"STRUCTURE AND LIGAND-BINDING PROPERTIES OF ABNORMAL HUMAN ALBUMINS"

by

Paul Matejtschuk, B.Sc.(York)

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Warwick in the Department of Chemistry.

January 1986

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To Dad, Mom and Sue,

with love

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DECLARATION

The work described herein was carried out in the Department of Chemistry at the University of Warwick, between October 1982 and October 1985 and was funded by the Science and Engineering Research Council. It is the original work of the author, except where specific acknowledgement is made or implied. This thesis has not been submitted for any other degree.

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I would like to thank all those members of C421 past and present who have contributed useful ideas and a convivial atmosphere in which to work and also my wife for her patience and support during the time of study.

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ABSTRACT

Albumins Redhill, Warwick-1 and Carlisle are monomeric slow albumin variants discovered in sera obtained from patients in unrelated families resident in the U.K.

Albumin Redhill had previously been studied in this laboratory and was here purified by fast protein liquid chromatrography (FPLC) and was subsequently submitted for amino acid sequencing by the solid-phase Edman process. It was found that Albumin Redhill has an extra N-terminal arginine residue, and this places it into a new class of albumin variants.

The binding of nickel and copper was studied in greater depth than previously, and the binding of these metal ions at the primary N-terminal site confirmed these as being significantly inhibited due to the inclusion of the extra basic amino acid residue. The binding of warfarin to Albumin Redhill is reduced compared to normal albumin.

Albumin Carlisle, a heat-stable variant, was found in three members of a family of English origin from Carlisle. The binding of a range of dyes and the electrophoretic mobility on a series of media were assessed. The variant Albumin Carlisle was purified to homogeneity by FPLC chromatofocusing and was shown to be antigenically indistinguishable from albumin A, although it does have a more basic isoelectric point (5.74 compared to 5.63 for normal albumin). The evidence from both electrophoretic and chromatographic procedures are consistent with an acid → neutral amino acid mutation, and studies of the CNBr fragments of the variant suggest that the site of mutation is in the region 329-548 residues.

Reverse-phase HPLC has been used to pinpoint a difference in the profile of the tryptic digest of the variant albumin from the normal, and it may be that this technique could be utilised to obtain molecular data on the mutation.

The ligand binding properties of metal ions, bilirubin, palmitate and warfarin were assessed and it was shown that Albumin Carlisle has increased warfarin binding but decreased bilirubin affinity, although the binding of metal ions and palmitate was unaffected.

PUBLICATIONS

(1) Albumins Warwick-1 and Warwick-2, Two Human Albumin Variants
Au, H. Y. N., Brand, S., Hutchinson, D. W. and Matejtschuk, P.
I.R.C.S. Med. Sci., (1984) 12, 56-57

(2) Structures and Ligand-Binding Properties of Human Serum Albumins
Au, H. Y. N., Brand, S., Hutchinson, D. W. and Matejtschuk, P.
Biochem. Soc. Trans., (1984) 12, 659-60

(3) The N-Terminal Amino Acid Sequence of Albumin Redhill
Hutchinson, D. W. and Matejtschuk, P.
FEBS Lett., (1985) 193, 211-212

CHAPTER ONE

INTRODUCTION

1.1 HUMAN SERUM ALBUMIN

1.1.1 Occurrence

Serum albumin has been one of the most studied proteins; it is ubiquitous in mammals and birds and is found in several classes of reptiles. Albumin (derived from albus, the Latin for white, i.e., egg-white) was first used to describe the soluble fraction of slightly acidified serum, more rigorous definitions having successively been devised as the physical properties that characterise the protein have been elucidated. One of the best definitions was proposed by Peters (1970) who suggested the following five criteria:

(a) high solubility - even in half saturated 2 M ammonium sulphate, 23°C, pH 6.0;

(b) high net negative charge, -19 at neutral pH, albumin is the most anodic of the major proteins of serum;

(c) a molecular weight ≈ 66,000 daltons;

(d) lacking any carbohydrate substituents, by customary tests (recently normal albumin has been shown to be at least 10% glycosylated (Shaklai, 1984));

(e) the major protein component of serum.

The most unambiguous definition of albumin is its amino acid sequence which has subsequently been discovered.

Albumin comprises more than 50% of the total protein content of serum (usually ≈ 43 g/L); its ability to bind reversibly many different ligands makes it unique in both biological and medical uses. Albumin provides 70% of the colloidal osmotic pressure of the blood, it is important for the maintenance of circulation and its high affinity for long chain fatty acids makes it an ideal transporter of fatty acids in the blood, increasing their effective solubility by several orders of magnitude as it carries them from their site of absorption in the intestine to the liver.

Albumin also binds bilirubin very strongly and removes this potentially toxic metabolite from the blood, so protecting the newborn from kernicterus and the possibility of brain damage, due to the accumulation of bilirubin in its tissues. Albumin acts as an overflow carrier of steroid and thyroid hormones, binds divalent heavy metal ions, and is of great interest to pharmacologists due to its wide range of hydrophobic drug and dye binding, playing an important role in the pharmacodynamics of drug treatment. Albumin can act as a nutritional reserve, its catabolism providing amino acids for the peripheral tissues; albumin concentration has been used as a

marker for malnutrition and cellular damage, as it drops markedly after surgery or fasting. Albumin has been used as a diagnostic marker of vascular and renal integrity and its rate of synthesis as a measure of liver function. Albumin has been used as a standard protein in many assays and biochemical measurements and is also used by immunologists as a model antigen. It has often been used to protect valuable macromolecules from possible degradation and is added to many immobilisation reactions to block excess reactive sites.

In the light of its many uses, and its great abundance in serum, it is surprising to discover that the amino acid sequences of the serum albumins were only determined comparatively recently.

1.1.2 Primary Structure

Human serum albumin (HSA) is a protein of 65,000 molecular weight, with a total of 17 disulphide bridges and 35 cysteine residues in total. The length of the albumin peptide chain (585 amino acids in a continuous single polypeptide) has long discouraged efforts to elucidate its complete sequence. The N- and C-terminal amino acids were discovered in 1960 (Ikenaka, 1960) and some proteolytic studies were made using trypsin, chymotrypsin, cathepsin or pepsin. However, the tryptic digest alone should give rise to 82 peptides and the sequencing and alignment of such a number of fragments was not achieved until

the more modern protein sequencing techniques of the 1970's were available. Some peptides were sequenced earlier, such as that around the free SH group (Witter and Tuppy, 1960) and the N-terminal peptic fragment (Bradshaw and Peters, 1969).

In the early 1970's several groups worked independently on the sequencing of human albumin, using gram quantities of protein and different methodologies. Brown, *et al.*, published the first full sequence of human serum albumin in 1975 (Behrens, *et al.*, 1975), having purified both chymotryptic and tryptic fragments, and by comparison of the results obtained a complete sequence which agreed with existing data (see Fig. 1.1).

At the same time Meloun, *et al.* (1975) published a complete amino acid sequence; however, their approach had been different in that they had first cleaved albumin with cyanogen bromide and then proteolytically digested the resultant large fragments. The two sequences were in 93% general agreement.

In 1982 the mRNA derived cDNA clone was sequenced by Dugaiczyk, *et al.* (1982) using *in vitro* methods to express the liver mRNA (Fig. 1.2). As well as encoding the sequence of albumin itself, the 2078 nucleotide mRNA included additional bases which confirmed the presence of an amino acid propeptide and a further 18 amino acid prepropeptide whose

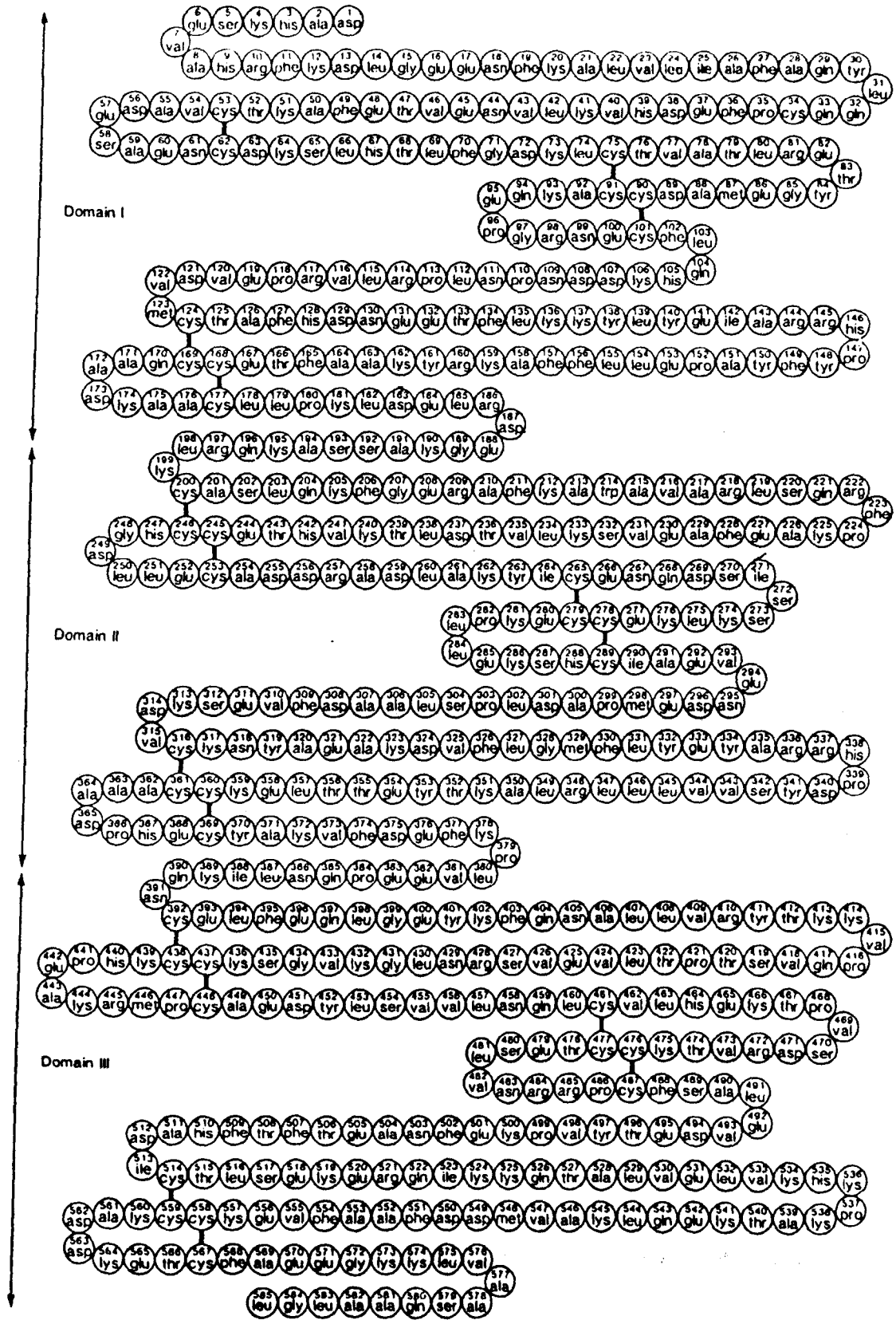


Fig. 1.1 The complete amino acid sequence of human serum albumin showing disulphide bridges and domain structure (from Dugaiczyk, *et al.*, 1982).

```
                                              -18         p r e           -10
                                              Met lys trp val thr phe ile ser leu leu phe leu phe ser
(A)GCTTTTCTCTTCTGTCAACCCCACACGCCTTTGGCACA ATG AAG TGG GTA ACC TTT ATT TCC CTT CTT TTT CTC TTT AGC  (80)

        -1  -6    p r o     -1  1                           10                          20
ser ala tyr ser arg gly val phe arg arg asp ala his lys ser glu val ala his arg phe lys asp leu gly glu glu asn phe lys
TCG GCT TAT TCC AGG GGT GTG TTT CGT CGA GAT GCA CAC AAG AGT GAG GTT GCT CAT CGG TTT AAA GAT TTG GGA GAA GAA AAT TTC AAA (170)

21                                  30          34                  40                                      50
ala leu val leu ile ala phe ala gln tyr leu gln gln cys pro phe glu asp his val lys leu val asn glu val thr glu phe ala
GCC TTG GTG TTG ATT GCC TTT GCT CAG TAT CTT CAG CAG TGT CCA TTT GAA GAT CAT GTA AAA TTA GTG AAT GAA GTA ACT GAA TTT GCA (260)

51      53                          60      62                          70                  75                  80
lys thr cys val ala asp glu ser ala glu asn cys asp lys ser leu his thr leu phe gly asp lys leu cys thr val ala thr leu
AAA ACA TGT GTT GCT GAT GAG TCA GCT GAA AAT TGT GAC AAA TCA CTT CAT ACC CTT TTT GGA GAC AAA TTA TGC ACA GTT GCA ACT CTT (350)

81                                  90  91                          100 101                                     110
arg glu thr tyr gly glu met ala asp cys cys ala lys gln glu pro gly arg asn glu cys phe leu gln his lys asp asp asn pro
CGT GAA ACC TAT GGT GAA ATG GCT GAC TGC TGT GCA AAA CAA GAA CCT GGG AGA AAT GAA TGC TTC TTG CAA CAC AAA GAT GAC AAC CCA (440)

111                                 120         124                 130                                     140
asn leu pro arg leu val arg pro glu val asp val met cys thr ala phe his asp asn glu glu thr phe leu lys lys tyr leu tyr
AAC CTC CCC CGA TTG GTG AGA CCA GAG GTT GAT GTG ATG TGC ACT GCT TTT CAT GAC AAT GAA GAG ACA TTT TTG AAA AAA TAC TTA TAT (530)

141                                 150                                 160                         168 169 170
glu ile ala arg arg his pro tyr phe tyr ala pro glu leu leu phe phe ala lys arg tyr lys ala ala phe thr glu cys cys gln
GAA ATT GCC AGA AGA CAT CCT TAC TTT TAT GCC CCG GAA CTC CTT TTC TTT GCT AAA AGG TAT AAA GCT GCT TTT ACA GAA TGT TGC CAA (620)

171                     177         180                                 190                                     200
ala ala asp lys ala ala cys leu leu pro lys leu asp glu leu arg asp glu gly lys ala ser ser ala lys gln arg leu lys cys
GCT GCT GAT AAA GCT GCC TGC CTG TTG CCA AAG CTC GAT GAA CTT CGG GAT GAA GGG AAG GCT TCG TCT GCC AAA CAG AGA CTC AAG TGT (710)

201                                 210                                 220                                     230
ala ser leu gln lys phe gly glu arg ala phe lys ala trp ala val ala arg leu ser gln arg phe pro lys ala glu phe ala glu
GCC AGT CTC CAA AAA TTT GGA GAA AGA GCT TTC AAA GCA TGG GCA GTA GCT CGC CTG AGC CAG AGA TTT CCC AAA GCT GAG TTT GCA GAA (800)

231                                 240                 245 246         250     253                             260
val ser lys leu val thr asp leu thr lys val his thr glu cys cys his gly asp leu leu glu cys ala asp asp arg ala asp leu
GTT TCC AAG TTA GTG ACA GAT CTT ACC AAA GTC CAC ACG GAA TGC TGC CAT GGA GAT CTG CTT GAA TGT GCT GAT GAC AGG GCG GAC CTT (890)

261             265                 270                         278 279 280                             289 290
ala lys tyr ile cys glu asn gln asp ser ile ser ser lys leu lys glu cys cys glu lys pro leu leu glu lys ser his cys ile
GCC AAG TAT ATC TGT GAA AAT CAA GAT TCG ATC TCC AGT AAA CTG AAG GAA TGC TGT GAA AAA CCT CTG TTG GAA AAA TCC CAC TGC ATT (980)

291                                 300                                 310                 316                 320
ala glu val glu asn asp glu met pro ala asp leu pro ser leu ala ala asp phe val glu ser lys asp val cys lys asn tyr ala
GCC GAA GTG GAA AAT GAT GAG ATG CCT GCT GAC TTG CCT TCA TTA GCT GCT GAT TTT GTT GAA AGT AAG GAT GTT TGC AAA AAC TAT GCT(1070)

321                                 330                                 340                                     350
glu ala lys asp val phe leu gly met phe leu tyr glu tyr ala arg arg his pro asp tyr ser val val leu leu leu arg leu ala
GAG GCA AAG GAT GTC TTC TTG GGC ATG TTT TTG TAT GAA TAT GCA AGA AGG CAT CCT GAT TAC TCT GTC GTG CTG CTG CTG AGA CTT GCC(1160)

351                                 360 361                         369 370                                     380
lys thr tyr glu thr thr leu glu lys cys cys ala ala ala asp pro his glu cys tyr ala lys val phe asp glu phe lys pro leu
AAG ACA TAT GAA ACC ACT CTA GAG AAG TGC TGT GCC GCT GCA GAT CCT CAT GAA TGC TAT GCC AAA GTG TTC GAT GAA TTT AAA CCT CTT(1250)

381                                 390     392                         400                                     410
val glu glu pro gln asn leu ile lys gln asn cys glu leu phe glu gln leu gly glu tyr lys phe gln asn ala leu leu val arg
GTG GAA GAG CCT CAG AAT TTA ATC AAA CAA AAT TGT GAG CTT TTT GAG CAG CTT GGA GAG TAC AAA TTC CAG AAT GCG CTG TTA GTT CGT(1340)

411                                 420                                 430                         437 438     440
tyr thr lys lys val pro gln val ser thr pro thr leu val glu val ser arg asn leu gly lys val gly ser lys cys cys lys his
TAC ACC AAG AAA GTA CCC CAA GTG TCA ACT CCA ACT CTT GTA GAG GTC TCA AGA AAC CTA GGA AAA GTG GGC AGC AAA TGT TGT AAA CAT(1430)

441                         448     450                                 460 461                                 470
pro glu ala lys arg met pro cys ala glu asp tyr leu ser val val leu asn gln leu cys val leu his glu lys thr pro val ser
CCT GAA GCA AAA AGA ATG CCC TGT GCA GAA GAC TAT CTA TCC GTG GTC CTG AAC CAG TTA TGT GTG TTG CAT GAG AAA ACG CCA GTA AGT(1520)

471                 476 477     480                                 490                                     500
asp arg val thr lys cys cys thr glu ser leu val asn arg arg pro cys phe ser ala leu glu val asp glu thr tyr val pro lys
GAC AGA GTC ACC AAA TGC TGC ACA GAA TCC TTG GTG AAC AGG CGA CCA TGC TTT TCA GCT CTG GAA GTC GAT GAA ACA TAC GTT CCC AAA(1610)

501                                 510         514                 520                                     530
glu phe asn ala glu thr phe thr phe his ala asp ile cys thr leu ser glu lys glu arg gln ile lys lys gln thr ala leu val
GAG TTT AAT GCT GAA ACA TTC ACC TTC CAT GCA GAT ATA TGC ACA CTT TCT GAG AAG GAG AGA CAA ATC AAG AAA CAA ACT GCA CTT GTT(1700)

531                                 540                                 550                         558 559 560
glu leu val lys his lys pro lys ala thr lys glu gln leu lys ala val met asp asp phe ala ala phe val glu lys cys cys lys
GAG CTC GTG AAA CAC AAG CCC AAG GCA ACA AAA GAG CAA CTG AAA GCT GTT ATG GAT GAT TTC GCT GCT TTT GTA GAG AAG TGC TGC AAG(1790)

561                     567     570                                 580
ala asp asp lys glu thr cys phe ala glu glu gly lys lys leu val ala ala ser gln ala ala leu gly leu ter          ter
GCT GAC GAT AAG GAG ACC TGC TTT GCC GAG GAG GGT AAA AAA CTT GTT GCT GCA AGT CAA GCT GCC TTA GGC TTA TAA CATCACATTTAAAAG(1883)

               ter   ter
CATCTCAGCCTACCATGAGAATAAGAGAAAGAAAATGAAGATCAAAAGCTTATTCATCTGTTTTTCTTTTTCGTTGGTGTAAAGCCAACACCCTGTCTAAAAAACATAAATTTCTTTAA(2002)

TCATTTTGCCTCTTTTCTCTGTGCTTCAATTAATAAAAAATGGAAAGAATCTAA ...... 20 ...... AA (2078)
```

Fig. 1.2 The complete nucleotide sequence of human serum albumin mRNA as determined from cloned cDNA, showing the presence of the pro- and prepro-peptides (from Dugaiczyk, *et al.*, 1982).

existence had already been suggested by previous workers (see Section 1.1.6).

1.1.3 Secondary Structure

Once the amino acid sequence was known, predictions of the secondary structure were soon made. Both teams responsible for sequencing HSA noticed the regular occurrence of adjacent cys-cys residues; these form the basis of the postulated repeating loop structure (see Fig. 1.3). The nine loops held together by these disulphide bridges give rise to three domains, each with characteristic binding properties. There is some 50-55% α helix by circular dichroic measurements and 15-18% β sheet (Peters, 1975) which are in good agreement with the values of 46% α helix and 16% β sheet predicted from the structure of bovine serum albumin (BSA). From predictions based on the methods of Chou and Fasman (1978) the long loops appear to be helical with some β sheet in loops one, three, six and seven. In the shorter tight loops the α helical content is less, due to the steric restrictions imposed by the disulphide bridges. Eight of the tips of the loops have β turns, seven with cystine residues and one with the free thiol of cys-34.

Brown (1977) has proposed a structure with six uniform helical regions; each consisting of a long and a short loop. The final structure (see Fig. 1.4)

can be viewed as consisting of three domains of the repeated unit (large loop)-(small loop)-(connecting segment)-(large loop) which can be broken down into three sub-domains. This structure, with its strong disulphide bridges, explains the stability of albumin to denaturing conditions such as acid, base, heat and 6 M urea. Each segment is held in close proximity by the S-S bridges and so secondary structure readily forms. Even after fragmentation serum albumin retains helical structure in those fragments which contain disulphide bonds. However, the stability of the conformation is not due entirely to disulphide bridges as after complete reduction of these bonds the fragments and even the whole protein will slowly refold to restore its antigenic, circular dichroic and fatty acid binding properties.

1.1.4 Tertiary Structure

Albumin has been shown to exist in four interchangeable conformations, dependent upon pH. Foster (1960) showed the existence of a normal (N) form at pH 5-7 and a fast migrating form (F) at pH 3-4, which appears to be due to expansion of the molecule and results in increased anodic mobility on electrophoresis. Below pH 3 a further conformational change occurs, resulting in the expanded form, exposing many of the hydrophobic residues to the solvent. Above pH 7 several other conformations exist; a slow transition

to the A form occurs above pH 8, resulting in a slower migration on electrophoresis (pI = 5.45 cf. 5.2 for normal form). This transformation appears to involve the rearrangement of some disulphide bonds, since it is suppressed by alkylating agents, yet accelerated by thiol compounds. Wallevik (1976) has shown that the A form is broken down more rapidly *in vivo* than the normal. Transformation to the A form becomes irreversible around pH 10. Between pH 7 and 8 a second slow form can exist especially in the presence of calcium ions. This B form also appears to involve the free thiol group and a slight loss of helical content has been noted (Zurawski and Foster (1974) (Fig. 1.5).)

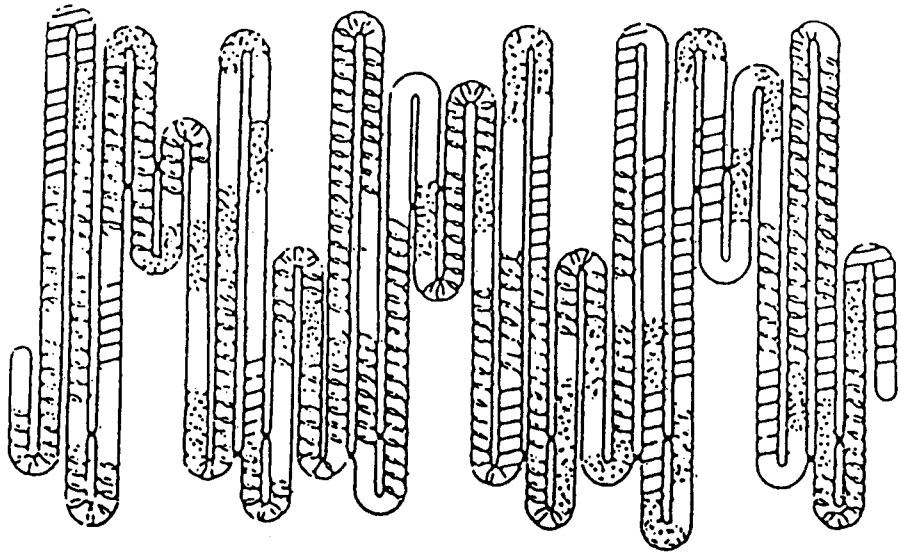


Fig. 1.3 Distribution of secondary structure in BSA (Peters and Reed, 1977).
Key: α-helix
β-pleated sheet
β-turns

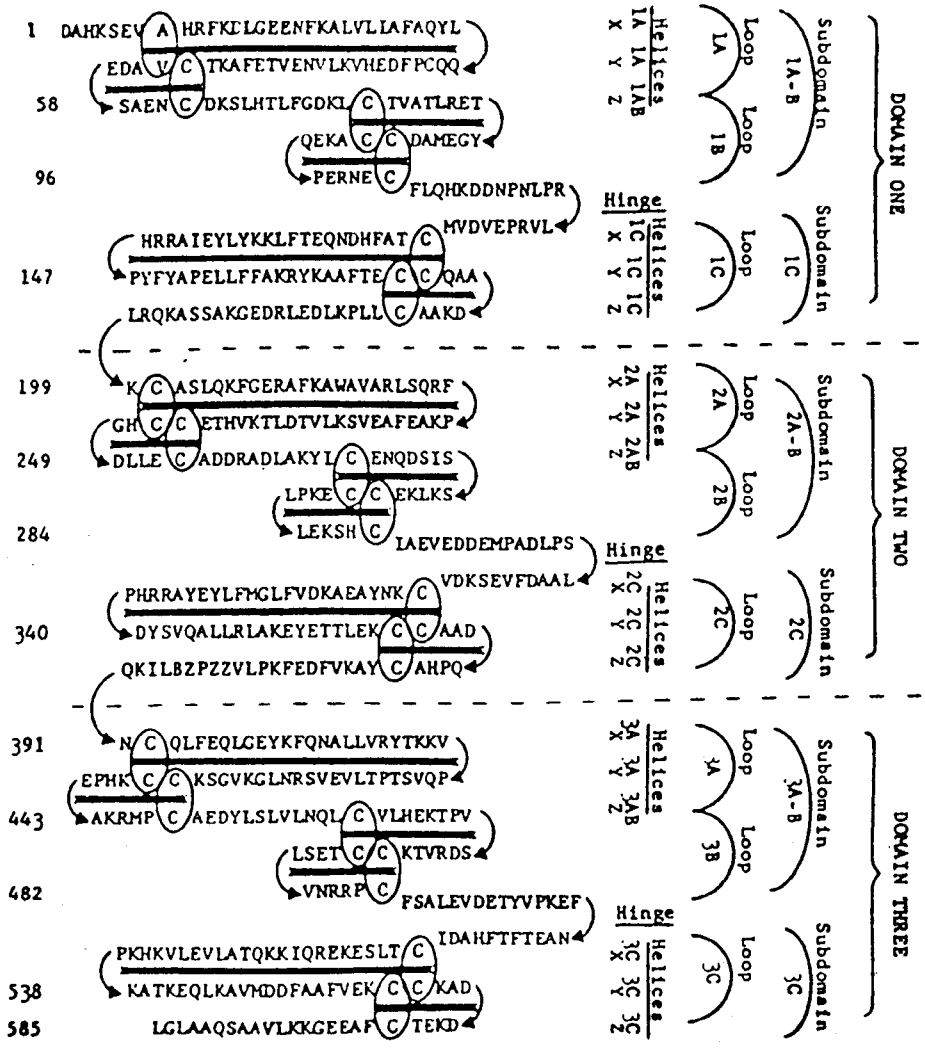


Fig. 1.4 Structural organisation of HSA showing subdomain and loop structures (from Brown, *et al.*, 1979).

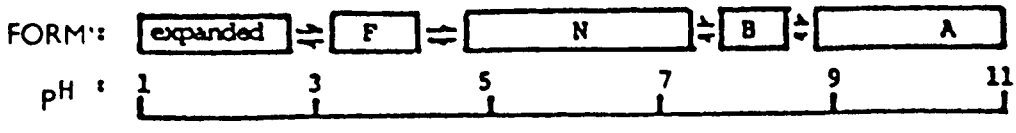


Fig. 1.5 The conformations of serum albumins at differing pH values.

The free thiol group of albumin, cysteine-34, has aroused much interest in that *in vivo* it appears to be partially blocked by thiol-containing ligands such as glutathione or cysteine. The mercapto and non-mercapt-albumins have been extensively studied and there appears to be ≈ 0.6 mole free thiol group/mole albumin, although this can be restored to one mole by addition of reducing agents. Albumin has been proposed to exist to some degree as a dimer in serum, and partial blocking of the thiol residue aids the peptic digestion of the protein.

The three dimensional structure of albumin is thought to be an ellipsoid molecule approximately 141 x 42 Å (Wright and Thompson, 1975). Other studies suggest that there are three globular subunits of diameters 38, 53 and 38 Å (Bloomfield, 1966) and this gains some support from 1,amino-8-naphthalene sulphonic acid (ANS) binding studies (Fig. 1.6). The N-terminus appears to be more compact than the C-terminus and undergoes a lesser degree of unfolding during N → F transformations, indeed when isolated the N-terminal fragment 1-385 of BSA does not undergo the N → F transition (Kahn and Salahuddin, 1984). Sakata and Atassi (1980) have demonstrated five antigenic sites on human albumin.

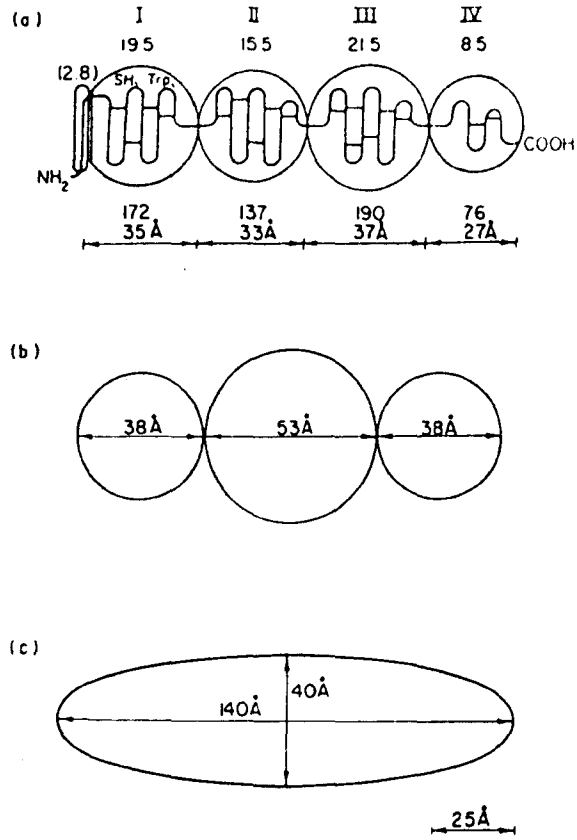


Fig. 1.6 Three dimensional models of serum albumin (Peters, 1970).
(a) Four domain model of Peters,
(b) Three sphere model (Bloomfield, 1966),
(c) Ellipsoid structure (Squire, *et al.*, 1968).

1.1.5 Domain Homology

The three domains are not only similar in conformation but have regions of homologous sequence. The cysteine residues appear invariant in all mammalian albumins and this indicates that they all share a similar domain type structure. There is internal homology between all three domains; 48 residues are identical in domains one and two (25% identity), the longest continuous homologous sequence being four amino acids. Domains two and three have 41 positions the same (21% identity) and one and three have 35 positions (18% identity), demonstrating that domains one and two are more

similar to each other than either is to domain three (Brown, 1976). This similarity has led Brown to propose that albumin originated as a single domain (residues 504-81) which underwent gene duplication and subsequent deletion several times to arrive at the present structure (Brown, et al 1979). The degree of domain homology is similar to that seen between myoglobin and haemoglobin or the immunoglobin light and heavy chains. Human and bovine albumins have 20-23% difference in amino acid sequence. Brown maintains that the proposed 'primordial domain' residues 504-581, has some homology with sperm whale myoglobin in sequence and structure; however, McLachlan and Walker (1977), although confirming the intramolecular homology did not find homology between HSA and myoglobin, as although some regions did show good matches they were all typical surface helices, with one side exposed to the aqueous environment and the other buried in the protein.

1.1.6 Albumin Synthesis and Degradation

Serum albumin is synthesised in the liver, where it can comprise up to 50% of the total protein produced by that organ. The complex balance of synthesis and degradation has been reviewed by Rothschild and Oratz (1977). DNA directed mRNA synthesis in the hepatocyte nuclei is followed by cytoplasmic translation of the mRNA attached to the

ribosomes of the endoplasmic reticulum. Synthesis commences on the 40S ribosome subunit attached to the mRNA and the peptide chain is inserted through the 60S subunit into the endoplasmic reticulum, extending as each amino acyl tRNA donates its amino acid in the order specified in the mRNA, one mRNA accommodating as many as 20 ribosomes. Albumin passes through the endoplasmic reticulum to the Golgi apparatus and from there in vesicles to the cell membrane for secretion. Using labelled amino acids it has been shown that from their incorporation to location in the endoplasmic reticulum requires 3-6 minutes and the label reaches the Golgi apparatus in 15-20 minutes.

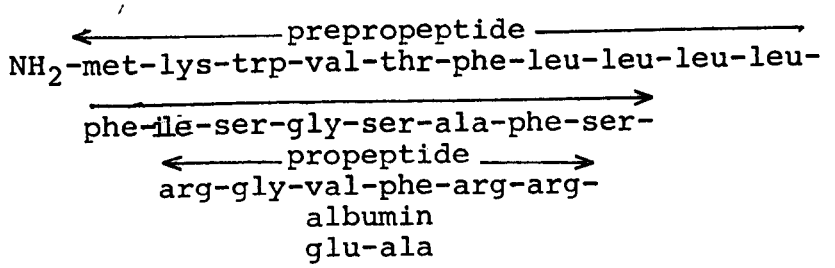
Urban, *et al.* (1974) showed the presence of a propeptide at the N-terminus of albumin isolated from hepatocyte microsomes and the sequence of this hexapeptide (Russell and Geller, 1975) is given in Fig. 1.7. This was confirmed by Quinn, *et al.* (1975) who showed that the propeptide could be excised *in vitro* using trypsin to give normal albumin. Edwards, *et al.* (1976) traced the course of the proalbumin and showed that it was the predominant form in the endoplasmic reticulum, but that it constituted only 45% of the albumin in the Golgi and that in the secretory vesicles the serum form only was observed. Strauss, *et al.* (1977) expressed rat liver mRNA *in vitro* to show the presence of the additional 18 amino acid prepropeptide, and obtained

part of its sequence which consisted of at least 38% hydrophobic residues and was similar in length, half-life, intracellular localisation and possibly function to sequences in parathyroid hormone and proinsulin. Subsequently, Dugaiczyk (1982) demonstrated that the human propeptide was also similar with a high percentage of hydrophobic residues.

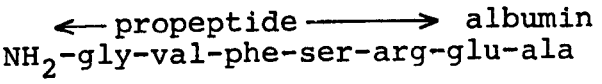
The probable function of the prepropeptide is to enable the protein to enter the endoplasmic reticulum on synthesis, the hydrophobic section acting as a signal peptide passing through the non-polar membrane. The signal sequence is excised and it is thought that the propeptide acts in an analogous manner directing the albumin through the endoplasmic reticulum and to the Golgi for incorporation into secretory vesicles. It is at this stage that it too is proteolytically cleaved, leaving the serum form of albumin to be exported from the cell.

Albumin has been found in the liver at as little as 32 days gestation; however, it would seem that in the foetus α-foetoprotein performs the major role in ligand transport (Hervé, *et al.*, 1984). Albumin synthesis rises soon after birth and continues to increase, attaining a plateau value in adulthood of around 130-200 mg/kg/day. The level of albumin is slightly higher in men than women, ≈ 40 ± 5 g/L. Between thirty and forty percent of the exchangeable albumin pool occurs in the plasma, the rest being

Rat Preproalbumin



Possible Rat Proalbumin Variant (Urban, *et al.*, 1974)



Human Preproalbumin (Dugaiczyk, *et al.*, 1982)

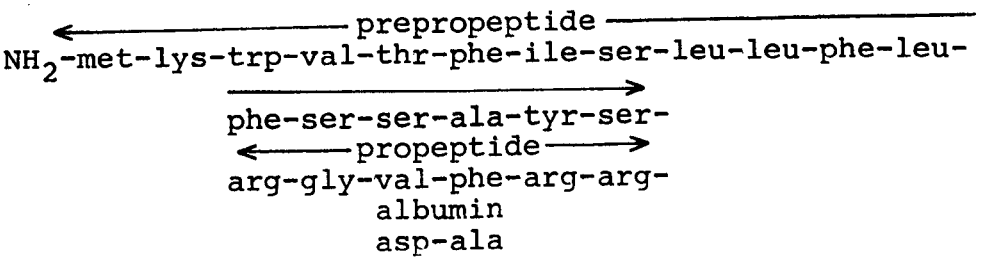


Fig. 1.7 Pro- and Prepro-peptides from Human and Rat Serum Albumins

located in the extravascular tissue such as muscle and skin. The latter contains 30-40% of the extravascular pool although only being 6% of the total body weight. The two pools are in equilibrium with around 75% of the albumin exchanging every two days. Albumin is present in all secretions and excretions that have been tested and is the major constituent in oedema.

Albumin synthesis is stimulated by both thyroid hormone and cortisone, but the most important factor for its control is the nutritional state. In starvation, albumin synthesis is rapidly decreased and remains depressed until the deficiency state is corrected. The level of albumin in the extra-vascular pool is depleted, and degradative rates also decrease in order to conserve it.

Albumin breakdown is a random process, it is poorly understood but proceeds at a high rate, presumably due to pinocytosis and subsequent digestion in the lysosomes. No single organ can be defined as predominantly responsible for albumin degradation. Muscle must account for a major part due to its relative abundance, and though the kidneys and intestine both degrade albumin in disease states neither play a significant role in healthy patients.

1.2 LIGAND BINDING

Many studies have been carried out into the ability of albumin to bind a wide range of ligands both of physiological importance and of pharmacological interest. Several excellent reviews have been written (Brown and Shockley (1982), Vallner (1977), Sellers and Koch-Weser (1977) and Kragh Hansen (1981)).

Each of the three domains of albumin is specialised to bind a particular type of ligand.

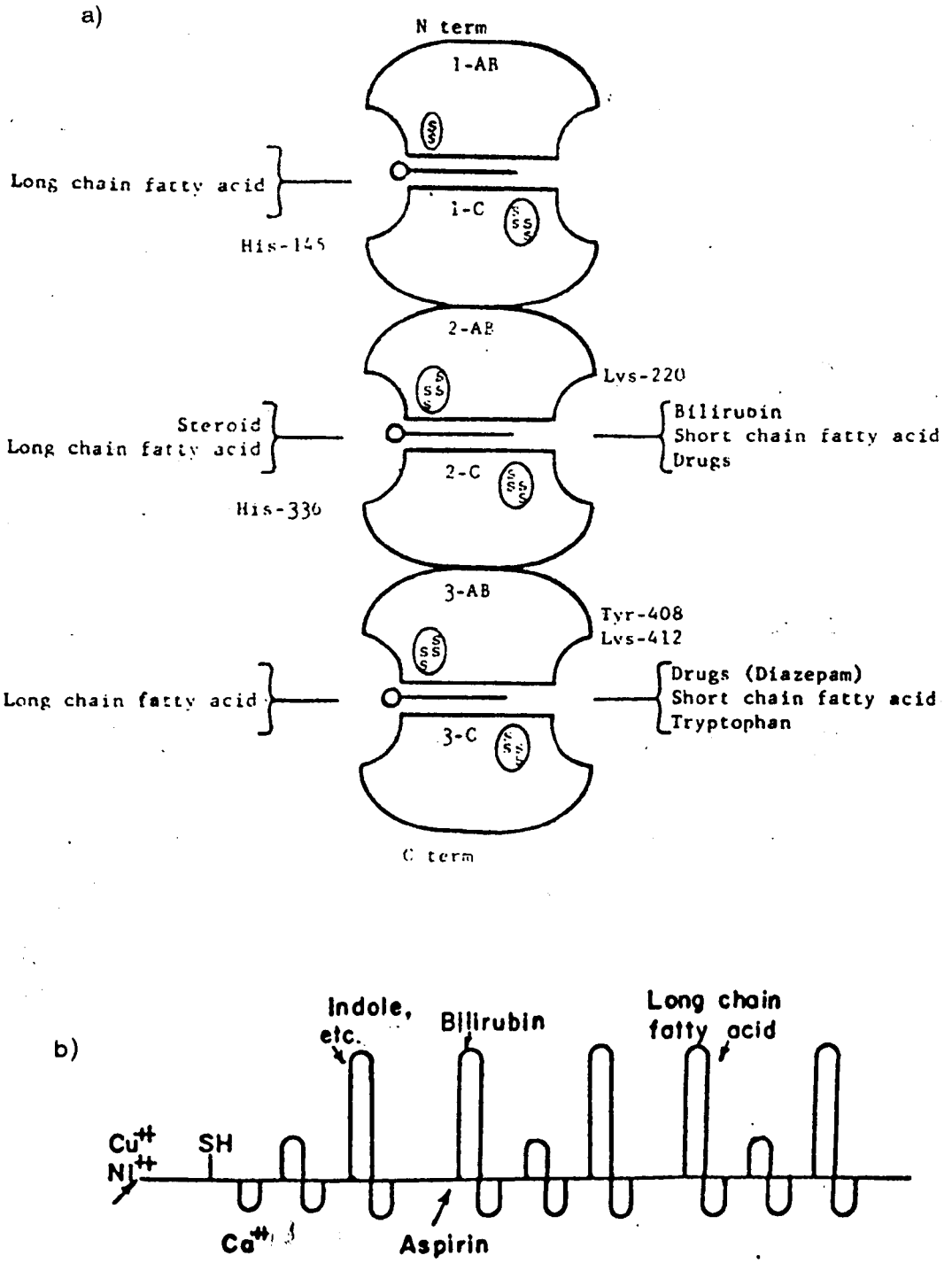


Fig. 1.8 Two representations of the ligand binding sites on HSA.
(a) Brown and Shockley (1982)
(b) Peters and Reed (1977)

A summary of these sites is shown in Fig. 1.8. Each domain has been split by Brown and Shockley (1982) into three subdomains; however, other reviews consider the binding of each ligand type individually, as will be done here (see Table 1.1).

1.2.1 Metal Ions

The amino terminus of the albumin molecule has a primary site for the binding of divalent metal ions such as Cu^{II} and Ni^{II}, in a 1:1 molar ratio to albumin (Sarkar, 1981). The binding affinities decline in the order $Cu^{2+} > Ni^{2+} > Zn^{2+}$ with K_a values from 9×10^6 to 5.7×10^2 M^{-1}. Other metal ions have been shown to bind to albumin including Cd^{2+} and Al^{3+}; however, this may occur at less specific secondary sites. Copper is an important trace element in mammals and although caeruloplasmin acts as the primary carrier, the binding of Cu^{II} by albumin does appear to be of physiological importance. Ni^{II} has not been shown to have any physiological role in man and may well be toxic (McNeely, *et al.*, 1972); the high affinity of albumin for it may well play a role in the removal of toxic amounts from the body and this is possibly true to a lesser extent for Cd^{2+} also. Zinc is bound to HSA and a deficiency of Zn^{II} results in impairment of growth, and in skin disorders (acrodermatitis enteropathica).

Glennon and Sarkar (1982) have studied the divalent metal site using a model tripeptide,

TABLE 1.1 LIGAND BINDING TO HUMAN SERUM ALBUMIN*

Ligand	n_1	Ka M^{-1}	n_2	Ka M^{-1}	Temperature ^{o}C
Bilirubin	1	1.4×10^8	2	5×10^5	37
Haemin	1	1.1×10^8			24
Palmitate	2	6.0×10^7	5	3.0×10^6	23
Linoleate	2	1.3×10^7	5	2.5×10^6	23
Oleate	2	1.1×10^8	5	4.0×10^6	23
L-Tryptophan	1	1.6×10^4			2
L-Thyroxine	1	1.6×10^6	3	6×10^4	24
Progesterone	1	3.6×10^5	8	6×10^3	4
Testosterone	1	2.38×10^4			25
Estradiol	1	1.0×10^5			5
Cortisol	2	5.0×10^3			37
Warfarin	2	8.9×10^4	4	6.7×10^3	27
Salicylate	1	2.2×10^5	5	1.6×10^3	37
Phenylbutazone	1	2.3×10^5	4	5.6×10^3	37
Digitoxin		6.9×10^4			20
Clofibrate	2	2.5×10^4	8	4.7×10^2	ca 20+
Sulfaethidole	1	1.5×10^5	3	1.6×10^3	25
Diazepam	1	4.9×10^5			22
O-methyl Red	1	1.99×10^5			37
Methyl Orange	2	2.8×10^3			37
Bromocresol Green	1	1.0×10^6	4	2.1×10^4	37

*Adapted from Kragh Hansen (1981) and Bowmer and Lindup (1980)

L-asp-L-ala-L-his N-methylamide, corresponding to the first three amino acids of HSA. They proposed that the binding geometry for copper ions was square planar and involved the α-amino nitrogen group, two deprotonated peptide nitrogens and the imidazole nitrogen (see Fig. 1.9). Laussac and Sarkar (1984) have since given support to this model from n.m.r. studies of the isolated 1-24 peptide residue of HSA. In their model they suggest that the carboxylate group of the aspartate residue stabilises the binding from above the plane of the ring. The penta-coordinated copper seems unusual and there may be further stabilisation from a water molecule beneath to attain the 6-coordinate state.

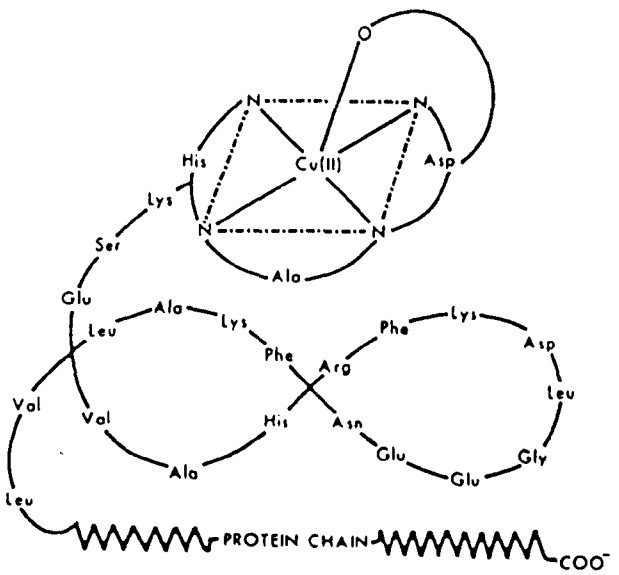


Fig.1.9 Proposed copper(II) transport site of human albumin.

Callan and Sunderman (1973) presented data for the binding of Ni^{II} to several mammalian albumins (summarised in Table 1.2). This emphasises the need for histidine at the third amino acid residue, as the affinity for Ni^{II} of canine and porcine albumins which lack histidine at residue three is significantly lower than those which possess it. This gives further evidence to Sarkar's model, and dogs and possibly pigs are more susceptible to nickel and copper poisoning than the other mammals in the table, as they can only bind metal ions at the weaker secondary sites.

1.2.2 Bilirubin

Bilirubin, the breakdown product of haem, is tightly bound by albumin, and thus rendered less toxic as already mentioned. The location of the primary binding site has been studied using covalently attached affinity labels and also from the properties of fragments from proteolytic digestion. Peptides 1-386, and 49-307 residues, both bind bilirubin similarly to intact HSA (Geisow and Beaven, 1977b). Using affinity labelling both lys 220 and lys 240 have been shown to be involved, as is the region designated subdomain 2AB in Brown's model (Brown and Shockley, 1982); residues 186-306 have been suggested to contain the primary binding site. Using ^{3}H-bilirubin, Hutchinson and Mutopo (1979) interpreted the labelling of the 1-124 CNBr fragment of HSA as being the primary

TABLE 1.2 Binding of $^{63}Ni^{II}$ to Serum Albumins*

Species of Albumin	Maximum Binding Sites	First Association Constant (L/mole)	% $^{63}Ni^{II}$ Bound to Albumin	NH_2-terminal Amino Acid Sequence
Human	15	3×10^5	87	NH_2-Asp-Ala-His
Rat	9	2×10^5	88	NH_2-Glu-Ala-His
Porcine	11	8×10^4	77	NH_2-Glu-Thr-Tyr
Canine	7	2.5×10^4	55	NH_2-Glu-Ala-Tyr

*0.05 mM $^{63}Ni^{II}$ and albumin solutions in 0.1 M tris-HCl 37°C, pH 7.4

Adapted from Callan and Sunderman (1973)

binding site, with a secondary site in residues 125-217; however, Gitzelmann – Cumarasamy et al. (1976) using similar methods had found the label in residues 129-297 and 446-547. This inconsistency may be due to the labelling of other reactive groups not associated with bilirubin binding, or of secondary bilirubin sites only.

The binding of bilirubin to its primary site may be influenced by fatty acids. The addition of two molar laurate increases the affinity for bilirubin and the binding sites for the first two moles of long chain fatty acids and bilirubin are distinct (Berde, *et al.*, 1979). However, the third mole of long chain fatty acid binds nearer to the bilirubin site and at concentrations of fatty acid > 5 M there is competition at or near the bilirubin primary site and bilirubin is displaced. Hsia et al. (1982) have shown the presence of one high affinity and two lower affinity stereospecific binding sites for bilirubin together with a series of non-stereospecific sites of low affinity.

The primary binding site has a high K_a value in the region 5.5×10^7 to 1×10^8 M^{-1} and the secondary sites approximately 10^6 M^{-1}, indicative of the importance of bilirubin in the body. Other ligands do bind at or near the bilirubin site and these include warfarin, salicylate, bromocresol green, bromophenol blue and phenol red; these may be responsible for displacing bilirubin when present in high concentrations.

1.2.3 Fatty Acid Binding

1.2.3.1 Introduction

Fatty acids bind to albumin with an affinity only exceeded by that of bilirubin; albumin is normally the only plasma protein to bind appreciable amounts of fatty acid and so plays an important role in their utilisation by the body. Fatty acids are released from the intestine complexed to albumin; being poorly soluble in aqueous media, binding to albumin increases their effective solubility and prevents the dimerisation of the C_{14-18} chain fatty acids as only the monomeric anion will bind. Albumin transports fatty acids to the liver and also from the liver to peripheral muscles, heart and other organs enhancing their uptake by reducing the diffusion distance, so increasing their relative concentration at cell membranes. Fatty acids can be grouped into two classes, long chain fatty acids C_{12-18} and short chain C_{10} and less; albumin appears to have distinct sites for each class.

1.2.3.2 Short Chain Fatty Acids (SCFA)

There appears to be a primary binding site for SCFA up to decanoate in the subdomain 3AB, no binding occurring to any extent elsewhere until this is saturated (Brown and Shockley, 1982). The site is linked to residues Trp 214 and Lys 220 and there is a strong preference for SCFA reflected in the K_a

values, although this may also be a secondary site for long chain fatty acids. A secondary site for SCFA occurs at subdomain 2AB near Trp 214, and Lys 220 is involved as it is protected from modification by octanoate. The site is probably identical to the bilirubin primary binding site and again although long chain fatty acids will bind there is a preference for SCFA.

1.2.3.3 Long Chain Fatty Acids (LCFA)

Long chain fatty acids bind to at least three sites on albumin. The primary site ($K_a \approx 10^7\ M^{-1}$) is near loop 7 residues $T_{377-505}$ (subdomain 3C) and no other ligands compete at this site. Secondary sites still with high affinity ($K_a \approx 10^6\ M^{-1}$) occur in subdomains 1C and 2C; although when isolated these fragments do not show palmitate binding this is probably due to a loss of the structural stability of the sites in aqueous solution. Spin label studies reveal that palmitate binds to a tight hydrophobic cleft and that the carboxyl group is relatively immobile, suggesting an electrostatic bond to a positively charged residue such as lysine.

Models of the primary fatty acid binding site have been constructed with six helices positioned so as to create a hydrophobic pocket in the centre ideal for the binding of the lipophilic carbon chain of long chain fatty acids (Brown and Shockley, 1982). It is known that the hydrophobic forces are the major driving force in binding fatty acids although the

electrostatic charge does make a contribution.

The binding of long chain fatty acids requires a multiple site model with at least six high affinity sites and three or four weaker ones with as many as twenty low affinity binding sites also occurring. The association constants decrease in sequence from $K_{a_1} = 91.4 \times 10^7\ M^{-1}$ to $K_{a_8} = 2.2 \times 10^7\ M^{-1}$ for palmitate (Spector and Fletcher, 1977). The Scatchard approximation of binding does not allow for the creation of new sites or for co-operativity between sites and so the generalised equation of Spector and Fletcher (1977) is preferred. The presence of a first double bond in the hydro-carbon chain increases binding, but the second double bond reduces it to below that of the saturated fatty acid, for example C 18:1 > C 18:0 > C 18:2.

At physiological levels of fatty acid (0.3-1.2 mM) the binding of two moles of long chain fatty acid has no effect on other ligand binding; however, the distribution of fatty acids between albumin molecules is not homogenous. At a 1:1 molar ratio of stearate:albumin only 43% of the fatty acid is in a 1:1 complex, with 43% being bound to albumins at a 2:1 ratio and 12% at a 3:1 stearate:albumin ratio. This trend is similar for other physiological long chain fatty acids suggesting that the lower affinity sites may be occupied even at low molar ratios in a percentage of albumin molecules.

Fatty acids can compete with other ligands for binding to albumin due to their many secondary sites. L-tryptophan can be displaced by quite low levels of oleate (McMenamy, 1965) and this may play a role in the control of tryptophan and hence over serotonin levels in the brain (Curzon *et al.* 1973). Thyroxine is also significantly displaced by high levels of fatty acids, although this is not physiologically important due to its binding primarily to more specific globulins. The only ligands known to displace fatty acids from albumin are salicylates in high concentration and Clofibrate (an anti-cholesterolemic drug), probably acting via a conformational change (Spector, 1975).

1.2.4 Other Physiological Ligands

1.2.4.1 Indole Derivatives

The primary indole binding site seems to be located near an active tyrosine residue as it can be inhibited by the nitration of tyrosine; the site may be composed of several distinct regions of the molecule brought into close proximity (Brown and Shockley, 1982). Geisow and Beaven (1977b) showed the primary site to be located in residues 307-86 and this is consistent with the findings of Reed, *et al.* (1976), but Sjodin, *et al.* (1977) and Gambhir and McMenamy (1973) have placed the site nearer the N-terminus. Gerig and Klinkenborg (1980) found evidence for two sites by n.m.r. and from the peptic

fragments of King (1973) it would appear that a primary site occurs in the 3AB region with secondary binding near the N-terminus, incorporating his 146.

