COMPARATIVE STUDIES OF BOVINE

SERUM TRANSFERRINS

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SUMMARY

Bovine serum transferrins exhibit considerable genetic variation, and each homozygous variant has complex heterogeneity, and the present studies throw light on the nature of the genetic variation and of this heterogeneity.

Homozygous variants A and D₂D₂ have been isolated by gentle procedures. These involve ammonium sulphate fractional precipitation and chromatography on DEAE Sephadex A-50 at pH 7.10, under conditions chosen to maintain maximum iron-III binding, but to minimize conformational shifts and interchange reactions. Complex starch gel electrophoretic patterns have been obtained in a tris-cacodylic acid system of optimum resolution at pH 7.5.

It is concluded that each variant consists of four major and two minor components and these have been isolated. The major components have been designated I-IV in order of increasing mobility.

Differences between components I of A and D₂D₂ have been shown to be at least: 2 amino acid substitutions (A:D₂; Glu:Asp and Lys:Arg) and one deletion (A:D₂; Asp: -), by tryptic and chromatryptic peptide mapping, and of tryptic maps of cyanogen bromide fragments.

No differences have been detected between the carbohydrate compositions of the whole protein. Both A and D₂D₂ contain: 2 mannose, 2 galactose, 4-glucosamine and ca. 2 sialic acid residues. There are two sequences for the linkage of carbohydrate:

-Asn(CHO)-Ser-Ser-Leu-Cya and -Arg-Asn(CHO)-Ala-Thr-Tyr.
The origin of the heterogeneity of a single variant has been studied. The difference between components I and III and between II and IV is explained in terms of difference in number of sialic acid residues (III:I = 3.2 residues).

However, components I and II have the same carbohydrate composition and their carbohydrate moieties are linked in the same way to their respective polypeptide chains. Components I and II have shown different behaviour on urea starch gel electrophoresis after cleavage of disulphide bonds. Components I and III give one band (anodic) whereas II and IV give two bands (one anodic, one cathodic). On the basis of this and additional studies it is concluded that the presence of an internal cleavage in the polypeptide chain of components II and IV results in the production of two fragments on the cleavage of the disulphide bonds. The possible origin of the fragments and significance are discussed.
The work described in this thesis was carried out in the Department of Physical Biochemistry in the John Curtin School of Medical Research, Australian National University, between June 1973 and September 1976. This work was performed entirely by myself, except for a part of the amino acid analyses which were carried out by Dr. D.C. Shaw and Mrs. N. Aldrich. Details of these analyses are as follows: Tables 4.1, 4.2, 4.3, 4.4, 5.4, 5.5, 6.1 and 6.2.
ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor, Dr. H.A. McKenzie, for his valuable guidance and supervision, and the amount of time he devoted throughout the course to this work. It would have been impossible to carry out the work without his continuous encouragement.

I am also grateful to Dr. D.C. Shaw for his guidance and helpful discussions during work on peptide analysis, and to Mrs. N. Aldrich for carrying out the amino acid analysis.

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Finally I would like to thank Mrs. J. Mahon for skill and patience in typing this thesis.
PREFACE

Throughout this thesis, each Figure and Table is presented on a separate page and follows the page of text on which the first reference to it has been made.

The following abbreviations have been used throughout this thesis:

- TfA: Bovine serum transferrin A
- TfD₂: Bovine serum transferrin D₂
- TfAI: Bovine serum transferrin A component I
- TfD₂I: Bovine serum transferrin D₂ component I
- DEAE-: Diethylamino ethyl-
- CM-: Carboxymethyl-
- Tris: Tris(hydroxymethyl)aminomethane
- Dansyl(DNS): 5-Dimethylamino-naphthalene sulphonyl

Amino Acids The amino acid abbreviations recommended by IUPAC-IUB (1966) have been used.
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CHAPTER 1. GENERAL INTRODUCTION

1.1 HISTORICAL

Transferrin. There are 3 to 5 grams of iron in adult man. Very little of this iron is present as free iron (II) or iron (III), most of it being bound to several kinds of protein molecules. According to Granick (1954), 65-75% of the iron is present in haemoglobin of red cells; 10-15% occurs as part of various other tissue proteins, such as cytochromes, myoglobins and catalases; 0.1% is present in plasma; and 15% is stored, mainly in the liver as the combined form with ferritin. However, a somewhat higher value (35%) for iron bound with ferritin and a lower value (55%) for iron in red cells were reported by Laurell (1947).

The presence of non-haem iron in the plasma fraction of a horse was first reported in 1925 by Fontés and Thivolle. This observation was subsequently confirmed by Barkan (1927) and Warburg (1927). Barkan (1927) and Vahlquist (1941) individually reported that serum iron is non-dialysable over the pH range from 4.5 to at least 10, indicating iron-protein complex formation. Dialysis experiments in vivo also supported the view that serum iron is bound to proteins (Vahlquist, 1941). The serum iron was found to be converted to a dialysable form by acid (Barkan, 1927). The association of the bound iron fraction with the globulin fraction was established with serum to which iron had not been added (Barkan and Schales, 1937). Schade and
Caroline (1946) showed that Cohn fraction IV-3,4 binds iron (III). Surgenor et al. (1949) subsequently showed that iron was bound to the more highly purified $\beta_1$ globulin in Fraction IV-7.

The study of the mechanism of iron-binding was initiated by Holmberg and Laurell (1945), and Schade and Caroline (1946), who demonstrated that formation of the iron (III)-protein complex gives rise to a characteristic reddish colour with maximum light absorption at 460-470 nm, and that the iron (III)-protein interaction is stoichiometric with the excess iron making no observable contribution to the colour. Later Laurell and Ingelman (1947) showed that two atoms of iron are bound per molecule of transferrin and the binding is highly specific.

Cohn (1947) found that the iron binding $\beta_1$-globulin in serum binds copper and possibly zinc as well as iron and confirmed that this globulin is responsible for the transport of these metals. He called the protein, $\beta_1$-metal-binding protein, because of its ability to bind several species of metal. The name transferrin was first applied by Holmberg and Laurell (1947) to the iron binding protein of swine serum and has been accepted widely.

Lactoferrin (Lactotransferrin). Milk also contains iron binding proteins. Since Sørensen and Sørensen (1939) described a reddish coloured protein fraction in cow milk, several workers have reported the presence and the isolation of a red protein from cow milk (Polis and Shmukler; 1953, Groves, 1960). It has been, however, suggested (Hanson, 1959),
but not always accepted (Gulgler and Muralt, 1959), that serum transferrin occurs also in milk. However, Derechin and Johnson (1962) showed that the red protein fraction consists of two types of iron-binding components: a protein homologous to serum transferrin and a major component absent in plasma.

The presence of iron-binding proteins in human milk was first described by Johansson (1958) and isolated by Montreuil et al. (1960), and Johansson (1960). These two iron-binding proteins show the same iron binding capacity and absorption spectrum (Johansson, 1958 and 1960) but are immunologically different (Montreuil et al. 1960). The same authors also noted many chemical differences between the two proteins. Johansson (1960) reported the stability difference of iron complexes in acid pH environment. The iron-binding protein synthesised in the mammary gland was designated lactotransferrin by Montreuil et al. (1960). The name "lactoferrin" was proposed by Blanc and Isliker (1961).

Lactoferrin constitutes the major part of the red fraction from many, but not all, varieties of milk. On the basis of electrophoretic pattern of the two proteins Masson and Heremans (1970) concluded that milk from rats, rabbits and dogs was devoid of lactoferrin. Rabbit and rat milks were found to be rich in transferrin, but dog milk was practically devoid of any iron-binding proteins, except for traces of transferrin. The same authors reassessed the results of Ezekiel et al. (1963) and suggested that iron-
binding protein present in albino rat or quokka milk also appear to correspond to transferrin.

Despite its name, lactoferrin is not specific to milk. It has been demonstrated in nearly all human secretions except serum or sweat. It is secreted by the lachrymal, bronchial and salivary glands (Masson and Heremans, 1966; Masson et al., 1968a), mucosa of the endometrium (Masson et al., 1968b) and seminal vesicles (Heckman and Rümké, 1968). The presence of the protein in human tears (Broekhuyse, 1974), pancreatic juice (Colomb et al., 1974), synovial fluid (Bennett et al., 1973), urine (Masson and Heremans, 1966a) and hepatic bile (Masson and Heremans, 1966) was also established.

Ovotransferrin (Conalbumin). Conalbumin comprises 3-16% of the eggwhite from various species of birds (Bain and Deutsch, 1947; Clark et al., 1963), and was the first of the transferrins to be separated and identified as an individual protein. The name conalbumin was first applied to the fraction of egg white by Osborne and Campbell (1900). This protein was later investigated extensively by physical methods. In 1944, Schade and Caroline found that raw eggwhite contains a factor which inhibits the growth of certain micro-organisms by fixing the iron in a form nutritionally unavailable to the bacteria. This antibacterial protein was shown by Alderton et al. (1946) to be identical to the conalbumin and to be very similar to serum transferrin in almost all respects. The extensive study of iron-binding property of conalbumin was made by Fraenkel-Conrat and
5

Feeney (1950). Structural similarity of the protein parts of conalbumin and transferrin of domestic hen was indicated by Williams (1962).

Nomenclature for the Iron-Binding Proteins. Various names have been proposed for the iron-binding proteins and these are summarised in Table 1.1.

1.2 BIOSYNTHESIS AND FUNCTION

Biosynthesis. There is evidence for transferrin synthesis by liver, spleen, bone marrow, lymph nodes, thymus and peritoneal macrophages in the rat, (Stecher and Thorbecke, 1967; Phillips and Thorbecke, 1966); and by some of these tissues, as well as the liver, in the mouse, rabbit, guinea pig, monkey and human (Smith et al., 1967). However, the primary site of synthesis of human and rat transferrin appears to be in the liver (Dancis et al., 1957; Wise and Oliver, 1966).

The site of synthesis of ovotransferrin (conalbumin) and lactoferrin have not been established as firmly as those of serum transferrin. However, oviduct and secreting tissues (especially the mammary gland) are believed to be the most likely site of synthesis of ovotransferrin and lactoferrin respectively.

Function. There is little doubt that the main function of blood serum transferrin is the transport of iron, from where it is stored or released, to tissues where it is used.

Upon absorption by gut mucosal cells, iron is stored within ferritin molecules of liver and spleen
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(Farrant, 1954; Granick, 1954). Of the 27 mg of iron entering the blood stream each day some 20 mg are derived more or less directly from catabolism of red cells and the remainder is derived primarily from storage iron (ferritin) with a few per cent coming from ingested iron. Approximately 20 mg of iron in the blood is used for haemoglobin synthesis in bone marrow. It is well established that most or probably all of the iron acquired by immature erythroid cells and used for haemoglobin synthesis is delivered from plasma transferrin. Schade (1955) reviewed the earlier studies on the role of serum transferrin in iron transport. It was shown that iron is more readily taken up by the reticulocyte when it is bound to serum transferrin than bound to ferritin or to inorganic compounds (Laurell, 1947; Zschocke and Bezkorovainy, 1974). Jandl et al. (1959) found that iron bound to serum transferrin is readily and preferentially transferred to immature, but not to mature mammalian red cells. Fig. 1.1 is a schematic diagram showing some possible pathways of iron in the body.

There is little doubt that the first step in the transfer of iron from the serum transferrin to reticulocytes, and probably to the other erythropoietic cells, is the binding of transferrin to the cells (Jandl and Katz, 1963; Morgan, 1964). However, the exact process of iron uptake from serum transferrin by the reticulocytes is not yet determined. There is electron microscopic evidence that transferrin molecules penetrate into reticulocyte cells (Morgan and Appleton, 1969), probably via surface endocytosis.
Figure 1.1  Some possible pathways of iron in the body. Erythroid and non-erythroid iron turnover are represented by the upper and lower parts of the figure, respectively. (from Zchocke and Bezkorovainy, 1974)

Tf : Transferrin
RE : Reticuloendothelial

Numbers indicate average iron turnover in mg per 100 ml whole blood per day (according to Cook et al., 1970; Finch et al. 1970)
or microtubular membranes (Hemmaplarch et al., 1974). On the other hand, others believe that iron is removed from transferrin while the latter is attached to a reticulocyte surface receptor (Jandl and Katz, 1963; Speyer and Fielding, 1974), whereby iron combines with a specific receptor found in the reticulocyte stroma (Garrett et al., 1973) and is then mobilized from the stroma by an intercellular iron binding protein, having an approximate molecular weight of 5,000 (Workman and Bates, 1974).

Neither function has been yet demonstrated for lactoferrin nor ovotransferrin. Since Schade and Caroline (1944) found the antibacterial role of raw ovotransferrin, the same inhibitor activity was demonstrated also in serum transferrin (Schade, 1963; Bullen and Rogers, 1969) and lactoferrin (Masson et al., 1966b; Oram and Reiter, 1966 and 1968). There appears to be little question that the iron-binding proteins play an antibacterial role in vitro by fixing iron ion nutritionally unavailable to bacteria. It has not been demonstrated unequivocally, however, that this phenomenon is important also in vivo. The importance of the iron binding property of ovotransferrin has been shown in the prevention of infection of the egg by gram negative bacteria at the time the egg is laid (Feeney and Nagy, 1952; Garibaldi and Bayne, 1962).
1.3 THE BINDING OF IRON

The mechanism of iron-binding has been studied with a variety of methods. Holmberg and Laurell (1947) found that transferrin binds Fe and Cu, but the affinity for Fe is much greater than for Cu. In addition to Fe and Cu other metal ions can bind to the protein with various affinities. The relative stability of the transition metal-ovotransferrin complexes is in the following order: Fe(III) > Cr(III), Cu(II) > Mn(II), Co(II), Cd(II) > Zn(II) > Ni(II) (Tan and Woodworth, 1969). Some of the metals confer characteristic colours to the complexes owing to absorption in the visible region: Fe(III) $\lambda_{\text{max}}$ 465 nm salmon pink; Cu(II) $\lambda_{\text{max}}$ 440 nm yellow; Cr(III) $\lambda_{\text{max}}$ 610 and 435 nm, light blue-green, Co(III) $\lambda_{\text{max}}$ 405 nm; Mn(III) $\lambda_{\text{max}}$ 430 nm yellow (Feeney and Komatsu, 1966).

Ovotransferrin (Fraenkel-Conrat and Feeney, 1950) and lactoferrin (Schade et al., 1949) share these properties with serum transferrin, although lactoferrin-iron complex is more stable at low pH than transferrin-iron complex (Johansson, 1960). Aisen and Leibman (1972) reported that the binding constant for iron at pH 6.4-6.7 is 300 times larger for lactoferrin than for transferrin.

Affinity of Two Metal Binding Sites. A controversial question in the chemistry of the transferrins is whether the two specific sites of the proteins bind metal ions pairwise or randomly. Warner and Weber (1953) obtained a value of
$1 \times 29 \text{ M}^{-1}$ for the association constant of Fe(III) in ovo-
transferrin and proposed that the binding of the first iron facilitated the binding of the second ion ($K_2 = 100 K_1$) such that both sites on any one molecule were effectively occupied together. By using equilibrium dialysis Davis et al. (1962) concluded that $K_2$ is several hundredfold larger than $K_1$. On the other hand, Aasa et al. (1963), using the same method, concluded that the sites were equivalent and independent, where the transferrin solution half saturated with iron would contain the protein complexes with two and one iron atom, and the iron free protein. The most recent determination of the relative affinities of the two sites by equilibrium dialysis gave $K_1 = 12K_2$ (Aisen and Leibman, 1968).

Apparent support for the equivalence of the association constants and for a random order of binding of metal ion was obtained from electrophoretic experiments on partially iron-saturated transferrin, which demonstrated the existence of the protein complexes with two and one iron atoms, and the iron free protein (Stratil, 1967; Aisen et al., 1966). On the contrary, the view that the metal atoms bind only in pairs to the protein was also obtained from electrophoresis experiments (Giblett, 1968).

However, the bulk of recent evidence from various studies, including protein fluorescence studies (Evans and Holbrook, 1975), EPR and Mössbauer spectra (Aisen et al., 1973) favour the different affinity of the two metal binding sites (probably $K_1 > K_2$), even though the local environments
of the two metal binding sites may be the same. There appears to be little interaction between the two iron-binding sites (Aisen et al., 1966).

The Nature of a Metal Binding Site. The nature of the metal binding site has been investigated by many workers. Several observations indicate that the metal binding site may contain three tyrosine and two histidine residues. The liberation of three protons accompanying by the binding of iron (III) to the protein (Warner and Weber, 1953); chemical modification of tyrosine residues by iodine (Phillips and Azari, 1972), N-acetylimidazole (Komatsu and Feeney, 1967) and tetrannitromethane (Line et al., 1967); spectrophotometric titration (Wishnia et al., 1961); fluorescence spectroscopy (Chun, 1971); nuclear magnetic resonance spectra (Woodworth et al., 1970) and circular dichroism spectra (Nagy and Lehrer, 1972) have provided convincing arguments for the role of tyrosine residues in the binding of iron(III) by transferrin. The possible participation of two histidine side chains in the binding of each iron atom has been indicated by chemical modification of histidine residues (Line et al., 1967) and electron spin resonance studies (Aasa and Aisen, 1968).

Anion Binding. The specific binding of iron to transferrin occurs only when a suitable anion is also bound (Schade et al., 1949; Bates and Schlaback, 1973), although several authors reported the formation of the transferrin-iron(III) complex in the absence of bicarbonate and its substitutes (Aisen et al., 1967; Price and Gibson, 1972). The anion
binding with transferrin in blood is believed to be bicarbonate (or carbonate) but, in its absence a wide variety of polyfunctional carboxylic acid (e.g. oxalate, nitrilotriacetate) may fulfil this role (Aisén et al., 1967).

**Hypothetical Structure for a Metal Binding Site.** On the basis of the above arguments, several hypothetical structures have been proposed for an iron binding site of transferrin. Fig. 1.2 shows the early structure proposed by Warner (1953) and the later one of Windle et al. (1963).

**Comparative Properties of the Metal-Free and Metal Complexes of Transferrins.** Apart from the visible development of colour that occurs when Fe(III) binds to apotransferrin, various other changes have been demonstrated. It is known that iron transferrin is much more stable than apotransferrin towards denaturation by heat, urea and extremes of pH (Warner and Weber, 1953; Azari and Feeney, 1958; Glazer and McKenzie, 1963), and towards chemical and enzymatic treatment (Azari and Feeney, 1961; Fraenkel-Conrat, 1950). It has been concluded that transferrin becomes more elongated and more compact when binding with iron from hydrogen-tritium exchange (Ulmer, 1969) and ultracentrifugal studies (Bezkorovainy, 1966; Charlwood, 1971).

**Iron-Binding Fragments.** Recently two groups of workers successfully used the resistance of iron-transferrin complex against trypsin digestion in order to obtain iron binding fragments from domestic hen ovotransferrin (Williams, 1974 and 1975) and cattle transferrin (Brock et al., 1976). A similar iron-binding fragment was also obtained from CNBr digest of hen ovotransferrin (Tsao et al., 1974). These results are summarised in Table 1.2.
Figure 1.2 Diagrammatic representation of an iron(III) binding site of transferrin proposed by Warner (1953), top, and Windle et al. (1963), bottom.
<table>
<thead>
<tr>
<th>Source</th>
<th>Digestion</th>
<th>No. of Fe(III) Bound Peptide</th>
<th>MW of Peptide</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe(III) Saturated Ovotransferrin</td>
<td>Trypsin</td>
<td>1</td>
<td>35,000 (N-terminal half)</td>
<td>Williams (1974)</td>
</tr>
<tr>
<td>Fe(III) Saturated Ovotransferrin</td>
<td>Subtilisin</td>
<td>1</td>
<td>35,000 (C-terminal half)</td>
<td>Williams (1975)</td>
</tr>
<tr>
<td>30% Fe(III) Saturated Ovotransferrin</td>
<td>Trypsin</td>
<td>1</td>
<td>35,000 (C-terminal half)</td>
<td>Williams (1975)</td>
</tr>
<tr>
<td>Fe(III) Saturated Bovine Transferrin</td>
<td>Trypsin</td>
<td>1</td>
<td>32,800</td>
<td>Brock et al. (1976)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>38,500</td>
<td></td>
</tr>
<tr>
<td>Fe(III) Free Ovotransferrin</td>
<td>CNBr</td>
<td>1</td>
<td>36,000 (N-terminal half)</td>
<td>Tsao et al. (1974)</td>
</tr>
</tbody>
</table>
1.4 PHYSICAL AND CHEMICAL PROPERTIES

Molecular Weight. There were large discrepancies in molecular weight of the various transferrins obtained in the earlier studies. However, with improvement in purification and in measurement of molecular weight, 76,000-77,000 has been established as the most likely values. The earlier studies of serum transferrin indicated a higher molecular weight in the region of 87,000-90,000 (Laurell and Ingelman, 1947; Oncley et al., 1947). Similar values of 87,000 and 89,000 were reported for conalbumin (Bain and Deutsch, 1948) and lactoferrin (Montreuil et al. 1960) respectively. However, in recent years Palmour and Sutton (1971) reported values of 75,000-79,000 for various transferrins based on sedimentation equilibrium studies. Similar values were obtained by Hudson et al. (1973) and Greene and Feeney (1968). Spik (1968) also found a similar value for lactoferrin from various measurements. Some recent authors have reported somewhat higher values (Bezkorovainy et al., 1968; Eijk et al., 1972). On the other hand, some workers have reported lower molecular weight when the proteins were measured in a concentrated urea or guanidine hydrochloride solution than found in an aqueous solution (Jeppsson, 1967a; Efremov et al., 1971; Richardson et al., 1973), suggesting possible dissociation of transferrin molecule. The physical characters of the iron-binding proteins are summarised in Table 1.3.
<table>
<thead>
<tr>
<th>Property</th>
<th>Transferrin</th>
<th>Lactoferrin</th>
<th>Ovotransferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight</td>
<td>77,000&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76,000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>77,000&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sedimentation Coefficient ( (s_{20, w}^o) )</td>
<td>5.0&lt;sup&gt;d&lt;/sup&gt;, 5.4&lt;sup&gt;e&lt;/sup&gt;, 5.1&lt;sup&gt;f&lt;/sup&gt;, 4.7&lt;sup&gt;g&lt;/sup&gt;, 4.9&lt;sup&gt;h&lt;/sup&gt;</td>
<td>4.7&lt;sup&gt;i&lt;/sup&gt;</td>
<td>4.8&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diffusion Coefficient at 20°C ( (\times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}) )</td>
<td>8.4&lt;sup&gt;e&lt;/sup&gt;, 5.8&lt;sup&gt;h&lt;/sup&gt;</td>
<td>4.6&lt;sup&gt;i&lt;/sup&gt;</td>
<td>5.6&lt;sup&gt;k&lt;/sup&gt;</td>
</tr>
<tr>
<td>Absorption Maxima (iron complex) ( (\text{nm}) )</td>
<td>278,465</td>
<td>280,452&lt;sup&gt;i&lt;/sup&gt;</td>
<td>280&lt;sup&gt;j&lt;/sup&gt;, 470&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>Extinction Coefficients ( (A_{0.01 \text{ gcm}^{-3}}) )</td>
<td>11.7(278)&lt;sup&gt;k&lt;/sup&gt;</td>
<td>11.7(280)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>11.3(280)&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>( (\text{cm}) )</td>
<td>0.600(465)&lt;sup&gt;m&lt;/sup&gt;</td>
<td>0.500(452)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0.621(470)&lt;sup&gt;j&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iron-Binding Capacity ( (\mu g \text{ iron per mg protein}) )</td>
<td>1.51&lt;sup&gt;h&lt;/sup&gt;</td>
<td>1.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.59&lt;sup&gt;n&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atoms of Iron Bound per Molecule Protein</td>
<td>2&lt;sup&gt;o&lt;/sup&gt;</td>
<td>2&lt;sup&gt;b,p&lt;/sup&gt;</td>
<td>2&lt;sup&gt;n&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Palmour and Sutton (1971), Hudson et al. (1973), Greene and Feeney (1968);  
<sup>b</sup>Spik (1968);  
<sup>c</sup>Greene and Feeney (1968);  
<sup>d</sup>Oncley et al. (1947);  
<sup>e</sup>Bain and Deutsch (1948);  
<sup>f</sup>Charlwood (1963);  
<sup>g</sup>Phelps and Cann (1956);  
<sup>h</sup>Roberts et al. (1966);  
<sup>i</sup>Montreuil et al. (1960);  
<sup>j</sup>Glazer and McKenzie (1963);  
<sup>k</sup>Fuller and Briggs (1956);  
<sup>l</sup>Bezkorovainy et al. (1963);  
<sup>m</sup>Inman et al. (1961);  
<sup>n</sup>Warner and Weber (1951);  
<sup>o</sup>Laurell and Ingelmann (1947), Surgenor et al. (1949);  
<sup>p</sup>Blanc and Isliker (1961).
Number of Polypeptide Chains. One of the major concerns has been the number of polypeptide chains in transferrin. Molecules as large as the transferrins tend to be composed of subunits. Furthermore, the binding of two atoms iron per molecule, the possession of two equal size carbohydrate moieties (Graham and Williams, 1975), and the observation of fewer spots on peptide maps in number than would be expected (Jeppsson, 1967a; Baker et al., 1968) gave rise to the suggestion of a dual structure consisting of at least two subunits on a single duplicate structure. Cleavage of disulphide bonds in concentrated urea or guanidinium chloride solution has also occasionally yielded products interpreted as subunits of the transferrin (Jeppsson, 1967a; Efremov et al., 1971). In spite of various efforts to detect subunits, it now seems certain that transferrins are made up of a single polypeptide chain (Greene and Feeney, 1968; Fish et al., 1969).

An unique approach was made to examine the possible peptide duplication in domestic hen ovotransferrin by Elleman and Williams (1970). They identified 34 unique cysteic acid containing peptides in the protein. Since hen ovotransferrin contains 31 residues of half-cystine per molecule, it was concluded that hen ovotransferrin does not consist of two identical halves or subunits. Supporting evidence for a single polypeptide chain structure of transferrins is provided by the presence of only one N-terminal amino acid residue in the transferrins of various species. These results are summarised in Table 1.4.
<table>
<thead>
<tr>
<th>Species</th>
<th>N-Terminus</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Transferrin</td>
<td>Val</td>
</tr>
<tr>
<td>Human</td>
<td>Lactoferrin</td>
<td>Gly</td>
</tr>
<tr>
<td>Bovine</td>
<td>Transferrin</td>
<td>Ala</td>
</tr>
<tr>
<td>Bovine</td>
<td>Lactoferrin</td>
<td>Ala</td>
</tr>
<tr>
<td>Baboon</td>
<td>Transferrin</td>
<td>Val</td>
</tr>
<tr>
<td>Pig</td>
<td>Transferrin</td>
<td>-</td>
</tr>
<tr>
<td>Hen</td>
<td>Ovotransferrin</td>
<td>Ala</td>
</tr>
<tr>
<td>Duck</td>
<td>Ovotransferrin</td>
<td>Ala</td>
</tr>
</tbody>
</table>

Is often sparsely distributed in a protein, CMR degradation produces only a few peptide fragments even from a large molecule such as a transferrin. Bezkorovainy and Grohlich (1974) used CMR degradation products of various transferrins from several species to examine similarities or dissimilarities of the primary structures of the proteins so that their evolution could be traced throughout the phylogenetic tree.
Polypeptide Chain. The transferrin from several species have been extensively studied with regard to amino acid composition. Some representative amino acid compositions are shown in Table 1.5. The amino acid compositions are generally similar, but some significant variations in the reported values for the individual amino acids are apparent. The presence of less common amino acid has not so far been reported.

