to induce protective immunity. The HA2 molecule would be a potential candidate for vaccine development. It has been shown to be unique in the database thereby avoiding the problem of cross-reactivity with other organisms.

7.4 CONCLUSION

The results from this thesis suggest that the putative pathogen *P. gingivalis* has an integrated strategy for obtaining essential iron and porphyrin. Incidental bleeding provides an environment that could promote the growth and virulence potential of *P. gingivalis* resulting in an enhanced production and release of the Rgp/Kgp family of gingipains. The gingipains have the capacity to actively fulfill the essential porphyrin and iron requirements of the organism by diffusing into the connective tissue of the pocket wall and actively disrupting pre-formed vascular networks. The HA2 domain would be integral at this point to specifically bind to the propionate face of the released haemoglobin molecules to capture essential porphyrin for the organism. This strategy complements the well accepted role of the gingipains as important multi-functional proteins enabling vascular permeability, aggregation and lysis of erythrocytes, clot disruption, binding and degradation of haemoglobin to obtain porphyrin and iron.

BIBLIOGRAPHY

- Abe, N., T. Kadowaki, K. Okamoto, K. Nakayama, M. Ohishi, and K. Yamamoto. 1998. Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease. *J. Biochem.* **123**:305-312.
- Aduse-Opoku, J., J. Muir, J. M. Slaney, M. Rangarajan, and M. A. Curtis. 1995. Characterisation, genetic analysis, and expression of a protease antigen (PrpRI) of *Porphyromonas gingivalis* W50. *Infect. Immun.* **63**:4744-4754.
- Aduse-Opoku, J., J. M. Slaney, M. Rangarajan, J. Muir, K. E. Young, and M. A. Curtis. 1997. The Tla protein of *Porphyromonas gingivalis* W50: a homolog of the R1 protease precursor (PrpR1) is an outer membrane receptor required for growth on low levels of haemin. *J. Bacteriol.* **179**:4778-4788.
- Aisen, P., and A. Leibman. 1972. Lactoferrin and transferrin : a comparative study. *Biochim. Biophys. Acta.* **257**:314-323.
- Aisen, P., and E. B. Brown. 1975. Structure and function of transferrin. *Prog. Haematol.* **9**:25-56.
- Albandar, J. M., L. J. Brown, and H. Löe. 1997. Putative periodontal pathogens in subgingival plaque of young adults with and without early-onset periodontitis. *J. Periodontol.* **68**:973-81.
- Altman, L. C., R. C. Page, J. L. Ebersole, and G. E. Vandersteen. 1982. Assessment of host defenses and serum antibodies to suspected periodontal pathogens in patients with various types of periodontitis. *J. Periodontol. Res.* **17**:495-497.
- Altschul, S., W. Gish, W. Miller, E. Myers, and D. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403-10.
- Amano, A., M. Kuboniwa, K. Kataoka, K. Tazaki, E. Inoshita, H. Nagata, T. Hiroo, and S. Shizukuishi. 1995. Binding of haemoglobin by *Porphyromonas gingivalis*. *FEMS Microbiol. Lett.* **134**:63-67.
- Arnoux, P., R. Haser, N. Izadi, A. Lecroisey, M. Delepierre, C. Wandersman, and M. Czjzek. 1999. The crystal structure of HasA, a haemophore secreted by *Serratia marcescens*. *Nat. Struct. Biol.* **6**:516-20.
- Auerbach, R., W. Auerbach, and I. Polakowski. 1991. Assays for angiogenesis: a review. *Pharmacol. Ther.* **51**:1-11.

- Ausprunk, D., and J. Folkman.** 1977. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumour angiogenesis. *Microvasc. Res.* 1453-1465.
- Barua, P. K., D. W. Dyer, and M. E. Neiders.** 1990. Effect of iron limitation on *Bacteroides gingivalis*. *Oral Microbiol. Immunol.* 5:263-268.
- Beck, J., R. Garcia, G. Heiss, P. S. Vokonas, and S. Offenbacher.** 1996. Periodontal disease and cardiovascular disease. *J. Periodontol.* 67:1123-1137.
- Bedi, G. S., and T. Williams.** 1994. Purification and characterisation of a collagen-degrading protease from *Porphyromonas gingivalis*. *J. Biol. Chem.* 269:599-606.
- Benjamin, P. A., P. A. Rogers, J. N. W. U S, M. F. Cole, and M. A. Curtis.** 1997. Increased titre and avidity of IgG antibodies to whole cells and a cell surface protein in subjects with adult periodontitis. *J. Periodontol. Res.* 32:31-9.
- Biberstein, E., P. Mini, and M. Gills.** 1963. Action of *Haemophilus* cultures on δ -aminolevulinic acid. *J. Bacteriol.* 86:814-819.
- Bird, P. S., E. Gemmell, B. Polak, R. G. Paton, W. Sosroseno, and G. J. Seymour.** 1995. Protective immunity to *Porphyromonas gingivalis* infection in a murine model. *J. Periodontol.* 66:351-362.
- Blum, G., M. Ott, A. Lischewski, A. Ritter, H. Imrich, H. Tschäpe, and J. Hacker.** 1994. Excision of large DNA regions termed pathogenicity islands from tRNA-specific loci in the chromosome of an *Escherichia coli* wild-type pathogen. *Infect. Immun.* 62:606-614.
- Booth, V., F. P. Ashley, and T. Lehner.** 1996. Passive immunisation with monoclonal antibodies against *Porphyromonas gingivalis* in patients with periodontitis. *Infect. Immun.* 64:422-427.
- Bouts, E. A., T. Koseki, T. Nishihara, and I. Ishikawa.** 1996. Characterisation of the immunodominant antigens of *Porphyromonas gingivalis* 381 in high-responder patients. *Oral Microbiol. Immunol.* 11:236-41.
- Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Bramanti, T. E., and S. C. Holt.** 1990. Iron-regulated outer membrane proteins in the periodontopathic bacterium, *Bacteroides gingivalis*. *Biochem. Biophys. Res. Commun.* 166:1146-1154.

Bramanti, T. E., and S. C. Holt. 1991. Roles of porphyrins and host iron transport proteins in regulation of growth of *Porphyromonas gingivalis* W50. *J. Bacteriol.* **173**:7330-7339.

Bramanti, T. E., and S. Holt. 1992. Effect of porphyrins and host iron transport proteins on outer membrane protein expression in *Porphyromonas (Bacteroides) gingivalis*: identification of a novel 26 kDa haemin-repressible surface protein. *Microb. Pathog.* **13**:61-73.

Bramanti, T. E., and S. Holt. 1993. Haemin uptake in *Porphyromonas gingivalis*: Omp26 is a haemin-binding surface protein. *J. Bacteriol.* **175**:7413-7420.

Bramanti, T. E., S. C. Holt, J. L. Ebersole, and T. van Dyke. 1993. Regulation of *Porphyromonas gingivalis* virulence: haemin limitation effects on the outer membrane protein (OMP) expression and biological activity. *J. Periodontol. Res.* **28**:464-466.

Bujard, H., R. Gentz, M. Lanzer, D. Stüber, M. Müller, I. Ibrahimi, M. T. Häuptle, and B. Dobberstein. 1987. A T5 promoter based transcription-translation system for the analysis of proteins *in-vivo* and *in-vitro*. *Methods Enzymol.* **155**:416-433.

Carmen, R. J., M. D. Ramakrishnan, and F. H. Harper. 1990a. Haemin levels in culture medium of *Porphyromonas (Bacteroides) gingivalis* regulate both haemin binding and trypsin-like protease production. *Infect. Immun.* **58**:4016-4019.

Carmen, R. J., M. D. Ramakrishnan, F. H. Harper, and M. A. Curtis. 1990b. *Porphyromonas (Bacteroides) gingivalis*: the role of haemin and iron in the aetiology of destructive periodontitis, p. 161-167. In S. P. Borriello (ed.), *Clinical and Molecular Aspects of Anaerobes*. Wrightson Biomedical Publishing, Petersfield, U.K.

Carter, K. C. 1987. , p. 161, *Essays of Robert Koch*, vol. xvii-xix. Greenwood Press, New York.