L-tryptophan is bound with one hundred times the affinity of the D form (1.6×10^4 M^{-1}) and can be displaced by fatty acids, the site seeming to be associated with that for thyroxine and a secondary salicylate site.

1.2.4.2 Thyroid Hormone

Albumin binds thyroxine only as a secondary carrier, however it may be of physiological importance at high levels of the hormone. There is a primary site of high affinity (1.6×10^6 M^{-1}) and a number of secondary sites (Steiner, *et al.*, 1966, Cheng, *et al.*, 1981). The primary site is thought to occur in subdomain 2AB.

1.2.4.3 Steroid Hormones

Steroid hormones are primarily carried by a specific α-globulin but can also bind to albumin, which has a higher capacity though of lower affinity. A primary site has been assigned to the subdomain 2C (residues 307-85) by Pearlman and Fong (1972). This site also binds bilirubin, and Soltys and Hsia (1978) have shown competition between nine steroids and bilirubin for this site.

1.2.4.4 Haemin

Haemin, a breakdown product of haemoglobin, binds to albumin with a high association constant

and is not displaced even by a 10:1 oleate:HSA level (Adams and Berman, 1980). The location is not exactly known but appears to be in the sequence 124-298 residues, although it seems that the C-terminus of the molecule is required for the total binding affinity (Hrkal, *et al.*, 1978). The binding of haemin is presumably instrumental to its catabolism.

1.2.5 Drug Binding

1.2.5.1 Introduction

Pharmacological interest in albumin is due mainly to its ability to tightly bind a large range of drugs. These drugs can be placed into two categories: those that are negatively charged organic acids or with electronegative centres, and bind mainly by non-covalent hydrophobic interactions, and those which bind irreversibly due to the formation of amide or similar linkages. Excellent reviews of drug binding to albumin have been written by Vallner (1977) and Sellers and Koch-Weser (1977) and more recently, by Kragh-Hansen (1981). Most drugs form only weak ionic interactions with albumin and so their hydrophobic interactions are the driving force for binding, although electrostatic interactions help to strengthen the binding.

Albumin acts as a reservoir for unbound drugs, the free form is usually the only active one.

However, binding to albumin ensures that the drug is carried throughout the vascular system and so decreases the effective dose and concentration and the rate of elimination (see Fig. 1.10). The distribution of the drug is dependent upon the degree to which it binds to albumin and this may vary from one tissue to another. Many experiments have examined the binding of drugs to albumin but their results are only valid where near physiological conditions have applied. Kragh-Hansen (1981) defines three major binding sites for drugs; the benzodiazepines which bind to the indole site, other drugs such as warfarin and Phenylbutazone which bind to the bilirubin site, and others which appeared to occupy an independent site. The anti-rheumatoid pain relievers Ibuprofen and Naproxen seem to bind to subdomain 3AB as does Clofibrate.

The kidneys remove drugs which bind to albumin and their metabolites. If the drug is tightly bound then its elimination from the body is a slow process, the drug-albumin complex will not diffuse through the glomerulus and only the free drug is eliminated; however, turnover is quicker in the kidney tubules.

1.2.5.2 Warfarin

Warfarin is used with other coumarin derivatives as an anticoagulant and it is > 99% bound to albumin at physiological concentrations. Over 95% of the bound drug is associated with the one or two primary sites ($K_a \approx 10^5\ M^{-1}$), the rest is spread

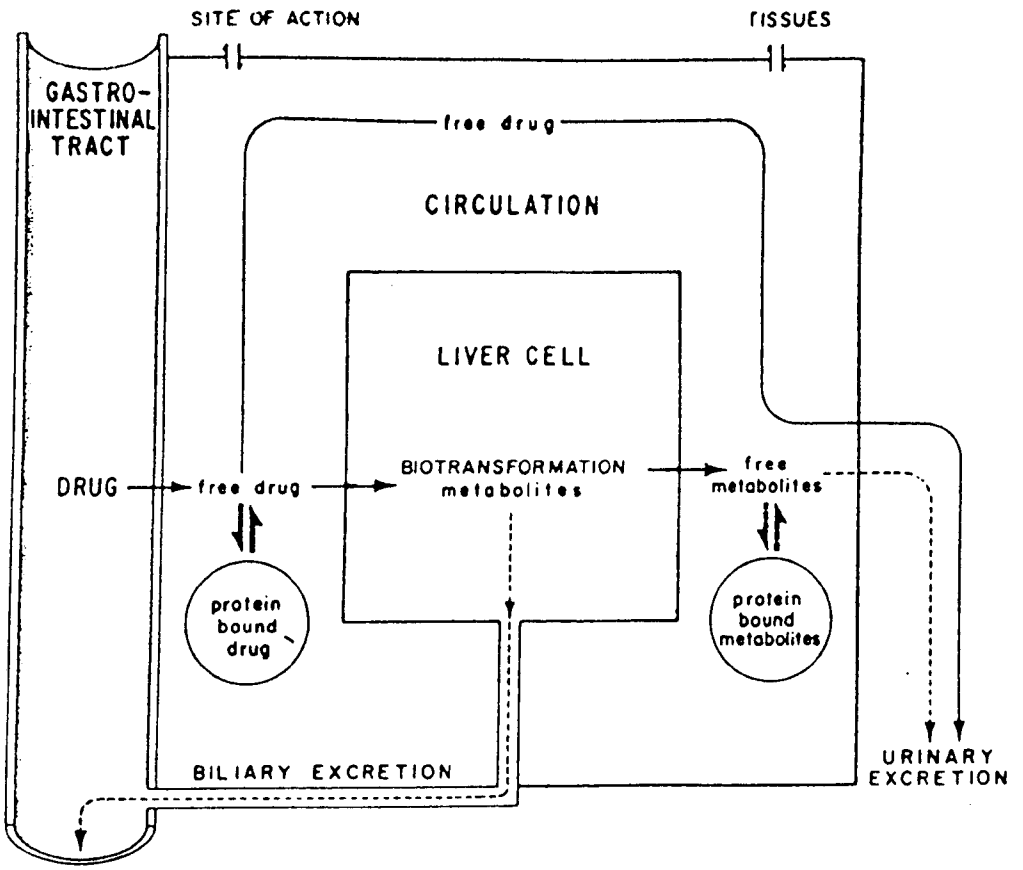


Fig. 1.10 The effect of drug-albumin binding on drug metabolism (Sellers and Koch-Weser 1977)

over the three or four secondary ones ($K_a \approx 5 \times 10^3\ M^{-1}$) (Oester, *et al.*, 1976, Maes, *et al.* 1982). Warfarin is bound primarily as the anion, and binding is dependent upon fatty acid concentration. Binding of long chain fatty acids up to a 3:1 molar ratio enhance warfarin binding by causing conformational changes to the albumin but at molar ratios > 3:1 fatty acid:albumin they decrease warfarin binding, presumably by direct competition (Sebille, *et al.*, 1984).

The albumin molecule must be intact for high affinity warfarin binding, as binding to isolated

subdomains is only one-tenth that of the whole molecule. The warfarin binding site is primarily hydrophobic; however, hydrogen binding is important as the hydroxy form of warfarin is bound poorly. Feshke, *et al.* (1979) have shown the warfarin primary binding site to include Trp 214. Warfarin can be displaced by Clofibrate, Phenylbutazone and salicylate. However, differences in the type of binding between warfarin and salicylate imply that they do not simply compete for the same site (Oester, *et al.*, 1976).

1.2.5.3 Benzodiazepines

The benzodiazepines, widely used tranquilisers and anti-depressants, bind to albumin with $K_a \simeq 10^5$-10^6 M^{-1} (Sjodin, *et al.*, 1976). Electronegative and halogen substituents increase the binding and positive groups inhibit it. Wanwimolruk, *et al.* (1983) have shown the binding site to be a hydrophobic cleft 16 Å deep by 8 Å wide with a cationic group near the surface. The site could possibly be identical with the short chain fatty acid binding site, Sudlow, *et al.* (1976) defining benzodiazepine binding requirements as being

a hydrophobic molecule such as an aromatic carboxylic acid with a negatively charged group distinct from the hydrophobic region.

1.2.5.4 Salicylate

The binding of salicylate may be to a separate site as Kragh-Hansen (1981) can fit it into

none of the five sites he describes. The secondary site is most likely identical to the high affinity thyroxine site. Vallner (1977) suggests four primary sites with $K_a \simeq 2 \times 10^5\ M^{-1}$, and its high degree of binding may implicate it in influencing the binding of other drugs administered simultaneously.

1.2.5.5 Acetylsalicyclic Acid (Aspirin)

Hawkins, *et al.* (1968) showed that acetylsalicylic acid acetylates albumin, and this was pinpointed by Walker (1976) to the ε-amino of lysine 199 (Fig. 1.11).

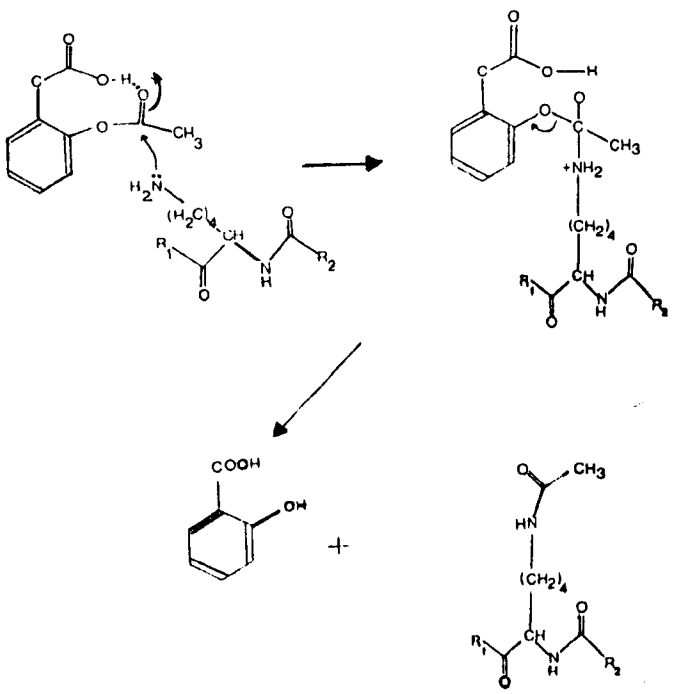


Fig. 1.11 The acetylation of albumin by acetyl-salicylic acid.

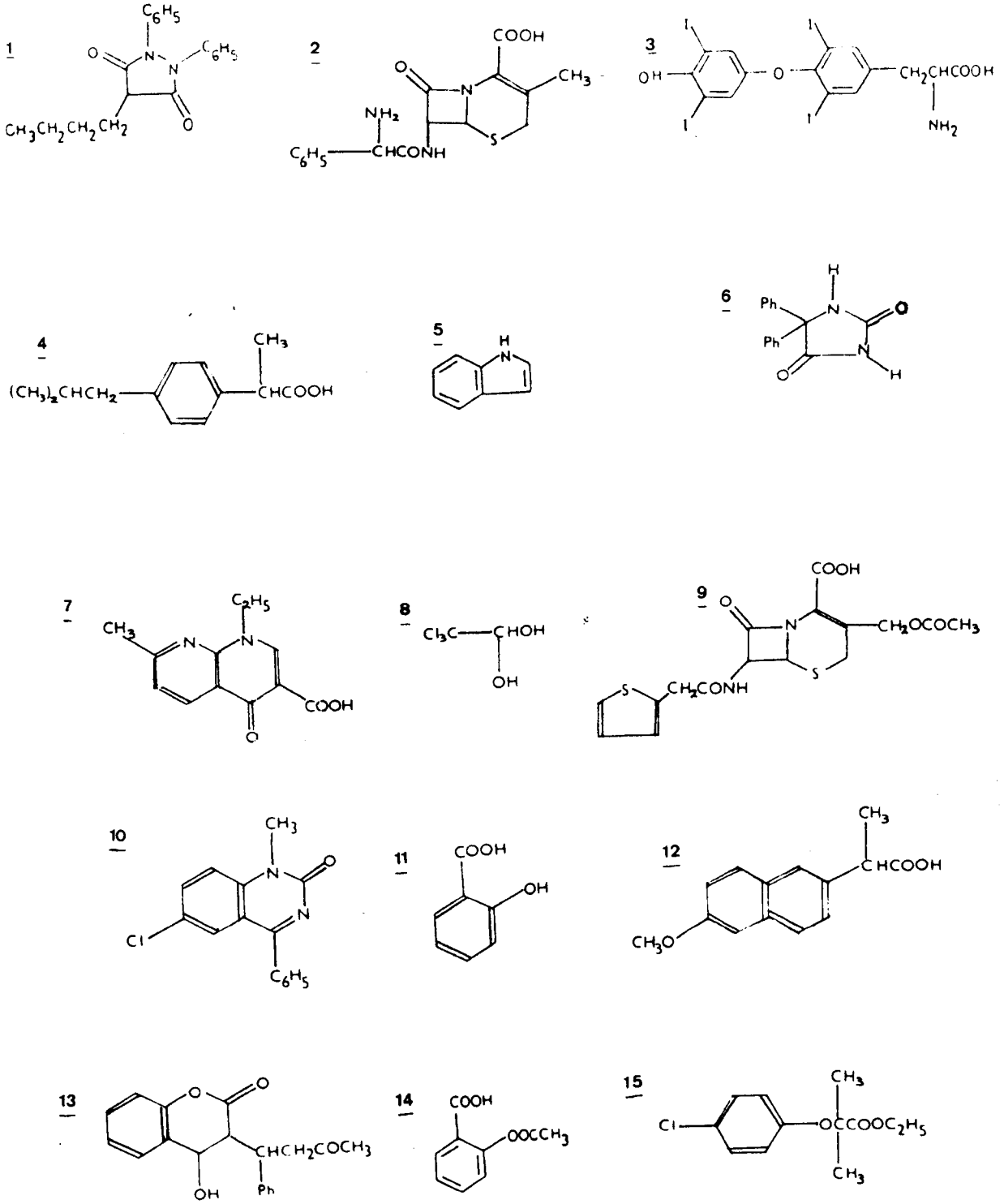


Fig. 1.12 Structures of some ligands which bind to albumin: 1) Phenylbutazone, 2) Cephalexin, 3) thyroxine, 4) Ibuprofen, 5) Indole, 6) Phenytoin, 7) Tolbutamide, 8) chloral hydrate, 9) Cephalothin, 10) Diazepam, 11) salicylic Acid, 12) Naproxen, 13) warfarin, 14) acetyl-salicylic acid, 15) Clofibrate.

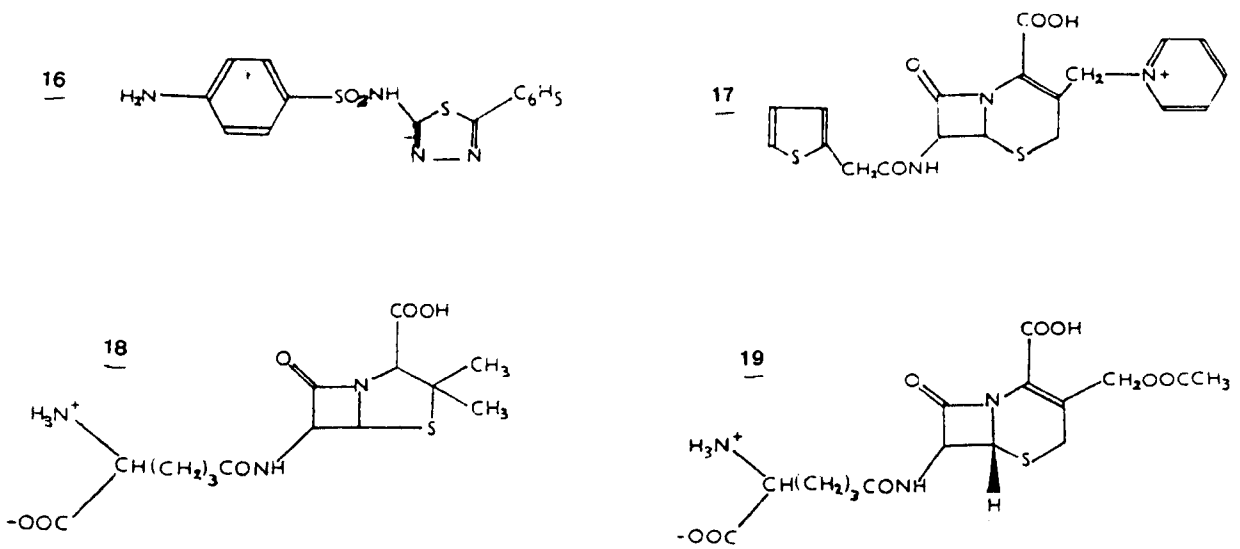


Fig. 1.12 Continued. 16) Sulphathidole, 17) Cephaloridine, 18) Penicillin N, 19) Cephalosporin C.

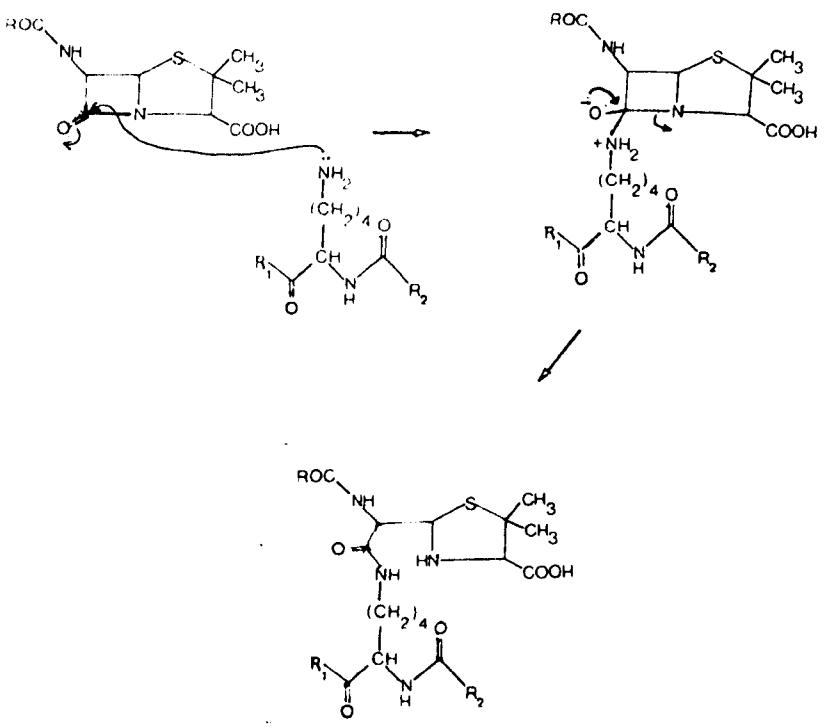


Fig. 1.13 The acetylation of HSA by penicillin.

1.2.5.6 Antibiotics

Sulphonamides bind with a fairly high affinity ($K_a = 10^2$-10^3 M^{-1}) to albumin (Hsu, *et al.*, 1974) at two or three main sites. Phillips et al. 1973 described a hydrophobic cleft for the primary binding site of penicillins; he noted that binding was due to a covalent attachment to a lysine residue which brought about a conformational change on binding four molar equivalents of penicillin. Hsu, *et al.* (1974) estimated the association constant as $K_a \simeq 3 \times 10^3$ M^{-1} and showed that halogen, methyl, benzene and methoxy substituents increased binding but amino groups inhibited it. Several penicillins have been shown to acetylate an ε-amino lysine residue and this destroys the β-lactam ring (Bundgaard, 1977) (Fig. 1.13).

Cephalosporins appear to bind at a hydrophobic site and again a cationic amino acid has been implicated, binding constants are $\simeq 10^3$ M^{-1}. Bruderlein, *et al.* (1981) have shown a similar acylation of a lysine residue, resulting in loss of the β-lactam ring in a series of antibiotics.

1.2.5.7 Implications of Drug Binding to HSA

Due to the wide range of ligands binding to albumin there is inevitably competition. Bilirubin can be displaced by sulphonamides and so potentiates its toxicity especially in the neonate where many drugs are only poorly bound and so effective drug concentrations may vary. An important factor

when considering drug competition is the amount of drug bound by albumin. A 1% displacement of a drug only 50% bound has little effect, but if a drug is 99% bound there is an effective doubling of the free drug concentration. This can have serious consequences; warfarin can be displaced by Phenylbutazone or Clofibrate to cause hyperprothrombinemia and spontaneous haemorrhages due to the raised level of the anti-coagulant. Bone marrow toxicity may result if metho-trexate is displaced by salicylates and sulphonamides. The volume in which the drug is contained is an important factor in the rise in effective drug concentration; the level of albumin synthesis is also important - in hypoalbuminemia (low albumin concentration) the percentage free drug is increased and so in liver disease or kidney failure the albumin binding of drugs is reduced and thus the effective free drug concentration is increased to possibly toxic levels. For example, the effective level of Phenytoin (an anti-convulsant) can rise by 30% in viral hepatitis. Drug treatment in hypoalbuminemia and combinations of drugs in therapy should be dictated by the free drug concentrations in the individual patients.

1.2.6 Dye Binding

Peters (1970) describes dye binding to albumin as being weaker than for fatty acids or bilirubin, with a number of sites which vary for different dyes. Possibly the most studied class of

dyes binding to albumin are the sulphophthaleins with $K_a \simeq 10^6\ M^{-1}$, such as phenolsulphonephthalein (phenol red), bromophenol blue, and bromosulphophthalein. Other dyes which are tightly bound include Evans Blue, Trypan Blue, Congo Red, and azobenzoates such as methyl red. Dye binding causes a configurational change in albumin, protecting it from denaturation and proteolysis. 1-Anilino-naphthalene-8-sulphonic acid (ANS) also binds to albumin in a funnel shaped primary site in subdomain 1C and at four other sites on the inner aspects of the three domains of albumin, and this dye has been used as a probe to follow fragmentation studies.

Bowmer and Lindup (1980) have calculated association constants for methyl orange, o-methyl red, and bromocresol green (see Table 1.1); Kragh-Hansen (1981) has shown that phenol red and bromophenol blue bind to the primary bilirubin site, and that they displaced each other and bromocresol green and bilirubin from this binding site. Sulphonobromophthalein binds to a different site, possibly at the secondary bilirubin site (Kawisaka, *et al.*, 1974) and it is feasible for a region to bind one molecule of Trypan Blue but two of ANS.

The binding of immobilised Cibacron Blue F3GA to albumin has been used in affinity purification of the protein from serum and appears to bind at the primary bilirubin binding site, and a more general conformational

change may occur (Lagercrantz and Larsson, 1983). The binding of bromocresol green has been incorporated into an albumin concentration assay (Doümas, *et al.*, 1971) although the complexation must be studied immediately to avoid interactions with other serum proteins. The concentration of albumin may be under-estimated due to the presence of Phenylbutazone and Clofibrate or in uremic patients (Calvo *et al.*, 1985). An improved assay using bromocresol purple has recently come into use.

1.3 VARIANTS OF SERUM PROTEINS

1.3.1 Introduction

Several blood proteins have been shown to have genetically inherited variant forms, some causing pathological deficiencies (see Table 1.3). Three such proteins will be considered here as examples of the nature of such mutations and their effect on function.

1.3.2 α_1-Protease Inhibitor (α_1-Antitrypsin)

The third most abundant class of plasma proteins by weight are the protease inhibitors and the best characterised of these is α_1-protease inhibitor (α_1-antitrypsin). Serum protease inhibitors control a variety of events associated with connective tissue turnover, coagulation, fibrinolysis,

Plasma protein	Genetic variants or polymorphism	Hereditary deficiencies*	Some conditions associated with abnormal plasma concentration	
			Decreased	Increased
Albumin	At least 20 variants	Analbuminemia	Nephrotic syndrome hepatic cirrhosis, glomerulonephritis, edema, kwashiorkor, malnutrition, trauma	Dehydration
α_1-Acid-glycoprotein	At least 3 variants		Inflammatory syndrome	Trauma, inflammation, rheumatoid arthritis, some malignancies
α_1-Antitrypsin	23 recognized alleles	Pulmonary emphysema, hepatic *cirrhosis* in children		Inflammation, infection
α_1-Fetoglobulin				Acute hepatitis, hepatic cirrhosis hepatoma pregnancy
Ceruloplasmin	At least 6 variants	Wilson's disease		
α_2-Macroglobulin	At least 2 variants, Xm system			Children have ~2.5 times the plasma concentration of adults
Haptoglobin	3 common and some rare variants	Hypo- and anhaptoglobinemia		Inflammation, infection, rheumatic fever, metastatic cancer
Transferrin	At least 20 variants	Atransferrinemia	Acute and chronic infections, liver disease	Pregnancy and chronic iron deficiency
Hemopexin			Hemolytic diseases, e.g., sickle-cell anemia, pernicious anemia, and paroxysmal nocturnal hemoglobinuria	Injection of porphyrogenic drugs (Chap. 3)
C-reactive protein				Acute infection
Lipoproteins	Several variants	Hypobetalipoproteinemia (low LDL), abetalipoproteinemia (*no* LDL), Tangier disease (no HDL), familial lecithin-cholesterol acyltransferase deficiency		Hyperlipidemias (Chap. G20)
Immunoglobulin	Several variants of γ, α, and μ heavy chains and κ and λ light chains	Agammaglobulinemias of IgG, IgA, IgM		Many infectious disorders

Table 1.3 Some serum protein variants and their effects.

complement activation and inflammatory reactions. As the name suggests, α_1-antitrypsin was first noticed due to its inhibitory effect on trypsin. However, it is effective against a range of serum proteases, its greatest inhibitory effect being on neutrophil elastase. It has for this reason been renamed α_1-protease inhibitor (α_1-PI).

α_1-Protease inhibitor is a single polypeptide of 53,000 molecular weight, with 10-15% carbohydrate content. At least twenty variant forms are known, the common form being called M and the phenotype $P_{I_{MM}}$. Variants are detected as slow or fast migrating components on electrophoresis and the slowest known variant $P_{I_{ZZ}}$ was discovered by Laurell and Erichsson (1963) and occurs in 1-2% of North Europeans but has a higher frequency in Maoris (8.2%) and Iranians (2.2%). Another variant is the S form and both these have been shown to cause reduced α_1-PI concentration in serum. There is a variant $P_{I_{(--)}}$ which is very rare with only twenty known cases, and which results in no α_1-PI synthesis. The α_1-PI concentrations for the various genotypes are: $P_{I_{MM}}$ 100%, $P_{I_{SS}}$ 54%, $P_{I_{MS}}$ 80%, $P_{I_{M-}}$ 50%, $P_{I_{MZ}}$ 60%, $P_{I_{SZ}}$ 33%, $P_{I_{ZZ}}$ 12% and $P_{I__}$ 0%.

In patients with less than 35% $\alpha_I P_I$, the occurrence of emphysema is very high and is thought to be caused by a lack of active inhibitor in serum; neutrophil elastase, being free to digest connective tissue, causes damage to the alveoli which appear to be

especially susceptible to elastolytic attack. In PI_{ZZ} individuals the probability of developing emphysema is twenty times that of normal individuals and amongst those PI_{ZZ} individuals who smoke, the mortality rate is 70% by the age of 50 years, due to the further inhibitory effect of the oxidants in cigarette smoke upon the small amount of active α_1-PI. Large amounts of α_1-PI are found in the hepatocytes of PI_{ZZ} individuals and it would appear that a point mutation in the protein sequence, the substitution of lysine for glutamate (a C → T transition) at residue 53, prevents 85% of the protein from obtaining the correct conformation and instead it aggregates to form inclusion bodies in the hepatocytes. In the PI_{SS} variant a glutamate to valine substitution at residue 131 occurs due to a T → A transversion in the mRNA. However, the reduction in serum levels of active inhibitors is less severe in this case.

Twenty per cent of PI_{ZZ} individuals also develop hepatitis, possibly due to the intracellular levels of non-functional Z variant α_1-PI, and rheumatoid arthritis may also result from this condition. The heterozygous individuals do not suffer any ill effects as the level of α_1-PI in the serum is not as drastically reduced.

1.3.3 Transferrin

Transferrin is the primary carrier of free

ferric ions in serum, and is responsible for their transport from the site of haemoglobin catabolism to the bone marrow and to storage organs such as the liver, so providing an efficient turnover of metabolic iron and preventing iron toxicity. Transferrin, previously termed siderophilin, has a molecular weight of around 77,000 daltons and is a single polypeptide chain with two binding sites for ferric ions, one at the N-terminal domain and one at the C-terminal domain, which appear to function independently. Transferrin is synthesised by the liver and has a plasma concentration of ≈ 2.5 g/L. Variants of transferrin have been detected in at least twenty cases, those with charge differences being disclosed as fast or slow migrating bands in the β-globulin region on electrophoresis. The variants are fairly equally divided between fast forms (Tf_B) and slow ones (Tf_D), compared to the common type (Tf_C). Several transferrin variants have been sequenced, the variant Tf_{D_1} has two glycine residues which have been converted to aspartate and asparagine respectively, and Tf_D, another slow variant, results from an arginine to lysine mutation (Putnam, 1975). No mutant form has yet been associated with a pathological condition although Evans, *et al.* (1982) found a variant with a defective C-terminal iron binding site. The isolated C-terminus appeared to be less stable than the normal and to have only one-tenth the association constant for Fe^{III}, though not influencing

the N-terminal binding (Evans, *et al.*, 1984). This variant may function as a monoferric transferrin but no pathological effects have yet been ascribed to this condition.

Atransferrinemia, the congenital absence of transferrin, has only rarely been reported. Heilmeyer, *et al.* (1961) detailed such a case with severe anaemia, frequent infections, impaired growth and the absence of immunologically detectable transferrin. Radio-labelled ^{59}Fe was cleared from plasma abnormally quickly and became localised in the liver, little being used for haemoglobin synthesis. Death resulted from hemosiderosis of heart muscle and visceral organs. Hypotransferrinemia may be found with other clinical conditions, for example, extreme proteinuria, but no genetic basis has as yet been postulated.

1.3.4 Haemoglobin

1.3.4.1 Introduction

Haemoglobin is not a plasma protein, being incorporated into the erythrocytes. However, haemoglobin variants have probably been the most studied of all variant proteins with over 300 types characterised. Consequently, the methodology used in the studies of these variants is of great use in considering other variant proteins, and they can provide a great deal of information about the types of mutation which can occur.

Haemoglobin is a tetramer consisting of monomeric globular polypeptides, around 140 amino acids in length with a subunit molecular weight of 16,000 daltons. Adult haemoglobin is made from two α and two β-subunits although other monomer subunits exist, the ζ and ε chains being formed in the early foetus and the γ chain in later development and in the neonate. The α and β-chains have seven and eight helices respectively, lettered A to H; the iron containing haem ring is positioned between the E and F helices, and one molecule of oxygen binds to each subunit haem group. Allosteric effects between the subunits result in sigmoidal binding affinity, releasing oxygen at low pressure in the tissues and binding it tightly in the relatively greater oxygen pressure of the lungs. There are several classes of haemoglobin mutation which result in altered physiological state and these are called haemoglobinopathies (Smith and Hill, 1983).

1.3.4.2 Haemoglobins with Altered Solubility

Sickle cell anaemia is probably the best documented disease caused by an alteration to the haemoglobin (Hb) molecule. The sickle cell haemoglobin (HbS), which contains a glu → val transformation at the sixth residue of the β-chain, aggregates on deoxygenation, resulting in the deformity of the red cells known as sickling, and rendering them susceptible to lysis, resulting in a haemolytic anaemia. The aggregation is thought to be due to the generation of a hydrophobic region

at the site of mutation which then adheres to a complementary hydrophobic region, residues 78-88, of the β-chain and results in clumping of the chains. Several other variants of this type are known. The heterozygote for HbS, having 50% normal haemoglobin, is not seriously anaemic but does have a degree of resistance to the malarial parasite, *Plasmodium falciparum*, which cannot survive in the sickled cells of the patient's sera.

1.3.4.3 Inclusion Body Haemolytic Anaemia

The formation of amorphous intra-cellular aggregates which deform the cells and predispose them to lysis can occur in haemoglobin variants such as $Hb_{Hammersmith}$. The aggregates, termed 'inclusion' or 'Heinz' bodies are caused due to the destabilisation of the oxyhaemoglobin state, resulting in reduced oxygen affinity and opening of the haem binding pocket with loss of the porphyrin group and subsequent aggregation of the free globin chains. This is termed inclusion body haemolytic anaemia, and in the above-mentioned variant is due to a phe → ser mutation at the β-chain 42nd residue.

1.3.4.4 Haemoglobins with Altered Oxygen Affinity

Haemoglobin variants exhibiting abnormally high oxygen affinity cause an absolute increase in the mass of red cells, termed polycythemia. This condition is due to the destabilisation of the deoxy-Hb form by disruption of the inter-subunit

interaction, for example in $Hb_{Kempsey}$, $asp\beta_{99}$ is replaced by asn and this prevents formation of a stabilising hydrogen bond between residue 99 and $tyr\alpha_{42}$ in the α chain. Similarly, destabilisation of the oxy-Hb state results in reduced oxygen affinity and cyanosis - a bluish discolouration due to the depletion of the oxygen supply in skin and peripheral tissues.

Five abnormal haemoglobins result in methaemoglobinemia where high concentrations of MetHb, which can no longer bind oxygen, are formed. Mutation of the distal or proximal histidines (α58 and α87) to tyrosine in HbM_{Boston} and HbM_{Iwate} results in this condition where the oxygen coordination site is blocked by a water or hydroxyl group. The homozygous state is unknown and is presumably fatal.

α Chain

					140								
A	...	Thr	Ser	Lys	Tyr	Arg	Term						
		ACC	UCC	AAA	UAC	CGU	UAA						
Constant Spring	..	Thr	Ser	Lys	Tyr	Arg	Gln	Ala	Gly	Ala	Ser	Val	Ala
			UCU	AAA	UAC	CGU	CAA	GCU	GGA	GCC	UCG	GUA	GCU
			Ser	Asn	Thr	Val	Lys	Leu	Glu	Pro	Arg	Term	
			UCC	AAU	ACG	GUU	AAG	CUG	GAG	CGU	CGG	UAG	

Fig. 1.14 Chain elongation in $Hb_{Constant\ Spring}$ due to a mutation in the termination codon (residue 142).

1.3.4.5 Types of Mutation

Variant proteins such as those causing the haemoglobinopathies can be the result of several types of error; deletion of the β141 residue, for example, results in the variant $Hb_{Coventry}$. Addition or removal of amino acids from the globin chain can be the result of a point mutation converting a termination codon into one specifying an amino acid and *vice versa*. In $Hb_{Constant\ Spring}$ the termination codon UAA is converted to CAA coding for glutamine and the chain is extended by 16 residues (Fig. 1.14). Several extended variants are listed in Table 1.4. Deletion of a single nucleotide can result in a "frame shift" mutation which again can result in different amino acid chains being constructed as in Hb_{Wayne} (see Table 1.4). In Hb_{Grady} duplication of part of the reading frame results in a tripeptide insertion in the sequence. Fusion of the different genes for Hb can occur due to unequal crossing-over and this can give rise to variants such as Hb_{Lepore} where the first third of the sequence is that of Hb δ-chain and the rest is like the β-chain (Winslow and Andersson, 1983).

The hereditary anaemias α and β-thalassemia are caused by defective chain synthesis due to deletions in the globin genes. In α-thalassemia deletions in both the α-genes encoded on a chromosome are common and a homozygote will usually die in infancy

Residue	*Name*	*Inserted residue*
α142–172	Constant Spring	Glu-Ala-Gly-Ala-Ser Val-Ala-Val-Pro-Pro-Ala-Arg-Trp-Ala-Ser-Gln-Arg-Ala-Leu-Leu-Pro-Ser-Leu-His-Arg-Pro-Phe-Leu-Val-Phe-Glu
α115–118	Grady	Gly-Thr-Phe repeated
α139–146	Wayne	Asn-Thr-Val-Lys-Leu-Glu-Pro-Arg

Table 1.4 Some extended haemoglobin variants.

due to lack of a functional α-globin subunit. In α-thalassemia$_{II}$ only one α-gene is usually affected and the condition is less severe ($Hb_{Constant\ Spring}$ is of this type). β-Thalassemia, resulting from deletions in the β-globin gene, is widespread in the Middle East, Mediterranean, Africa and Asia, and although the heterozygote is assymptomatic the homozygous condition is usually fatal if regular transfusions are not received and iron toxicity and secondary complications are also a problem. A milder form of β-thalassemia can occur where both α and β-thalassemias are co-inherited or where the foetal globins are still synthesised in adult life (WaiKan, 1983).

Consideration of the types of mutation known to occur in haemoglobins may be of assistance in

understanding variation in other plasma proteins.

1.4 VARIANTS OF HUMAN SERUM ALBUMIN

1.4.1 Introduction

Variants of serum albumin were first demonstrated by Scheurlen (1955) who noticed that the electrophoretic pattern of a Swiss German patient with unstable diabetes contained two albumin bands during periods of coma and precoma, though these coalesced on return to normal health. Knedel (1957) described this condition of two albumins in the serum as "Bisalbuminemia" and he showed it to be an hereditary condition and described two 'slow' variants (Knedel, 1958). Albumin variants, as with several other plasma proteins are classified by their mobility on electrophoresis compared to the normal band; hence a more anodic band is termed 'fast' and a more cathodic band, 'slow'. Nennstiel and Becht (1957) reported the first fast albumin variant. Wuhrmann (1959) re-examined Scheurlen's patient and found that this variant was now a permanent characteristic of her serum, and was also present in her father, brother and son. The early transient nature of the band was probably due to the inadequate separations obtained on filter paper electrophoresis, the double albumin being more apparent in the acidosis which accompanied the comatous state.

Earle, *et al.* (1959) described another slow variant bisalbumin in a Norwegian/American family;

they isolated the variant and performed peptide mapping, concluding that an acidic residue, aspartate or glutamate had been replaced by a basic one, probably lysine; the decrease in net negative charge giving rise to a cathodic variant (D. Gitlin, *et al.*, 1961). During the following few years other cases were discovered in families of various European nationalities, most being slow variants similar to that discovered by Earle. However, two fast variants were found, Albumin Reading (Tarnoky and Lestas, 1964) in an English/Welsh family and Albumin Gent in a Belgian one (Wieme, 1960). These variants were all at low frequency in the population (one in several thousand). However, in 1966 Melartin and Blumberg (1966a) reported a fast variant albumin among American Indians which was present in a higher frequency. The variant was originally found in Naskapi Indians and so was named Albumin Naskapi. The tribe was small and inbred and homozygous individuals, possessing only the variant albumin, were found for the first time. The term bisalbuminemia was no longer appropriate and Melartin (1967) proposed the name alloalbuminemia to describe the condition and this has subsequently replaced the previous terms, bisalbumin, para-albumin and double or split albumin, which were really only accurate in the heterozygous state. A second polymorphic variant, this time of the slow variety, was discovered in Mexican Indians (Melartin and Blumberg,

1966b) and was also shown to occur in the homozygous state.

1.4.2 Albumin Variant Classification

There are over eighty known albumin variants in the literature and an exhaustive account of their discovery and properties will not be attempted here, the field being reviewed excellently already by Schell and Blumberg (1977) and Tárnoky (1980). A systematic nomenclature has been proposed, the common form of albumin being called Albumin A and the slow variant of Earle et al (1959) being given the name Albumin B. Other variants are named after their ethnic, geographic or laboratory origin using the standard nomenclature used in several other serum protein variants, for example, Albumin Stirling and Albumin Gent. In other cases, the genotype is expressed as a superscript, for example, an albumin Naskapi heterozygote would be termed $Al^{A}Al^{Na}$. The many Italian variants use the location of the laboratory making the discovery and the family's place of origin, with the lettering system of Italian car number plates, for example Al.SO/BS for Albumin Sondrio/Brescia.

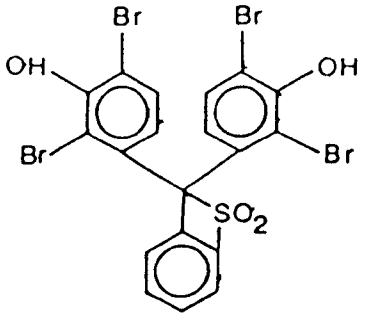
The qualitative terminology 'very slow, slow, normal, fast and very fast' first used to describe the migration of variants, soon became inadequate and Weitkamp, *et al.* (1973) devised a starch gel electrophoretic procedure

for characterising variant forms at three pH ranges; sodium acetate/EDTA pH 5, Tris/lithium/succinate/citrate pH 6 and Tris/EDTA/borate pH 6.9. Some of the later variants differ in mobility in only one of the three systems, and it would seem that fast variants separate better at pH 5 and slow ones at higher pH, migration distance being with respect to the origin or an artificially enhanced transferrin peak.

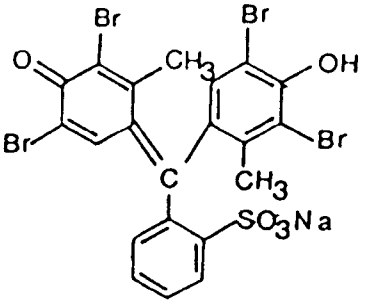
1.4.3 Genetics of Alloalbumins

More recently Tárnoky (Curnow, *et al.*, 1978) has proposed a dye-binding procedure which utilises six ligands in several media; this allows for the detection of variants differing only in their dye binding affinities and has now been used to more fully characterise a number of variants (Fig. 1.15).

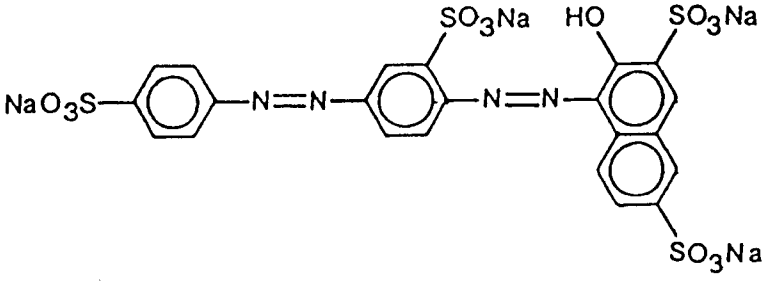
From those variants where it has been possible to trace ancestries it has been shown that alloalbuminemia is a co-dominant autosomal trait, inherited in a simple Mendelian manner with unreduced expression of both genes, resulting in 50:50 ratios of variant to normal in most heterozygotes. The only exceptions known are Albumin Luarca which falls to 29% on freezing (Izquierdo, *et al.*, 1971), some dimeric mutants, where the percentage of variant to normal can vary and Albumin Vancouver (Frohlich, *et al.*, 1978) where the ratio $Al^{A}:Al^{Va}$ is 35:65. Linkage studies performed on albumin indicate that only the Group specific component (Gc) is



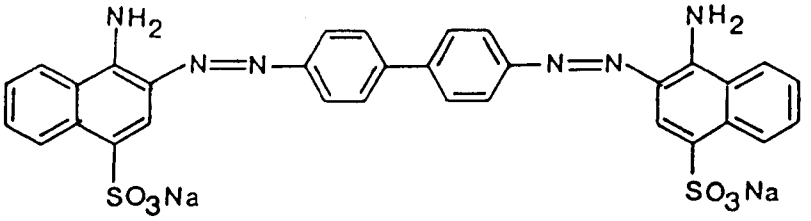
bromophenol blue



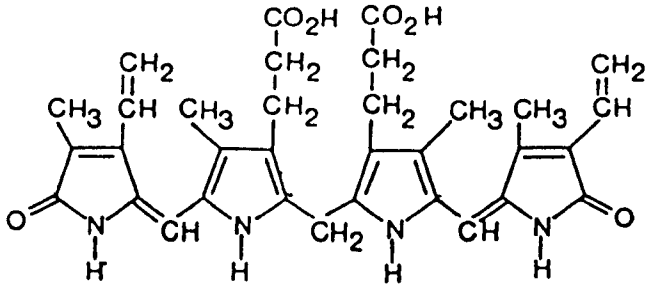
bromocresol green



Ponceau S



Congo Red



bilirubin

Fig. 1.15 Classification of variants by dye binding: structures of the RBH test dyes.

co-inherited to any degree.

1.4.4 Immunology

Most experiments to determine the antigenicity of albumin variants have shown them to be identical to the normal form, using double immunodiffusion, immunoelectrophoresis and two-dimensional immunoelectrophoresis with mono-specific antihuman albumin antibody from a number of mammals. The only contradictory report is that of Robbins, *et al.* (1963), who reported antigenic identity at a 1:1 ratio antibody:albumin, but two separate arcs if the serum was diluted 1 to 30. However, this may be an artefact due to excess antibody, (Payne and Dickinson, 1967). In addition, Margni, *et al.* (1970) found slightly different antigenic properties in two alloalbuminemic forms.

More recently Lapresle and Doyen (1983) have raised monoclonal antibodies to a proteolytically derived 6,000 dalton fragment of albumin, comprising the last small loop (residues 558-67), the disulphide bridge 514-59 and residue 570. Using this antibody they have shown that it was necessary to add 120 times the amount of albumin B compared to albumin A to obtain 50% inhibition of labelled-albumin detection, indicating that residue 570, which is different in albumin B, is important in the monoclonal antibody binding site. It therefore seems that monoclonal antibodies may be

able to distinguish between normal and variant albumins.

1.4.5 Albumin Variant Polymorphism and Distribution (Table 1.5)

1.4.5.1 Amerindian Variants

Genetic polymorphism is the simultaneous occurrence in the same habitat of two or more discontinuous forms of a species in such proportions that the rarest of them cannot be maintained merely by recurrent mutation (Ford, 1965). Albumin variants are classed as polymorphic if they have a frequency > 1% in a population. There are four albumin variants which can be regarded as polymorphic, Albumins Mexico, Naskapi, Makiritare and Yanomama-2. These four variants occur almost exclusively in the American continents and represent two types of polymorphism:

(a) Yanomama-2 and Makiritare reach polymorphic frequencies only in the ethnic groups in which they occur and are thus termed restricted variants.

(b) Albumins Naskapi and Mexico are classed as dispersed variants as they occur in frequencies above 1% in several ethnic populations over a wide geographical area (Schell and Blumberg, 1977). Albumin Naskapi is found predominantly in Northern Amerindians, occurring at high frequencies among the Naskapi and Montagnais Indians of Quebec, also among some Albertan and Alaskan

TABLE 1.5 Some albumin variants and their distribution (Adapted from Sellers and Koch-Weser (1977) and Tárnoky (1980)

Name of Variant	Ethnic Origin	Properties
Ann Arbor	European	Slow migrating. Mutation located
B	European	Slow, may have subtypes
Birmingham	Indian	Slow, similar to Kashmir
Christchurch	New Zealand	Slow, proalbumin variant
Gainesville	European (U.S.A.)	Slow, identical to Christchurch
Gent	European	Fast migrating
Kashmir	Indian	Slow, identical to Afghanistan
Lille	French	Slow, proalbumin variant
Luarca	Spanish	Slow, unstable to freeze-thawing
Makiritaire	S. American Indian	Slow, dimeric
Mexico	N. American Indian	Slow, polymorphic, mutation pinpointed
Mi/Fg	Italian	Fast, homozygote
Naskapi	N. American Indian	Fast, polymorphic
Oliphant	European	Slow, identical to Ann Arbor
Parklands	New Zealand	Slow, mutation located
Pollibauer	European	Slow, identical to Lille
Reading	European	Fast,migrating
Stirling	Scottish	Slow, ratio in serum variable
Warao	S. American Indian	Slow, dimeric
Yanomama	S. American Indian	Slow, diemric

Indian groups and at lower frequencies in several other Indian tribes in the United States and Canada. Albumin Mexico is distributed amongst the Middle American and South Western United States Indians, although occurring in other tribes at lower frequencies. Albumins Naskapi and Mexico are both found in some southwestern tribes such as the Apache and Navajo Indians.

These variants have been studied extensively in order to elucidate the anthropological origins of the tribes and the findings are consistent with archaelogical evidence for the relationships between the tribes. Franklin, *et al.* (1980b) have shown that Albumin Naskapi is identical to a variant found in a community of Eti Turks in Asia Minor; this variant was previously called Albumin Mersin. In view of the limited distribution of Albumin Naskapi, which has not been found to occur in other racial groups the suggestion is made that these two communities may have descended from a common ancestral stock; skeletal, morphological and genetic evidence support the hypothesis that American Indians may have descended from a Mongoloid population which entered the New World from Central or Eastern Asia over the Bering Straits.

Several other Amerindian variants are known, including Belem I, II and III (I and II slow, III fast); Belem III however, is indistinguishable from two other Amerindian albumins, Maku and

Makiritare-2 (Salazano, *et al.*, 1974). Helena, *et al.* (1975) described ten rare Brazillian variants and compared them to seven known variants, several showing unique mobilities on electrophoresis.

1.4.5.2 European Variants

The frequency of albumin variants in Europe is low (1 in 1,000 to 1 in 10,000); however, slow variants of the B type occur in several countries, for example, in the region of Stuttgart where there are slight differences which are classified as sub-types within albumin B (Ott, *et al.*, 1975). A fast variant, Albumin Syracuse is detectable only on electrophoresis at pH 5.4. Italy has the highest number of classified variants in Europe including two homozygotes, Albumin MI/TN (Milano/Trento) a slow variant, and Albumin MI/FG (Milano/Foggia) a fast one (Vanzetti, *et al.*, 1979).

1.4.5.3 Southern Asia

Alloalbuminemia has been found in natives of Kashmir (Tarnoky & Dowding, 1969), Afghanistan, the Punjab (Dash, *et al.*, 1982), Vellore (Hill, 1975) and other parts of India as well as occurring in Indian, Malayan and Chinese populations in other countries (Welch and Lie Injo, 1972). Several Japanese variants are known (Nishimukai, *et al.*, 1982), the inhabitants of Hiroshima and Nagasaki showing a frequency fifteen times that in other parts of Japan. The explanation for this may not be straight-

forward as no increase in variant albumins is seen in those exposed to fall-out from nuclear tests in the Marshall Islands (Neel, *et al.*, 1976). Albumin New Guinea has the same migration as Albumin Reading.

1.4.5.4 African Variants

Few African variants are known, Albumin Cayemite, a fast Haitian Negroid variant (Weitkamp, *et al.*, 1969) and two or three more being the only ones recorded. Smith, *et al.* (1976) may have found a silent variant of African origin.

1.4.6 Structural Properties of Variant Albumins

1.4.6.1 Assessment of New Variant Albumins

Over 80 albumin variants are known (Schell and Blumberg, 1977). These are, however, unlikely to be all unique; the data suffers from ambiguities such as those variants which have been renamed, those which are rediscoveries of existing variants, those which are not given a name or are used in comparative studies only and are not fully classified individually. Schell and Blumberg (1977) compared variants, giving geographical location, synonyms, and ethnic origins.

Weitkamp, *et al.* (1973) listed twenty three types of variant and their population distributions, all of which were separable using the three buffer starch gel electrophoretic system. The number of new types has grown since then, however it is difficult to assess a new variant without the whole range of known

variants.

1.4.6.2 Types of Variant Albumin

The majority of variants are monomeric, probably arising from a single point mutation at a single amino acid. Several dimeric variants do exist, although the percentage in the dimer form is usually low, suggesting that the mutation is in a monomeric variant which subsequently tends to aggregate. Examples of this are Albumins Makiritare (Warao) and Yanomama. The molecular weight of variants can be assessed on SDS-PAGE, some dimeric albumins reverting to monomer with 0.1 M mercaptoethanol but others being resistant to such reduction requiring 8 M urea to dissociate them (Jamieson and Ganguly, 1969). Most variants occur in a 50:50 ratio and are stable to heating at 56°C and repeated freeze-thawing. The percentage of dimeric variants often varies under these conditions and this has been used as a diagnostic test, Albumin CN/BL (Vacca, *et al.*, 1974) constituting only 21% of the total albumin and Norwegian variant RIH (Chapman, *et al.*, 1978) occurring in a 64:36 ratio Alb^{A}:Alb^{RIH}. The Scottish variant Albumin Stirling (Curnow,et al 1978) varies from 33-41% dependent upon drug treatment whereas the Fijian variant, Albumin Vancouver (Frohlich, *et al.*, 1978) occurred in the ratio Al^{A}:Al^{Va} 35:65; differential synthesis has been proposed to explain this effect.

Izquierdo, *et al.* (1971) has reported a

Spanish variant, Albumin Luarca, which is unstable to freezing, falling from 44 to 27% on prolonged storage and is also decreased by rapid freeze-thawing. Albumin Makiritare (Warao) has been shown to constitute 30% of serum albumin (Arends, *et al.*, 1969), but reverted to the mobility of normal albumin on heating to 56°C for one to two hours, this suggesting the variant band was due to dimer.

Electrophoretic techniques characterise only those variants which have an altered charge from normal albumin. Nei and Chakraborty (1976) have postulated that a change in net charge occurs only in one-quarter to one-third of all substitutions, so that the number of silent albumin variants, that is, those not causing any difference in charge, could be two or three times the number detectable by electrophoresis. Two possible examples of the silent variants may have been found by Smith, *et al.* (1976) and Walsh, *et al.* (1983). Smith, *et al.* reported a family with high plasma zinc level whose excess zinc was albumin-bound, possibly due to an electrophoretically silent albumin variant, as 80% of serum zinc binds to albumin. A similar result has been reviewed by Tárnoky (1980) for a raised indocyanine green binding, coupled to normal binding of bromosulphophthalein described by Kawasaki, *et al.*, 1973. A variant albumin, IMVS was reported by Walsh, *et al.* (1983) solely on the basis of increased bromocresol green and bromocresol

purple binding in the presence of excess Pegosperse detergent and merthiolate. They noticed decreased HABA binding and found six other patients with similar findings in 21 months. They isolated the abnormal dye-binding albumin from a protein with normal binding on DEAE-Sepharose. Albumin IMVS was not detectable on gel electrophoresis but was present in several members of the family.

1.4.6.3 Sequence Analysis

The albumin B variant of Norwegian/American origin has been shown by Gitlin, *et al.* (1961) to have an equivalent of two extra protons, which was thought to be an aspartate/glutamate to lysine mutation. Winter, *et al.* (1972) followed a similar regime to obtain sequence data on two B type slow variants, Albumin Ann Arbor (a Danish variant) and Albumin Oliphant (of German origin). Variant albumins were isolated by ion-exchange chromatography and the abnormal peptides, located by tryptic/ chymotryptic digestion and peptide mapping, were sequenced by the Edman procedure. The abnormal peptide was found to be Ala-Lys-Glu-Gly-Lys-Lys-Leu compared to Ala-Glu-Glu-Gly-Lys-Lys-Leu in the normal albumin. This mutation Glu → Lys has been subsequently located in the albumin sequence at residue 570. Both albumin variants showed the same mutation and in view of the similarity to Earle's variant this has become the accepted mutation resulting in albumin B.