Comparative studies on the primary structure of transferrin have not been made in general. However, there have been some comparisons on the basis of their gross chemical properties. Attempts to establish the primary structure have been hampered, because the polypeptide chain of a transferrin is large for direct sequence studies. Cyanogen bromide (CNBr), which splits peptide chains at methionine residues, was first applied to structural studies on the transferrins by Jeppsson (1967b). Since methionine is often sparsely distributed in a protein, CNBr degradation produces only a few peptide fragments even from a large molecule such as a transferrin. Bezkorovainy and Grohlich (1974) used CNBr degradation products of serum transferrins from several species to examine similarities or dissimilarities of the primary structures of the proteins so that their evolution could be traced throughout the phylogenetic tree.

The successful CNBr cleavage of transferrin molecules into several small size of peptides encouraged several workers to initiate the investigation of complete amino acid sequence of transferrins. Sutton et al. (1975) have so far
Table 1.5 Amino Acid Compositions of Transferrins.

<table>
<thead>
<tr>
<th></th>
<th>Human Transferrin (A)</th>
<th>Human Lactoferrin (B)</th>
<th>Rabbit Transferrin (C)</th>
<th>Hen Ovo-transferrin (D)</th>
<th>Hen Ovo-transferrin (E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp+Asn</td>
<td>81</td>
<td>76</td>
<td>67</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>58</td>
<td>61</td>
<td>69</td>
<td>63</td>
<td>65</td>
</tr>
<tr>
<td>His</td>
<td>19</td>
<td>19</td>
<td>10</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>Arg</td>
<td>24</td>
<td>26</td>
<td>38</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td>Lys</td>
<td>51</td>
<td>58</td>
<td>40</td>
<td>48</td>
<td>58</td>
</tr>
<tr>
<td>Gly</td>
<td>54</td>
<td>46</td>
<td>50</td>
<td>48</td>
<td>51</td>
</tr>
<tr>
<td>Ser</td>
<td>40</td>
<td>38</td>
<td>47</td>
<td>42</td>
<td>37</td>
</tr>
<tr>
<td>Thr</td>
<td>30</td>
<td>27</td>
<td>34</td>
<td>35</td>
<td>36</td>
</tr>
<tr>
<td>Ala</td>
<td>59</td>
<td>54</td>
<td>57</td>
<td>51</td>
<td>52</td>
</tr>
<tr>
<td>Cys/2</td>
<td>35</td>
<td>38</td>
<td>27</td>
<td>36</td>
<td>30</td>
</tr>
<tr>
<td>Met</td>
<td>4</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Val</td>
<td>43</td>
<td>41</td>
<td>44</td>
<td>50</td>
<td>49</td>
</tr>
<tr>
<td>Leu</td>
<td>57</td>
<td>56</td>
<td>55</td>
<td>53</td>
<td>46</td>
</tr>
<tr>
<td>Ile</td>
<td>14</td>
<td>15</td>
<td>16</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>Pro</td>
<td>30</td>
<td>30</td>
<td>34</td>
<td>38</td>
<td>26</td>
</tr>
<tr>
<td>Phe</td>
<td>28</td>
<td>27</td>
<td>29</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Tyr</td>
<td>24</td>
<td>21</td>
<td>21</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>Trp</td>
<td>9</td>
<td>-</td>
<td>11</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

The number of amino acid residues was obtained by recalculating the author's results so that polypeptide chains of transferrins possess a molecular weight of ca. 74,800 (see 4.2.1).

(A) Mann et al. (1970)
(B) Bezkorovainy and Grohlich (1974)
(C) Querinjean et al. (1971)
(D) Hudson et al. (1973)
(E) Williams (1975)
(F) Eijk et al. (1972)
Horse  Pig  Rat  Baboon  Cattle  
Transferrin  Transferrin  Transferrin  Transferrin  Transferrin  
(D)           (D)           (D')          (B)              (B)              

|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 78 | 87 | 76 | 78 | 81 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 64 | 66 | 42 | 61 | 64 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 15 | 13 | 18 | 19 | 16 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 25 | 26 | 27 | 25 | 23 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 47 | 46 | 47 | 53 | 59 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 45 | 39 | 58 | 45 | 46 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 42 | 39 | 47 | 42 | 49 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 34 | 28 | 36 | 29 | 41 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 54 | 53 | 53 | 59 | 51 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 35 | 37 | 33 | 38 | 29 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|  4 |  6 |  4 |  9 |  7 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 47 | 46 | 51 | 38 | 34 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 58 | 59 | 57 | 60 | 50 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 17 | 19 | 21 | 12 | 15 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 45 | 40 | 32 | 31 | 33 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 21 | 26 | 32 | 27 | 27 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 22 | 18 | 18 | 20 | 22 |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| 10 | 10 | -  | -  | -  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

**Note:** The table above represents the transferrin levels in various species. The numbers indicate the level of similarity in their protein structure, with higher numbers indicating greater similarity. This data was compared with transferrin levels in other species and used to infer evolutionary relationships.
completed nearly half of the sequential analysis of human serum transferrin, using CNBr peptide fragments. Although final conclusions about the presence of internal homology can not be made until the complete sequential analysis is finished, interesting similarities between some regions of sequence were reported. An example is shown below.

<table>
<thead>
<tr>
<th>CN-1 Peptide</th>
<th>Tyr-Leu-Gly—Glu-Tyr-Val-Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN-4 Peptide</td>
<td>Tyr-Leu-Gly-Tyr-Glu-Tyr-Val-Thr</td>
</tr>
</tbody>
</table>

Structural similarity between serum transferrin and lactoferrin of human was examined by comparing the size of the CNBr peptides (Montreuil and Spik, 1975). It was revealed that methionine residues are differently distributed in the polypeptide chains. On the other hand comparison of some cysteine peptides from human lactoferrin with those from serum transferrin shows a reasonable degree of similarity in their primary structures (Deconinck et al., 1974). This was interpreted as indicating that lactoferrin and transferrin were originally derived from a common ancestral iron-binding protein.

Carbohydrate Moiety. Since the presence of carbohydrate in human serum transferrin was first reported by Surgenor et al. (1949), many workers investigated the carbohydrate portion of transferrins. They agree on the presence of galactose, mannose, N-acetylglucosamine and sialic acid in the proteins. The presence of fucose has not been generally confirmed. Several authors (Hudson et al., 1973; Hatton et al., 1974;
Eijk et al., 1972) compared the carbohydrate composition of serum transferrin from various mammals and observed similarities in the monosaccharides present, but striking differences in their amount. Table 1.6 shows carbohydrate compositions of transferrin from various sources obtained by several workers. The carbohydrate compositions of ovo-transferrin and serum transferrin of chicken, and comparison of lactoferrin and serum transferrin of human are also compared in Table 1.6. Hudson et al. (1973) postulated that the large variation observed in monosaccharide content of the various transferrin is a result of differing numbers of heteropolysaccharide chains per molecule polypeptide chain. Thus they predicted the presence of one carbohydrate chain in bovine transferrin, two in rabbit and equine transferrin and four in porcine transferrin.

Structure for the heterosaccharide chain in human transferrin were proposed by Jamieson et al. (1971) and Montreuil and Spik (1975), but they are not identical.

Glycopeptide have been prepared from various transferrins and their structures have been studied. The comparison of glycopeptides obtained from the ovo-transferrin and serum transferrin of the domestic hen was made by Williams (1968). The amino acid sequence of the glycopeptides were found to be the same in both proteins, indicating that both proteins have similar amino acid sequences and that the carbohydrate groups are linked to the same amino acids on the protein molecules. In contrast, serum transferrin and lactoferrin of human have quite different amino acid sequence
Table 1.6(A) Carbohydrate Compositions of Serum Transferrins

Comparison of the Results from Several Authors.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Cattle</th>
<th>Rabbit</th>
<th>Pig</th>
<th>Horse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Jamieson (1965a)</td>
<td>Montreuil et al. (1965)</td>
<td>Hudson et al. (1973)</td>
<td>Hudson et al. (1973)</td>
<td>Hudson et al. (1973)</td>
</tr>
<tr>
<td>MW used</td>
<td>90,000</td>
<td>90,000</td>
<td>77,300</td>
<td>76,700</td>
<td>76,400</td>
</tr>
<tr>
<td>Total carbohydrate</td>
<td>5.9%</td>
<td>5.7%</td>
<td>3.04%</td>
<td>5.48%</td>
<td>11.8%</td>
</tr>
<tr>
<td>Total Hexoses</td>
<td>2.6%</td>
<td>2.35%</td>
<td>1.21%</td>
<td>2.07%</td>
<td>3.89%</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.74% (3.7)</td>
<td>1.2% (5.8)</td>
<td>0.49% (2.1)</td>
<td>0.97% (4.1)</td>
<td>1.82% (7.7)</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.85% (9.3)</td>
<td>1.2% (5.8)</td>
<td>0.72% (3.0)</td>
<td>1.10% (4.6)</td>
<td>2.07% (8.8)</td>
</tr>
<tr>
<td>Glucosamine¹</td>
<td>2% (8.1)</td>
<td>2% (8.1)</td>
<td>1.02% (3.5)</td>
<td>1.60% (5.5)</td>
<td>4.15% (14.3)</td>
</tr>
<tr>
<td>Fucose</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
<td>trace</td>
<td>0.74% (3.4)</td>
</tr>
<tr>
<td>Sialic Acid²</td>
<td>1.3% (3.8)</td>
<td>1.37% (3.9)</td>
<td>0.74% (1.8)</td>
<td>1.71% (4.2)</td>
<td>3.01% (7.4)</td>
</tr>
</tbody>
</table>

1. As N-acetyl derivative.
2. As N-acetylneuraminic acid.

The numbers in brackets express the number of sugar residues per molecule of protein.
Table 1.6(B)  Comparison of Carbohydrate Composition of Domestic Hen Serum Transferrin and Ovotranferrin, and of Human Serum Transferrin and Lactoferrin.

<table>
<thead>
<tr>
<th></th>
<th>Domestic Hen¹</th>
<th></th>
<th>Human²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ovo-transferrin</td>
<td>Serum Transferrin</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>MW used</td>
<td>90,000</td>
<td>90,000</td>
<td>95,000</td>
</tr>
<tr>
<td>Total carbohydrate(%)</td>
<td>2.2%</td>
<td>4.6%</td>
<td>7.1%</td>
</tr>
<tr>
<td>Total hexoses(%)</td>
<td>0.8%</td>
<td>2.8%</td>
<td>3.9%</td>
</tr>
<tr>
<td>Galactose(%)</td>
<td>(4.0)</td>
<td>(10.5)</td>
<td>(12.2)</td>
</tr>
<tr>
<td>Mannose(%)</td>
<td></td>
<td></td>
<td>(8.5)</td>
</tr>
<tr>
<td>N-acetylglucosamine(%)</td>
<td>1.4% (5.7)</td>
<td>1.4% (5.7)</td>
<td>2.4%</td>
</tr>
<tr>
<td>Fucose(%)</td>
<td></td>
<td></td>
<td>(10.3)</td>
</tr>
<tr>
<td>N-acetylneuraminic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sialic Acid(%)</td>
<td></td>
<td>0.35% (1.0)</td>
<td>0.87%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.6</td>
</tr>
</tbody>
</table>

1. Williams (1962)
2. Montreuil et al. (1965)
3. As N-acetyl derivative.
4. As N-acetylneuraminic acid.

The numbers in brackets express the number of sugar residues per molecule of protein.
of the polypeptide regions carrying carbohydrate moieties but possess the same basic structures of carbohydrate moieties (Montreuil and Spik, 1975).

The existence of two carbohydrate chains in transferrins from various sources has been reported by several authors (Jamieson, 1965; Graham and Williams, 1975). These two carbohydrate chains from a transferrin molecule appear to be identical in carbohydrate composition, but linked to different amino acids of the polypeptide chain. The two glycopeptides prepared from various transferrins were distinguished as type A and type B by Graham and Williams.

\[
\begin{align*}
\text{type A} & : \quad \text{CHO} \\
& : \quad \text{Asn - basic amino acid}
\end{align*}
\]

\[
\begin{align*}
\text{type B} & : \quad \text{CHO} \\
& : \quad \text{Asn - neutral aliphatic amino acid}
\end{align*}
\]

Relatively little similarities between the amino acid sequence of glycopeptides from various species was found and this was interpreted as reflecting the dissimilarity of transferrin from various species in the amino acid sequence of the polypeptide chains.

In all the glycopeptides so far prepared from the transferrins, the carbohydrate moieties are linked to the polypeptide chain by an asparaginyl-N-acetylglucosamine bond. The presence of a carbohydrate moiety which is O-glycosidically linked to the polypeptide chain of lactoferrin (Spik and Montreuil, 1969) is unlikely (Montreuil and Spik, 1975).
1.5 GENETIC VARIATION

lactoferrin (Lactotransferrin). There have not been sufficient studies on the polymorphism of lactoferrins to draw any definite conclusions. Several types of electrophoretic patterns of lactoferrin in milk from individual cows were observed by Groves and Peterson (1965) and they suggested the possible existence of genetically controlled variations in cow lactoferrin analogous to the serum transferrin system. However, it was shown that the variation in electrophoretic patterns of human lactoferrin is not due to genetic variation, but due to difference in sialic acid content (Wolfson, 1971).

Ovotransferrin (Conalbumin). Since the existence of three types of genetically controlled ovotransferrin in domestic hen egg was reported by Lush (1961), this observation has been confirmed by several workers (Williams, 1962; Ogden et al., 1962; Feeney et al., 1963). Ogden et al. also found that variation in ovotransferrin parallels that in the transferrins. No studies have been made on the structural differences between the variants.

Human Serum Transferrin. Genetically controlled serum transferrin variation has been reported in many vertebrates. Among these the variation of human serum transferrin has been especially well established and some twenty electrophoretic variants have been so far described (Sutton, 1972) (see Table 1.7). All variants except transferrin C have low gene frequencies and limited geographical distribution.
Table 1.7 The Human Transferrins Listed in Order of Electrophoretic Mobility.

<table>
<thead>
<tr>
<th>Transferrins</th>
<th>References</th>
<th>Population Source</th>
<th>First Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>B_{Lae}</td>
<td>Lai (1963)</td>
<td>New Guinea native</td>
<td></td>
</tr>
<tr>
<td>B_0</td>
<td>Giblett et al. (1959)</td>
<td>American Caucasian</td>
<td></td>
</tr>
<tr>
<td>B_{0-1}</td>
<td>Parker and Bearn (1961a)</td>
<td>Navajo Indian</td>
<td></td>
</tr>
<tr>
<td>B_{Atalanti}</td>
<td>Murray et al. (1964)</td>
<td>Greek</td>
<td></td>
</tr>
<tr>
<td>B_1</td>
<td>Harris et al. (1958)</td>
<td>English Caucasian</td>
<td></td>
</tr>
<tr>
<td>B_{1-2}</td>
<td>Arends et al. (1962)</td>
<td>Italo-African</td>
<td></td>
</tr>
<tr>
<td>B_2</td>
<td>Smithies (1958)</td>
<td>Canadian Caucasian</td>
<td></td>
</tr>
<tr>
<td>B_3</td>
<td>Parker and Bearn (1961b)</td>
<td>Japanese</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Poulik and Smithies (1958)</td>
<td>Common variant (all population)</td>
<td></td>
</tr>
<tr>
<td>D_{Adelaide}</td>
<td>Cooper et al. (1964)</td>
<td>Australian Caucasian</td>
<td></td>
</tr>
<tr>
<td>D_0</td>
<td>Giblett et al. (1959)</td>
<td>American Negro</td>
<td></td>
</tr>
<tr>
<td>D_{Wigan}</td>
<td>Glenn-Bott et al. (1964)</td>
<td>English Caucasian</td>
<td></td>
</tr>
<tr>
<td>D_{0-1}</td>
<td>Harris et al. (1960)</td>
<td>English Caucasian</td>
<td></td>
</tr>
<tr>
<td>D_{Montreal}</td>
<td>Parker and Bearn (1962b)</td>
<td>Canadian Caucasian</td>
<td></td>
</tr>
<tr>
<td>D_{Chi}</td>
<td>Parker and Bearn (1961b)</td>
<td>Chinese</td>
<td></td>
</tr>
<tr>
<td>D_1</td>
<td>Smithies (1958)</td>
<td>American Negro</td>
<td></td>
</tr>
<tr>
<td>D_{Rainford}</td>
<td>Roop and Putnam (1967)</td>
<td>Caucasian</td>
<td></td>
</tr>
<tr>
<td>D_{Finland}</td>
<td>Seppälä (1965)</td>
<td>Finnish Caucasian</td>
<td></td>
</tr>
<tr>
<td>D_2</td>
<td>Harris et al. (1958)</td>
<td>African Negro</td>
<td></td>
</tr>
<tr>
<td>D_3</td>
<td>Giblett et al. (1959)</td>
<td>American Negro</td>
<td></td>
</tr>
</tbody>
</table>

In the table, B_{Lae} has the fastest mobility on zone electrophoresis, at pH 8.8.
Type C is the common variant, and detected in all populations sampled. None has been reported to be associated with any disease condition and selective differences have been detected.

Since Smithies (1957) first demonstrated the existence of genetic variation of transferrin, there have been several attempts to demonstrate difference in amino acid composition among the different phenotypes. Parker and Bearn (1962a) performed total amino acid analyses on isolated transferrin C, B2, D1 and D3, but did not find any clear differences. Further investigation has been made by the peptide mapping of the variants. The results so far obtained are shown in Table 1.8.

In transferrin variants D1, DChi and B2, one amino acid substitution detected was sufficient to explain the relative electrophoretic mobility compared with that of transferrin C, the common variant. Amino acid substitution of D1 and DChi (against C) appears to occur in the same polypeptide region (Howard et al., 1968).

Cattle Serum Transferrin. Genetically controlled differences in the ß-globulins of British breeds of dairy cattle have been described by Smithies and Hickman (1958) and Ashton (1958). Giblett et al. (1959) showed that these ß-globulins are transferrins. It was shown that each genotype from the transferrin alleles (TfA, TfB, ...) gives only one phenotype (TfA, TfB ...). Thus the homozygous phenotype expressed by allele TfA is described as TfA or TfAA. At least eleven alleles have been so far recognised in cattle serum. They are shown in Table 1.9.
Table 1.8 Amino Acid Difference in Human Transferrin Variants (comparison is with transferrin C).

<table>
<thead>
<tr>
<th>Variant</th>
<th>Enzyme Used</th>
<th>Amino Acid Substitution (variant : TfC)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_1$</td>
<td>Chymotrypsin</td>
<td>Gly:Asp</td>
<td>Wang and Sutton</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1965)</td>
</tr>
<tr>
<td>$D_{Chi}$</td>
<td>Trypsin</td>
<td>Arg:His</td>
<td>Wang et al. (1967)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Howard et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1968)</td>
</tr>
<tr>
<td>$B_2$</td>
<td>Trypsin</td>
<td>Glu:Gly</td>
<td>Wang et al. (1966)</td>
</tr>
<tr>
<td>$D_3$</td>
<td>Trypsin</td>
<td>Three additional peptides (no further analysis)</td>
<td>Sutton and Bowman (1962)</td>
</tr>
<tr>
<td>$D_{Reinford}$</td>
<td>Trypsin</td>
<td>One additional peptide (no further analysis)</td>
<td>Roop and Putnam (1967)</td>
</tr>
</tbody>
</table>
Table 1.9 Genetic Variants of Bovine Serum Transferrin.

<table>
<thead>
<tr>
<th>Type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Smithies and Hickman (1958), Ashton (1958)</td>
</tr>
<tr>
<td>B</td>
<td>Ashton (1959)</td>
</tr>
<tr>
<td>D₁</td>
<td>Kristjansson and Hickman (1965), Ashton (1958)</td>
</tr>
<tr>
<td>D₂</td>
<td>Kristjansson and Hickman (1965), Ashton (1958)</td>
</tr>
<tr>
<td>E</td>
<td>Smithies and Hickman (1958), Ashton (1958)</td>
</tr>
<tr>
<td>F</td>
<td>Ashton (1959)</td>
</tr>
<tr>
<td>Gₖetna</td>
<td>Ashton and Lampkin (1965)</td>
</tr>
<tr>
<td>Gₕ.S.Africa</td>
<td>Osterhoff (1964)</td>
</tr>
<tr>
<td>H</td>
<td>Sartore and Bernoco (1966)</td>
</tr>
<tr>
<td>Iₕungary</td>
<td>Soos et al. (1972)</td>
</tr>
<tr>
<td>N</td>
<td>Gahne (1961)</td>
</tr>
</tbody>
</table>
D₁ and D₂ alleles were originally assigned as D (Ashton, 1958), but D was later found to be divided into two types of allele, D₁ and D₂, by use of a buffer of higher resolution in electrophoresis (Kristjansson and Hickman, 1965). Among these types, A, D₁, D₂ and E are widespread in domestic breeds, whereas the other alleles are in some rare breeds and absent in others. As an example type B and F exist only in Zebu cattle, but not in Jersey, Hereford or Shorthorn breeds (Ashton 1959). The relative mobilities of the four common homozygous phenotypes on starch gel electrophoresis at pH 7.8 are shown in Fig.1.3, together with those of B and F.

Unlike the human transferrins the precise chemical differences among bovine transferrin variants have not been so far described.

The present work aims at elucidating the nature of the chemical differences of two homozygous variants of bovine serum transferrin, AA and D₂D₂.

1.6 ELECTROPHORETIC HETEROGENEITY OF TRANSFERRIN

The complexity of the electrophoretic pattern produced by a single transferrin allele has been observed in transferrins from varieties of vertebrates. The differences giving rise to the multiple components has naturally become a problem of importance to be solved.

It is known that electrophoretic heterogeneity of a given glycoprotein is often caused by several types of variation in the carbohydrate moieties, and most commonly
Figure 1.3 Relative mobility in starch gel of common homozygous bovine transferrin variants (according to Ashton (1959). The anode is at the top of the diagram.
it is due to variation in the carbohydrate groups of glycoproteins produced by partial substitution of sugar residues, particularly sialic acid, on a basically similar core structure.

Serum transferrins of the majority of vertebrate species so far examined contain sialic acid (Palmour and Sutton, 1971; Williams, 1962; Stratil and Spooner, 1971; Hudson et al., 1973; Stratil and Kůbek, 1974; Spooner et al., 1975). Relatively few exceptions to this were observed. For examples, in marine lamprey (Boffa et al., 1966) sialic acid could not be detected and neuraminidase did not affect the electrophoretic mobility. Similarly sialic acid was absent in carp transferrin (Valenta et al., 1975).

The relation of sialic acid content to the electrophoretic heterogeneity of transferrin has been studied by numerous workers. In some species sialic acid accounts for all of the heterogeneity (e.g. domestic hen: Williams, 1962; sheep: Spooner et al., 1975) in some other species it is responsible only for a part of the heterogeneity (e.g. pig: Stratil and Kůbek, 1974). The heterogeneity of carp (Valenta et al., 1975) and marine lamprey (Boffa et al., 1966) transferrins is not caused by sialic acid, as the latter is absent in these transferrins.

Bovine Transferrins. The presence of four distinct protein bands in a homozygous bovine transferrin zone was first reported by Smithies and Hickman (1958), and was subsequently confirmed by several workers (Raush et al., 1965; Datta et al., 1965; Jamieson, 1965a). An increased number of
bovine transferrin bands on electrophoresis has been observed on modification of the buffer system. Makarechian and Howell (1966) concluded that each transferrin allele is in fact responsible for the synthesis of six distinct protein bands. More recently Stratil and Spooner (1971) postulated the presence of twelve components in a homozygous transferrin variant.

The nature of the heterogeneity of cattle transferrin has been investigated by several workers. In view of obvious resemblance to lactate dehydrogenase, where the complex electrophoretic pattern is produced by different combination of two kinds of subunits into a tetramer (Vesell, 1965), and of the fairly large molecular size of transferrin (77,000) as a single polypeptide chain, several attempts were made to demonstrate subunits in bovine transferrin (Chen and Sutton, 1967; Richardson et al., 1973). The weight of evidence from the results of these authors clearly favours a single polypeptide structure of bovine transferrin. However, the possible presence of subunits in transferrin was also suggested by ultra centrifugation and gel electrophoresis studies of the disulphide bond cleaved bovine transferrin (Richardson et al., 1973; Efremov et al., 1971).

Attempts were made to attribute the occurrence of the multiple components of bovine serum transferrin solely to the difference in the number of sialic acid residues contained in the individual components. The removal of sialic acid residues from the bovine serum transferrin by the action of neuraminidase did not abolish completely the complex pattern, although the complexity was reduced markedly (Chen
and Sutton, 1967; Parker and Bearn, 1962a; Efremov et al., 1971, Scharmann et al., 1971). This is shown schematically in Fig.1.4. Stratil and Spooner (1971) showed clearly, with the use of the isolated individual components, that removal of sialic acid residues leads the six individual components to separation into two groups, having different electrophoretic mobility (Fig.1.4). This suggests that the multiplicity brought about by the extent of addition of sialic acid residues does not account for all the electrophoretic behaviour of bovine serum transferrin.

Possible differences in the polypeptide chain was examined by amino acid analyses and the peptide mapping of the isolated individual components (Chen and Sutton, 1967; Richardson et al., 1973). No significant differences were observed, suggesting that the primary structures of the peptide chain of the components resemble one another.

Thus, the nature of the electrophoretic complexity exhibited by a single homogeneous variant has not been elucidated completely. In this thesis a further study is made to find causes which give rise to the multiple components.

1.7 THE PURPOSE OF THE PRESENT INVESTIGATION

From the foregoing discussion of the transferrins it is evident that bovine serum transferrin occurs in a wide variety of genetic variants. Each of the homozygous variants exhibits heterogeneity. The extent to which this heterogeneity is real or apparent has not been completely resolved.
BOVINE TRANSFERRIN AA

Comparison of Electrophoresis Patterns

Figure 1.4  Effect of neuraminidase treatment on gel electrophoretic patterns of bovine transferrin. Comparison of the results of Parker and Bearn (1962a); Chen and Sutton (1967); Scharman et al. (1970); Efremov et al. (1971); Stratil and Spooner (1971).
Furthermore the nature of the differences between the genetic variants has not been elucidated. It is obviously important, if we are to understand the structure and function of the transferrins more completely, that some of the problems of the heterogeneity of cattle transferrin be resolved. In the present study a comparison of two bovine transferrins, A and D₂, has been undertaken, and also the individual components of a single variant have been compared.

Efforts have been made to answer the following specific questions.

(1) To what extent is the heterogeneity of a single variant real or apparent?
How many components are present in a single variant?
Is it possible to isolate the individual components in native state at a pH near the natural pH?

(2) What is the difference between the two genetic variants, A and D₂?
How many amino acid residue differences are present?
Is there any variation in the carbohydrate moiety?

(3) What is the difference between the multiple components of a single variant?
To what extent does sialic acid contribute to the heterogeneity of a single variant?
Is there any other factor in the carbohydrate moiety causing the complexity in addition to sialic acid?
Is there any difference in the polypeptide portion?
(4) How many carbohydrate moieties does each molecule of transferrin have?

To which amino acid residues are they linked?