Chapple, C. C., R. K. Kumar, and N. Hunter. 2000. Vascular remodelling in chronic inflammatory periodontal disease. *J. Oral Pathol. Med.* **29**:500-6.

Chen, Z., J. Potempa, A. Polanowski, S. Renwert, W. Wikstrom, and J. Travis. 1991. Stimulation of proteinase and amidase activities in *Porphyromonas (Bacteroides) gingivalis* by amino acids and dipeptides. *Infect. Immun.* **59**:2846-2850.

Chen, C.-K. C., A. DeNardin, D. W. Dyer, and R. J. Genco. 1991. Human immunoglobulin G antibody response to iron-repressible and other membrane proteins of *Porphyromonas (Bacteroides) gingivalis*. *Infect. Immun.* **59**:2427-2433.

- Chen, Z., J. Potempa, A. Polanowski, M. Wikstrom, and J. Travis.** 1992. Purification and characterisation of a 50-kDa cysteine proteinase (gingipain) from *Porphyromonas gingivalis*. *J. Biol. Chem.* **267**:18896-18901.
- Choi, B. K., S. H. Park, Y. J. Yoo, S. H. Choi, J. K. Chai, K. S. Cho, and C. K. Kim.** 2000. Detection of major putative periodontopathogens in Korean advanced adult periodontitis patients using a nucleic acid-based approach. *J. Periodontol.* **71**:1387-94.
- Chu, L., T. E. Bramanti, J. L. Ebersole, and S. C. Holt.** 1991. Haemolytic activity in the periodontopathogen *Porphyromonas gingivalis*: kinetics of enzyme release and localisation. *Infect. Immun.* **59**:1932-1940.
- Ciborowski, P., M. Nishikata, R. D. Allen, and M. S. Lantz.** 1994. Purification and characterisation of two forms of a high-molecular weight cysteine proteinase (porphypain) from *Porphyromonas gingivalis*. *J. Bacteriol.* **176**:4549-4557.
- Clerehugh, V., G. J. Seymour, P. S. Bird, M. Cullinan, D. B. Drucker, and H. V. Worthington.** 1997. The detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* using an ELISA in an adolescent population with early periodontitis. *J. Clin. Periodontol.* **24**:57-64.
- Collinson, L. M., M. Rangarajan, and M. A. Curtis.** 1998. Altered expression and modification of proteases from an avirulent mutant of *Porphyromonas gingivalis* W50 (W50/BE1). *Microbiol.* **144**:2487-96.
- Coulton, J., and J. Pang.** 1983. Transport of haemin by *Haemophilus influenzae* Type b. *Curr. Microbiol.* **9**:93-98.
- Courant, P. R., and H. Bader.** 1966. *Bacteroides melaninogenicus* and its products in the gingiva of man. *Periodontics.* **4**:131-6.
- Curtis, M. A., J. Aduse-Opoku, J. M. Slaney, M. Rangarajan, V. Booth, J. Cridland, and P. Shepherd.** 1996. Characterisation of an adherence and antigenic determinant of the ArgI protease of *Porphyromonas gingivalis* which is present on multiple gene products. *Infect. Immun.* **64**:2532-9.
- Cutler, C. W., J. R. Kalmar, and R. R. Arnold.** 1991. Phagocytosis of virulent *Porphyromonas gingivalis* by human polymorphonuclear leukocytes requires specific immunoglobulin G. *Infect. Immun.* **59**:2097-104.
- Cutler, C. W., J. R. Kalmer, and C. A. Genco.** 1995. Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. *Trends Microbiol.* **3**:45-51.
- D'Amore, P. A., and R. W. Thompson.** 1987. Mechanisms of angiogenesis. *Annu. Rev. Physiol.* **49**:453-484.

DeCarlo, A. A., and G. J. Harber. 1997. Haemagglutinin activity and heterogeneity of related *Porphyromonas gingivalis* proteinases. *Oral Microbiol. Immunol.* **12**:47-56.

DeCarlo, A. A., M. Paramaesvaran, P. L. Yun, C. Collyer, and N. Hunter. 1999. Porphyrin-mediated binding to haemoglobin by the HA2 domain of cysteine proteinases (gingipains) and haemagglutinins from the periodontal pathogen *Porphyromonas gingivalis*. *J. Bacteriol.* **181**:3784-3791.

Delvos, U., C. Gajdusek, H. Sage, L. A. Harker, and S. M. Schwartz. 1982. Interactions of vascular wall cells with collagen gels. *Lab. Invest.* **46**:61-72.

Deshpande, R. G., M. B. Khan, and C. A. Genco. 1998. Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. *Infect. Immun.* **66**:5337-5343.

DeStefano, F., R. F. Anda, H. S. Kahn, D. F. Williamson, and C. M. Russell. 1993. Dental disease and risk of coronary heart disease and mortality. *B.M.J.* **306**:688-91.

Discipio, R. G., P. J. Daffern, M. Kawahara, R. Pike, J. Travis, and T. E. Hugli. 1996. Cleavage of human complement component C5 by cysteine proteinase (gingipain-1) from *Porphyromonas gingivalis*. Prior oxidation of C5 augments proteinase digestion of C5. *Immunol.* **87**:660-7.

Dorn, B. R., W. A. J. Dunn, and A. Progulske-Fox. 1999. Invasion of human coronary artery cell by periodontal pathogens. *Infect. Immun.* **67**:5792-8.

Dreyer, W. P., and N. J. Basson. 1992. *Porphyromonas gingivalis* as a putative pathogen in ovine periodontitis. *J. Dent. Assoc. S. Afr.* **47**:513-6.

Dvorak, H. F., V. S. Harvey, P. Estrella, L. F. Brown, J. McDonagh, and A. M. Dvorak. 1987. Fibrin containing gels induce angiogenesis. Implications for tumour stroma generation and wound healing. *Lab. Invest.* **57**:673-686.

Eaves, D. J., J. Grove, W. Staudenmann, P. James, R. K. Poole, S. A. White, I. Griffiths, and J. A. Cole. 1998. Involvement of products of the *nrfEFG* genes in the covalent attachment of haem c to a novel cysteine-lysine motif in the cytochrome c552 nitrite reductase from *Escherichia coli*. *Mol. Microbiol.* **28**:205-16.

Ebersole, J. L., M. A. Taubman, D. J. Smith, and S. S. Socransky. 1982. Humoral immune responses and diagnosis of human periodontal disease. *J. Periodontol.* **17**:478-480.

Ebersole, J. L., M. A. Taubman, and D. J. Smith. 1985. Gingival crevicular fluid antibody to oral microorganisms. II. Distribution and specificity of local antibody responses. *J. Periodontol. Res.* **20**:349-356.

- Ebersole, J. L., M. A. Taubman, D. J. Smith, and D. E. Frey. 1986. Human immune responses to oral microorganisms : patterns of systemic antibody levels to *Bacteroides* spp. *Infect. Immun.* **51**:507-513.
- Fan, Q., T. J. Sims, T. Nakagawa, and R. C. Page. 2000. Antigenic cross-reactivity among *Porphyromonas gingivalis* serotypes. *Oral Microbiol. Immunol.* **15**:158-65.
- Finlay, B. B., and S. Falkow. 1997. Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* **61**:136-69.
- Fleishman, R., M. Adams, O. White, R. Clayton, E. Kirkness, A. Kerlavage, C. Bult, J. Tomb, B. Dougherty, and J. M. e. al. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science.* **269**:496-512.
- Folkman, J., and C. Haudenschield. 1980. Angiogenesis *in vitro*. *Nature.* **288**:551-556.
- Form, D. M., B. M. Pratt, and J. A. Madri. 1986. Endothelial cell proliferation during angiogenesis; *in-vitro* modulation by basement membrane components. *Lab. Invest.* **55**:521-530.
- Fujimura, S., Y. Shibata, K. Hirai, and T. Nakamura. 1996. Binding of haemoglobin in the envelope of *Porphyromonas gingivalis* and isolation of the haemoglobin-binding protein. *Infect. Immun.* **64**:2339-2342.
- Fujimura, S., Y. Shibata, K. Hirai, and N. Takeshi. 1995. Some binding properties of the envelope of *Porphyromonas gingivalis* to haemoglobin. *FEMS Immunol. Med. Microbiol.* **10**:109-114.
- Fujino, E., T. Fujino, S. Karita, K. Sakka, and K. Ohmiya. 1995. Cloning and sequencing of some genes responsible for porphyrin biosynthesis from the anaerobic bacterium *Clostridium josei*. *J. Bacteriol.* **177**:5169-75.
- Fujiwara, S., H. Wiedemann, R. Timpl, A. Lustig, and J. Engel. 1984. Structure and interactions of heparan sulfate proteoglycans from a mouse tumour basement membrane. *Eur. J. Biochem.* **143**:145.
- Gamble, J. R., L. J. Matthias, G. Meyer, P. Kaur, G. Russ, R. Faull, M. C. Berndt, and M. A. Vadas. 1993. Regulation of *in-vitro* capillary tube formation by anti-integrin antibodies. *J. Cell Biol.* **121**:931-943.
- Genco, C. A., C. W. Cutler, D. Kapczynski, H. Maloney, and R. R. Arnold. 1991. A novel mouse model to study the virulence of and host response to *Porphyromonas (Bacteroides) gingivalis*. *Infect. Immun.* **59**:1255-63.