Franklin, *et al.* (1980a) reported a new slow albumin variant in a sub-class of Albumin Mexico - termed Mexico-2. By use of cyanogen bromide fragmentation and acid-Triton X 100-urea gel electrophoresis the mutant fragment was isolated and sequenced. They found the variant was due to a mutation from aspartate to glycine at residue 550, part of the long chain fatty acid binding site.

In the same year Franklin, *et al.* (1980b) isolated a mutant fragment from the fast variant Albumin Naskapi, and located the mutant in the region residues 373-89. They also pinpointed the mutation site of a slow Turkish variant, Albumin Adana, in the region 447-548 and hence distinct from the albumin B site.

A New Zealand variant, Albumin Christchurch, was characterised as a slow variant by Brennan and Carrell (1978). On sequencing the albumin variant they found the proalbumin hexapeptide sequence was still intact, but there had been a mutation in the last residue from Arg-Gly-Val-Phe-Arg-Arg to Arg-Gly-Val-Phe-Arg-Gln. The hexapeptide, normally excised by an Arg-Arg specific protease, possibly Cathepsin B, (Judah and Quinn, 1978) had not been recognised and removed in Albumin Christchurch, the proalbumin being secreted into the bloodstream. In the heterozygote, equal amounts of normal and variant albumins were present in blood. Further support for the proposed theory of propeptide

cleavage deficiency was gained when another proalbumin was discovered by Rousseaux's group (Abdo, *et al.*, 1981) in a serum sample from Lille, France. The slow variant was separated by preparative cellulose acetate electrophoresis and found on sequencing to have an N-terminal peptide - Arg-Gly-Val-Phe-His-Arg - differing from the normal albumin at residue -2, an arginine to histidine mutation; the arg-arg sequence having changed, the propeptide remained intact and the proalbumin was secreted intact into the bloodstream. Limited digestion with trypsin *in vitro* resulted in removal of the propeptide, using a less specific proteolysis (Fig. 1.16).

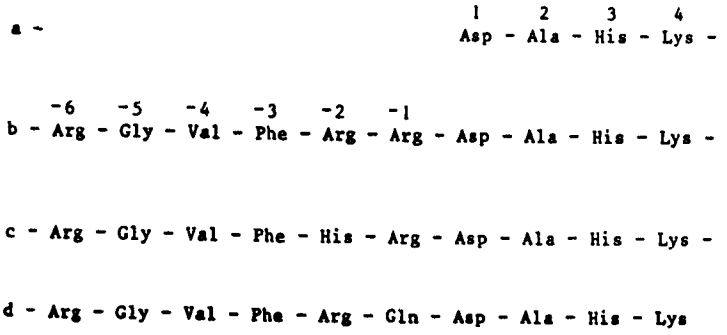


Fig. 1.16 Comparison of human proalbumin variants.
(a) Normal albumin N-terminal sequence.
(b) Sequence of normal human proalbumin (Dugaiczyk, *et al.*, 1982).
(c) Sequence of Proalbumin Lille (Abdo, *et al.*, 1981).
(d) Sequence of Proalbumin Christchurch (Brennan and Carrell, 1978).

Subsequent to the discoveries of Albumins Christchurch and Lille two other variants of different ethnic origins have been sequenced and shown to be identical to them. Fine *et al.* (1983) reported the amino acid sequence of Albumin Gainsville, an American variant of Irish descent, as being the same as that of Albumin Christchurch and the slow albumin variant Pollibauer, of Austrian descent, was identical to that of Proalbumin Lille (Galliano, *et al.*, 1984). The same mutation occurring twice in distinct populations may suggest that some other variants in the list of Schell and Blumberg may be identical also.

More recently, P. Iadorola, *et al.* (1984) isolated a mutant peptide from the serum of a patient with the Italian variant Mi/Fg (Vanzetti, *et al.*, 1979). Sequencing of the variant revealed a mutation of Lys → Glu at residue 573, resulting in an increase in net negative charge and giving rise to a more anodic fast variant (Iadarola, *et al.*, 1985).

During the compilation of this review a new variant has been reported in the literature. Brennan (1985) has isolated a slow variant, Albumin Parklands, from a 77 year old male Caucasian heterozygote by chromatography on DEAE-Sephadex eluting with a pH gradient from 5.2 to 4.5. After tryptic digestion of the S-carboxymethylated albumin and using reverse-phase high performance liquid chromatography to separate the fragments, Brennan discovered a variant peptide consisting of residues

360-72, which had a histidine substituted for aspartate at residue 365. This mutation was not shown to cause any ill effects in the patient but did show resistance to partial acid hydrolysis due to loss of the susceptible asp-pro sequence.

In Table 1.6, the point mutations of known variant albumins are presented with the nucleotide transformations in each case. In each of these assignments the mutation requiring a single base change has been assumed to be the one which has occurred. All but Albumin Parklands are thought to be the result of transitions, G → A in all the slow variants, and A → G in the fast Mi/Fg variant. Albumin Parklands is the only case of a transversion yet discovered, from G → C. Whether this trend is significant cannot be determined until further data on albumin mutants can be obtained.

1.4.7 Abnormal Properties of Albumin Variants

1.4.7.1 Disease States

No disease or clinical condition has been linked to the possession of a variant albumin, even in the homozygous state. Variants are often found initially during hospitalisation or treatment for some medical problem. However, these are not restricted only to those possessing a variant albumin, for example, a hand defect found in two bisalbuminemic members of a family was also found in those members with

Genetic Mutations In Known Variants

Albumin Variant	Amino-Acid Residue	Amino-Acid alteration		Codon alteration	
		Normal	Variant	Normal	Variant
B	570	glu	lys	GAG	AAG
Christchurch	-1	arg	gln	CGA	CAA
Lille	-2	arg	his	CGT	CAT
Mi/Fg	573	lys	glu	AAA	GAA
Mexico-2	550	asp	gly	GAT	GGT
Parklands	365	asp	his	GAT	CAT

Table 1.6 Genetic mutation of human variant albumins.

only normal albumin (Franglen, *et al.*, 1960).

Several incomplete accounts of diabetes mellitus and also raised serum cholesterol levels have been associated to alloalbuminemia, although chance association cannot be excluded. Tárnoky (1980) referred to a platelet defect in an Australian family which segregated in the same members as a slow alloalbumin with raised thyroxine binding and decreased binding of bromophenol blue (Clancy and Firkin, 1974). This is unlikely to be due to chance, but whether the conditions are genetically linked or actually caused one

by the other is not clear.

1.4.7.2 Differences in Ligand Binding

1.4.7.2.1 Metal Ions

Defects in the N-terminus of albumin have been shown to result in Cu^{II} and Ni^{II} binding deficiencies. Brennan and Carrell have shown that this primary binding site is missing in Proalbumin Christchurch and Fine, *et al.* (1983) showed that Albumins Gainesville and Pollibauer have defective binding of ^{63}Ni. This defect in the divalent metal ion site is not seen in any other alloalbumin type other than proalbumins and so it would seem that the elongation of the N-terminus blocks metal ion binding at the primary site.

1.4.7.2.2 Drugs

Wilding, *et al.* (1977) have shown that the binding of warfarin to heterozygote and homozygote patients, in both sera and isolated protein, was greater for albumin B than albumin A but less for both Albumins Naskapi and Mexico. The differences were sufficient to have a pharmacological effect and care is needed in the administration of drugs to such patients. Schell and Blumberg (1977) suggest that the reduced binding of warfarin to albumin variants may have been an advantage in prolonged exposure to hallucinogens over generations.

Blumberg (1969) has suggested that neonatal kernicterus might be a problem in those patients with albumin variants; several dye binding studies

Royal Berkshire Hospital (Curnow et al 1978)

after the Tarnoky RBH schedule have been performed on various variants some proteins showing slight differences. The quantitative work of Lorey et al.(1984)on Yanomama-2 showed these Indians to have decreased binding capacity for bilirubin and hence increased likelihood of bilirubin toxicity. Sarcione and Aungst (1962) found an albumin variant which bound high levels of ^{131}I-thyroxine in all those affected by it; Tarnoky and Lestas (1964) have also found Albumin Reading to bind more thyroxine than albumin A. Barbaree and Decker (1971) showed that normal albumin bound more thyroxine than their variant. Albumins Naskapi and Mexico bind thyroxine in a manner which closely resembles that of normal albumin.

THYROXINE BINDING OF ALBUMIN BANDS IN BISALBUMINEMIC SERA*

Albumins	Thyroxine binding
Slow Al, A	S only
A, Reading	A < Re
A, Naskapi	A = Na
Mexico, A	Me = A
A, fast Al	A > F
Slow Al, A	S = A
Slow Al, A	S = A
Vancouver, A	Va = A

* The results are taken from published papers on studies where test conditions may differ. Cathodic (slow) albumins are printed to the left, fast variants to the right, of albumin A.

TABLE 1.7 Thyroxine binding: properties of some variant albumins.

Competition for albumin sites has already been referred to as important in drug therapy. However, the effects of variants on drug therapy requires further study, although it may well be significant especially for polymorphic variants.

1.4.7.2.3 Dye Binding

The binding of dyes to albumin has been used extensively to characterise variant types. The ligands of the Royal Berkshire Hospital (RBH) test include; Ponceau S, bromocresol green, HABA, bromophenol blue, congo red and bilirubin, and the qualitative binding of these drugs provides a more effective method of classifying variant types than electrophoretic mobilities alone, the binding of these ligands indicating the relative affinities of the different binding sites. Tárnoky has proposed that bromophenol blue binds less to slow variants and more to fast variants than to the normal albumin, however, Wieme, *et al.* (1975) has criticised these findings as being an artefact of the streaming of bromophenol blue to the anode. The binding of a ligand to variant albumins may vary in different media and pH values and so direct comparison of results must be made cautiously. However, some variants have been shown to have differing dye affinities to albumin A.

1.4.7.3 Metabolic Studies

Bianchi, *et al.* (1974) performed simultaneous metabolic turnover studies of the two albumins from a

bisalbuminemic patient, labelling albumin A with ^{131}I and the albumin variant with ^{125}I. They found that the turnover of the albumins over eight days was almost identical for normal and variant forms.

1.4.8 Transient Bisalbuminemia

1.4.8.1 Introduction

In 1964 Gabl and Huber described the case of an eleven year old boy with ascites who had a fast migrating albumin variant as well as albumin A. The fast variant was immunologically albumin and monomeric but occurred in serum in the ratio Alb^A:Alb^F 3:1 and in the ascitic fluid as Alb^A:Alb^F 1:2, but did not occur in the urine. After treatment for the ascites the fast variant disappeared and was absent in subsequent checks; the albumin did not occur in any other members of the family and the cause of this 'transient' bisalbuminemia was not established.

1.4.8.2 Penicillin Therapy

The binding of some ligands to albumin can alter the electrophoretic mobility of the albumin band and the transient band is often present as less than 50% of the total albumin. Several such cases are known (Tárnoky, 1980) and the best defined is that for penicillin. Arvan, *et al.* (1968) discovered that patients receiving high dosages of penicillin G showed a transient fast albumin band on screening of their serum; this effect was reproducible *in vitro* and the

term 'transient bisalbuminemia' was used to describe it. Formation of a fast band *in vitro* did not occur if the penicillin was pre-treated with penicillinase, but the penicillinase had no effect if used after the albumin complex was formed. It would therefore seem that the β-lactam ring is necessary for the interaction with albumin. The condition is not associated with permanent alloalbuminemia; heterozygous Naskapi serum when treated gave four bands. The albumin-bound penicillin is inactive although other binding properties of the albumin are not affected. Porta, *et al.* (1974) standardised the conditions for *in vitro* production of penicillin-albumin complexes and showed that penicillinase present as a ratio 1:4 penicillinase:penicillin prevented formation of the fast band; penicillinase-producing bacteria have a similar effect, and the inhibition of albumin-penicillin complexes has been proposed as an assay for penicillinases in pathogens (Porta, *et al.*, 1976). The binding of penicillin is thought to be by covalent modification of the lysine residue with simultaneous destruction of the β-lactam ring (Bundgaard, 1977). Lapresle and Wal (1979) separated normal and penicillin-induced fast albumin from a patient and showed that penicilloyl functions occurred in far greater numbers on the fast variant. The amount of penicillin involved in such binding is small and appears to have no serious effect on the

dosage in the patient.

1.4.8.3 Pancreatitis

The presence of fast albumin has been reported in cases where plasma amylase is high. Stoodley and Rowe (1970) described bisalbuminemia in a patient with hyperamylasemia and pancreatitis where the fast band was not immunoelectrophoretically albumin and faded over the course of treatment. Tárnoky (1980) reviewed the case of a fast band due to pancreatitis where the albumin pattern obtained was consistent to that of tryptic digestion, and it may be that the transient albumins detected in such conditions are the product of proteolytic digestion of normal albumin by pancreatic enzymes. Rousseaux, *et al.* (1976) showed differences in the C-terminii of albumin A and a transient albumin consistent with proteolytic digestion by elastase or chymotrypsin. Vaysee, *et al.* (1981) described a case of pancreatitis in a bisalbuminemic patient giving rise to three albumin bands, the modified slow band becoming merged with albumin A.

1.4.8.4 Albumins in Other Diseases

There are several reports of albumin modification in disease states; bisalbuminemia was shown in two patients with immunological abnormalities, one suffering from myasthesia gravis and the other from myeloma. Although no definite explanation of this concurrence could be given,

proteolytic effects may have caused one or both abnormal protein bands.

Albumin is not usually heavily glycosylated. However, Candiano, *et al.* (1984) showed extensive microheterogeneity in diabetic patients due to the glycosylation of up to twenty amino acids; however, the degree to which glycosylation occurs normally is a matter of recent speculation. Gentou and Plazzonet (1978) studied a slow variant in a 49 year old man dying with reticulosarcoma, present at 44% in serum and cerebrospinal fluid. On amino acid analysis the slow band had ten glu → lys modifications and five ala → pro causing a more cathodic albumin. This may have been due to inversion of a segment of cistron (see Fig. 1.17). Borisenko and Troitskii (1984) have also reported modifications in the albumin of cancer patients, with reduced numbers of unmodified tyrosine hydroxyl groups and with alteration of the free thiol residue causing inhibition of polymerisation. They also found that 50% of the molecules had a glycosylated N-terminus, and a modified lysine at the fourth residue also occurred. In both these reports the changes are probably not inherited and have most likely been due to the serious pathological conditions of the patients;

Foster (1977) described albumin

microheterogeneity in normal patients and the causes of this must be determined before an accurate assessment of the two cases discussed can be made.

1.4.9 Analbuminemia

Analbuminemia, the apparent absence of albumin from the serum, has been described in only twenty cases since its discovery by Bennhold, *et al.* in 1954. This discovery was in a 31 year old woman who had a genetically inherited absence of serum albumin. Most cases have been in the White population though this may merely reflect differences in the availability of medical care. The lack of albumin has been partly balanced by increases in globulins to three or four times their normal level. High cholesterol and triglyceride levels have been found and serum calcium is also reduced in some analbuminemics. In the absence of albumin fatty acids can bind to other serum proteins with a lower affinity although bilirubin binding is more difficult to replace.

Some very reduced levels of albumin are detectable by rocket immunoelectrophoresis, being around 150-400 mg/L. Analbuminemia can be the result of two different conditions. Christen and Franglen (1972) found a patient with only 38 mg/L free albumin. However, on gel filtration and lyophilisation a large amount of albumin was found which had been complexed to macroglobulin and the total level of albumin was approximately normal, although whether the complexed

Fig. 1.17 Inversion of cistron possibly the cause of the variant albumin of Gentou and Plazzonet (1978).

Intron H1

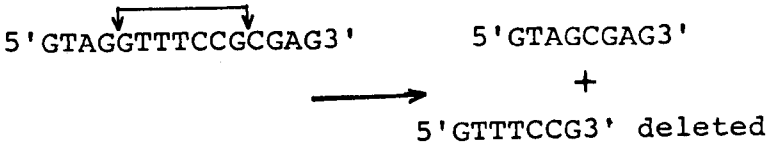


Fig. 1.18 Deletion in analbuminemic rat DNA (Esumi, *et al.*, 1983).

albumin was physiologically functional was not known. This type of masking of albumin may occur in other cases of analbuminemia but genetically reduced synthesis of albumin may be the correct explanation of others. The small amount of free albumin appears to have a half-life six times that of normal albumin in the body; if levels are increased to normal by transfusion reversion to the normal rate of catabolism will occur in some but not all patients (Gitlin and Gitlin, 1975).

Avery, *et al.* (1983) have shown that the DNA of an analbuminemic patient did not differ in its gross structure from that of normal patients and so no major deletion of the gene had occurred as in the thalassemias. Nagase has succeeded in breeding a strain of analbuminemic rats (NAR) and studies of these have shown there to be a seven base pair deletion in the intron H1 of the gene for albumin (see Fig. 1.18). This is thought to block mRNA splicing and prevent expression of the albumin gene (Esumi, *et al.*, 1983). No similar changes in the human gene have yet been reported. The condition is thought to have autosomal recessive inheritance because of its rarity and consanguinous occurrence. Clinical symptoms of analbuminemia are surprisingly few and this has led Cohen (1965) to suggest that albumin is not an essential protein. The main effect found is Oedema due to drop in osmotic pressure, and other symptoms may include fatigue, diarrhoea and fainting spells. Several patients have had more serious illnesses but these have been unassociated to the albumin defect. The skin contains 11% of the total

body albumin and dermatitis might be expected and is noted as occurring by Tárnoky (1980).

Studies of Nagase analbuminemic rats (NAR) have also shown no severe pathological effects. Plasma clearance of bilirubin is increased fourfold over normal rats, due to a significant reduction in serum binding capacity (Inoue, *et al.*, 1985). Takikawa, *et al.* (1985) have shown a marked decrease in bile acid levels in NAR, the unbound fraction in serum being ten fold greater than for normal Sprague Dawley rats (SDR). This also suggests reduced serum binding in the absence of albumin. Shiraishi, *et al.* (1984) have shown that serum zinc (which is normally 60% bound to albumin) is unaffected in NAR. Hirate, *et al.* (1984) studying the metabolism of ^{14}C-salicylic acid showed that the distribution volume was three to four times greater in NAR and implicated as the cause the absence of albumin binding to control the free drug concentration.

These results suggest that further studies are needed to determine the consequences of analbuminemia in drug clearance and metabolism of physiological ligands; although there is no severe pathology apparent, more subtle changes may be important in the administration of drug therapies or the possibility of bilirubin toxicity in the newborn. It is not known how well the NAR condition reflects human analbuminemia and further studies of human individuals with the condition is necessary before a more conclusive understanding of the effects

of the vastly reduced albumin levels can be attempted.

CHAPTER TWO

AIMS OF THIS PROJECT

2.1 INTRODUCTION

The occurrence and properties of several serum albumin variants have been studied in this laboratory over a number of years. During this time four albumin variants, all inherited and of the slow type, have been found, their properties studied and attempts made to purify the variant forms and specify the points of mutation. Much of the earlier work has been performed by two previous students and has been reported in two theses [Au (1982) and Brand (1983)], and briefly in three papers (Au, *et al.*, 1984a, b; and Brand, *et al.*, 1984). Details of the variants characterised are outlined below.

2.2 ALBUMIN WARWICK-1

Albumin Warwick-1 was found in a family of Punjabi descent, and was first identified in a young male with ulcerative colitis. Subsequent studies revealed that another brother, a sister and the mother of the first case were shown to carry two albumins but not the father or the two other sisters. Purification of the variant was carried out and two possible sites

of mutation indicated by cyanogen bromide (CNBr) cleavage, thought to be in residues 1-87 and 447-548. A reduction in the binding of bilirubin was noted and an isoelectric point (pI) of approximately 0.1 pH units higher than normal was estimated for the variant. It is a heat-stable monomeric variant indistinguishable from albumin A by immunological tests, with dye-binding properties similar to Albumin Kashmir by the RBH schedule (Au, 1982).

2.3 ALBUMIN WARWICK-2

Albumin Warwick-2, which is a slow migrating variant, was found in a heterozygous Indian woman living in the Warwick locality. It has been shown to have an amino acid substitution in the N-terminal region, residues 1-87 but is not a proalbumin. Albumin Warwick-2 is present in slightly less than 50% of the total albumin in serum and has a pI value of ≈5.01, compared to 4.9 for normal albumin. It has normal palmitate, Cu^{II} and Ni^{II} binding but has reduced bilirubin binding. Its dye binding properties have also been ascertained. It is a heat stable monomer.

3.4 ALBUMIN REDHILL

Albumin Redhill was discovered in a

Caucasian mother and her son from Redhill, Surrey. Initial evidence suggested that this monomeric variant might be a proalbumin, as the N-terminal amino acid was shown to be arginine and not the aspartate of normal albumin. However, limited tryptic digestion gave an ambiguous result. The nickel and copper binding of the variant was reduced and this also suggested that a mutation had occurred in the N-terminus. There might also be a mutation near the C-terminus, in residues 503-585, possibly due to a short insertion of 10-12 amino acids which gave an increased molecular weight on SDS PAGE. The pI value of Albumin Redhill was shown to be 4.75 compared to ≈ 4.9 for normal albumin and was indistinguishable from albumin A by immunological tests. It is a heat-stable variant (Brand, *et al.*, 1984).

2.5 ALBUMIN CARLISLE

This variant was reported to our laboratory in Spring 1984, having been discovered by Dr. C. Lord of the Department of Pathology at the Cumberland Infirmary, Carlisle. Albumin Carlisle was first found in August 1983 in a 36-year old Caucasian woman of British descent who had presented with slight weakness and aching in the left leg. Nine months previously she had a short-lived rash diagnosed as chickenpox (Variocella) associated with the sudden onset of

weakness first in the left leg and later in the left arm; some numbness of the left side of the face, and reduction of sensation in the left arm and leg were also noted at that time. These abnormalities had resolved slowly and no unusual symptoms were noted on presentation. In the past, she had required emergency surgery for ruptured ectopic pregnancy. Haematological, thyroid, renal and liver tests all gave normal results. However, bisalbuminemia was noted in the serum with a slow variant, visible after cellulose acetate electrophoresis of serum.

Familial studies showed that the bisalbuminemia was present in the youngest daughter (7 years old) and 16 years old son of the index patient but not in the other two daughters (17 and 11 years old). The mother and one brother of the index patient also had bisalbuminemia, although other members of the family were not studied (see Fig. 2.1). Two of the index patient's offspring had suffered with childhood eczema.

2.6 OBJECTIVES OF THIS PROJECT

The objectives of this study are outlined below.

(a) The establishment of a high yield purification process for the isolation of albumin variants.

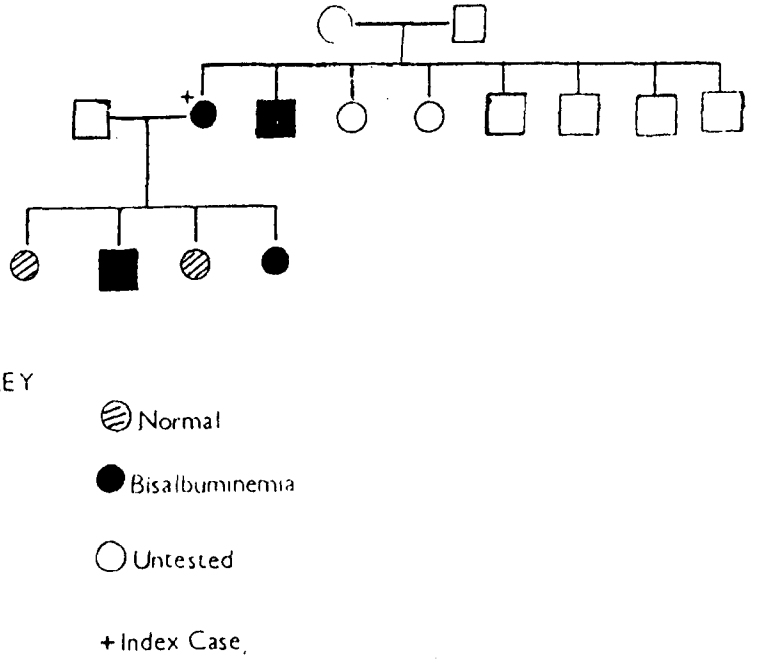


Fig. 2.1 Family tree of the family with Albumin Carlisle showing inheritance of the variant through three generations.

(b) The purification and N-terminal sequence of Albumin Redhill in order to elucidate the point of mutation, and further study the decreased affinity of the Nickel and copper binding site.

(c) The characterisation of the new albumin variant, Albumin Carlisle, isolation of the variant albumin and studies of the possible point of mutation.

(d) The comparative analysis of the binding to the variant albumin of a number of ligands.

2.7 EXPERIMENTAL

The experimental work is divided into several sections.

(i) The initial classification of Albumin Carlisle, its isoelectric point, thermal and freezing stability and immunological character were deduced.

(ii) Various methodologies were employed to isolate the albumins from serum and purify them to homogeneity in order to obtain pure variant albumin Carlisle.

(iii) Albumin Redhill was purified from double albumin in a similar manner to Albumin Carlisle and the isolated Albumin Redhill was used to obtain an N-terminal sequence of the variant albumin.

(iv) Peptide mapping was performed by both proteolytic and chemical methods in order to locate regions of mutation in Albumin Carlisle.

(v) Ligand binding studies were performed in order to qualitatively compare the affinity of normal and variant albumins to a range of ligands.

Each of these steps will be considered individually and the results of each section recorded and conclusions presented from the research performed.

CHAPTER THREE

THE CHARACTERISATION OF ALBUMIN CARLISLE

3.1 INTRODUCTION

Albumin Carlisle was first discovered in 1984 as previously mentioned and studies commenced upon it in this laboratory in April of that year. The initial step was to quantify its electrophoretic behaviour and dye binding properties and to establish the other criteria drawn-up for the characterisation of albumin variants as described in Section 1.4.6.2. The following is a list of the stages in this characterisation:

(a) Estimation of the total protein concentration of the serum.

(b) Estimation of the total albumin concentration of the serum.

(c) Qualification of the electrophoretic mobility of the albumin variant on the five media of RBH schedule.

(d) Estimation of the ligand-binding properties of the normal and variant albumins using the RBH schedule.

(e) Determination of the relative proportions of normal to variant albumins in the serum.

(f) Determination of the stability of the variant

with respect to repeated freeze-thawing.

(g) Determination of the thermal stability of the variant albumin.

(h) Elucidation of the molecular weight of the variant albumin.

(i) Identification of any proalbumin variant by limited tryptic digest.

(j) Calculation of the isoelectric point (pI) of the variant albumin.

(k) Determination of the immunological identity of the variant albumin.

The third and fourth criteria were carried out by Dr. A. L. Tárnoky and Mrs. J. V. Curnow of the Royal Berkshire Hospital, Reading.

3.2 EXPERIMENTAL

3.2.1 Materials

A list of the special chemicals is given in Appendix I. All other chemicals were freely obtainable and were of the highest quality available and used without further purification.

3.2.2 Determination of Total Protein in Serum

The total protein content of a known volume of serum was determined by the Biuret method (Gornall, *et al.*, 1949).

3.2.2.1 Biuret Reagent

The Biuret reagent consisted of copper sulphate pentahydrate (1.5 g), sodium potassium tartrate tetrahydrate (6.0 g), sodium hydroxide (10% w/v, 300 ml) and potassium iodide (1.0 g) made up to one litre with double distilled water. A plastic bottle was used to store the reagent in which it was stable indefinitely provided no red or black precipitate formed.

3.2.2.2 Standard Albumin Solutions

Human serum albumin was used as received and a concentration of 0-14 mg used to construct a standard curve, each sample being made up to a final volume of 1 ml in distilled water. Aliquots (4 ml) of the Biuret reagent were added with vortexing to the range of standards in duplicate and the absorbances at 540 nm were read after leaving the tubes to stand 30 minutes to allow stabilisation of the colour formation. The 540nm. absorbance was plotted against known albumin concentration, determined spectrophotometrically from the absorbance at 280 nm using $E^{1\%}_{280\ nm} = 5.31$ (Hunter and McDuffie, 1959).

3.2.2.3 Preparation of the Serum Samples

Sera were defatted essentially by the method of McFarlane (1942) except that sera (diluted 1:20 in water) was extracted with ether at room temperature and then centrifuged, the resultant organic phase being discarded, and the aqueous phase (1 ml) used for the

Table 3.1 Standard conditions for the determination of electrophoretic mobility by the RBH Schedule

Medium	Electrophoresis buffer	Current (mA)	Voltage (V)	Time
Cellulose acetate (78 x 150 mm) Shandon Celagram	Barbitone, Oxoid 0.1 M, pH 8.6	7.5		1 hr 20 mins
Helena Titan III Zip Zone Cellulose acetate plates 2 3/8" x 3"	Barbitone 0.05 M, pH 8.8		110	35 mins
Filter paper Whatman 3 MM (36 x 5 cm)	Barbitone 0.05 M, pH 8.6	3.5		7.5 hr
Disc-PAGE (7%, pH 8.3 → 8.9 → 8.3)	*tris* (0.05 M)-glycine (0.38 M) pH 8.3	2-3 mA/gel		
Agar (Oxoid, 1%) (8.5 x 8.5 cm)	Barbitone 0.05 M, pH 8.6		200	1.5 hr
Agarose (1%) (Corning ACI) sucrose (5%) EDTA (0.035%) in barbital (0.065 M, pH 8.6)	Barbitone 0.1 M, pH 8.6		90	45 mins

Biuret assay, as with the standard solutions above.

3.2.3 Determination of Total Albumin in Serum

The total albumin concentration of serum was determined by the bromocresol green binding assay (Doumas, *et al.*, 1971, Gustaffson, 1976). The reagent dye consisted of bromocresol green (0.15 mM), sodium succinate (75 mM), 'Brij 35' surfactant (30% w/v, 4 ml) per litre pH 4.2 ± 0.05 with NaOH. Reagent (2 ml) was added to serum (10 µl) or HSA standard solutions callibrated by absorbance at 280 nm. The solution was vortexed briefly and the absorbance read at 629 nm after 10 seconds and at 10 second intervals thereafter for 2-3 minutes. From these values the absorbance at time zero could be extrapolated for each of a range of albumin concentrations ranging from 1-10 g/dL, and a standard curve constructed from which to extrapolate the concentration of albumin in the serum samples.

3.2.4 Electrophoretic Mobility by the RBH Schedule

The system of the Royal Berkshire Hospital (RBH) laboratory (Tárnoky, 1980) comparing the mobility of albumin variants on five media was employed (electrophoresis on filter paper was omitted). The details of the electrophoretic conditions are given in Table 3.1. The systems used for Albumin Carlisle were cellulose acetate (two different media), agar,

agarose and disc PAGE. Staining was with Ponceau S (0.2% w/v) in TCA (3% w/v) and destaining in acetic acid (7% v/v).

3.2.5 Ligand Binding by the RBH Schedule

Ligand binding was approximated for five dyes, bromocresol green, Ponceau S, Congo Red, bromophenol blue and bilirubin. Incubation of the ligand (0.05% w/v, 50 µl) with serum (100 µl) in a 2:1 molar excess of dye:albumin for ten minutes at 37°C was followed by analysis by the electrophoretic means already used to quantify mobility. The only exception was that for disc PAGE, serum-sucrose solution (0.3 ml) was incubated with each of the dye solutions (50 µl) and the total volume loaded over the spacer gel.

At the end of each run qualitative estimation of the amount of ligand bound was made by eye and the positions of the albumin bands and any unbound ligand noted, and compared to the results after staining and destaining which were as described in Section 3.2.4.

3.2.6 Ratios of Normal and Variant Albumins in Serum

The albumins were separated by cellulose acetate electrophoresis in barbitone buffer (0.075 M, pH 8.6) at 8 v/cm until the marker dye had nearly eluted. The strip was stained using Ponseau S [(0.9 g) in TCA (13.4 g), sulphosalicylic acid (13.4 g)

per litre of water] for 10-20 minutes and destained in acetic acid (5% v/v), and rinsed with water to remove the acid. The normal and variant bands were individually excised and the dye eluted using NaOH (0.4 M, 1 ml) with agitation for several hours. The red colour was restored by addition of acetic acid (40% v/v, 100 µl) and the fragments of cellulose acetate removed by centrifugation. The absorbance of the supernatant was measured at 512 nm against a reference solution of 5% acetic acid and the ratio of normal to variant albumin estimated from the relative absorbances.

3.2.7 Stability to Freeze-Thawing (Izquierdo, *et al.*, 1971)

In order to determine the stability of the variant albumin to freeze-thawing, aliquots (20 µl) were twice frozen to -20°C for several hours and thawed to 37°C for a similar period. Similar aliquots were repeatedly frozen and thawed ten times during 6-8 hours and further samples were rapidly frozen and thawed 35 times over a period of several days.

Any effects upon the sera due to these treatments were assessed by disc PAGE in 7.5% non-denaturing gels with untreated normal and Carlisle sera run for comparison (see Section 3.2.10).

3.2.8 Thermal Stability

Due to the thermal instability of some

variants (Arends, *et al.*, 1969) samples of Carlisle sera were heated at 56°C for 30 minutes and 2 hours and any effect on the albumins determined by cellulose acetate electrophoresis.

3.2.9 Molecular Weight of the Variant Albumin

The molecular weight of the variant and normal albumins were determined by SDS-PAGE using purified, isolated double albumins from Carlisle sera and also pooled normal and Carlisle sera. The gel systems used were of two types;

(a) The discontinuous buffer system of Laemmli (1970), employing a 12% separating gel (16 x 16 x 0.15 cm) with tris-HCl (0.375 M), SDS (0.1% w/v), pH 8.85 (T = 12%, C = 2.6%) and a 3% stacking gel with tris-HCl (0.125 M), SDS (0.1% w/v), pH 6.8. It was helpful to include sucrose (40% w/v) in the stacking gel so as to increase its mechanical strength. The electrophoresis buffer was tris (0.05 M), glycine (0.384 M), SDS (0.1% w/v) with the pH corrected to 8.3 with HCl. Samples for electrophoresis were boiled for 5 minutes in loading buffer tris-HCl (0.0625 M), glycerol (10% v/v), SDS (2% w/v), 2-mercaptoethanol (5% v/v), bromophenol blue (0.01%) pH 6.8), and protein equivalent to 10-20 µg per sample well were loaded and electrophoresis carried out at 50-60 V constant voltage (C.V.) while the tracking dye focused in the stacking gel followed by 100-120 V C.V. for the

remainder of the run or at 50 V C.V. overnight. Electrophoresis was stopped when the tracking dye had migrated to within 1 cm of the bottom of the gel and the gel was simultaneously fixed and stained in PAGE Blue G-90 [(0.25% w/v) in methanol (45% v/v), acetic acid (10% v/v)] overnight and destained in the same concentrations of methanol/acetic acid at 40°C for one to two days with successive changes of the destaining solution.

(b) A second gel system employed was that outlined in the BDH booklet "Molecular weight markers for SDS-PAGE (BDH Chemicals Ltd., Poole, U.K.) using a similar buffer system but with a 5-20% gradient of acrylamide.

Loading, running and staining conditions were as for the 12% gels and in both systems commercial HSA and molecular weight markers (12,300-78,000) were used in order to estimate the molecular weights of the samples under study.

3.2.10 Detection of Proalbumin by Limited Proteolysis

Albumin Carlisle serum was tested by the procedure of Rousseaux, *et al.* (1982) in order to detect any proalbumin. TPCK-treated trypsin was dissolved in ammonium hydrogen carbonate (0.1 M) with calcium chloride (0.1 mM) pH 8.0 at a concentration of 3 mg/ml. Serum (60 µl) was taken, trypsin (30 µg, 10 µl) added to it and the digest incubated at 37°C,

aliquots (20 µl) being removed for analysis at 2 hours, 4 hours and finally 6 hours after addition of the enzyme. Soyabean trypsin inhibitor (1.2 mg of a 60 mg/ml solution in water) was added to the aliquots in order to stop further digestion and the samples subsequently frozen at -20°C. Analysis of the effects of proteolytic digestion was performed by electrophoresis at two pH values. Electrophoresis at pH 8.6 was performed in non-denaturing disc PAGE (Davis, 1967) using a T = 7.5%, C = 2.6% separating gel containing tris-HCl (0.375 M, pH 8.85) and a 3% stacking gel (0.125 M tris, pH 6.8) with a reservoir buffer comprising tris-HCl (0.05 M) with glycine (0.384 M), pH 8.3.

Aliquots of the digested serum samples (10 µl) were thawed and loading buffer (20 µl, 40% w/v sucrose, 0.01% w/v bromophenol blue) added and the mixture briefly vortexed before application to the gel. Electrophoresis was performed at ≈ 100 V C.V. until the bromophenol blue had eluted. Staining and destaining were essentially as described in Section 3.2.9.

Electrophoresis at pH 5.0 was performed on cellulose acetate strips (150 x 78 mm, Sartorious) pre-equilibrated in sodium acetate buffer (0.031 M) with EDTA (4 mM (pH 5.0) by soaking and blotting off the excess buffer. Serum samples (10 µl aliquots) were diluted with loading buffer (20 µl) as before and applied using a microsyringe around 2 cm from the cathode.

Electrophoresis was carried out in the sodium acetate buffer for 6.5 hours at 14 V/cm and the strip subsequently stained in Amido Black 10B/methanol/ acetic acid (1:90:10 v/v/v) overnight and destained in methanol:acetic acid (9:1 v/v) for several hours with repeated changes of destaining solution (Fine, *et al.*, 1983).

In both systems undigested normal serum and Carlisle serum, containing both albumins, were used as controls, together with commercial HSA, purified double Albumin Redhill, trypsin and trypsin inhibitor, also run for comparison.

3.2.11 Determination of Isoelectric Point (pI) of Variant Albumin

Purified albumin Carlisle and double albumin were analysed together with commercial HSA using both isoelectric focusing gel rods in the pH interval 4-6.5 and pre-cast thin layer gel slabs in the pH internal 3.5-9.5. In both systems the pH gradient in the gel was determined by means of pI standard markers.

3.2.11.1 .1 Isoelectric Focusing (IEF) in Thin Layer Polyacrylamide Gel Slabs (T = 5%, C = 3%)

Pre-cast slabs were a gift from LKB and contained ampholyte in the range pH 3.5 to 9.5. The experimental details were basically as outlined in the LKB booklet number 1804 accompanying the gels and

as described in the LKB application note 250 "Analytical Electrofocusing in Thin Layers of Polyacrylamide Gels". The pre-cast gel was unwrapped and placed upon the water-cooled platten of an LKB 2117 Multiphor apparatus, using a small amount of liquid paraffin to ensure good contact and taking care to eliminate air bubbles. The appropriate electrolyte solutions were made up (see Table 3.2.) and absorbent wicks, cut to size so as to be slightly shorter than the gel width, were soaked in the electrolytes, with slight swelling, and blotted on filter paper to remove excess fluid. The strips were then applied on to the gel in line with the Multiphor electrodes. An initial experiment was performed across the width of half a gel and a subsequent experiment was carried out across the length of another gel.

pH Range	Anolyte (+)	Catholyte (-)
3.5-9.5	H_3PO_4 (0.1 M)	NaOH (1.0M)
4-6.5	(DL) glutamic acid (0.01 M)	L-histidine (0.01 M)

Table 3.2 Electrolyte solutions for IEF experiments.

3.2.11.1.2 Preparation of Samples

Samples of normal albumin, Albumin Carlisle, and double Albumin Carlisle were dissolved in distilled water, and were defatted by the method of Chen (1967). Aliquots of each sample (≃ 10 µg) were absorbed onto small pieces of Whatman 3 MM filter paper (1 x 0.5 cm) and placed onto the gel surface positioned about 3 cm from the cathode according to the grid guide-line situated beneath the gel, so as to ensure no overlapping of the tracks occurred on electrophoresis. The exact position of the sample origin on the gel is unimportant as the proteins will migrate in the ampholyte gradient to their isoelectric point irrespectively providing the gel is run for sufficient time. A vial of pI marker proteins (Pharmacia) were reconstituted according to the manufacturer's instructions and loaded either side of the albumin samples.

3.2.11.1.3 Electrophoresis, Staining and Destaining

The samples having been applied using the filter paper strip, the lid of the apparatus was put in place and the electrophoresis commenced using an LKB 2197 constant power unit set at the following conditions;

Across the width of the gel: 600 V max. voltage, 20 mA max. current, 10 W maximum power. The electrofocusing was performed for 5½ hours, with cooling by a continuous flow of water through the platten.

Across the length of the gel: electrophoresis was carried out as above, but for a total of

5250 volt hours (5 hours at 600 V and 3 hours at 750 V).

All sample applicator strips were removed after ½-1 hour when the pH gradient was deemed to have formed and the samples had migrated into the gel. Once electrophoresis was completed, the gels were removed from the apparatus and, still supported on their thin plastic backsheet, were fixed in trichloro-acetic acid w/v (TCA) (11.5% w/v), sulphosalicylic acid (3.45% w/v), in distilled water for 0.5-1 hour, and then briefly soaked in destaining solution (ethanol 25% v/v, acetic acid 8% v/v in distilled water) in order to remove the unfixed ampholyte molecules from the gel before staining. The gels were stained in PAGE Blue G-90 (0.460 g in 400 ml destaining solution) for ten minutes or so at 60°C, and subsequently destained in the destaining solution for several hours with changes of buffer until the background was clear.

3.2.11.2 Isoelectric Focusing in Polyacrylamide Gel Rods (T = 5%, C = 3%)

Isoelectric focusing was performed on double and normal albumins by electrophoresis in polyacrylamide gel rods (9 x 0.8 cm i.d.) which contained Pharmalyte in the pH range 4-6.5 according to the details outlined in "Isoelectric Focusing, Principles and Methods" from Pharmacia Fine Chemicals (Milton Keynes, U.K.). The glass rods were cleaned thoroughly and sealed at one end with Parafilm.

Acrylamide gels (T = 5%, C = 3%) containing 13.3% v/v glycerol and 6.3% v/v Pharmalyte were cast into the glass rods and the rods placed into the gel rod apparatus, the electrolyte buffers being as stated in Table 3.2.

Samples of albumin were dissolved in a 1:15 solution of Pharmalyte (of the same interval as in the gel) in water containing sucrose (15%. w/v) and a trace amount of methyl red in order to follow the course of the electrophoresis. Loads equivalent to 5-10 µg protein per band were added and electrophoresis carried out at 500 V max. voltage, 6 mA max. current for seven hours, until the current was negligible.

The gels were carefully excised from the glass tubes and fixed in trichloroacetic acid (11.5% w/v) sulphosalicylic acid (3.45 w/v) in methanol:water (3:7 v/v) for one hour and then stained overnight in PAGE Blue G-90 (0.115 g in methanol (500 ml), acetic acid (160 ml) diluted to 2 L in water). Destaining was in repeated changes of the same solution, but without the dye, until a clear background was obtained.

The migration of proteins of known pI contained in the Pharmacia low pI calibration kit was used to calibrate the pH gradient in the gel rods as before, a vial was resuspended in the Pharmalyte/sucrose loading solution (100 µl) and aliquots (50 µl) loaded onto the surface of the appropriate gels. The migration distances were plotted against the tabulated pI values

(see Table 3.3) and the pI of the albumin samples extrapolated from the resultant graph.

Protein	pI' (24°C ± 1.5°C)
pepsinogen	2.80
amyloglucosidase	3.50
methyl red (dye)**	3.75
glucose oxidase	4.15
soybean trypsin inhibitor	4.55
β-lactoglobulin A	5.20
bovine carbonic anhydrase B	5.85
human carbonic anhydrase B	6.55

** Methyl red is a dye and does not appear in final stained gel.

Table 3.3 List of pI marker standards used to calibrate pH gradient in IEF.

3.2.12 Determination of Immunological Identity

Albumin Carlisle was compared to normal albumin by two immunological methods; by the double immunodiffusion procedure of Ouchterlony (1949) and by immunoelectrophoresis; using goat anti-human albumin A antibodies (3.5 mg/ml) in both processes.

3.2.12.1 Ouchterlony Double Immunodiffusion

3.2.12.1.1 Preparation of Agar Gel (Clausen, 1981)

Agar Noble (electrophoretic grade) was used as received and dissolved with heating at a 1.5% concentration in aqueous NaCl (0.83% w/v),

sodium azide (0.1% w/v). The agar solution was boiled at 100°C for 40 minutes with the flask loosely stoppered by an inverted beaker to prevent excess evaporation.

Microscope slides were washed with alcohol and then diethyl ether and then covered with the molten agar solution (≈ 3 ml) using a pipette, and the agar allowed to set at room temperature. Wells (approximate volume 10 µl) were punched into the agar with a truncated Pasteur pipette using a template so that eight wells were cut out at a (8.5 cm) radius around a central well; the agar being sucked out under vacuum by attaching the pipette to a water pump. The plates were kept at a constant humidity in petri dishes containing a moist piece of filter paper and the plates stored at 4°C until use.

3.2.12.1.2 Determination of a Balanced Immunological System

Initial experiments were performed to ascertain a balanced proportion of antigen and antibody which gave a strong precipitin line without artefactual spurs. Two trials were set up:

(i) A fixed quantity of antigen diffusing against a series of antibody dilutions

Pure albumin A (10 µl, 1 mg/ml) or pure albumin Carlisle (10 µl, 1mg/ml) was applied to the central well of an agar plate and antiserum, diluted in the ratios, undiluted, 1:1, 1:2, 1:3, 1:4, 1:5, 1:10, and 1:15 with water, was applied in 10 µl aliquots to the surrounding wells using a microsyringe.

The slides were replaced in a Petri dish humidity chamber and diffusion allowed to occur for 16 hours at 4°C.

(ii) A fixed quantity of antibody diffusing against a series of antigen dilutions

Undiluted antiserum (10 µl) was applied to the central well of another agar plate and pure albumin A or pure Albumin Carlisle (1 mg/ml), diluted with water in the same ratios as above, was applied to the surrounding wells, and diffusion performed as above.

3.2.12.1.3 Gel Staining

The gels were washed for 24 hours after the diffusion was completed using phosphate buffered saline (0.05 M disodium hydrogen phosphate pH 7.0 with NaCl (0.8 w/v) to remove unreacted antigen and antibody. The agar was washed free of the salts with distilled water for several hours and then stained with Amido Black 10B (0.1% w/v in acetic acid (5.4% v/v), sodium acetate (6.72% w/v), glycerol (1% v/v)) for 10 minutes and destained in acetic acid (5% v/v) for several hours. Gels could be dried by careful blotting with filter paper (Whatman number 542) or by leaving open to the air at room temperature for two days.

3.2.12.1.4 Immunodiffusion of Albumin Carlisle

A balanced immunological system having been determined for normal albumin and Albumin Carlisle, this ratio was used to set up a further experiment

where normal albumin, Albumin Carlisle, and albumin A isolated from Carlisle serum were applied undiluted as 1 mg/ml solutions to wells surrounding undiluted anti-albumin A antiserum (10 µl), and also in a second experiment where the albumin A was diluted 1:1 with distilled water. Diffusion and subsequent washing, staining and destaining were as described previously (Section 3.2.12.2 and 3.2.12.3).

3.2.12.2 Immunoelectrophoresis (Scheidegger, 1955)

3.2.12.2.1 Preparation of the Agar Gel (Clausen, 1981)

Agar Noble (electrophoresis grade, 1% w/v) was dissolved in sodium veronal-HCl buffer (0.05 M, pH 8.4) containing sodium azide (0.1% w/v) by heating at 100°C, again loosely covering the mouth of the conical flask to prevent evaporation. Alcohol-washed microscope slides were covered with the molten buffered agar (2-3 ml) and the agar allowed to set. Two wells were cut in parallel approximately 2 cm from the one end of the slide and one filled with Carlisle serum (2 µl) and the other with normal pooled serum (2 µl).

3.2.12.2.2 Immunoelectrophoresis in the Agar Gel

The agar plate was placed in a Shandon cellulose acetate electrophoresis apparatus with filter paper wicks (Whatman number 1) positioned on either side and electrophoresis carried out longitudinally in sodium veronal-HCl buffer (0.05 M pH 8.4) at 80 V C.V. for 3½ hours. The slide was removed from the apparatus, a narrow longitudinal channel cut equidistant

between the two sample wells with a scalpel and the agar strip removed. Antiserum (40 µl) was spread equally along the channel and immunodiffusion proceeded for 16 hours at 4°C in a Petri dish humidity chamber.

The gel was washed and the immunoprecipitation lines stained using the method previously described (Section 3.2.12.1.3). A second gel was run in parallel up to the end of the electrophoresis step and then stained to show the extent of the separation of the normal and variant albumins before the immunodiffusion was performed.

3.3 RESULTS AND DISCUSSION

The serum of the mother who carried the Carlisle variant as well as normal albumin was used in the tests described in Section 3.2. The total protein concentration in her blood was estimated by the Biuret method to be 59 g/L by interpolation from the graph of the standard curve (Fig. 3.1). Evaluation by the RBH schedule gave a total protein concentration of 80 g/L. Serum protein levels may vary with disease and other nutritional factors and this may explain the discrepancy in these values which were taken over a six month period. The nature of the bisalbuminemia remained unaltered over this period. The normal range of total serum protein is 60-80 g/L and so Albumin Carlisle serum does not significantly differ from normal serum

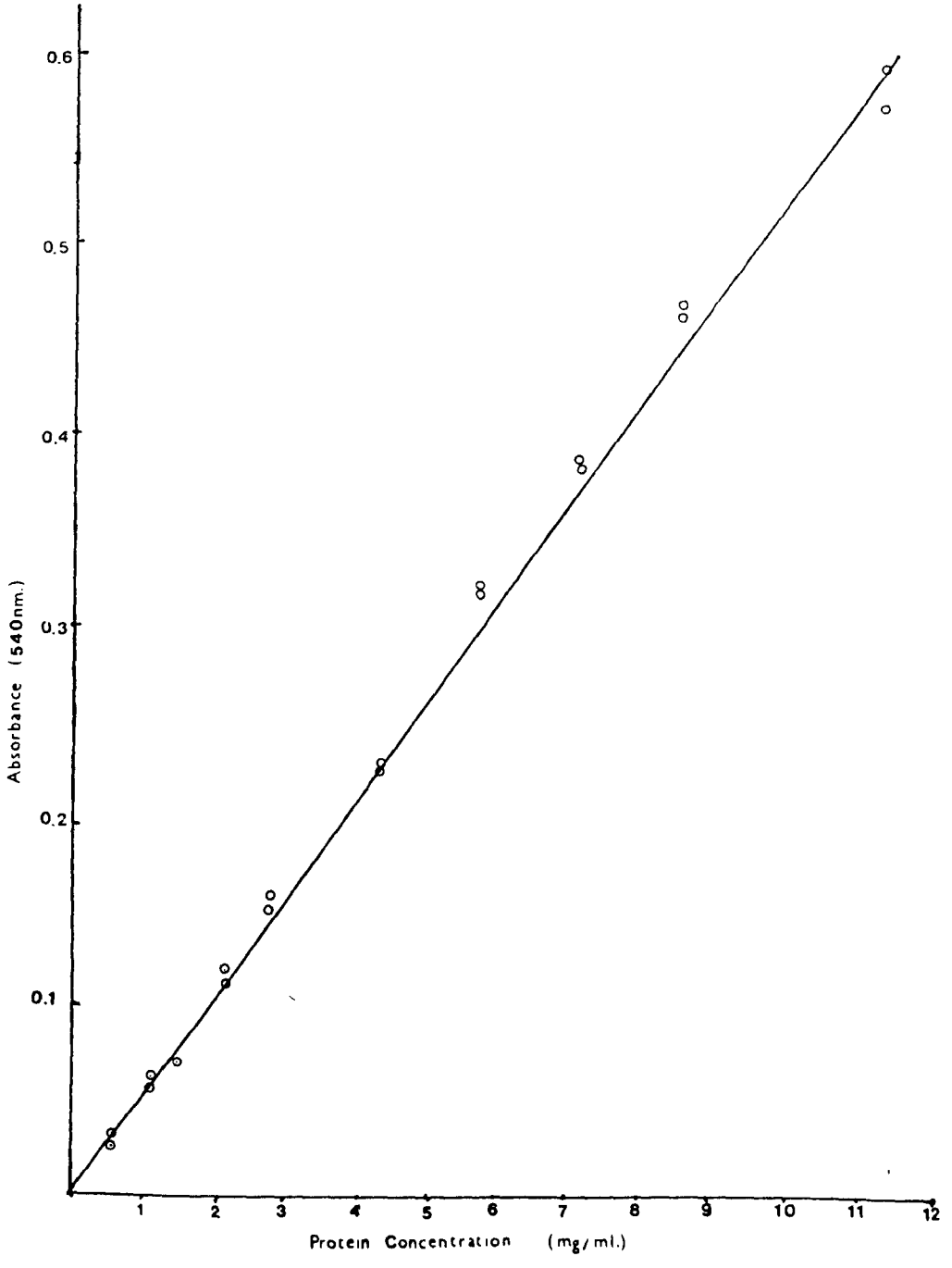


Fig. 3.1 Protein concentration: Biuret standard curve.