What is the amino acid sequence around each amino acid residue to which the moiety is linked?
CHAPTER 2. MATERIALS AND METHODS

2.1 BLOOD COLLECTION

The genetic variants of transferrin were isolated from the blood of cattle which were known to be homozygous with respect to this protein. Blood samples homozygous in transferrin AA were collected from Droughtmaster animals, and those homozygous in transferrin D₂D₂ were collected from a Charolais X, at the University of Queensland, Veterinary School Farm, Moggill. Blood (850 ml) was directly drawn into the glass bottle containing 150 ml acid-citrate solution, prepared from 22 g sodium citrate and 8 g citric acid in one litre water, pH 4.9. In order to avoid the possible contamination in transferrin fraction, dextrose was not used since one of the objects of this study is carbohydrate determination. Immediately after collection, the blood cells were separated from the plasma and 220 g solid (NH₄)₂SO₄ was added slowly to 1 litre plasma-acid-citrate solution, with gentle stirring. Plasma-acid-citrate, containing 220 g l⁻¹ (NH₄)₂SO₄, was transferred to Canberra in a thermos flask at 18°C. The sample collection and preliminary treatment with (NH₄)₂SO₄ were carried out by members of the Veterinary Blood Grouping Laboratory, School of Veterinary Science, University of Queensland.

2.2 WATER, GLASSWARE AND DIALYSIS CASING

Throughout this study, precautions were taken to avoid contamination by trace metals, that have been shown to bind with transferrin (Tan and Woodworth, 1969).
Water was distilled twice, the second distillation being carried out in a pyrex all-glass still.

Laboratory glassware was of Pyrex type. It was soaked in \( \text{H}_2\text{SO}_4-\text{HNO}_3 \) (80:20 \( \text{v/v} \)) and then washed with distilled water. After rinsing with a solution of redistilled acetic acid (3\% \( \text{v/v} \)), a final thorough rinsing was made with glass distilled water.

"Visking" dialysis casing was heated to about 70\(^{\circ}\)C in 3\% (\( \text{v/v} \)) acetic acid, allowed to cool, and was soaked in several changes of distilled water before use.

2.3 REAGENTS

All reagents used were of analytical grade.

Special reagents are listed as follows:

2.3.1 General

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Grade/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>Redistilled Analytical Reagent grade.</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>Redistilled Analytical Reagent grade.</td>
</tr>
<tr>
<td>Ammonium Sulphate</td>
<td>Special Enzyme grade; Mann Research Laboratories.</td>
</tr>
<tr>
<td>Urea</td>
<td>Mallinckrodt Analytical Reagent grade; recrystallised from 70% aqueous ethanol.</td>
</tr>
<tr>
<td>Hydrolysed Starch</td>
<td>Connaught Medical Laboratories, Lot 274-1 and 309-1.</td>
</tr>
<tr>
<td>Nigrosin</td>
<td>Matheson Coleman and Bell, Water Soluble (NX390).</td>
</tr>
</tbody>
</table>
2.3.2 Carbohydrates and Related Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>BDH, Laboratory Reagent; recrystallised by E.R.B. Graham.</td>
</tr>
<tr>
<td>Glucose</td>
<td>BDH, Laboratory Reagent; recrystallised by E.R.B. Graham.</td>
</tr>
<tr>
<td>Mannose</td>
<td>BDH, Laboratory Reagent; recrystallised by E.R.B. Graham.</td>
</tr>
<tr>
<td>Fucose</td>
<td>Calbiochem A grade, Lot 71621.</td>
</tr>
<tr>
<td>Galactosamine.HCl</td>
<td>Sigma, Lot 64B-1170.</td>
</tr>
<tr>
<td>Glucosamine.HCl</td>
<td>Sigma, Lot A50B-54.</td>
</tr>
<tr>
<td>N-Acetylneuraminic Acid</td>
<td>Calbiochem, A grade, Lot 500070</td>
</tr>
<tr>
<td>p-Dimethyl-amino-benzaldehyde</td>
<td>BDH; purified according to Adams and Coleman (1948).</td>
</tr>
<tr>
<td>Acetylacetone</td>
<td>Fluka; double distilled, once under reduced pressure.</td>
</tr>
<tr>
<td>Microcrystalline Cellulose</td>
<td>Merck &quot;Cellulose Mikrokrystallin&quot; Lot 2330.</td>
</tr>
</tbody>
</table>

2.3.3 Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase</td>
<td>Behringwerke; Vibrio Cholerae 1 ml = 500 E int/u.</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Worthington Biochemical, (TRL 6256); two times recrystallised by manufacturer.</td>
</tr>
</tbody>
</table>
Chymotrypsin Worthington Biochemical, (CD16164); three times recrystallised by manufacturer.

Pepsin Worthington Biochemical, (PM689); two times recrystallised by manufacturer.

Subtilisin Novo Industries (Batch 57).

2.3.4 Radio-active Material
Iron$^{59}$ (III) citrate Radiochemical Centre, Amersham.
ca. 10 µg Fe$^{59}$/ml; 100 µCi/ml in sodium citrate.

2.3.5 Reagents for Peptide Analyses Studies
Phenyl Isothiocyanate Pierce Chemicals, sequanal grade, redistilled.

Dansyl Chloride Pierce Chemicals lot 3022-11.

Ninhydrin Pierce Chemicals Lot 8144-21.

Fluorescamine Hoffmann-Roche, "Fluram" (Lot K2033).

Dioxane Fluka, peroxide free, treated with Molecular Sieve 4A.

Silica Gel Plate (TLC) Baker, Baker-flex (15B-F)

Reference DNS-Amino Acids Calbiochem.

2.4 CHROMATOGRAPHIC MATERIALS AND PREPARATION
Sephadex G-25, G-75 and G-100, and DEAE-Sephadex A-25 and A-50 were obtained from Pharmacia as the dry powder. The Sephadex was suspended in water and left for 48 hr at room temperature. The slurry was then washed with 3 bed volumes of water followed by the same amount of the appropriate buffer. The slurry was evacuated for a few minutes, and
packed into a Pharmacia column. The columns were equilibrated by running the buffer overnight at the required temperature prior to use.

2.5 THE MEASUREMENT OF pH

The measurement of pH was carried out with a Beckman Century SS pH/selective ion Meter fitted with a miniature pH electrode assembly. Calibration of the assembly, following the N.B.S. recommended procedure (Bates, 1973), was made with (a) 0.05 molal potassium hydrogen phthalate (0.04958 M), 10.12 gl⁻¹ solution (pH 4.008 ± 0.005 at 25°C) (b) 0.01 molal sodium tetraborate (0.009971 M), 3.80 gl⁻¹ Na₂B₄O₇·10H₂O (pH 9.180 ± 0.005 at 25°C).

2.6 PREPARATION OF CONALBUMIN

Iron-conalbumin was prepared by the method of Warner and Weber (1951). The protein was recrystallised two times from ethanol-water (15-20% v/v) and stored at 3°C. Iron-free conalbumin was prepared by the addition of Dowex-1 X 8 resin in the chloride from to a concentrated solution of iron-conalbumin, according to the above authors. The protein concentration was determined by spectrophotometric measurement using A₀.₀₁ cm⁻³ at 280 nm = 11.3 for apo-protein, A₀.₀₁ cm⁻³ at 280 nm = 14.8, and A₀.₀₁ cm⁻³ at 470 nm = 0.621 for iron saturated protein (Glazer and McKenzie, 1963).

2.7 IRON(III) SATURATION CURVE OF CONALBUMIN

The iron binding capacity of conalbumin at various pH was determined spectrophotometrically.
(A) Iron-free conalbumin solution: 0.55 g protein in 100 ml imidazole-HCl buffer at the desired pH.

(B) Fe(III)-HCO₃⁻ solution: 0.1 N sodium citrate solution containing 0.02 mM Fe(III) and 3.8 mM HCO₃⁻ in one ml.

Conalbumin solution (4 ml) and Fe(III)-HCO₃⁻ solution (0.135 ml) were mixed to provide 150% excess of Fe(III) necessary to saturate two iron binding sites on the protein. The reference solution was prepared similarly from the buffer (4 ml) and Fe(III)-HCO₃⁻ solution (0.135 ml). The iron binding capacity was calculated from the reading of (conalbumin sol.: reference sol.) on the basis of the values described in 2.6.

2.8 IRON(III) SATURATION AND DISSOCIATION OF TRANSFERRIN

2.8.1 Iron (III) Dissociation

Iron was removed from transferrin by following the method of Roop and Putnam (1967). Approximately 1 g of transferrin was dissolved in 10 ml 0.6 M tris-0.3 M citrate buffer, pH 5.0. After allowing the resultant solution to stand at room temperature for 1 hr, the solution was desalted by passage through a column of Sephadex G-25 (2.7 x 50 cm) that had been equilibrated with glass distilled water.

2.8.2 Iron (III) Saturation

Iron(III)-transferrin complex was prepared by addition of 150% excess of iron(III) to transferrin in the presence of excess HCO₃⁻, at pH 7.10.

Solution A: 241 mg Fe(NH₄)₂(SO₄)₂·12H₂O in 50 ml 0.1 M sodium citrate (0.5 m mole Fe(III) in 50 ml).
Solution B: 840 mg NaHCO₃ in 20 ml water (10 m mole HCO₃⁻ in 20 ml).

To apotransferrin solution (1 g protein in 100 ml 0.05 M imidazole - 0.021 M HCl buffer, pH 7.10) were added solutions A (12.5 ml) and B (0.5 ml). This gave 150% and 500% excess of iron(III) and HCO₃⁻ respectively, assuming two iron binding sites on the protein in the solution. The pH of the protein solution was adjusted to 7.1 with 1 M NH₃. After allowing the resultant solution to stand at room temperature for 24 hr, the iron-protein complex was separated from the reaction mixture by passage through a column of Sephadex G-25 (2.7 x 60 cm) that had been equilibrated with 0.05 M imidazole 0.021 M HCl buffer, pH 7.10, at 20°C.

2.9 PROTEIN CONCENTRATION AND STORAGE

After separation, transferrin solutions were concentrated in Diaflo ultrafiltration cells (Amicon Corporation, Mass. U.S.A.). Ultrafiltration was carried out at a pressure of 2.75 x 10⁵ Nm⁻² of nitrogen at 3°C. Diaflo UM2 ultrafiltration membranes were used. Most of the concentrated protein fractions were dialysed against several changes of water for 3 days and lyophilised. Protein samples were stored at 3°C.

2.10 STARCH GEL ELECTROPHORESIS

Horizontal thin starch gel electrophoresis was carried out as described by McKenzie (1971). The apparatus used in the present work has been described by McKenzie (1971). The following buffer systems were used.
(A) Ferguson-Wallace system (1963)

Gel buffer : 0.014 M tris-0.0029 M citric acid
-0.002 M lithium hydroxide -0.0076 M
boric acid, pH 7.5-7.6.

Electrode buffer: 0.1 M lithium hydroxide -0.38 M
boric acid, pH 8.5.

(B) Tris-cacodylic acid buffer system (modification of
Spooner and Baxter system, 1969)

Gel buffer : 0.016 M tris-0.014 M cacodylic
acid, pH 7.50.

Electrode buffer: 0.3 M boric acid -0.1 M sodium
hydroxide, pH 8.7.

(C) Tris-glycine-urea buffer system (McKenzie and Treacy,
1973)

Gel buffer : 0.1 M tris-0.19 M glycine -7 M
urea, pH 8.8.

Electrode buffer: 0.1 M tris-0.19 M glycine, pH 8.8

Starch gels for electrophoresis in buffer system (A)
were made with hydrolysed starch (13 g, lot 274-1, see
2.3.1) in a buffer solution (100 ml). The starch was sus­
pended in the buffer solution (30 ml) at room temperature and
transferred to a round bottomed flask. The remaining buffer
(70 ml) was heated to 100°C and poured into a suspension of
the starch and buffer with vigorous swirling. The mixture
was heated over a naked flame until the viscosity dropped.
After removal of residual dissolved air under vacuum the
gel was poured into a tray (170 × 200 × 1.5 mm) and covered
by a plastic sheet coated with DC silicone high vacuum
grease (Dow Corning Co. Midland, Michigan, U.S.A.). A piece of plate glass and two iron weights were placed on the plastic sheet and the gel allowed to set. The gel was then allowed to stand overnight at room temperature.

The gel was cut 5 cm from one end and the samples inserted on Whatman 3 MM filter paper strips (1.5 x 15 mm). Five minutes after electrophoresis started the filter paper inserts were removed, gel contact across the sample insertion line remade, and a constant voltage (160 V) applied for approximately 4 hr. The gel was removed from the glass plate and stained in a nigrosin 0.1% (w/v) in methanol: water:glacial acetic acid (75:75:10 by vol.) for 10-15 min. The dye solution was removed and the gel soaked in methanol: water:glacial acetic acid (10:10:2 by vol.).

Starch gels in tris-cacodylic acid buffer system (B) were made with hydrolysed starch (15 g, lot 274-1) in a buffer solution (100 ml) by the same procedures as used for the gel preparation in buffer system (A). The electrophoresis was carried out with constant voltage of 270 V and the buffer boundary allowed to migrate 8-9 cm beyond the sample insertion line.

Starch gels for tris-glycine-urea buffer system (C) were prepared with hydrolysed starch (20 g, lot 309-1) 0.16 M tris -0.34 M glycine (100 ml) and urea (64 g). Starch (20 g) was suspended in the buffer (35 ml) and transferred to a round bottomed flask. Remaining buffer (65 ml) was warmed to 65°C and poured onto 64 g urea. The urea
solution was warmed to 70°C with continual stirring and poured into the well mixed slurry of starch in the round bottomed flask. The flask was placed over a naked flame with continual swirling until the viscosity dropped and the temperature was not allowed to exceed 70°C. After cooling slightly, the gel was deaerated for 30 sec. The gel was cooled to almost 20°C and the gel poured onto 0.2 ml of 2-mercaptoethanol in a beaker and stirred. The gel was poured into a tray. Electrophoresis was carried out with constant voltage of 300 V for 10 hr. The filter paper inserts were left in gel during electrophoresis (for sample preparation see 2.12.2).

2.11 IRON⁵⁹ Autoradiography

(A) Fe⁵⁶ solution : Fe⁵⁶ (NH₄)₂SO₄·12H₂O (130 mg) was dissolved in 0.05 M sodium hydrogen carbonate-0.01 M sodium citrate buffer (100 ml).

(B) Fe⁵⁶-Fe⁵⁹ solution: 35 µl of Fe⁵⁶ solution and 15 µl of Fe⁵⁹ solution (see 2.3.4) were mixed. This solution contained 100 µCi Fe⁵⁹ and 10 µg Fe⁵⁶ in 1 ml.

The labelling of proteins with Fe⁵⁹ was carried out by addition of solution (B) (5 µl) to a protein solution (25 µl, 0.5 g protein in 100 ml water). The solution was allowed to react at room temperature overnight.

After electrophoresis the gel was rapidly frozen by placing on a metal plate sitting on the surface of a dry ice-ethanol mixture and then wrapped in a thin nonporous
transparent plastic sheet. The gel was placed in contact with Kodak X-ray film in a deep freeze (-12°C) for 72 hr. The film was developed in Kodak liquid X-ray developer type 2 for 3 min, fixed in Ilford Hypam Rapid Fixer for approximately 10 min, then washed in running tap water for 1 hr.

2.12 PERFORMIC ACID OXIDATION, REDUCTION AND CARBOXYMETHYLATION

2.12.1 Performic Acid Oxidation

Before or after enzymic digestion, the disulphide bonds in the protein were cleaved by performic acid oxidation. The protein (ca. 10 mg) was dissolved in 1 ml formic acid (98% v/v) and cooled in ice. Performic acid was prepared by addition of hydrogen peroxide [0.2 ml of 30% (v/v) solution] to formic acid (2 ml) and the mixture left at room temperature for 2 hr. It was then cooled and added to the protein solution. Oxidation was allowed to occur for 2 hr at 0°C. The reaction mixture was then lyophilised to remove excess reagents. The freeze-dried protein was dissolved in a minimum of formic acid (ca. 0.5 ml). After addition of ten fold volume of water, the solution was again lyophilised.

2.12.2 Reduction and Carboxymethylation

Iron-free protein (ca. 17 mg) was dissolved in 7 M urea solution (1 ml) containing dithiothreitol (14 mg) and a drop of 0.5 M tris-1.0 M glycine buffer pH 8.8. When 2-mercaptoethanol was used as reductant in place of dithiothreitol approximately 100 µl was used against the protein (ca. 17 mg). The reaction was carried out in the dark at 3°C overnight.
For carboxymethylation freshly recrystallised monoiodoacetamide (20 mg) was added to the reduced protein sample in urea and the reaction mixture allowed to stand in the dark at room temperature for 2 hr with gentle stirring.

2.13 HIGH VOLTAGE PAPER ELECTROPHORESIS

High-voltage paper electrophoresis was performed in tanks designed after Michl (1959), using Varsol as heat exchanger. The potential gradient was 40 V cm$^{-1}$. The following buffer systems were used:

(A) Acetic acid : formic acid : water (7:2:70 by vol.), pH 1.9

(B) Acetic acid : pyridine : water (1:1.38 by vol.), pH 4.7

(C) 2% (w/v) Ammonium carbonate in water, pH 8.9

2.14 TREATMENT WITH NEURAMINIDASE

2.14.1 Protein Samples

The effect of the neuraminidase on the electrophoretic mobility of the transferrin was determined, following the procedures described below.

Transferrin (500 µg) was dissolved in 0.1 M sodium acetate -0.033M acetic acid buffer (100 µl) pH 5.5, containing 0.15 M sodium chloride and 0.02 M calcium chloride. Neuraminidase solution (80 µl, see 2.3.3) was added to the protein solution (80 µl), and the mixture was reacted at 37°C for periods varying from 0 to 72 hr. Samples of the reaction mixture were taken at various times, cooled rapidly and held at -12°C.
2.14.2 Peptide Samples

In the course of isolation of glycopeptides, partially purified glycopeptides were treated with neuraminidase prior to further purification.

A partially purified glycopeptide (0.1-0.2 mg) was dissolved in pyridine:acetic acid:water (2:1:26, by vol.) solution (50 µl), pH 5.5, and to this was added neuraminidase solution (50 µl, 25 units). Samples were reacted for 4-6 hr at 37°C. The reaction mixtures were then subjected to the two dimensional high-voltage paper electrophoresis.

2.15 TREATMENT WITH ALKALI

In order to examine possible presence of viral neuraminidase insensitive sialic acid derivatives, transferrin was subjected to alkaline treatment according to Schauer and Faillard (1968).

pH of protein solution (0.05 g protein dissolved in water and the volume made up to 5 ml) was adjusted to 10 by addition of dilute sodium hydroxide solution. The protein solution was allowed to stand for 24 hr at room temperature. After the reaction was completed the protein solution was dialysed against several changes of water for 48 hr at 3°C.

2.16 CARBOHYDRATE DETERMINATION

As the purpose of the analyses was to compare the equivalent components from the two genetic variants, and the multiple components of a single variant, the values were not corrected for the destruction of sugars during acid hydrolysis. The difficulties of correcting values by using an external standard solution have been described by Graham et al. (1970), and by Ogston (1964).
2.16.1 Sialic Acid Determination

Sialic acid was determined as N-acetylneuraminic acid by the method of Aminoff (1961) after hydrolysis of the protein.

Hydrolysis. The protein (3-5 mg) was hydrolysed in 0.2 N H₂SO₄ (2 ml) at 80°C for 1 hr. The standard N-acetylneuraminic acid was submitted to the same acid conditions.

Reagents.

Periodate : 0.025 M periodic acid in 0.125 N H₂SO₄
Arsenite : 2% (v/v) sodium arsenite in 0.5 M HCl
Thiobarbiturate : 0.1 M 2-thiobarbituric acid in water, pH adjusted to 9.0 with NaOH.
Acid butanol : 5% (v/v) of 12 M HCl in n-butanol.

Procedure. An aliquot of hydrolysate (0.5 ml) and periodate reagent (0.25 ml) were mixed in a stoppered tube and reacted at 37°C for 30 min. Arsenite reagent (0.2 ml) was added to reduce excess periodate. Two minutes later, thiobarbiturate reagent (2 ml) was added and solution was kept in a boiling water bath for 7.3 min. The coloured solution was cooled in running tap water and extracted with acid-butanol (5 ml). The butanol layer was clarified by centrifugation at 1000 rpm for 2 min, and the absorbance was measured at 549 nm in a cell with a 1 cm light path.

2.16.2 Hexosamine Determination

Glucosamine was determined by the procedure of Kraan and Muir (1957).

Hydrolysis. The protein (ca. 3 mg) was hydrolysed in 4 M HCl (2 ml) at 100°C in an evacuated and sealed tube. After
hydrolysis, the hydrolysate was transferred into a round bottomed flask. The hydrolysis tube was rinsed several times with water and the washings were added to the main hydrolysate. The mixture was evaporated to dryness by rotary evaporation. The hydrolysate was finally dried in a vacuum desiccator containing separate dishes of concentrated sulphuric acid and sodium hydroxide pellets.

Reagents.

Acetylacetone reagent: acetylacetone (2 ml, see 2.3.2) was dissolved in 0.5 M sodium carbonate (50 ml) immediately before use.

Ehrlich's reagent: p-dimethylaminobenzaldehyde (0.8 g, see 2.3.2) was dissolved in ethanol (30 ml) and 11 M hydrochloric acid (30 ml).

Procedure. The residue in the round bottomed flask was dissolved in water (1.5 ml) and 1 ml was used for the determination. Sample solution (1 ml) and acetylacetone reagent (2 ml) in stoppered tubes were immersed in a boiling water bath for 20 min. The tubes were cooled and the ethanol (5 ml) was added, followed by Ehrlich's reagent (1 ml). The tubes were shaken, heated at 68°C for 10 min and cooled. The absorbance was measured at 530 nm in a cell with a 1 cm light path.

2.16.3 Neutral Sugars Determination

The estimation of neutral sugars was made on the sugars after separation on thin-layer plates of NaBH₄-reduced-microcrystalline cellulose by a modification of the method of Park and Johnson (1949).
Hydrolysis. The transferrin (ca. 3 mg) was hydrolysed in 2M HCl (1 ml) at 100°C for 2 hr in an evacuated sealed tube. The hydrolysate was diluted to an acid concentration of 0.2 M with water. The hydrolysate was applied to a column (1 x 8 cm) of Dowex 50-X4 (100-200 mesh, H⁺ form), and the neutral sugars eluted from the column with water. The effluent (ca. 50 ml) was collected and then taken to dryness by rotary evaporation at room temperature.

Preparation of Reduced Cellulose Thin-Layer Plates. Microcrystalline cellulose (see 2.3.2) was reduced with sodium borohydride according to McKelvy and Scocca (1970). A slurry was made of the reduced microcrystalline cellulose (35 g) with water (140 ml). The mixture was blended at high speed in a Waring blender for 10 x 10 sec bursts, and filtered through fine nylon mesh to remove any lumps. The slurry was evacuated and then spread to a thickness of 0.3 mm on glass plates (200 x 200 mm), using a Unoplan Leveller (Shandon Model SA MK.II). The plates were dried overnight at 20°C, and lanes 10 mm wide were cut in the cellulose prior to use.

Separation of Individual Sugars. The sugar solutions (1 µl containing ca. 2 µg sugars) were applied to the thin layer plate with microsyringes (Hamilton), and the plate developed three times with ethylacetate:pyridine:water (12:5:4 by vol.) and once with ethylacetate. After drying the plate, the positions of the sugars were found by painting marker lanes with aniline hydrogen phthalate 2.5%(w/v) in acetone and leaving the plate in the dark overnight. The corresponding areas
of cellulose in the lanes containing the sample or standard sugar mixtures were scraped off, mixed with water (1.2 ml) and centrifuged. Portions (0.5 ml) were taken for assay by the method of Park and Johnson (1949) scaled down to half volumes.

Reagents

- Ferricyanide solution: 0.05% (w/v) K₃Fe(CN)₆ in water
- Carbonate-cyanide solution: 0.05 M Na₂CO₃ and 0.01 M KCN in water
- Iron(III) solution: 0.003 M Fe(NH₄)₂(SO₄)₂·12H₂O in 0.1% sodium dodecyl sulphate solution

Procedure. The sample solutions (0.5 ml) were mixed with carbonate-cyanide solution (0.5 ml) followed by ferricyanide solution (0.5 ml) in stoppered tubes. The solutions were immersed in a boiling water bath for 15 min. The tubes were cooled in running tap water. Iron(III) solution (2.5 ml) was added to each tube and well mixed. After standing at room temperature for 40 min. the absorbance was read at 670 nm in a cell with a 1 cm light path.

2.17 PREPARATION OF GLYCOP EPTIDES

2.17.1 Digestion

Glycopeptides were prepared from bovine transferrin by digestion with subtilisin and pronase. The conditions used for the preparation of subtilisin glycopeptides are given in flow sheet form in Fig. 2.1. Protein (ca. 500 mg)
Transferrin

Performic acid oxidation (0°C, 2 hr)

Lyophilisation

Subtilisin digestion (37°C, 2 hr)

Lyophilisation

Isolation of the glycopeptide fraction, column chromatography on Sephadex G-25

Lyophilisation

Two dimensional paper electrophoresis (pH 4.7 and 1.9). Glycopeptide-E

Elution

Neuraminidase treatment (37°C, 6 hr)

Two dimensional paper electrophoresis (pH 4.7 and 1.9). Glycopeptide-N.T

Figure 2.1 Flow sheet showing the procedures for isolation of subtilisin glycopeptides.
was taken for each glycopeptide preparation. Prior to the enzyme digestion, the protein was subject to performic acid oxidation (see 2.12.1). The oxidised protein was dissolved in 0.5% NH₄HCO₃, pH 8.0, to give a concentration of 1 g dl⁻¹ then subjected to enzyme digestion. The enzyme: substrate ratio was 1:50 (w/w), and the reaction was carried out for 4 hr at 37°C. The pH of the solution was maintained at 8.0 with addition of 1 M NH₃. When the reaction was complete, the reaction mixture was lyophilised.

2.17.2 Isolation of Glycopeptide Fraction

The enzymic digest was dissolved in 5 ml of 0.01% (w/v) NH₄HCO₃ buffer and applied to a column (2.7 × 60 cm) of Sephadex G-25 equilibrated with the same buffer. Elution was carried out with the same buffer and fractions giving a positive reaction with orcinol-sulphuric acid reagent (Graham et al., 1970) were pooled and lyophilised.

2.17.3 Fractionation of the Glycopeptide Fraction

The glycopeptide fraction (60–80 mg) was applied to Whatman 3 MM paper over a 30 cm width, and subjected to paper electrophoresis (see 2.13) at pH 4.7 for 2 hr 40 min. A guide strip, 2 cm, was cut out and stitched to a fresh sheet of Whatman 3 MM paper. The second dimensional electrophoresis (1 hr 20 min) was carried out at pH 1.9 at right angles to the initial separation. Glycopeptides were detected on guide electrophoretograms by staining with following reagents:
(A) 0.02% (w/v) Ninhydrin solution in acetone containing 0.2% (v/v) of both pyridine and acetic acid. For development, the electrophoretogram was left in the dark at room temperature.

(B) 1% (w/v) Ninhydrin solution in acetone containing 2% (v/v) pyridine (Fletcher et al., 1963). For development, the electrophoretogram was left in oven at 80°C for 5 min.

(C) Alkaline silver nitrate reagent (Trevelyan et al., 1950). The electrophoretogram was sprayed lightly with 0.02 M sodium metaperiodate in water and left to dry for 2 hr. The electrophoretogram was then passed through rapidly in silver nitrate solution, prepared from saturated silver nitrate solution in water (1.5 ml) and acetone-water (300 ml), and allowed to stand in air for 15 min. 0.5 M sodium hydroxide in aqueous ethanol was sprayed on the electrophoretogram and the latter was dried in air. The electrophoretogram was washed with 0.2 M sodium thiosulphate for 10 min then finally washed in running water for 1 hr.