Genco, C. A., B. M. Odusanya, and G. Brown. 1994. Binding and accumulation of haemin in *Porphyromonas gingivalis* are induced by haemin. *Infect. Immun.* **62**:2885-2892.

Genco, C. A., W. Simpson, R. Y. Forng, M. Egal, and B. M. Odusanya. 1995. Characterisation of a Tn4351-generated haemin uptake mutant of *Porphyromonas gingivalis*: evidence for the coordinate regulation of virulence factors by haemin. *Infect. Immun.* **63**:2459-2466.

Genco, C. A., B. M. Odusanya, J. Potempa, J. Mikolajczyk-Pawlinska, and J. Travis. 1998. A peptide domain on gingipain R which confers immunity against *Porphyromonas gingivalis* infection in mice. *Infect. Immun.* **66**:4108-14.

Genco, C. A., J. Potempa, J. Mikolajczyk-Pawlinska, and J. Travis. 1999. Role of gingipains R in the pathogenesis of *Porphyromonas gingivalis*-mediated periodontal disease. *Clin. Infect. Dis.* **28**:456-65.

Genetics Computer Group, I. 1995. Program Manual for the Wisconsin Package, Version 8.1, 575 Science Drive, Madison, Wisconsin, USA 53711.

Genetics Computer Group, I. 1996. Program Manual for the EGCG Package, Version 8.1.0. Coordinating ed., P. Rice. The Sanger Centre, Hinxton Hall, Cambridge, CB10 1RQ, England.

Gibbons, R. J., and J. B. Macdonald. 1960. Haemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. *J. Bacteriol.* **80**:164-170.

Gimbrone, M. A. J., R. S. Cotran, and J. Folkman. 1974. Human vascular endothelial cells in culture. *J. Cell Biol.* **60**:673-684.

Grant, D. S., P. I. Lelkes, K. Fukuda, and H. K. Kleinman. 1991. Intracellular mechanisms involved in basement membrane induced blood vessel differentiation *in-vitro*. *In Vitro Cell Dev. Biol.* **27A**:327-36.

Greenburg, G., and E. Hay. 1982. Epithelia suspended in collagen gels can lose polarity and express characteristics of migrating mesenchymal cells. *J. Cell Biol.* **95**:333-339.

Grenier, D., and D. Mayrand. 1987. Functional characterisation of extracellular vesicles produced by *Bacteroides gingivalis*. *Infect. Immun.* **55**:111-117.

Grenier, D. 1991. Haemin-binding property of *Porphyromonas gingivalis* outer membranes. *FEMS Microbiol. Lett.* **77**:45-50.

- Griffen, A. L., M. R. Becker, S. R. Lyons, M. L. Moeschberger, and E. J. Leys.** 1998. Prevalence of *Porphyromonas gingivalis* and periodontal health status. *J. Clin. Microbiol.* **36**:3239-42.
- Griffen, A. L., S. R. Lyons, M. R. Becker, M. L. Moeschberger, and E. J. Leys.** 1999. *Porphyromonas gingivalis* strain variability and periodontitis. *J. Clin. Microbiol.* **37**:4028-33.
- Grimm, B., A. Bull, and V. Breu.** 1991. Structural genes of glutamate 1-semialdehyde aminotransferase for porphyrin synthesis in a cyanobacterium and *Escherichia coli*. *Mol. Gen. Genet.* **225**:1-10.
- Haapasalmi, K., M. Mäkelä, O. Oksala, J. Heino, K. M. Yamada, V.-J. Uitto, and H. Larjava.** 1995. Expression of epithelial adhesion proteins and integrins in chronic inflammation. *Am. J. Pathol.* **147**:193-206.
- Hacker, J., L. Bender, M. Ott, J. Wingender, B. Lund, R. Marre, and W. Goebel.** 1990. Deletions of chromosomal regions encoding for fimbriae and haemolysins occur *in vivo* and *in vitro* in various extraintestinal *Escherichia coli* isolates. *Microb. Pathog.* **8**:213-225.
- Hacker, J., G. Blum-Ochler, I. Mühldorfer, and H. Tschäpe.** 1997. Pathogenicity islands of virulent bacteria : structure, function and impact on microbial evolution. *Mol. Microbiol.* **23**:1089-1097.
- Haffajee, A. D., and S. S. Socransky.** 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontol.* **2000**. **5**:78-111.
- Han, N., J. Whitlock, and A. Progulske-Fox.** 1996. The haemagglutinin gene (hagA) of *Porphyromonas gingivalis* 381 contains four large, contiguous, direct repeats. *Infect. Immun.* **64**:4000-4007.
- Hanley, S. A., J. Aduse-Opoku, and M. A. Curtis.** 1999. A 55-kilodalton immunodominant antigen of *Porphyromonas gingivalis* W50 has arisen via horizontal gene transfer. *Infect. Immun.* **67**:1157-71.
- Haralabopoulos, G. C., D. S. Grant, K. H.K., P. I. Lelkes, S. P. Papaioannou, and M. E. Maragoudakis.** 1994. Inhibitors of basement membrane collagen synthesis prevent endothelial cell alignment in matrigel *in vitro* and angiogenesis *in vivo*. *Lab. Invest.* **71**:575-582.
- Haraszthy, V. I., J. J. Zambon, M. Trevisan, M. Zeid, and R. J. Genco.** 2000. Identification of periodontal pathogens in atheromatous plaques. *J. Periodontol.* **71**:1554-1560.

Hochuli, E., H. Dobeli, and A. Schacher. 1987. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. Chromatog.* **411**:177-184.

Holt, S. C., J. Ebersole, J. Felton, M. Brunsvold, and K. S. Kornman. 1988. Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. *Science*. **239**:55-57.

Hoover, C. I., and F. Yoshimura. 1994. Transposon-induced pigment-deficient mutants of *Porphyromonas gingivalis*. *FEMS Microbiol. Letts.* **124**:43-8.

Imamura, T., R. N. Pike, J. Potempa, and J. Travis. 1994. Pathogenesis of periodontitis: a major arginine-specific cysteine proteinase from *Porphyromonas gingivalis* induces vascular permeability enhancement through activation of the kallikrein/kinin pathway. *J. Clin. Invest.* **94**:361-7.

Imamura, T., J. Potempa, R. E. Pike, and J. Travis. 1995a. Dependence of vascular permeability enhancement on cysteine proteinases in vesicles of *Porphyromonas gingivalis*. *Infect. Immun.* **63**:1999-2003.

Imamura, T., J. Potempa, R. N. Pike, J. N. Moore, M. H. Barton, and J. Travis. 1995b. Effect of free and vesicle-bound cysteine proteinases of *Porphyromonas gingivalis* on plasma clot formation: implications for bleeding tendency at periodontitis sites. *Infect. Immun.* **63**:4877-4882.

Imamura, T., J. Potempa, S. Tanase, and J. Travis. 1997. Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from *Porphyromonas gingivalis*. *J. Biol. Chem.* **272**:16062-7.