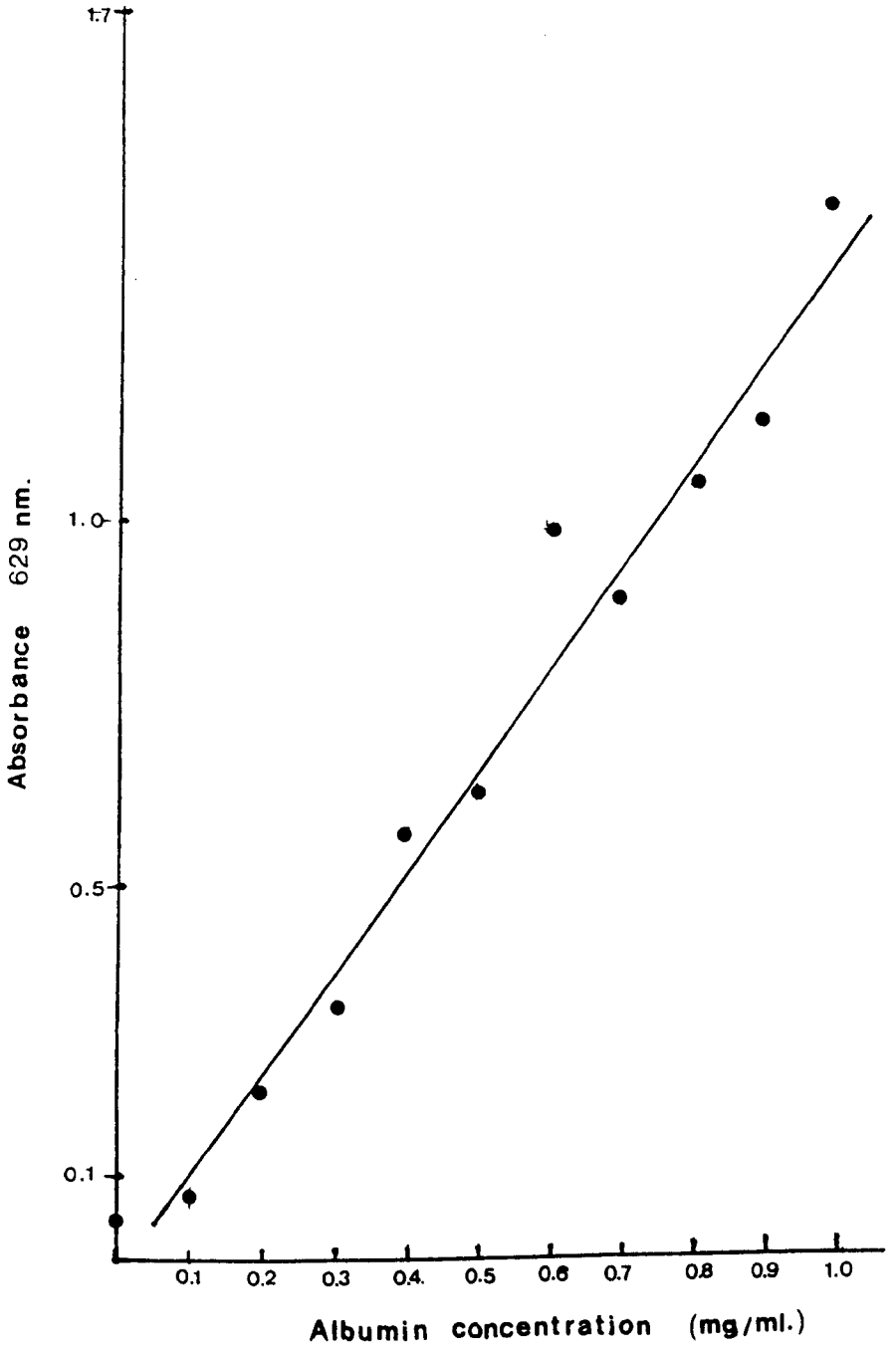


Fig. 3.2 Albumin concentration: bromocresol green standard curve.

in this respect.

The total albumin level for the serum of the mother with Albumin Carlisle was estimated by the bromocresol green method to be 47-50 g/L (80% of the total protein) derived from the values of the constructed standard curve (see Fig. 3.2). The level in the initial sample of the maternal serum received six months earlier had been higher (53-59 g/L) possibly due to the effects of the recent illness. By the densiometric scanning of serum samples of the maternal serum, the RBH test schedule estimated the total albumin concentration to be 66-74% of total protein, dependent on different electrophoretic conditions. It may be that the bromocresol green method had over-estimated the actual albumin concentration, which would explain the discrepancy between these two methods, or than the densiometric scanning had overlooked minor protein constituents. The son and daughter of the patient had serum albumin levels of 35-38 g/L and 46-49 g/L respectively, the levels being lower as might be expected in children. However, the level in the son is low compared to the average value of 40 $\pm$ 5 g/L, and it may be related to some medical condition or illness at the time the sample was given.

The electrophoretic mobility of Albumin Carlisle using the RBH schedule is given in Table 3.4. The mobility of Albumin Carlisle is compared to that of 12 other slow variants reported using the RBH test

TABLE 3.4 RELATIVE MOBILITIES OF ALBUMIN VARIANTS

Albumin Variant	C.A.	Helena	Disc	New Agar	Agarose
Carlisle	90:100	90:100		82 92 -89 : -100	84 95 -89 : -100
Redhill (Brand 1983)	Incomplete Separation	94 97 -96 : -100	84 92 -91 : -100		Incomplete Separation
Warwick-1 (Au 1982)	90:100	93:100		82 93 -92 : 100	85 94 -91 : -100
Warwick-2 (Brand 1983)	88:100	91:100	82 94 -91 : -100	78 89 -87 : 100	84 94 -90 : 100
Vancouver (Frohlich, *et al.*, 1978)	89.5:100	89.5:100	84.5 94 -93 : -100	92:100	81- 90.5 89.5 : -100
Jaffna (Bayliss *et al.*, 1983)	94:100	88:100	88 -91 :100		85 -93 :100
Stirling (Curnow, *et al.*, 1978)	89:100	89- 96- 94 : 100	86 92 -90 : -100	85 92 -89 : -100	84 91 -89 : -100
Yorkshire (Curnow, *et al.*, 1978)	90:100	89 95- -94 : 100	86 94 -92 : -100	84 95 -93 : -100	83 93 -89 : -100
RIH (Chapman, *et al.*, 1978)	91:100	87.5 96 -92 : -100		86 92 -92 : -100	81 91 -90 : -100
Amsterdam (Sa nders, *et al.*, 1979)	86:100	86 94 -92 : -100	84 92- -89 : 100	71 88 -81 : -100	76 91 -85 : -100
Kashmir (from H. Au, 1982)	90:100	93:100		81 92 -91 : -100	84 93.5 -90.5 : 100
Hussain (from H. Au, 1982)	89:100	92:100		84 94- -92 : 100	84 93 -90.5 : -100
Birmingham Bradwell, *et al.*, 1975)	92:100			92:100	94:100

*All distances expressed on a 0-100 scale where 0 is the cathodic trailing edge of the protein pattern and 100 is the leading edge of albumin A.

previously. It can be seen that Albumin Carlisle has a unique electrophoretic profile, although it appears most similar to Albumins Warwick-2 and Kashmir, both of which are of Asian origin. The other European variants compared, Albumins RIH, Yorkshire, Amsterdam, Stirling, and Redhill, differed in their electrophoretic profile in most of the systems used. No direct comparison with albumin B was possible but Tárnoky made no indication that Albumin Carlisle was identical to any variant in his collection.

Albumin Carlisle is different to the three albumin variants previously studied in this laboratory, being slower than Albumin Redhill (see Fig. 3.3) and less slow than Albumin Warwick-2. Albumin Warwick-1 was of similar mobility on cellulose acetate, agar and agarose but comparison of sera of the two variants on disc PAGE indicated that both albumin bands on Warwick-1 migrated further than the equivalent bands in Albumin Carlisle sera and were broader, suggesting that the similarities may be due to the poorer resolving powers of the previous media.

The qualitative ligand binding studies on Albumin Carlisle from serum suggests (Table 3.5) that Albumin Carlisle binds less bromophenol blue than normal albumin in all the media tested and that its binding of Ponceau S might be reduced also. The binding of bromocresol green differed only in the disc PAGE system and the binding of Congo Red may also be slightly less

TABLE 3.5 DYE BINDING PROPERTIES OF ALBUMIN CARLISLE

Medium:	Ligand:	Bromophenol Blue	Ponceau S	Bromocresol Green	Bilirubin	Congo Red
Cellulose acetate		S < A	O,O	S = A	S = A	O,O
Cellulose acetate Helena		S < A	O,O	S = A	S = A	O,O
Disc PAGE		S < A	S < A	S < A	S = A*	S = A*
New Agar		S < A	O,O	O,O	S < A	S = A
Agarose		S < A	O,O	O,O	S < A	-

KEY:

- not determined
S = Albumin Carlisle
A = albumin A
O,O no binding

*In these cases dye was also found streaming ahead which suggests lower binding capacity by albumin variant.

than that of albumin A from the same serum. The binding of bilirubin is less for Albumin Carlisle in both agar and agarose systems; ligand streaming ahead to the anode was observed for both bilirubin and Congo Red on disc PAGE. This has been interpreted by Tårnoky as being due to reduced binding by the variant. However, Wieme, *et al.* (1975) have criticised this method as previously recorded in Section 1.4.7.2.3 and so whether this evidence represents real differences at the molecular level is open to question. However, the initial appearance of the serum of Albumin Carlisle was intensely yellow, which might indicate excessive levels of free bilirubin due to reduced binding to albumin.

On comparison of the binding displayed by Albumin Carlisle to that of other variants studied in this laboratory and other variants for which data is recorded, it was found (Table 3.6) that Albumin Carlisle was most similar in its dye binding to Albumin Amsterdam and Albumin Warwick-2. There are differences even between these variants, however, especially in the binding in different media. Albumin Carlisle is quite dissimilar to Albumin Redhill, or Albumins Stirling and Yorkshire, all British variants.

It would seem that Albumin Carlisle is different to those variants already classified by the RBH schedule in electrophoretic mobility and dye binding, and these differences may be due to changes

TABLE 3.6 COMPARISON OF DYE BINDING PROPERTIES OF VARIANT ALBUMINS

Variant Ligand:	Bromophenol Blue	Ponceau S	Bromocresol Green	Bilirubin	Congo Red
Carlisle	$S < A_{(A-E)}$	$S < A_{(C)}$	$S < A_{(C)}$ $S = A_{(A,B)}$	$S = A_{(A,B)}$ $S < A_{(C-E)}$	$S < A_{(C)}$ $S = A_{(D)}$
Redhill (Brand, 1983)	$S < A_{(A-E)}$	$S < A_{(C)}$	$S < A_{(A,C)}$ $S = A_{(B,D)}$	$S < A_{(A-E)}$	$S < A_{(C,E)}$
Warwick-One (Au, 1982)	$S < A_{(A-E)}$	$S < A_{(B,C)}$	$S < A$ $S = A_{(B)}$	$S = A_{(A,C,E)}$ $S < A_{(B)}$	$S < A_{(D,E)}$ $S < A_{(C)}$
Warwick-Two (Brand, 1983)	$S < A_{(A-E)}$	$S < A_{(C)}$	$S = A_{(B,D)}$ $S < A_{(A,C)}$	$S = A_{(B,D,E)}$ $S < A_{(A,C)}$	$S < A_{(C)}$ $S < A_{(D,E)}$
Birmingham	$S < A_{(A-E)}$	(-)	$S = A_{(D)}$	$S = A_{(A,D)}$	$S = A_{(D)}$
RIH	$S < A_{(A,C-E)}$ $S = A_{(B)}$	S only$_{(C)}$	$S < A_{(A-C)}$ A only$_{(E)}$	A only (A,C) $S < A_{(E)}$	$S = A_{(A,C)}$ $S < A_{(E)}$
Amsterdam	$S < A_{(A-E)}$	$S < A_{(C)}$	$S < A_{(D,E)}$ $S = A_{(C)}$	$S < A_{(C)}$ $S = A_{(A,B,E)}$	$S = A_{(C)}$ $S < A_{(...,E)}$
Stirling	$S < A_{(A-C,E)}$ $S = A_{(D)}$	$S < A_{(C)}$	$S < A_{(A-C)}$ A only$_{(E)}$	$S < A_{(A-E)}$	$S < A_{(C-E)}$
Yorkshire	$S < A_{(A-E)}$	A only$_{(B,C)}$	$A < S_{(A-C,E)}$	$S < A_{(A-C,E)}$	$S < A_{(C-E)}$

A Cellulose acetate celagram
B Cellulose acetate Helena
C disc PAGE
D New Agar
E Agarose
- Undetermined

at the amino acid level.

The ratio of variant to normal albumin in the Carlisle serum was derived from the relative ratios of eluted Ponceau S to be within experimental error 1:1 (48:52) variant:normal and the data from the RBH tests support this finding.

Albumin Carlisle was unaffected by repeated freeze-thawing of serum over 30 times, no significant difference being visible on electrophoresis. This was confirmed by Tárnoky and Curnow (private communication to Dr. D. W. Hutchinson), and they also reported Albumin Carlisle to be stable to heating at 56°C for two hours. No change in the electrophoretic properties of Albumin Carlisle was noted over eighteen months of storage at -20°C. It therefore would seem unlikely that Albumin Carlisle is a transient or dimeric variant, as these have been found to be unstable under these conditions (see Section 1.4.6.2).

The molecular weight of Albumin Carlisle by SDS PAGE was indistinguishable from that of normal albumin ($\approx$ 65,000) using double Albumin Carlisle in serum and purified double Albumin Carlisle (10 µg) in both 12% SDS PAGE and 5-20% gradient SDS PAGE. An extra band was detected on some gels with increased loading but was absent at lower concentrations on the same gel. It can be inferred that Albumin Carlisle is a monomeric variant essentially of the same molecular weight as HSA.

Limited proteolysis with trypsin gave no apparent difference in the electrophoretic pattern of Albumin Carlisle, at pH 5.0 or 8.6. Although anodic bands were present ahead of the albumin, they were identified as being due to the excess trypsin inhibitor. The relative ratios of variant and normal albumins was unaltered by tryptic digestion when compared to untreated normal and Carlisle sera (see Fig. 3.3). This suggests that Albumin Carlisle is not a proalbumin variant as it has no trypsin cleavable propeptide.

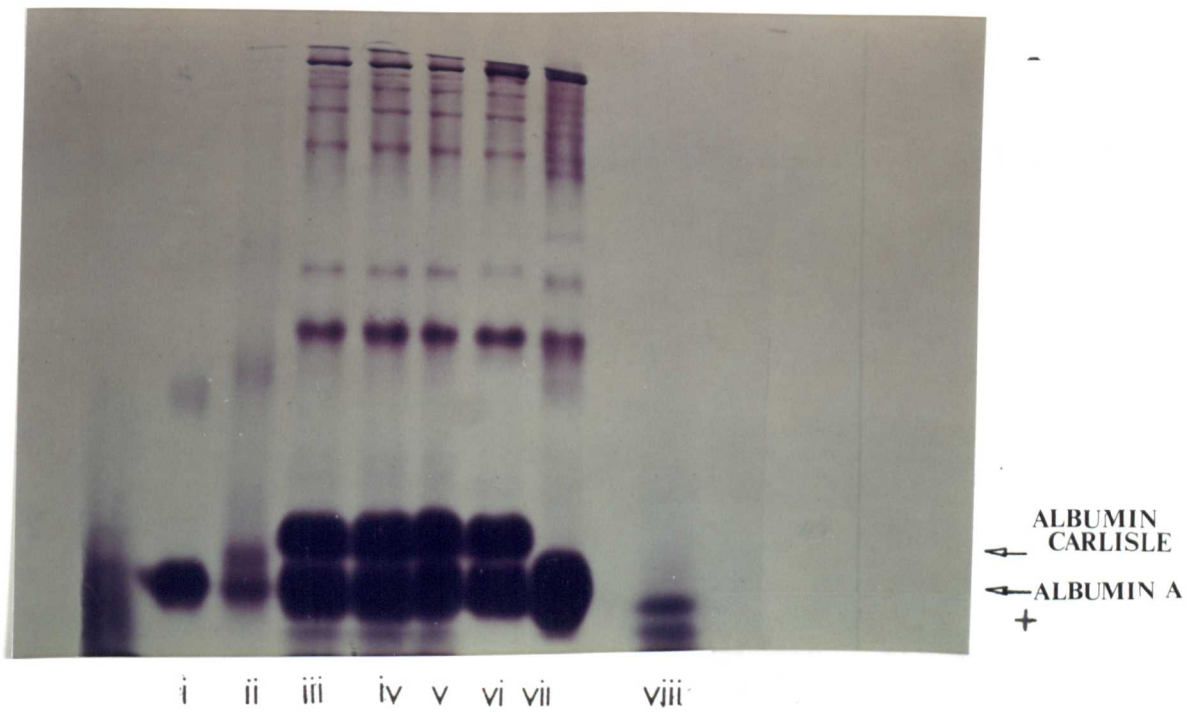


Fig. 3.3 Limited tryptic digestion of double Albumin Carlisle in serum. Analytical disc-PAGE of two (v), four (iv) and six hour (iii) tryptic digestions of Carlisle serum was carried out. HSA (i), normal serum (vii), double Albumin Redhill (ii), Carlisle serum (vi) and trypsin inhibitor (viii) were run for comparison.

The isoelectric point (pI) of Albumin Carlisle was determined to be 5.74 for defatted albumin compared to 5.63 for normal albumin on a long isoelectric focusing gel slab, and 4.75 compared to 4.45 for HSA when run widthways, both in the pH 3.5-9.5 range. Isoelectric focusing in the narrow pH range 4-6.5 in gel rods gave the pI value of 5.8 for double Carlisle, 5.4 for HSA but 5.26 for purified abnormal albumin. The higher pI value is consistent with the elution profile of Albumin Carlisle on chromatofocusing and so it can only be assumed that the lower value is due to the presence of bound ligand reducing the pI of the albumin. It may be possible that some of the Polybuffer used in chromatofocusing (see Section 4.4.2.2.1) was still attached to the albumin, despite attempts to remove it.

Normal albumin has been shown to be extensively heterogeneous on isoelectric focusing (Gianazza, *et al.*, 1984 and Candiano, *et al.*, 1984) and this is probably due to endogenous ligands still being bound as defatted HSA has a pI ≈ 5.7 but native albumin one ≈ 4.7 due to the presence of fatty acids and bilirubin. Brand (1983) reported a low pI band (4.65) in all normal and variant albumin samples electrofocused on the pH range 4-6.5.

In the present experiments, thin layer IEF revealed 6-7 bands in the defatted commercial albumin (some presumably being due to dimeric forms) and 3-4 bands in the defatted pure Carlisle although most

of these were comparatively minor components. Less heterogeneity was noticed in the rod gels due to their lower sensitivity.

From the results obtained on Albumin Carlisle it would appear that it has an isoelectric point 0.1 to 0.3 pH units higher than albumin A.

Albumin Carlisle and normal HSA were reacted with anti-human antibodies raised in goat sera. From trial studies to establish a balanced immunological system, dilutions of antibody gave strong preciptin lines at dilutions to 1:2 antibody:water for pure Carlisle and to 1:3 for commercial HSA. Subsequently, a system with undiluted antibody and Albumin Carlisle solution (1 mg/ml) but with both undiluted and diluted (1:1) commercial HSA solution was implemented for the immunodiffusion experiment. Pure Albumin Carlisle, commercial HSA and albumin A from Carlisle sera (stock solutions of 1 mg/ml) were cross-reacted with undiluted anti-albumin antiserum (Fig. 3.4). The precipitin line obtained was continuous for HSA and for Albumin Carlisle indicating overall antigenic identity. The preparation of albumin A from Carlisle serum gave slightly ambiguous results possibly due to contaminants remaining from the purification process. Slight differences in the distance between the central well and the precipitin line are most likely due to inaccuracies caused by using a manual procedure to cut the wells and do not seem to indicate

a significant immunological trend.

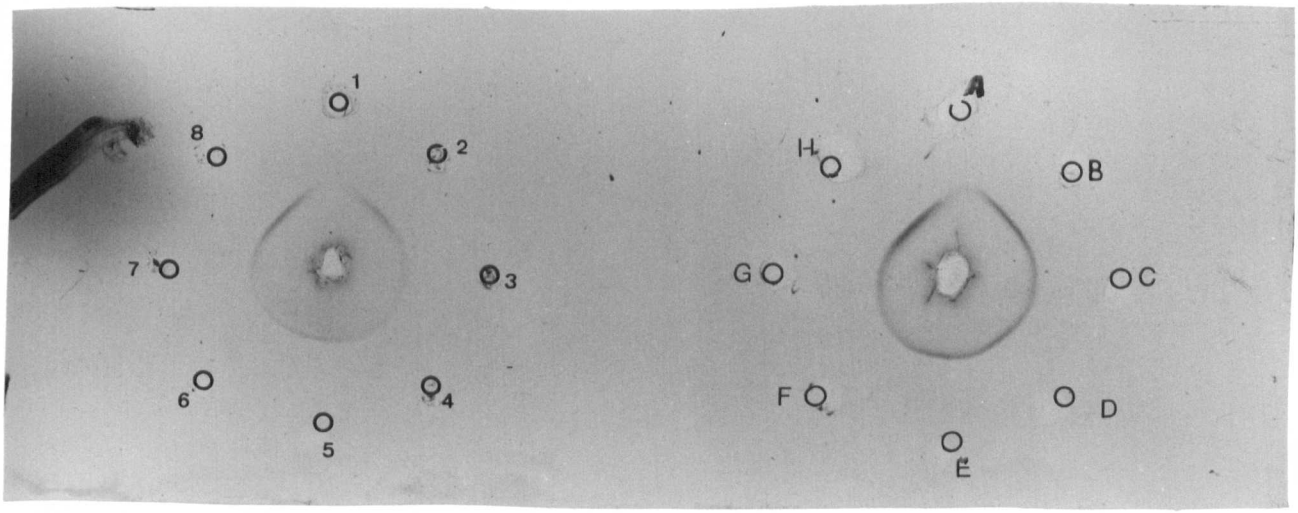


Fig. 3.4 Double immunodiffusion of Albumin Carlisle and albumin A. Undiluted anti-albumin A antibody was added to the central wells, HSA was added to the outside wells 5-8 (1 mg/ml) and B-E (0.5 mg/ml), Albumin Carlisle to wells 2-4 and F-H (1 mg/ml) and Carlisle serum normal albumin to wells 1 and A (1 mg/ml).

Immunoelectrophoresis of Carlisle serum containing variant and normal albumins supported the conclusion that Albumin Carlisle is antigenically indistinguishable from albumin A. Both proteins, after separation by electrophoresis, reacted to the anti-albumin antibody and gave an elongated precipitin arc which encompassed both albumin bands, other non-antigenically identical proteins having been washed out of the gel during staining (Fig. 3.5). No appreciable spur formation was seen in the arc which could have indicated small degrees of antigenic

difference, although this does not mean that all the antigenic sites are necessarily identical in the variant albumin. Doyen, *et al.* (1985) have proposed thirteen different epitopes on normal albumin using monoclonal antibodies raised against albumin polypeptide fragments. It has been noted in Section 1.4.4 that it is possible to distinguish some albumin variants by use of monoclonal antibodies and such studies if applied to Albumin Carlisle might show small differences in its antigenic determinants not detectable with the polyclonal antibodies used in these experiments.

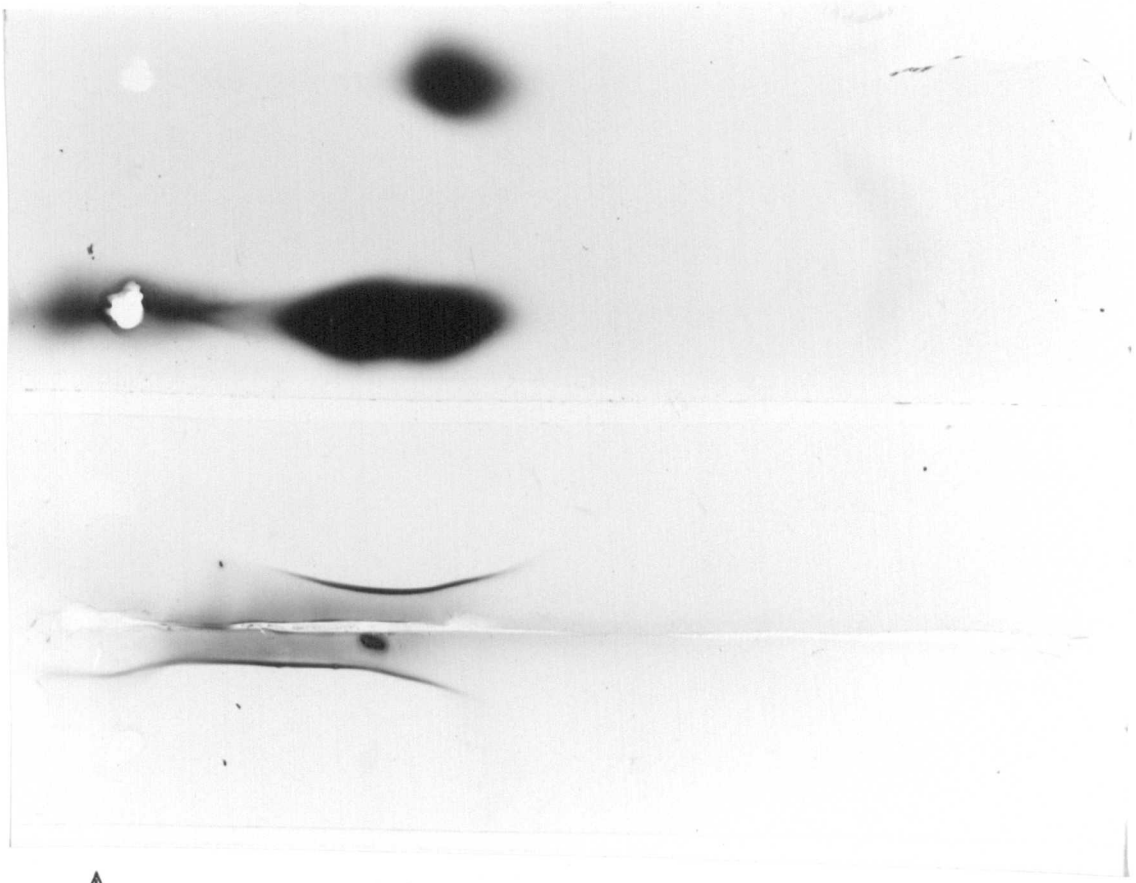


Fig. 3.5 Immunoelectrophoresis of normal (i) and Carlisle (ii) sera; (a) after electrophoresis only (A) and after subsequent immunodiffusion (B).

3.4 SUMMARY

Albumin Carlisle appears to be a heat stable monomeric slow (cathodic) variant with unique electrophoretic properties as far as can be ascertained. It is inherited in the two offspring of the initial carrier and appears to be antigenically indistinguishable from normal albumin. Albumin Carlisle has an isoelectric point (pI) at approximately 5.74 compared to normal albumin (5.63) and this is consistent with it being more cathodic on electrophoresis. Levels of the albumin in serum indicate that it contributes just under half of the total albumin and the total levels are within the normal range. Albumin Carlisle may have slightly decreased affinity for bilirubin and Congo Red, possibly caused by some mutation in the polypeptide chain. However, Albumin Carlisle does not appear to be a proalbumin variant.

CHAPTER FOUR

PURIFICATION OF ALBUMIN VARIANTS

4.1.1 Introduction

The properties of normal albumin and variant albumins are for the most part very similar; molecular weight and antigenicity, for example, vary little if at all. There are three possible properties where the differences, caused mostly by a single amino acid mutation, are sufficient to be utilised for separating the albumin forms. These are:

(i) net charge (ion exchange chromatography and preparative electrophoresis);

(ii) pI values (chromatofocusing and preparative isoelectric focusing);

(iii) ligand binding (affinity chromatography).

This Chapter outlines the various separative methods employed over a two year period in order to obtain pure variant albumin from Carlisle and Redhill sera.

4.1.2 Methods Used for Variant Purification

Methods which have previously been used successfully to purify albumin variants are listed in Table 4.1. The easiest approach is to screen sufficient patients to locate an individual homozygous for the variant albumin. Alloalbuminemia is generally

TABLE 4.1 METHODS OF ALBUMIN ISOLATION

	Method of Isolation	Author	Variant
1	Homozygote obtained	Franklin *et al.* (1980b)	Naskapi
		Vanzetti *et al.* (1979)	Mi/Fg
2	Ion Exchange Chromatography		
	(i) DEAE Sephadex, sodium phosphate buffer 0.2 M pH 5.75	Winter *et al.* (1972)	Oliphant/Ann Arbor
	DEAE Sephadex, phosphate buffer 0.1 M pH 5.75	Galliano *et al.* (1984)	Pollibauer
	DEAE Sephadex, phosphate buffer 0.15 M pH 5.75	Lapresle (1977)	Gainesville
	DEAE Sephadex, phosphate buffer 0.15 M pH 5.75	Lapresle and Wal (1979)	Penicillin transient
	DEAE Sephadex A50, sodium phosphate buffer 0.4 M pH 5.75	Rousseaux *et al.* (1976)	Penicillin transient
	(ii) DEAE Cellulose, phosphate buffer 0.02 M pH 7.0 with 0-1 M NaCl gradient	Bradwell *et al.* (1975)	Birmingham
3	Ion Exchange Chromatography with pH Gradient		
	(i) DEAE Sephadex A50, sodium acetate buffer 25 mM pH 5.2 to 4.5	Brennan and Carrell (1978)	Christchurch
	DEAE Sephadex A50, sodium acetate buffer 25 mM pH 5.2 to 4.5	Brennan (1985)	Parklands
	(ii) DEAE Sepharose CL6B, sodium acetate buffer 25 mM pH 5 to 4.65	Walsh *et al.* (1983)	IMVS
	(iii) QAE Sephadex A50, sodium acetate buffer 0.1 M pH 5.1 to 4.6	Bradley and Hornbeck (1974)	Unnamed slow and fast variant
4	Preparative Electrophoresis		
	(i) Starch block electrophoresis, veronal buffer 0.1 M pH 8.6	Gitlin *et al.* (1961)	B
	(ii) Agar electrophoresis, veronal buffer 0.1 M pH 8.8	Gentou and Plazzonet (1978)	Unnamed variant
	(iii) Cellulose acetate electrophoresis, tris veronal buffer 0.06 M pH 8.6	Abdo *et al.* (1981)	Lille
	(iv) Agarose electrophoresis, barbital buffer 0.02 M pH 8.6	Bianchi *et al.* (1974)	Unnamed variant
	(v) PAGE	Franklin *et al.* (1980a)	Mexico-2
	PAGE	Au *et al.* (1984)	Warwick-1 and 2
	PAGE	Brand (1983)	Redhill
5	Chromatofocusing		
	(i) Start buffer; histidine HCl 25 mM pH 6.2	Tiliyer *et al.* (1982)	Kashmir
	(ii) Eluting buffer; Polybuffer 74-HCl (1:12 in water pH 4.0)	Tiliyer *et al.* (1984)	Jaffna
	(some experiments with piperazine HCl 25 mM pH 5.5)	Brand *et al.* (1984)	Redhill
6	FPLC Chromatofocusing		
	(i) Start buffer; piperazine-HCl (25 mM pH 6.3)	This work	Redhill
	(ii) Eluting buffer; Polybuffer 74-HCl (1:10 dilution in water pH 4.0)		Carlisle

considered to be a co-dominant trait but its incidence is still comparatively rare(1 in 1,000 to 1 in 10,000 in Europeans) in most populations, so that this solution to the problem of obtaining pure variant albumin has been possible mainly for polymorphic variants such as Albumins Naskapi (Franklin *et al.*, 1980b) and Mexico-2 (Franklin *et al.*, 1980a), although the Italian variant Mi/Fg has also been found in a homozygote condition (Vanzetti *et al.*, 1979). In the absence of homozygous individuals variants must be purified from heterozygotes by either ion-exchange chromatography, chromatofocusing or preparative electrophoresis.

4.1.3 Ion Exchange Chromatography

Ion exchange chromatography separates molecules on the basis of differences in net charge and can distinguish even small variations between proteins such as albumin, which have a high negative charge at near neutral pH and so bind tightly to anionic exchangers such as DEAE (diethyl-amino-ethyl) and QAE (quaternary amino-ethyl) Sephadex. Elution of bound protein can then be achieved by raising the ionic strength of the eluant until the proteins are desorbed. The degree of separation possible with ion-exchange chromatography is dependent primarily upon the differences in affinity for the column matrix of the proteins to be separated. Therefore albumins which differ substantially in their

net charge from albumin A may well separate from it on ion-exchange chromatography. An example of such an albumin is the Ann Arbor/Oliphant variant (Winter, *et al.*, 1972) and also Albumins Gainesville (Lapresle, 1977) and Pollibauer (Galliano, *et al.*, 1984). Two penicilloylated transient variants are also separable for normal albumin by this means (Table 4.1). Albumin Birmingham was also separated on DEAE cellulose by a fairly low salt concentration gradient (Bradwell, *et al.*, 1975).

4.1.4 Use of Ion Exchange Chromatography with a pH Gradient

By using a pH gradient rather than a salt gradient Brennan and Carrell (1978) separated proalbumin Christchurch from albumin A in the sera of a heterozygous patient. The gradient was formed from two buffer reservoirs at the pH values of the two extremes of pH and the underlying principle of the technique is that at values above its pI a protein is negatively charged and so is bound to the column but that below its pI the protein is positively charged and is desorbed from the column. The pH interval of the gradient is chosen so as to encompass the pI values of both albumins and elution should occur in the order of decreasing pI. However, the pH gradient formed in this manner is not entirely smooth.

This method has been successfully used to isolate other variants including Albumins Parklands

(Brennan, 1985), IMVS (Walsh, *et al.*, 1983) and using a QAE Sephadex exchanger for the separation of a fast and slow variant by Bradley and Hornbeck (1974).

4.1.5 Preparative Electrophoresis

Electrophoresis has been the main method used for identifying albumin variants and as such it might at first seem to be an ideal method for their isolation. However, although some variants have been separated in this way there are severe limitations. The maximum loading of albumins is far lower than for chromatographic methods (only 1-10 mg dependent on the type of electrophoresis) and resolution is often lost when scaling up to preparative systems, reducing the maximum loading still further. Contaminants from the media may also prove troublesome if the isolated protein is to be used in other studies.

Some variants have been purified by this technique including albumin B (Gitlin, *et al.*, 1961) using starch block electrophoresis, proalbumin Lille (Abdo, *et al.*, 1981) using cellulose acetate electrophoresis and two unnamed variants by agar and agarose electrophoresis (Table 4.1); all using veronal buffer. Preparative polyacrylamide gel electrophoresis (prep-PAGE) has been used by Franklin, *et al.* (1980a) to isolate the variant fragment from Albumin Mexico-2 and in this laboratory to isolate Albumins Warwick-1 and 2 and Albumin Redhill (Au, 1982, Brand, 1983).

4.1.6 Chromatofocusing

4.1.6.1 Introduction

Chromatofocusing is a relatively new technique (Sluyterman, L. A. Æ. and Elgersma, O. (1978)). Separation is achieved according to the isoelectric point of the proteins to be separated and as such it has some similarities to ion exchange chromatography with a pH gradient (Section 4.1.4). However, the special ion-exchange resins used in chromatofocusing (Polybuffer Exchanger PBE94 and 118 (Pharmacia Ltd.,UK.) and the elution media (Polybuffer 74 or 96) consist of a large number of buffering species so as to give an even buffering capacity over a wide pH range and so give a smoother and more reliable pH gradient than is possible using ion exchange chromatography.

The pH gradient is self-generating and so the need for a gradient mixer with two solvent reservoirs is eliminated. The exchangers have a high capacity and the technique is simple to use; theoretically bands of 0.05 pH units can be generated and so the proteins are eluted in tightly focused bands.

4.1.6.2 Principles of Operation

The pH interval of the experiment is chosen so that the isoelectric points of the proteins to be separated are mid-way between the two extremes of pH, the actual range of the pH interval being as narrow as possible so as to give maximal resolution.

The PBE exchanger is equilibrated at a pH slightly above the higher pH limit. A small volume (5-10 ml) of Polybuffer, appropriately diluted and brought to the value of the lower pH limit with HCl, is added so as to commence gradient formation before the protein sample is applied. After sample application, the column is eluted with Polybuffer and the pH gradient formed down the column until the bottom of the column is at the same pH as that of the top. In order to prevent any artefacts due to dissolved CO_2 the buffers are all thoroughly degassed before correcting pH and subsequent use.

4.1.6.3 Protein Separation by Chromatofocusing

The protein is applied at a pH above its pI and so is negatively charged and will be rapidly bound to the exchanger. As the pH of their environment decreases below the pI values of the protein components they become positively charged and are desorbed and migrate down the column until they are reabsorbed at pH values above their pI values. In this manner proteins are progressively eluted in the order of their pI values and will leave the column in the same order. Elution may not be at the pI value of some proteins due to their insolubility and hence precipitation at this value only to be resolubilised at slightly lower pH. As separation is dependent on pI values, proteins applied to the column later will overtake those initially loaded if their pI values are higher.

4.1.6.4 Application of Chromatofocusing

Tiliyer, *et al.* (1982) used chromatofocusing for the isolation of Albumin Kashmir from double albumins. However, in a later paper (Tiliyer, *et al.*, 1984) the same authors reported that another electrophoretically more cathodic variant was not separable by chromatofocusing from albumin A, although another Indian variant, Albumin Jaffna was isolated successfully. For both these isolated variants a repeated chromatofocusing run was necessary on the partially purified peak before completely pure variant was obtained. A similar finding was reported by Brand (1983) for Albumin Redhill, and the yields in these purifications were low (typically 20-25%) with unexplained losses. There is considerable time and expense involved in repeated runs of conventional chromatofocusing and these factors, combined with the unpredictable outcome (Tiliyer, *et al.*, 1984) detract from its usefulness as a viable means of isolating variant albumins.

4.1.7 FPLC Chromatofocusing

The development of high-performance liquid chromatography (HPLC) has revolutionised separation processes for proteins and peptides in the last five years (Hearn, *et al.*, 1983). The main advantages are:

(i) greatly improved resolution, even of multi-component mixtures;

(ii) much reduced experimental run times from many hours or days to minutes;

(iii) easy recovery of isolated polypeptides in much smaller elution volumes.

Fast protein liquid chromatography (FPLC) is a modification of HPLC; the latter was initially developed for small organic molecules and later used for PTH-amino acids and small peptides. Traditional HPLC column matrices were made from derivatised silicas of uniform small pore size and so required pressures of 500-1500 p.s.i. in order to elute them at a reasonable flow rate. This necessitated the use of stainless steel pump heads and tubing in order to withstand the high pressures. The resolution of larger polypeptides and proteins on such columns was often poor.

FPLC has advantages over HPLC in the following respects:

(1) High speed separations equivalent or faster than those obtainable on HPLC but with high recovery of protein, unlike traditional HPLC where often inexplicable losses occurred.

(2) High resolution of components using far lower pressure than conventional HPLC, resulting in cheaper glass columns, and the protection of sensitive samples from high pressure conditions.

(3) High biocompatibility with separations being achieved without contact with metal ions which may well inactivate some sensitive enzymes.

Several FPLC matrices are constructed from new synthetic polymers which have been developed from the more traditional resins but with a small highly uniform bead size so that surface area has been greatly increased, dead space reduced and flow rate improved. This provides better separation than was possible with conventional soft gels.

The application of FPLC technology to chromatofocusing gives improved resolution in far shorter experimental run times (one hour compared to 10-20 hours).

4.2 EXPERIMENTAL

4.2.1 Isolation of Double Albumin from Serum

4.2.1.1 Introduction

Several methods have been used to isolate albumins from serum, the most successful being that of Cohn, *et al.* (1946) who used ethanol (40% v/v, pH 4.8, -5°C) to precipitate albumin fraction V from human serum. Commercial fraction V albumin is prepared by an essentially similar process, and is often contaminated by a number of impurities which can be removed by recrystallisation to give a product of 99% purity but which still contains substantial levels of dimer. Foster (1977) criticised the recrystallisation process for introducing unusual contaminants into the preparation.

Dimers and any contaminant proteins can be

removed by gel filtration. Foster recommended gel filtration, ion exchange chromatography, ammonium sulphate precipitation and electrophoresis as being the only four processes which in his opinion did not modify albumin.

More recently, Travis, *et al.* (1976) have used affinity chromatography with Cibacron Blue F3GA immobilised on Sepharose 6LB to obtain high purity albumin. The albumin binds tightly to the column, the other protein contaminants of serum are eluted and the albumin may be desorbed afterwards. Dimers are still presumably present in such a preparation and indeed form spontaneously especially in defatted lyophilised albumin and this dimerisation is catalysed by Cu^{II} ions (Foster, 1977). Spencer and King (1971) have proposed a means of obtaining pure monomeric albumin after blocking the free thiol residues with cystine.

Ammonium sulphate precipitation at 45% and 75% saturation followed by gel filtration was chosen as a suitable purification process to obtain double albumins. It is simple to perform, requires only 1-2 days and is inexpensive. Aging of albumin preparations due to amidation of carboxylate residues and similar transformations can occur and it is wise to avoid such artefacts by prompt use of samples after purification.

4.2.1.2 Ammonium Sulphate Precipitation (McMenamy, *et al.*, 1971)

Blood was collected from patients without addition of an anti-coagulant, serum was obtained by centrifugation and stored frozen at -20°C until required. An initial ammonium sulphate precipitation was performed by dissolving ammonium sulphate (45% saturation (277 g/L)) in serum by stirring at 0°C (and correcting the pH to 6.5). Stirring was continued for 20 minutes and the soluble fraction including albumin removed by centrifugation at 7,000 r.p.m. (MSE 18,16 x 15 ml rotor) at 4°C for 30 minutes and the supernatant collected. Saturation of the supernatant was increased to 75% by further addition of ammonium sulphate (final concentration = 516 g/L) and the pH corrected to 4.5. The solution was stirred on ice for 10-20 minutes then centrifuged for 0.5-1 hour at 12,000 r.p.m. (MSE 18,16 x 15 ml rotor) at 4°C. The supernatant was discarded and the albumin-containing precipitate resuspended in a minimum of tris-HCl (0.01 M,pH 7.0 with NaCl (0.05 M)).

4.2.1.3 Gel Filtration of the Second Ammonium Sulphate Precipitate

The pH of the solution was checked and corrected if necessary to pH 7.0 before it was loaded onto a column of Sephadex G100 (84 x 1.6 cm) equilibriated in tris-HCl (0.01 M, pH 7 with 0.05 M NaCl). The column was eluted in the same solution at 14 ml/hour and the eluate monitored at 280 nm. Approximately 4 ml fractions were collected and albumin-containing

fractions pooled into two peaks and dialysed against distilled water at 4°C. Analysis of the purity of the albumin fractions was made by non-denaturing disc-PAGE (Section 3.2.10).

4.2.1.4 Defatting of Double Albumins

Double albumins, obtained from serum as described above, were defatted by the method of Chen (1967). Albumins were dissolved in a minimal volume of water, activated charcoal (½ the weight of protein) was added and the pH brought to 3.0 with HCl. The solution was stirred on ice for one hour then centrifuged in a bench centrifuge to remove the charcoal. The supernatant was restored to pH 7.0 with NaOH and the purified albumins dialysed against distilled water overnight at 4°C and lyophilised.

4.3 PURIFICATION OF ALBUMIN REDHILL

4.3.1 Introduction

Purification was attempted by the procedures recommended by Brand (1983) and also by several other HPLC and affinity methods.

4.3.2 Chromatofocusing

4.3.2.1 Chromatofocusing on Soft Gels

Chromatofocusing was performed essentially as described by Tiliyer, *et al.* (1982) with the modifications of Brand, *et al.* (1984). Double Albumin Redhill

was applied in start buffer (histidine-HCl, 25 mM, pH 6.2) and elution with Polybuffer 74-HCl (diluted 1 in 12 with distilled water, pH 4.0) was performed, collecting 2 ml fractions and analysing the eluate at 280 nm. Albumin-containing peaks were redissolved in a minimal volume of tris/NaCl buffer and freed of Polybuffer by elution on Sephadex G100, equilibriated and eluted in tris-HCl (0.01 M tris, 0.05 M NaCl, pH 7.0).

4.3.2.2 FPLC Chromatofocusing of Double Albumin Redhill

FPLC chromatofocusing was performed on a Pharmacia FPLC system with two P500 pump units, a UV-2 absorbance monitor, an injection valve, chart recorder and FRAC 100 fraction collector and the chromatography was controlled by a GP250 gradient programmer. The chromatofocusing column (HR5/20 mono 200 x 5 mm Pharmacia U.K.) was used according to the manufacturer's instructions, and all solvents were thoroughly degassed and filtered through a 0.5 µm filter before use.

The column was equilibriated in piperazine-HCl (25 mM, pH 6.3) diluted to this concentration with MilliQ water. Double Albumin Redhill was dissolved in start buffer at a nominal concentration of 20 mg/ml (w/v) and centrifuged briefly to remove solid contaminants. The sample was filtered to remove large particles and applied to the column in 500 µl injections. Elution with Polybuffer (diluted 1:10 in MilliQ water, degassed, filtered and corrected to pH 4.0 with HCl) was commenced at 1 ml/min at room temperature.

After 40 minutes the pH gradient was completed and the column was purged with NaCl (2 M, 1.2 ml) to remove any bound material before re-equilibration in the start buffer. A complete experimental run took ≈ 1 hour. Fractions (≈ 1 ml) were collected and a new fraction commenced automatically for every peak.

4.3.2.3 Purification of Isolated Albumins

The fractions from a single run were passed through Sephadex G25, (6 x 1 cm i.d.) equilibrated and eluted in distilled water, in order to desalt the proteins and fractions (1 ml) were collected. Aliquots (10 µl) of each were spotted onto a silica t.l.c. plate (Merck) and protein-containing fractions detected by spraying with ANS (0.03% w/v Mg^{2+} salt in sodium dihydrogen phosphate 0.1 M, pH 6.8) and observing any fluorescence at 350 nm. Protein-containing peaks were lyophilised, resuspended in distilled water (100 µl) and aliquots (20 µl) diluted in load buffer (30 µl) and applied to a 7.5% non-denaturing PAGE for analysis.

From the results obtained, the fractions from each run that contained only pure Albumin Redhill were pooled and Polybuffer removed by gel filtration on Sephadex G 75 (35 x 0.6 cm i.d.) equilibriated in NaCl (0.9% w/v in distilled water). The eluate was measured at both 280 and 254 nm. Fractions (1-2 ml) were collected and the albumin peaks pooled and dialysed against distilled water before lyophilisation.

4.3.3 Other Separative Techniques

A number of other purification methods were tried in order to isolate Albumin Redhill before the successful use of FPLC chromatofocusing. These are briefly described below.

4.3.3.1 Preparative PAGE

Preparative PAGE (T = 7.5%, C = 2.6%) was performed in non-denaturing discontinuous gels (18 x 16 x 1.5 cm) essentially as outlined in Section 3.2.10. Samples of double Albumin Redhill (5-10 mg) were dissolved in bromophenol blue/sucrose 0.01%/37%, w/w in water. Electrophoresis was carried out at 150 V C.V. until the bromophenol blue tracking dye had eluted. After electrophoresis, albumin bands were visualised, without fixing and staining in PAGE 90, by one of the three methods listed below:

(i) The gel was viewed over a F_{254} fluorescent silica t.l.c. plate (Merck) under $UC_{254\ nm}$ illumination (Brand, 1983). Proteins appeared as absorbent bands and so quenched the fluorescence.

(ii) Gels were sprayed briefly with ANS (0.03% w/v Mg^{2+} salt in NaH_2PO_4, 0.1 M, pH 6.8) and viewed under $UV_{354\ nm}$ illumination. Protein bands fluoresced, especially after exposure to HCl fumes.

(iii) The bromophenol blue concentration in the loading solution was increased so that some was retained, bound to the albumin bands, which were then detectable under illumination.

The excised albumin A and Albumin Redhill bands were individually stained briefly in bromophenol blue and placed over a second 7.5% gel. A 3% acrylamide stacking gel was cast around the gel band as described by Mendel-Hartvig (1982) and reverse electrophoresis performed. Protein was collected at the top of the gel in a layer of glycerol, overlayed with NaCl (2 M). After electrophoresis was judged to be complete this layer was removed, dialysed against distilled water at 4°C and lyophilised.

Analysis of the extracted albumin fractions was performed on disc-PAGE as before, against normal albumin and double Albumin Redhill.

4.3.3.2 Preparative Cellulose Acetate Electrophoresis

Cellulose acetate electrophoresis was performed essentially as described by Abdo *et al.* (1981) but using a veronal buffer composed of sodium barbitone (15.4 g/L) and 5',5'-diethyl barbituric acid (2.76 g/L) pH 8.6. Double albumins (1-5 mg) were loaded in sucrose/bromophenol blue solution as before and electrophoresis carried out at 10-20 V/cm for 4-6 hours so as to obtain maximum separation of the albumin bands. The individual bands were detected by their binding of bromophenol blue and excised. The pooled strips from several runs were eluted and purified as described by Abdo, *et al.* (1981). Analysis of purified albumin fractions was by disc-PAGE as previously described.

4.3.3.3 HPLC

The separation of double albumins was attempted by HPLC without success. The following systems were used:

(i) Ion-exchange chromatography was performed on a TSK 5PW DEAE anion exchange column (75 x 7.5 mm i.d.) equilibrated in tris-HCl (50 mM, pH 7.4) with a 0-0.5 M NaCl gradient over 30 minutes at 1 ml/min using an LKB chromatographic system.

(ii) Ion-exchange chromatography was performed on a MonoQ (HR 5/5, 50 x 5 mm i.d.) column (Pharmacia) equilibrated in piperazine-HCl (20 mM, pH 5.5) using a 0-0.35 M NaCl gradient over 10 minutes at 2 ml/min on a Pharmacia FPLC system.

(iii) Reverse phase HPLC was performed on a pro-RPC column (HR 5/10, 50 x 5 mm i.d.) (Pharmacia) equilibrated in trifluoroacetic acid (TFA 0.1% v/v aqueous) and applying 0-70% gradient of TFA (0.1% v/v) in acetonitrile over 30 minutes at a flow rate of 0.7 ml/min on a Pharmacia FPLC system.

All solvents were thoroughly degassed and filtered before use and the double albumins (typical loading 200 µl of a 1 mg/ml solution) were filtered through a 0.45 µm filter before injection onto the column.

4.3.3.4 Affinity Chromatography

Albumin Redhill had been reported to have reduced affinity for Ni^{II} and Cu^{II} ions (Brand, *et al.*, 1984) and it was thought this could be utilised to achieve separation of the variant and normal albumins by Cu^{II} or Ni^{II} affinity chromatography.

4.3.3.4.1 Preparation of Affinity Column

(i) Chelex-100 (Bio Rad, 5 g) was resuspended in nickel(II) chloride solution (0.1 M) and a column (5 x 1 cm i.d.) was filled with this resin and washed with the nickel(II) chloride solution (50 ml) and then with water until no further Ni^{II} was removed. The column was then equilibrated in buffer (N-ethylmorpholine 0.1 M, pH 7.8 or NaCl 0.15 M). A Cu^{II}-Chelex-100 column prepared in a similar manner, was also used.

(ii) Chelating Sepharose 6B (Pharmacia) was a gift from Mr. E. Groundwater of Pharmacia, and was prepared as directed in the booklet "Affinity Chromatography on Chelating Sepharose 6B" from Pharmacia using a solution of nickel(II) chloride (5 mg/ml in water, 50 ml). Excess Ni^{II} was removed by repeated washing in water and then in buffer and the column (4 x 1 cm i.d.) equilibrated in either disodium hydrogen phosphate (0.05 M, pH 7.5) or HEPES (0.05 M, pH 7.5) both of which contained NaCl (0.5 M) to overcome any ionic interactions.

4.3.3.4.2 Elution of Double Albumins on Affinity Columns

Small amounts (1-5 mg) of double Albumin Redhill were applied to the affinity columns in a minimal volume of buffer. Samples for chelating Sepharose 6B chromatography were deionised before use, on Chelex-100 as described in Section 7.

(i) Ni-Chelex-100. After application of double albumins, the column was eluted at 10-20 ml/hour in the same buffers used for equilibrating the column. The eluate was monitored at 280 nm and fractions (≈ 1 ml) were collected.

(ii) Cu-Chelex-100. Solutions of double albumins were loaded and the column was initially eluted at ≈ 20 ml/hour in NaCl (0.01 M) and subsequently purged with copper(II) chloride (0.05 M) to remove any bound protein. The eluate was monitored at 280 nm and fractions (≈ 1 ml) were collected.

(iii) Ni-Chelating Sepharose 6B. Double albumins were applied and the column was eluted at ≈ 20 ml/hour in the initial equilibration buffers (phosphate or HEPES) and then with one of the following solutions to displace the bound proteins:

(a) Phosphate with a gradient of glycine (0-20 mg/ml).

(b) Phosphate with a gradient of EDTA (0-10 mM).

(c) HEPES with a gradient of nickel(II) chloride (0-10 mg/ml).

Eluate was monitored at 280 nm and fractions (1 ml) were collected and analysed, after desalting, on disc-PAGE.

4.4 PURIFICATION OF ALBUMIN CARLISLE

4.4.1 Introduction

Albumin Carlisle was thought to be a new variant and a wide range of previously successful techniques (Table 4.1) were used in order to attempt to isolate it, some of which have already been described.

4.4.2 Experimental

4.4.2.1 Chromatofocusing

Soft gel chromatofocusing was carried out on double Albumin Carlisle as outlined in Section 4.1.6.4. Double Albumin Carlisle (≈ 10 mg) was loaded and elution performed at ≈ 12 ml/min until the pH of the eluate was the same as that of the Polybuffer 74 eluant. Fractions (2 ml) were collected and the pH and the absorbance at 280 nm were measured. Peaks were analysed, after purification, by disc-PAGE.

4.4.2.2.1 FPLC Chromatofocusing

FPLC chromatofocusing was performed on double Albumin Carlisle at Guy's Hospital Medical School essentially as previously described in Section 4.3.2.2. Solutions of double albumins in start buffer (nominally 20 mg/ml w/v) were loaded in aliquots (0.5 or 1.0 ml) and a flow rate of 1.0 or 0.5 ml/min was used. Fractions were collected as before and aliquots (10 µl) used without purification and loaded onto disc-PAGE in bromophenol blue/sucrose loading solution (40 µl) for analysis against normal albumin and double Albumin Carlisle.

4.4.2.2.2 Purification of Albumin Fractions

Purification of Albumin Carlisle isolated by FPLC from Polybuffer contaminants was attempted by chromatography on hydroxyapatite (Bio Gel HTP, Bio Rad.) after the method of Satyanarayana and Klein (1984). A column (15 x 0.4 cm i.d.) of hydroxy-apatite was equilibrated

in potassium dihydrogen phosphate (0.01 M, pH 7.45) and the sample of albumin and Polybuffer applied. Elution was at ≈ 15 ml/hour and the eluate was monitored at 280 and 254 nm, by collecting 2 ml fractions and reading manually in a spectrophotometer. After 30-50 ml of buffer had eluted, a stepwise gradient was formed by eluting with potassium dihydrogen phosphate (0.1 M, pH 7.45) and elution continued using 50-100 ml of this buffer. Purified protein was dialysed against distilled water and lyophilised.

4.4.2.3 Ion Exchange Chromatography

Separation of double Albumin Carlisle was attempted unsuccessfully by ion exchange chromatography using the following systems.

(i) DEAE cellulose (DE52, Whatman) equilibrated in sodium phosphate buffer (0.2 M, pH 5.75) and eluted according to the method of Winter, *et al.* (1972).

(ii) DEAE cellulose equilibrated in tris-HCl (0.01 M, pH 8.6) and eluted with a gradient of 0-0.5 M NaCl after the method of Bradwell, *et al.* (1975).

(iii) Ion exchange HPLC using a Partisil SAX anion exchange column (25 x 0.46 cm i.d., Whatman) with a 0.01-0.5 M gradient of potassium dihydrogen phosphate (pH 7.0) eluting at 1 ml/min over 30 minutes followed by a gradient up to 1.5 M KH_2PO_4, using a Waters HPLC system (one 6000A pump, one M45 pump, and a 600 series gradient programmer with U6K injector and 441 absorbance detector, monitoring at 280 nm). Fractions were collected manually.

4.4.2.4 Ion Exchange Chromatography with pH Gradient

Double Albumin Carlisle (10 mg) was applied to a DE52 column and a gradient of pH from 5.5 to 4.5 applied using solutions of sodium acetate (25 mM) as eluant (essentially after Brennan and Carrell, 1978) at a flow rate of 27 ml/hour. Eluate was monitored at 280 nm and fractions (5 ml) were collected.