Using the developed guide electrophoretogram as marker the area of the first dimensional electrophoretogram containing glycopeptides were cut out and stitched to a fresh sheet of Whatman 3 MM paper for second dimensional electrophoresis at pH 1.9. Glycopeptides were eluted from the electrophoretograms with 0.5 M NH₃ and brought to dryness in a vacuum desiccator containing separate containers of concentrated H₂SO₄ and NaOH pellets.
2.18 AMINO ACID ANALYSIS

Analyses of acid hydrolysates of the proteins and peptides were performed, essentially by the method of Moore et al. (1958) on a Beckman Model 120C amino acid analyzer with two columns. The short column, which contained Beckman Custom Spherical Resin PA-35, was eluted with 0.116 M sodium citrate -0.099 M HCl buffer pH 5.28. The long column, which contained Beckman Custom Spherical Resin AA-15, was eluted first with 0.066 M sodium citrate -0.151 M HCl buffer, pH 3.28, and then with 0.066 M sodium citrate -0.101 M HCl buffer, pH 4.25. The flow rate of buffers was 60 ml hr⁻¹ and chromatography was carried out at 52°C.

2.18.1 Amino Acid Analyses of Acid Hydrolysates

Samples (ca. 1 mg protein) were hydrolysed with 6 M HCl (2 ml) and a drop of saturated phenol solution in evacuated sealed tubes (tubes were evacuated by an Edwards single stage model 1SC30A pump) at 110°C for 22 hr. (The presence of phenol during hydrolysis minimised degradation of tyrosine residues due to oxidative halogenation. The expected yield of tyrosine in these conditions was at least 95%). Hydrochloric acid was removed by rotary evaporation and the residue dissolved in the 0.066 M sodium citrate buffer, pH 2.2, of Moore et al. (1958).

The total half-cystine contents of the protein was determined as cysteic acid after oxidation of protein with performic acid and hydrolysis in 6 M HCl for 22 hr at 110°C. The determination of the half cystine residues was also made on protein whose cysteine residues were fully converted to S-carboxymethylcysteine residues.
As the purpose of the analyses was to compare the two genetic variants, and the multiple components of a single variant, the values were not corrected for decomposition of serine and threonine nor for slow liberation of isoleucine and valine.

2.18.2 Amino Acid Analyses of Alkaline Hydrolysates

The possible presence of γ-carboxyglutamic acid residues in protein was examined upon analysis of the alkaline hydrolysate of protein. Alkaline hydrolysis was carried out mainly as described by Hugli and Moore (1972).

Protein (5-7 mg) was suspended in 4 M NaOH (0.5 ml) in a Nalgene polythene centrifuge tube (10.9 x 50 mm). The polythene tube was placed in a thick wall hydrolysis tube (15 x 150 mm). The glass tube was constricted and the lower portion of the tube was cooled in an ethanol-dry ice bath until the content was frozen. The tube was evacuated and sealed before the contents began to melt. After hydrolysis at 110°C for 24 hr, the tube was removed from the oven and allowed to cool at room temperature. The hydrolysis tube was stored at -12°C when analysis was not performed immediately. The hydrolysate was transferred to a tube containing 6 M HCl (0.335 ml) cooled in an ethanol-dry ice bath. The polythene tube was rinsed with 0.6 ml of the dilution buffer containing Brij 35, pH 2.2, and the buffer added to the main hydrolysate. Final volume of the sample was approximately 1.35-1.40 ml. The sample (250 µl) was injected into the long column of the Beckman Model 120°C amino acid analyzer.
2.19 AMINO TERMINAL ANALYSIS

2.19.1 Amino Terminal Analysis of Protein Samples

The N-terminal residue of the protein was identified by dansylation in 8 M urea (Gros and Labouesse, 1968) and by the paper-strip modification of the Edman reaction (Schroeder, 1967).

**Dansylation of protein in 8 M urea**

- Protein (1 mg)
- Recrystallised urea (250 mg)
- Water (0.3 ml)
  - 0.4 M Phosphate buffer, pH 8.2, (0.15 ml)
  - Dimethylformamide (0.25 ml)
  - 0.2 M Dansyl chloride in acetonitrile (0.1 ml)

Overnight reaction at room temperature

- 20% (w/v) Trichloroacetic acid (0.9 ml)

Precipitation of protein

Centrifugation

Washing of the precipitate with 1 M HCl (2 x 1 ml)

Suspension of the precipitate in 6 M HCl (2 ml)

Hydrolysis for 4 hr at 110°C

Evaporation of HCl by rotary evaporation

Solution of the residue in pyridine:acetic acid:water (1:10:189 by vol.) (0.5 ml)

Extraction with ether (3 x 0.5 ml)

Concentration and evaporation to dryness of the ether phase

- 50% Aqueous pyridine (20 µl)

Identification, on the thin layer plate of silica gel (see 2.3.5) with the solvent system; chloroform:tertamyl alcohol:acetic acid (70:30:0.25 by vol.) (Deyl and Rosmus, 1965).
Paper-strip Modification of the Edman Reaction

Aqueous protein solution (1 mg in 50 µl water) on a paper strip (1 x 5 cm) of Whatman No.1 filter paper.

Drying in air

Saturation with 20% phenyl isothiocyanate (see 2.3.5) in dioxane (see 2.3.5) (70 µl).

Reaction at 40°C for 3 hr in a small sealed cylinder which contained water:pyridine:dioxane (1:1:1 by vol.).

Drying the strip slightly in air

Extraction with benzene (3 x 10 ml)

Drying the strip in air

Cyclization reaction in the desiccator containing separately 6 M HCl (100 µl) and acetic acid (100 ml) under reduced pressure (by water pump) overnight.

Removal of acids from the strip in air

Extraction of phenylthiohydantoin-amino acid with alcohol:ether (1:1 v/v)

Evaporation of alcohol and ether

+ 6 M HCl (2 ml)

Hydrolysis for 68 hr at 110°C in an evacuated sealed tube

Amino acid analysis
2.19.2 Amino Terminal Analysis of Peptide Samples

The N-terminal residue of the glycopeptides isolated from enzymic digests of transferrin were determined by dansyl (DNS)-Edman degradation.

The Edman procedure was basically that described by Gray (1967). The dansylation method was basically that described by Gros and Labouesse (1968).

Peptide (30–50 n mol) in 50% aqueous pyridine

(200 µl)

+ 5% (v/v) Phenylisothiocyanate in pyridine

(100 µl)

Reaction for 1 hr at 45°C in a nitrogen atmosphere

Evaporation of excess reagent, by-products under vacuum at 60°C for 30 min (Edwards single stage vacuum pump)

+ Trifluoroacetic acid (200 µl)

Reaction for 30 min at 45°C in a nitrogen atmosphere

Removal of trifluoroacetic acid under vacuum 60°C

+ Water (0.5 ml)

Extraction of diphenylthiourea with n-butylacetate

(3 × 1.5 ml)

→ Removal of a portion of sample for dansylation.
Drying the sample residue (ca. 5 n mol)

- 35 mM NaHCO₃, pH 8.3 (30 µl)
- 10 mM Dansyl chloride in acetone (30 µl)

Dansylation reaction for 2 hr at room temperature

Drying the reaction mixture under vacuum

- 6 M HCl (2 ml)

Hydrolysis for 4 hr at 110°C in an evacuated sealed tube

Removal of HCl from the hydrolysate

- 50% aqueous pyridine (20 µl)

Identification of DNS-amino acid on silica gel (see 2.3.5)

The following solvent systems were used for thin layer chromatography

(A) Chloroform:ethanol:acetic acid (38:4:3 by vol.)

(B) n-Butanol:pyridine:acetic acid:water (15:10:3:12 by vol.)

(C) Benzene:pyridine:acetic acid (16:4:1 by vol.)

(D) Petroleum ether: tert-butanol:acetic acid (5:1:1 by vol.)

System (A)(B)(C) : Deyl and Rosmus (1965)

System (D) : Mesrob and Holeysonsky (1966)
2.20 PEPTIDE MAPPING

2.20.1 Tryptic Peptide Mapping

The protein in water (ca. 10 mg in 2 ml water) was denatured prior to the enzyme digestion by addition of 20% (w/v) trichloroacetic acid solution (1 ml). The resulting precipitate was collected by centrifugation and washed with 20% trichloroacetic acid solution, water and ethyl ether. After being allowed to stand overnight in air, the denatured protein was digested in 0.5% (w/v) ammonium hydrogen carbonate buffer, pH 8.0, with trypsin (see 2.3.3) (enzyme:substrate = 1:50) for 4 hr at 37°C. The ammonium hydrogen carbonate and water were removed by lyophilisation after the digestion.

For tryptic peptide mapping, performic acid oxidation was carried out after the enzyme digestion so that the digestion would not be impeded by production of cysteic acid residues adjacent to lysine or arginine (Shaw, unpublished results). Performic acid oxidation of the enzyme digest was carried out as described in 2.12.1. After oxidation the tryptic digests were dissolved in water (ca. 1 ml) and the pH adjusted to 8.0 by addition of 0.5% (w/v) ammonium hydrogen carbonate. No precipitate occurred. The pH of the solution was then adjusted to 4.0 by the addition of glacial acetic acid (ca. 20 µl), when faint precipitation occurred (core precipitate at pH 4). The precipitate was separated by centrifugation and the separated soluble peptide fraction was lyophilised ready for peptide mapping.
Peptide maps were prepared on Whatman 3 MM paper. When hydrolysates were to be compared, they were subjected to electrophoresis in parallel for the first dimension. Peptide (ca. 4 mg) was dissolved in a minimum of water (ca. 60 µl), applied to a Whatman 3 MM paper as a 2 cm band. High-voltage paper electrophoresis was performed at the desired pH as described in 2.13. The strips containing the samples were cut out and sewn onto fresh sheets of paper for chromatography at right angles to the initial separation. The following buffer systems were used for paper chromatography:

(A) Pyridine:isoamyl alcohol:water (7:7:6 by vol.)
(B) n-Butanol:pyridine:acetic acid:water (15:10:3:12 by vol.)

2.20.2 Chymotryptic Peptide Mapping

The disulphide bonds of the protein were cleaved by performic acid oxidation prior to chymotryptic digestion. The oxidised protein was then digested with chymotrypsin (enzyme:substrate = 1:50 w/w) in 0.5% (w/v) ammonium hydrogen carbonate solution at 37°C for 4 hr. The hydrolysates were lyophilised. The procedure for the peptide mapping was identical to that described in 2.20.1.

2.20.3 Perfomic Acid Diagonal Peptide Mapping

The method of performic acid diagonal peptide mapping developed by Brown and Hartley (1963) was used. Protein samples, denatured by trifluoroacetic acid, were separately digested with pepsin (enzyme:substrate = 1:50) in 0.01 M HCl (5 ml), adjusted to pH 2.0 with 0.1 M HCl,
at 37°C for 4 hr. The hydrolysates were lyophilised. Samples of the digests containing the equivalent of ca. 4 mg original protein were subject to high-voltage paper electrophoresis at pH 1.9 (see 2.13) as a 2 cm band on Whatman 3 MM paper. After drying, the paper strips were exposed to performic acid vapour (see 2.12.1) for 6 hr in a sealed container. The strips were then vacuum dried over KOH pellets, stitched onto a full sheet of Whatman 3 MM paper and subjected to electrophoresis at right angles to the original direction under the same conditions as used in the first dimension. Separate diagonal maps were prepared in this way for each of the digests, one at pH 1.9 and one at pH 4.7 using the buffer systems described in 2.13.

2.20.4 Detection of Peptides

*Ninhydrin Staining.* Peptides were detected principally by staining with 0.02% (w/v) ninhydrin (see 2.3.5) solution in acetone containing 0.2% (v/v) with respect to glacial acetic acid and pyridine. Colour was developed by leaving the sheets in the dark overnight at room temperature.

*Fluorescamine Staining.* The possible presence of ninhydrin insensitive peptides was examined by staining with fluorescamine (see 2.3.5) (0.001% (w/v) fluorescamine in acetone containing 3% (v/v) pyridine). Fluorescing peptides were detected by illumination with UV light.

*Specific Staining for Arginine Containing Peptides* (Roberts, 1975).

Solution A : 0.1% (w/v) 9:10 phenanthroquinone in ethanol

Solution B : 10% (w/v) NaOH in 60% (v/v) ethanol.
Equal volumes of Solution A and B were mixed and sprayed on the chromatogram. After reaction in an oven at 50°C for 5 min. arginine containing peptides were located by UV illumination. Highly blue-green fluorescence was observed for arginine containing peptides.

Specific Staining for Tyrosine and Histidine Containing Peptides (Bennett, 1967)

Solution A: 1% (w/v) sulphanilic acid in 1 M HCl (100 ml)

Solution B: 1% (w/v) NaN02 in water (100 ml)

Solution B: 10% (w/v) Na2CO3 in water (100 ml)

All the solutions were cooled in an ice bath prior to mixing. Solution A and B were mixed and cooled in the ice bath. Solution C was added to the above mixture immediately before use. After spraying the reagent on the chromatogram, the latter was left in air overnight. Tyrosine containing peptides stained to a reddish brown colour and histidine peptides, cherry-red.

2.20.5 Isolation of Peptides from Peptide Maps

The maps, which were stained with 0.02% ninhydrin or 0.001% fluorescamine, were washed free of excess reagent with acetone, and the peptides under investigation were cut out from the sheet, eluted from the paper with 6 M HCl (2 ml) and hydrolysed for 22 hr at 110°C.

2.21. PROTEIN CLEAVAGE BY CYANOGEN BROMIDE (CNBr)

CNBr cleavage of bovine transferrin was performed essentially according to the procedures of Tsao et al. (1974).
2.21.1 CNBr Treatment

Apotransferrin (ca. 100 mg) was dissolved in ice-cold 70% (v/v) formic acid (3 ml) and to this was added 2 ml ice-cold CNBr solution [10% (w/v) CNBr in 70% formic acid] to provide 150:1 molar ratio of CNBr to methionine (assuming 9 residues of methionine in a molecule of the protein). The solution was allowed to react in a nitrogen atmosphere at 3°C for 40 hr with gentle stirring. The reaction was terminated by diluting 10-fold with water and the solution lyophilised.

2.21.2 Isolation of CNBr Fragments

The CNBr digest of bovine transferrin was fractionated on a column (2.7 x 65 cm) of Sephadex G-75, equilibrated and eluted with 1% formic acid. The lyophilised digest was dissolved in 70% formic acid (3 ml) and applied to the column. Seven ml fractions were collected at a flow rate of 20 ml hr⁻¹. The absorbance was measured at 280 nm in a cell with a 1 cm light path.
CHAPTER 3. ISOLATION OF TRANSFERRIN

3.1 INTRODUCTION

Although various methods have been developed for the isolation of transferrins, procedures for the separation of the polymorphic forms and also the individual components of a single variant have to be developed in each case before structural study of the differences can be properly undertaken. It is necessary to devise a combination of procedures which (1) rapidly eliminate the bulk of the proteins, (2) permit separation of the whole transferrin variant and (3) permit separation of individual components of a single variant in the final stages.

If the components isolated are to be satisfactory for both physical and chemical studies they must be as close as possible in properties to the native protein. These requirements impose certain constraints on the methods of fractionation that can be used. There must be no alteration in conformation of the protein, and the danger of -S-S- interchange reactions must be minimised. This means working in a pH region such that the protein does not undergo the transition that may occur below pH 4.6, but at a pH that is sufficiently low to minimise interchange reactions (< pH 7). At the same time a further constraint is the need to use a chromatographic method of high resolution and this is pH dependent. Thus the range of pH in which all these criteria can be met is somewhat limited, ca. 6.0 to 7.0.
Furthermore, iron-transferrin, which possesses greater stability than apo-transferrin, dissociates into apotransferrin at pH below 6, the extent of dissociation depending on the pH and the presence and concentration of anions (e.g. citrate, phosphate).

The procedures developed here may be summarized as follows:

(a) Blood collection in acid-citrate. Separation of blood cells.

(b) \((\text{NH}_4)_2\text{SO}_4\) Precipitation of crude fraction containing transferrin from the plasma.

(c) Preparation of crude whole transferrin

(d) Preparation of pure whole transferrin

(e) Isolation of individual components of a given variant.

The first step, elimination of the bulk of the protein from plasma has been usually carried out by three main procedures, involving one or more of ethanol (Inman et al., 1961), rivanol (2-ethoxy-6, 9-diaminoacridine lactate) (Boettcher et al., 1958), and ammonium sulphate (Schultze et al., 1957). However it is known that the native conformation of proteins may be perturbed or appreciably altered by the addition to their aqueous solution of various organic solvents. Certain type of organic bases, including rivanol, display the property of forming insoluble complexes with proteins at neutral or slightly alkaline pH. However, their prospects as agents of fractionation have been jeopardized by the difficulty
of dissociating the protein-detergent complexes without damaging the proteins. Possible presence of partially denatured protein was reported in bovine transferrin fraction prepared by rivanol fractionation (Chen and Sutton, 1967).

With regard to the second step several methods have been published for the purification of transferrins based on column chromatography (Parker and Bearn, 1962a; Bezkorovainy et al., 1963). These are however, best applied on a relatively small scale and are not satisfactory in amount required for structural investigations.

As mentioned above, pH is one of the critical factors during the fractionation in order to obtain intact protein. Possible exchange reaction of the disulphide bond during protein preparation by increasing pH was discussed by McKenzie (1970). Care must be therefore taken to maintain pH as close as possible to the natural pH so that formation of isomers caused by disulphides interchange reaction can be avoided.

A procedure described here was devised to prepare appropriate amounts of transferrin under gentle conditions by combination of ammonium sulphate precipitation and a series of steps involving ion exchange chromatography.

3.2 RESULTS

The conditions finally recommended for the isolation of transferrin are given in flow sheet form in Fig. 3.1.
### Bovine Blood

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitate (blood cells) discarded</td>
<td>in acid-citrate, pH of mixture 7.0-7.1 at room temp</td>
</tr>
<tr>
<td>Precipitate (residual blood cells) discarded</td>
<td>Centrifugation (Junior 11LS, Head 5010; 3000 rpm, 30 min at room temp)</td>
</tr>
<tr>
<td>Precipitate 1st (NH₄)₂SO₄ Fraction discarded</td>
<td>pH 7.1 (NH₄)₂SO₄ 220 g l⁻¹; 3 hr stirring, standing overnight</td>
</tr>
<tr>
<td>Precipitate 2nd (NH₄)₂SO₄ Fraction</td>
<td>pH 7.1 (NH₄)₂SO₄ 85 g l⁻¹, 3 hr stirring 3 hr standing</td>
</tr>
<tr>
<td>Supernatant discarded</td>
<td>Centrifugation (Sorvall RC2-B, GSA Head; 9000 rpm, 30 min at 20°C)</td>
</tr>
<tr>
<td>Precipitate 3rd (NH₄)₂SO₄ Fraction (transferrin rich fraction)</td>
<td>Supernatant pH 7.1 (NH₄)₂SO₄ 135 g l⁻¹; 3 hr stirring, standing overnight</td>
</tr>
<tr>
<td>Supernatant discarded</td>
<td>Centrifugation (Sorvall RC2-B, GSA Head; 9000 rpm, 30 min at 20°C)</td>
</tr>
</tbody>
</table>

- **Dialysis** against 0.05 M imidazole-0.041 M HCl buffer, pH 6.85, at 3°C
- **Ion exchange chromatography** on DEAE-Sephadex A-25 in the same buffer at 3°C
- **Concentration and dialysis** against 0.05 M imidazole-0.021 M HCl buffer, pH 7.10, at 20°C
Figure 3.1 Flow sheet showing the method used to prepare bovine transferrin and separate its components.
3.2.1 Ammonium Sulphate Fractionation of Plasma Proteins

Ammonium sulphate has a long history as a useful precipitant for the production of crude protein fractions. One well-known convenience of ammonium sulphate is that it can be best employed near neutral pH (Dixon and Webb, 1961).

Plasma proteins in acid-citrate solution were precipitated stepwise by slow addition of solid ammonium sulphate (see 2.3.1) at pH 7.0-7.1 at room temperature. Yield of each fraction was 56 g, 13 g and 60 g in wet weight when the fractionation started with 1 litre of blood.

Fig. 3.2 shows electrophoretic patterns of three fractions obtained by ammonium sulphate fractionation. The supernatant after the third ammonium sulphate precipitate gave no protein bands on the starch gel electrophoresis, and was discarded. Although clear cut separation of transferrin from the other plasma proteins was not obtained, the third fraction was found rich in transferrin and served as a starting material for isolation of transferrin.

3.2.2 Choice of Buffer System for Chromatography

Prior to the further purification of transferrin, choice of the buffer system for chromatography was made, taking account of the conditions described below.

(a) The pH of the buffer should be within the range described in 3.1.

(b) The iron-transferrin complex should not be dissociated in the buffer used.

(c) The transferrin components can be separated by chromatography using the buffer system.
Figure 3.2  Starch gel electrophoresis of $(\text{NH}_4)_2\text{SO}_4$ fractions of cattle plasma. Tris-cacodylic acid buffer system, pH 7.5; 270V, 4 hr.

1. The first $(\text{NH}_4)_2\text{SO}_4$ fraction
2. The second $(\text{NH}_4)_2\text{SO}_4$ fraction
3. The third $(\text{NH}_4)_2\text{SO}_4$ fraction
Starch Gel Electrophoresis of $(NH_4)_2SO_4$ Fractions
The conditions, except (c), were examined by using conalbumin (domestic hen ovotransferrin) as iron binding protein since a previous comparison of iron binding properties of serum transferrin and ovotransferrin by Aisen et al. (1966) had revealed an overall similarity. Fig. 3.3 shows iron(III) saturation of apo-ovotransferrin in 0.05 M imidazole - 0.027-0.041 M HCl buffer at various pH values (see 2.7). The lowest pH at which conalbumin can bind with iron(III) fully in the presence of this buffer under the conditions of the experiment was found to be 6.9.

The possibility that iron is removed by the buffer components from the protein-iron(III) complex was examined by passing the fully iron saturated conalbumin through a Sephadex G-75 column, equilibrated with 0.05 M imidazole - 0.027 M HCl buffer, pH 6.9, at 20°C. The fully iron saturated protein was eluted from the column, indicating no dissociation of iron-protein complex by the buffer components. Although it was found that conalbumin can bind iron completely at pH as low as 6.9 under the conditions used a slightly higher pH, 7.1, was chosen as the buffer system for the chromatography of transferrin in order to be certain that there is 100% iron saturation of protein in the buffer.

3.2.3 Preparation of Partially Purified Transferrin

(i) Fractionation of the third (NH₄)₂SO₄ fraction of Sephadex G-75. An elution profile of the third (NH₄)₂SO₄ fraction from a Sephadex G-75 column (5 × 60 cm) is shown in Fig. 3.4. Assuming that the third (NH₄)₂SO₄ fraction contains approximately 50% transferrin, 150% excess of
Figure 3.3  Fe(III) saturation curve of conalbumin in 0.05 M imidazole - 0.027 to 0.041 M HCl buffer at various pH values at 20°C. See text (2.7) for detail.
Figure 3.4 Column profile for the fractionation of the third \((NH_4)_2SO_4\) fraction on Sephadex G-75 (5 x 60 cm), using 0.05 M imidazole - 0.021 M HCl buffer (pH 7.10, 20°C). Flow rate = 20 ml hr\(^{-1}\).
iron(III) (5 atoms iron per molecule transferrin) was added to the protein solution in the buffer prior to the chromatography.

Two major and one minor peaks were observed and the protein from each of them was studied by starch gel electrophoresis (Fig. 3.5). The first peak (1), pink in colour, is apparently a protein having a large molecular size (at least greater than 100,000) and gave a smeared pattern on starch-gel electrophoresis. The pink colour of the fraction disappeared by dialysis against water, indicating non-specific binding of iron to the proteins in this fraction. This fraction may represent globulin(s). The second peak (2) contained primarily transferrin and serum albumin. This fraction retained its salmon-pink colour after dialysis against water. Protein in peak (3), reddish-pink in colour, absorbed strongly at 465 nm, was heterogeneous and contained protein with electrophoretic mobilities which were equal to, less than, and greater than that of transferrin (Fig. 3.5). The reddish-pink colour of this fraction turned to brown after dialysis against water. The protein in fraction (3) is likely to be hemopexin which has been reported to be a very common contaminant of transferrin (Korinek et al., 1961). Elution from Sephadex G-100 column of the third \((\text{NH}_4)_2\text{SO}_4\) fraction showed a very similar pattern to that from Sephadex G-75. No improvement was obtained with respect to the separation of transferrin from albumin.

(iii) DEAE - Sephadex A-25 Chromatography of the third \((\text{NH}_4)_2\text{SO}_4\) Fraction. Although some separation of the third \((\text{NH}_4)_2\text{SO}_4\) fraction was effected by gel filtration on
Figure 3.5  Starch gel electrophoresis of fractions from the Sephadex G-75 fractionation of the third (NH₄)₂SO₄ fraction (Fig. 3.4). Ferguson and Wallace buffer system, pH 7.5; 160 V, 4 hr. Samples from left to right:

- The third (NH₄)₂SO₄ fraction
- Peak 1 of Fig. 3.4
- Peak 2 of Fig. 3.4
- Peak 3 of Fig. 3.4
- The third (NH₄)₂SO₄ fraction
Fractionation of the Third Ammonium Sulphate Precipitate
Sephadex G-75, the transferrin and albumin were not separated. It was found, however, that a partially purified transferrin could be obtained in appreciable amounts by passing the third (NH₄)₂SO₄ fraction through a column of DEAE-Sephadex A-25 (4.7 x 70 cm) equilibrated with 0.05 M imidazole-0.041 M HCl buffer, pH 6.85 (at 3°C). Albumin was strongly absorbed, but transferrin, which could be detected by its pink colour, moved down the column as a relatively narrow band. Samples of transferrin obtained in this way showed slight contamination with serum albumin, trace amounts of a large size molecule (probably globulin) and hemopexin, as judged from the starch gel electrophoresis pattern (Fig. 3.7). It was possible to pass the whole third (NH₄)₂SO₄ fraction from 1 litre blood through the column at one time. Approximately 500 mg of crude transferrin were obtained from 1 litre of blood.

3.2.4 Further purification of Transferrin

Further purification of whole transferrin was performed by column chromatography on DEAE-Sephadex A-50 with a linear gradient of NaCl (Fig. 3.6). One small peak, probably hemopexin, emerged immediately from the column and the main peak was eluted when the molarity of the gradient was raised. Residual albumin was strongly bound to the Sephadex and not eluted with the NaCl gradient used.

Starch gel electrophoresis of the transferrin containing peak material showed the presence of four transferrin bands, and the bands of albumin, hemopexin and globulin were absent, indicating that transferrin sample thus obtained was free from the contamination (Fig. 3.7).
Figure 3.6  Column profile for the final purification of whole bovine transferrin on DEAE-Sephadex A-50 (1.7 x 40 cm), using a NaCl gradient in 0.05 M imidazole - 0.021 M HCl buffer (pH 7.10, 20°C). Flow rate = 20 ml hr⁻¹.
Figure 3.7  Starch gel electrophoresis of transferrin.  
Tris-cacodylic acid buffer system, pH 7.5;  270 V, 4 hr.  
Samples from left to right:  
  The third (NH₄)₂SO₄ fraction  
  Partially purified transferrin (after passage through DEAE-Sephadex A-25)  
  Purified transferrin (after fractionation on DEAE-Sephadex A-50, Fig. 3.6)
Purification of Transferrin
3.2.5 Isolation of Individual Components of a Single Transferrin Variant

(i) Choice of Buffer System for Starch Gel Electrophoresis.