Imamura, T., J. Potempa, and J. Travis. 2000. Comparison of pathogenic properties between two types of arginine-specific cysteine proteinases (gingipains-R) from *Porphyromonas gingivalis*. *Microb. Pathog.* **29**:155-163.

Ingber, D., and J. Folkman. 1988. Inhibition of angiogenesis through modulation of collagen metabolism. *Lab. Invest.* **59**:44-51.

Ingber, D. E., and J. Folkman. 1989. Mechano-chemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis in vitro: Role of extracellular matrix. *J. Cell Biol.* **109**:317-330.

Inoshita, E., K. Iwakura, A. Amano, H. Tamagawa, and S. Shizukuishi. 1991. Effect of transferrin on the growth of *Porphyromonas gingivalis*. *J. Dent. Res.* **70**:1258-1261.

Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* **52**:2745-2756.

Jackson, C. J., I. Giles, A. Knop, A. Nethery, and L. Schreiber. 1994. Sulphated polysaccharides are required for collagen-induced vascular tube formation. *Exp. Cell Res.* **215**:294-302.

Johansson, K., and S. Kalfas. 1998. Characterisation of the proteinase-dependent cytotoxicity of *Porphyromonas gingivalis*. *Eur. J. Oral Sci.* **106**:863-71.

Jordan, H. V., and P. H. Keyes. 1964. Aerobic, Gram-positive, filamentous bacteria as etiologic agents of experimental periodontal disease in hamsters. *Arch. Oral Biol.* **9**:401-414.

Jose, J., F. Jahnig, and T. F. Meyer. 1995. Common structural features of IgA1 protease-like outer membrane protein autotransporters. *Mol. Microbiol.* **18**:377-82.

Kadowaki, T., M. Yoneda, K. Okamoto, K. Maeda, and K. Yamamoto. 1994. Purification and characterisation of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. *J. Biol. Chem.* **269**:21371-21378.

Kagan, J. M. 1980. Local immunity to *Bacteroides gingivalis* in periodontal disease. *J. Dent. Res.* **59**:1750-1756.

Kane, J. F. 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* **6**:494-500.

Karunakaran, T., and S. C. Holt. 1993. Cloning of two distinct haemolysin genes from *Porphyromonas (Bacteroides) gingivalis* in *Escherichia coli*. *Microb. Pathog.* **15**:37-49.

Katz, J., D. C. Ward, and S. M. Michalek. 1996. Effect of host response on the pathogenicity of strains of *Porphyromonas gingivalis*. *Oral Microbiol. Immunol.* **5**:309-18.

Katz, J., K. P. Black, and S. M. Michalek. 1999. Host responses to recombinant haemagglutinin B of *Porphyromonas gingivalis* in an experimental rat model. *Infect. Immun.* **67**:4352-4359.

Keil, B. 1971. Trypsin, p. 249-275. In P. D. Boyer (ed.), The enzymes, vol. 3. Academic Press, New York.

Keyes, P. H., and H. V. Jordan. 1964. Periodontal lesions in the Syrian hamster.III. Findings related to an infectious and transmissible component. *Arch Oral Biol.* **9**:377-400.

Kim, S.-J., L. Chu, and S. C. Holt. 1996. Isolation and characterisation of a haemin-binding cell envelope protein from *Porphyromonas gingivalis*. *Microb. Pathog.* **21**:65-70.

Kimura, S., A. Nagai, T. Onitsuka, T. Koga, T. Fujiwara, H. Kaya, and S. Hamada. 2000. Induction of experimental periodontitis in mice with *Porphyromonas gingivalis*-adhered ligatures. *J. Periodontol.* **71**:1167-73.

Kinsella, J. L., D. S. Grant, B. S. Weeks, and H. K. Kleinman. 1992. Protein kinase c regulates endothelial cell tube formation on basement membrane matrix, Matrigel. *Exp. Cell Res.* **199**:56-62.

Klagsbrun, M., D. Knighton, and J. Folkman. 1976. Tumour angiogenesis activity in cells grown in tissue culture. *Cancer Res.* **36**:110-114.

Klauser, T. M., J. Pohlner, and T. F. Meyer. 1993. The secretion pathway of IgA protease-type proteins in gram-negative bacteria. *Bioassays.* **15**:799-805.

Kleinman, H. K., M. L. Garvey, L. A. Liotta, and P. G. Robbey et al. 1982. Isolation and characterisation of type IV procollagen, laminin and heparan sulfate proteoglycan from the EHS sarcoma. *Biochem.* **21**:6188.

Kleinman, H. K., M. L. McGarvey, J. R. Hassell, and V. L. Star et al. 1986. Basement membrane complexes with biological activities. *Biochem.* **25**:312.

Kontani, M., H. Ono, H. Shibata, Y. Okamura, T. Tanaka, T. Fujiwara, S. Kimura, and S. Hamada. 1996. Cysteine protease of *Porphyromonas gingivalis* 381 enhances binding of fimbriae to cultured human fibroblasts and matrix proteins. *Infect. Immun.* **64**:756-762.

Kubinowa, M., A. Amano, and S. Shizukuishi. 1998. Haemoglobin-binding protein from *Porphyromonas gingivalis* is identical to lysine-specific cysteine proteinase (lys-gingipain). *Biochem.Biophys.Res.Com.* **249**:38-43.

Kubota, Y., H. K. Kleinman, G. R. Martin, and T. J. Lawley. 1988. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* **107**:1589-1598.

Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin et al. 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature.* **390**:249-56.

Kurihara, H., F. Nishimura, T. Nakamura, M. Nakagawa, I. Tanimoto, Y. Nomura, S. Koikeguchi, K. Kato, and Y. Murayama. 1991. Humoral immune response to an antigen from *Porphyromonas gingivalis* 381 in periodontal disease. *Infect. Immun.* **59**:2758-62.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature.* **227**:680-685.

Lagergard, T., M. Purven, and A. Frisk. 1993. Evidence of *Haemophilus ducreyi* adherence to and cytotoxin destruction of human epithelial cells. *Microb. Pathog.* **14**:417-31.

Lamont, R. J., and H. F. Jenkinson. 1998. Life below the gum line : pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev.* **62**:1244-1263.

Laughon, B. E., S. A. Syed, and W. J. Loesche. 1982. API ZYM system for identification of *Bacteroides* spp., *Capnocytophaga* spp., and *Spirochetes* of oral origin. *J. Clin. Microbiol.* **15**:97-102.

Lawson, D. A., and T. F. Meyer. 1992. Biochemical characterisation of *Porphyromonas (Bacteroides) gingivalis* collagenase. *Infect. Immun.* **60**:1524-1529.

Lee, B. C. 1992b. Isolation of an outer membrane haemin-binding protein of *Haemophilus influenzae* type b. *Infect. Immun.* **60**:810-6.

Lee, B. C. 1992a. Isolation of haemin-binding proteins of *Neisseria gonorrhoeae*. *J. Med. Microbiol.* **36**:121-7.

Lee, P. T., A. Y. Hsu, H. T. Ha, and C. F. Clarke. 1997. A *c*-methyltransferase involved in both ubiquinone and menaquinone biosynthesis : isolation and identification of the *Escherichia coli* ubiE gene. *J. Bacteriol.* **179**:1748-1754.

Lembariti, B. S., M. A. van't Hof, T. Pilot, and W. H. van Palenstein-Helderman. 1997. Clinical parameters associated with periodontitis in untreated persons. *East Afr. Med. J.* **74**:427-30.

Létoffé, S., J. M. Ghigo, and C. Wandersan. 1994. Iron acquisition from haem and haemoglobin by *Serratia marcescens* extracellular protein. *Proc. Natl. Acad. Sci. USA.* **91**:9876-9880.

Létoffé, S., V. Redeker, and C. Wandersman. 1998. Isolation and characterisation of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. *Mol. Microbiol.* **28**:1223-1234.