A similar gradient was used on QAE Sephadex A25 essentially after the method of Bradley and Hornbeck (1974) and an analogous HPLC experiment performed on a Partisil SAX anion exchanger using a pH gradient from pH 5.1 to 4.5 in potassium dihydrogen phosphate (0.1 M). Any separation was estimated by disc-PAGE.

4.4.2.5 Preparative Electrophoresis

Preparative electrophoresis on cellulose acetate and polyacrylamide was performed as described in Sections 4.3.3.1 and 4.3.3.2 using the reverse elution electrophoresis method of Mendel-Hartvig (1982) and the cellulose acetate method of Abdo, *et al.* (1981).

Fractions from each method were analysed on disc-PAGE against double Albumin Carlisle and normal albumin.

4.5 POLYACRYLAMIDE GEL TITRATION CURVE FOR ALBUMIN CARLISLE

4.5.1 Principle

Double Albumin Carlisle was analysed using a pH titration curve method in polyacrylamide gel

containing carrier ampholyte (pH 3-10, Pharmacia). A pH gradient was formed in a square polyacrylamide thin layer slab gel by electrophoresis of the ampholytes until they reached their isoelectric points and were stationary. A sample of double albumin could then be applied to a central well cut longitudinally across the pH gradient and electrophoresis carried out at 90° to the initial focusing. The protein mixture was thus electrophoresed at different pH values along the gradient and the degree of separation possible at each pH value was seen by staining the gel with Coomassie Blue. A titration curve for the normal and variant albumin was obtained, their subsequent mobilities reflecting the charge make-up of the proteins at each pH, along a continuous curve.

4.5.2 Experimental

The method used was as described in the booklet "Isoelectric Focusing, Principles and Practice" by Pharmacia and was performed for us by Dr. R. W. Evans of Guy's Hospital Medical School, London. A polyacrylamide gel (10 x 10 cm x 1 mm thick) was cast containing ampholyte in the pH range 3-10 (T = 5%, C = 3%), as for isoelectric focusing in thin layer slabs, only with a sample notch former already in place. Isoelectric focusing and electrophoresis were performed at 4°C on a cooling plate. The anode electrolyte was H_2SO_4 (0.05 M) and the cathode electrolyte NaOH (1 M). Isoelectric

focusing was performed in the first dimension at 15 W with a limiting voltage of 2000 V (unlimited current) and the gel focused for 800 volthours.

After focusing the gel was rotated through 90°, fresh electrode strips positioned and double albumin (≃1-2mg) in water (50 µl) applied to the sample trough. Electrophoresis was carried out at 15 W (limiting voltage 1000 V) in the same buffers for 250 volthours (using a Pharmacia power supply and flat-bed apparatus such as the FBE3000).

Fixing, staining and destaining were as described previously for isoelectric focusing (Section 3.2.11.1).

4.6 RESULTS

4.6.1 Albumin Redhill Purification

Albumin Redhill and normal albumin co-precipitated in the second ammonium sulphate precipitation step. The double albumins chromatographed together on G-100 (Fig. 4.1) giving two major peaks, one at 60 ml and the other at 175 ml of buffer eluted. Non-denaturing disc-PAGE showed these to contain primarily the monomeric albumins with small amounts of dimer and polymeric albumin. The dimeric content of the first peak may have been higher than the second, also it possibly contained more lipid. A small amount of a protein running behind the double albumins was seen in the same preparations and may

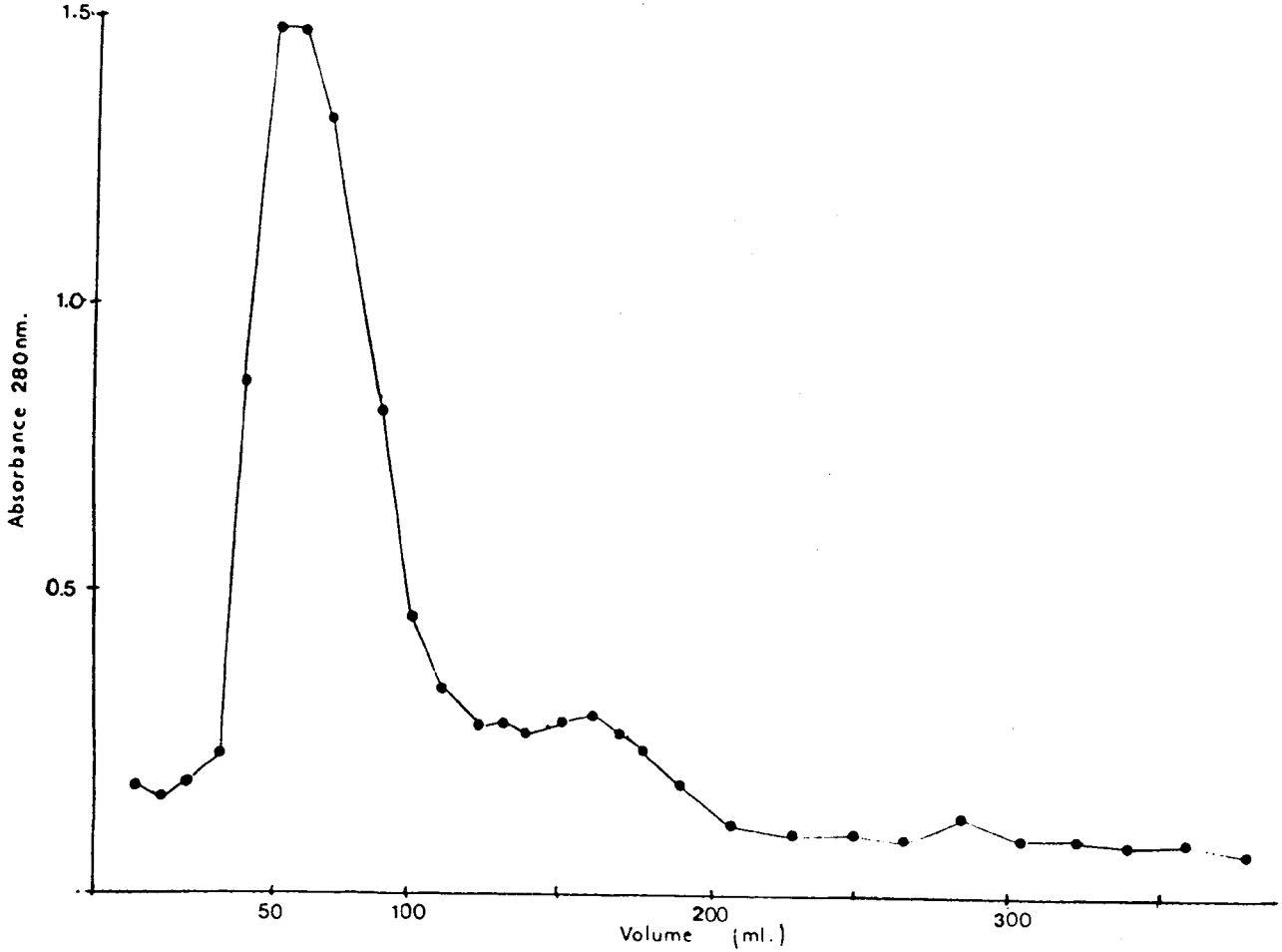


Fig. 4.1 Gel Filtration of double Albumin Redhill on Sephadex G-100.
(Second ammonium sulphate precipitate applied)

have been transferrin, or possibly an albumin degradation product due to storage. Double albumins were defatted by the method of Chen as described and after dialysis and lyophilisation gave a white fluffy powder, which was used without further purification in the experiments to isolate pure Albumin Redhill.

Previously successful methods such as preparative PAGE and chromatofocusing in soft gels gave disappointing results. On preparative PAGE, although albumins A and Redhill separated, there was considerable difficulty in being able to visualise the distinct bands and only the method of pre-staining with bromophenol blue gave discernably separate bands. Albumin Redhill obtained from reverse elution did not give a recognisable 280 nm absorbance even after dialysis and non-dialysable contaminants with a high UV absorption were present, possibly due to acrylamide monomer, or other gel contaminants. Despite attempted purification on DE52 mini-columns eluting with a NaCl gradient, the contaminants were still present and electrophoretic analysis of the isolated albumin gave a diffuse band from which it was impossible to tell the degree of purification from Albumin A. The actual recovery of total protein also seemed to be very low compared to the amount initially loaded. Although Brand (1983) gave photographic evidence of pure Albumin Redhill by this method, it is possible that UV contaminants may have resulted in over-estimation of the albumin concentration and hence

explain why sequence analysis of such material failed to reveal any albumin.

Preparative cellulose acetate was similarly unsuccessful in yielding pure variant. The separation of Albumin Redhill from albumin A is poor on this medium and despite long run times the isolated Albumin Redhill bands still contained some albumin A on analysis by disc-PAGE. The electrophoretic pattern of this isolated variant was also diffuse and this may have been due to the presence of excessive salts or to molecular aging of the albumin during extraction.

Ion-exchange HPLC failed to resolve the double albumins, yielding only double albumins on analysis of the peaks obtained. The several peaks obtained may have been attributable to dimeric albumins or albumins with endogenous ligands still tightly bound and so possessing different net ionic charges.

Reverse phase HPLC also failed to resolve the variant and normal albumins which co-eluted in a single peak. It would appear that the net differences in charge or hydrophobicity between Albumin Redhill and normal albumin are insufficient for these techniques to be able to resolve them.

Nickel and copper affinity chromatography also failed to resolve the two albumins. On Cu-Chelex-100 two peaks were obtained, the latter containing just albumin A and the former, although slow migrating on PAGE, was shown to consist of both albumins by cellulose

acetate electrophoresis. The experiments using Ni-Chelex-100 gave no significant separation as both albumins were eluted without binding. A reason for this may have been that the metal was bound too closely to the matrix and was thus inaccessible to the albumin. Chelating Sepharose was therefore used where the metal ion is bound to a side chain at a greater distance from the matrix. Two peaks were obtained on chromatography of the double albumins with either a glycine gradient in a phosphate buffer or with a Ni^{II} gradient in a HEPES buffer. The first peak contained pure albumin A, the second a mixture of the two albumins. With a Ni^{II} gradient in a HEPES buffer albumin A was the major component even in the second peak. This result seemed contrary to expectations as one would expect albumin A to be bound to the column and Albumin Redhill to be eluted. However, instead the albumins were eluted together or albumin A on its own, no band containing just Albumin Redhill could be found during several runs.

Chromatofocusing on a soft gel gave a poor result - 10 hours or more were needed to develop the full gradient, and although a main peak with two shoulders was eluted early in the experiment, the pH at this point was between 6.5 and 7.0. Two peaks were eluted much later but they contained little or no albumin by PAGE. Due to the excessive time taken and poor pH gradient formation a second round of chromatofocusing was not attempted.

FPLC chromatofocusing yielded two sets of

peaks at the start and end of the gradient, and three peaks in the middle of the gradient which contained the albumins that had separated (Fig. 4.2). The first peak contained protein not bound to the column at pH 6.3 and the last peak contained proteins eluted from the column with the salt wash, and otherwise still bound at pH 4.0. The total $OD_{280\ nm}$ of these regions was far higher than that of the three middle peaks. The three peaks consisted of pure normal albumin, double albumins, and pure Albumin Redhill. The total yield of pure Albumin Redhill was quite low (≈ 5% by relative 280 nm peak heights). The reason for there being three peaks may be due to aggregation of the normal and variant forms to give a mixed dimer resulting in the middle peak, or alternatively, incomplete resolution at the pI value due to precipitation and mixing on resoluilisation may have occurred.

Although the yield of variant was very low the run time was short and so the pooled peaks from several runs could be taken. The analytical PAGE of fractions from a run (Fig. 4.3) revealed that the third peak contained pure Albumin Redhill.

Application of the pooled pure Albumin Redhill to gel filtration resulted in the albumin being eluted quickly but the contaminant Polybuffer far more slowly. The UV_{max} of the albumin was at 280 nm and that of the Polybuffer at 254 nm (Fig. 4.4). However, despite dialysis and gel filtration of the 'pure' albumin on Sephadex G-25

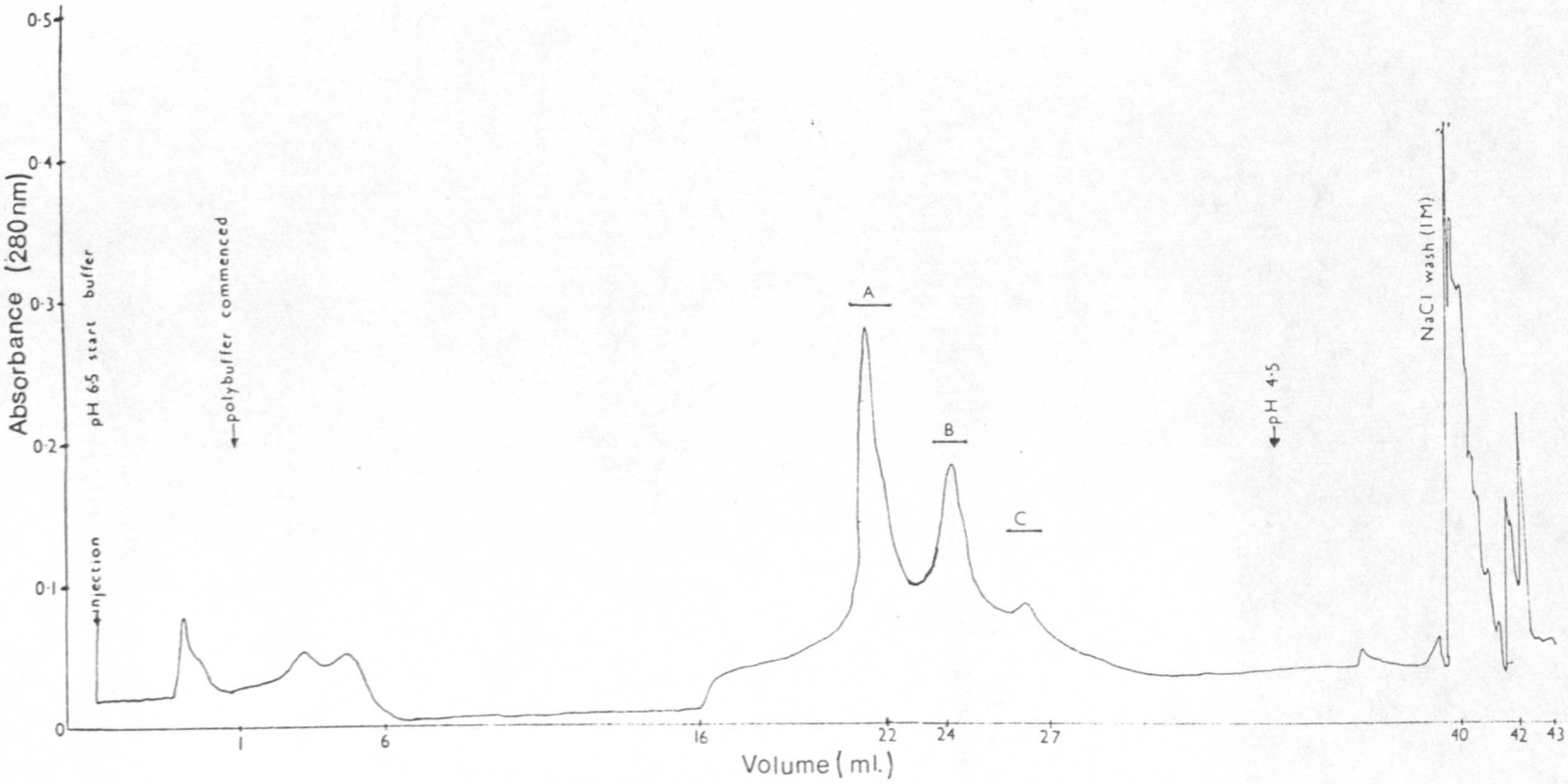


Fig. 4.2 FPLC Chromatofocusing of double Albumin Redhill on a mono P column.

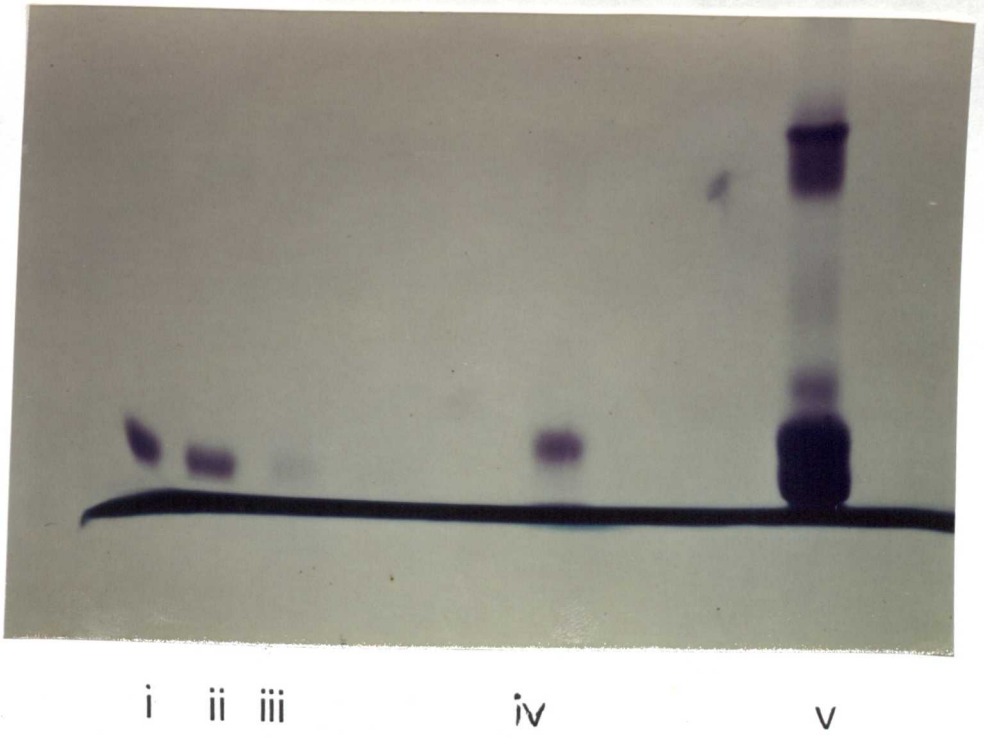


Fig. 4.3 PAGE of albumin fractions: (i, ii & iii) HSA, (iv) Peak C from chromatofocusing (v) double Albumin Redhill.

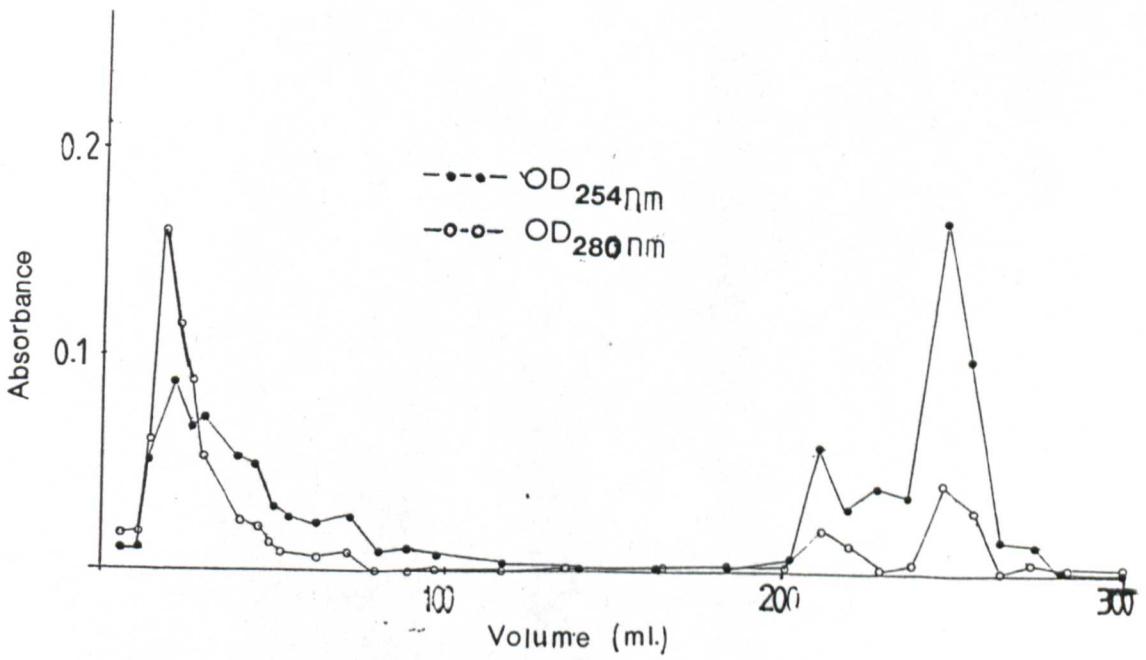


Fig. 4.4 Gel filtration of fractions from chromatofocusing (fig.4.2) in order to remove Polybuffer, on Sephadex G75.

in water, UV contaminants were still present. These may be due to some component of Polybuffer, which is thought to have a large molecular weight of several thousand. The isolated Albumin Redhill was pooled and lyophilised.

4.6.2 Purification of Albumin Carlisle

Albumin Carlisle was isolated from serum together with albumin A with an elution profile on G-100 essentially the same as that of Albumin Redhill (Fig. 4.5, Section 4.6.1). The albumin containing fractions were pooled, dialysed and lyophilised. Small traces of EDTA were included in the dialysis to chelate any metal ions and retard aggregation. Double Albumin Carlisle was defatted as previously described and the resultant powder shown to be 70-80% pure albumin by Coomassie Blue protein assay (Bradford, 1976) and free of any major contaminant on gel electrophoresis, though a small amount of transferrin may have been present in some runs.

A simple elution on DE52 (Winter, *et al.*, 1972) did not resolve the variant from the normal albumin. Gradient elution on DE52 used to successfully purify Albumin Birmingham (Bradwell, *et al.*, 1975) was also unsuccessful. HPLC ion exchange chromatography did not give improved resolution of the albumins and it appeared that albumin bound on the column and was only later desorbed at higher salt concentration.

None of the examined

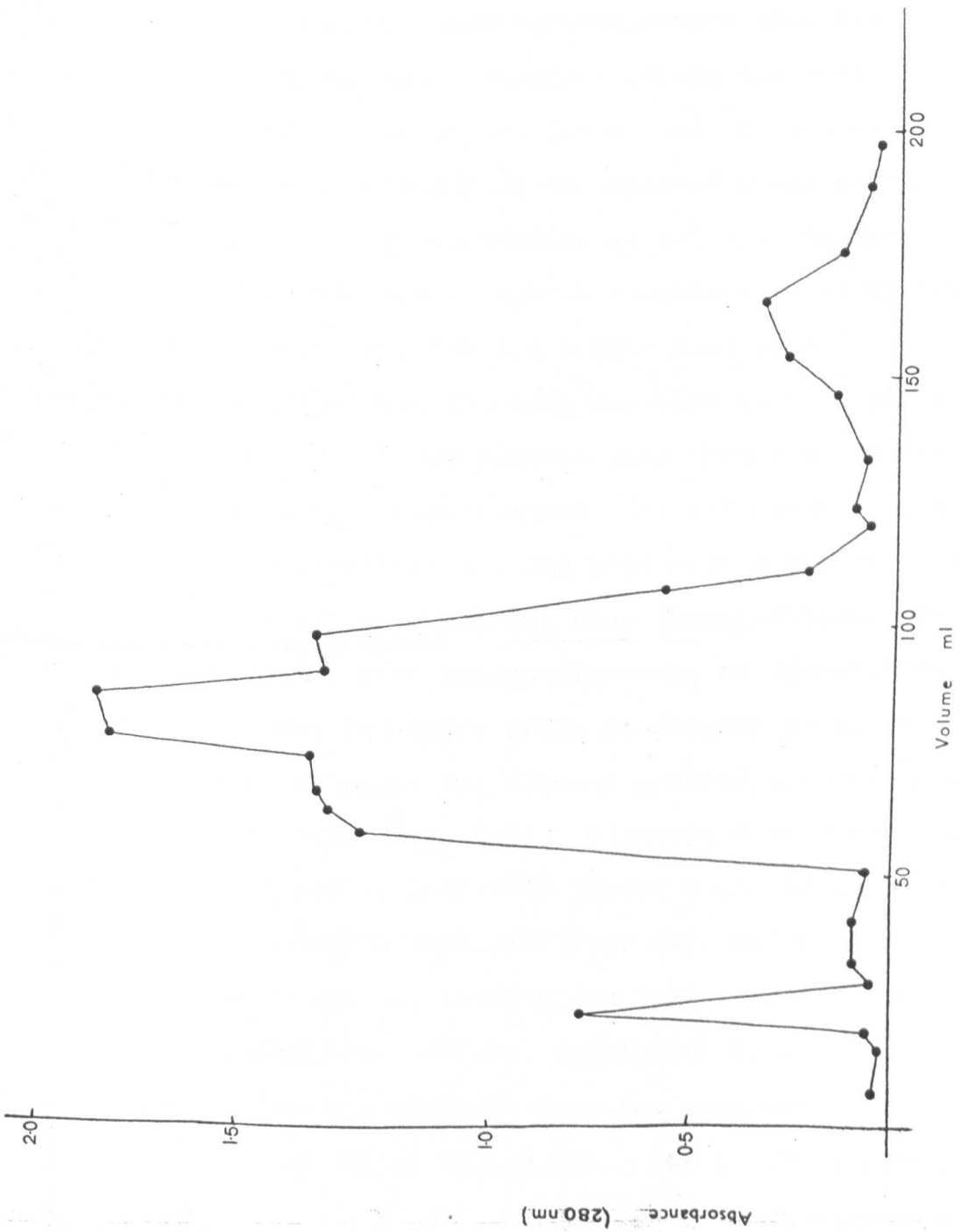


Fig. 4.5 Gel filtration of double Albumin Carlisle on Sephadex G-100.
(Ammonium sulphate second precipitate applied.)

peaks contained pure albumin variant. The use of a pH gradient on an ion-exchanger was also completely unable to separate variant from normal albumin, both on soft gel or HPLC. In a similar experiment with QAE Sephadex no separation was achieved either.

Attempts to purify Albumin Carlisle by preparative electrophoresis were also frustrated due to inconclusive analysis of the extracted bands. The glycerol containing layers and those from cellulose acetate showed high absorbances which blotted out any protein assignments at 280 nm. Analysis on disc-PAGE was ambiguous, sometimes yielding two bands and at other times a single band which migrated over the same distance as both albumins.

These efforts were abandoned in favour of chromatofocusing. Conventional soft gel chromatofocusing required long run times and the resultant peaks were very broad and were in large volumes thus giving ambiguous results. However, FPLC chromatofocusing of double Albumin Carlisle yielded two major peaks of roughly equal 280 nm absorbance as well as peaks for unbound protein and that eluted in the salt wash (Fig. 4.6). Aliquots from these peaks analysed on disc-PAGE showed that the first major peak contained Albumin Carlisle (Fig. 4.7) and the other albumin A. Variations in loading and flow rate affected resolution, better separation being achieved at 0.5 ml/min. although baseline separation of the peaks did not occur in any run. The yield of pure Albumin

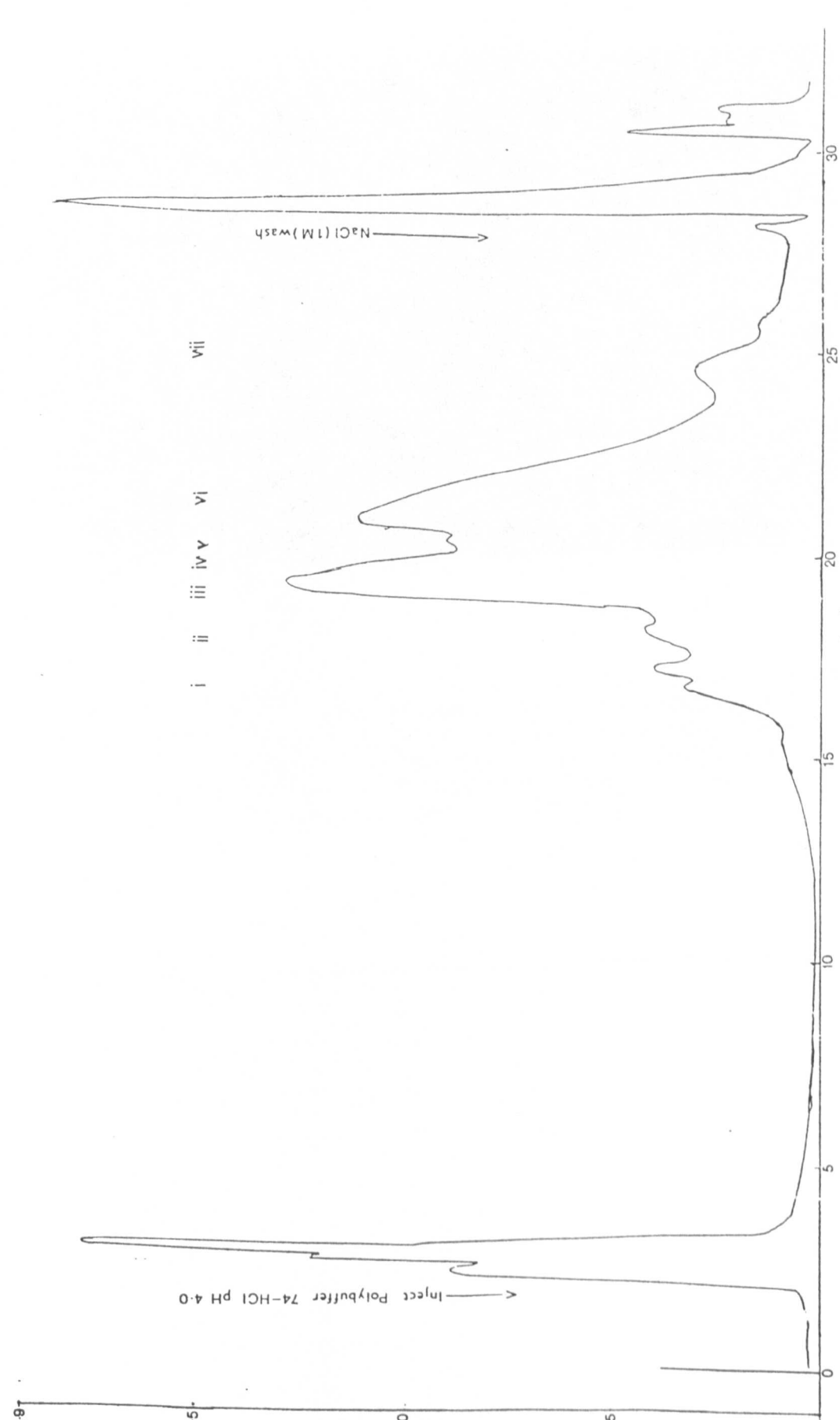


Fig. 4.6 FPLC Chromatofocusing of double Albumin Carlisle on a mono P column.

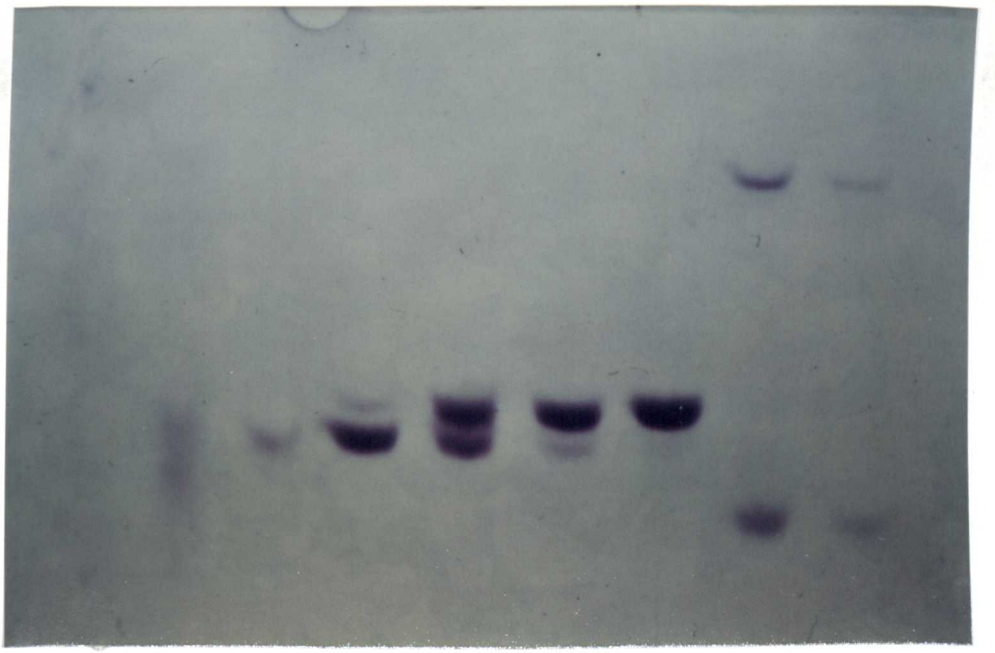


Fig. 4.7 PAGE of FPLC fractions from the chromatofocusing of double Albumin Carlisle (numbered as in Fig. 4.6): (i & ii) non-albumin proteins, (iii) Albumin Carlisle, (iv) mainly Albumin Carlisle, (v) double albumins, (vi) mainly albumin A, (vii) albumin A.

Carlisle was slightly larger than for albumin A and eluted before albumin A in the pH gradient, consistent with its pI being higher than that of normal albumin. The minor peaks contained dimers (first two peaks) and normal albumin in the small fifth peak (see Fig. 4.7).

Although only ≈ 16% pure Albumin Carlisle was obtained (as a % of total 280 nm absorbance) on a single run, because the experimental run time was short and the technique far less expensive in solvents than traditional chromatofocusing, FPLC chromatofocusing was the technique of first choice in purifying Albumin Carlisle.

From analysis of the pH titration curve (Fig. 4.8) it is clearly seen that Albumin Carlisle and albumin A are most distinctly separated at pH values in the region pH 6.0-7.5 and that the resolution between them is very poor at pH values of pH 4.0 or below. Thus, the pH range for the ion exchange separations was correct for separating the variants in most cases and a separation in the pH range 6-7.5 should theoretically be possible. Any further intensive ion-exchange studies, if carried out, should be in this pH range. From the reduced mobility of Albumin Carlisle at neutral and slightly alkaline pH values it would seem that Albumin Carlisle is caused by an acid to neutral mutation, from the predictions of Righetti et al. (1978)

The isoelectric point of Albumin Carlisle (calculated from the point of no net

migration of the protein) was shown to be higher than for albumin A (pI = 5.22 for Albumin Carlisle compared to 5.09 for albumin A), although accurate values would have to be calculated using a pH electrode to define the gradient in the gel, which would also be true for obtaining accurate values of the optimal pH for ion exchange separation.

4.7 CONCLUSIONS

A variety of well established separative techniques were unsuccessfully attempted in order to isolate Albumins Carlisle and Redhill from sera also containing albumin A. However, FPLC chromatofocusing was successful for both variants although with differing elution profiles and recoveries. The technique is rapid, simple to perform and inexpensive. The relative effectiveness of the technique was shown to be variable and so complete trials would need to be performed on each variant to be purified by this method. Both albumin variants were obtained in $\geq$ 90% purity from traces of albumin A and with further modifications to run conditions higher yields and greater purity may be possible.

Albumin Carlisle was shown to have a higher pI value than albumin A by chromatofocusing and a pH titration curve, but Albumin Redhill appears to have a lower pI value than normal albumin from its elution profile. The pH titration profile is consistent with

an acid to neutral amino acid transformation in the Albumin Carlisle molecule.

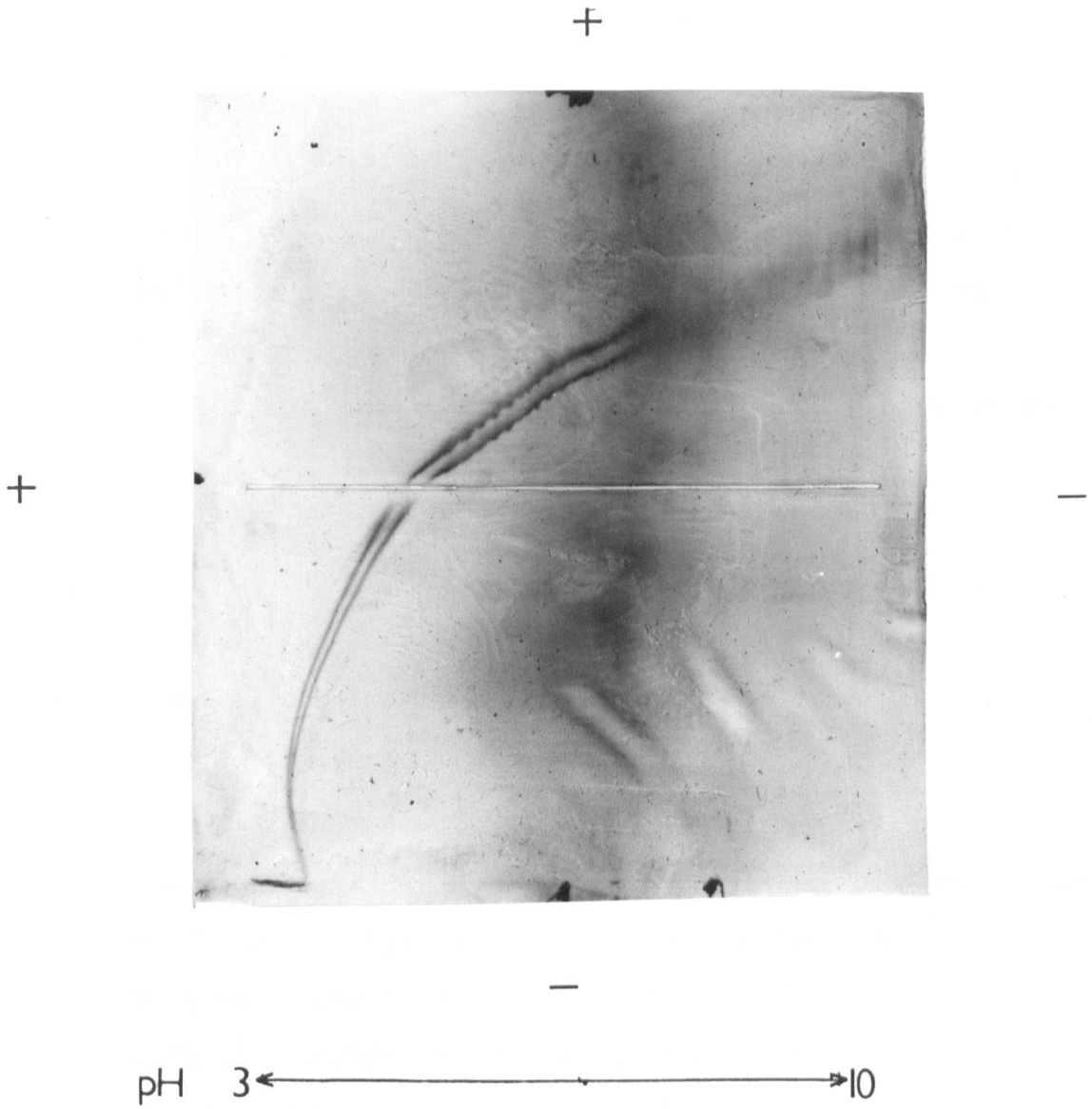


Fig. 4.8 pI titration curve of double Albumin Carlisle.

CHAPTER FIVE

PEPTIDE MAPPING OF ALBUMIN VARIANTS

5.1.1 Peptide Mapping

The chemical or enzymatic digestion of a protein and the subsequent separation of the fragments has been a popular method of structural analysis for nearly 30 years and has been termed "finger-printing" or peptide mapping. Several excellent reviews of the techniques used in these processes have been compiled by James (1980) and Hermodson (1983).

The earliest two-dimensional peptide maps were reported in the late 1940's. In 1959 Ingram pinpointed, by use of a combination of chromatographic and electrophoretic separations of a tryptic digest, a mutant peptide in the haemoglobin variant responsible for causing sickle cell anaemia, having a glu → val mutation. Subsequently, peptide mapping has been used to identify mutations in protein variants, to carry out comparative biochemistry and to classify the subunit structures of viral proteins. Proteins to be studied are often either S-carboxymethylated after reduction with thiol reagents, or are reduced and oxidised with performic acid.

Enzymes used to digest proteins include trypsin, with specificity for lysine and arginine

residues; chymotrypsin, which cleaves after hydrophobic residues such as phenylalanine, and several less widely used enzymes such as thermolysin (which cleaves at the amino-terminal side of hydrophobic residues), clostripain (specific for arginine residues), and mouse submaxillary proteases (also specific for arginine residues). Less specific enzymes, which are occasionally used include papain, subtilisin, pepsin, pronase and Staphylococcal protease.

Chemical cleavages can be classified into three main groups:

(i) Cleavage at methionine - using cyanogen bromide (CNBr) (Gross and Witkop, 1961). This is the most specific chemical cleavage method, and due to the relative scarcity of methionine residues, it usually gives rise to a small number of relatively large peptides.

(ii) Cleavage at tryptophan - using a variety of reagents including N-bromo-succinimide, N-chloro-succinimide, o-iodosobenzoic acid, BNPS-skatole and CNBr after the modification of all methionine residues.

(iii) Cleavage at other residues - including cysteine using 2-nitro-5-thiocyanobenzoic acid (Stark, 1977). These cleavages are less specific and so suffer from cross-reaction contaminants due to undesired

cleavages at other residues. The asp-pro bond is particularly sensitive to acid hydrolysis and this has also been used to analyse protein structure (Marcus, 1985).

5.1.2 Peptide Resolution

The peptides produced by proteolytic or chemical means may be separated by several different procedures. The first system used for the analysis of tryptic peptides was chromatography followed by high-voltage electrophoresis on Whatman filter paper (Bennett, 1967). This was replaced in the late-1960's by thin-layer methods, mainly on silica or cellulose, which required less sample for detection of the peptides and gave improved resolution and reduced run times. A typical solvent used for chromatography was a combination of acetic acid, pyridine, butan-1-ol and water, followed by electrophoresis in pyridine-acetate buffers, although more non-polar solvent systems are now often used for silica thin-layer experiments (James, 1980). Two-dimensional chromatographic separations have also been used successfully with the dansylated peptides of proteolytic digests (Gerday, *et al.*, 1968; Tichy, 1975).

More modern methods for peptide separation have included isoelectric focusing in polyacrylamide gels, SDS-PAGE and a variety of chromatographic

systems. SDS-PAGE has been used by Cleveland, *et al.* (1977) to analyse the peptides produced by enzymatic digests in the presence of SDS, and it has been applied to proteins still contained within excised polyacrylamide gel bands. This work has since been extended to include chemical cleavages of proteins still contained within gel slices with subsequent analysis by SDS-PAGE (Lam and Kasper, 1980). Possibly one of the most popular new methods of peptide separation has been the use of reverse phase and ion exchange HPLC (see Section 5.3).

5.1.3 Peptide Localisation and Elution

Early peptide maps were developed using ninhydrin sprays or dips, often containing cadmium acetate to increase sensitivity or 2,4,6-collidine to give different coloured spots for various amino acids. The use of dansyl chloride (Gray and Hartley, 1963) to derivatise the amino-terminal residues of peptides to give fluorescent amino acid derivatives on hydrolysis has been widely used and is extremely sensitive.

Other detection methods have included the use of o-phthaldehyde (Benson and Hare, 1975) and more recently fluorescamine (Stephens, 1978) which can be used to detect picomoles of peptide as fluorescent moieties (Fig. 5.1). A highly sensitive method of peptide mapping is to label the peptides

with ^{125}I or to incorporate ^{14}C or ^{32}P which can then be easily detected by autoradiography. Although the radioactive hazard is a disadvantage this method is often used when only small amounts of protein are to be digested (Jay, 1984).

5.2 METHODS OF PROTEIN CLEAVAGE

5.2.1 Aims

Human albumin has six methionine residues, one tryptophan and a single asp-pro sequence at residues 365-6. The methods for structural analysis of the protein chosen were dictated by cleavages at these residues and so CNBr, NCS and similar agents were used to generate large fragments of normal and variant albumins. The N and C-termini of the variant albumin were determined and tryptic and other enzymatic digestions performed, which generated a large number of much smaller fragments.

5.2.2.1 Reduction of Disulphide Bridges

The sites of cleavage of many chemical and enzymatic agents are often buried in the tertiary structure of the protein. It is therefore necessary to disrupt the disulphide bonds and unfold the protein before fragmentation can take place. Once reduced, refolding of the protein can be prevented by performic acid oxidation or S-carboxymethylation.

5.2.2.2 Performic Acid Oxidation

Performic acid oxidation is used to denature albumin before determination of the N-terminus by dansylation and prior to tryptic digestion. As well as converting cystine residues to cysteic acid, performic acid oxidation also causes oxidation of methionine residues to the sulphone and so prohibits subsequent CNBr degradation without prior reduction of the methionine sulphone to its original form. Tryptophan residues are also modified by performic acid to N-formylkynurenine, with cleavage of the indole ring. This prevents further cleavage at this residue with tryptophan-specific reagents (Fig. 5.2).

5.2.2.3 Reduction and S-Carboxymethylation

A more useful technique for denaturing proteins if they are to be subsequently cleaved by other agents is to reduce the disulphide bonds and S-carboxymethylate the resultant thiol residues. Reduction of the disulphide bridges can be achieved using 2-mercaptoethanol (Hirs, 1967) or dithiothreitol/ dithioerythreitol (Cleland, 1964) usually anaerobically with excess reagent present at alkaline pH. Reduction is usually carried out in the presence of denaturants such as guanidinium hydrochloride or urea. Once denatured and reduced the thiol resudues are blocked with either iodoacetamide or iodoacetic acid, the latter causing an increase in the net negative charge of the protein (Fig. 5.3).

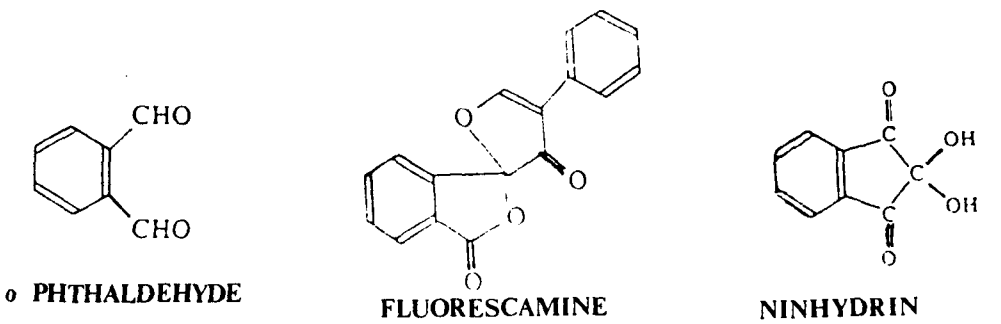


Fig. 5.1 Some N-terminal labelling reagents.

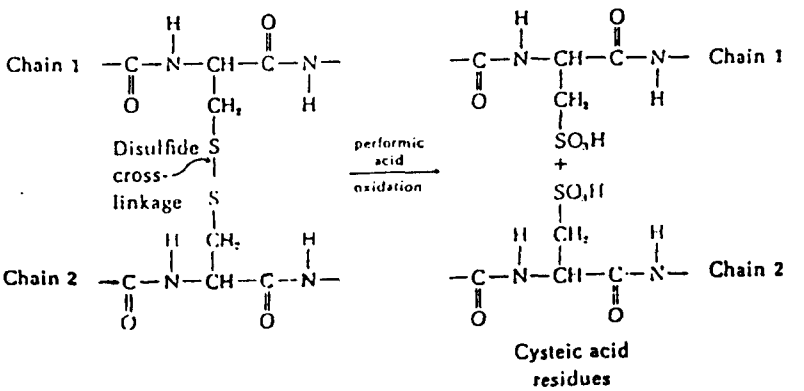


Fig. 5.2 Performic acid oxidation of proteins.

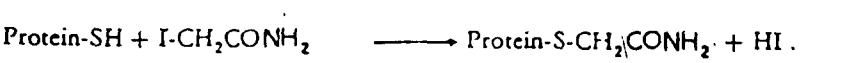


Fig. 5.3 S-Carboxymethylation of proteins.

5.2.3.1. Determination of N-terminal Amino Acid

N-terminal residues were first detected by Sanger (1945) using fluorodinitribenzene (FDNB), to generate a yellow amino acid derivative which could be identified by chromatography. A far better method is that of Gray and Hartley using dansyl chloride, described in Gray, 1972, although other procedures have been devised including:

(i) Phenylisothiocyanate - Edman's reagent (1950) used in the stepwise sequencing of proteins.

(ii) Dimethylaminoazobenzene-4-isothiocyanate (DABITC) introduced by Chang, *et al.* (1976) and producing strongly coloured derivatives.

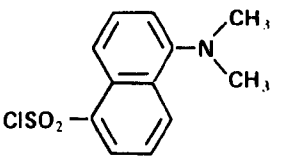
(iii) Cyanate (Stark, 1972a).

(iv) Aminopeptidases for the enzymatic cleavage of N-terminal residues (Light, 1972).

Dansyl chloride is probably the most popular of these methods and it has been used here because of its high sensitivity, requiring only 1-5 nmol of protein, and its comparatively simple protocol for use (Fig. 5.4).

5.2.3.2 Mechanism of Reaction

Dansyl chloride reacts with primary amino groups such as those of N-terminal residues and ε-lysine residues and also with secondary amines. Other side chains are modified in the order cys > tyr > lys > his (Gray, 1972); however, the cysteinyl



(5-Dimethylaminonaphthalene-1-sulfonyl Chloride)

Fig. 5.4 Structure of dansyl chloride.

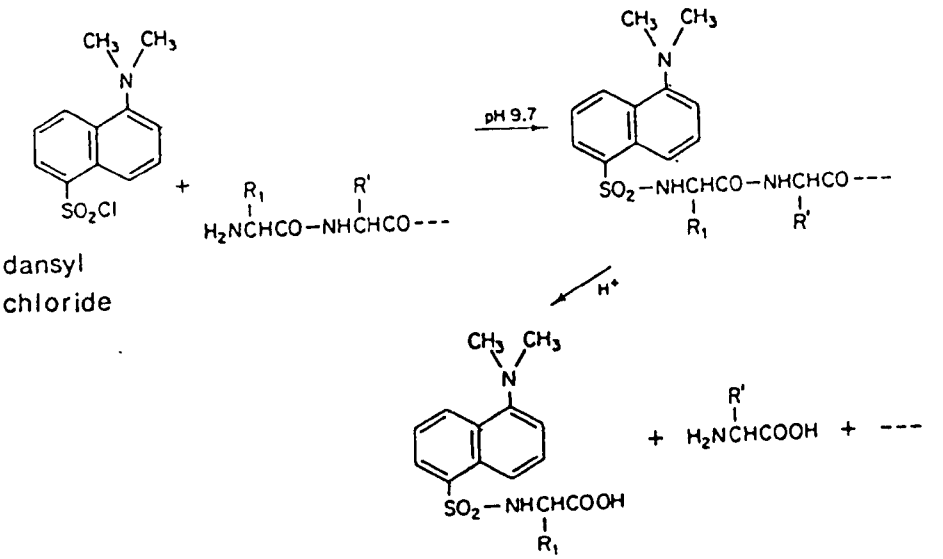


Fig. 5.5 Coupling of dansyl chloride to the N-terminal residue of a protein.

and histidinyl side-chain derivatives are unstable under the acidic conditions of peptide hydrolysis.

The coupling reaction (Fig. 5.5) must occur at alkaline pH, as only the NH_2 group is attacked and not the protonated $\overset{+}{N}H_3$ form, and the buffer is usually chosen between pH 8-10. A ratio of dansyl chloride: protein of 5:1 is usually adequate and SDS and urea may be added to improve peptide solubility without interfering with the dansylation process. Once dansylation has occurred (about one hour), acid hydrolysis at 105^{o}C is used to cleave all the peptide bonds. Hydrolysis is normally continued for 16 hours; however, some residues are more susceptible than others; DNS-proline being a secondary sulphonamide is rapidly broken down, DNS-threonine, DNS-cysteine and DNS-serine all decompose to 1,dimethyl naphthalene-1-sulphonamide (DNS-NH_2), and DNS-tryptophan is also destroyed. Other dansyl derivatives such as DNS-valine, DNS-leucine and DNS-alanine are stable to such hydrolysis. The dansylated N-terminal amino acid can be determined after hydrolysis by two-dimensional chromatography against known standards.

5.2.4 Determination of C-Terminal Residue

Methods of determination used for C-terminal analysis are less satisfactory than those for the N-terminus. Hydrazinolysis has been used by

Schroeder (1972) and C-terminal tritium labelling by Matsuo, *et al.* (1966); other methods include hydantoin formation (Stark, 1972b) and digestion with carboxypeptidases (Ambler, 1972). The latter has been the method chosen here as it is a far simpler procedure than the others. However, one problem encountered with carboxypeptidases is their differing specificities. Carboxypeptidase A releases non-polar residues rapidly from the C-terminus, but acidic or glycyl residues are released very slowly and proline or arginine not at all. The nature of the penultimate residue also affects the rate of release of the C-terminal residue, and difficulties arise if the C-terminal residue is released slowly but the penultimate residue quickly. Endopeptidase contamination is a common problem and must be inhibited with pepstatin A. Carboxypeptidase B removes arginine and lysine residues from the C-terminal of peptides and proteins without difficulty but is not very stable. Carboxypeptidase Y (Hayashi, 1977) from baker's yeast has a broad specificity but with a preference for hydrophobic residues. Glycine at the penultimate residue reduces the rate of C-terminal hydrolysis. The enzyme is stable at -20^{o}C and at 20^{o}C for 8 hours, but is inhibited by heavy metal ions; Lee and Riordan (1978) have given details of its practical use. Once released the C-terminal amino acid can be detected by chromatographic means

against standards, or by amino acid analysis.

5.2.5 Cleavage at Methionine with CNBr

Cyanogen bromide (CNBr) was introduced by Gross and Witkop (1961) and is the most specific of the chemical cleavages available. It causes cleavage at the carboxyl side of methionine under strong acid at room temperature, giving rise to a homoserine residue which is interconvertible with homoserine lactone (Ambler, 1965) (Fig. 5.6).

Cleavage occurs to a lesser extent at met-ser and met-thr bonds. Met-cys bonds may fail to be cleaved due to formation of a homoserine residue without peptide bond lysis. Doyen and Lapresle (1979) have shown that met 123 in HSA is only partially susceptible to CNBr cleavage due to its neighbouring cystine residue, as conversion to a homoserine residue can take place without bond breakage, so giving rise to a possible eight CNBr fragments.