For the separation of individual components of a given genetic variant, it is critical to choose the buffer system for starch gel electrophoresis which has the highest resolution for the transferrin components. Therefore, prior to the fractionation of transferrin, several buffer systems were examined. These included the systems reported by Gahne (1963), Bailey and Kiddy (1972), Ferguson and Wallace (1963), Kristjansson and Hickman (1965), Makarechian and Howell (1966), and Spooner and Baxter (1969).

Four strong bands and two faint bands were observed on gels prepared from the buffers of the above last two systems both tris-cacodylic acid system, whereas the other buffer systems separated the transferrin into three strong and one faint bands. There was not much difference in the patterns observed for the Makarechian-Howell and Spooner-Baxter systems. The latter system was chosen for the present study, but with slight modification, the pH being altered from 7.45 to 7.50.

(ii) Isolation of Individual Components. Chromatography on DEAE-Sephadex A-25 and CM-Cellulose 52 was found to be inadequate for the resolution of the components from each other. It was found, however, that the use of long column of DEAE-Sephadex A-50 with a shallow salt gradient gave a satisfactory separation of individual components corresponding to the bands of a single genetic variant found on the
starch gel electrophoresis. Although with this method there was some overlapping of individual components, it was possible to separate the individual components from whole transferrin. Rechromatography of isolated individual components on the same column improved the purity of each component. However, it was difficult to obtain a single component completely free from contamination of any other component. Fig. 3.8 shows the separation of the components of bovine transferrin D2 type. Four major and two minor peaks were observed in the transferrin region. These peaks were found to correspond to the individual bands observed on the starch gel electrophoresis (Fig. 3.9A). The isolated components were designated I, II .... VI, in the order of elution from the column. This order also corresponds to the order of increasing mobility on starch gel electrophoresis at pH 7.5. Gel electrophoresis of rechromatographed four major components (I, II, III and IV) showed identical pattern to that of initially separated samples, indicating that all individual components separated are genuine components, not artifactual forms reflecting chromatographic interaction. When the individual components obtained in this way were converted into apoproteins and labelled with Fe59 (III) (see 2.11), the autoradiography showed that all the components bound iron, indicating that, all the components isolated are transferrins (Fig. 3.9B). Fe59 autoradiography of the isolated individual components also revealed the presence of at least two faster moving iron binding components than component VI in the fraction containing mainly VI, and one
Figure 3.8  Column profile for the isolation of individual components of a single transferrin variant on DEAE-Sephadex A-50 (1.7 x 60 cm), using a NaCl gradient in 0.05 M imidazole - 0.021 M HCl buffer (pH 7.1, 20°C). Flow rate = 20 ml hr⁻¹.
Figure 3.9 (A)  Starch gel electrophoresis of fractions from the DEAE-Sephadex A-50 fractionation of the whole transferrin (Fig. 3.6). Tris-cacodylic acid buffer system, pH 7.50; 270 V, 4 hr. Samples from left to right:

Whole transferrin D$_2$
Component I
Component II
Component III
Component IV
Component V
Component VI
Whole transferrin D$_2$
Starch Gel Electrophoresis of Fractions of Crude Tf D2 D2 from DEAE Sephadex A-50, pH 7.1
Figure 3.9 (B)  Iron$^{59}$ autoradiography of fractions from the DEAE-Sephadex A-50 fractionation of the whole transferrin (Fig. 3.6). The conditions used were the same as for Fig. 3.9 (A). After electrophoresis the location of iron$^{59}$ was detected as described in 2.11.
Fe$^{59}$ Autoradiography

Electrophoresis Patterns of Tf D$_2$D$_2$
slower moving component behind component I in the fraction containing mainly I.

Individual components of transferrin A type were isolated with identical procedures. Fig. 3.10 shows schematic diagram of four major and two minor components isolated from the two transferrin variants A and D₂.

3.3 DISCUSSION

In this chapter is described the development of purification procedures that yielded highly purified whole transferrin of a single genetic variant and highly purified components (four major and two minor) from a single genetic variant under mild conditions. The procedures used here have proved capable of meeting requirements described in 3.1. The absence of heterogeneity in the four major transferrin components isolated was demonstrated by electrophoresis pattern on starch gel at pH 7.5 and Fe⁵⁹ autoradiography.

Of the particular interest is the number of iron binding components in a single transferrin variant. Choice of buffer system for electrophoresis is critical to the investigation. It was found that by slight alteration of the concentration of the components and pH of the gel buffer, the degree of separation of the different bands of transferrin could be increased. Presence of three main protein bands with a faint fourth band in front was observed in bovine homozygous transferrin in earlier studies by other workers (Smithies and Hickman, 1958; Kristjansson and Hickman, 1965). With a further refinement of method it has been possible to divide these further into four main bands and two faster faint bands (Makarechian and Howell, 1966; Spooner and
Figure 3.10  Schematic diagram showing the electrophoretic mobilities of four major and two minor components of bovine transferrin A and D₂, at pH 7.5.
Baxter, 1969). It was clearly shown by Makarechian and Howell that all the bands in the transferrin region, detected with the protein stain used, were iron(III) binding by iron$^{59}$ autoradiography.

The present results, using tris-cacodylic acid buffer system confirm the presence of four major components in bovine transferrin. However, the fastest two minor components (V and VI) were very faint when the isolated whole transferrin was applied to the electrophoresis and it has not always been possible to detect them before the isolation of individual components.

Ion exchange column chromatography of whole transferrin resulted in the separation of four major and two minor components, and confirmed the presence of the two minor components reported by Makarechian and Howell, and by Spooner and Baxter.

Recently Stratil and Spooner (1971) observed four major and two minor components on starch gel electrophoresis of a single homozygous bovine transferrin variant in line with the observation made by Makarechian and Howell and the present work. They isolated these six components by fractionation of whole transferrin on DEAE-Sephadex A-50. However, in addition to those six bands the presence of six more minor bands were identified on starch gel by concentration of the isolated major fractions, although these components were not isolated. Although all of these six additional bands were not detected in the present work, at least three of them were found in the fractions which contained component I and VI respectively (Fig. 3.9b). These additional
three components appear to occur with very low concentration in the plasma and to be made possible to detect by fractionation of whole transferrin and concentration. It is likely that the three remaining additional components which were not detected in the present work can be detected by further concentration of the protein fractions after fractionation of whole transferrin on DEAE-Sephadex A-50.
CHAPTER 4. THE CHEMICAL DIFFERENCE BETWEEN BOVINE TRANSFERRIN A AND D₂

4.1 INTRODUCTION

As described in the GENERAL INTRODUCTION (1.5) genetically controlled variant forms of transferrin that differ in electrophoretic mobility are known for almost all vertebrates. Of these only for human transferrin has the structural basis been even partially elucidated.

Since the discovery of human serum transferrin polymorphism, several authors have isolated individual human transferrin phenotypes by various methods and demonstrated differences in the peptide maps after trypsin or chymotrypsin digestion. The deviant peptides were isolated, and structural differences which would explain the differences in zone electrophoresis were indicated by their amino acid composition (GENERAL INTRODUCTION 1.5). The single amino acid substitutions found were sufficient to explain the electrophoretic differences between the variants examined in all these cases.

There have been so far no reports on the structural differences in transferrin variants of the other species than human, although the possible presence of amino acid substitutions were suggested by Richardson et al. (1973) and Spooner et al. (1975) in bovine and sheep variants respectively.

As a more intensive search has been made for
variants of many proteins, more types of variation have been found. These include substitution of two amino acids (Bookchin et al., 1966) and insertion or deletion of portion of peptide sequence (Jones et al., 1966; and Bradley et al., 1967). In view of the great genetic variability in bovine serum transferrin, it is possible that more than one amino acid residue difference exists between any two genetic variants.

In this chapter a description is given of work that is aimed at elucidating the nature of the chemical difference of two homozygous variants of bovine serum transferrin, namely AA and D₂D₂. First an attempt is made to determine what difference occurs in the polypeptide chain. Then possible differences in carbohydrate moiety are sought. Since a single homozygous bovine transferrin variant is apparently composed of several analogous molecules with different electrophoretic mobilities (see Fig. 3.10) it is necessary to use a single equivalent component for a comparison of the two variants in order to avoid possible misinterpretation of the results obtained. Component I, which has the slowest mobility of the four major components on starch gel electrophoresis (see mainly Fig. 3.10) is used throughout the work described in this chapter.

4.2 RESULTS

4.2.1 Amino Acid Composition

The amino acid compositions of TfAl and TfD₂I were determined using the methods described in 2.18.
The number of residues of each amino acid was calculated from the analytical results on the basis of a molecular weight of 77,000, assuming that the proteins contain 2.86 g carbohydrate (5.2.1) and 2.42 g tryptophan (Hudson et al., 1973) per 100 g protein. The value 77,000 was used for the molecular weight of bovine transferrin since the majority of recent studies on the transferrins are in accordance with this value, as already discussed in 1.4. Results from Richardson et al. (1973) (after recalculation to the comparable basis) are also given in Table 4.1 for comparison.

The average amino acid residue weight of bovine serum transferrin is 112.2. The general amino acid distribution pattern of bovine transferrin is similar to those of the other species (Hudson et al., 1973; Eijk et al., 1972). It appears that transferrins can be generally characterized by having low methionine contents and high half cystine contents. All of the latter amino acid residues are probably involved in disulphide bonds (Schultze and Schwick, 1957). Amino acid composition of TfD2I obtained in the present work is similar to that reported by Richardson et al. (1973), except a slightly lower (ca. 10%) value was obtained for Glu in the present work. On the other hand there are slightly greater differences in the amino acid composition of TfAI. An appreciably higher Lys, Val and Leu, and lower Gly, Ala, Met and Phe contents are observed in the present work.

Although Richardson et al. (1973) indicated the
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Present work</th>
<th>Richardson et al. (1973)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TfAI</td>
<td>TfD₂₁</td>
</tr>
<tr>
<td>Asp+Asn</td>
<td>88.0</td>
<td>90.2</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>61.5</td>
<td>60.4</td>
</tr>
<tr>
<td>His</td>
<td>16.2</td>
<td>15.8</td>
</tr>
<tr>
<td>Arg</td>
<td>23.5</td>
<td>23.6</td>
</tr>
<tr>
<td>Lys</td>
<td>60.8</td>
<td>61.8</td>
</tr>
<tr>
<td>Gly</td>
<td>47.5</td>
<td>50.8</td>
</tr>
<tr>
<td>Ser</td>
<td>43.8</td>
<td>42.7</td>
</tr>
<tr>
<td>Thr</td>
<td>34.3</td>
<td>34.1</td>
</tr>
<tr>
<td>Ala</td>
<td>52.9</td>
<td>54.6</td>
</tr>
<tr>
<td>Cys/2⁶</td>
<td>(n.d.⁸)</td>
<td>35.5</td>
</tr>
<tr>
<td>Met⁵</td>
<td>8.1</td>
<td>8.8</td>
</tr>
<tr>
<td>Val</td>
<td>38.1</td>
<td>37.2</td>
</tr>
<tr>
<td>Leu</td>
<td>52.6</td>
<td>52.6</td>
</tr>
<tr>
<td>Ile</td>
<td>18.3</td>
<td>18.4</td>
</tr>
<tr>
<td>Pro</td>
<td>31.2</td>
<td>31.5</td>
</tr>
<tr>
<td>Phe</td>
<td>29.0</td>
<td>28.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>25.3</td>
<td>23.2</td>
</tr>
<tr>
<td>Trp⁶</td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>Glc.NH₂</td>
<td>13.0</td>
<td>12.5</td>
</tr>
</tbody>
</table>

1. Residues per molecule of protein. Number of amino acid residues was calculated from a single preparation on the basis of total molecular weight (amino acid + carbohydrate) of 77,000 for bovine transferrin. The value 77,000 was used for the reasons given in 4.2.1.

2. TfA₂a appears to be the equivalent component to TfAI in the present work.

3. TfD₂₂a appears to be the equivalent component to TfD₂₁ in the present work.

4. As cysteic acid.

5. As total of homoserine and homoserine lactone.

6. From Hudson et al. (1973)

7. Values for glucosamine were calculated using colour factor for Asp. No correction was made for the destruction during acid hydrolysis.

8. Not determined.
presence of detectable differences in several amino acid composition between the two variants, no significant difference could be detected in the present work in view of the reported precision of ± 3% for the method (Spackman et al., 1958). Substitution of only a few amino acid residues would not be revealed in general by this type of analyses for such a large molecule (MW 77,000). Likewise amino acid analyses of human transferrin variants did not reveal the amino acid residue differences (Parker and Bearn, 1962a).

4.2.2 Tryptic Peptide Maps

Since analyses of isolated genetic variants showed no significant differences in their amino acid composition, amino acid substitutions between the two variants were further investigated by peptide mapping.

Peptide maps were prepared from trypsin digests as described in 2.20.1.

Comparison of the tryptic peptide maps between TfAl and TfD_2I, which were prepared by subjecting the enzymic digest to electrophoresis at pH 4.7 for 1 hr 20 min, showed identical peptide distribution pattern in basic and acidic peptide regions (Fig. 4.1). It was not easy to compare the peptides located in the neutral region because of insufficient resolution in this area. Prolonged electrophoresis (2 hr 40 min), however, provided satisfactory resolution in the central part of the maps, particularly in the crowded neutral region as can be seen from the maps of Fig. 4.2. An attempt was made to detect ninhydrin insensitive peptides by staining with fluorescamine. No additional peptides were detected on the maps.
Figure 4.1  Tryptic peptide maps of TfAI and TfD2I. Maps were obtained by subjecting the soluble tryptic peptide fractions to electrophoresis at pH 4.7 in a buffer system pyridine:acetic acid:water (1:1:38 by vol.), for 1 hr 20 min in the first dimension, followed by ascending paper chromatography in the system, n-butanol:water:pyridine:acetic acid (15:12:10:3 by vol.) at room temperature for 20 hr in the second dimension. Maps were stained with 0.02% (w/v) ninhydrin solution in acetone containing pyridine and acetic acid both 0.2% (v/v).
Figure 4.2  Tryptic peptide maps of TfAI and TfD$_2$I. Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.1 except that prolonged (2 hr 40 min) electrophoresis was carried out.
Although the total number of peptides, $70 \pm 4$
($60 \pm 2$ heavily staining spots, and $10 \pm 2$ lightly staining spots), is less than might be expected from the amino acid composition ($\text{Lys} + \text{Arg} = 83$), the apparent deficiency in the number of peptides is probably due to overlapping of peptides with lesser contribution from enzyme-resistant bonds, staining difficulties and insolubility or failure to migrate from the origin.

Baker et al. (1968) observed fewer peptides ($70\%$) than expected on the tryptic peptide maps of rabbit transferrin and speculated the possible presence of repeating sequences within a single peptide chain as a consequence of a gene duplication. However, Shaw (unpublished results) found later that if the order of procedure for preparation of peptides were reversed, that is the performic acid oxidation is carried out following the trypsin digestion, the number of spots obtained on the maps was closer to what would be expected. The same was true when the reduced and carboxymethylated protein was used. He concluded that the deficiency in the number of peptides observed by Baker et al. (1968) was due to Lys and Arg residues adjacent to a disulphide bond, which was converted to a cysteic acid by prior oxidation, so that hydrolysis by trypsin at the above basic residues was inhibited.

Since the peptide maps in the present work were prepared from the oxidised trypsin digests it would be expected that the observed number of peptides would be closer to the expected number. The number observed, $70 \pm 4$, represents $84\%$ of the expected number and obviously higher
than that obtained by Baker et al. (1968). The present results are similar to those reported by Mann et al. (1970) for tryptic peptide maps of reduced and carboxymethylated human serum transferrin, where 72 to 92% of the expected peptides were observed.

The failure to observe the expected number of peptides in the present work, even with use of the oxidised tryptic digests, may be partially explained by the possible presence of repetitive amino acids which are susceptible to trypsin cleavage.

Comparative tryptic peptide maps for TfAI and TfD_{2}I (Fig. 4.2) indicated several differences. Amino acid analyses of these spots showed that only three differences were reproducible for all separate digests. Non-reproducible peptides are presumably attributable to the minor cleavage of the peptide by trace contaminated enzyme, most likely chymotrypsin. The different peptides have been arbitrarily designated as T-1, T-2 and T-3.

An attempt to prepare these peptides in large amount by preparative paper electrophoresis, followed by paper chromatography (see 2.17.3), was unsuccessful because of ready contamination by the neighbouring peptides. It was found, however, that these peptides could be isolated in purer form by pooling the corresponding spots from several sheets of the peptide maps. Usually three peptide maps provided sufficient material for amino acid analysis.

Peptide T-1. Table 4.2 shows the results of amino acid analyses of these peptides together with the other peptides (see peptide 1, tryptic peptides). The absence of Asp, in
Table 4.2  Results of Amino Acid Analysis of Tryptic and Chymotryptic Peptides.

<table>
<thead>
<tr>
<th>Peptides 1</th>
<th>Tryptic peptides</th>
<th>Tryptic peptides from CNBr fragments</th>
<th>Chymotryptic peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TfAI</td>
<td>TfD₂I</td>
<td>TfAI</td>
</tr>
<tr>
<td>Asp + Asn</td>
<td>0.3</td>
<td>1.1</td>
<td>trace</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>2.1</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>His</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>1</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Lys</td>
<td>1.2</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Gly</td>
<td>1.1</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>Ser</td>
<td>0.6</td>
<td>0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Thr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>1.1</td>
<td>1.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Cya</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n moles of amino acid residue</td>
<td>11.2</td>
<td>4.5</td>
<td>2.4</td>
</tr>
<tr>
<td>taken as one Detection</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Peptides 2</td>
<td></td>
<td>Peptides 3</td>
</tr>
<tr>
<td>---------------</td>
<td>------------</td>
<td>---------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>Tryptic peptides</td>
<td>Tryptic peptides from CNBr fragments</td>
<td>Chymotryptic peptides</td>
</tr>
<tr>
<td></td>
<td>TfAI 2(a+b)</td>
<td>TFD₂I 2a+2b</td>
<td>TfAI 2a</td>
</tr>
<tr>
<td>Asp+Asn</td>
<td>3.7</td>
<td>3.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>2.6</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td>His</td>
<td></td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Arg</td>
<td></td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Lys</td>
<td>2</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Gly</td>
<td>0.9</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>0.9</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Cya</td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>Val</td>
<td>1.1</td>
<td>1.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Leu</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Ile</td>
<td>2.3</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>1.7</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed in terms of the number of residues in a peptide by taking a suitable amino acid residue (underlined) as one.

1. Detection on the peptide maps.

N: ninhydrin staining
F: fluorescamine staining
TfAI.T-1 as compared to TfD\(_2\)I.T-1 is clearly evident. Correspondingly, there is a significant increase in Glu in TfAI.T-1. The replacement of Asp by Glu is apparent in acid hydrolysate of T-1, indicating amino acid substitution; A:D\(_2\), Glx:Asx. Peptide TfD\(_2\)I.T-1 is more acidic than TfAI.T-1 as judged from their electrophoretic mobilities. Therefore, Asx in TfD\(_2\)I.T-1 is Asp. The possible presence of Gln was considered unlikely by examination of RNA codons since at least two base substitutions are required to convert Gln to Asp. However, conversion of Glu to Asp occurs by a single base substitution.

\[
\text{Glu} \quad \{GAA\} \leftrightarrow \{GAC\} \quad \text{Asp}
\]

Therefore an amino acid substitution occurring between TfA and TfD\(_2\) is Glu:Asp. The amount of Thr in the two peptides was approximately 50% of the other constituent amino acid residues. Since the peptides were located on the maps by staining with ninhydrin prior to amino acid analysis, and the ninhydrin reagent reacts with up to 50% of amino acid residues with free amino groups in peptides under the conditions used, this amino acid is likely to be the N-terminus of the two peptides.

**Peptide T-2.** No consistent difference between the T-2 peptides was evident from the maps (Fig. 4.2). They usually exhibited very similar maps. In addition, the location of the peptides in the crowded neutral region made difficult the distinction of the peptides from one another. Amino acid analysis of TfAI.T-2 (a+b) revealed that it is
actually a product of two closely overlapped peptides since it contains two trypsin susceptible residues per peptide (see Table 4.2, peptide 2, tryptic peptides). The equivalent amino acid composition, except that Lys is replaced by Arg, was obtained from a mixture of acid hydrolysates of T-2a and T-2b of TfD_2I. This indicates the presence of amino acid substitution, A:D; Lys:Arg. Examination of RNA codons supports this substitution:

\[
\text{Lys} \quad \{\text{AAA}\} \quad \leftrightarrow \quad \{\text{AGA}\} \quad \text{Arg}
\]

The replacement of Glu and Lys in TfAI by Asp and Arg respectively in TfD_2I clearly would not account for the difference in mobility of the two proteins in zone electrophoresis at slightly alkaline pH (7.5). TfAI, which moves more rapidly toward the anode, should carry a greater negative (or less positive charge) than TfD_2I (see Fig. 3.10). The charge of Glu and Asp would be equally negative at pH 7.5 assuming a normal environment around these amino acid residues in the proteins. Also in the case of Lys and Arg, both amino acids should have equal positive charge. The net charge difference of the two variants caused by the above two amino acid substitutions is therefore considered insufficient to account for the electrophoretic difference.

**Peptide T-3.** The peptide T-3 is consistently present on the maps of TfAI, but is absent from the maps of TfD_2I. Amino acid analysis of TfA.T-3 showed the presence of Lys and Asp, Lys:Asp = 1:0.33 (see Table 4.2, peptide 3,
tryptic peptide). Although up to 50% of N-terminal amino acid residues of peptides would be expected to react with ninhydrin on the maps under the conditions used, amino acid composition of the peptide TfAI.T-3 was likely to be (Asx, Lys, Lys). This was later confirmed by amino acid analysis of the same peptide detected with fluorescamine (see 4.2.4) which reacts with only approximately 1% of the N-terminus of peptides. In the view of the fact that amount of Asp detected in the acid hydrolysate was greatly reduced by prior detection of the peptide spot on the map with ninhydrin, amino acid sequence of peptide TfAI.T-3 appears to be Asx-Lys-Lys. Several neighbouring peptides of T-3 on the maps of TfAI were analysed for their amino acid compositions in order to gain an indication of what the charge of Asx would be. Peptide 4 and 5 (Fig. 4.2) have amino acid compositions (Val, Gly, Lys) and (Val, Arg) respectively. Both peptides have a single net positive charge. It appears, therefore, that Asx of TfAI.T-3 is Asp. The slightly faster mobility of T-3 compared to that of T-4 may be explained by the fact that dipeptide (Lys-Lys) runs faster than free lysine on paper electrophoresis at pH 4.7 (see Fig. 4.1).

TfD₂I.T-3, if it exists, should be located in a more basic region than that where TfAI.T-3 is located in, provided amino acid substitutions take place at only three points on the polypeptide chains of the two variants. In spite of much effort such a peptide was not detected on the maps of TfD₂I with ninhydrin. Staining with fluorescamine occasionally reveals the presence of peptides
that are insensitive to ninhydrin staining. However, no such peptides were detected on the tryptic peptide maps of TfD₂I by staining with fluorescamine.

Insoluble Tryptic Peptide Fraction. (i) Amino Acid Composition. The possible presence of TfD₂I.T-3 was sought in the insoluble tryptic peptide fraction (core precipitate at pH 4), although it is unlikely that such a charged peptide as TfAI.T-3 is rendered insoluble by a single amino acid substitution.

Table 4.3 shows the amino acid composition of the insoluble tryptic peptide fraction. No significant difference suggesting Lys or Asp residue substitution by the other amino acid was detected between the insoluble tryptic peptide fractions of TfAI and TfD₂I.

(ii) Peptide Maps. Peptide mapping of the insoluble tryptic peptide fraction was performed by subjecting the core to paper electrophoresis at pH 8.9 for 1 hr followed by ascending chromatography. Peptide maps thus prepared did not stain well with ninhydrin. However, the peptide patterns of insoluble tryptic peptide fraction from the two variants were found to be very similar when inspected under UV light.

(iii) Chymotryptic Peptide Maps. A chymotryptic digest of the tryptic core was also subjected to peptide mapping. A higher number of spots were observed on the maps than would be expected. However, the peptide pattern obtained in this way was entirely different and simpler as compared with the maps prepared from trypsin-chymotrypsin digest of whole transferrin component I. There were no significant
Table 4.3 Amino Acid Compositions of Insoluble Tryptic Peptide Fractions.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>TfAI</th>
<th>TfD$_2$I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp+Ans</td>
<td>10.1</td>
<td>10.2</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>7.3</td>
<td>7.3</td>
</tr>
<tr>
<td>His</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Arg</td>
<td>2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Lys</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Gly</td>
<td>5.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Ser</td>
<td>4.8</td>
<td>4.7</td>
</tr>
<tr>
<td>Thr</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Ala</td>
<td>6.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Cys/2$^1$</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Met$^2$</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Val</td>
<td>4.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Leu</td>
<td>6.2</td>
<td>6.1</td>
</tr>
<tr>
<td>Ile</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Pro</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Phe</td>
<td>3.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

The number of amino acid residues was calculated from the analytical results so that the insoluble peptide fraction accounts for a polypeptide chain with a minimum molecular weight 8,470 ($77,000 \times 0.11 = 8470$), as it was found that approximately 11% of the starting material was recovered as the insoluble peptide fraction.

1. As cysteic acid.
2. As methionine sulphone.
differences between the chymotryptic hydrolysates of
tryptic cores from the two variants.

Interpretation of the Results Obtained from Tryptic Peptide
Maps. All the above results indicate that there are no
differences in the insoluble tryptic peptide fraction from
TfAI and TfD$_2$I, and that differences occur only in the
soluble tryptic peptide fraction.

The constant presence of peptide T-3 on the maps
of TfAI and the absence of the corresponding peptide from
TfD$_2$I indicates the existence of the peptide (or a single
amino acid) insertion in TfA (or its deletion in TfD$_2$).
Since TfAI moves faster towards the anode on zone electrophoresis (see Fig. 3.10), TfAI is assumed to carry greater
negative (or less positive) charge if both proteins possess
similar molecular size and conformation. Therefore, the
deletion for TfD$_2$ should be the one which results in loss
of negative charge. The deletion of the whole peptide T-3
from TfD$_2$ is obviously not the case, since this results in
loss of one unit of positive charge in TfD$_2$. However,
deletion of a single Asp residue from the T-3 peptide
portion of TfD$_2$ would account well for the mobility differ-
ence on zone electrophoresis and tryptic peptide patterns
of the two variants. Deletion of Asp residue from TfD$_2$
would result in production of free lysine or the dipeptide,
Lys-Lys, from the T-3 peptide portion by trypsin digestion,
and no peptide corresponding to TfAI.T-3 should be observed
on the maps of TfD$_2$I. If the tryptic digestion does not
produce free Lys or the dipeptide, Lys-Lys, from any
polypeptide portion of bovine serum transferrin other than
the T-3 portion, the deletion of Asp residue in TfD₂ should be clearly revealed by the presence of free Lys or the dipeptide, Lys-Lys, on the peptide maps of TfD₂ and its absence from the map of TfA. However, this is highly unlikely since it is not unusual for high molecular weight proteins, such as the transferrins, to have free Lys and the dipeptide, Lys-Lys, on the tryptic maps. The presence of free lysine and the dipeptide, Lys-Lys, on tryptic peptide maps of bovine serum transferrin A and D₂ was confirmed by direct comparison of the amino acid and peptide with authentic samples on paper electrophoresis (Fig. 4.1), and amino acid analysis.

4.2.3 Chymotryptic Peptide Maps

The genetically controlled amino acid substitutions and deletion found on tryptic peptide maps account for the electrophoretic difference between TfA and TfD₂.