- Lewis, J. P., and F. L. Macrina.** 1998. IS195, an insertion sequence-like element associated with protease genes in *Porphyromonas gingivalis*. *Infect. Immun.* **66**:3035-42.
- Lewis, J. P., J. A. Dawson, J. C. Hannis, D. Muddiman, and F. L. Macrina.** 1999a. Haemoglobinase activity of the lysine gingipain protease (*Kgp*) of *Porphyromonas gingivalis* W83. *J. Bacteriol.* **181**:4905-13.
- Lewis, J. P., and F. L. Macrina.** 1999b. Localisation of HArep-containing genes on the chromosome of *Porphyromonas gingivalis* W83. *Infect. Immun.* **67**:2619-23.
- Leys, E. J., J. H. Smith, S. R. Lyons, and A. L. Griffen.** 1999. Identification of *Porphyromonas gingivalis* strains by heteroduplex analysis and detection of multiple strains. *J. Clin. Microbiol.* **37**:3906-11.
- Lindhe, J.** 1989. Textbook of Clinical Periodontology, 2nd Edition ed. Munksgaard, Copenhagen.
- Lindhe, J., S.-E. Hamp, and H. Löe.** 1975. Plaque induced periodontal disease in beagle dogs. A 4-year clinical, roentgenographical and histometrical study. *J. Periodontol.* **10**:243-55.
- Löe, H.** 1965. Physiology of the gingival pocket. *Acad. Rev. Californian Acad. Periodontol.* **13**:6-14.
- Löe, H., E. Theilade, and S. B. Jensen.** 1965. Experimental gingivitis in man. *J. Periodontol.* **36**:177-187.
- Löe, H., A. Anerud, H. Boysen, and E. Morrison.** 1986. Natural history of periodontal disease in man. Rapid, moderate and no loss of attachment in Sri Lankan laborers 14 to 46 years of age. *J. Clin. Periodontol.* **13**:431-440.
- Loesche, W. J.** 1976. Chemotherapy of dental plaque infection. *Oral Sci. Rev.* **9**:65-107.
- Loesche, W. J., S. A. Syed, E. C. Morrison, B. Laughon, and N. S. Grossman.** 1981. Treatment of periodontal infections due to anaerobic infections due to anaerobic bacteria with short-term treatment with metranidazole. *J. Clin. Periodontol.* **8**:29-44.
- Loos, B. G., D. Mayrand, R. J. Genco, and D. P. Dickinson.** 1990. Genetic heterogeneity of *Porphyromonas (Bacteroides) gingivalis* by genomic DNA fingerprinting. *J. Dent. Res.* **69**:1488-93.

- Loos, B. G., D. W. Dyer, T. S. Whittam, and R. K. Selander.** 1993. Genetic structure of populations of *Porphyromonas gingivalis* associated with periodontitis and other oral infections. *Infect. Immun.* **61**:204-212.
- Lopez, N. J.** 2000. Occurrence of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Prevotella intermedia* in progressive adult periodontitis. *J. Periodontol.* **71**:948-54.
- Lyons, S. R., A. L. Griffen, and E. J. Leys.** 2000. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *J. Clin. Microbiol.* **38**:2362-5.
- MacDonald, J. B., R. M. Sutton, M. L. Knoll, E. M. Madlener, and R. M. Grainger.** 1956. The pathogenic components of an experimental fusospirochetal infection. *J. Infect. Dis.* **98**:15-20.
- Maciag, T., J. Kadish, L. Wilkins, M. B. Stemerman, and R. Weinstein.** 1982. Organisational behaviour of human umbilical vein endothelial cells. *J. Cell Biol.* **94**:511-520.
- Mahanonda, R., G. J. Seymour, L. W. Powell, M. F. Good, and J. W. Halliday.** 1991. Effect of initial treatment of chronic inflammatory periodontal disease on the frequency of peripheral blood T-lymphocytes specific to periodontopathic bacteria. *Oral Microbiol Immunol.* **6**:221-227.
- Marsh, P. D., A. S. McDermid, A. S. McKee, and A. Baskerville.** 1994. The effect of growth rate and haemin on the virulence and proteolytic activity of *Porphyromonas gingivalis* W50. *Microbiol.* **140**:861-5.
- Matsson, M., D. Tolstoy, R. Aasa, and L. Hederstedt.** 2000. The distal haem centre in *Bacillus subtilis succinate* : quinone reductase is crucial for electron transfer to menaquinone. *Biochem.* **39**:8617-24.
- McBride, B. C., U. Singh, and A. Joe.** 1993. *Porphyromonas gingivalis*: Gene cloning of determinants of pathogenicity, p. 552-568. In M. Sebald (ed.), *Genetics and Molecular Biology of Anaerobic Bacteria*. Springer-Verlag.
- McClung, C., J. Somerville, M. Guerinot, and B. Chelm.** 1987. Structure of the *Bradyrhizobium japonicum* gene HemaA encoding 5-aminolevulinic acid synthase. *Gene.* **54** (1):133-9.
- McKee, A. S., A. S. McDermid, A. Baskerville, A. B. Dowsett, D. C. Ellwood, and P. D. Marsh.** 1986. Effect of haemin on the physiology and virulence of *Bacteroides gingivalis*. *Infect. Immun.* **52**:349-355.

- McKee, A. S., A. S. McDermid, R. Wait, A. Baskerville, and P. D. Marsh.** 1988. Isolation of colonial variants of *Bacteroides gingivalis* W50 with a reduced virulence. *J. Med. Microbiol.* **27**:59-64.
- Menard, C., and C. Mouton.** 1995. Clonal diversity of the taxon *Porphyromonas gingivalis* assessed by random amplified polymorphic DNA finger-printing. *Infect. Immun.* **63**:2522-2531.
- Mikolajczyk-Pawlinska, J., T. Kordula, N. Pavloff, P. A. Pemberton, W. C. Chen, J. Travis, and J. Potempa.** 1998. Genetic variation of *Porphyromonas gingivalis* genes encoding gingipains, cysteine proteinases with arginine or lysine specificity. *Biol. Chem.* **379**:205-11.
- Minhas, T., J. Greenman, and A. G. Schaffer.** 1993. The influence of haemin levels on growth and enzyme production by *Porphyromonas gingivalis* in continuous culture. *Microbios.* **76**:105-114.
- Montesano, R., L. Orci, and P. Vassalli.** 1983. *In-vitro* rapid organisation of endothelial cells into capillary-like networks is promoted by collagen matrices. *J. Cell. Biol.* **97**:1648-1652.
- Montesano, R., and L. Orci.** 1985a. Tumour-promoting phorbol esters induce angiogenesis *in-vitro*. *Cell.* **442**:469-477.
- Montesano, R., P. Mouron, and L. Orci.** 1985b. Vascular outgrowths from tissue explants embedded in fibrin or collagen gels: A simple *in vitro* model of angiogenesis. *Cell Biol. Int. Rep.* **9**:869-875.
- Montesano, R., J.-D. Vassalli, A. Baird, R. Guillemain, and L. Orci.** 1986. Basic fibroblast growth factor induces angiogenesis in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **83**:7297-7301.
- Mooney, J., E. Adonogianaki, M. P. Riggio, K. Takahashi, A. Haerian, and D. F. Kinane.** 1995. Initial serum antibody titre to *Porphyromonas gingivalis* influences development of antibody avidity and success of therapy for chronic periodontitis. *Infect. Immun.* **63**:3411-6.
- Moritz, A. J., D. Cappelli, M. S. Lantz, S. C. Holt, and J. L. Ebersole.** 1998. Immunisation with *Porphyromonas gingivalis* cysteine protease : effects on experimental gingivitis and ligature-induced periodontitis in *Macaca fascicularis*. *J. Periodontol.* **69**:686-97.
- Mouton, C., P. G. Hammond, J. Slots, and R. J. Genco.** 1981. Serum antibodies to oral *Bacteroides asaccharolyticus* (*Bacteroides gingivalis*) : relationship to age and periodontal disease. *Infect. Immun.* **31**:182-192.