The oxidation of methionine to methionine sulphone or sulphoxide prevents cleavage and any such auto-oxidation must be reversed using thiols before treatment with CNBr (Neumann, 1972). Oxidative destruction of the tryptophan residue is possible if methionine residues are already modified and CNBr has been used to cleave tryptophan in proteins with previously modified methionines (Huang, *et al.*, 1983).

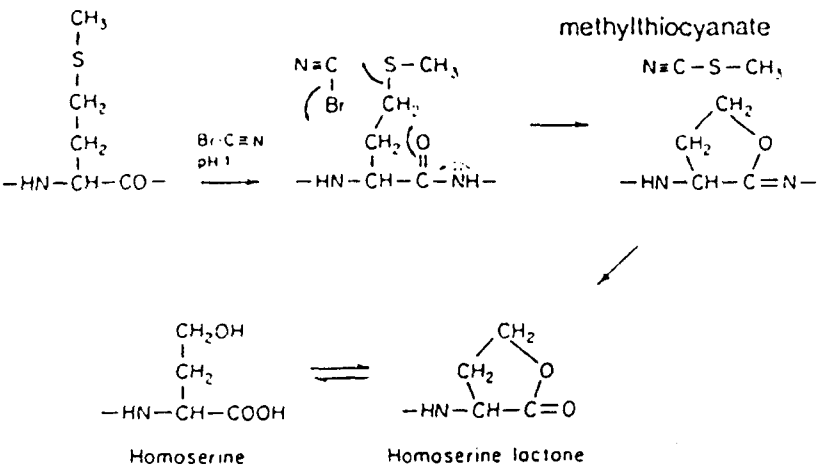


Fig. 5.6 Cleavage at methionine with CNBr.

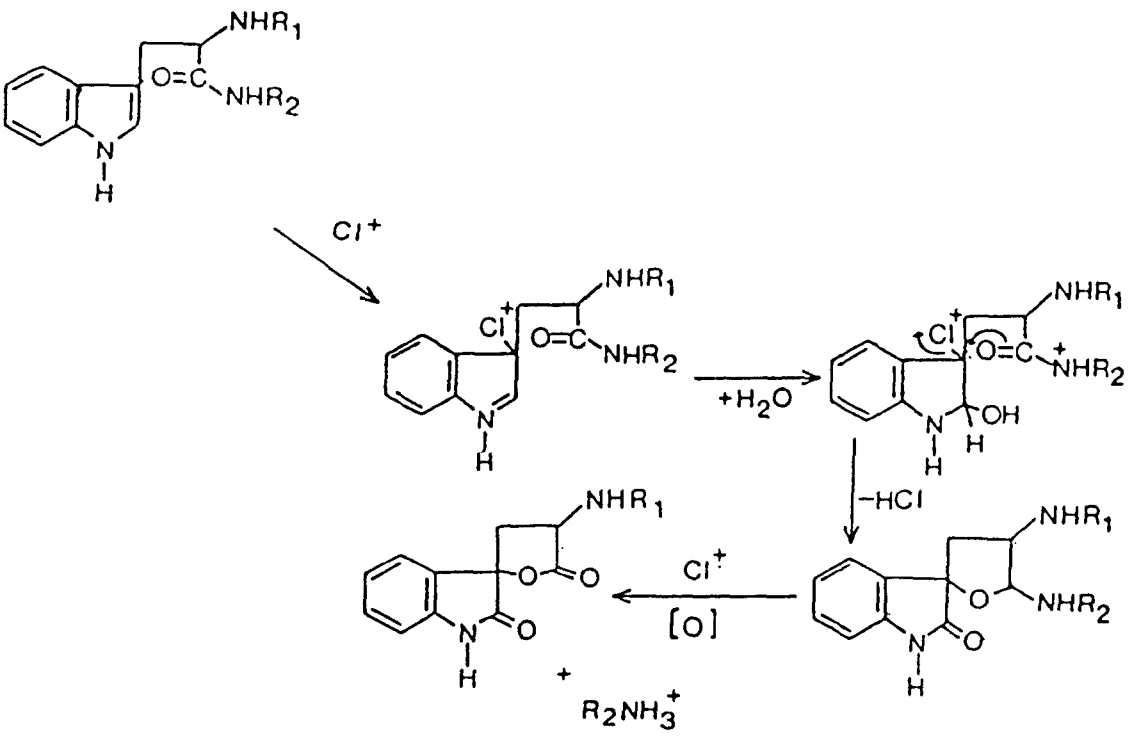


Fig. 5.7 Cleavage at tryptophan with NCS.

5.2.6 Cleavage at Tryptophan

5.2.6.1 N-Bromo- and N-Chloro-Succinimides (NBS, NCS)

HSA has a single tryptophan at residue 214 and this can be cleaved to give two peptides, an N-terminal fragment of ≈ 24,000 MW and a 42,000 MW C-terminal fragment. Oxidative cleavage at tryptophan using bromine or N-bromo-succinimide are not of great value in sequence determination as they give low yield cleavages with many side reactions, also attacking tyrosine and histidine residues. N-Chloro-succinimide is milder and more specific and has been used in several cases (Shechter, *et al.*, 1976), (Lischwe and Sung, 1977) and has been used to cleave proteins in polyacrylamide gel slices (Lischwe and Ochs, 1982).

NCS appears to be more specific than NBS and cleavage of the tryptophan is the only major reaction; however, the yield of tryptophanyl bond cleavage is slightly less than for NBS (20-60% Shechter, *et al.*, 1976). It is sometimes necessary to reduce secondary structure before the protein tryptophan residues are exposed and the inclusion of 4-5 M urea, although not altering the cleavage reaction, does cause substantial unfolding of the protein. Optimal conditions appear to be a 10 M excess of reagent over substrate, with cleavage allowed to proceed for 30 minutes at room temperature in acidic solution. A mechanism (Fig. 5.7) has been proposed for the

cleavage and the apparent specificity is thought to be due to the absence of molecular chlorine (Lischwe and Sung, 1977). The tryptophanyl residue is oxidised to the oxindole residue and this promotes peptide bond cleavage. Two molar equivalents of chlorine are required per mole of tryptophanyl residue cleaved.

5.2.6.2 O-Iodosobenzoic Acid

O-Iodosobenzoic acid was introduced by Mahoney and Hermodson (1979) as a tryptophan-cleaving reagent which cleaved in moderate to high yields (Fig. 5.8). However, iodoxybenzoic acid ($IO_2C_6H_4COOH$) may be a contaminant in the commercially prepared reagent, causing some additional cleavage at tyrosine residues. In order to protect these tyrosine residues p-cresol was introduced (Mahoney, *et al.*, 1981) as a scavenger of any iodoxybenzoic acid. Fontana, *et al.* (1983) have shown from amino acid analysis that some methionine residues are also oxidised and cystine residues converted to cysteic acid in the presence of halides, the order of reactivity being met > trp > cys > tyr > his.

The usual reagent conditions include 4 M guanidinium hydrochloride with 80% v/v acetic acid in order to denature the protein. The reagent may be purified from iodoxybenzoic acid by either gel filtration in 10% v/v acetic acid or ion-exchange chromatography with a salt gradient.

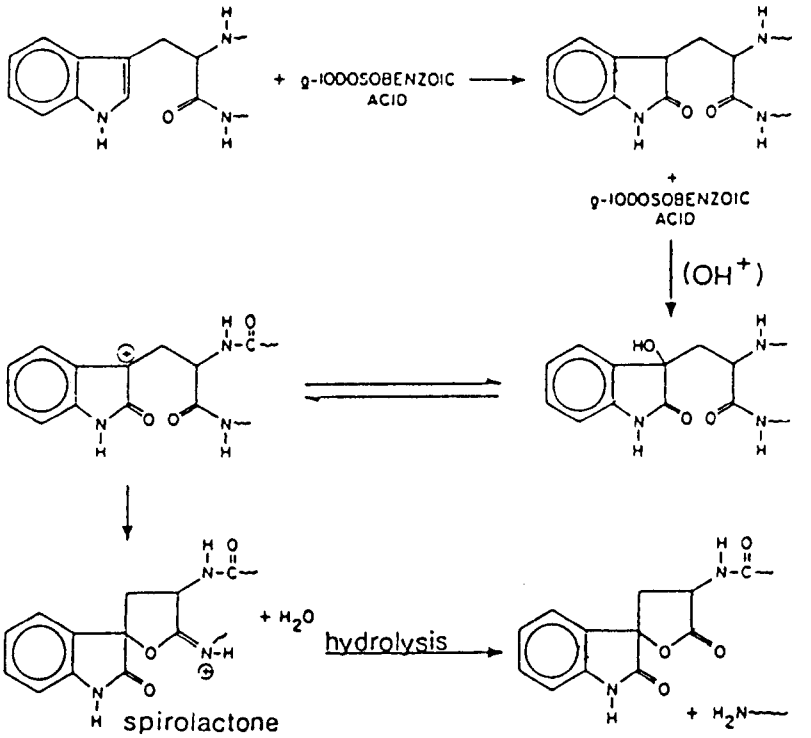


Fig. 5.8 Cleavage at tryptophan with O-iodoso-benzoic acid.

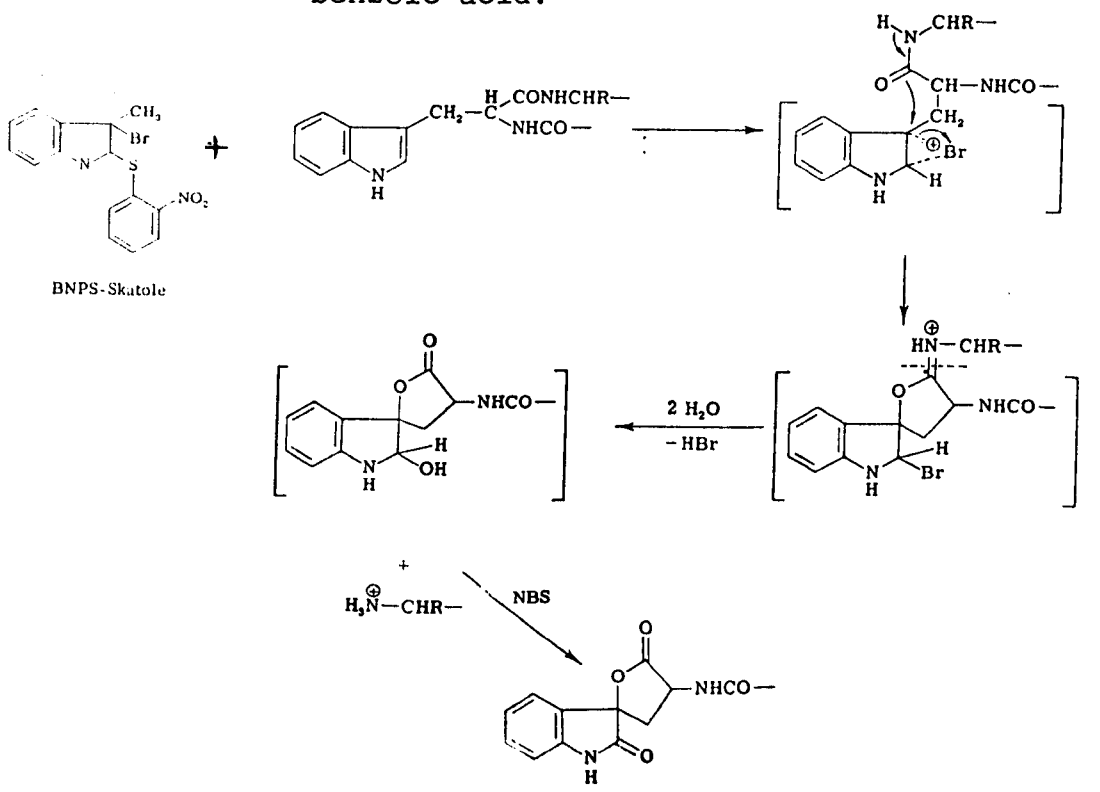


Fig. 5.9 Cleavage at tryptophan with BNPS-skatole.

5.2.6.3 BNPS-Skatole

The reagent 2-(2-nitrophenylsulphenyl)-3-methyl-3-bromo-indolenine (BNPS-Skatole) was introduced by Omenn, *et al.* (1970) and has been used extensively for tryptophanyl bond cleavage in proteins. BNPS-Skatole is used in aqueous acetic acid at several-fold molar excess over the total tryptophan concentration of the protein. Solutions must be made up freshly as the reagent rapidly decomposes, releasing bromine, and should be kept refrigerated when not in use. If used correctly the only side reactions which occur are the oxidation of methionine to the sulphone and cysteine to cystine. Tyrosine residues can be protected by the addition of phenolic scavengers and the tryptophanyl bond cleavage yield is then in the range 50-70% (Fig. 5.9).

5.2.6.4 Other Methods of Tryptophanyl Bond Cleavage

Mild oxidation of polypeptides in acetic acid/12 N HCl/DMSO followed by more vigorous oxidation with 48% v/v HBr will cleave the tryptophanyl bonds in 60% yield, the only other residues to be modified being methionine and cysteine (Savige and Fontana, 1977). However, yields are lower than for BNPS-skatole and the harsh acidic conditions may promote non-specific acid hydrolysis of peptide bonds.

Another similar method of tryptophan bond cleavage is that of Huang, *et al.* (1983) who treated reduced and alkylated protein with DMSO/HCl to oxidise the tryptophan residues to oxindolylalanine and methionine

residues to methionine sulphone. The solution was then partially neutralised with ammonia and CNBr in formic acid added to cleave on the carboxyl side of the oxindolylalanine. This method gave near quantitative cleavage with no significant modification of other residues (Fig. 5.10).

5.2.7 Peptide Mapping in SDS Gels

Enzymatic digestion of proteins in SDS-containing buffers followed by direct analysis on SDS-PAGE was introduced by Cleveland, *et al.* (1977). Albumin is digested using α-chymotrypsin and papain among other enzymes and the results analysed by electrophoresis. Papain is a thiol protease, requiring reducing conditions for full activity and of a general specificity although dipeptide sequences after phenylalanine residues are cleaved with some preference. α-Chymotrypsin cleaves at the carboxyl side of tryptophan, tyrosine, phenylalanine and other hydrophobic residues, but is inhibited by a proline residue after the cleavage site, and its activity is reduced for acidic residues and increased for basic ones.

The enzymes are used in molar excess to degrade the albumin dissolved in SDS buffer and the resultant fragments separated by their molecular weight on SDS-PAGE.

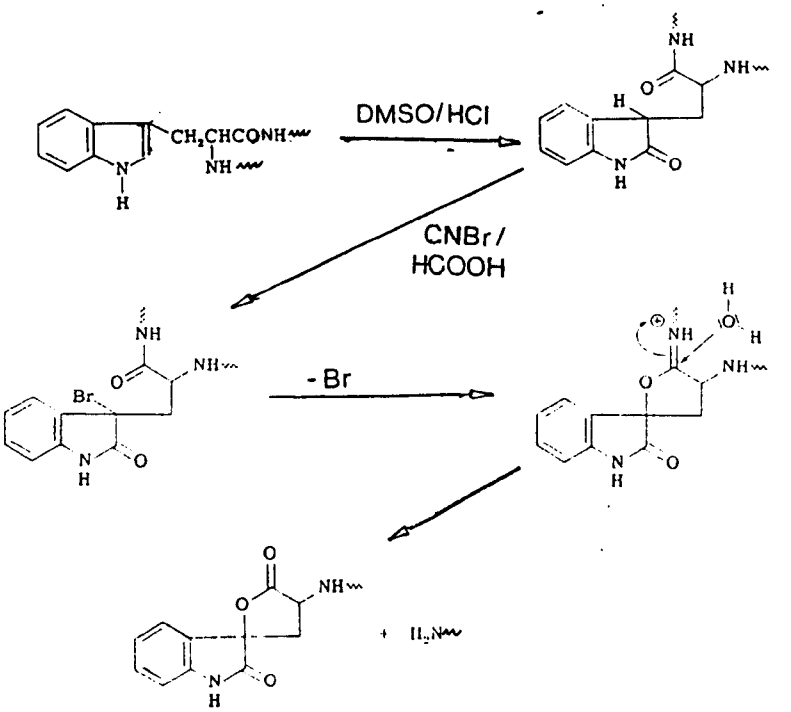


Fig. 5.10 Cleavage at tryptophan with HCl/DMSO/ CNBr.

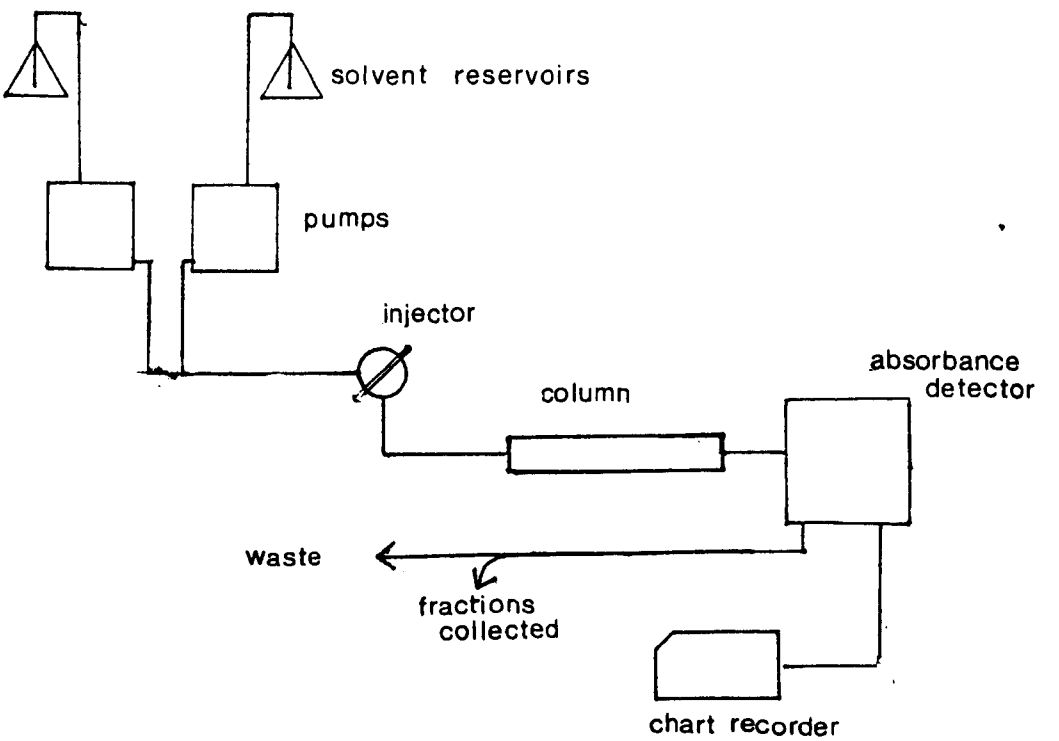


Fig. 5.11 Schematic representation of an HPLC system, suitable for gradient elution.

5.2.8 Digestion with Pepsin

Bradshaw and Peters (1969) cleaved albumin with pepsin and studied the isolated fragments. Geisow and Beaven (1977a) have further studied the action of pepsin on albumin and analysed the results by SDS-PAGE and so more recently have Ledden, *et al.* (1982).

The specificity of pepsin is wide, though cleavage of the protein chain adjacent to hydrophobic residues is especially favoured, and at the site of two adjacent hydrophobic residues cleavage occurs in between them (Fruton, 1974). The prediction of cleavage sites is difficult but by controlled digestions 44,000 and 29,000 MW fragments have been isolated (Geisow and Beaven, 1977b).

Fragments are often held together by disulphide bonds and the inclusion of 2-mercaptoethanol will alter the pattern of peptides obtained on electrophoresis.

5.2.9 Digestion with Trypsin

Trypsin has been one of the most widely used enzymes in protein sequencing and is the most specific of the commonly used proteases cleaving after arginine or lysine residues, depending upon reaction conditions. The initial protein sequencing of human albumin was achieved by the combination of tryptic and chymotryptic digestion, and an albumin variant (albumin B) was analysed by this method (Winter, *et al.*, 1972).

Lysine or arginine followed by proline are poorly cleaved and the rate of cleavage is variable, being reduced by acidic residues. Trypsin is usually pre-treated with N-tosyl-L-phenylalanyl-chloromethyl ketone (TPCK) to inhibit any contaminant chymotryptic activity.

5.3 THE USE OF HPLC IN PEPTIDE MAPPING

5.3.1 Introduction

The traditional methods of peptide separation (ion exchange and gel filtration chromatography and two-dimensional finger-printing) have in the last decade largely been replaced by HPLC either in ion exchange or reverse phase (RP) modes. Several excellent reviews of the techniques and their applications have been written (Hughes and Wilson, 1983), (Regnier, 1983) and at least one symposium compiled (Hearn, *et al.*, 1983).

The main advantages of HPLC are that it gives vastly improved resolution of peptides due to the improved matrices, which have a smaller, more uniform particle size giving rise to greater surface area. The experimental time required per run is greatly decreased and the method allows simple collection of isolated peptides and can be used semi-preparatively, allowing greater loadings and easier recoveries than

would be possible by two-dimensional chromatography.

Ion exchange HPLC (IEX-HPLC) is in theory an improvement of existing ion-exchange chromatography. However, reverse phase (RP) has been used mainly as an HPLC technique although it is analogous in some respects to hydrophobic interaction chromatography.

Typically, both IEX-HPLC and RP-HPLC require gradient elution techniques with one or two pumps, a sample injector, the column and an absorbance detector or other means of peptide identification positioned after it (see Fig. 5.11).

5.3.2 Reverse Phase HPLC (RP-HPLC)

Reverse phase HPLC is usually performed on silica-based columns with alkyl chain substituents of varying sizes. The common chain lengths are octyl (C_8) or octadecyl (C_{18}) and excess Si-OH groups are normally blocked (referred to as "capping"). The principle of the separation depends primarily upon hydrophobic interactions between the alkyl side chain and the proteins or peptides to be separated. The mobile phase is provided by a solution of gradually increasing non-polarity and usually a gradient of either methanol, acetonitrile or propan-2-ol is used. The peptides or proteins elute in the order of their increasing hydrophobicity, as they remain bound to the column until their interactions with it become less than those with the mobile phase, where upon they

are eluted. The polar aqueous buffer used initially is usually acidified (pH 2-6.5) and acid at the same concentration is included in the organic buffer, resulting in a consistent pH from one experiment to the next. Trifluoroacetic acid (TFA) is the most commonly used acid as it is an excellent solvent for polypeptides and alters the mobilities of peptides on the column by an ion-pairing effect (Acharya, *et al.*, 1983).

The separation achieved is little affected by column length but the solvent system, pore size of the column, and the degree of "capping" are all important. Due to the harsh solvent conditions of RP-HPLC some enzymes are permanently inactivated by it although others seem to be unaffected. For peptide mapping, where no residual activity is required, RP-HPLC is a popular choice and the digested fragments of an ever increasing number of proteins are being separated in this manner.

5.3.3 Application of RP-HPLC to Albumin Variants

RP-HPLC has already been widely used for the isolation of mutant peptides of variant haemoglobins (Wilson, *et al.*, 1979) but has not been applied to the problem of albumin variants until recently. Iadarola, *et al.* (1984) separated the CNBr fragments of an Italian albumin variant by RP-HPLC and more

recently Brennan (1985) used RP-HPLC to separate the tryptic fragments of Albumin Parklands. The use of HPLC in the separation of such mutant fragments may well become more widespread in the future.

5.4 EXPERIMENTAL

5.4.1 Determination of the N-Terminal Residue

5.4.1.1 Performic Acid Oxidation

Performic acid was prepared by the addition of formic acid (8 volumes, 99%, v/v, AR grade, distilled) to hydrogen peroxide (2 volumes, 30%, v/v), allowing the mixture to stand at room temperature for 2 hours in a sealed vessel, at which time the concentration of performic acid was at a maximum. The performic acid solution was cooled on ice for 15 minutes, and HSA and double Albumin Carlisle (1-2 mg dissolved in formic acid (99%, v/v, 0.5 ml) were chilled on ice for a similar length of time. Performic acid (0.5 ml) was then added to each protein solution and oxidation allowed to proceed for 2.5 hours at $0^{o}C$.

The oxidised proteins were precipitated using TCA (80%, w/v, 0.5 ml) and centrifuged for 5 minutes in a bench centrifuge. The precipitate was

washed three times with ethanol, and once with diethyl ether before being dried *in vacuo* over NaOH.

5.4.1.2 Labelling with Dansyl Chloride (Gray 1972)

The precipitate was redissolved in ammonium hydrogen carbonate (0.2 M, with 0.1%, w/v, SDS, 500 µl) to neutralise any residual acid and then lyophilised. The lyophilised material was resuspended in SDS (1%, w/v, 150 µl) and heated at 100°C for 5 minutes, before allowing to cool at room temperature. N-ethyl morpholine (50 µl) was added and the solution vortexed; the pH was then estimated with indicator paper, and further corrected if necessary until the pH was ≈ 9.0.

Dansyl chloride (25 mg/ml, 75 µl) in anhydrous dimethylformamide was added and any precipitate re-dissolved with SDS (1%, w/v). Dansylation was allowed to proceed for 4 hours in the dark at room temperature. Acetone (0.5 ml) was added and the protein precipitated using a bench centrifuge. The precipitate was washed with acetone (80% v/v aqueous, 0.5 ml) and centrifuged again before drying *in vacuo* over NaOH pellets.

5.4.1.3 Analysis of Dansylated Amino Acids

The dried sample was resuspended in HCl (6.1 M, 1-200 µl) and transferred to a constricted glass vial which had been pre-cleaned by heating to 500°C overnight. The sample was frozen in

liquid nitrogen and the vial flame-sealed. Hydrolysis was performed at 105° ± 10°C for 16 hours and the vials were then opened and dried *in vacuo* over NaOH pellets.

The dansyl derivative was redissolved in aqueous pyridine (50% v/v, 10 µl) and spotted onto a 15 x 15 cm double-sided polyamide t.l.c. plate (Cheng Chin Trading Co.) and resolved in two dimensions using four buffer systems.

First Dimension:

(i) Water:formic acid (90% v/v), (200:3, v/v)

Second Dimension:

(ii) Toluene:acetic acid (9:1, v/v)

(iii) Ethyl acetate:methanol:acetic acid (20:1:1, v/v/v)

(iv) Ammonia:ethanol (1:1 v/v).

The dansyl amino acids were identified by their fluorescence under UV light at 365 nm and their mobilities compared to those of dansyl amino acid standards run on the reverse side of the plates. DNS-arginine, DNS-aspartate, DNS-ε-lysine and di-DNS-lysine were run as standards together with DNS-glycine and N-O-di-DNS-tyrosine.

5.4.2 Determination of C-Terminal Residue

5.4.2.1 Reaction with Carboxypeptidase Y (Lee and Riordan, 1978)

Double Albumin Carlisle (1-2 mg) and an equal amount of albumin A were resuspended in

MES buffer (2-(N-morpholino)-ethane sulphonic acid) (10 mM, pH 6.8, 200 µl). Carboxypeptidase Y (5 µg aliquots of 12.5 µmol/mg protein, pH 6.75, 25°C) were resuspended in water (20 µl) overnight at 0°C before use. Endopeptidase activity was inhibited by incubation with pepstatin A (7 µg) in MES buffer (20 µl) for 20 minutes.

Enzyme was then added to each of the albumin solutions and digestion carried out at 37°C for 7 and 100 minutes. The reaction was stopped by lyophilisation. Analysis was by the method detailed in Section 5.4.2.3.

5.4.2.2 Digestion with Carboxypeptidase A

The method of Geisow and Beaven (1977a) was used. Carboxypeptidase A (2.2 mg/ml) was incubated at 0°C overnight in water. Albumins (0.5 mg) were dissolved in N-ethylmorpholine buffer (0.2 M, pH 8.5, 50 µl) and enzyme added (1 µl, 20 µg) and the digestion allowed to proceed for 3 hours, after which time the solutions were lyophilised to stop the reaction. Digests were analysed as described in Section 5.3.2.3.

5.4.2.3 Analysis of Carboxypeptidase Digests

The samples were resuspended in MES buffer (10 mM, 40 µl) and applied to a 20 x 20 cm silica gel t.l.c. plate (Merck BDH). Ascending chromatography was performed in chloroform:methanol:ammonia (2:2:1 v/v/v), until the solvent front had migrated

to within 2 cm of the top of the plate. The plate was then air-dried and sprayed with ninhydrin (0.2% w/v in 95% ethanol:acetic acid 20:1, v/v) and heated at 60°C for 15-30 minutes in order to visualise the amino acid spots. Standard solutions of L-leucine, L-alanine, and glycine were chromatographed simultaneously.

5.4.3 Cleavage at Methionine

5.4.3.1 Digestion at Methionine with CNBr

The method used was essentially that of Franklin, *et al.* (1980a,b). Normal and double albumins were dissolved in N_2-saturated formic acid (70% v/v, 2 ml) and CNBr (a weight equal to that of the protein) was added, dissolved in formic acid (70% v/v). The reaction was allowed to proceed for 24 hours in the dark at room temperature. The mixture was then diluted with 15 volumes of distilled water and lyophilised. Lyophilisation was repeated to ensure complete removal of excess reagents.

Alternatively, normal and heterozygous sera (25-100 µl) were taken and diluted with formic acid (70% v/v, 200 µl). An equivalent weight of CNBr dissolved in formic acid (70% v/v, 10 mg/ml) was added and digestion allowed to proceed as described above.

5.4.3.2 Reduction and S-Carboxymethylation of CNBr Fragments

Reduction and S-carboxymethylation was performed in triethanolamine acetate buffer (0.2 M, pH 8.6, with urea, 8 M, methylamine, 25 mM, dithiothreitol, 60 mM and EDTA, 10 mM). The albumins were resuspended in this buffer (300 µl) and heated at 37°C for 4 hours in order to fully reduce the disulphide bridges. Iodoacetamide was then added to a concentration of 130 mM and alkylation allowed to proceed at room temperature in the dark for 30 minutes. The reaction was stopped by the addition of excess dithiothreitol and the fragments diluted with distilled water (5 volumes) and precipitated by addition of TCA to a concentration of 20% w/v. The peptides were centrifuged and the supernatants discarded. The precipitates were washed with acetone:HCl (100:0.2 v/v) and acetone and then dried *in vacuo* over NaOH. In all the stages of precipitation each solution contained 1% thiodiglycol (v/v).

5.4.3.3 Analysis of CNBr Fragments by Triton-Urea PAGE

Analysis of the CNBr peptides was on Triton X-100 urea gels (T = 12%, C = 0.66%) with Triton X-100 (5 mM), acetic acid (6% v/v) and urea (6 M) as described by Zweidler (1978). The fragments were dissolved in loading buffer (urea (8 M), thiodiglycol (1% v/v) and dithiothreitol (20 mM)) at 37°C for 90 minutes before electrophoresis.

The Triton-urea gels were subjected to pre-electrophoresis for 3.5 hours, overlaying the gel surface with acetic acid (5% v/v) with the same urea and detergent content as the gel, using a solution of acetic acid (5% v/v) as the electrode buffer. A further half-an-hour of pre-electrophoresis was carried out with the same overlaying solution but with the addition of 2-mercaptoethanol (0.5 M). The electrode buffer was replaced with a fresh solution of acetic acid and the CNBr fragments (≈ 100 µg) were loaded and electrophoresis performed at 50-100 V C.V. overnight. Electrophoresis was stopped when the tracking dye (basic fuschin (0.01%, w/v) dissolved in the load buffer) had almost eluted from the gel. Peptides were fixed and stained in PAGE Blue G90 (0.25% w/v in methanol, 45% v/v, acetic acid, 10% v/v) and destained in the same solution without the dye.

5.4.4 Cleavage at Tryptophan

5.4.4.1 Cleavage with N-Chloro-Succinimide (NCS) (Shechter, *et al.*, 1976)

Albumin A and double Albumin Carlisle (≈ 1 mg each) were dissolved in acetic acid (80% v/v, 50 µl) and NCS (2 mg/ml in dimethylformamide) was added in 10-fold molar excess over the total tryptophan content of the protein. The reaction was allowed to proceed for 40 minutes at room temperature and then residual reagent was destroyed by the addition of excess L-meth-

ionine. The samples were diluted 1:5 with water and the protein fragments precipitated by the addition of TCA to 20% (w/v) and collected by centrifugation. The precipitate was washed with acetone containing HCl (0.2% v/v) and then with acetone alone and dried *in vacuo* over NaOH. A control was performed without the addition of NCS and analysis of the digestion was on 10% SDS-PAGE, performed as previously described (Section 3.2.9).

5.4.4.2 Cleavage with BNPS-Skatole (Fontana, 1972)

Normal and double albumins (≈ 1 mg) were taken and dissolved in acetic acid (66% v/v, 100 µl). Freshly prepared BNPS-Skatole in acetic acid (80% v/v) was added so as to give a 100-fold molar excess of reagent over total tryptophan content and cleavage was allowed to proceed at room temperature in the dark for 28 hours, with continual stirring. The solutions were then lyophilised and stored frozen until analysed on SDS-PAGE (either T = 7.5% or T = 10-20% gradient PAGE).

5.4.5 Proteolysis in SDS (Cleveland, *et al.*, 1977)

Normal and double albumins (≈ 1 mg) were dissolved in sample buffer (tris-HCl (125 mM, pH 6.8) with SDS (0.5% w/v), glycerol (10% v/v), and bromophenol blue (0.001% w/v), 400 µl) and boiled for five minutes to fully denature the proteins. After cooling, papain (36 µg, Sigma) or chymotrypsin (133

µg, Sigma) was added to denatured solutions of albumin A, double Albumins Redhill and Carlisle and heterozygous Albumin Warwick-1 sera (50 µl). Digestion was allowed to proceed for 30 minutes and then 2-mercaptoethanol and SDS were added to final concentrations of 10% v/v and 2% w/v respectively and the samples boiled for five minutes to stop the digestion.

Aliquots (≃ 100 µg) of the digests were analysed directly on SDS-PAGE (T = 20%, C = 2.5%) and electrophoresis performed at 100 V C.V. until the bromophenol blue tracking dye had almost eluted from the gel. Electrophoresis buffer was as described in Section 3.2.9.

5.4.6 Digestion with Pepsin

Digestion was performed as outlined in Ledden, *et al.* (1982). Monomeric albumins were formed from double Albumin Carlisle and albumin A (1 mg of each) by the half-cystinylisation process of King and Spencer (1971). The albumins were dissolved in tris-HCl (0.1 M, pH 7.96, 100 µl) and 6.24 x molar excess of cystine, dissolved in the same buffer, was added. Disulphide exchange was carried out for 17 hours at room temperature by which stage all albumin present should essentially be monomeric (King and Spencer, 1971) and the samples were desalted using a Centricon ultrafiltration apparatus,

and lyophilised. Digestion with pepsin was performed by resuspending the albumins in ammonium formate buffer (0.1 M, pH 3.7, 200 µl) with 4.2 mM octanoic acid and digestion with pepsin was allowed to proceed for 12 minutes at a ratio of enzyme:substrate 1:1000. Resultant digestion was analysed by SDS-PAGE (T = 7.5%, C = 2.6%) using a constant voltage of 50 V C.V. overnight essentially as described for SDS-PAGE in Section 3.2.9. Peptic digestions of HSA, double Albumins Carlisle and Redhill and also Albumin Warwick-1 serum were performed.

5.4.7 Digestion with Trypsin

Albumin A and double Albumin Carlisle were digested with trypsin by the method of Behrens (1975).

5.4.7.1 Performic Acid Oxidation

Normal and double albumins (5 mg of each) were oxidised using a modified performic acid reagent (Behrens, 1975). Formic acid (88% v/v), hydrogen peroxide (30% v/v) and phenol (90% v/v in water) were mixed in the ratios 100:10:6 and stirred at room temperature for one hour before chilling on ice. Albumins were dissolved at 50 mg/ml concentration in formic acid (88% v/v) and an equal volume of the performic acid reagent was added and allowed to react for 1.5 hours at $0^{o}C$. The oxidised proteins were precipitated with diethyl ether (8 volumes) and the precipitateswashed with diethyl ether

twice and finally once with cold acetone and dried *in vacuo*.

5.4.7.2 Tryptic Digestion

The precipitates were ground up and dissolved in ammonium bicarbonate (0.5% w/v, pH 8.2, 1-2 ml). Trypsin (TPCK-treated, Sigma) was added at a ratio of 1:50 and digestion allowed to proceed for 4½ hours at room temperature, with periodic stirring. The digestion was stopped by bringing the pH to 2.0 with 88% formic acid, and after allowing any carbon dioxide to be released, the digests were frozen until further use.

5.4.7.3 Peptide Mapping

Tryptic digestions were resuspended and chromatographed in butan-1-ol:pyridine:acetic acid: H_2O, 90:60:18:72 v/v/v/v (Bennet, 1967), loading 1-2 mg of albumin for each map. Chromatography was performed on 20 x 20 cm t.l.c. cellulose plates (made from Avicell microcrystalline-cellulose (Merck)) with repeated application of the sample about 4 cm in from the left hand side, approximately 1 cm up the plate. Chromatography was stopped when the solvent front had migrated to within 2 cm of the top of the plate, and the plate was then thoroughly air-dried.

Thin layer electrophoresis was performed at 90° to the chromatographic separation in a Shandon cellulose acetate apparatus at 300 V C.V. for 4-5 hours, the greater distance being that to the cathode. The plate was thoroughly air-dried and sprayed with

ninhydrin, as made up in Section 5.4.2.3, or with the ninhydrin-cadmium acetate reagent (Heilmann, *et al.*, 1957). Identical runs were performed for albumin A and double Albumin Carlisle.

5.4.8 Peptide Mapping by HPLC

Organic solvents used were of HPLC grade (BDH Ltd., U.K.) and were of the lowest UV absorbance obtainable. All solvents were thoroughly degassed and filtered through a 0.5 µm filter before use.

5.4.8.1 CNBr Fragments

Albumin A and double Albumin Carlisle were digested with CNBr and reduced and S-carboxymethylated essentially as described by Iadarola, *et al.* (1984) or Franklin, *et al.* (1980a).

Initial experiments were performed on CNBr-treated S-carboxymethylated albumin A in order to devise a suitable separative system for the CNBr fragments on a Waters HPLC system (as described in Section 4.4.2.3) with a µC_{18} Bondapak (30 x 0.8 cm i.d.) column (Waters Associates Inc.). A variety of RP-HPLC solvent systems were tried and samples (≈ 500 µg) applied with peptides being detected by ultraviolet absorbance at 280 or 214 nm.

The cleavage method of Iadarola, *et al.* (1984) was followed and separation of the CNBr fragments thus generated was attempted on the µC_{18} Bondapak column at 1 ml/min, using a 20-60% gradient of

propan-2-ol:acetonitrile 1:2 v/v with 0.05% v/v TFA over aqueous TFA (0.05% v/v) in 30 minutes. Fractions from repeated runs were collected, pooled and analysed after lyophilisation. Fractions were resuspended in loading buffer and applied to 10% Triton X-100/ urea gels as detailed in Section 5.4.3.2.

5.4.8.2 Tryptic Fragments

Tryptic digestions were performed essentially after the method of Behrens (1975). Aliquots of the digested albumin solutions (50-100 µl of a nominal concentration of 3-5 mg/ml) were taken, after centrifugation to remove undigested material, and applied to RP-HPLC using a range of gradients of acetonitrile: propan-2-ol (2:1, v/v) containing 0.05%, v/v TFA over aqueous TFA (0.05%, v/v) to elute the peptides from the column. A flow rate of 1 ml/min was used and the absorbance followed at 214 nm.

5.4.8.3 Peptic Fragments

Albumin A, pure abnormal Albumin Carlisle and double Albumin Carlisle (≃ 1 mg of each) were digested with pepsin (0.2 mg/ml in 1 mM, HCl, Worthington Enzymes) in the ratio of 1000:1, substrate:enzyme in a total volume of ammonium formate buffer (0.1 M, pH 2.5, 500 µl). Digestions were allowed to proceed at room temperature for 0, 35, 80, 105, 135, 225, 285, and 470 minutes and for an overnight period. Aliquots (50 µl) were taken after each time interval and the reaction

stopped by addition of 2 M tris until the pH was > 8.0. Fractions were frozen at -20°C until loaded and analysis performed on 10% non-SDS-PAGE and by RP-HPLC on a Waters HPLC system as before on a μC_{18} Bondapak column. A 0-100% gradient of acetonitrile:propan-2-ol (2:1, v/v) with 0.05% (v/v TFA) over aqueous TFA (0.05%, v/v) was used at 1 ml/min. flow rate, monitoring absorbance at 214 nm, 1.0 AUFS.

Samples of HSA and double Albumin Carlisle were also digested for 35 minutes and then analysed by the HPLC as above.

5.5 RESULTS

The N-terminii of Albumin Carlisle and albumin A were determined by dansylation of the N-terminal residues. After acid hydrolysis, the dansyl amino acids obtained were compared to known standards on two-dimensional polyamide plates. The dansyl amino acids derived from the hydrolysis of double Albumin Carlisle when chromatographed in the first three solvents gave a single dansyl amino acid-DNS aspartate (Fig. 5.12). No DNS-arginine could be detected in the hydrolysate indicating that the N-terminal residue of Albumin Carlisle is aspartate, the same as that of albumin A and that it is not a proalbumin variant (which have arginine at the N-terminus). It was possible to use a fourth solvent system, in

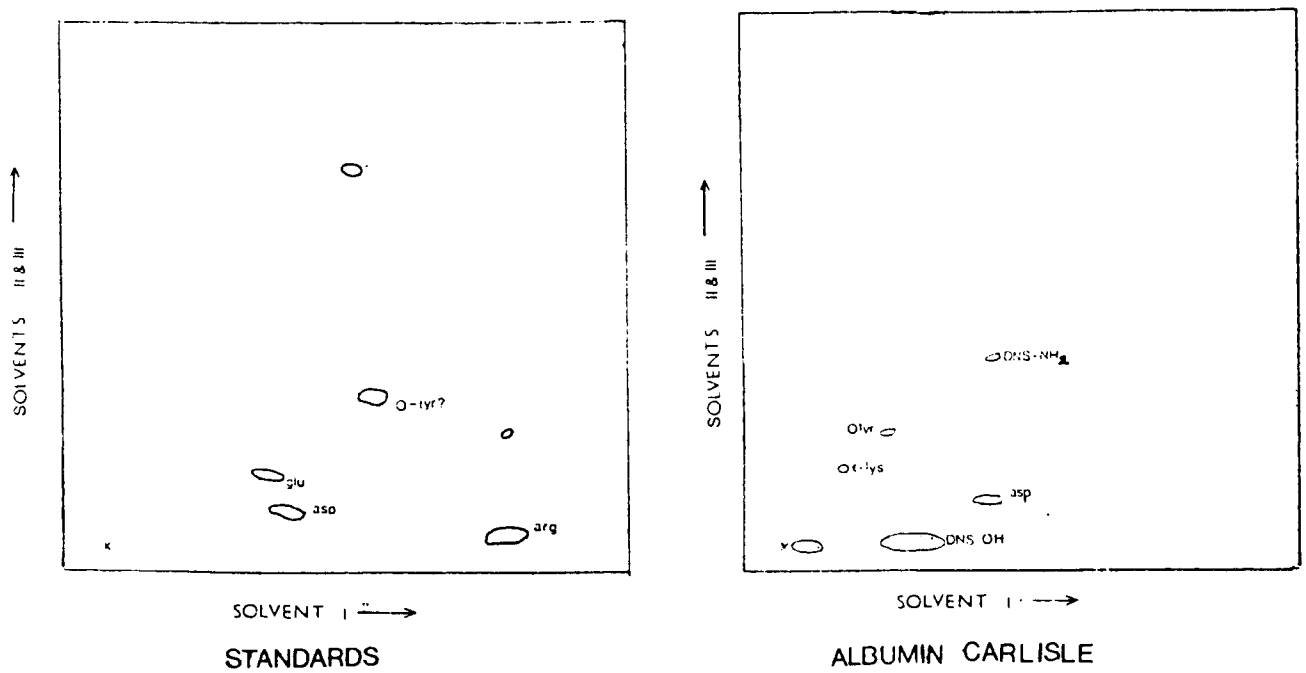


Fig. 5.12 Identification of N-terminal residue of Albumin Carlisle.

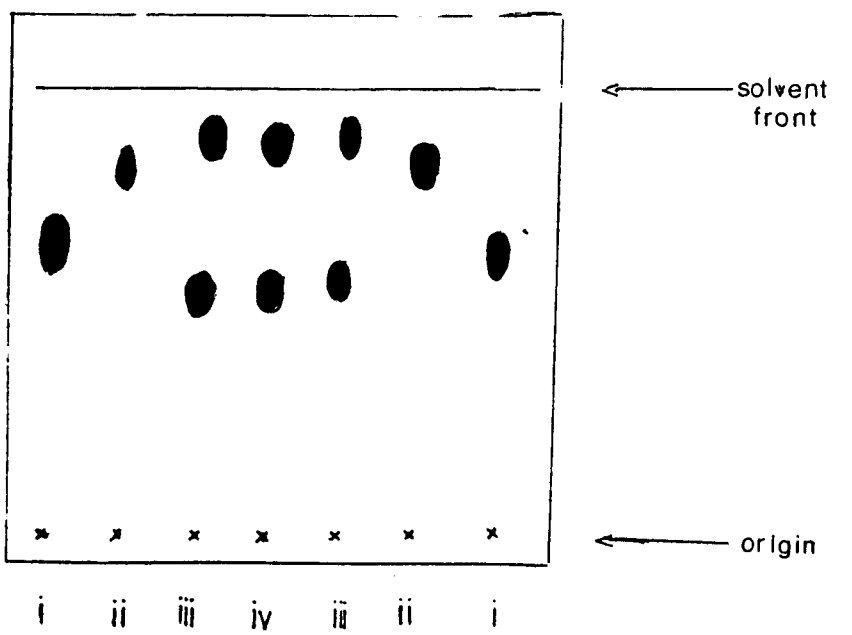


Fig. 5.13 Determination of C-terminal residue of Albumin Carlisle by t.l.c. after digestion with carboxypeptidase Y. Tracks (i), glycine; (ii) leucine; (iii) double Albumin Carlisle; and (iv) HSA.

the second dimension, in order to clarify poorly separated dansyl amino acids. This was unnecessary in this instance and did not alter the conclusion obtained from the three solvent system results.

The C-terminus of Albumin Carlisle was determined using both carboxypeptidase A and Y. Both albumin A and Albumin Carlisle gave leucine and glycine ninhydrin-positive spots on t.l.c. when run against amino acid standards. The C-terminus of albumin A was already known to be gly-leu-COOH and the pattern of released amino acids was identical using carboxypeptidase A for both double Albumin Carlisle and albumin A. This would indicate that the C-terminal leucine and penultimate glycine residue were excised from the albumins by carboxypeptidase A after a 5-10 minutes digestion (Fig. 5.13).

In some experiments with carboxypeptidase Y only glycine was released from both albumin A and double Albumin Carlisle, probably because of the slow release of the leucine residue by this enzyme. However, digestions for longer periods (100 minutes) showed that both leucine and glycine were released from both samples.

The incubation of double Albumin Carlisle and normal albumin with CNBr and subsequent electrophoretic analysis revealed that double Albumin Carlisle showed an extra band on gel electrophoresis, although several other bands present in normal albumin

were absent. The pattern of bands was consistent on two independently run gels (see Fig. 5.14). Franklin, *et al.* (1980a,b) have characterised the fragments generated under these conditions and from correlations to their results it would appear that the extra peptide band corresponds to CNBr fragment V or VI (residues 329-446 or 446-548). Double Albumin Carlisle appeared to lack any modified fragment III (residues 123-298) or fragment I (residues 1-87). These latter differences may be due to variations in the degree of amino acid modification (cysteine oxidation and lysine alkylation) in the different preparations of CNBr digests.

Cleavage at tryptophan using both N-chloro-succinimide and BNPS-Skatole (Fig. 5.15) yielded two anodic fragments on SDS-PAGE. From their migration compared to known molecular weight markers, these bands are consistent with the larger C-terminal fragment (residues 215-585) and the smaller N-terminal fragment (residues 1-214). In neither gel could any discernable extra band be noticed with the C-terminal fragments, suggesting that the point mutation in Albumin Carlisle does not alter the properties of these large peptides sufficiently to separate them from the normal fragments on SDS-PAGE.

The percentage cleavage with BNPS Skatole was far higher than for the NCS digestion although the fragments from the latter were clearly visible.

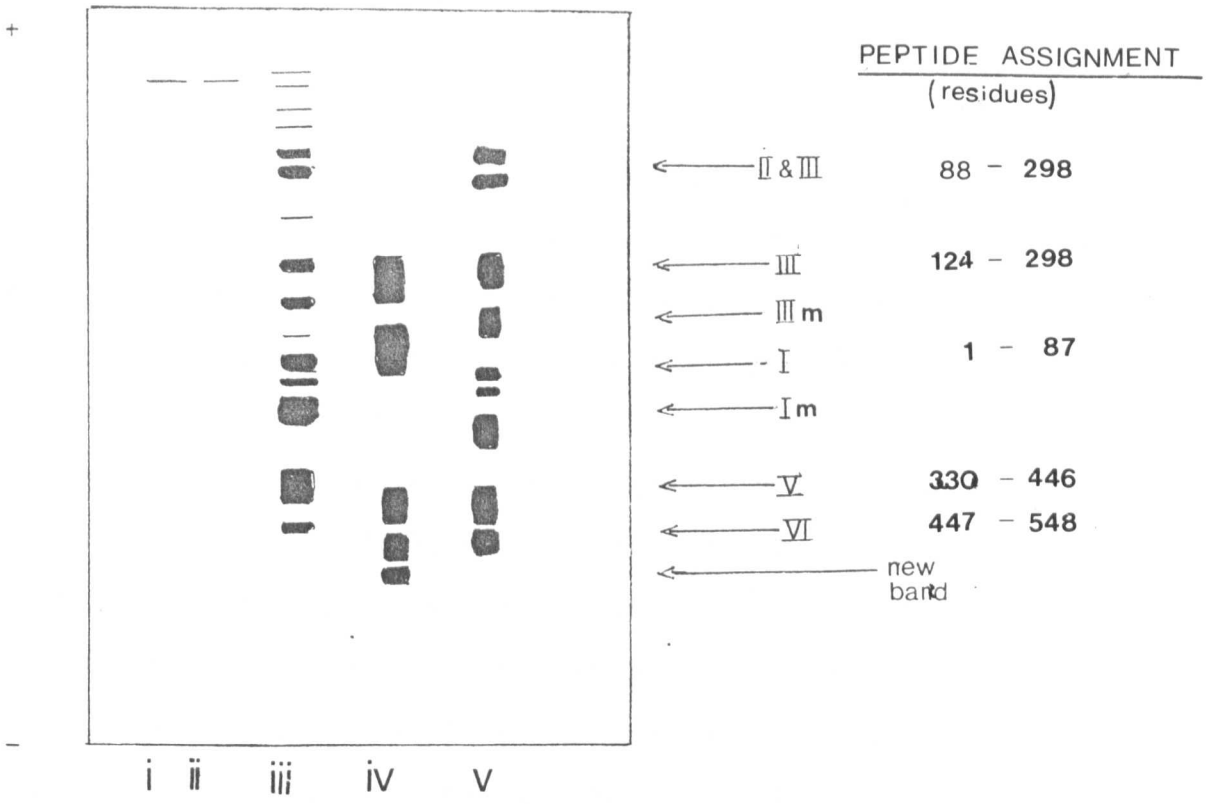


Fig. 5.14 Electrophoresis of the CNBr digestions of double Albumin Carlisle and HSA on Triton-Urea PAGE. (i) HSA, (ii) double Albumin Carlisle, (iii) and (v) CNBr digested HSA, (iv) CNBr digested double Albumin Carlisle.

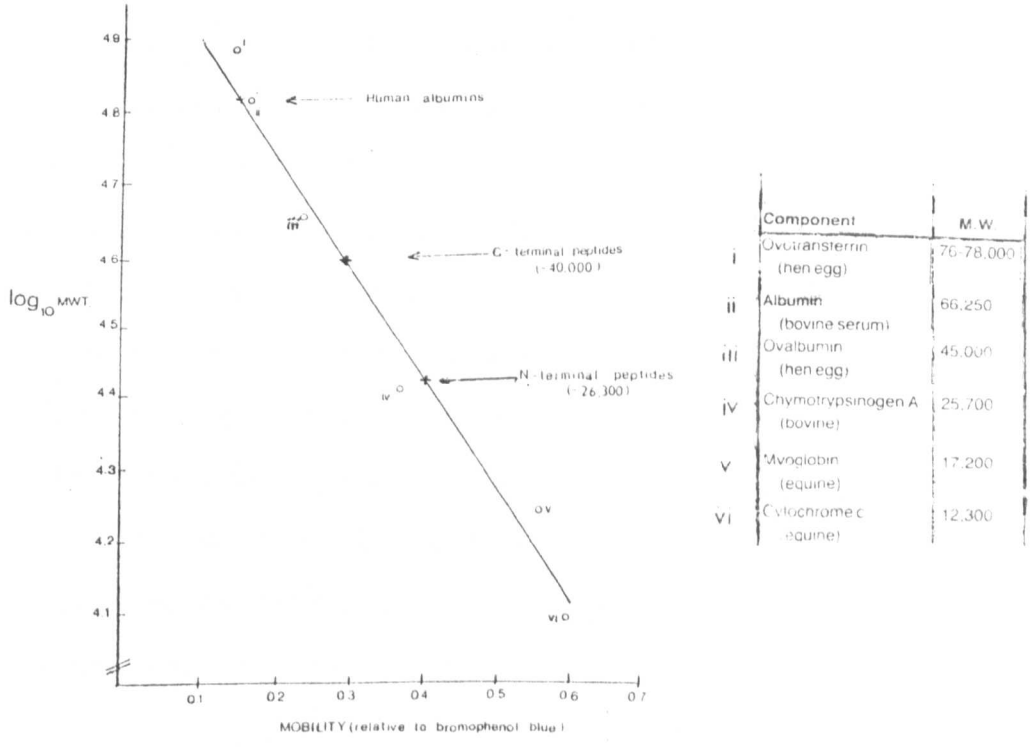


Fig. 5.15 Electrophoresis of the BNPS skatole digestions of albumin variants. Determination of molecular weight of fragments by SDS-PAGE.

Cleavage with o-iodosobenzoic acid gave poor results and one reason for this may be due to loss of peptides in the supernatant on TCA precipitation.

Cleavage with pepsin in the presence of octanoic acid, as detected by 7.5% SDS-PAGE gave essentially the same number of fragments for double Albumin Carlisle and albumin A; with two bands, one at ≈ 42,000 MW and the other at ≈ 36,000 MW. Double Albumin Redhill, however, contained two components instead of one for the first band, at 44,000 and 40,000 MW. This may be due to the increased MW of Albumin Redhill variant, giving rise to fragments of higher molecular weight. Peptic digestion and analysis were performed after the method of Ledden, *et al.* (1982) who also reported two fragments under reducing conditions, of 50,000 and 30,000 MW. Electrophoresis in 10% non-SDS gels revealed four anodic bands ahead of the albumin and these were again similar for double Albumin Carlisle and normal HSA.