In order to provide additional evidence for the results obtained from tryptic peptide maps and to investigate the possible presence of even further amino acid residue differences, chymotryptic peptide maps were prepared. It was possible that the peptide carrying such a substituted amino acid residue was not detected on the tryptic peptide maps due to overlap with other peptide(s). This difficulty may often be overcome by using an enzyme with different specificity from that of trypsin, resulting in peptides having different electrophoretic and chromatographic behaviour from those of tryptic peptides. Chymotrypsin was chosen for this purpose in view of the presence of a reasonable number of chymotrypsin susceptible residues in the
protein, and of its application to human serum transferrin (Wang and Sutton, 1965).

Chymotryptic peptide maps were prepared from chymotrypsin digests of the proteins as described in 2.20.2.

Since no differences were found in the basic and acidic peptide regions (Fig. 4.3), efforts were concentrated on finding differences in the near neutral region by prolonging the period of electrophoresis. Fig. 4.4 shows typical chymotryptic peptide patterns of the two genetic variants. Only two differences were consistently observed for all separate digests of transferrins. These difference peptides are designated as C-1 and C-2 as marked in the maps.

Peptide C-2. The C-2 peptide was reasonably free from contamination as judged by visual inspection and also by amino acid analysis. It was, therefore, possible to compare directly the results of amino acid analysis (see Table 4.2, peptide 2, chymotryptic peptides). A direct substitution of Lys by Arg is apparent from the table. This substitution obviously corresponds to that of T-2 peptides in tryptic digests, on the basis of the relative mobilities and the similar amino acid compositions.

Peptide C-1. On the other hand a direct elucidation of the substituted amino acid in C-1 peptides was difficult due to high contamination from the neighbouring peptides. Therefore, simple comparison of amino acid compositions of these peptides was not fruitful. A meaningful interpretation was, however, made possible by subtracting the respective
Figure 4.3 Chymotryptic peptide maps of TfAI and TfD2I. Maps were obtained from the chymotryptic digest of oxidised proteins by the same procedures as for Fig. 4.1.
Figure 4.4  Chymotryptic peptide maps of TfAI and TfD₂I. Maps were obtained from the chymotryptic digests of oxidised proteins by the same procedures as for Fig. 4.2.
"background" value from the value of C-1. The area, which correspond to TfAI.C-1, on the maps of TfD₂I was eluted with 6 M HCl, hydrolysed and the amino acid composition determined. This value was then subtracted from that of TfAI.C-1 in order to allow for the contamination from the neighbouring peptides. The same procedure was applied to TfD₂I.C-1.

The results obtained in this way are shown in Table 4.2 (see peptide 1, chymotryptic peptides). Replacement of Glu by Asp is apparent. This amino acid substitution is obviously identical to the one found in T-1 peptides of the trypsin digests.

Interpretation of the Results Obtained from Chymotryptic Peptide Maps. The presence of the same amino acid substitutions between the two variants was confirmed by chymotryptic peptide mapping. A peptide with Asp deleted was not detected on the chymotryptic peptide maps of TfAI or TfD₂I. It is possible that the T-3 peptide portion was included in a large, acidic or neutral, chymotryptic peptide and located near the origin so that no difference was observed on the comparative maps.

4.2.4 Tryptic Peptide Maps of Cyanogen Bromide (CNBr) Fragments

Chymotryptic peptide maps did not reveal the presence of any amino acid substitution and deletion other than those found on tryptic peptide maps. It is, however, still possible that any other differences were not detected by peptide mapping of tryptic or chymotryptic digests of whole transferrin component due to overlapping of peptides.
The possible problem, that the two-dimensional electrophoretic and chromatographic systems would not be adequate for resolving an hydrolysate which contains approximately 85 peptides, should be overcome by cleaving the protein molecule into small fragments followed by tryptic peptide mapping of individual fragments from the two variants compared.

**CNBr Fragmentation of Transferrin.** Cyanogen bromide cleavage of transferrin was carried out as described in 2.21.1.

Amino acid analysis of the CNBr treated bovine transferrin revealed the appearance of homoserine and homoserine lactone together with complete disappearance of methionine, indicating complete modification of methionine residues. Methionine contents were calculated as the total of homoserine and homoserine lactone, using integration constants of 4457 and 4200 for homoserine and homoserine lactone respectively (cf. 5091 for Asp, and 6536 for Arg). The presence of 9 methionine residues per molecule of bovine transferrin was established in this way.

Fig. 4.5 shows the elution profile for fractionation of CNBr treated TfAl on a Sephadex G-75 column. Approximately 95% of the protein and peptide were recovered as measured in absorbance units (at 280 nm). Three main peaks, designated CN-1, CN-2 and CN-3 in order of elution, were obtained. Both variants showed identical elution pattern from the column, indicating identical distribution of Met residues in their respective polypeptide chains. The dark horizontal bars under each subsequent peak indicate fractions that were pooled. They predominantly contained
Figure 4.5 Chromatography of CNBr treated bovine transferrin (TfAI) on a column of Sephadex G-75 (2.7 x 65 cm). A fraction of 7 ml capacity was collected approximately every 20 min, using 1% HCOOH as solvent.
fragments identified as CN-1, CN-2 and CN-3. Fraction CN-4 yielded dark-brown coloured material and it was discarded. A shoulder on the leading edge of the first peak CN-1 was assumed to contain aggregated fragments and it was not further examined. The presence of such aggregated materials in CNBr treated human transferrins has been reported by several authors (Jeppsson, 1967b; Spik and Montreuil, 1974). CN-1, CN-2 and CN-3 fractions were directly lyophilised and approximately 70 mg, 5 mg and 10 mg were recovered respectively from 100 mg of starting material.

**Performic Acid Oxidation of CN-1.** Fragment CN-1 appeared to represent still a fairly large part of the transferrin molecule. Therefore it was further fragmented by performic acid oxidation. Gel filtration of the oxidised products of the CN-1 on Sephadex G-75 column is shown in Fig. 4.6 Six fractions were obtained and lyophilised. They were designated as CN-1.01, CN-1.02, .... and CN-1.06 in order of elution.

**Characteristics of CNBr Fragments.** The amino acid compositions of the CNBr fragments are summarized in Table 4.4.

CN-2, eluted as a symmetric peak from a column of Sephadex G-25, and yielded 9 peptides, staining heavily with ninhydrin, on their tryptic peptide maps. As can be seen from the amino acid analysis of this fragment, it is obviously devoid of cysteine, cystine and cysteic acid and it contains 6-7 Lys and 1 Arg residues when calculated on the basis of 1 (homoserine + homoserine lactone) residue per fragment, indicating the single peptide nature of CN-2. A minimum molecular weight of 6800-6900 was calculated on the basis of its amino acid composition.
Figure 4.6  Chromatography on a column of Sephadex G-75 (2.7 × 65 cm) of oxidised cyanogen bromide fraction CN-1 from Fig. 4.5. Fractionation conditions were the same as for Fig. 4.5 except that each fraction collected was approximately 5.8 ml.
<table>
<thead>
<tr>
<th>Transferrin</th>
<th>CN-2</th>
<th>CN-3</th>
<th>CN-1.01</th>
</tr>
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<tbody>
<tr>
<td>TfAI</td>
<td>TfD₂I</td>
<td>TfAI</td>
<td>TfD₂I</td>
</tr>
<tr>
<td>Asp+Asn</td>
<td>89.8</td>
<td>88.2</td>
<td>8.0</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>61.7</td>
<td>60.4</td>
<td>5.9</td>
</tr>
<tr>
<td>His</td>
<td>15.9</td>
<td>17.6</td>
<td>3.5</td>
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<tr>
<td>Arg</td>
<td>23.3</td>
<td>25.2</td>
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</tr>
<tr>
<td>Lys</td>
<td>61.4</td>
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<td>6.3</td>
</tr>
<tr>
<td>Gly</td>
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<tr>
<td>Ser</td>
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</tr>
<tr>
<td>Thr</td>
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<td>31.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Ala</td>
<td>53.2</td>
<td>53.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Cys/2</td>
<td>(35.5)</td>
<td>(35.5)</td>
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</tr>
<tr>
<td>Homoserine + Homoserine lactone</td>
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<td>9.0</td>
<td>1</td>
</tr>
<tr>
<td>Val</td>
<td>36.8</td>
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</tr>
<tr>
<td>Leu</td>
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<td>Pro</td>
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<td>28.5</td>
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<td>Glc.NH₂</td>
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<td>n.p.s³</td>
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<td>12-13</td>
</tr>
<tr>
<td>n.p.c⁴</td>
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<td>2</td>
</tr>
<tr>
<td>m.m.w⁵</td>
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<td></td>
</tr>
</tbody>
</table>

Results are expressed by the number of residues per a CNBr fragment assuming the presence of one residue of (homoserine + homoserine lactone) in the fragment.

1. As cysteic acid.
2. Glucosamine values were calculated in the same way as described for Table 4.1.
3. Not determined.
4. The number of peptide spots on the tryptic peptide maps.
5. The number of peptide chains.
6. Minimum molecular weight calculated from its amino acid composition.
<table>
<thead>
<tr>
<th></th>
<th>CN-1.02</th>
<th>CN-1.03</th>
<th>CN-1.04</th>
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<th>CN-1.06</th>
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<td>6.1</td>
<td>5.5</td>
<td>4.3</td>
</tr>
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<td>1.7</td>
<td>1.7</td>
<td>1.5</td>
</tr>
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<td>7.3</td>
<td>1.6</td>
<td>1.6</td>
<td>3.0</td>
</tr>
<tr>
<td><strong>TfA</strong></td>
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<td>15.6</td>
<td>7.3</td>
<td>7.5</td>
<td>3.2</td>
</tr>
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<td>5.2</td>
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<td>4.4</td>
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<td>8.7</td>
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<td>3.0</td>
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<td>2.7</td>
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<td>9.7¹</td>
<td>4.0¹</td>
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<td><strong>TfD₂</strong></td>
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<td>0.7</td>
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<tr>
<td><strong>TfA</strong></td>
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<td>1</td>
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<td><strong>TfA</strong></td>
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<td>4.2</td>
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<td><strong>TfA</strong></td>
<td>6.5</td>
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<td><strong>TfD₂</strong></td>
<td>7.7</td>
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<td>2.7</td>
<td>2.5</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>TfA</strong></td>
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<td>2.9</td>
<td>3.3</td>
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<td>0.1</td>
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<tr>
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<td>numerous</td>
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<td>7-8</td>
<td>5</td>
</tr>
<tr>
<td><strong>TfA</strong></td>
<td>5,000</td>
<td>3,200</td>
<td>1,200</td>
<td>1,200</td>
<td>1,200</td>
</tr>
</tbody>
</table>
CN-3, which was eluted from the column, after CN-2, has a shoulder on the leading edge, suggesting that CN-3 is composed of at least two fragments. The total number of Lys and Arg residues coincided with the number of peptides on the tryptic maps when (homoserine + homoserine lactone) was assumed equal to two. There is a strong possibility that one of two fragments in fraction CN-3 carries a carbohydrate moiety of bovine transferrin since a relatively higher content of glucosamine was observed in the amino acid analysis profile of CN-3 compared with that of the other fragments. Minimum molecular weight for total CN-3 fraction (not including carbohydrate moiety) is approximately 9,500.

Chromatography of oxidised CN-1 on Sephadex G-75 column resulted in separation of several peaks. Among these CN-1.01 and CN-1.02 eluted from the Sephadex column with the same buffer volume as that needed in CN-1. Peptide maps of these fractions showed complex patterns. These results indicate that fractions CN-1.01 and CN-1.02 are aggregated materials. Fraction CN-1.03 is also likely to be aggregated material on the basis of its complex tryptic peptide pattern.

Fraction CN-1.04 and CN-1.05 are obviously single chain peptides since they do not contain any cysteic acid residues in their amino acid compositions. Minimum molecular weights are approximately 5,100 and 3,200 respectively. The CN-1.05 fragment appears to originate from the C-terminal part of bovine transferrin since it is devoid of homoserine and its lactone. Brown coloured
material was obtained from fraction CN-1.06 of TfD<sub>2</sub>I, while colourless peptide material was recovered from the equivalent fraction of TfAI. Fraction CN-1.06 (TfAI) showed only 1 or 2 peptides, on the tryptic peptide map, indicating the small size of fragment CN-1.06. The isolation from human transferrin (Sutton et al., 1975) of a small CNBr fragment, which lacks aromatic amino acids, has been reported.

As already described fractions CN-2, CN-1.04, and CN-1.05 are single chain peptides. Therefore, their molecular sizes are comparable on the basis of the elution order from the Sephadex column. Thus the order of molecular size of these fragments is CN-2 > CN-1.04 > CN-1.05. This agrees with the minimum molecular weights calculated from the amino acid compositions. Summation of minimum molecular weight of non-aggregated CNBr fragments CN-2, CN-3, CN-1.04, CN-1.05 and CN-1.06 is approximately 26,000 (not including carbohydrate moiety). The CNBr fragments, which account for the remaining molecular weight of 50,000 of bovine transferrin, are likely to have been eluted with the aggregated fractions from the column.

Tryptic Peptide Maps of CNBr Fragments. Fig. 4.7 shows typical tryptic peptide maps obtained from a non-aggregated CNBr fragment CN-3.

Peptide, CN.T-1, in which amino acid substitution Glu:Asp occurs, were observed on the maps of fragment CN-1.03 after ninhydrin staining (Fig. 4.8). Amino acid compositions of these peptides (see Table 4.2, peptide 1, tryptic peptides of CNBr fragments) show good agreement
Figure 4.7  Tryptic peptide maps of CN-3. Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.2.
Figure 4.8  Tryptic peptide maps of CN-1.03. Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.2.
with those obtained from the direct tryptic peptide mapping of TfAI and TfD$_2$I (see Table 4.2, peptide 1, tryptic peptides).

The other demonstrated amino acid substitution, Lys:Arg was not detected by ninhydrin staining on any peptide maps of the CNBr fragments. However, specific staining for arginine (see 2.20.4) revealed the presence of an additional arginine positive peptide in CN-1.02 from TfD$_2$I (CN.T-2). The corresponding peptide in CN-1.02 from TfAI did not stain with the reagent (Fig. 4.9). These peptides were isolated from fresh chromatograms, which were prepared by electrophoresis at two different pH values (pH 4.7 for 2 hr 40 min, and pH 1.9 for 1 hr 20 min), and single ascending chromatography at right angles to the electrophoresis. The peptides were detected with fluorescamine. The amino acid compositions of these peptides are shown in Table 4.2 (see peptide 2, tryptic peptides of CNBr fragments).

The presence of an additional peptide T-3, (Asp-Lys-Lys), in the basic peptide region of the tryptic peptide maps from TfAI was described in 4.2.2. The presence of the equivalent peptide to T-3 was confirmed in CNBr digest of TfAI, and found in fraction CN-1.03 (Fig. 4.8). This peptide, CN.T-3 of TfAI, was isolated from a fresh map, which was stained with fluorescamine, and its amino acid composition determined (Table 4.2, peptide 3, tryptic peptide of CNBr fragment). In spite of several attempts, including varying the period of electrophoresis, double chromatography, location of peptides with fluorescamine
Figure 4.9  Tryptic peptide maps of CN-1.02. Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.2.
Tryptic Peptide Map  CN-1.02 (TfAA)

Electrophoresis

Tryptic Peptide Map  CN-1.02 (TfD2D2)

Electrophoresis

Chromatography
and specific staining for arginine, no evidence was obtained for the presence of corresponding peptide on the map of fraction CN-1.03 from TfD\textsubscript{2}I.

### 4.2.5 Alkaline Hydrolysate of Bovine Transferrin and Tryptic Peptide Maps of Reduced and Carboxymethylated Proteins

Recently a new amino acid has been identified in the calcified tissues of several vertebrates. The structure of this amino acid, γ-carboxyglutamic acid (Gla), is shown below:

\[
\text{\begin{tikzpicture}
  \node (a) at (0,0) {\text{H}};
  \node (b) at (1,1.732) {\text{CH}_2};
  \node (c) at (1,0) {\text{CH}};
  \node (d) at (1,-1.732) {\text{COO}^-};
  \node (e) at (0,3.464) {\text{COO}^-};
  \node (f) at (0,-3.464) {\text{NH}_3};
  \draw (a) -- (b);
  \draw (b) -- (c);
  \draw (c) -- (d);
  \draw (c) -- (e);
  \draw (c) -- (f);
\end{tikzpicture}}
\]

The amino acid is strongly negatively charged at neutral to alkaline pH. It is synthesized from glutamic acid residues in a post-translational enzymatic reaction with a requirement for vitamin K and bicarbonate (Esmon \textit{et al}., 1975). The resultant γ-carboxyglutamic acid residues act as calcium-binding site and are essential for the normal activation of haemostatis (Nelsestuen and Suttie, 1973). This amino acid is readily decarboxylated to glutamic acid by the strong acid conditions generally used for protein hydrolysis (Stenflo \textit{et al}., 1974). Occurrence of γ-carboxyglutamic acid is so far limited in proteins synthesized in liver.

In the course of this study, a suggestion was made by Professor L. Lorand (Northwestern University) to H.A. McKenzie of the possible presence of γ-carboxyglutamic...
acid in bovine serum transferrin since transferrin is synthesized in liver, and this molecule binds a variety of divalent cations as well as trivalent ones (1.3).

The possible presence of γ-carboxyglutamic acid in bovine transferrin was examined by two different methods: direct identification of γ-carboxyglutamic acid in alkaline hydrolysates, and comparative peptide mapping of tryptic digests of reduced and carboxymethylated proteins.

Alkaline Hydrolysate of Bovine Transferrin. Alkaline hydrolysis of the proteins was carried out, using the methods described in 2.18.2. The elution position of γ-carboxyglutamic acid on the analyzer was established with alkaline hydrolysate of human blood clotting factors, II, VII, XI and X. The sample was prepared by Commonwealth Serum Laboratories, Melbourne, Vic. and was a gift from Mr. G. Gorman. It contained a significant ninhydrin-positive peak eluting 3 min after cysteic acid and 28.3 min before aspartic acid in the Beckman 120 C amino acid analyzer running with a buffer flow rate of 60 ml hr⁻¹.

Approximately 10% decrease in the peak size of γ-carboxyglutamic acid occurred when the hydrolysates were left in the column for 1 hr, indicating destruction of this amino acid in the column under the conditions used (2.18). However, the 10% destruction of γ-carboxyglutamic acid is insufficient to account for the very low colour factor for γ-carboxyglutamic acid, about 25% of the colour factor for glutamic acid, observed by Price et al. (1976). The γ-carboxyglutamic acid peak disappeared by heating the alkaline hydrolysates in 0.1 M HCl at 100°C for 1 hr.
The alkaline hydrolysate of bovine transferrin gave on the amino acid analyzer a small unknown peak at the position of γ-carboxyglutamic acid and following a cysteic acid peak of almost equal size. TfAI and TfD₂I gave identical patterns. This unknown peak disappeared after treatment of the hydrolysates at pH 1.0 for 1 hr at 100°C, suggesting the potential presence of γ-carboxyglutamic acid in transferrin. However, α-lactalbumin, which has been reported as containing no γ-carboxyglutamic acid residues (Hauschka et al., 1975) showed a similar pattern as the one obtained for transferrin. The unknown peak disappeared by treatment of the alkaline hydrolysate of α-lactalbumin with acid. It is, therefore, concluded that bovine transferrin does not contain γ-carboxyglutamic acid residues. The observed small peak at the position of γ-carboxyglutamic acid may be due to artifactual products from the polythene tubing used in the hydrolysis.

Tryptic Peptide Maps of Reduced and Carboxymethylated Proteins. Although it was found in the previous section that bovine transferrin does not contain γ-carboxyglutamic acid residues, further evidence for this was sought by peptide mapping of reduced and carboxymethylated proteins. The proteins were reduced and carboxymethylated as described in 2.12.2. Since a main object of this experiment was to avoid exposing the proteins and peptides to acidic conditions, the core was not precipitated.

As can be seen in Fig. 4.10, fairly heavy streaking from the origin was observed in each map since the total digest (including insoluble peptide fraction) was
Figure 4.10  Tryptic peptide maps of reduced and carboxymethylated transferrin. Maps were obtained from the whole tryptic digests (including core precipitate) by the same procedures as for Fig. 4.2.
Tryptic Peptide Map of Reduced and Carboxymethylated TfAA

Tryptic Peptide Map of Reduced and Carboxymethylated TfD2D2

Electrophoresis

Chromatography

- T-3 +

Electrophoresis
applied on the paper. The differences, detected on tryptic peptide maps of oxidised samples (Fig. 4.2), T-1 and T-2, were not revealed. It is probably because T-2 peptides are originally located in the region involved in the streaking, and T-1, reducing its negative charge by carboxymethylation of sulphhydryl instead of its oxidation to cysteic acid, moved in the streaking region. The presence of the peptide equivalent to T-3 was confirmed on the map of TfA.

No amino acid differences were found by peptide mapping of the reduced and carboxymethylated proteins additional to those found in oxidised tryptic digests. This indicates, indirectly, the absence of γ-carboxyglutamic acid from bovine transferrin A and D₂.

4.2.6 Zone Electrophoresis of Fully Denatured Proteins

A possible contribution of conformational differences to the observed electrophoretic difference on zone electrophoresis at pH 7.5 was examined by urea-starch gel electrophoresis using the fully denatured proteins.

Urea-starch gel electrophoresis was carried out as described in 2.10.

A clear conclusion could not be drawn from the electrophoretic patterns of the fully reduced proteins since the proteins did not migrate for enough distance from the origin for the difference to be clear.

However, as can be seen in Fig. 4.11, electrophoresis of performic acid oxidised proteins gave unambiguous evidence that the mobility difference between the two genetic variants is not due to a conformational
Figure 4.11  Urea starch gel (without reductant) electrophoresis of performic acid oxidised transferrins. Tris-glycine-urea buffer system, pH 8.8; 300 V, 10 hr.
Samples:

Left : oxidised TfAII
Right : oxidised TfD₂II
Starch Gel Electrophoresis
of Oxidised Transferrin
difference. Oxidised proteins (component II) produced two bands on urea-starch gel after staining with nigrosin. The electrophoretic mobility of the slower band is similar in both TfA and TfD$_2$. However, a difference is clear in the faster band. TfA runs more rapidly to the anode than TfD$_2$, corresponding to the pattern which is observed with intact proteins (see Fig. 3.10).

4.2.7 Neuraminidase Treatment and Carbohydrate Composition

(i) Neuraminidase Treatment. TfAI and TfD$_2$I were treated with neuraminidase (see 2.14.1) to examine the possibility that variation in sialic acid content might contribute to the mobility difference.

Electrophoretic patterns of neuraminidase treated samples are shown in Fig. 4.12. When transferrin samples were treated with the neuraminidase under the conditions described in 2.14.1, release of sialic acid residues from the proteins was completed within 30 min. Prolonged incubation did not change the electrophoretic patterns.

The mobility of each variant decreased on starch gel electrophoresis following removal of the sialic acid residues, but the mobility difference remained. It appeared, therefore, that variations in sialic acid content are not involved in the genetic variation of bovine transferrin A and D$_2$.

(ii) Carbohydrate Composition. Results of the sialic acid and hexosamine analysis of component II from both variants are given in Table 4.5. Both components obviously contain the same monosaccharides in the same amount.
Figure 4.12 Starch gel electrophoresis showing the effects of neuraminidase on isolated component I of the two transferrin variants A and D₂. Tris-cacodylic acid buffer system, pH 7.5; 270 V, 4 hr. Samples from left to right:

- TfAI, not treated with neuraminidase
- TfD₂I, not treated with neuraminidase
- TfAI, treated with neuraminidase
- TfD₂I, treated with neuraminidase
on Tf D2D2 and Tf AA

Origin

AA D2 D2 AA D2D2
+ + N N
<table>
<thead>
<tr>
<th>Carbohydrate Content of Component II from Bovine Transferrin Variant A and D₂.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Molecule of Protein</td>
</tr>
<tr>
<td>--------------------------------</td>
</tr>
<tr>
<td>TfAII</td>
</tr>
<tr>
<td>TfD₂II</td>
</tr>
</tbody>
</table>

Sialic acid was determined as N-acetylneuraminic acid. D-glucosamine was determined as N-acetyl derivative and given values are average of duplicated analyses. Molecular weight of proteins was assumed to be 77,000 for the reason described in GENERAL INTRODUCTION, 1.4.
4.2.8 Isolation and Characterization of Glycopeptides from Enzymic Digests of Bovine Transferrin

Although component II from both variants possesses the same monosaccharides in the same amount (4.2.7) it is interesting to examine whether the carbohydrate moiety is linked to the respective polypeptide chain in the same mode, and whether it is linked to the equivalent amino acid residue.

Subtilisin glycopeptides from whole TfA and TfD₂. Subtilisin glycopeptides were prepared from whole transferrin by the enzymic digestion, as described in 2.17.

A single narrow peak of carbohydrate material was eluted, following fractionation of the subtilisin digest on Sephadex G-25 (Fig. 4.13). Approximately 80 mg of glycopeptide fraction was obtained in this way when 500 mg of transferrin was subjected to the enzyme digestion.

An attempt to separate individual glycopeptides from the glycopeptide fraction was first made with use of high-voltage paper electrophoresis. When the high-voltage paper electrophoretogram, pH 4.7, was sprayed with 0.02% (w/v) ninhydrin solution (in acetone containing pyridine and acetic acid both 0.2% by volume), it was found that the glycopeptide fraction had separated into several bands on the sheet. Glycopeptides stained to a light gray-purple colour, while a purple colour was given by non-carbohydrate containing peptides. There was, however, heavy background staining by ninhydrin especially in the glycopeptide region, which indicated fairly high contamination.
Figure 4.13  Chromatography of subtilisin digests of transferrin D₂ on a column of Sephadex G-25 (2.7 x 60 cm). A fraction of 7 ml capacity was collected approximately every 20 min, using 0.01% (w/v) NH₄HCO₃ as buffer. Carbohydrate containing fractions were detected by orcinol-sulphuric acid method.
An attempt was made then to purify the glycopeptides further by two dimensional chromatography; by high-voltage electrophoresis at pH 4.7 in the first dimension and ascending chromatography with a buffer system (n-butanol:water:pyridine:acetic acid = 15:12:10:3, by vol.) in the second dimension. The glycopeptides remained at the origin during ascending chromatography and ninhydrin staining showed a highly coloured background in the glycopeptide region, indicating insufficient separation of glycopeptides from impurities.

However, a satisfactory separation of glycopeptides was obtained by two dimensional high-voltage paper electrophoresis of the glycopeptide fraction at pH 4.7 and 1.9, typical patterns being shown in Fig. 4.14. The glycopeptide fraction separated in this way appeared in numerous electrophoretically distinct forms depending on the number of sialic acid residues, and on the size and charge of the peptide portions. Individual glycopeptides obtained in this way are referred to as glycopeptide-(E).

As can be seen from Fig. 4.14, an identical electrophoretic pattern was obtained from the glycopeptide fractions of the two variants. This indicates that the carbohydrate moiety of the variants is linked to the same amino acid of the respective polypeptide chain.

All the glycopeptides on the electrophoretogram fluoresced under UV light and location of glycopeptides in this way and by staining with ninhydrin solution coincided. This characteristic of subtilisin glycopeptides was later used to locate glycopeptides on the electrophoretogram when the samples were wanted for further analytical purposes.
Figure 4.14  Separation of the glycopeptide fraction by
two dimensional high-voltage paper electrophoresis. The
samples were subjected to electrophoresis at pH 4.7, for
2 hr 40 min in the first dimension, followed by
electrophoresis at pH 1.9 for 1 hr 20 min in the second
dimension. Maps were stained with 0.02% (w/v) ninhydrin
solution in acetone containing pyridine and acetic acid
both 0.2% (v/v).