- Mukherjee, S.** 1985. The role of crevicular iron in periodontal disease. *J. Periodontol.* **56**:22-27.
- Muller, M., D. G. Lindmark, and J. McLaughlin.** 1977. Mode of action of metronidazole on anaerobic microorganisms. In *Metronidazole*(S. Finegold, ed.) Proceedings of the International Metronidazole Conference, Montreal, Quebec, Canada, May 26-28, 1976. Excerpta Medica 12-19.
- Nakayama, K., D. B. Ratnayake, T. Tsukubu, T. Kadowaki, K. Yamamoto, and S. Fujimura.** 1998. Haemoglobin receptor protein is intragenically encoded by the cysteine proteinase-encoding genes and the haemagglutinin-encoding gene of *Porphyromonas gingivalis*. *Mol. Microbiol.* **27**:51-61.
- Nehls, V., E. Schuchardt, and Drenckhahn.** 1994. The effect of fibroblasts, vascular smooth muscle cells and pericytes on sprout formation of endothelial cells in a fibrin gel angiogenesis system. *Microvasc. Res.* **48**:349-63.
- Neiders, M. E., P. B. Chen, H. S. Reynolds, J. J. Zambon, and R. J. Genco.** 1989. Heterogeneity of virulence among strains of *Bacteroides gingivalis*. *J. Periodontal. Res.* **24**:192-198.
- Neilands, J. B.** 1981. Microbial iron compounds. *Annu. Rev. Biochem.* **50**:715-731.
- Neilands, J. B.** 1982. Microbial envelope proteins related to iron. *Ann. Rev. Microbiol.* **36**:285-309.
- Nicosia, R. F., R. Tchao, and J. Leighton.** 1983. Angiogenesis-dependent tumor spread in reinforced fibrin clot culture. *Cancer Res.* **43**:2159-2166.
- Norris, J. M., and D. N. Love.** 1999. Associations amongst three feline *Porphyromonas gingivalis* species from the gingival margin of cats during periodontal health and disease. *Vet Microbiol.* **65**:195-207.
- O'Brien-Simpson, N. M., R. A. Paolini, and E. C. Reynolds.** 2000. RgpA-Kgp peptide based immunogens provide protection against *Porphyromonas gingivalis* challenge in a murine lesion model. *Infect. Immun.* **68**:4055-63.
- Offenbacher, S., V. Katz, G. Fertik, J. Collins, D. Boyd, G. Maynor, R. McKaig, and J. Beck.** 1996. Periodontal infection as a possible risk factor for pre-term low birth weight. *J. Periodontol.* **67**:1103-1113.
- Page, R. C., and H. E. Schroeder.** 1976. Pathogenesis of inflammatory periodontal disease- a summary of current work. *Lab. Invest.* **33**:235-249.
- Page, R. C., and H. E. Schroeder.** 1982. Periodontitis in Man and Other Animals. S.Karger Publishers, Basel, Switzerland.

Page, R. C., and J. D. Beck. 1997a. Risk assessment for periodontal diseases. *Int. Dent. J.* **47**:61-67.

Page, R. C., S. Offenbacher, H. E. Schroeder, G. J. Seymour, and K. S. Kornman. 1997b. Advances in the pathogenesis of periodontitis. Summary of developments, clinical implications and future directions. *Periodontol.* **2000**. **14**:216-246.

Page, R. C. 1999. Milestones in periodontal research and the remaining critical issues. *J. Periodontol. Res.* **34**:331-9.

Papapanou, P. N., V. Baelum, W. M. Luan, P. N. Madianos, X. Chen, O. Fejerskov, and G. Dahlen. 1997. Subgingival microbiota in adult Chinese : prevalence and relation to periodontal disease progression. *J. Periodontol.* **68**:651-66.

Pavloff, N., J. Potempa, R. N. Pike, V. Prochazka, M. C. Kiefer, J. Travis, and P. J. Barr. 1995. Molecular cloning and structural characterisation of the arg-gingipain proteinase of *Porphyromonas gingivalis*. *J. Biol. Chem.* **270**:1007-1010.

Pietrzak, E. R., B. Polak, L. J. Walsh, N. W. Savage, and G. J. Seymour. 1998. Characterisation of serum antibodies to individuals with and without periodontitis. *Oral Microbiol. Immunol.* **13**:65-72.

Pike, R., W. McGraw, J. Potempa, and J. Travis. 1994. Lysine- and Arginine-specific proteinases from *Porphyromonas gingivalis*. *J. Biol. Chem.* **269**:406-411.

Pike, R. N., J. Potempa, W. McGraw, T. H. T. Coetzer, and J. Travis. 1996. Characterisation of the binding activities of proteinase-adhesin complexes from *Porphyromonas gingivalis*. *J. Bacteriol.* **178**:2876-2882.

Pinchback, J. S., B. A. Taylor, J. R. Gibbins, and N. Hunter. 1996. Microvascular angiopathy in advanced periodontal disease. *J. Pathol.* **179**:204-209.

Polak, B., M. A. Peck, J. K. Dyer, P. S. Bird, R. A. Reinhardt, and G. J. Seymour. 1995. Purification and characterisation of *Porphyromonas gingivalis* outer membrane antigens. *Arch. Oral Biol.* **40**:905-912.

Polverini, P. J. 1995. The pathophysiology of angiogenesis. *Crit. Rev. Oral Biol. Med.* **6**:230-247.

Potempa, J., R. Pike, and J. Travis. 1995a. The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of Arg-gingipain or Lys-gingipain. *Infect. Immun.* **63**:1176-1182.

Potempa, J., N. Pavloff, and J. Travis. 1995b. *Porphyromonas gingivalis*: a proteinase / gene accounting audit. *Trends Microbiol.* **3**:430-434.

Potempa, J., A. Banbula, and J. Travis. 2000. Role of bacterial proteinases in matrix destruction and modulation of host response. *Periodontol.* 2000. **24**:153-192.

Quinn, F. D., R. S. Weyant, F. J. Candal, and E. W. Ades. 1994. Destruction of human microvascular endothelial cell capillary-like microtubules by Brazilian purpuric fever-associated *Haemophilus influenzae* biogroup aegyptius. *Pathobiol.* **62**:109-112.

Renvert, S., G. Dahlen, and M. Wikstrom. 1996. Treatment of periodontal disease based on microbiological diagnosis. Relation between microbiological and clinical parameters during 5 years. *J. Periodontol.* **67**:562-71.

Rizza, V., P. R. Sinclair, D. C. White, and P. R. Cuorant. 1968. Electron transport system of the protoheme-requiring anaerobe *Bacteroides melaninogenicus*. *J. Bacteriol.* **96**:665-671.

Roper, J. M., E. Raux, A. A. Brindley, H. L. Schubert, S. E. Gharbia, H. N. Shah, and M. J. Warren. 2000. The enigma of cobalamin (vitamin B12) biosynthesis in *Porphyromonas gingivalis*. *J. Bacteriol.* **275**:40316-40323.

Rumpf, R. W., A. L. Griffen, and E. J. Leys. 2000. Phylogeny of *Porphyromonas gingivalis* by ribosomal intergenic spacer region analysis. *J. Clin. Microbiol.* **38**:1807-10.

Saglie, F. R. 1988. Scanning electron microscope and intragingival microorganisms in periodontal disease. *Scanning Microsc.* **2**:1535-1540.

Saito, S., M. Hayakawa, H. Takiguchi, and Y. Abiko. 1999. Opsonophagocytic effect of antibody against recombinant conserved 40-kDa outer membrane protein of *Porphyromonas gingivalis*. *J. Periodontol.* **70**:610-7.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning : a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sanz, M., A. J. van Winkelhoff, D. Herrera, N. Dellemijn-Kippuw, R. Simon, and E. Winkel. 2000. Differences in the composition of the subgingival microbiota of two periodontitis populations of different geographical origin. A comparison between Spain and the Netherlands. *Eur. J. Oral Science.* **108**:383-92.

Saparamadu, K. D. 1996. Preventive dentistry-a priority in Sri Lanka. *Int. Dent. J.* **46**:97-102.

Sastry, S. K., and A. F. Horwitz. 1993. Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signalling. *Curr. Opin. Cell Biol.* **5**:819-831.