Pepsin appears to digest albumin fairly non-specifically from the C-terminal end and to leave a number of fragments consisting of the first three hundred residues of the protein (Geisow and Beaven, 1977a). If the mutation in Albumin Carlisle was in the last 100-200 amino acid residues then no differences would be expected in the fragments from peptic digestion, as the mutation would most

likely have already been excised.

Digestion with papain, which is a fairly non-specific protease, revealed gross homology between the generated peptides of double Albumin Carlisle and those of HSA (Fig. 5.16). Several differences do occur in the electrophoretic patterns on 20% SDS-PAGE but it was not possible at present to pinpoint these to a mutation at the molecular level.

Digestion with α-chymotrypsin also revealed gross homologies between the double albumins and albumin A. However, double Albumin Carlisle showed two extra bands and other bands of different mobility to the other albumins. The bands in the double Albumin Redhill were all also present in albumin A but some had differing mobilities, possibly indicating a mutation in these peptides. Double Albumin Warwick-1 showed a similar pattern of bands to albumin A but again with some differences in mobility. Differences at the cathodic end of the tracks may reflect varying degrees of completion of the proteolytic process.

In the hope of obtaining better resolution RP-HPLC was used to separate fragments derived from CNBr cleavage and also peptic and tryptic digestions. The separation of all the CNBr-derived fragments would have been a major advantage in locating the point of mutation. Therefore much time was spent on assessing different solvent systems for such a

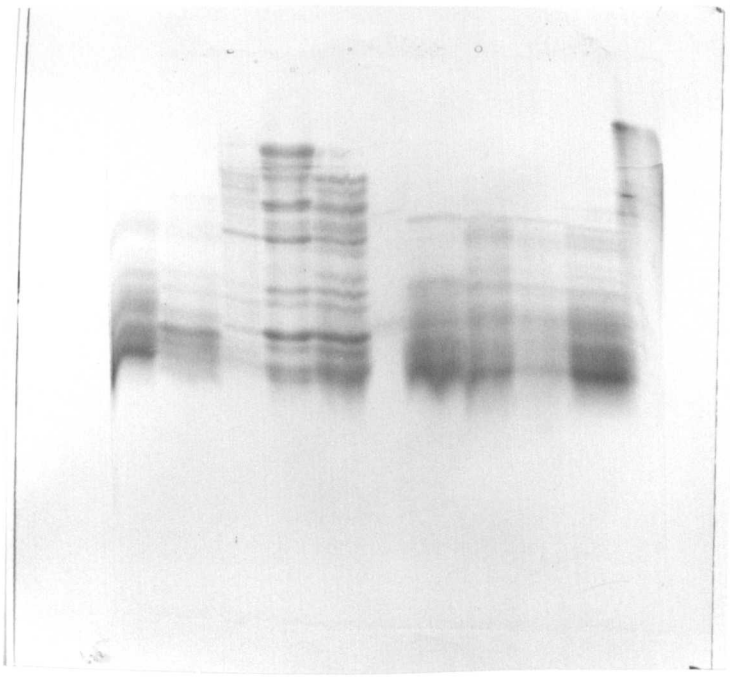


Fig. 5.16 SDS-PAGE of proteolytically digested double albumin variants. Chymotryptic digests of Albumin Warwick-1 (ii), Albumin Redhill (iii), albumin A (iv), and Albumin Carlisle (v). Papain digests of Warwick-1 (vi), HSA (vii), Redhill (viii) and Carlisle (i & ix). Undigested HSA (x).

	Solvent System	Conditions	Resolution Achieved
1	Trifluoroacetic acid (TFA)/ methanol + 0.1% v/v TFA	Isocratic Elution	Single peak only in methanol wash
2	10% v/v acetic acid/ methanol + 10% v/v acetic acid	0-60%methanol in 20 minutes 60-80% in 20 minutes	Single peak at 80% methanol
3	0.009 M H_2SO_4/aqueous acetonitrile + 0.009 M H_2SO_4	0-60% acetonitrile in 40 minutes	Several peaks resolved
4	0.05% v/v TFA/60% v/v acetonitrole + 0.05% v/v TFA	40-48% aceto-nitrile in 60 minutes	Several peaks resolved but separation of fragments incomplete
5	0.05% v/v TFA/aceto-nitrile: propan-2-ol 2:1 (v/v) + 0.05% v/v TFA	20-60% aceto-nitrile:propan-2-ol in 50 minutes	Several peaks resolved but separation of CNBr fragments not substantially improved.

TABLE 5.1 Solvent systems used for separation of CNBr fragments of albumin by RP-HPLC.

separation (see Table 5.1). However, reduced and alkylated CNBr fragments were poorly soluble in aqueous TFA buffers and therefore separation of non-alkylated fragments was also attempted.

The best separation was obtained with a gradient of 30-48% v/v acetonitrile with 0.1% v/v TFA over 0.1% v/v aqueous TFA; however, even then resolution was poor and the individual fragments were not isolated, although the three more cathodic fragments (on gels) were separable from the other fragments in some runs.

During late 1984, Iadarola, *et al.* (1984) published a method of separating the CNBr fragments of albumin using HPLC on two different columns. One, a Vydac C_{18}, was a large pore size column (≈ 300 Å) and so allowed better interaction with the column matrix for the peptides chromatographed; the other was the smaller pore size (≈ 100 Å) μC_{18} Bondapak column. The acetonitrile:propan-2-ol gradients used were as described previously (Section 5.4.8.1).

Attempts to repeat the results of Iadarola *et al.* using similar conditions on a μC_{18} Bondapak alone met with no success. The elution profile obtained was very different and the separation of the peptides was not significantly improved, and their identification on gels was hindered by their low concentration. The probable reason for this poor result was the small pore size of the Bondapak

column. Small pore sizes have been correlated to non-specific retention of peptides and poor elution profiles with variable peptide recovery. In their paper Iadarola, *et al.* (1984) reported that they were unable to separate all the albumin CNBr fragments in a single run and that the μC_{18} Bondapak column gave poorer resolution than the 300 Å pore size Vydac column. They used the μC_{18} Bondapak column to separate fragments I and V and to analyse the tryptic digest of the isolated CNBr fragment (Iadarola, *et al.*, 1985). This evidence would suggest that a separation under the conditions used above was unlikely to give good resolution.

The HPLC of 35 minutes peptic digests of HSA revealed two peaks after 10 minutes (17% solvent B), a large peak after 35 minutes at ≈ 58% B, and several peaks which were eluted afterwards. The first two peaks also appeared in double Albumin Carlisle and the only discernable differences were in the fine structure of the peaks eluted after 35 minutes of the gradient.

Controlled-time digests of HSA with pepsin revealed a gradual progression; the main peak at 35 minutes was absent in subsequent experiments, a major peak was noticed at 45 minutes (≈ 90% B) and the peak gradually decayed with time. Fine structure both before and after this increased with time and was presumably due to the peptic fragments generated; these peaks were maximal at 30 hours

after digestion.

Peptic digestion of purified abnormal Albumin Carlisle was also studied over a similar time course. Although some extra peaks were present by about 6 hours, they were different and less distinct than those for albumin A. Aliquots were simultaneously collected and run on non-SDS gels and the digested albumin bands were most distinct after ≈ 4 hours.

Tryptic Digests

The tryptic digests analysed by two-dimensional peptide mapping gave inconclusive results on thin layer cellulose and so RP-HPLC was attempted in order to analyse the fragments.

Initially a 0-100% gradient of acetonitrile: propan-2-ol, 2:1 was used over 45 minutes with gradient 3 on the Waters 660 gradient programmer. The pattern of peaks obtained was too constricted for good resolution and other systems were tried until a 20-80% gradient of solvent B over 50 minutes using gradient 3 was found to be optimal; with a low concentration of organic solvent maintained initially to elute the polar fragments and a gradient to higher concentrations being formed later to elute the other fragments.

Comparative runs of double Albumin Carlisle and albumin A (Fig. 5.17) were repeated several times and a consistent difference in the relative heights of a quartet of peaks was noticed for the Albumin

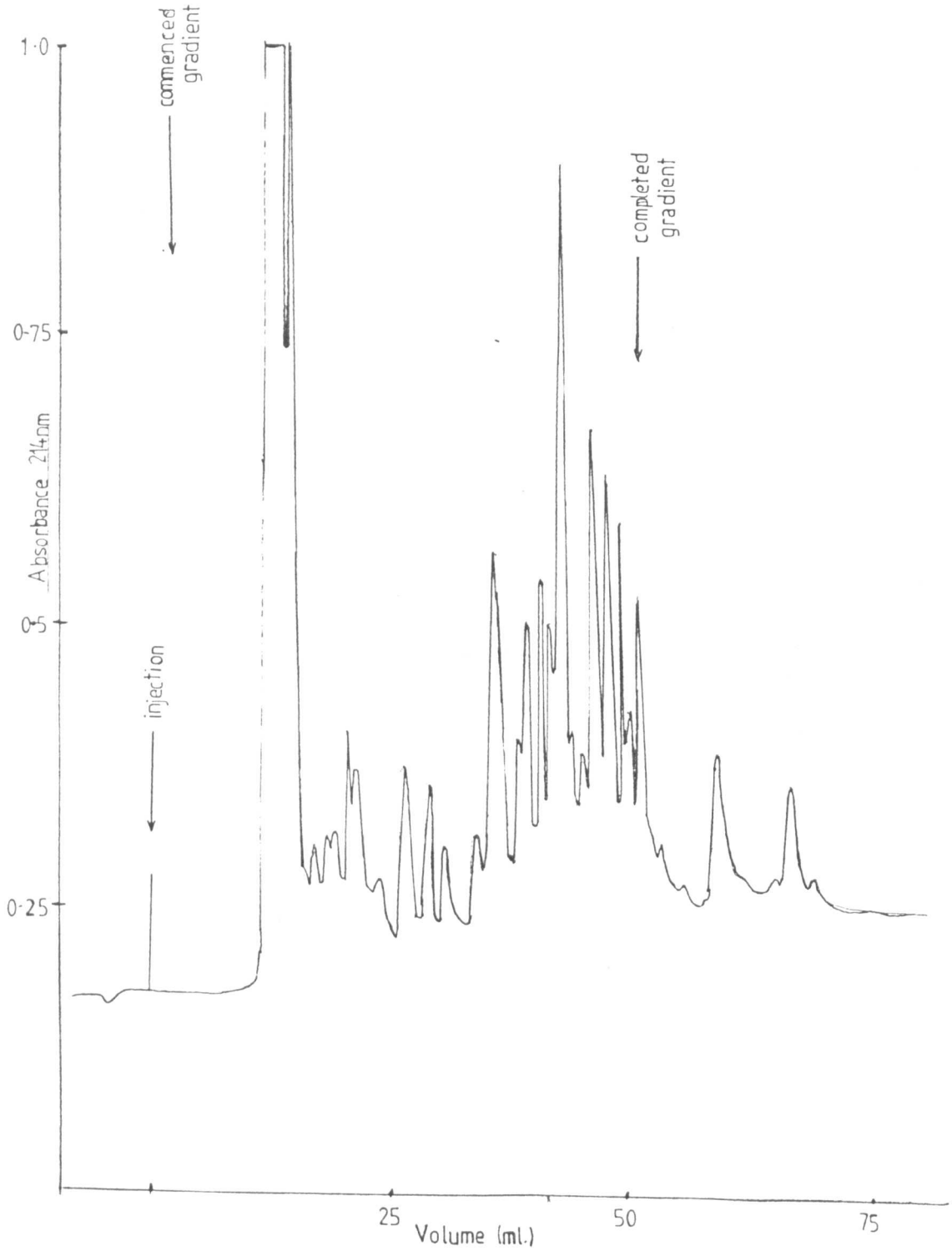


Fig. 5.17(i) RP-HPLC separation of the tryptic digest of albumin A.

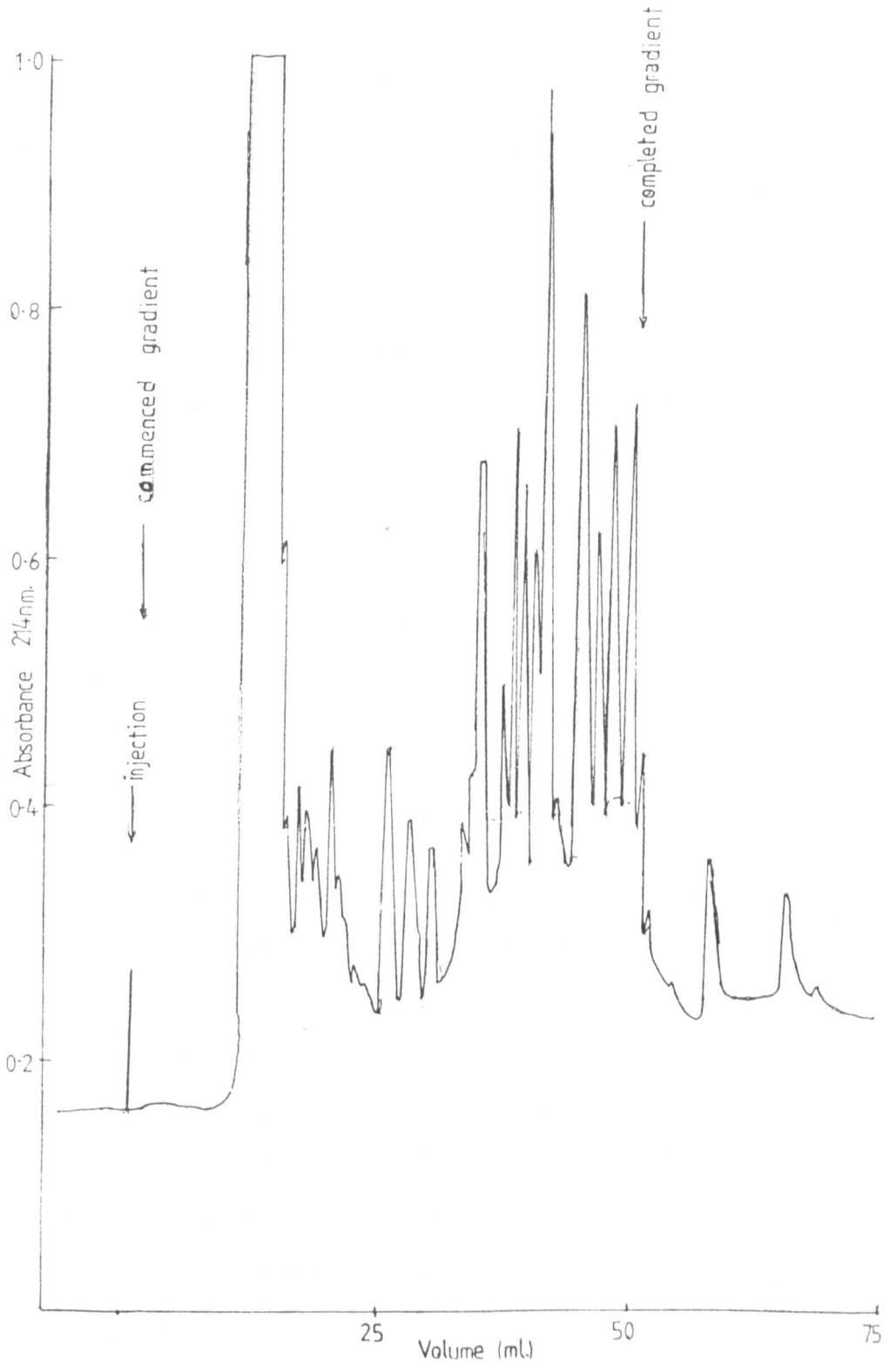


Fig. 5.17(ii) RP-HPLC separation of the tryptic digest of double Albumin Carlisle.

Carlisle samples after ≈ 43 ml of elution (≈ 80% B). The new pattern was seen only in chromatograms of double Albumin Carlisle, not in those of albumin A, and was similar to the type of difference found by Brennan (1985) for the tryptic digest of Albumin Parklands, and for similar variant proteins analysed by HPLC. As double albumin was used a total absence of the normal peaks would not be possible and so the difference generated by the albumin mutation would most likely be detected as a change in relative peak intensities.

The hydrophilic fragments eluted early on in the gradient were poorly resolved and there may be other differences in the albumins masked in this area, which further study might elucidate.

Due to lack of time the repeated pooling of these peaks was not possible, but this would seem the most logical progression in order to obtain sufficient material to attempt peptide sequencing.

It may be relevant at this stage to point out that Iadarola, *et al.* (1985), working with albumin from a homozygous patient, noted no differences in the HPLC profile of the tryptic fragments of the variant CNBr peptide and only pinpointed the mutation in Albumin Mi/Fg by subsequent collection and sequence analysis of the separated fragments.

5.6 CONCLUSION

Albumin Carlisle has been studied by peptide mapping on polyacrylamide and cellulose and by HPLC. The N and C-terminii are identical to those described for albumin A and digestion with CNBr revealed a possible mutation in the region residues 329-548. This is consistent with the evidence of peptic digestions which could reveal no difference in the albumin fragments generated, most of which have been digested from the C-terminus. Hence, it seems likely that the mutation has been excised by the peptic digestion, and as such is localised in the C-terminal region.

Digestion with papain and chymotrypsin and subsequent peptide mapping revealed differences between Albumin Carlisle and albumin A,which possibly implies differences in the susceptibility to proteolytic attack at certain residues within Albumin Carlisle, caused by an amino acid mutation.

Although RP-HPLC of CNBr fragments did not give satisfactory separation on the columns available enzymatic digestions using pepsin and trypsin were subsequently resolved. RP-HPLC does appear to be a powerful technique for the analysis of peptides generated by these means, provided that a range of columns of differing properties are available for use.

RP-HPLC of the tryptic digestion of double Albumin Carlisle revealed a consistent difference in the elution profile of a quartet of peaks and, had time allowed, further analysis of the amino acid composition of these peaks may have elucidated important details about the amino acid mutation in Albumin Carlisle.

CHAPTER SIX

AMINO ACID SEQUENCING OF ALBUMIN REDHILL

6.1 INTRODUCTION

6.1.1 Sequencing Methodology

The amino acid sequence of a protein is the most important data for comparing normal and variant forms. It is the most basic information about protein structure from which secondary and tertiary structure can be predicted and ligand binding, or enzymatic functions understood at a molecular level. As such it is not surprising that much effort has been put into developing modern and efficient protein sequencing methods. Over thirty years ago Sanger and Tuppy (1951) deduced the amino acid structure of insulin B chain using fluoro-dinitrobenzene (FDNB) to label the N-terminus and identifying the dinitrophenyl amino acids by paper-chromatography. Since then a number of methods for protein sequencing have been devised and improved so that by 1979 160,000 residues and 1,100 complete protein sequences were known, and the number has grown considerably since then. There are several methods that can be used to obtain amino acid sequences:

(1) The Edman Degradation;

(2) Mass Spectrometry;

(3) Interpretation from the cDNA sequence;

(4) Other degradative methods, including amino-peptidases and other amino-terminal cleaving reagents.

Of these the Edman method is probably the most well known, although in the past decade vast numbers of DNA sequences have been derived.

6.1.1.1 Edman Degradation

This technique is the most important for the determination of amino acid sequences of peptides. It was developed by Edman (1950) as a manual procedure and later by Edman and Begg (1967) in an automated form, and it has been constantly improved upon since then. The basic process is in three stages; firstly, the amino-terminal residue of the protein is coupled to the Edman reagent (phenylisothiocyanate, PITC). Secondly, this residue is then cleaved off the chain using strong acid to give, via cyclisation, an anilinothiazolinone residue. The third step is to convert the unstable anilinothiazolinone to a phenylthiohydantoin amino acid (PTH-amino acid) which can be compared to PTH-amino acid standards by chromatography.

The technique is probably the widest used of all protein sequencing methods and can by repeated cycles identify 20-70 residues from the N-terminus of a peptide or protein.

6.1.1.2 Mass Spectrometry

In principle mass spectrometry, by deriving precise molecular weights for small peptides cleaved

from the total chain, can be used to give amino acid sequences. However, the equipment is expensive and the data complicated so that it has been utilised by few laboratories. One main disadvantage is that for electron impact (EI) mass spectrometry the peptides must be volatilised by derivatisation before a sequence can be obtained. This problem has been overcome by use of Fast Atom Bombardment Mass Spectrometry (FAB-MS) where the sample, applied to the probe in a glycerol matrix, does not need to be volatile. In this technique the sample is bombarded with accelerated Xenon atoms and ionisation occurs to give quasi-molecular ions which can then fragment . The mass spectra from such bombardments can be used to determine the peptide sequence, as amino acids are sequentially lost by fragmentation, or FAB-MS can be performed on dipeptides released sequentially from the peptide by dipeptidyl aminopeptidases.

Although still less common than the Edman method, mass spectrometry is able to detect glycosylations and other post-translational modifications of amino acids which often prevent Edman sequencing methods from successfully obtaining a sequence. The large number of proteins showing post-translational modifications may well result in mass spectrometric sequencing becoming more widely used. A more complete review of the uses of mass spectrometry in peptide sequencing can be found in Elzinga (1983).

6.1.1.3 DNA Sequencing

Although DNA sequencing does not directly give

the amino acid sequence, this can be derived using the genetic code if the appropriate regions of DNA can be identified. The cloning of complementary DNA (cDNA) encoding the mRNA sequence has become an established technique and it can be both quicker and simpler to determine the sequence of the protein by these indirect methods (Sanger *et al.* 1977), Maxam and Gilbert, 1980). Often, if sufficient residues of a protein are known, then a synthetic oligonucleotide can be synthesised and can then be used as a probe for the DNA sequence encoding the protein. In this way many proteins which occur in such minute amounts that obtaining sufficient protein for sequencing is an almost insurmountable task may be sequenced from their DNA. However, there are disadvantages in sequences obtained in this way as no information about any post translational modifications is given and a loss of a base pair in the DNA sequencing may result in the wrong assignment of amino acids for a long section of the chain. DNA sequencing can reveal little about the disulphide bond assignments and positions, being unable to distinguish between cystine and cysteine residues. It does not detect the presence of glycosylations, acylations or other modifications to the amino acids which can be detected by combined protein sequencing methods. Any post-translation chain cleavage, such as occurs in the insulin molecule, could not be deduced from the DNA sequence alone. DNA sequencing in areas of repetitive sequence is also prone to difficulties as is the protein sequencing. Therefore DNA sequencing is

best performed in conjunction with modern highly sensitive protein sequencing methods so as to avoid such difficulties and present a complete picture of the post-translational gene product.

6.1.1.4 Other Methods

Other reagents for cleaving amino acids from the N-terminus have been developed since the widespread introduction of the Edman reagent; however, they have not attained routine use. Thioacetylthioglycolic acid (TATG) may be used, and this generates the free amino acid in mild conditions after cleavage (Doolittle, 1983). Enzymic analysis of the N-terminus may be achieved with amino dipeptidases (Light, 1972) such as leucine aminopeptidase, which preferentially releases hydrophobic amino acids, or aminopeptidase M, which has a broader specificity. Such enzymes are common in mammalian cells and their use,combined with mass spectrometry, may be a powerful means of sequencing blocked N-terminal and other modified proteins (D. W. Hutchinson, private communication).

Another possibility is the determination of C-terminal residues using the carboxypeptidases (see Section 5.2.4). Although primarily used for the identification of the C-terminal amino acid it may also be possible to utilise such enzymes to determine short regions of sequence from the C-termini of peptides and proteins.

6.1.2 The Edman Degradation

There are presently three main formats for using the Edman degradation, they are:

(i) manual liquid phase;

(ii) automated (spinning cup) liquid phase;

(iii) automated solid phase.

Each of the three use the same basic reaction and so the chemistry of the degradation will be considered first. A more complete analysis has been reported by Tarr (1977).

6.1.2.1 The Degradative Process

6.1.2.1.1 Coupling

As previously stated, the Edman degradation has three major steps, the first of which is coupling of the PITC to the amino-terminal residue. The coupling reaction (Fig. 6.1) takes place in alkaline solution between pH 8-10, so as to allow the amino group to be in the unprotonated form, but not too alkaline so as to result in the hydrolysis of the PITC to aniline, so competing with the Edman process. The attachment to ε-amino residues is far slower at pH 8 and although different N-termini react at different rates all should have been coupled after 30 minutes at 50°C using a solution of PITC in aqueous pyridine or similar solvent system.

Aldehydes in the buffer solution can result in partial blocking of the free amino group: reagents used should therefore be pure and molecular oxygen and other oxidants should be excluded as they may cause desulphurisation and stop further degradation (Isle and

Edman, 1963). Other reasons for low percentage coupling may include insufficient mixing or acidic contaminants of the peptide reducing the pH. Prolonged reaction times and higher temperatures may also produce undesirable side reactions.

Coupling gives rise to a phenyl thiocarbamyl (PTC) peptide, and solvents and by-products are removed by solvent extraction using either an organic solvent for solid phase or a two-phase aqueous/organic solvent for the liquid phase. The aqueous layer is retained and dried over P_2O_5 and NaOH *in vacuo* before the cleavage step can occur.

6.1.2.1.2 Cleavage (Cyclisation)

Most procedures for the cleavage reaction (Fig. 6.2) employ anhydrous perfluorinated carboxylic acids usually trifluoroacetic acid or heptafluorobutyric acid. Edman initially used HCl/nitromethane (Edman, 1950) and HCl/acetic acid has also been used. Water is generally avoided due to the danger of acid-catalysed peptide bond hydrolysis, although heptafluorobutyric acid with 4% water gave better results than the anhydrous acid. Other possible problems include esterification of hydroxyl groups on serine or threonine, as these are rapidly transferred to the α-NH_2 group in the alkaline media used for coupling when these residues become N-terminal and so block further degradation. Also, the acetylated PTH derivatives are more susceptible to β-elimination which complicates their detection.

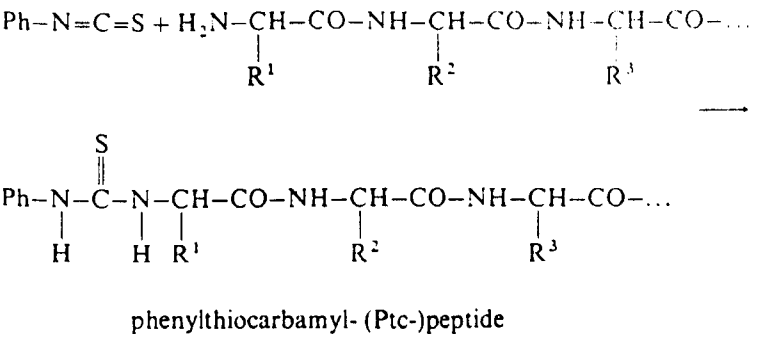


Fig. 6.1 Edman Degradation: coupling reaction.

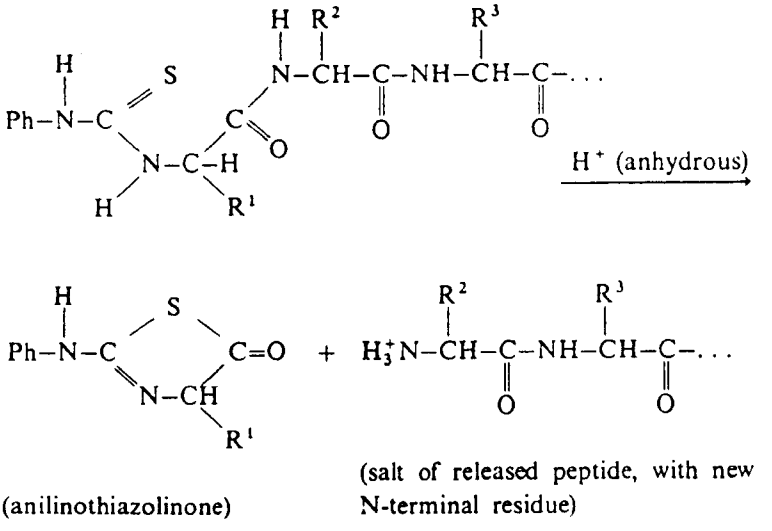


Fig. 6.2 Edman Degradation: cleavage reaction.

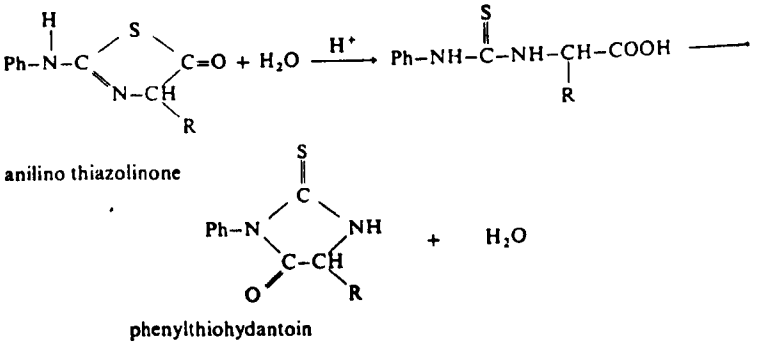


Fig. 6.3 Edman Degradation: conversion reaction.

After cleavage, volatile acid is removed *in vacuo* and the anilinothiazolinone derivative formed is extracted from the salt of the residual peptide by two-phase extraction, although any remaining acid will enhance the solubility of peptides in the butyl acetate and so increase losses for the next cycle. The organic phase containing the anilinothiazolinone derivative is dried over nitrogen and conversion to the PTH-amino acid carried out.

6.1.2.1.3 Conversion

The anilinothiazolinone derivatives of amino acids are not sufficiently stable to be reliably chromatographed and so conversion to the PTH derivatives is carried out before chromatographic identification (Fig. 6.3). A commonly used method is heating at 80°C for 10 minutes in 1.0 M HCl under N_2. The thiazolinone ring opens to give the intermediate phenylthiocarbamyl (PTC) amino acid which then cyclises in a second step to the phenylthiohydantoin (PTH) amino acid. All derivatives but glycine readily cyclise in this time (Isle and Edman, 1963). Partial hydrolysis of asparginyl and glutaminyl derivatives occurs, as does conversion of glutamate and aspartate to their methyl esters. To avoid such problems the aminothiazolinones can be reacted with methylamine and the phenylthiocarbamyl amino acid methylamides formed can be chromatographed against standards.

6.1.2.1.4 Identification of PTH-Amino Acids

(i) Edman (1950) identified his PTH-amino acids by back hydrolysis with barium hydroxide to

give the free amino acids, and then performed paper chromatography against amino acid standards. However, the method is inefficient and several amino acids are difficult to identify. Hydrolysis with $HCl/SnCl_2$ has replaced this method (Mendez and Lai, 1975), but the procedure is time-consuming and the technique is usually used only when other means of identification have failed.

(ii) The chromatography of PTH-amino acids on paper was proposed in 1953 by Sjoquist and was capable of detecting 50 nmol of PTH-amino acid. Since then thin layer chromatography has been used successfully. Silica gel fluorescent plates (Merck) have been used in a two-solvent system to detect 5-10 nmol of PTH-amino acid run against PTH-amino acid standards (Allen, 1981). The discrimination of the hydrophobic amino acids requires careful comparison as they resolve poorly from each other, and spraying with reagents, producing different colours for the various amino acids, has been used to aid identification. Two-dimensional t.l.c. of PTH-amino acids can detect up to 0.5-1.0 nmol of PTH-amino acid as absorbant areas on fluorescent silica (Kulbe, 1974).

(iii) Gas-liquid chromatography was popular for a long time in the 1960's and 1970's as being the most important method of quantitatively identifying PTH-amino acids. However, the more polar PTH-amino acids cannot be identified directly, requiring conversion to more volatile derivatives, and the PTH derivatives of arginine and cysteic acid are not identifiable at all. Pisano (1972) gives experimental details of the procedure but it has been

superceded by HPLC.

(iv) In the 1970's reverse phase HPLC was used to separate PTH-amino acids and this technique has largely been adopted for most automated sequencing. Reverse phase chromatography is performed on octyl or octadecyl silica with either isocratic or gradient elution of acetonitrile or methanol over an acidic aqueous phase usually at elevated temperature (30-50^{o}C). All of the PTH-amino acids are separable using gradient elution although good resolution is also achieved with the simple isocratic elutions. Detection is usually by absorbance at 254 nm though other wavelengths have been used. Although the equipment is initially expensive it provides separations in 20 minutes using 1-10 nmol of PTH-amino acid and sequences in the pmol.range have been reported.

6.1.2.1.5 Difficulties with the Edman Degradation

There are a number of competing side reactions and other difficulties which limit the theoretically infinite number of cycles of the Edman procedure which can be performed. In practice only 20-50 steps can usually be performed before the identification of the PTH-amino acid becomes impossible.

There are a number of problems which cause this loss of efficiency:

(i) The increase in proportion of preceding residue due to a gradual decrease in the cleavage efficiency. Although yields are optimally 98%, as the degradation proceeds through many cycles an 'overlap' arises due to

incomplete coupling and cleavage reactions. The newly produced PTH-amino acid eventually becomes undetectable against the increasing background.

(ii) Acid sensitive non-specific peptide bond cleavage also results in an increase in the background PTH-amino acids produced.

(iii) Small amounts of peptides may be lost at the two-phase extraction steps. This reduces the amount of material available for the next cleavage and serves to aggravate the problem of background noise.

(iv) The presence of blocked N-terminal residues due to the migration of acyl groups on serine and threonine, or of acyl groups naturally occurring in the peptide chain, prevents further degradation or reduces the yield of PTH-amino acid.

(v) Difficulties are sometimes encountered with sequences bearing O-glycosyl groups on serine and threonine. The residue produced is often difficult to characterise and may not extract into the organic phase after cleavage. Special methods are needed for such glycosylated residues.

(vi) Asparaginyl-glycine bonds. Under acidic conditions asn-gly sequences are converted to an imide structure, which forms a β-aspartyl peptide bond under alkaline conditions and so blocks further degradation, or allows it to proceed with very low yield.

(vii) N-terminal glutamine residues may cyclise under acidic conditions, although this can be avoided by strict timing at each stage. Similarly, PTC-histidine peptides are very rapidly cleaved and thus partial

coupling may occur of the next residue and so two residues are detected on analysis. This preview of the following residue can also be controlled as it does not occur in non-volatile buffers. Tryptophan oxidation can also lead to blockages in manual processes if the exclusion of molecular oxygen is not complete.

6.1.2.2 Modes of Operation for the Edman Degradation:

6.1.2.2.1 Manual Liquid Phase Sequencing

Modern manual methods typically require 50-250 nmol of peptide and several procedures have been described (Tarr, 1983; Zalut, 1983). The initial procedure of Edman (1950) has been modified subsequently so as to increase the sensitivity, also several peptides can be manually sequenced at the same time and the resultant PTH-amino acids dereacted by HPLC or t.l.c. Although the technique has difficulties (the need for oxygen-free environments and high vacuum together with small sample volume requires some dexterity on the part of the operator) several modifications have made it an invaluable and less expensive alternative to automated sequencing.

6.1.2.2.1.1 Dansyl Edman Method

This is an indirect method whereby a small aliquot of the peptide is removed after each cycle and reacted with dansyl chloride. As only 0.2 nmol. are required the repetitive yield is only slightly reduced and 10-30 residues may be identified with 2-50 nmol of peptide. Allen (1981) describes a methodology for a dansyl Edman manual procedure. The major problem with the dansyl Edman procedure is the poor differentiation of the amide and acid groups, and the loss of tryptophan

residues which are not identified.

6.1.2.2.1.2 Microsequencing with DABITC

Chang, *et al.* (1978) used an alternative isothiocyanate which gave intensely coloured PTH-residues. The reagent, 4-N,N-dimethylamino-azobenzene-4'-isothiocyanate (DABITC), yields thiohydantoic amino acid derivatives (DABTH-amino acids) whose properties have been characterised by Chang, *et al.* and the technique can be used for a solid phase sequencing procedure (Chang, 1979). The DABTH-amino acids are separable by 2-dimensional t.l.c. on polyamide plates against DABTH-amino acid standards. Although the derivatives appear as a faint yellow colour on chromatography a strong red is produced on exposure to concentrated HCl. Separation of DABTH-leu and DABTH-ile is poor. By coupling first with a small amount of DABITC and then with excess PITC, Chang, *et al.* (1978) succeeded in driving the otherwise slow coloured reaction to completion. The use of DABITC is now widespread and can be used for automated as well as manual procedures. By use of pre-column derivatisation and HPLC detection of the DABTH-amino acid at 436 nm, it is possible to sequence peptides with as little as 5 pmol. of material (Chang, *et al.*, 1983).

6.1.2.2.2 Automated Liquid Phase Sequencing

The first automated system for liquid phase Edman degradation was designed by Edman and Begg (1967). The spinning-cup device became the model for all liquid phase sequencers thereafter, although many mechanical

modifications have subsequently been made resulting in greater sensitivity with 20-50 residues being located at 24 cycles per day. The protein is dissolved in heptafluorobutyric acid and introduced into the spinning cup; the solvent is then evaporated under vacuum to leave the protein as a film around the sides of the cup. PITC is then introduced in a coupling buffer, usually Quadrol (N,N,N',N'-tetrakis (2-hydroxypropyl)-ethylenediamine) in aqueous propan-1-ol. Coupling occurs for ≈ 30 minutes at 50°C and then excess reagents are removed *in vacuo* and the Quadrol and by-products extracted with benzene and ethyl acetate. The film of PTC-protein is dried thoroughly and heptafluorobutyric acid added. Cleavage is allowed to proceed for three minutes and then most of the acid is removed *in vacuo*. The cleaved anilinothiazolinone derivative is extracted in 1-chlorobutane and collected. The dried protein film is then ready for another cycle. Due to the improvements in sequenator technology over recent years it is possible to sequence quite long peptides at the subnanomolar level (Hunkapiller and Hood, 1980). The sequenator must be cleaned regularly and correctly serviced for the sensitivity to be maintained.

To be effectively removed Quadrol has to be dissolved in a polar solvent, normally ethyl acetate; however, some loss of coupled peptides into this solvent is possible, thereby reducing the number of cycles that can be performed. Two possible ways of overcoming this problem have been proposed; dimethyl-

allylamine (Niall, *et al.*, 1969) which is volatile, and dimethyl benzylamine (Hermodson, *et al.*, 1972) which is soluble in benzene have been used in place of Quadrol so as to reduce peptide losses. A second approach has been to render the peptides more hydrophilic. Several chemical means have been proposed, including sulphonated isothiocyanates which react with the lysyl ε-NH_2 groups (Braunitzer, *et al.*, 1971), and carbodiimide coupling of carboxyl groups with hydrophilic amines (Foster, *et al.*, 1973). However, the most effective method has been found to be the addition of film-stabilising substances such as the polycationic Polybrene (1,5-dimethyl-1,5-diazundecamethylene polymethobromide) which anchors peptides in the aqueous phase and so reduces protein and peptide losses in two-phase partitions.

6.1.2.2.3 Gas Phase Sequencing

In 1981 Hewick, *et al.* reported a gas-liquid phase protein sequencer which was capable of picomole level sequencing. The polypeptide is embedded in a matrix of Polybrene dried onto porous glass fibre in a small cartridge-style reaction cell. Although not covalently attached, the sample is effectively anchored throughout the degradative cycle and is only exposed to relatively non-polar extraction solvents. Only a very minimal amount of trifluoroacetic acid is used and the coupling is performed in trimethylamine/water vapour and the cleavage in trifluoroacetic acid vapour. The reaction chamber is much reduced in size compared to the spinning cup (Hunkapiller, *et al.*, 1983) and

picomole sequences are reported.

6.1.2.2.4 Solid Phase Sequencing

As an alternative method of dealing with the problems encountered with peptide loss during two-phase extractions, covalent attachment of the peptide to an insoluble support was devised (Laursen, 1966). Efficient extractions are now possible without loss of peptide, and the process may be readily automated. The insoluble support is placed in a column, fitted with lines for the sequential delivery of reagents and collection of derivatised amino acids. The automated system has been commercially produced and several good reviews of the operation and characteristics of solid phase sequencing have been written (Previero and Coletti, Previero, 1977, Laursen and Machleidt, 1980).

The solid phase sequencing procedure was a logical extension of the solid phase peptide synthesis of Merrifield (1963) and the earliest support was made of modified polystyrene resin. These resins are often used but have distinct disadvantages, with differential swelling and small pore size giving very low attachment efficiency for larger polypeptides. These problems have been in part overcome by the use of a large excess of glass beads to pack the column.

6.1.2.2.4.1 SupportingMedia

Supports should be chemically stable to the Edman reagents, be accessible to the peptides and able to be coupled to them, and they should resist fracturing or gross deformation. Two types of resin have been

produced with this in mind; synthetic resin supports and glass bead supports.

6.1.2.2.4.2 Synthetic Resin Supports

Polystyrene resins give poor results with large peptides or proteins since they swell considerably. Two other types of polymeric support which have found wide application for protein immobilisation are cross-linked agaroses (Porath, 1974) and polyacrylamide (Inman, 1974). Polyacrylamide has been utilised in the Edman degradation but agarose is labile to trifluoroacetic acid. Some modified polyacrylamides have been used, but have not been fully studied, although proteins can be attached in reasonable yield.

6.1.2.2.4.3 Glass Supports

The most useful glass supports are prepared from treatment with aminoalkyl silanes. Control pore glass is usually chosen because of its high internal surface area.

Two types of control pore glass (CPG) have been used; 3-aminopropyl glass (APG) and N(2-aminoethyl)-3-aminopropyl glass (AEAPG).

APG is formed from the reaction of controlled pore glass with 3-aminopropyl triethoxysilane (Wachter, *et al.*, 1973) (Fig. 6.4).

AEAPG is obtained by derivatising CPG with N(2-aminoethyl)-3-aminopropyl trimethoxysilane (Bridgen, 1975). The CPG used has a nominal pore diameter of 75 Å, although larger pore diameters may be better for larger proteins.

Aminoalkyl glasses can also be prepared. CPG

is accessible to solvents of all kinds. The beads are filtered and washed with a non-polar organic solvent and then methanol and dried *in vacuo*. Iodoacetyl glass can be prepared by treating APG with iodoacetic acid and dicyclohexylcarbodiimide, and then washed with aqueous solvents and drying *in vacuo*.

6.1.2.2.4.4 Attachment of Peptides to Solid Support

The ideal point of attachment to CPG would be via the C-terminal residue. It is sometimes necessary to block the α-amino group to prevent side reactions, using BOC (t-Butyloxycarbonyl) or BOC-ON (2-t-Butoxycarbonyl oxyimino-2-phenyl-acetonitrile). Isothiocyanates have also been used (reviewed in Laursen and Machleidt, 1980). When trying to activate the peptide C-terminus the COOH groups of glutamyl and aspartyl residues are also often activated, possibly resulting in reduced sequencing efficiency. There may be a difference between the various carboxyls which can be exploited.

Carbonyldiimidazole gives high attachment yields but must be used in a solvent such as dimethylformamide which is a poor peptide solvent. It is also essential that the anhydrous conditions are maintained. When sequencing a modified glutamyl residue it does not appear as a PTH-amino acid, as the thiazolinone remains bound to the resin. The same problem at aspartyl residues prevents further sequencing.

Carbodiimides are the most widely used reagents for the formation of amide bonds. They react only slowly

with water in neutral and alkaline pH and so attachment can be made in aqueous solutions so that no peptide solubility problems occur. Aspartate and glutamate residues can be sequenced normally when short coupling times are used.

Homoserine residues created at the C-terminus of CNBr fragments can be coupled to amino resins after treatment with trifluoroacetic acid to give the homoserine lactone. This yields > 70% coupling of the C-terminus to the resin.

p-Phenylene diisothiocyanate can be used to activate peptide amino groups allowing attachment through the amino residue, so circumventing the problem of coupling through the carboxyl group (Laursen, *et al.*, 1972). This causes modifications of the terminal α-amino as well as the lysine ε-amino groups but treatment with acid under conditions of the Edman reaction cleaves the first peptide bond to liberate a new amino group. The first PTH-amino acid remains bound to the resin and must be detected by dansylation, or by differences in the composition of the peptide.

C-Terminal lysine residues as in tryptic peptides may be attached through the ε-NH_2 groups of these residues to isothiocyanato glass or resins. Cysteine residues may be bound to iodoacetyl glass (Chang, *et al.*, 1977) in reducing conditions in the dark, under N_2, for 30 minutes.

Choice of the coupling reagent is very important and a wide range of methods have been developed (Laursen and Machleidt, 1980).

6.1.2.2.4.5 Uses and Limitations of Solid Phase Sequencing

Essentially, solid phase sequencing is similar

to the liquid phase but a greater choice of solvents is possible as the peptide is covalently fixed to the support so that a relatively polar solvent such as methanol may be used to completely remove excess reagents and by-products. The main problems with solid phase sequencing arise from three areas:

(i) detection;

(ii) background;

(iii) repetitive yield.

(i) Detection Most analysis of automated sequencing monitors PTH-amino acids by HPLC at 254 nm. However, the maximum absorbance is nearer 269 nm and analysis at this wavelength can improve detection by 8-50% (Horn and Bonner, 1983). Another improvement in the detection limit can be brought about by using ^{35}S radio-labelled PITC, although the purity of commercially available reagents is generally a limiting factor. The use of DABITC in solid phase sequencing has further increased levels of detection; monitoring absorbance in the visible rather than UV spectrum reduces background and allows for picomole levels of DABTH-amino acid to be detected. Chang, *et al.* (1983) have discussed the use of pre-column derivatisation with DABITC or dimethylaminoazobenzene sulphonyl chloride (DABS-Cl) to improve sequencing to picomole levels by manual or automated means.

(ii) Background The background in solid phase sequencing is mainly due to break up of the support generating UV-absorbing fragments. However, these problems can be alleviated by thorough pre-treatment of the

support before use. Isothiocyanates are often used to block excess reactive groups on supports and can become a problem if further isothiocyanate-blocking reagents are not added. This problem can also be minimised by judicial choice of conditions. As reagents and solvents are not coated around the supporting medium they do not contribute significantly to problems with background absorbance. Pyridinium salts can arise due to interaction of the support with acidic pyridine buffers but this also can be corrected for by choice of a different buffer system.

(iii) Repetitive Yield The repetitive yield (RY), that is the yield from one cycle to the next, can be a problem. Theoretically 98% RY is possible, but in reality values are nearer 90-94%. The noted decrease in RY is not due to solvent or reagent impurities but to the interaction between the support and the polypeptide. The higher the polypeptide loading on the support the higher is the repetitive yield. However, overloading the support generates overlap in the sequence analysis.

Despite these limitations, Salnikow, *et al.* (1983) reported sequences of up to 34 residues using only 1-10 nmol. of peptide routinely with some peptides being sequenced at lower levels. L'Italien and Laursen (1983) reported 22 cycles per day of solid phase sequencing on as little as 0.5 nmol of proteolytically-derived peptides.

Solid phase automated Edman sequencing has become an effective method of analysing very small amounts of peptides, and sequencing limits are continually

being pushed further back as new innovations are applied to the basic regimen.

6.2 EXPERIMENTAL

6.2.1 Albumin Purification

Albumin Redhill was isolated as outlined in Section 4.3.2. The albumin was desalted on Sephadex G-25 and exhaustively dialysed overnight against double distilled water before lyophilisation.

Protein concentration was estimated by the method of Bradford (1976) using Coomassie Brilliant Blue G-250, against a standard curve of 0-100 µg HSA Fraction V (Sigma).

6.2.2 Protein Sequencing

Albumin Redhill (300 µg, 4.6 nmol) was submitted to the S.E.R.C. Protein Sequencing Facility at the University of Leeds, Department of Biochemistry. Dr. J. B. C. Findlay sequenced the protein by a solid phase Edman procedure using an unreported non-commercial method. Full sequence data was obtained including amino acids identified, peak areas, and amounts of each residue in picomoles.

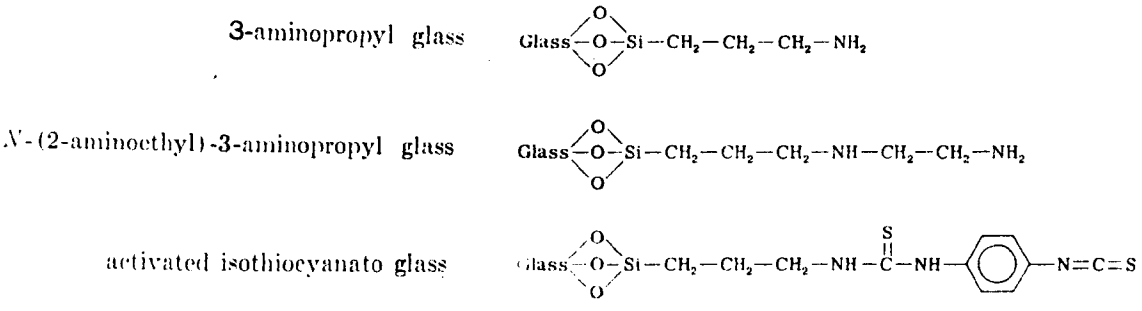


Fig. 6.4 Some common glass supports for solid phase sequencing.

	-6	-5	-4	-3	-2	-1	1	2	3	4
(a)							Asp	Ala	His	Lys
(b)						Arg	Asp	Ala	His	Lys
(c)	Arg	Gly	Val	Phe	Arg	Arg	Asp	Ala	His	Lys
(d)	Arg	Gly	Val	Phe	His	Arg	Asp	Ala	His	Lys
(e)	Arg	Gly	Val	Phe	Arg	Gln	Asp	Ala	His	Lys

(a) albumin A

(b) Albumin Redhill,

(c) human proalbumin

(d) Albumin Lille

(e) Albumin Christchurch

Fig. 6.5 Comparison of the sequences of Albumin Redhill and known proalbumin variants.

6.3 RESULTS

The sequence data revealed a major component (Albumin Redhill) and a minor component (identical to albumin A) (see Table 6.1). This minor albumin component was not detectable on PAGE of the initially analysed Albumin Redhill, but may have occurred due to the accumulation of normal albumin over the pooling of fractions from several runs; this albumin was not visualised by the PAGE Blue-90 staining method.

The minor component (the first 30 amino acids) was of identical sequence to that found for albumin A by Dugaiczyk, *et al.* (1982). The detector was set to 0.02 AUFS (absorbance units full scale) and the levels of PTH-amino acid were from 600-45 picomoles. The peak areas were approximately 58% of those for Albumin Redhill. The residues 16 and 17 of HSA were not detected although by analogy with the other known sequences they were probably glutamate residues. Residues 23 onwards were marked by a number of very low yields and residue 28 was also not determined. However, the sequence of the first thirty residues from 5 nmol. of protein is a fairly good sequence result even by solid phase methods. The repetitive yield for residues 8 to 21 of 90.7%, although good for such a distance in to the protein chain, was already too low for sequencing to proceed many more cycles into the protein.

Albumin Redhill (sequenced on two occasions) gave a reliable sequence over 30 residues with only residue 16 being undetermined. The yield of PTH-amino acids

REDHILL 3/C (SP 414) MAJOR

Cycle	PTH	Area	Fraction applied	AUFS	p mole
1	ARG	114524	0.250	0.020	1266
2	ASP	114419	0.250	0.020	1034
3	ALA	98210	0.250	0.020	846
4	HIS	100278	0.250	0.020	1050
5	LYS	180237	0.250	0.020	1036
6	SER +	11609	0.250	0.020	114
7	GLU	89454	0.250	0.020	799
8	VAL	178311	0.375	0.020	1009
9	ALA	157863	0.375	0.020	907
10	HIS	85912	0.375	0.020	599
11	ARG	119699	0.375	0.020	882
12	PHE	113604	0.375	0.020	602
13	LYS	225770	0.375	0.020	865
14	ASP	101357	0.375	0.020	610
15	LEU	110311	0.375	0.020	640
16	UNK				
17	GLU	56727	0.375	0.020	337
18	GLU	72476	0.375	0.020	431
19	ASN	56051	0.375	0.020	301
20	PHE	66229	0.375	0.020	350
21	LYS	141047	0.375	0.020	540
22	ALA	49754	0.375	0.020	286
23	LEU	53274	0.500	0.020	232
24	VAL	134406	0.500	0.020	570
25	LEU	95963	0.500	0.020	418
26	ILE	57200	0.500	0.020	231
27	ALA	28976	0.500	0.020	124
28	PHE	47196	0.500	0.020	187
29	ALA	56692	0.500	0.020	244
30	GLN	49284	0.500	0.020	212

Key : + ... Residue confirmed at 313 nm
? ..... Identification tentative
UNK ..... Not identified
(CM) ..... S-Carboxymethyl

Repetitive yield
VAL-8 to VAL-24 is
96.5 %

Repetitive yield
ALA-9 to ALA-27 is
89.6 %

Table 6.1(i) Amino acid sequence of Albumin Redhill. (Residues 1-30).

MINOR

Cycle	PTH	Area	Fraction applied	AUFS	p mole
1	ASP	66674	0.250	0.020	602
2	ALA	68871	0.250	0.020	593
3	HIS	37498	0.250	0.020	392
4	LYS	66390	0.250	0.020	381
5	SER *	6225	0.250	0.020	61
6	GLU	54495	0.250	0.020	487
7	VAL	48108	0.250	0.020	408
8	ALA	78397	0.375	0.020	450
9	HIS	25000	0.375	0.020	174
10	ARG	56769	0.375	0.020	418
11	PHE	50170	0.375	0.020	265
12	LYS	79114	0.375	0.020	303
13	ASP	49357	0.375	0.020	297
14	LEU	44983	0.375	0.020	261
15	GLY	35754	0.375	0.020	241
16	UNK				
17	UNK				
18	ASN	25217	0.375	0.020	135
19	PHE	23302	0.375	0.020	123
20	LYS	28139	0.375	0.020	107
21	ALA	21975	0.375	0.020	126
22	LEU	17312	0.375	0.020	100
23	VAL ?	27672	0.500	0.020	117
24	LEU ?	1	0.500	0.020	0
25	ILE	21584	0.500	0.020	87
26	ALA ?	1	0.500	0.020	0
27	PHE	20000	0.500	0.020	79
28	UNK				
29	GLN	28276	0.500	0.020	121
30	TYR ?	10076	0.500	0.020	45

Key : * ..... Residue confirmed at 313 nm
? ..... Identification tentative
UNK ..... Not identified
(CM) ..... S-Carboxymethyl

Repetitive yield
ALA-8 to ALA-21 is
90.7 %

Table 6.1(ii) Amino acid sequence of albumin A impurity. (Residues 1-30).

was still good (> 100 pmol) even at residues 27-28, and as might be expected the repetitive yield was 96.5% for residues 8-24 and 89.6% over residues 9 to 27. The later cycles therefore seemed to be proceeding at reduced yields, and sequencing further than 30 residues would probably have produced progressively more ambiguous results.

The residue 16, equivalent to residue 15 in albumin A, was a glycine residue in the minor component. It is possible that the solid phase coupling mechanism used may have been responsible for the inability to identify these three residues (one in Albumin Redhill and two in albumin A).