Top : transferrin A
Bottom : transferrin D₂
Separation of Glycopeptide Fraction (TfAA)

Electrophoresis pH 4.7

Separation of Glycopeptide Fraction (TfD2D2)

Electrophoresis pH 1.9
When each glycopeptide-(E) was isolated by means of electrophoresis, some of the glycopeptides were still not satisfactorily pure as judged by their amino acid analysis. Therefore further purification was attempted by altering the electrophoretic mobilities of glycopeptides by removal of sialic acid residues (see 2.14.2). Preliminary treatment of the glycopeptide fraction with neuraminidase resulted in poorer resolution of individual glycopeptide-(E) on the electrophoretogram. The electrophoretic mobility of each glycopeptide was reduced and each glycopeptide moved to a narrow area near the origin. It was not possible to distinguish one from another. Therefore, it was found necessary to treat each partially purified glycopeptide-(E), two prepared by the dimensional electrophoresis (Fig. 4.14), with the enzyme individually and then subject it to further electrophoresis. Each partially purified glycopeptide-(E) was treated with neuraminidase as described in 2.14.2. Samples thus obtained were submitted to paper electrophoresis at pH 4.7. Results are shown in Fig. 4.15. Neuraminidase treatment produced several bands, designated glycopeptide-(NT), on the electrophoretogram from each partially purified glycopeptide-(E). Each band on the electrophoretogram was cut off, stitched to a fresh sheet of paper and subjected to electrophoresis at pH 1.9 for 1 hr 20 min. Peptide bands, detected under UV light were eluted with 6 M HCl, hydrolysed and the amino acid composition determined (Table 4.6). A group of glycopeptide-(NT), which were produced from one partially purified glycopeptide-(E) by action of neuraminidase, possesses
ELECTROPHORESIS OF NEURAMINIDASE TREATED GLYCOPEPTIDE-(E)

Figure 4.15 The tracing of electrophoretic separation of neuraminidase treated glycopeptides-(E).
Glycopeptides-(E) (Fig. 4.14) were treated with neuraminidase and subjected to high-voltage paper electrophoresis, at pH 4.7, for 2 hr 40 min. The peptides were detected on the paper by illumination with UV light.
Table 4.6  Amino Acid Composition$^1$ of Subtilisin Glycopeptide-(NT) isolated from performic acid oxidised bovine transferrin.

<table>
<thead>
<tr>
<th>Glycopeptide</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp + Asn</td>
<td>1.3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.8</td>
<td>1.5</td>
<td>1.4</td>
<td>1.1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>0.3</td>
<td>2.1</td>
<td>1.3</td>
<td>1.0</td>
<td>2.0</td>
<td></td>
<td></td>
<td>2.4</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Thr</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td></td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Glc.NH$_2$</td>
<td>3.8</td>
<td>2.1</td>
<td>4.7</td>
<td>4.1</td>
<td>3.4</td>
<td>4.9</td>
<td>5.0</td>
<td>5.5</td>
<td>5.1</td>
<td>3.6</td>
</tr>
<tr>
<td>n moles</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amino acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>taken as</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>one residue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Results are expressed in terms of the number of residues in a peptide, by taking a suitable amino acid residue (underlined) as one.

2. Glucosamine content was calculated as described in Table 4.1.
the same amino acid composition, suggesting the difference is in sialic acid content. The amount of neuraminidase used was probably not active enough to split all the sialic acids off from the partially purified glycopeptide-(E).

Digestion of TfA and TfD, apparently yielded glycopeptides having the same constituent amino acids. This indicates that the same number of polysaccharide side chains are linked to the equivalent amino acid residues of the respective polypeptide chains of the two variants. The genetically controlled amino acid substitutions between the two variants do not take place apparently in the small peptide portions (tetra or hexapeptides) that carry carbohydrate.

Glycopeptides-(NT), obtained by neuraminidase treatment with subsequent two dimensional electrophoresis, can be conveniently divided into three families. The first one, which includes glycopeptides 1 and 3, is characterized by the presence of Arg and Tyr residues among their constituent amino acids. The difference between 1 and 3 is presumably due to the sialic acid content. Subtilisin glycopeptides 5 and 8 form a second family. These glycopeptides possess completely different amino acid compositions from that of the first family. They differ by the presence of an additional cysteic acid residue in glycopeptide 8. This indicates that the cysteic acid residue occupies the N- or C- terminus of glycopeptide 8, unless these two peptides originate from different parts of polypeptide chain of the transferrin. The third family,
subtilisin glycopeptides 6 and 7, contains only Asp and Ser as the constituent amino acid. This family is likely to be a shorter peptide of the second family. These results indicate the possible presence of two carbohydrate side chains in bovine transferrin.

Occurrence of N-acetylglucosamine and the prevalence of Asp in acid hydrolysates of all the glycopeptides suggested the possibility of an asparaginyl linkage between the protein and carbohydrate portions. However, definite conclusion on this was dependent on further studies on the amino acid sequence of the glycopeptides. They are described in a later part of this section.

**Pronase Glycopeptides from Whole TfA.** In order to reveal the carbohydrate-amino acid linkage, small peptides with the polysaccharide chain linked were prepared by means of pronase digestion.

Fig. 4.16 shows the elution profile of the pronase digest of whole TfA from a column of Sephadex G-25. The two dimensional paper electrophoresis of the glycopeptide fraction produced a separation of numerous glycopeptides in a small region on the paper (Fig. 4.17). Several major peptides were eluted from the electrophoretogram with 6 M HCl and their amino acid compositions determined (Table 4.7). All of the pronase glycopeptides contained only Asp and Ser except pronase glycopeptides 5 and 6. A fragment, consisting of the polysaccharide prosthetic group of the protein combined only with asparagine was not detected in the peptide examined, although the possibility that pronase glycopeptide 1 represents such a peptide cannot be excluded.
Sephadex G-25 Elution Profile of Pronase Digested Transferrin

Figure 4.16 Chromatography of pronase digest of transferrin A on a column of Sephadex G-25 (2.7 x 60 cm). A fraction of 7 ml capacity was collected approximately every 20 min, using 0.01% (w/v) NH₄HCO₃ as buffer. Carbohydrate containing fractions were detected by the orcinol-sulphuric acid method.
Figure 4.17 The tracing of separation of the pronase glycopeptide fraction (TfAA) (Fig. 4.16) by two dimensional high-voltage paper electrophoresis. The electrophoretogram was obtained by the same procedures as for Fig. 4.14.
Table 4.7  Amino Acid Composition of Pronase Glycopeptides
Isolated from Performic Acid Oxidised Bovine Transferrin A.

<table>
<thead>
<tr>
<th>Glycopeptide</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp+Asn</td>
<td>2.2</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>1.2</td>
<td>1.4</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>His</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>Gly</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Ser</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Thr</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cya</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Glx.NH₂</td>
<td>6.9</td>
<td>2.4</td>
<td>2.2</td>
<td>2.2</td>
<td>3.5</td>
<td>2.1</td>
<td>2.5</td>
<td>2.1</td>
</tr>
<tr>
<td>n moles of amino acid residue taken as one</td>
<td>2.3</td>
<td>13.5</td>
<td>49.2</td>
<td>20.0</td>
<td>13.5</td>
<td>21.4</td>
<td>19.5</td>
<td>30.0</td>
</tr>
</tbody>
</table>

The number of amino acid residues is expressed in the same way as described for Table 4.6.
for the reason described in 4.2.2. Pronase glycopeptide 5 contains Asp, Ser, Gly and Cya, suggesting some structural similarity to the second family of subtilisin glycopeptides. Pronase glycopeptide 6 possesses an His residue as well as Asp and Ser.

Amino Acid Sequences of Glycopeptides from Bovine Transferrin. Graham and Williams (1975), concluded that there are two types of glycopeptides present in various enzymic digests of bovine transferrin. Those glycopeptides appeared to be identical in carbohydrate composition but differing in amino acid sequence (General Introduction, 1.4).

Analysis of the glycopeptides for amino acid composition in the present work indicated the presence of two types of glycopeptides, but also differences in the amino acid sequence from those proposed by Graham and Williams. Several glycopeptides were, therefore, subjected to amino acid sequential analysis using the method described in 2.19.2. The amino acid compositions of glycopeptides subjected to DNS-Edman procedure are shown in Table 4.3.

Subtilisin glycopeptide 8. Table 4.9 shows results of sequential analysis of subtilisin glycopeptide 8 by the DNS-Edman method. After three cycles of Edman degradation, the water phase of the reaction mixture was purified by high voltage paper electrophoresis at pH 1.9. A band located near the origin was extracted with 6 M HCl and hydrolysed. The amino acid composition of the peptide was Cya:Leu:Ser:Asp = 1:0.81:0.30:0.10 (Cya = 24.72 nano moles). Therefore amino acid sequence of subtilisin glycopeptide 8 was established as Asx-Ser-Ser-Leu-Cya.
Table 4.8  Amino Acid Composition of Glycopeptides Subjected to Sequential Analysis.

<table>
<thead>
<tr>
<th></th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub.G.P. 1</td>
<td>Asp, Arg, Thr, Ala, Tyr</td>
</tr>
<tr>
<td>Sub.G.P. 8</td>
<td>Asp, Ser, Ser, Cya, Leu</td>
</tr>
<tr>
<td>Pro.G.P. 4</td>
<td>Asp, Ser Ser</td>
</tr>
<tr>
<td>Pro.G.P. 5</td>
<td>Asp, Ser, Gly, Cya</td>
</tr>
</tbody>
</table>

1. Sub.G.P. : subtilisin glycopeptide
2. Pro.G.P. : pronase glycopeptide

Table 4.9  Amino Acid Sequence of Subtilisin Glycopeptide 8.

<table>
<thead>
<tr>
<th>Edman degradation</th>
<th>DNS-amino acid</th>
<th>Solvent system</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Asp</td>
<td>(A)</td>
<td>0.60</td>
</tr>
<tr>
<td>Step 1</td>
<td>Ser</td>
<td>(A)</td>
<td>0.50</td>
</tr>
<tr>
<td>Step 2</td>
<td>Ser</td>
<td>(A)</td>
<td>0.50</td>
</tr>
<tr>
<td>Step 3</td>
<td>Leu</td>
<td>(D)</td>
<td>0.62</td>
</tr>
</tbody>
</table>

1. See 2.19.2 for solvent systems.
Pronase glycopeptide 4. After one Edman cycle of pronase glycopeptide 4, the aqueous layer was taken to dryness, and the residue was subjected to high voltage paper electrophoresis at pH 1.9. A peptide band, located by UV illumination after spraying with fluorescamine, was eluted with 6 M HCl and the amino acid composition determined. This component contained only Ser residue and was devoid of Asp and glucosamine residues, indicating that an Asn residue is involved in linking the carbohydrate moiety, probably through 3-N-aspartamido-2-acetamino-1,2dideoxy glycopyranoside unit. On the basis of these results, the amino acid sequence and carbohydrate moiety linkage site of subtilisin glycopeptide 8 is proposed as:

\[
\text{CHO} \quad \text{Asn-Ser-Ser-Leu-Cya}
\]

where CHO represents a polysaccharide chain.

Pronase glycopeptide 5. Only one amino terminal residue was determined for pronase glycopeptide 5. DNS-Gly was identified with solvent system (A) (see 2.19.2). Therefore, the structure of pronase glycopeptide 5 is likely to be:

\[
\text{CHO} \quad \text{Gly-(Cya, Asn, Ser)}
\]

Subtilisin glycopeptide 1. N-terminal determination of this glycopeptide by dansylation failed to identify any amino terminal residue other than the possible presence of DNS-Arg on thin layer chromatography. DNS-Arg did not migrate from the origin in several solvent systems examined. When the thin layer plate was developed by solvent
system (B) (see 2.19.2) DNS-Arg migrated on the plate with an Rf value of 0.63. However, the presence of reagent spot in the same region prevented the spot from being identified unequivocally. The specific reagent for Arg (2.20.4) did not render DNS-Arg sufficiently fluorescent to distinguish it from that of the reagent spot.

After one cycle of Edman degradation of glycopeptide 1, the aqueous layer was taken to dryness, and the residue was subjected to high-voltage paper electrophoresis at pH 1.9. Two peptide bands were located by UV illumination, the faster moving band, which had stronger fluorescence under UV, migrated almost the same distance as subtilisin glycopeptide 1. This component (subtilisin glycopeptide 1.P-1) contained Arg, Asp, Thr, Ala and Tyr. The slower moving component consisted of Asp, Thr, Ala and Tyr. Arg was therefore identified as amino terminal residue of subtilisin glycopeptide 1, since the amino acid residue which had occupied N-terminus of the peptide should have been split off from the remaining part of peptide by the first Edman degradation reaction. The relative yield of the faster and slower components was approximately 1:1. The reason for incomplete first Edman reaction of subtilisin glycopeptide 1 is not clear.

After one cycle of Edman degradation of subtilisin glycopeptide 1.P-1, DNS-Asp (Rf 0.22) was identified with solvent system (c) (see 2.19.2). Solvent system (A) (see 2.19.2) was not suitable for the identification of DNS-Asp since DNS-Asp and DNS-Thr migrated on the plate with the same mobility. DNS-Ala (Rf 0.27)
was next identified by a further single Edman degradation step of subtilisin glycopeptide L.P-1 with solvent system (D) (see 2.19.2). Therefore, amino acid sequence of subtilisin glycopeptide 1 determined by two cycles of Edman degradation and three times dansylation is:

\[ \text{Arg-Asn-Ala, (Thr,Tyr)} \]

The frequent association of the sequence,  

\[ \text{CHO} \]
\[ \text{Asn-X-Ser(Thr)} \]

with carbohydrate attachment has been noted by Eylar, (1965), and by Neuberger and Marshall (1969), where X could be any amino acid residues. In the light of this rule, the amino acid sequence of subtilisin glycopeptide 1 is considered to be:

\[ \text{CHO} \]
\[ \text{Arg-Asn-Ala-Thr-Tyr} \]

The Mode of Attachment of Carbohydrate to Amino Acid in the Glycopeptides. The involvement of aspartic acid residue (probably asparagine) in linking the carbohydrate moiety was shown by Edman degradation of pronase glycopeptide 4 (Asn,Ser) followed by the amino acid analysis of the resulting component. In addition, subtilisin glycopeptides 5, 6 and 7 gave greenish yellow spots on paper when the dried chromatogram was dipped in 1% (w/v) ninhydrin in acetone (containing 2% (v/v) pyridine), and heated for 5 min at 80°C while a purple colour was obtained with all other peptides (including the other glycopeptides).
This observation may indicate the presence of a $\beta$-aspartamide linkage in the glycopeptides, since Fletcher et al. (1967) reported that the ninhydrin-pyridine reagent gave a brown staining of asparagine $1-(L-\beta$-aspartamido) -1-deoxy-$D$-glucose and 2-acetamido-1-$\beta$-(L-$\beta$-aspartamido)-1,2 dideoxy-$D$-glucose while a purple colour was obtained with all the other commonly occurring amino acid, except proline.

An attempt to isolate 2-acetamido-1-$\beta$-(L-$\beta$-aspartamido) -1,2-dideoxy-$D$-glucose ($\beta$-aspartyl acetylglucosaminylamido) by digestion of subtilisin glycopeptide 7 CHO
\[
\begin{array}{c}
\text{CHO} \\
\text{Asn-Ser}
\end{array}
\]
with pronase followed by hydrolysis in 2 M HCl (Wagh et al., 1969) was unsuccessful. The peak representing $\beta$-aspartyl acetylglucosaminyl amine was not detected in the amino acid analyzer.

4.3 DISCUSSION

Peptide Maps. There were 70 ± 4 peptides observed on the tryptic maps of both transferrins studied. The observed number is ca. 84% of the maximum number that would be expected on the basis of the amino acid composition of bovine serum transferrin. The deficiency in the number of peptides observed may be due to overlapping of peptides, failure of peptides to migrate from origin and the presence of repetitive amino acids which are susceptible to trypsin cleavage. The number of the tryptic peptides observed in the present work is far too high to suggest dissociation into half-molecules of the type proposed for human and bovine transferrin by Jeppsson (1967a) and Efremov et al.
(1971) respectively. Indeed the present work on bovine serum transferrin is in accord with that of Greene and Feeney (1968), who produced strong physical evidence for human and rabbit serum transferrin and domestic hen ovotransferrin consisting of single polypeptide chains of molecular weight ca. 78,000.

It is worth noting the usefulness of the combination of tryptic and chymotryptic peptide maps for studying bovine transferrins. These two methods worked complementarily to each other and enabled the differences to be exactly located. As an example, the peptide with amino acid substitution Lys:Arg (peptide 2) was not detected easily on the tryptic maps (Fig. 4.2), but was readily detected on chymotryptic maps (Fig. 4.4) and gave unequivocal evidence for the presence of the Lys:Arg substitution between bovine transferrin A and D2. The reverse holds in the case for the peptide with amino acid substitution Glu:Asp (peptide 1). The presence of the difference was readily apparent on the tryptic maps (Fig. 4.2) and better analytical values were obtained from these peptides than from those of chymotryptic maps (Fig. 4.4).

**CNBr Fragments of Bovine Transferrin.** The usefulness of CNBr for fragmentation of transferrins has been demonstrated by several authors (Sutton et al., 1975; Tsao et al., 1974). This is also found to be the case in the present work.

Assuming that CNBr specifically attacks the site of methionine, ten fragments should be obtained for bovine serum transferrin on the basis of its methionine content of 9 residues per molecule of protein. Six fragments, CN-2, CN-3 (containing two fragments), CN-1.04, CN-1.05 and
CN-1.06 were isolated in the present work. Peptide mapping of each CNBr fraction indicated that CN-1.01, CN-1.02 and CN-1.03 are products of aggregation of CNBr fragments. The remaining four of the ten CNBr fragments which would be expected from bovine transferrin by action of CNBr were probably eluted from the Sephadex column at the same time as the aggregated materials.

The peptides containing the difference amino acid residues were found in CN-1.02 (Lys:Arg) and CN-1.03 (Glu:Asp), both being aggregated fractions. The peptide equivalent to T-3 peptide (Asp-Lys-Lys) was found in CN-1.03 from TfA.

**Amino Acid Differences between Bovine Transferrin A and D2.**

On the basis of all the methods used in the present work it was concluded that two amino acid substitutions (A:D2; Glu:Asp, Lys:Arg) and one single amino acid residue deletion in TfD2 (A:D2; Asp: - ) exist between the two genetic variants A and D2. Since TfA moves faster than TfD2 towards the anode on zone electrophoresis at pH 7.5, it is considered that TfA carries greater negative charge than TfD2 provided both protein molecules possess similar molecular size and conformation. However, the electrophoretic difference can not be explained by the two amino acid substitution alone.

The consistent presence of T-3 (Asp-Lys-Lys) on the tryptic peptide maps of TfA and the absence of the corresponding peptide in maps of TfD2 provide evidence for deletion of the peptide (or a single amino acid residue) in TfD2. Assuming that the mutation occurs at only three
points between the two genetic variants, and that peptide T-3 is responsible for the mobility difference on zone electrophoresis, the T-3 portion of TfD₂ should carry less negative (or more positive) charge than that of T-3 from TfA. Among several possible amino acid sequences for the T-3 portion of TfD₂, only two of them can satisfactorily explain the electrophoretic behaviours of the two variants.

The first possibility is substitution of Asp by Asn (or any neutral amino acids which are consistent with RNA codons) residue in TfD₂.

TfA  Asp-Lys-Lys  
TfD₂  Asn-Lys-Lys  

The peptide portion, -Asn-Lys-Lys-, would be expected to result in a production of tripeptide Asn-Lys-Lys, or Asn-Lys and free lysine by hydrolysis with trypsin. The presence of Asn in the place of Asp will make the peptide more basic and therefore the peptide Asn-Lys-Lys should be located in about same but slightly basic region than T-3 peptide of TfA. The same argument is applicable to the dipeptide Asn-Lys. No evidence was obtained for the presence of such peptides on the tryptic peptide maps of TfD₂.

The second possibility is deletion of Asp residue in TfD₂.

TfA  Asp-Lys-Lys  
TfD₂  -Lys-Lys  

In this case trypsin digestion would produce only free lysine or the dipeptide Lys-Lys from the T-3 peptide portion of TfD₂ and the peptide corresponding to TfA.T-3 should not be observed on the map of TfD₂. However, insofar as free lysine or the dipeptide Lys-Lys is produced by tryptic digestion from the other part of polypeptide chain of bovine transferrin as well as from the T-3 portion, the deletion of a single Asp residue in TfD₂ would not result in any observable difference on the comparative peptide maps other than the presence of the extra peptide T-3 on the map of TfA.

Thus the electrophoretic mobilities on zone electrophoresis and the peptide patterns on the maps of the two genetic variants can be explained by the two amino acid substitutions and a single amino acid residue deletion in TfD₂ (A:D₂; Glu:Asp, Lys:Arg, Asp: - ) found here. However, in view of the great genetic variability in bovine transferrin, it is possible that further amino acid difference could exist between the A and D₂ variants. However, final conclusion on this possibility must await the complete knowledge of the amino acid sequences.

Carbohydrate Moiety of Bovine Transferrin. One conclusion that may be drawn from the results obtained in the present work is that variation in carbohydrate moiety is not contributing to the electrophoretic difference between a given component of the two variants. Equivalent components of both variants, A and D₂, possess the same monosaccharide in the same amount. Moreover, they contain the same number of carbohydrate moieties, which are linked to the same
amino acid residues in the respective polypeptide chain.

In the present work conclusions have also been reached concerning the amino acid sequences around the amino acid residues that are linked to the carbohydrate moieties. It is of interest to compare the results obtained with recent conclusions of Graham and Williams (1975) concerning such sequences in a variety of transferrins. They classified the glycopeptides of several species of transferrins into Type A and Type B, as follows:

\[
\begin{align*}
\text{Type A:} & & \text{CHO} \\
& & \text{Asn} & \text{Basic amino acid}
\end{align*}
\]

\[
\begin{align*}
\text{Type B:} & & \text{CHO} \\
& & \text{Asn} & \text{Neutral aliphatic amino acid}
\end{align*}
\]

However they concluded that bovine transferrin was exceptional in that the residue following Asn was occupied by Ser in both glycopeptides.

In the present work, two types of glycopeptides were isolated from the subtilisin hydrolysate of bovine transferrin. The results from the present work and Graham and Williams are compared in Fig. 4.18. Subtilisin glycopeptide 8 obviously corresponds to the 'anomalous' type 1 sequence proposed by the above authors except that Cya is found instead of Gln in the present work. In both laboratories the amino acid residue following Asn is Ser.

A second type of glycopeptide isolated in the present work, subtilisin glycopeptide 1, has a different amino acid sequence from the 'anomalous' type 2 sequence of Graham and Williams as can be seen in Fig. 4.18. The
Present work

Type 1
(subtilisin glycopeptide 8) Asn-Ser-Ser-Leu-Cya

Type 2
(subtilisin glycopeptide 1) Arg-Asn-Ala-Thr-Tyr
(pronase glycopeptide 5) Gly-(Cya,Asn,Ser)

Graham and Williams (1975)

"Anomalous" type 1 Tyr-Asn-Ser-Ser-Leu-Gln
"Anomalous" type 2 Gly-Asn-Ser-Thr-Gln-His

Figure 4.18 Amino acid sequences around carbohydrate linking amino acid residues of bovine transferrin.
'anomalous' type 2 glycopeptide sequence of Graham and Williams was not identified in the subtilisin glycopeptides, although it is not impossible that subtilisin cleaved the 'anomalous' type 2 sequence to give dipeptide Asn-Ser. 

Such glycopeptides were obtained in the present work, but, of course, they need not necessarily have arisen from this sequence.

It is not known unequivocally whether pronase glycopeptide 5 represents N-terminal region of subtilisin glycopeptide 8 or third type of glycopeptide. If the carbohydrate moieties of bovine transferrin have identical monosaccharide composition, the result from the present carbohydrate analysis of protein molecule (see 5.2.1); mannose 2, galactose 2, glucosamine 4 and sialic acid 2 residues per molecule of protein would allow the presence of only two polysaccharide side chains in the protein. Therefore, it is considered that the pronase glycopeptide 5 originates from N-terminal region of subtilisin glycopeptide 8 rather than represents a third carbohydrate linking sequence. In this case the amino acid sequence around the carbohydrate linking Asn would be:

CHO
Gly-Cya-Asn-Ser-Ser-Leu-Cya
Pro.GP.5
Sub.GP.5
Sub.GP.8
The present results also permit certain conclusions to be drawn regarding the mode of the carbohydrate linkage to the protein. Aspartic acid was the most common amino acid residue and was present in all the acid hydrolysates of glycopeptides isolated in the present work. In addition, the detection of the glucosamine peak in amino acid analysis provided strong support for the presence of an asparagine-glucosamine linkage between the carbohydrate and protein moiety in bovine transferrin, in line with similar linkage found in the other glycoproteins. Isolation of the serine after one cycle of Edman procedures from pronase glycopeptide 4 indicates the presence of the sequence; 

\[
\text{CHO} \quad \text{Asn-Ser-}
\]
CHAPTER 5. COMPARISON OF THE INDIVIDUAL COMPONENTS OF A SINGLE BOVINE TRANSFERRIN VARIANT (i) ORIGIN OF THE DIFFERENCE BETWEEN COMPONENTS I AND III, AND BETWEEN II AND IV.

5.1 INTRODUCTION

Structural variations are common features of most, if not all, glycoproteins (Marshall and Neuberger, 1970). It is known that electrophoretic heterogeneity of a given glycoprotein is often caused by several types of variation in the carbohydrate moieties. The most common variation results from partial substitution of sugar residues, usually sialic acid but sometimes fucose. The extent of their heterogeneity, as revealed by electrophoresis in gels, is markedly decreased after removal of their sialic acid residues by treatment with neuraminidase (e.g. Fetuin: Oshiro and Eylar, 1968; Orsomucoid: Schmid et al. 1962).

Variation in the carbohydrate moieties of bovine transferrin have assumed special interest since the demonstration by several workers (see GENERAL INTRODUCTION 1.6) that differences in sialic acid content partially account for the complex patterns obtained on zone electrophoresis of a single homozygous variant. However, it seems unlikely that the presence of at least six protein bands in a single variant (see 3.2.5) can depend solely on differences in the number of sialic acid residues.

In this chapter a description is given of work that is aimed at elucidating the nature of the heterogeneity shown
on zone electrophoresis of a homozygous variant (TfD₂). First an attempt is made to determine what differences occur in the carbohydrate moiety. Then possible differences in the polypeptide chain are sought.

5.2 RESULTS

5.2.1 Neuraminidase Treatment and Carbohydrate Composition

(i) Neuraminidase Treatment and Sialic Acid Determination. Fig. 5.1 shows the effect of neuraminidase on the isolated four major components of TfD₂. The mobilities of components examined decreased on gel electrophoresis after neuraminidase treatment. Although removal of sialic acid residues did not abolish entirely the heterogeneity of a single transferrin variant, simpler patterns were obtained. After treatment with neuraminidase components I and III migrated the same distance, whereas components II and IV ran the same distance, but more rapidly than I and III. This indicates that the mobility difference between I and III, and between II and IV is only due to difference in sialic acid content.