- Schenkein, H. A., H. M. Fletcher, M. Bodnar, and F. L. Macrina.** 1995. Increased opsonisation of a prtH-defective mutant of *Porphyromonas gingivalis* W83 is caused by reduced degradation of complement-derived opsonins. *J. Immunol.* **154**:5331-7.
- Schifferle, R. E., S. A. Shostad, M. T. Bayers-Thrsring, D. W. Dyer, and M. E. Neiders.** 1996. Effect of protoporphyrin IX limitation on *Porphyromonas gingivalis*. *J. Endo.* **22**:352-355.
- Schwartz, E., G. Scherrer, G. Hobom, and H. Kössel.** 1978. Nucleotide sequence of cro, cII and part of the O gene in phage lambda DNA. *Nature.* **272**:410-414.
- Scott, C. F., E. J. Whitaker, B. F. Hammond, and R. W. Colman.** 1993. Purification and characterisation of a potent 70-kDa thiol lysyl-proteinase (Lys-gingivain) from *Porphyromonas gingivalis* that cleaves kininogens and fibrinogens. *J. Biol. Chem.* **268**:7935-7942.
- Scragg, M. A., S. J. Cannon, M. Rangarajan, D. M. Williams, and M. A. Curtis.** 1999. Targeted disruption of fibronectin-integrin interactions in human gingival fibroblasts by the RI Protease of *Porphyromonas gingivalis* W50. *Infect. Immun.* **67**:1837-1843.
- Seddon, S. V., H. N. Shah, J. M. Hardie, and J. P. Robinson.** 1988. Chemically defined and minimal media for *Bacteroides gingivalis*. *Curr. Microbiol.* **17**:147-149.
- Shah, H. N., R. Bonnet, B. Mateen, and R. A. D. Williams.** 1979. The porphyrin pigmentation of subspecies of *Bacteroides melaninogenicus*. *Biochem. J.* **180**:45-50.
- Shah, H. N., and M. D. Collins.** 1988. Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. *Int. J. Syst. Bacteriol.* **38**:128-131.
- Shah, H. N., and S. E. Gharbia.** 1989. Ecological events in subgingival dental plaque with reference to *Bacteroides* and *Fusobacterium* specie. *Infection.* **17**:264-268.
- Shah, H. N., and S. E. Gharbia.** 1995. The biochemical milieu of the host in the selection of anaerobic species in the oral cavity. *Clin. Infect. Dis.* **20**(Suppl 2):S201-300.
- Shah, H. N., and S. E. Gharbia.** 1989a. Lysis of erythrocytes by the secreted cysteine proteinase of *Porphyromonas gingivalis* W83. *FEMS Microbiol. Lett.* **61**:213-218.
- Shah, H. N., S. V. Seddon, and S. E. Gharbia.** 1989b. Studies on the virulence properties and metabolism of pleiotropic mutants of *Porphyromonas gingivalis* (*Bacteroides gingivalis*) W50. *Oral Microbial. Immunol.* **4**:19-23.

Shah, H. N., S. E. Gharbia, and C. M. O'Toole. 1992. Assessment of the relative cytotoxicity of *Porphyromonas gingivalis* cells, products, and components on human epithelial cell lines. *J. Periodontol.* **63**:44-51.

Sharp, L., S. Poole, K. Reddi, J. Fletcher, S. Nair, M. Wilson, M. Curtis, B. Henderson, and P. Tabona. 1998. A lipid A-associated protein of *Porphyromonas gingivalis*, derived from the haemagglutinating domain of the RI protease gene family, is a potent stimulator of interleukin 6 synthesis. *Microbiol.* **144**:3019-3026.

Shi, Y., D. B. Ratnayake, K. Okamoto, K. Yamamoto, and K. Nakayama. 1999. Genetic analyses of proteolysis, haemoglobin binding, and haemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgp A*, *rgp B*, *kgp*, and *hagA*. *J. Biol. Chem.* **274**:17955-60.

Shi, Y., W. Kong, and K. Nakayama. 2000. Human lactoferrin binds and removes the haemoglobin receptor protein of the periodontopathogen *Porphyromonas gingivalis*. *J. Biol. Chem.* **275**:30002-8.

Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with Gene Fusions, Cold Spring Harbour Laboratory, Cold Spring Harbor N.Y.

Simpson, L. M., and J. D. Oliver. 1993. Regulation of proteolytic activity of *Vibrio vulnificus* by iron-containing compounds. *Microb. Pathog.* **14**:249-52.

Slakeski, N., P. S. Bhogal, N. M. O'Brien-Simpson, and E. C. Reynolds. 1998. Characterisation of a second-cell associated Arg-specific cysteine proteinase of *Porphyromonas gingivalis* and identification of an adhesin-binding motif involved in association of the prtR and prtK proteinases and adhesins into large complexes. *Microbiology.* **144**:1583-1592.

Slots, J. 1981. Enzymatic characterisation of some oral and non-oral Gram-negative bacteria with the API ZYM system. *J. Clin. Microbiol.* **14**:288-294.

Smalley, J. W., A. J. Birss, H. M. Kay, A. S. McKee, and P. D. Marsh. 1989. The distribution of trypsin-like enzyme activity in cultures of a virulent and an avirulent strain of *Bacteroides gingivalis* W50. *Oral Microbiol. Immunol.* **4**:178-81.

Smalley, J. W., A. J. Birss, A. S. McKee, and P. D. Marsh. 1991. Haemin-restriction influences haemin-binding, haemagglutination and protease activity of cells and extracellular membrane vesicles of *Porphyromonas gingivalis*. *FEMS Microbiol Lett.* **90**:63-68.

Smalley, J. W., A. J. Birss, A. S. McKee, and P. D. Marsh. 1996. Haemin binding as a factor in the virulence of *Porphyromonas gingivalis*. *FEMS Microbiol. Lett.* **141**:65-70.

Smalley, J. W., A. J. Birss, A. S. McKee, and P. D. Marsh. 1998a. Haemin regulation of haemoglobin binding by *Porphyromonas gingivalis*. *Curr. Microbiol.* **36**:102-106.

Smalley, J. W., J. Silver, P. J. Marsh, and A. J. Birss. 1998b. The periodontopathogen *Porphyromonas gingivalis* binds iron protoporphyrin IX in the μ -oxo dimeric form : an oxidative buffer and possible pathogenic mechanism. *Biochem. J.* **331**:681-685.

Socransky, S. S. 1977. Microbiology and periodontal disease: present status and future considerations. *J. Periodontol.* **48**:497.

Spinner, F., M. R. Cheesman, A. J. Thomson, T. Kaysser, R. B. Gennis, Q. Peng, and J. Peterson. 1995. The *haem* b558 component of the cytochrome bd quinol oxidase complex from *Escherichia coli* has histidine-methionine axial ligation. *Biochem. J.* **308**:614-4.

Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans : *Chlamydia trachomatis*. *Science*. **282**:754-9.

Stoebner, J. A., and S. M. Payne. 1988. Iron-regulated haemolysin production and utilisation of haem and haemoglobin by *Vibrio cholerae*. *Infect. Immun.* **56**:2891-5.

Stojiljkovic, I., and K. Hantke. 1992. Haemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. *EMBO Journal*. **11**:4359-67.

Stojiljkovic, I., V. Hwa, L. de Saint Martin, P. O'Gaora, X. Nassif, F. Heffron, and M. So. 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilisation and virulence. *Mol. Microbiol.* **15**:531-541.

Stubbs, S., J. A. Hobot, R. J. Waddington, G. Embery, and M. A. O. Lewis. 1999. Effect of environmental haemin upon the physiology and biochemistry of *Prevotella intermedia* R78. *Lett. Appl. Microbiol.* **29**:31-36.

Sutcliffe, J. G. 1979. Complete nucleotide sequence of the *E. coli* plasmid pBR322. Cold Spring Harb Symp Quant. Biol. **43**:77-90.

Takahashi, J., A. Saito, T. Nakagawa, S. Yamada, K. Ishihara, and K. Okuda. 1998. Dynamics of serum immunoglobulin G avidity for in adult periodontitis. *J. Periodontol.* **69**:367-73.