The yield of serine was comparatively poor compared to the other residues. Sequencing of serine and threonine residues often give poor results on Edman degradation.

The sequence of Albumin Redhill appears to be exactly the same as that of albumin A but with an extra arginine residue at the N-terminus. As the sequence was obtained twice and the assignments are unambiguous it appears that Albumin Redhill, residues 1-30, consists of an extra N-terminal arginine residue onto the normal albumin sequence. Albumin Redhill does not seem to have the modified hexapeptide proalbumin sequence found previously in Albumins Christchurch (Brennan and Carrell, 1978) and Lille (Abdo, *et al.*, 1981).

6.4 DISCUSSION

In the previously recorded N-terminal mutations of HSA mentioned above the hexapeptide proalbumin is left intact when mutations occur at residues -1 or -2. This has led to the suggestion that an arg-arg specific protease exists in the endoplasmic reticulum or Golgi apparatus which fails to cleave the mutant proalbumins in Albumins Christchurch and Lille (see Fig. 6.6). However, in Albumin Redhill only an N-terminal extra arginine is present. (Residue -1 of the proalbumin sequence is arg.) Thus, it would appear that Albumin Redhill is a new type of proalbumin variant in which five residues of the propeptide but not the final arg are cleaved from albumin.

It is interesting to note that an extra N-terminal residue, especially a positively charged residue, would disrupt the square planar arrangement of the divalent metal binding site, which would explain the defective Cu/Ni binding. Also, this would possibly explain why, although the N-terminal residue is arginine, no propeptide was removed on tryptic digestion; it may be that the extra arginine is poorly cleaved by trypsin to give a single amino acid and the "normal" Redhill albumin.

The sequence of normal human and bovine albumins is known to be asp-ala and that of rat serum albumin glu-ala. However, Leibowitz and Soffer (1971) have shown the existence of an arginylated albumin in commercial bovine serum albumin (BSA). The extra N-terminal arginine is

analogous to the present result but occurs in only ≤ 10% of the total albumin. The authors proposed that the extra amino acid is due to an acylation reaction catalysed by t-RNA protein transferase.

This may possibly be the case in BSA, as only ≤ 10% of the pooled BSA was affected, but this seems unlikely to be a valid explanation of Albumin Redhill as the variant form is ≈ 50% of the total serum albumin and occurs in two related individuals. Rather, it would seem to be that Albumin Redhill is caused by an inherited difference in N-terminal sequence. This may be due to:

(i) Lack of arg-arg specificity for the proalbumin cleavages. The sequence of the two previous proalbumins have resulted in the conclusion that the propeptide is cleaved by either an arg-arg specific protease, membrane bound in the Golgi apparatus, or endoplasmic reticulum. The sole arginine at residue -1 in Albumin Redhill may be due to a new earlier cleavage site being formed by residues -3 and -2. If one assumes that residue -2 is arginine, then on examination of the codon for the -3 residue it is not possible for mutation to an arginine residue to result from a single base alteration. However, leucine, valine, isoleucine, serine, tyrosine or cysteine can be produced by a single point mutation. Whether any of these residues together with arginine can give a suitable dipeptide cleavage site is unknown. There is no alternative cleavage site known for human albumin but in chicken serum albumin the propeptide

is quite different and has 7 residues ending in ala-arg (Hache, *et al.*, 1983). If this is also a recognised mutation site in humans then it may be that a combination of one of the above residues with arginine may also result in premature peptide cleavage and so give rise to proalbumin Redhill. This is, however, pure speculation at present.

(ii) It may be that due to a molecular defect in the cleavage enzyme the dipeptide arg-arg in Albumin Redhill is detected, but only the first arginyl residue is cleaved off with the rest of the propeptide. This would result in the extra N-terminal arginine seen in Albumin Redhill and the genetic fault may then lie not with the Albumin Redhill but with the cleavage enzyme of the patients.

(iii) It may be that acylation of 50% of the albumin has occurred, and that this is the cause of the extra residue in Albumin Redhill. However, it does seem rather unlikely that this hypothesis could explain such a high percentage of modification.

Whatever the reason, the sequence of Albumin Redhill describes a unique variant previously unreported, and as such is important for the understanding of albumin variants and their ligand binding properties. Albumin Redhill may be a unique type of variant and may or may not be due to an N-terminal mutation in the protein. The C-terminal mutation reported by Brand (1983) for Albumin Redhill has not been studied here and may also occur at the same time as the proalbumin mutation.

CHAPTER SEVEN

LIGAND BINDING PROPERTIES OF ALBUMIN VARIANTS

7.1.1 Introduction

The important role of albumin in the transport of ligands in the body has been outlined in Chapter 1 and qualitative differences in the binding of some ligands to albumin variants have been found (Section 1.4.7). Brand, *et al.* (1984) reported that Albumin Redhill displayed defective binding of nickel and copper ions and the ligand-binding properties of a range of dyes and other ligands have also been contrasted to normal albumin for those albumin variants available in this laboratory (Chapters 2 and 3).

7.1.2 Techniques Used for Measuring Ligand Binding

A wide range of techniques have been used to quantify the ligand binding properties of albumins. These techniques are similar to those in general use and while a brief outline of some of the methods will be given, this review is far from being exhaustive.

7.1.2.1 Equilibrium Dialysis

This is probably the most widely used technique for the measurement of ligand binding. It requires the ligand to be bound to be

dialysable but the protein to not be so. A solution of albumin is placed in a dialysis bag and suspended in a solution containing the ligand. The solution is allowed to attain equilibrium and the concentration of ligands inside and outside the dialysis bag is measured. If no ligand is bound the concentrations should be identical; however, if ligands are bound by the albumin then the concentration inside will be higher. By repeated experiments at varying concentrations a Scatchard analysis (1949) of the binding constant can be performed.

Though in widespread use in pharmacological studies, the process of equilibrium dialysis takes a long time to perform; however, by use of radio-labelled ligands analyses of the binding of a wide range of drugs and other molecules have been performed using this method.

7.1.2.2 Gel Filtration

This method for the analysis of ligand binding to proteins was devised by Hummel and Dreyer (1962) and is further outlined by Yoza (1977). A column of Sephadex, of sufficiently small pore size for the protein to be totally excluded, is equilibrated in buffer containing a known concentration of ligand. The protein, previously equilibrated in the ligand-containing buffer, is then applied and chromatography carried out. The amount of ligand in the eluate is monitored and is correlated

to the elution of the protein.

Although the protein is at equilibrium and so the interactions between protein and ligand are analogous to those in equilibrium dialysis, it has been shown that a Donnan effect occurs, due to the confinement of the macro-ion in the mobile phase resulting in a redistribution of small ions. However, Jordan, *et al.* (1974) devised a modification that corrects for the Donnan effect by increasing the amount of protein applied and obtaining a plateau rather than a peak for the concentration of bound ligand.

This technique is rapid and comparatively simple, however, possible errors due to the Donnan effect should be taken into account.

7.1.2.3 Electrophoresis of Bound Ligands

Tárnoky and colleagues (Curnow, *et al.*, 1978) introduced a series of simple dye binding tests with which to characterise variant albumins. These tests could be applied to other ligands that are bound to albumin and qualitative trends in ligand binding determined after electrophoretic separation of the variant and normal albumins. The method is qualitative and open to criticism (as the ligand is reversibly bound to the albumin and may dissociate on electrophoresis giving artefactual trends). However, it is quickly performed and is readily applicable to variant proteins, as it separates variants as well as measuring the amount of bound ligand to each. Cavalli-Sforza

et al. (1977) screened serum proteins for their affinities to a wide range of radioactive ligands by a similar procedure.

7.1.2.4 Fluorescence Quenching

The quenching of intrinsic protein fluorescence by the binding of a ligand to an adjacent site has been utilised with albumin to study the binding of thyroxine (Steiner, *et al.*, 1966) and bilirubin (Levine, 1977). The intrinsic fluorescence of albumins is due mainly to tryptophanyl residues. Conformational changes resulting from the binding of a ligand to an adjacent binding site causes a fractional reduction in the fluorescence, which can be related to the concentration of ligand bound and allowing a Scatchard analysis to be performed.

7.1.2.5 Spectroscopic Changes

The spectroscopic properties of labelled probes can be utilised in order to quantify their binding constants. Hsia, *et al.* (1982) used enantiomeric spin labels to quantify binding to the bilirubin binding site by electron spin resonance. Circular dichroism can also be used to measure binding by following the changes in the spectra of protein or ligand with sequential addition of ligand. Pfaff, *et al.* (1975) used a range of spectroscopic techniques to study the binding of bromosulphophthalein to albumin. The binding of bromocresol green to albumin has been

monitored by UV absorbance, and that of ANS by fluorescence (Reed, *et al.*, 1975).

7.2 EXPERIMENTAL

The binding of the divalent metal ions Cu^{II} and Ni^{II}, the drug warfarin, and the physiological ligands thyroxine and bilirubin, to variant albumins was studied by either gel filtration, electrophoresis or fluorescence quenching.

7.2.1 Metal Ion Binding

7.2.1.1 Removal of Trace Metal Ions from Serum

Sera or isolated double albumins, were purified from any contaminant heavy metal ions by chromatography on Chelex 100 (Bio Rad). A column (6 x 1 cm i.d.) of Chelex 100 equilibrated in sodium acetate (0.5 M, pH 6.5) was washed with 4-5 bed volumes of sodium acetate solution as above and then with 5 bed volumes of distilled water. The Chelex resin was thereby converted to the Na^+ form and serum (100 µl) or double albumins (1-10 mg) was applied in distilled water and eluted, collecting 1 ml fractions. The proteins were detected by spraying aliquots with ANS (as described in Section 4.3.2.3); the fractions were then pooled and lyophilised.

7.2.1.2 Electrophoresis on Cellulose Acetate (Brennan and Carrell, 1980)

Purified sera or double albumins (≈1mg) were

incubated with (^{63}Ni/nickel chloride, 1μCi, 300mCi/mmol., Amersham International) for 30 minutes at 37°C with a trace amount of bromophenol blue, and then applied to a cellulose acetate strip ([150 x 78 mm], Sartorius) and electrophoresis performed in veronal buffer as described in Section 3.2.4 at 8-10 V/cm C.V.

After electrophoresis the strip was thoroughly dried and incubated at room temperature with a sheet of LKB Ultrofilm in the dark for several days. The autoradiogram was developed using commercial developer and fixer solutions (Eastman-Kodak) and the cellulose acetate strip stained briefly in Ponceau S (as in Section 3.2.4) and destained in acetic acid (5% v/v in water). Electrophoresis of double albumins and normal albumins was performed in parallel for comparison .

7.2.1.3 Semi-quantitative Analysis

Electrophoresis on double Albumin Redhill and double Albumin Carlisle was performed as described in Section 7.2.1.2. However, only one albumin sample was run per strip and after electrophoresis two guide strips were cut, one at each side, and stained in Ponceau S as previously. Using the guide-strip the albumin peak was identified without staining and a series of fifteen 0.5 cm strips cut across the protein track encompassing the albumin band, and reference strips were also cut from the cathodic and anodic regions of the track. Each

strip was placed into a glass screw-cap scintillation vial which was subsequently filled with scintillation fluid (Optiphase (Fisons), 4 ml per vial) and counted on a Packard 2425 scintillation counter set to read in the ^{3}H window.

7.2.1.4 Gel Filtration on Sephadex (Yoza, 1977)

A more quantitative estimate of the nickel or copper binding to albumin A and double Albumins Redhill and Carlisle was made by the gel filtration method of Hummel and Dreyer (1962), as described by Yoza (1977).

A column of Sephadex G50 (46 x 1.6 cm i.d.) was equilibrated in 3 mg/L copper(II)nitrate (Spectrosol atomic absorbance grade, BDH Chemicals) in tris-HCl (0.01 M, pH 8.0). A sample of albumin (of known protein concentration by the method of Bradford,1976) was stripped of any contaminant Cu^{II} by passage through Chelex 100 (Section 7.2.1.1) and redissolved in the tris-Cu^{II} buffer (1-2 ml, for ≈ 3 mg albumin). The sample was applied to the column and eluted at a flow rate ≈ 40 ml/hr.

Fractions were collected (≈ 1-1.5 ml) and the absorbance at 280 nm monitored, and the concentration of Cu^{II} estimated by atomic absorbance on a Varian Techtron Atomic Absorbance Spectrophotometer using an oxidising flame (air:acetylene 7.5:3) at 325.1 nm,calibrating the copper concentration using a 0-10 µg/ml series of Cu^{II} solutions.

Similar experiments were performed using a nickel(II) chloride atomic absorbance grade solution (Spectrosol, BDH Chemicals) in tris-HCl buffer (0.01 M, pH 8.0) on a column of Sephadex G 50. Samples of albumin solutions, accurately measured by the method of Bradford (1976), were chromatographed and the absorbance at 280 nm monitored as before. Atomic absorbance was used to measure the concentration of Ni^{II} in the fractions collected using an oxidising flame (air:acetylene 8:3.5) and monitoring absorbance at 232.2 nm. A standard curve for solutions of 0-10 µg/ml Ni^{II} was constructed as before.

7.2.2 Thyroxine Binding

The binding of thyroxine to albumin was performed essentially as described in Section 7.2.1.2. ^{125}I-labelled thyroxine (0.1 µCi, 40 mCi/mmol. Amersham International) was incubated with each albumin (1 mg in 30 µl water) for 12 hours at room temperature before loading onto a cellulose acetate strip; electrophoresis was then carried out at 10 V/cm for 2.5 hours in 0.075 M sodium veronal buffer pH 8.6. The strips were dried, stained in Ponceau S as described previously, and the albumin bands excised together with anodic and cathodic strips to act as controls. The strip, cathodic to albumin A, equivalent to the slow alloalbumin, was excised from the normal albumin

runs. A similar pattern of strips was excised for double Albumins Warwick-1, Warwick-2, and double Albumin Redhill, excising the normal and variant albumins and anodic and cathodic strips as controls. A strip was excised cathodic to each albumin at the approximate migration distance of the β-globins in order to ascertain the binding to any specific thyroid binding protein still present. Excised bands were placed in plastic scintillation vials and counted on an LKB 1280 Ultrogamma counter.

7.2.3 Warfarin Binding

The binding of warfarin to albumin A and Albumins Carlisle and Redhill was measured by gel filtration essentially as described by Sun, *et al.* (1984) except by conventional chromatography rather than by HPLC.

A column of Sephadex G25 (40 x 1 cm i.d.) was equilibrated in sodium phosphate buffer (0.2 M) containing warfarin (27 mg/L). Albumin samples (of known concentration ≈ 3 mg) were dissolved in the warfarin buffer and loaded onto the column. Elution was at ≈ 0.5 ml/min and the fractions collected (≈ 1 ml/test tube) were monitored at 280 nm and 313 nm and the resultant profiles plotted. Calculations of ligand bound were made using the method outlined by Yoza (1978).

7.2.4 Bilirubin Binding

The binding of bilirubin to albumin A and to double Albumin Carlisle was compared by the method of Levine (1977). Bilirubin was used as received from Sigma or purified by the method of Fog (1964) by chromatography on anhydrous sodium sulphate. It was dissolved at a concentration of 20 μM in sodium phosphate buffer (125 mM, pH 7.4) just before use and was kept wrapped in foil.

Aliquots (2-10 μl) of the bilirubin solution were added to the albumin (2 ml of a known concentration ≈ 1 μM in sodium phosphate buffer 125 mM, pH 7.4) and the cell inverted several times before reading the fluorescence. Readings were taken on a Perkin Elmer fluorescence spectrophotometer, exciting at 294 nm and monitoring emission at 343 nm. Readings were performed in a darkened room to reduce photo-isomerisation of the bilirubin.

The degree of fluorescence quenching increased to a limiting value after which no further drop in fluorescence was observed. Similar experiments were performed for albumin A and double Albumin Carlisle, and the percentage fluorescence plotted against the molar ratio of bilirubin:albumin. Absorbance at 294 and 343 nm was noted at the end of each experiment in order to assess the inner filter effect.

7.2.5 Binding of ^{14}C-palmitate

The binding of fatty acids to HSA and variant albumins was assessed for Albumin Carlisle and Warwick-1, by electrophoresis using ^{14}C-palmitate (Amersham International). Serum (30 µl) was incubated with ^{14}C-palmitate (50mCi/mmol, 1 µl, 0.1 µCi) or double albumin (4 mg/with 25 µl) 2.5 µCi) for 30 minutes at 37°C. The samples were then loaded onto a cellulose acetate strip together with a trace of bromophenol blue to act as a marker.

Electrophoresis was carried out for 3-4 at 8-10 V/cm and after removing the strip it was air-dried and incubated with a strip of LKB Ultrofilm for autoradiography photographic film, and developed for several days in the dark. To prevent adhesion of any moist parts of the strip to the photographic film a thin layer of cling-film was inserted between them. After 7-14 days incubation, the film was developed and the strip stained in Ponceau S as described in Section 7.2.1.2. The two were re-aligned (most easily performed by marking a notch in the two while still clamped together) and the areas of exposed film compared to the bands stained by the Ponceau S dye on the cellulose acetate strip.

7.3 RESULTS

7.3.1 Metal Ion Binding

Serum samples were freed of contaminant metal ions after passage throgh a Chelex-100 column

and fractions (≈ 1 ml) collected and checked for protein content using the ANS method previously described (Section 4.3.2.3. The protein containing fractions were lyophilised and resuspended and used for the ^{63}Ni binding experiment.

After development of the autoradiograms both Albumin Warwick-1 and Albumin Carlisle bound the same amount of isotope as albumin A from the optical density of the exposed film. Both variant albumins showed strong exposed bands as well as the albumin A in each run, and HSA from normal serum also bound Ni^{63} in each experiment (Fig. 7.1).

The estimation of Ni^{63} binding by this method is only qualitative but supports the evidence already accumulated (in this work and in Au, 1982), that Albumins Carlisle and Warwick-1 are not pro-albumins and have no N-terminal mutations that would prevent Ni^{II} binding to them.

Cellulose acetate electrophoresis in the presence of Ni^{63} was also used to quantify Ni^{63} bound to Albumin Redhill and Albumin Carlisle. After identical incubations Ni^{63}-labelled double Albumins Carlisle and Redhill and normal HSA were subjected to electrophoresis so as to obtain maximal separation of the albumins. Each cellulose acetate strip was cut into strips widthways and each strip counted using a scintillation counter. The profiles of the albumins are given in Fig. 7.2. The areas correspond-

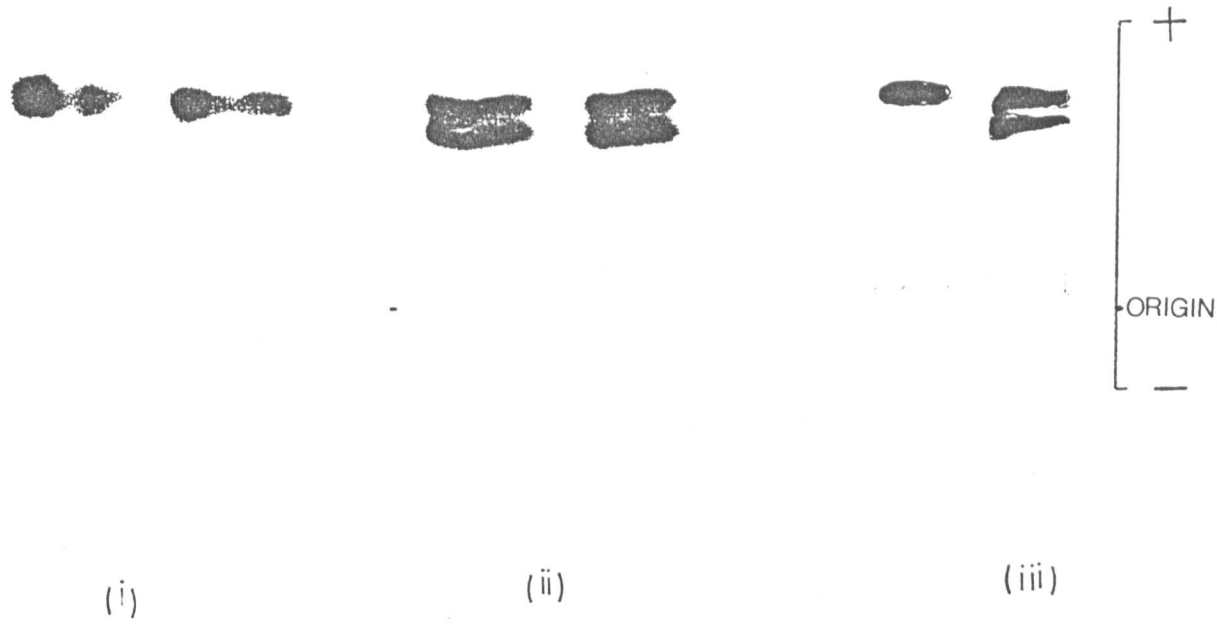


Fig. 7.1(i) Nickel Binding: autoradiogram: (i) normal serum, (ii) double Albumin Warwick-1, (iii) double Albumin Carlisle.

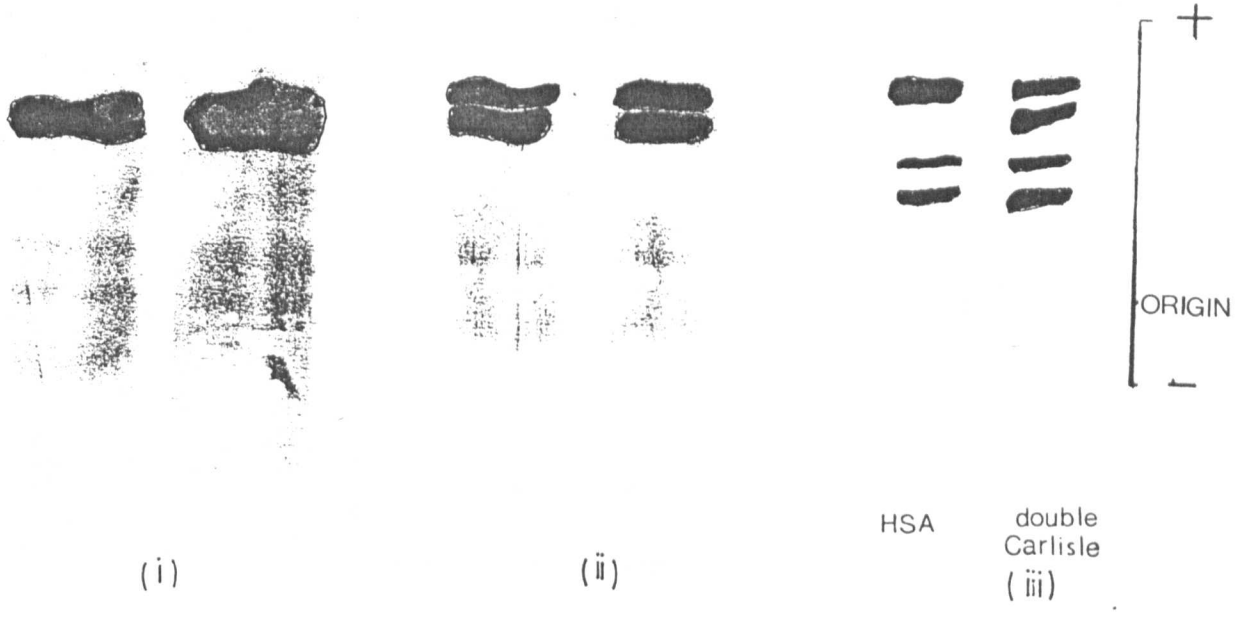
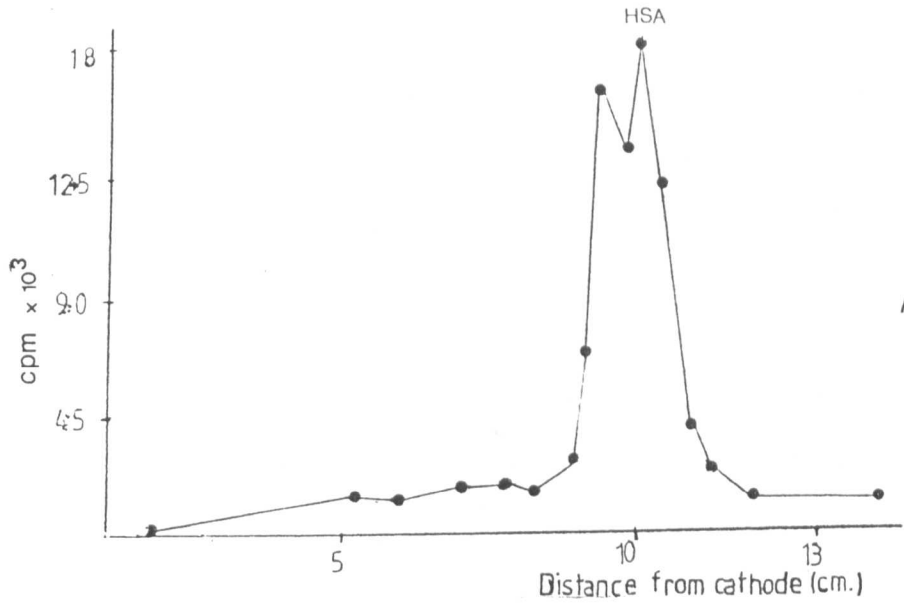
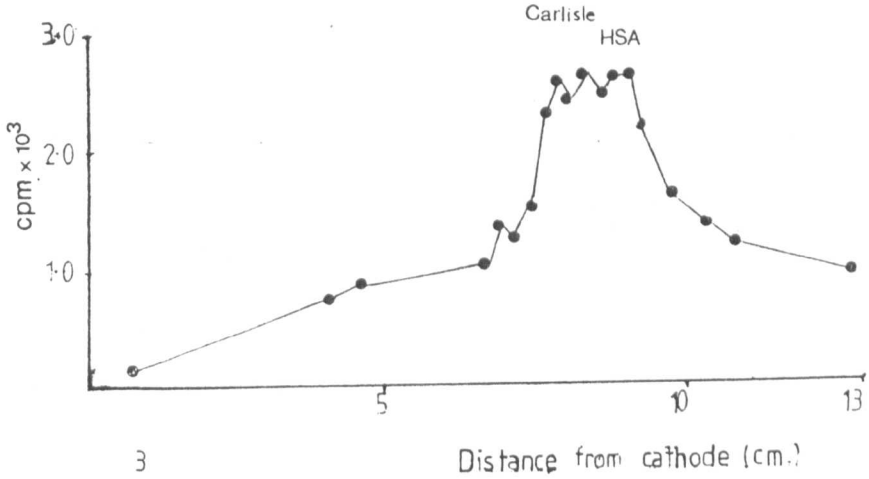


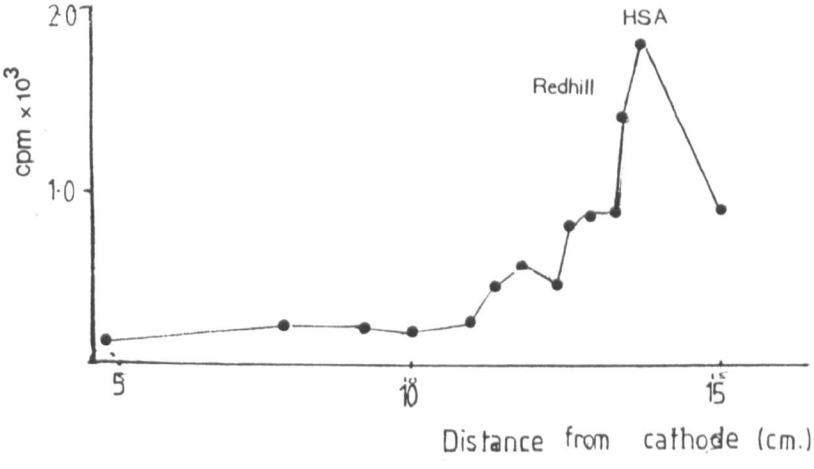
Fig. 7.1(ii) Nickel Binding: cellulose acetate electrophoretogram: (i) normal serum, (ii) double Albumin Warwick-1, (iii) double Albumin Carlisle.



ALBUMIN A



DOUBLE ALBUMIN
CARLISLE



DOUBLE ALBUMIN
REDHILL

Fig. 7.2 Profiles of the binding of ^{63}Ni to normal and variant albumins, separated by cellulose acetate electrophoresis.

ing to each albumin band were approximated and the binding was found to be essentially 1:1. However, duplicate experiments on double Albumin Redhill showed that Albumin Redhill bound only 40-50% of the $^{63}Ni^{II}$ bound by albumin A. These values differ from the results of Brand (1983) which by autoradiography showed no binding of $^{63}Ni^{II}$ to Albumin Redhill on cellulose acetate electrophoresis.

Gel filtration studies on albumins were carried out in order to characterise further the binding of Ni^{II} and Cu^{II} to variant and normal albumins.

Absorbance at 280 nm was used to monitor protein concentration, and atomic absorbance used to monitor the Cu^{II} or Ni^{II} concentrations. Fig. 7.3 shows a typical elution profile, the metal ion concentration rising with the elution of the albumin and dropping to form a trough afterwards. The peak area is correlated to the metal ion concentration bound to albumin and the trough area to the amount of metal ion removed from solution by binding to albumin.

The ratios of Ni^{II} and Cu^{II} bound to normal albumin and albumin variants assessed by these experiments is summarised in Table 7.1.

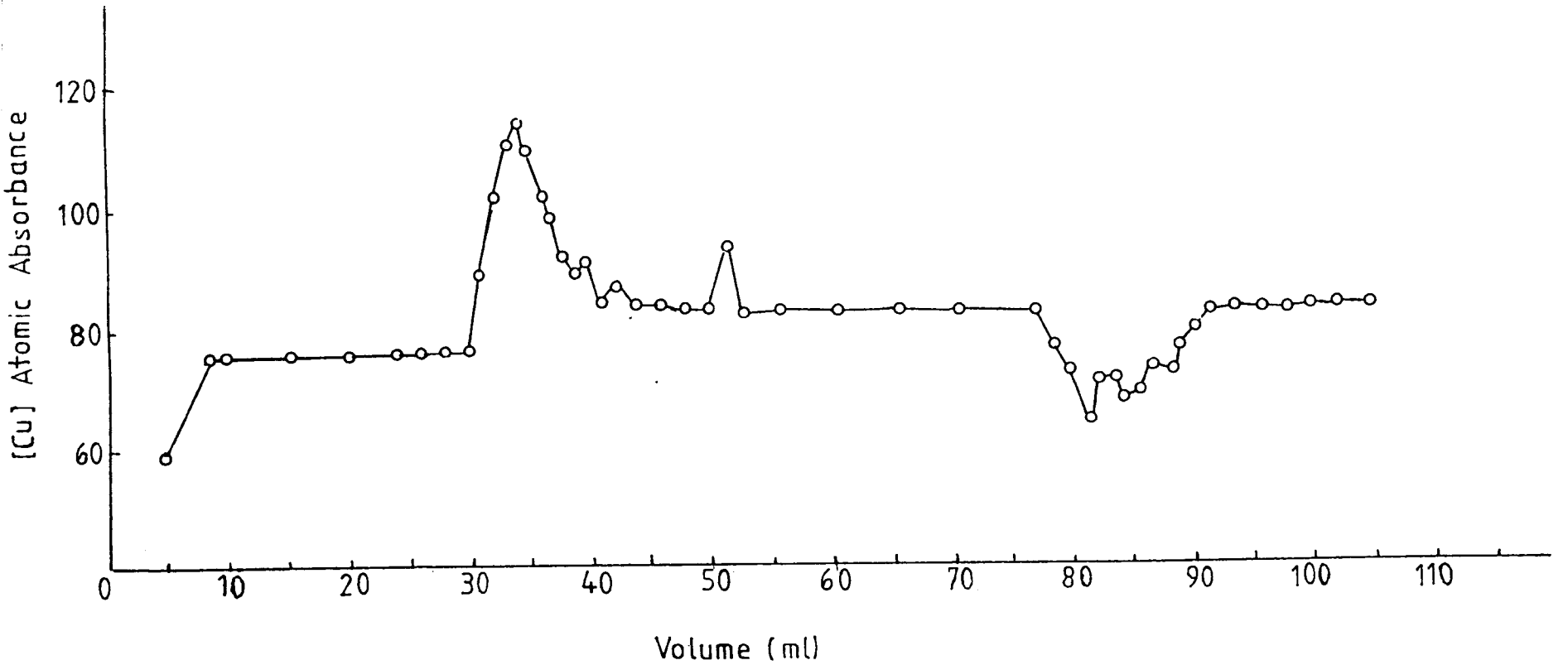


Fig. 7.3 Copper binding: atomic absorbance spectroscopy of albumin A chromatographed on Sephadex G50.

Copper Binding

Albumin A:	1.0
Double Albumin Warwick-1:	1.32
Double Albumin Carlisle:	0.77
Double Albumin Redhill:	0.39

Nickel Binding

Albumin A:	1.0
Double Albumin Carlisle:	1.52
Double Albumin Redhill:	0.60

TABLE 7.1 Ratios of metal ion binding to albumin variants by gel filtration.

The binding of Cu^{II} to double Albumin Redhill appears to be reduced to 30-50% of that for albumin A, suggesting that the binding to Albumin Redhill is negligible if one takes into account the 50% normal albumin present. However, results for the binding of Ni^{II} were more variable indicating that the binding to Albumin Redhill was ≈40% of that to albumin A. The wide variation in the results was perhaps due to the presence of varying amounts of dimeric albumin in different Redhill preparations. Another factor may be the rather unstable baseline in some experiments, as the measurement of Ni^{II} was near to the lower practical limit of detection.

The binding of Cu^{II} to double Albumin Carlisle was approximately the same as that for albumin A

(80-96%) and the binding of Ni^{II} was in fact greater than that for albumin A (1.52:1) though this may well be due to the factors already cited as possible sources of error in the Albumin Redhill determination. The binding of Cu^{II} to double Albumin Warwick-1 was approximately equal or slightly higher than that for albumin A (W1:A 1.32:1.0).

From the comparison of these results to those already obtained by the electrophoretic methods it would seem that the binding of Cu^{II} and Ni^{II} is normal for Albumin Carlisle and Albumin Warwick-1 but that they are reduced considerably for the binding to Albumin Redhill. Brand (1983) found a reduced Cu^{II} binding (≃ 28% of that for albumin A) and severely reduced Ni^{II} binding. These trends are consistent with the findings presented here with Cu^{II} binding considerably reduced for Albumin Redhill and also the Ni^{II} binding being substantially lower.

The effect of reduced binding on the Cu^{II} and Ni^{II} ion metabolism in patients with Albumin Redhill has not been studied; however, such studies may well be of physiological interest.

The reduction in the Ni^{II} and Cu^{II} binding by Albumin Redhill is most likely the result of the extra N-terminal arginine residue which may cause distortion of the square-planar binding configuration of the heavy metal ion binding site. The presence of a positively charged arginine residue may well

also cause charge repulsion of the cation and so result in the reduced binding.

7.3.2 Thyroxine Binding

The binding of thyroxine to Albumins Redhill, Warwick-1 and Warwick-2 was assessed by cellulose acetate electrophoresis, followed by gamma radiation counting.

The distribution of radioactivity over the electrophoretic tracks was determined. The double albumins were visualised by Ponceau S staining and the normal and abnormal albumins excised as strips together with four more cathodic strips in order to assess binding of thyroxine to the β-globulin thyroxine carrier, or albumin dimers.

Strips were counted and the background subtracted from the corrected c.p.m. The resultant ratios of normal:variant binding obtained in a series of experiments on each double albumin were calculated and are tabulated below (Table 7.2). Corrections for the ratio of normal:abnormal albumins in sera were made. The experiments were performed prior to receipt of Albumin Carlisle and so thyroxine binding studies have not been performed on it.

The binding of thyroxine to the albumin variants studied here indicates a generally increased binding by the variants up to twice that of albumin A. Whether this is a real increase or an artefact

TABLE 7.2

BINDING OF ^{125}I-THYROXINE TO ALBUMIN VARIANTS

Albumin Warwick-1 (raw data 10 experiments)	W1:A =	1.94:1 ± 1.59
(corrected (-2 lowest and highest values))	W1:A =	1.42:1 ± 0.43
Albumin Warwick-2 (2 experiments)	W2:A =	1.26 - 1.28:1
Albumin Redhill (3 observations)		1.96:1.0 ± 0.98

Rh:W1:W2:A = 1.96:1.94:1.27:1.0

of the electrophoresis can not be stated with complete confidence. However, in an artefact one would expect thyroxine to stream to the anode if at all and so give reduced binding by the variants, and not the increased binding seen. Others have reported increased thyroxine binding to albumin variants including Lalloz, *et al.* (1983) who reported a variant with thirty times increased thyroxine binding. The variants are all of the slow type, and it may be that the net decrease in negative charge encourages binding of the negatively charged thyroxine to the albumin.

Lalloz, *et al.* (1985) have reported a silent albumin variant solely on the basis of its different thyroxine binding. This would suggest that an electrophoretically discernable change in net charge is not necessary for thyroxine binding to be affected. The one patient described by Lalloz, *et al.* (1985) may have suffered from hyperthyroxemia and in each of the three cases known to them the affinity for thyroxine and tri-iodothyroxine was considerably increased.

The differences in thyroxine binding to albumin variants reported here are unlikely to have physiological effects, considering the fact that albumin has merely an excess thyroxine binding role, and two more specific carrier proteins occur in serum.

7.3.3. Warfarin Binding

The binding of warfarin to double Albumins

Carlisle and Redhill was compared to that for HSA (Sigma). Each sample was defatted and the concentration measured before loading onto the column. Elution profiles were constructed at 313 nm to follow the elution of warfarin and at 280 nm for that of the protein.

The binding of warfarin to each albumin is summarised below in Table 7.3.

Table 7.3 The Binding of Warfarin to Albumin Variants

Albumin	Ratio Warfarin:Albumin bound
HSA	0.695:1.0
HSA	0.69:1.0
Double Albumin Redhill	0.532:1.0
Double Albumin Carlisle	1.81:1.0

The results indicate that Albumin Carlisle binds more warfarin than normal albumin but that Albumin Redhill binds slightly less. These results must be viewed with caution, however, and represent merely initial trends as there are several possible sources of error:

(i) The absorbance of the baseline is very large compared to that of the peak, making assessment of the area of the warfarin-albumin complex difficult. Further experiments with more albumin would be required to give more conclusive results.

(ii) The method makes no allowances for Donnan effects on the column. Comparison of the K_a values obtained to those published by Sun, *et al.*, 1984, which are of the order of 10^5 to 10^6 for the primary binding site and 10^3-10^4 for the secondary, would seem to indicate that the method used here is approximating the two sites together or that the primary site is still blocked. It is not possible to quantitatively study the binding of warfarin by a single experiment; sufficient points to construct a Scatchard (1949) plot are needed. However, this was not possible at the time due to the substantial amount of protein required for such a study and hence only the qualitative trends can be reported. Albumin B has been shown to have increased warfarin binding (Wilding, *et al.*, 1977) and Albumins Naskapi and Mexico to have reduced binding. In the light of the importance of warfarin-type drugs in the treatment of thrombosis and related conditions, a more complete study of the role of variant albumins in controlling serum levels of warfarin may well yield important results.

7.3.4 Bilirubin Binding

The quenching of albumin solutions with bilirubin was followed over a bilirubin:albumin molar ratio of 0:1 to 1:1. Quenching was completed by ≈ 0.6:1.0 molar ratio of bilirubin:albumin. No improvement in the extent of quenching was achieved, despite vigorous defatting of the albumin and purification of the bilirubin (Fog, 1964).

This may be indicative of some tightly-bound endogenous ligand still being present. Despite this problem quench curves for double Albumin Carlisle and albumin A were compared in several experiments, and it seemed to be a consistent trend that the initial quenching of albumin A, proportional to the first mole of bilirubin binding at the primary binding site, was higher than for Albumin Carlisle. This seemed to indicate a tighter binding affinity for normal albumin than Albumin Carlisle at this site.

The secondary bilirubin sites represented lower down the quench curve also showed some reduced affinity in samples of Albumin Carlisle (Fig. 7.4).

As it has been thought that the primary bilirubin site and that of warfarin are equivalent or in close proximity the results may seem contradictory for those two ligands, with increased warfarin binding but reduced bilirubin affinity. However, if the primary bilirubin site was inaccessible it may be that the binding studies are revealing the properties of lower affinity sites. It is possible that other studies using the peroxidase method (Brodersen, 1974) may be of value in assessing these results.

7.3.5 Palmitate Binding

Electrophoresis on cellulose acetate was used to qualitatively assess the binding of palmitate, labelled with ^{14}C, to the albumin types. Both Albumin

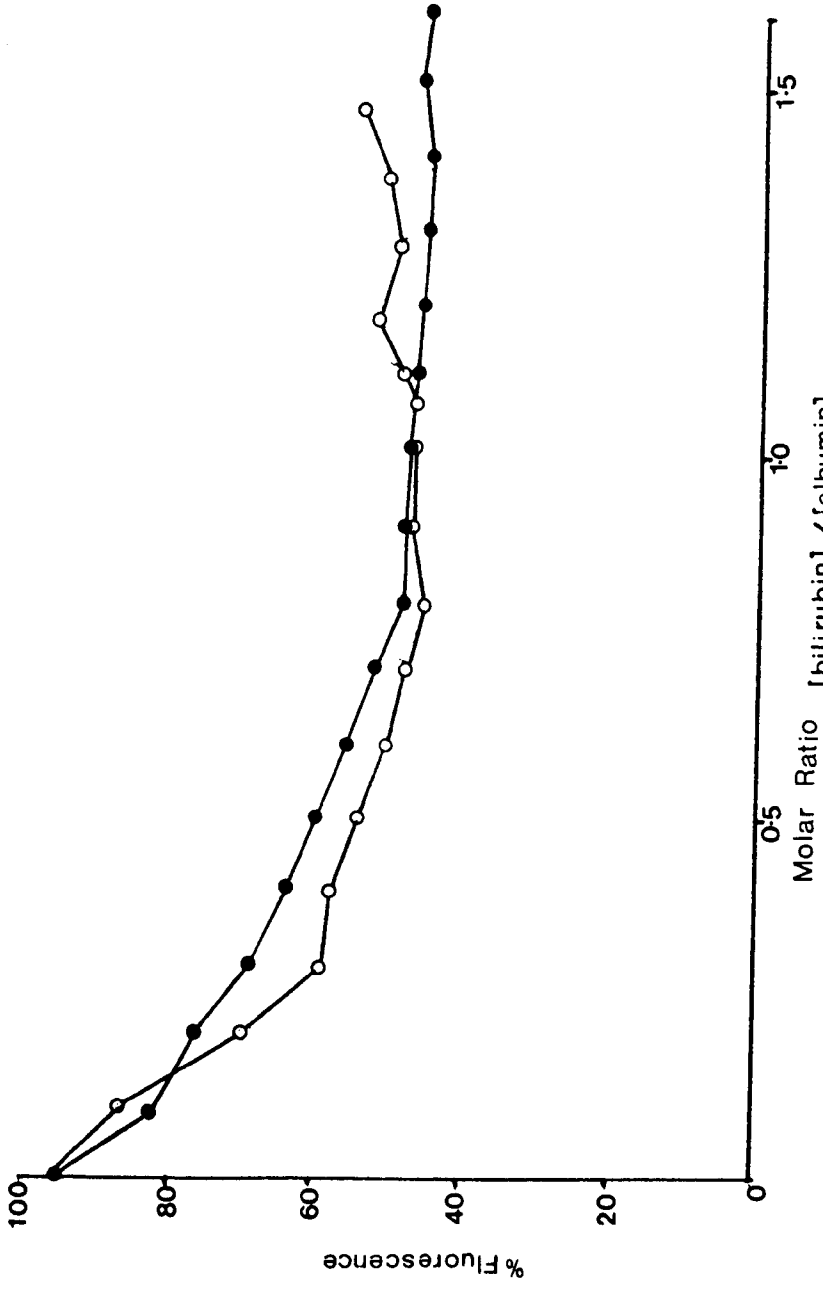


Fig. 7.4 Bilirubin binding: Fluorescence quenching curves for HSA (-o-o-) and double Albumin Carlisle (-●-●-●-).

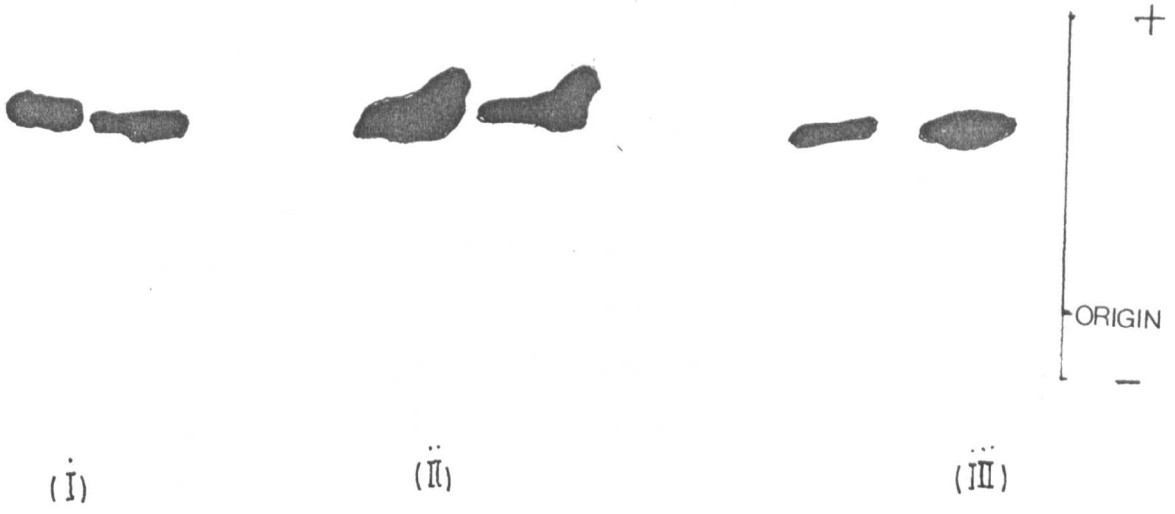


Fig. 7.5(i) Palmitate Binding: autoradiogram, (i) normal serum, (ii) double Albumin Warwick-1, (iii) double Albumin Carlisle.

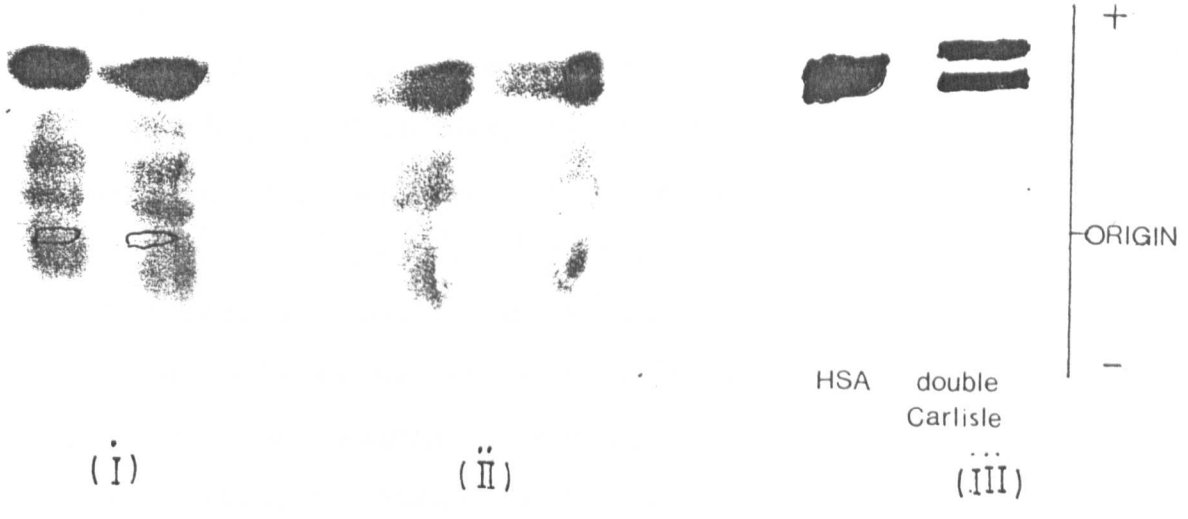


Fig. 7.5(ii) Palmitate Binding: cellulose acetate electrophoretogram, (i) normal serum, (ii) double Albumin Warwick-1, (iii) double Albumin Carlisle.

Warwick-1 and Albumin Carlisle showed two bands of equal intensity on autoradiography (Fig. 7.5). This suggests that both albumin variants have in broad terms similar fatty acid binding properties to that of albumin A which is consistent with no mutation having occurred at the C-terminal fatty acid binding site.' However, the results are only qualitative and as there are so many sites for weak fatty acid interactions (up to 60 (Spector, 1975)) a mutation causing a reduction in one of these sites would not be detected by this method.

If time had allowed, a quantitative binding study, such as that described by Spector and Fletcher (1977), would have been performed to investigate any such mutation.

7.4 CONCLUSION

The binding properties of Albumin Warwick-1 recorded here are consistent with its assignment as a non-N-terminal albumin variant with essentially normal Ni^{II}/Cu^{II} and fatty acid binding. The thyroxine binding, although nearly twice that of normal albumin, would probably be of little physiological consequence, as the role of albumin is only as an over-flow carrier for this ligand. The results obtained for other Indian variants have failed to show any gross ligand-binding deficiencies and this is also true for Albumin Warwick-1,

and so its inclusion with them as an Indian albumin variant seems justifiable.

The binding of metal ions to Albumin Redhill, initially shown to be defective by Brand (1983), has been confirmed in these studies. The ligand binding to double albumin has been reduced to ≤ 50% of the normal and this is consistent with metal ion binding to Albumin Redhill being largely deficient. However, less specific divalent metal binding sites exist and it may well be that although the N-terminal binding site is disrupted these lower affinity sites still do bind some Cu^{II}/Ni^{II}, and may explain why affinity chromatography on immobilised Ni^{II} was unable to separate the variant and normal albumins.

The sequence data (Chapter 6) lends considerable support to the hypothesis that the primary metal ion binding site is non-functional in Albumin Redhill. The incorporation of a positively charged N-terminal residue would disrupt not only the configuration but also the charge interactions in such a site.

Thyroxine binding in Albumin Redhill is elevated although the molecular explanation of this result must await further evaluation. The increased binding is not of the order noticed in the variants reported by Lalloz, *et al.* (1983, 1985) and

presumably has little if any physiological effect.

The binding of warfarin to Albumin Redhill may be slightly reduced from gel filtration experimental results and both this and the thyroxine binding differences may be a consequence of the second mutation site proposed by Brand (1983) in the C-terminal half of the molecule and thought to be a possible region of peptide duplication. As no molecular evidence for such a mutation exists any comments on the reason for these ligand binding changes must be purely speculative. It may be that as thyroxine is a hydrophobic ligand, an increase in the length of a hydrophobic pocket in the albumin molecule could increase its binding. Similarly, if a duplication of sequence has occurred it may have altered the conformation or environment at the warfarin binding site and so disrupted binding of this ligand.

Albumin Carlisle appears to bind metal ions normally, and to have qualitatively similar palmitate binding to normal albumin. A slightly decreased bilirubin binding but increased affinity for warfarin compared to normal albumin have also been shown. Albumin Yanomama-2 and possibly other polymorphic variants display reduced affinity for bilirubin, as does Albumins Redhill and Warwick-2 (Brand, 1983). Therefore, little evidence may be obtained for the classification of Albumin Carlisle from this result. The increased binding of warfarin noticed for Albumin Carlisle may be more informative.

Wilding, *et al.* (1977) have shown that albumin B has increased warfarin binding but that Albumins Naskapi and Mexico show reduced affinity. Thus in this respect Albumin Carlisle shows some similarity to albumin B. The evidence of Chapter 5, describing the probable site of mutation in Albumin Carlisle, may suggest a similar area of mutation to that in albumin B. However, further studies are necessary to locate the precise site of the mutation in Albumin Carlisle before it can be considered to be analogous to the variant described by Winter, *et al.* (1972), especially in the light of the electrophoretic heterogeneity occurring within the group of variants classed as albumin B by Ott, *et al.* (1975).

APPENDIX

Special equipment and chemicals were purchased from the suppliers listed below. All other reagents were readily available.

Chromatographic Materials

Sephadex gel filtration, chromatofocusing, ion-exchange and affinity column materials were purchased from Pharmacia Ltd., Milton Keynes, U.K. FPLC equipment was also from Pharmacia. Chelex-100 and hydroxyapatite HTP were from Bio Rad Laboratories Ltd., Watford, Herts, U.K. Filter papers, DE52 cellulose anion exchange media and the Partisil SAX anion exchange HPLC column were from Whatman Laboratory Sales Ltd., Maidstone, Kent, U.K.

HPLC grade solvents were purchased from Fisons plc, Loughborough, U.K. Filters for HPLC solvents were from Millipore UK Ltd., Harrow, Middlesex, U.K., and filtration/degassing apparatus from Whatman. The LKB HPLC system was from LKB Instruments Ltd., Hartford, Cheshire, U.K.

Polyamide t.l.c. plates were prepared by the Cheng Chin Trading Co and obtained through BDH Chemicals Ltd., Poole, Dorset, U.K. Avicell microcrystalline cellulose and pre-made silica G60

t.l.c. plates were made by Merck and were also obtained through BDH.

Electrophoretic Materials

Acrylamide, N,N'-methylene bisacrylamide and sodium dodecyl sulphate were of Electron grade and were purchased from BDH. IEF pre-cast PAG-plates were from LKB and an LKB Multiphor apparatus and power supply were used. The IEF pI standards kit and Pharmalyte were from Pharmacia and the molecular weight markers for SDS-PAGE were from BDH. Cellulose acetate strips were purchased from Sartorious Instruments Ltd., Belmont, Surrey, U.K.

Reagents and Miscellaneous Equipment

HSA was purchased from Sigma Fine Chemicals Ltd., Poole, Dorset, U.K. TPCK-treated trypsin, papain, α-chymotrypsin, and pepsin were also from Sigma as were the cyanogen bromide and o-iodosobenzoic acid. Carboxypeptidases and additional pepsin were obtained from Worthington Diagnostic Systems Inc., Freehold, New Jersey, U.S.A., BNPS-skatole was purchased from Fluka, Fluorochem Ltd., Glossop, Derbyshire, U.K. and N-chloro-succinimide from Aldrich Fine Chemicals, Poole, Dorset, U.K. Rabbit anti-human albumin anti-serum was purchased from Miles Laboratories Ltd., Slough, U.K.

Radio-labelled ligands (see Chapter Seven) were obtained from Amersham International plc, Amersham, Bucks., U.K. Centricon micro-concentration apparatus was supplied by Amicon Ltd., Stonehouse, Gloucs., U.K. Atomic absorbance readings were made using a Varian Techtron atomic absorbance spectrophotometer. Photographic chemicals were obtained from Kodak Ltd., Kirkby, Liverpool, U.K.

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