- Takahisa, I., J. Potempa, S. Tanase, and J. Travis.** 1997. Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain Rs) from *Porphyromonas gingivalis*. *J. Biol. Chem.* **272**:16062-7.
- Tanner, A., M. F. Maiden, P. J. Macuch, L. L. Murray, and R. L. Kent.** 1998. Microbiota of health, gingivitis and initial periodontitis. *J. Clin. Periodontol.* **25**:85-98.
- Tatusov, R. L., R. Mushegian, P. Bork, N. P. Brown, W. S. Hayes, M. Borodovsky, K. E. Rudd, and E. V. Koonin.** 1996. Metabolism and evolution of *Haemophilus influenzae* deduced from whole-genome comparison with *Escherichia coli*. *Curr. Biol.* **6**:279-291.
- Taub, M., Y. Wang, T. M. Szcesney, and H. K. Kleinman.** 1990. Epidermal growth factor or transforming growth factor alpha is required for kidney tubulogenesis in Matrigel cultures in serum-free medium. *Proc. Natl. Acad. Sci. USA.* **87**:4002.
- Tew, J. G., D. R. Marshall, J. A. Burmeister, and R. R. Ranney.** 1985. Relationship between gingival crevicular fluid and serum antibody titres in young adults with generalised and localised periodontitis. *Infect. Immun.* **49**:487-493.
- Tompkins, G. R., D. P. Wood, and K. R. Birchmeier.** 1997. Detection and comparison of specific haemin binding by *Porphyromonas gingivalis* and *Prevotella intermedia*. *J. Bacteriol.* **179**:620-626.
- Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA.* **76**:4350-4354.
- Tse, C. S., and D. M. Williams.** 1992. Inhibition of human endothelial cell proliferation *in-vitro* in response to n-butyrate and propionate. *J. Periodontol. Res.* **27**:506-510.
- Van Wunkelhoff, A. J., N. Kippuw, and J. De Graaff.** 1987. Cross-inhibition between black-pigmented *Bacteroides* species. *J. Dent. Res.* **66**:1663-7.
- Vernon, R. B., J. C. Angello, M. L. Iruela-Arispe, T. F. Lane, and E. H. Sage.** 1992. Reorganisation of basement membrane matrices by cellular traction promotes the formation of cellular networks *in-vitro*. *Lab. Invest.* **66**:536-547.
- Vernon, R. B., and E. H. Sage.** 1995. Between molecules and morphology. *Am J. Pathol.* **147**:873-883.
- Vrako, R., and E. P. Benditt.** 1970. Capillary basal lamina thickening. Its relationship to endothelial cell death and replacement. *J. Cell Biol.* **47**:281-5.

- Weidner, M.-F., D. Grenier, and D. Mayrand. 1996. Proteolytic artifacts in SDS-PAGE analysis of selected periodontal pathogens. *Oral Microbiol. Immunol.* **11**:103-108.
- Wilson, M., D. Lopatin, G. Osborne, and J. B. Kieser. 1993. Prevalence of *Treponema denticola* and *Porphyromonas gingivalis* in plaque from periodontally-healthy and periodontally-diseased sites. *J. Med. Microbiol.* **38**:406-10.
- Woese, C. R. 1987. Bacterial Evolution. *Microbiol. Rev.* **51**:221-271.
- Wingrove, J. A., R. G. DiScipio, Z. Chen, J. Potempa, J. Travis, and T. E. Hugli. 1992. Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas (Bacteroides) gingivalis*. *J. Biol. Chem.* **267**:18902-18907.
- Wyss, C. 1992. Growth of *Porphyromonas gingivalis*, *Treponema denticola*, *T. pectinovorum*, *T. socranski*, and *T. vincentii* in a chemically defined medium. *J. Clin. Microbiol.* **30**:2225-2229.
- Xie, Z., and S. Merchant. 1998. A novel pathway for cytochromes c biogenesis in chloroplasts. *Biochim. Biophys. Acta.* **1365**:309-18.
- Ximenez-Fyvie, L. A., A. D. Haffajee, and S. S. Socransky. 2000a. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. *J. Clin. Periodontol.* **27**:648-57.
- Ximenez-Fyvie, L. A., A. D. Haffajee, and S. S. Socransky. 2000b. Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis. *J. Clin. Periodontol.* **27**:722-32.
- Yano-Higuchi, K., N. Takamatsu, T. He, M. Umeda, and I. Ishikawa. 2000. Prevalence of *Bacteroides forsythus*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in subgingival microflora of Japanese patients with adult and rapidly progressive periodontitis. *J. Clin. Periodontol.* **27**:597-602.
- Yap, A. S., W. M. Brieher, and B. M. Gumbiner. 1997. Molecular and functional analysis of cadherin-based adherens junctions. *Annu. Rev Cell Dev Biol.* **13**:119-146.
- Ye, P., C. C. Chapple, R. K. Kumar, and N. Hunter. 2000. Expression patterns of E-cadherin, involucrin, and connexin gap junction proteins in the lining epithelia of inflamed gingiva. *J. Pathol.* **192**:58-66.
- Yun, P. L. W., A. A. DeCarlo, and N. Hunter. 1999. Modulation of major histocompatibility complex protein expression by human gamma interferon mediated by cysteine proteinase-adhesin polyproteins of *Porphyromonas gingivalis*. *Infect. Immun.* **67**:2986-2995.

Zoellner, H., and N. Hunter. 1989. Perivascular hyaline deposits in inflamed gingival tissues. *J. Oral Pathol. Med.* **18**:333-338.

Zoellner, H., and N. Hunter. 1991. Vascular expansion in chronic periodontitis. *J. Oral Pathol. Med.* **20**:433-7.

APPENDIX

A MICROBIOLOGY

A1 CDC adjusted media recipe

Per litre	
Trypticase soy broth	10 g
NaCl	5 g
Trypticase peptone	10 g
Yeast	10 g
L-cysteine	0.4 g
De-ionised water	1000 ml
(Agar if plates)	(20 g)
Autoclave for 15 min at 15p.s.i.	
Supplements (sterile filter)	
Haematin	1 ml/100 ml
Menadione	0.4 ml/100 ml
Horse serum	20 ml/L

Note :

Haematin stock (1 ml/100 ml)
50 mg haemin chloride
Dissolve in 1 ml 1 N NaOH
Make up to 100 ml with de-ionised water.
Store at 4°C.

Menadione stock (1 ml/100 ml)

50 mg menadione

Dissolve in 50 ml ethanol.

Make up to 100 ml with de-ionised water.

Store at 4°C in brown glass bottle.

A2 LB (Luria-Bertani) medium

Per litre :

To 950 ml de-ionised water add :

Bacto-tryptone 10 g

Bacto-yeast extract 5 g

NaCl 10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Adjust the volume of the solution to 1 litre with deionised water. Sterilise by autoclaving for 20 min at 15 lb/sq. in. on liquid cycle.

Note : For LB agar plates add 15 g/ml Agar

B TISSUE CULTURE

B1 Medium M199

Dissolve in water to make 1 litre :

1 sachet of M199 (modified) with Earle's salts.

29 ml sodium bicarbonate (7% solⁿ)

20 ml penicillin/streptomycin (5000 µg/ml-5000 I.U./ml)

20 ml sodium pyruvate (100 mM solⁿ)

Sterile filter

Store at 2-7°C

B2 HUVEC growth media

To make 100 ml combine :

80 ml medium M199

20 ml foetal bovine serum
30 µg/100 ml of endothelial growth supplement
1.0 ml heparin sodium at 10 Units/ml
250 µg/100ml fungizone
2 mM glutamine.
Sterile filter and store at 2-7°C.

B3 Hank's buffered salts solution (HBSS)

Dissolve in water to make 1 litre :
1 sachet of Hank's salts
0.35 g NaHCO₃
Adjust to pH 7.2
Sterile filter.
Store at 2-7°C

B4 Cord buffer

Dissolve in water to make 1 litre :
8.182 g NaCl
0.298 g KCl
1.982 g glucose
0.006 g KH₂PO₄
0.115 g Na₂HPO₄
Adjust to pH 7.2
Sterile filter.
Store at 2-7°C

B5 Gelatin solution

Gelatin was dissolved (2.0 g in water to make 1 litre) and autoclaved for 15 min at 15 p.s.i. and kept at 4°C.

B6 Trypsin/EDTA solution

To make EDTA stock combine :

200 mg ethylene diaminetetraacetic Acid

in 100 ml PBS= 0.2% stock.

Stock trypsin/EDTA :

50 mg trypsin

10 ml EDTA 0.2% stock

90 ml PBS

Makes 100 ml. Sterile filter and store at 2-7°C