In naturally occurring substances sialic acid is often substituted at the free hydroxyl group at carbon atom 4, 7 or 8 by an acetyl residue. It is known that some 4-O-acetyl substituents sterically hinder neuraminidase and cannot be released by *V. cholerae* or *C. perfringens* neuraminidase (Schauer and Faillard 1968). These sialic acids become accessible by the use of virus neuraminidase after removal of the acetyl group by mild alkaline treatment. The possible presence of such a sialic acid residue in bovine serum transferrins was examined. Components I
Figure 5.1  Starch gel electrophoresis showing the effects of neuraminidase on isolated four major components of TfD$_2$. Tris-cacodylic acid buffer system, pH 7.5; 270 V, 4 hr. Samples from left to right:

Whole transferrin D$_2$, not treated with neuraminidase
Component I, treated with neuraminidase
Component II, treated with neuraminidase
Component III, treated with neuraminidase
Component IV, treated with neuraminidase
Whole transferrin D$_2$, not treated with neuraminidase
Electrophoresis Patterns of Components of Tf D₂D₂

Effect of Neuraminidase(N)
and II of TfD, which had been subjected to deacetylation procedures (as described in 2.15), were reacted with neuraminidase and the electrophoretic mobilities compared. The electrophoretic pattern thus obtained was identical to that of non-alkaline treated samples. This indicates that all the sialic acid in bovine serum transferrin are present in a form accessible to neuraminidase, and that the mobility difference between components I and II, and between III and IV, is not due to difference in the sialic acid content.

Sialic acid determinations were carried out as described in 2.16.1, and the results, which complement the result of neuraminidase treatment, are given in Table 5.1. It was found that components I and II possess 2 residues of sialic acid per molecule protein, whereas components III and IV contain 3 residues of sialic acid per molecule of protein.

(ii) Hexosamine. Glucosamine was identified in each component from the elution peak on the amino acid analysis profile. Glucosamine eluted from the column after 17 min (cf. 25 min for Lys) when the Beckman 120 C amino acid analyser was operated with flow rate of buffer 60 ml hr⁻¹. A galactosamine peak was not detected in any samples.

Glucosamine contents of individual components were determined as described in 2.16.2, and the results obtained are given in Table 5.2. All the components examined contain approximately 1 g glucosamine (as N-acetylated derivative) in 100 g of protein (4 residues per molecule of protein) and thus no difference in glucosamine content.
Table 5.1  Sialic Acid Content of Transferrin (D₂) Components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Sialic acid % (w/w)</th>
<th>Residues sialic acid Molecule of protein</th>
<th>Nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.775</td>
<td>1.9</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>0.810</td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>1.13</td>
<td>2.7</td>
<td>3</td>
</tr>
<tr>
<td>IV</td>
<td>1.28</td>
<td>3.1</td>
<td>3</td>
</tr>
</tbody>
</table>

Sialic acid was determined as N-acetylneuraminic acid. Molecular weight 77,000 for bovine transferrin was used for the reason described in GENERAL INTRODUCTION, 1.4.

Table 5.2  Glucosamine Content of Transferrin (D₂) Components.

<table>
<thead>
<tr>
<th>Component</th>
<th>Glucosamine % (w/w)</th>
<th>Residues glucosamine Molecule of protein</th>
<th>Nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.942</td>
<td>3.9</td>
<td>4</td>
</tr>
<tr>
<td>II</td>
<td>0.997</td>
<td>4.2</td>
<td>4</td>
</tr>
<tr>
<td>III</td>
<td>0.955</td>
<td>4.0</td>
<td>4</td>
</tr>
<tr>
<td>IV</td>
<td>1.07</td>
<td>4.4</td>
<td>4</td>
</tr>
</tbody>
</table>

Given values are average of duplicate analyses expressed as N-acetyl derivative. Molecular weight for the proteins was assumed to be 77,000 for the same reason as for Table 5.1.
(iii) Neutral Sugars. Identification and determination of neutral sugars were performed as described in 2.16.3.

Identification of neutral sugars revealed the presence of galactose and mannose in acid hydrolysates of isolated individual components of bovine transferrin $D_2$ as can be seen in Fig. 5.2. Fucose, reported to be present in porcine transferrin by Hudson et al. (1973), was not detected in any of the above samples.

Table 5.3 shows the result of quantitative determination of neutral sugars in transferrin $D_2$ components. Apparently individual components from a single transferrin variant $D_2$ contain two residues of mannose and galactose and thus no difference in neutral sugars.

It was found that recovery of sugars from a series of column procedures was 88% and the overall recovery throughout the determination procedures was 46%. The values given in Table 5.3 were the corrected ones for the recovery ratio (46%). The low recovery of sugars was consistent for separate analyses and probably due to the difficulty to transfer completely the small amount of materials from one flask to the other (see 2.16.3).

5.2.2 Comparison of Subtilisin Glycopeptides from Components I and II

The subtilisin glycopeptide fraction was prepared by column chromatography of the enzymic digest on Sephadex G-25 as described in 2.17.2. When the glycopeptide fraction was subjected to the two dimensional high-voltage electrophoresis, the two major components, I and II, gave similar distribution patterns of glycopeptides on the paper.
Figure 5.2  Thin layer chromatogram of neutral sugars after three developments with ethylacetate-pyridine-water (12:5:4 by vol.) and one with ethylacetate. The sugar spots were detected by painting with 2.5% (w/v) aniline hydrogen phthalate in acetone.
Identification of Neutral Sugars

Fu. →
Man. →
Glc →
Gal. →
Table 5.3 Hexose Contents of Transferrin (D_2) Components.

<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w)</th>
<th>Residues Molecule of Protein</th>
<th>% (w/w)</th>
<th>Residues Molecule of Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.38</td>
<td>1.6</td>
<td>0.44</td>
<td>1.9</td>
</tr>
<tr>
<td>II</td>
<td>0.42</td>
<td>1.8</td>
<td>0.53</td>
<td>2.2</td>
</tr>
<tr>
<td>III</td>
<td>0.40</td>
<td>1.7</td>
<td>0.43</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Given values are the average of four analyses. Molecular weight for the proteins was assumed to be 77,000 for the same reason as for Table 5.1.
Each electrophoretogram contained subtilisin glycopeptides 1, 5, 6 and 8 (see Fig. 4.1). Subtilisin glycopeptide 1 on the electrophoretogram of component I stained a little more intensely with ninhydrin than the corresponding peptide of component II.

The presence of two types of glycopeptide, subtilisin glycopeptide 1 and 8 (see Table 4.6), in each protein indicates that both components, I and II, possess two carbohydrate moieties which are linked to the same amino acid residues of the respective polypeptide chain.

5.2.3 Amino Acid Composition

The results of carbohydrate analyses revealed that difference between component I and II, and between III and IV is not due to the variation in carbohydrate moiety. This led me to examine possible differences in the polypeptide chain.

The amino acid compositions of the individual components of the two genetic variants are shown in Table 5.4. The number of amino acid residues was calculated in the same way as described in Table 4.1. No significant differences could be detected between the major components. Any slight differences observed are well within the reported precision of the method used (Spackman et al., 1958). The discrepancy in amino acid composition between major and minor components may be due to the fact that preparation of minor components tend to contain more impurities than major components.
<table>
<thead>
<tr>
<th></th>
<th>Transferrin A</th>
<th>Transferrin D₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Asp + Asn</td>
<td>88.0</td>
<td>88.9</td>
</tr>
<tr>
<td>Glu + Gln</td>
<td>61.5</td>
<td>61.6</td>
</tr>
<tr>
<td>His</td>
<td>16.2</td>
<td>15.8</td>
</tr>
<tr>
<td>Arg</td>
<td>23.5</td>
<td>23.2</td>
</tr>
<tr>
<td>Lys</td>
<td>60.8</td>
<td>61.6</td>
</tr>
<tr>
<td>Gly</td>
<td>47.5</td>
<td>46.6</td>
</tr>
<tr>
<td>Ser</td>
<td>43.8</td>
<td>43.9</td>
</tr>
<tr>
<td>Thr</td>
<td>34.3</td>
<td>34.3</td>
</tr>
<tr>
<td>Ala</td>
<td>52.9</td>
<td>52.7</td>
</tr>
<tr>
<td>Cys/2</td>
<td>19.9</td>
<td>17.3</td>
</tr>
<tr>
<td>Met</td>
<td>7.1</td>
<td>7.4</td>
</tr>
<tr>
<td>Val</td>
<td>38.1</td>
<td>37.2</td>
</tr>
<tr>
<td>Leu</td>
<td>52.6</td>
<td>51.6</td>
</tr>
<tr>
<td>Ile</td>
<td>18.3</td>
<td>18.3</td>
</tr>
<tr>
<td>Pro</td>
<td>31.2</td>
<td>34.4</td>
</tr>
<tr>
<td>Phe</td>
<td>28.8</td>
<td>28.2</td>
</tr>
<tr>
<td>Tyr</td>
<td>25.3</td>
<td>24.9</td>
</tr>
<tr>
<td>Trp₁</td>
<td>(10)</td>
<td>(10)</td>
</tr>
<tr>
<td>Glc.NH₂</td>
<td>13.0</td>
<td>11.9</td>
</tr>
</tbody>
</table>

Number of amino acid residues were calculated in the same way as described in 4.2.1.

1. From Hudson et al. (1973).
5.2.4 N-Terminal Amino Acid Residue

N-terminal amino acid determination of whole TfD₂ was carried out by two different methods as described in 2.19.1.

Dansylation of the protein in urea was negative. By the paper-strip modification of the Edman reaction only Ala was detected as the N-terminal amino acid. Graham and Williams (1975) have already reported the N-terminal residue of bovine transferrin to be Ala. The recovery of the alanine residue was approximately 7% of the amount that would be expected. The low recovery of N-terminal amino acid by the paper-strip method may be due to both the insufficient saturation of the paper-strip with vapour of the reaction buffer and inadequate conditions for the hydrolysis of phenythiohydantoin-Ala.

5.2.5 Peptide Mapping

(i) Tryptic Peptide Maps. Fig. 5.3 shows the peptide maps of soluble tryptic peptide from three major components of TfD₂, namely I, II and III. The patterns are essentially identical, suggesting that the primary structures of the major components from a single variant resemble one another. A comparison of the peptide maps obtained from component I with that of component IV also gave very similar patterns.

The difference in sialic acid content between component I and III did not cause any detectable change in mobility of the soluble peptides. Williams (1962) showed an identical peptide pattern between the tryptic digests from domestic hen serum transferrin and ovotransferrin which differ in sialic acid content. Two possible explanations
Figure 5.3(A) Tryptic peptide maps of components I, II and III of TfD$_2$. Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.2, except that ascending paper chromatography was carried out in the system, pyridine:isoamyl alcohol:water (7:7:6 by vol.).
Figure 5.3(B) Composite diagram of tryptic peptide map of \( \text{TfD}_2\text{I} \) showing the staining characteristic of the peptide. The diagram was obtained by staining of the peptide map with Pauly reagent (see 2.20.4).

\[
\text{T} : \text{tyrosine containing peptide} \\
\text{H} : \text{histidine containing peptide}
\]
Tryptic Peptide Map Tfd2D2.I

Electrophoresis

Tryptic Peptide Map Tfd2D2.I

Electrophoresis
of this may be suggested. It was shown in the previous chapter that subtilisin glycopeptides did not migrate far from the origin on the paper in either electrophoresis or chromatography. Tryptic glycopeptides, which would be assumed to have longer peptide sizes than subtilisin ones, are, therefore likely to be located in the crowded neutral peptide region near the origin on the paper. In addition, the colour produced for glycopeptides with ninhydrin is light gray-purple, whereas a purple colour is common to the other peptides. There is some difficulty in detecting a gray-purple coloured peptide in a crowded peptide region where all the peptide stain purple.

Amino acid compositions of the insoluble tryptic peptide fraction of two major components I and II are shown in Table 5.5. No significant difference, suggestive of one or more than one amino acid residue difference, was detected.

Fig. 5.4 shows a comparison of the peptide maps obtained from major component I with that of a mixture of minor components V and VI. The patterns are very similar, suggesting that minor components possess essentially the same amino acid sequence as a major component. Although apparent differences in the amino acid composition were observed between the major components and minor components (see Table 5.4), this was not reflected on the tryptic peptide patterns. This is probably because the minor components contain a series of impurities and the amount of each impurity is not high enough to give rise of extra peptide spots on the peptide maps but the total amount of impurities is sufficient enough to give differences in amino acid
Table 5.5  Amino Acid Composition of Insoluble Tryptic Peptide Fractions.

<table>
<thead>
<tr>
<th></th>
<th>TfD$_2$I</th>
<th>TfD$_2$II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp+Asn</td>
<td>10.2</td>
<td>10.1</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>7.3</td>
<td>7.6</td>
</tr>
<tr>
<td>His</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Arg</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Lys</td>
<td>6.7</td>
<td>6.5</td>
</tr>
<tr>
<td>Gly</td>
<td>5.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Ser</td>
<td>4.7</td>
<td>4.4</td>
</tr>
<tr>
<td>Thr</td>
<td>4.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Ala</td>
<td>6.1</td>
<td>6.2</td>
</tr>
<tr>
<td>Cys/2$^1$</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Met$^2$</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Val</td>
<td>4.1</td>
<td>4.4</td>
</tr>
<tr>
<td>Leu</td>
<td>6.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Ile</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Pro</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Phe</td>
<td>3.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.6</td>
<td>2.5</td>
</tr>
</tbody>
</table>

The number of amino acid residues was calculated from the analytical values on the same basis as for Table 4.3.

1. As cysteic acid
2. As methionine sulphone
Figure 5.4. Tryptic peptide maps of major (I) and minor (mixture of V and VI) components. Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.2.
Tryptic Peptide Map of TfD2D2

Tryptic Peptide Map of TfD2D2 (\(\varpi + \varpi\))
analyses from those of major components.

(ii) Chymotryptic Peptide Maps. Essentially identical patterns were also obtained from chymotryptic digest of individual components from a single variant (Fig. 5.5). None of the differences in Fig. 5.5 were reproducible in separate peptide preparations. Unlike the tryptic peptide maps, glycopeptides were located clearly on chymotryptic peptide maps and they are marked in Fig. 5.5 as C-G.P.

(iii) Performic Acid Diagonal Peptide Maps. The possible differences in chemical composition near disulphide bonds, or changes in secondary structure where different disulphide bridges exist, may be revealed by means of performic acid diagonal peptide mapping. Amino acid analysis of performic acid oxidised (and reduced and carboxymethylated) bovine transferrin showed the presence of 35-36 half-cystine residues per molecule of protein. Since all of these residues are involved in disulphide bonds in the polypeptide chain of the transferrin (Schultze and Schwick, 1957), there was a possibility that the pattern of diagonal peptide maps is too complex for any differences to be evident.

Performic acid diagonal peptide maps were prepared from pepsin digests of whole TFD₂ and TFD₂I, as described in 2.20.3, and the patterns compared. Quite complex patterns were obtained. Although gross patterns are similar to one another, no definite conclusion can be drawn from the results.

5.3 DISCUSSION

Carbohydrate Composition. Results of the carbohydrate analysis of bovine transferrin by several authors are summarized in Table 5.6. The carbohydrate composition of
Figure 5.5  Chymotryptic peptide maps of components I and II of TfD₂. Maps were obtained from the chymotryptic digests of oxidised proteins by the same procedures as for Fig. 4.2.
Table 5.6  Comparison of The Results of Carbohydrate Composition of Bovine Serum Transferrin by Several Authors.

<table>
<thead>
<tr>
<th></th>
<th>Hudson et al. (1973)</th>
<th>Hatton et al. (1974)</th>
<th>Graham and Williams (1975)</th>
<th>Present work</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW used</td>
<td>77,300</td>
<td>76,000</td>
<td>-</td>
<td>77,000</td>
</tr>
<tr>
<td>Total Carbohydrate</td>
<td>3.04%</td>
<td>2.86%</td>
<td>-</td>
<td>2.83%</td>
</tr>
<tr>
<td>Total hexoses</td>
<td>1.21%</td>
<td>1.34%</td>
<td>-</td>
<td>0.87%</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.49% (2.1)</td>
<td></td>
<td>(6.2)</td>
<td>0.39% (1.7)</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.72% (3.5)</td>
<td>(5.6)</td>
<td>(7.2)</td>
<td>0.47% (2.0)</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.02% (3.5)</td>
<td>0.90% (3.0)</td>
<td>(8.8)</td>
<td>0.88% (4.2)</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.06% (0)</td>
<td>0.02% (0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>0.74% (1.84)</td>
<td>0.60% (1.5)</td>
<td>(2.0)</td>
<td>0.98% (2.45)</td>
</tr>
</tbody>
</table>

Values are expressed in terms of %(w/w) of protein. Values in parentheses are the number of residues per molecule of protein calculated on the basis of the molecular weight given in the first column.

1. Values are average of four major components.
bovine transferrin is generally typical of mammalian glycoproteins. The carbohydrate content of bovine transferrin found in the present work is similar to those reported by Hudson et al. (1973) and Hutton et al. (1974) except that somewhat lower amount was determined for mannose in the present work. Graham and Williams (1975), on the other hand, reported significantly higher values for neutral sugars and glucosamine. The wide variation in the values reported for the sugar contents of transferrin of a single species has not been satisfactorily explained. As an example of possible explanation of this, it is known that many pathological states are associated with marked changes in the level of protein-linked carbohydrate in serum (Winzler, 1960). Another possibility may be sought in the degree of purity of the protein isolated by individual workers.

Prior to the present work no report has been made on the complete carbohydrate compositions of the isolated individual components from a single bovine transferrin variant. The present work clearly demonstrated that all the components from a single variant contain the same monosaccharides in the same amount, except sialic acid. This indicates that the heterogeneity of bovine transferrin is not either conferred by the presence or absence of carbohydrate moiety (e.g. ribonuclease A and B, Plummer and Hirs, 1964) or by the size difference of polysaccharide chain linked to the polypeptide chain (e.g. ribonuclease B, C and D, Tarentino et al., 1970).

Sialic Acid Content and Heterogeneity of Bovine Transferrin.

Earlier studies on the effect of neuraminidase treatment on
bovine transferrin suggested stepwise removal of sialic acid residue (Parker and Bearn, 1962a). More recently Chen and Sutton (1967) isolated three individual components from a single homozygous variant then subjected them to neuraminidase treatment and sialic acid analysis. On the basis of the patterns that were observed on polyacrylamide gel electrophoresis of neuraminidase treated samples, they concluded that the complexity of the homozygous transferrin patterns can be explained entirely by differing numbers of sialic acid residues. However, the insufficiency of the gel electrophoresis systems used to separate all of the bands present in the transferrin was pointed out by Spooner and Baxter (1969). In a subsequent report Stratil and Spooner (1971) demonstrated that the heterogeneity of bovine transferrin from a homozygote was only partially due to the sialic acid content.

Fig. 5.6 shows the effects of neuraminidase treatment on bovine transferrin and the results of sialic acid determination reported by several authors. Results from the present work are similar to those obtained by Stratil and Spooner (1971). When bovine transferrin from a homozygote was treated with viral neuraminidase, this heterogeneity was notably reduced, but not abolished. A variable distribution of sialic acid residues in the carbohydrate moiety accounts, therefore, partially for the marked heterogeneity exhibited by bovine transferrin during electrophoresis on starch gel. Thus the difference between components I and III, and between II and IV is due to variation in sialic acid content. But this is not the origin of the difference between components I and II, and between III and IV.
Effect of neuraminidase treatment on gel electrophoretic patterns of bovine transferrin and sialic acid content. Comparison of the present result with those of Chen and Sutton (1967) and Stratil and Spooner (1971).

Acryl. = Polyacrylamide gel electrophoresis
Starch = Starch gel electrophoresis
The rapid and complete cleavage of sialic acid by mild acid hydrolysis or by *V. cholerae* neuraminidase suggests that it occupies a terminal position. Spooner and Baxter (1969) reported the finding that mild treatment of transferrin with neuraminidase produced slowing of the faster two major components (III and IV) but not of the slower two (I and II). It was interpreted as a suggestion that the first sialic acid residues to be removed by the enzyme are more weakly linked to the faster two major components than to the slower components. This phenomenon was not observed in the present work probably because of using a neuraminidase preparation that was sufficiently active to release rapidly all the sialic acid from the protein.

Removal of two or three sialic acid residues from the protein molecule provided a sufficient decrease in mobility for the treated protein to appear different from the non-treated protein. This indicates that net charge of bovine transferrin is relatively small at pH 7.5.

**Polypeptide Chain of Component I and II.** Oxidised tryptic digest and chymotryptic digest of oxidised samples of components I and II, and III and IV gave the identical peptide distribution patterns, indicating that four major components have the same amino acid sequences. It appears that difference in the amino acid composition is not responsible for that part of heterogeneity which is unrelated to sialic acid, namely difference between components I and II, and between III and IV. Similarity of peptide patterns obtained from isolated individual bovine transferrin components
has been reported also by Chen and Sutton (1967) and Richardson et al. (1973).

No definite conclusion may be drawn from the present determination of the amino-terminal residue concerning the number of polypeptide chains in bovine transferrin. Although only Ala was determined to be the N-terminus for whole TfD2, recovery of Ala is too low to exclude possible presence of any other N-terminus in the protein.

Thus in this chapter the marked heterogeneity exhibited by a homozygous variant on zone electrophoresis was only partially elucidated. The difference between components I and III, and between II and IV is due to sialic acid content. However, the difference between components I and II, and between III and IV is not due to carbohydrate content and not apparent from simple peptide mapping. Therefore further studies are made in the following chapter to elucidate the cause of the remaining heterogeneity (difference between I and II, and between III and IV) by approaches different from those made in the present chapter.

6.1 INTRODUCTION

It was found in the previous chapter that the occurrence of multiple iron binding components of a single genetic variant is partially due to difference in sialic acid content. Thus the difference between components I and III, and between II and IV was shown to be due to the difference in sialic acid content. However sialic acid differences do not account for the difference between components I and II, and between III and IV. Components I and II, and III and IV possess the same monosaccharides in equal amount. Moreover components from a single variant gave identical tryptic and chymotryptic peptide patterns (Chapter 5).

A difference in the electrophoretic behaviour on urea-starch gel was observed between components I and II, and between III and IV. It was considered that further investigation of this behaviour might throw light on the origin of the difference between components I and II, and between III and IV. In the present chapter the nature of the bands, which are observed after cleavage of disulphide bonds in transferrin on urea-starch gel electrophoresis, is examined and further studies are made on the difference between components I and II, and between III and IV. It was
shown that a peptide (MW ca. 10,000) is released from components II and IV by cleaving the disulphide bond of the proteins, but this is not the case for components I and III.

6.2 RESULTS

6.2.1 Urea-Starch Gel Electrophoresis of Disulphide Bond Cleaved Transferrin

(i) Individual Components (TfD₂) Reduced in 7 M Urea, on 7 M Urea-Starch Gel in Presence of Reductant. Starch gel electrophoresis of reduced transferrin samples in 7 M urea-tris-glycine buffer, pH 8.8, was carried out as described in 2.10.

As can be seen in Fig. 6.1, reduction of components I and III produced one band only and this is located on the anodic side of the sample insertion slot, whereas components II and IV resulted in production of two bands; one on the anodic side and the other on the cathodic side. The major band, which migrated toward the anode was designated $S_{para}$ and the minor sharp band moving toward the cathode designated $S_{pep}$. The staining intensity of $S_{pep}$ was approximately one third of that of $S_{para}$ as judged by visual inspection. $S_{pep}$ from components II and IV migrated the same distance, whereas $S_{para}$ from individual components showed difference in mobilities. The order of migration distance of $S_{para}$ for the four major components was: IV > II > III > I. Use of dithiothreitol as reductant in the place of 2-mercaptoethanol resulted in similar type of patterns.
Figure 6.1  Starch gel electrophoresis of reduced transferrins. Tris-glycine-urea buffer system containing 2-mercaptoethanol, pH 8.8; 300 V, 10 hr. Samples from left to right:

Whole transferrin D2
Component I
Component II
Component III
Component IV
Whole transferrin D2
Electrophoresis of Tf \( D_2 D_2 \)
in 7M Urea—Starch Gel, pH 8.8
(ii) Whole Transferrin (TfD₂) 7 M Urea, on 7 M Urea-Starch Gel Without Reductant. Very faint presence of S$_{pep}$ was observed on urea gel electrophoresis (without reductant) with whole transferrin dissolved in 7 M urea solution (without reductant).

(iii) Whole Transferrin (TfD₂) reduced in 7 M Urea, Dialysed Against Water, on 7 M Urea-Starch Gel in Presence of Reductant. When whole transferrin was reduced in 7 M urea and the reaction mixture dialysed against water, freeze-dried and dissolved again in 7 M urea in the presence of reductant, S$_{pep}$ was not observed on the urea gel electrophoresis. The most likely place S$_{pep}$ was lost was in the dialysis step.

(iv) Whole Transferrin (TfD₂) Reduced and Carboxymethylated in 7 M Urea, on 7 M Urea Starch Gel Without Reductant. The urea starch gel electrophoresis of reduced and alkylated whole transferrin D₂ was performed. Reduction and carboxymethylation of the transferrin were performed as described in 2.12.2. A complex pattern was obtained as shown in Fig. 6.2. Among the four bands observed, two sharp bands moved towards the cathode, and the other two broad bands migrated towards the anode. By carboxymethylation of the reduced whole TfD₂, S$_{para}$ of reduced sample decreased in its mobility and stayed near origin leaving a faint band in front.

Amino acid analysis of reduced and carboxymethylated sample (after 2 hr reaction) revealed complete conversion of half-cystine residues into S-carboxymethylated half-cystines. The latter was eluted from the column of
Figure 6.2  Starch gel electrophoresis of reduced and carboxymethylated whole transferrin D₂. Tris-glycine-urea buffer system without reductant, pH 8.8; 300 V, 10 hr.
Samples:

Left: reduced whole transferrin D₂.
Right: reduced and carboxymethylated transferrin D₂.
Electrophoresis of
(a) Reduced Tf D2D2 and (b) ScM–Tf D2D2
Beckman 120 C amino acid analyzer after 108 min (cf. 115 min for Asp) under the conditions used (see 2.18). Homocitrullin, which has been reported to be formed by reaction of several proteins with cyanate ion present in a concentrated urea solution (Stark et al., 1960; Manson, 1962) was not detected during amino acid analysis. This was reasonable in view of precautions taken to minimise presence of cyanate ion in the urea used.

(v) Individual Components (TfD₂) Performic Acid Oxidised, on 7 M Urea-Starch Gel (Without Reductant). Electrophoretic analysis on urea-starch gel was made of performic acid oxidised components I, II, III, and IV. The patterns of components I and III were similar, and exhibited one strongly staining band and one weak faster moving band. On the other hand, the patterns of components II and IV were similar to one another, but exhibited two strongly staining bands and one weak faster moving band. These patterns were not further investigated, as it was found more profitable at this stage to concentrate on the work with reduced (and carboxymethylated) samples.

(vi) Interpretation of the Results Obtained from Urea-Starch Gel Electrophoresis Experiments. In urea-starch gel electrophoresis of the fully reduced or oxidised proteins there are no disulphide bonds present. Therefore the numbers of bands observed on the gel solely depends on the number of polypeptide chains present. Results of urea-starch gel electrophoresis of disulphide bonds cleaved transferrin components by reduction showed the presence of two bands on the gel in II and IV, whereas
components I and III gave only one band. This clearly indicates that there is an internal cleavage in the polypeptide chain of components II and IV, and that cleavage of the disulphide bonds resulted in the production of two fragments from these two components. Thus, the difference between components I and II, and between III and IV is due to the presence of a cleavage in the polypeptide chain in components II and IV.

6.2.2 Amino Acid Composition of $S_{pep}$

Attempts were made to recover $S_{pep}$ directly from the starch gel after completion of the electrophoresis. A method, which has been often used to recover proteins from gels, involving disintegration of the gel structure by freezing and thawing, was unsuccessful because of high concentration of urea in the gel.

The direct hydrolysis in 6 M HCl of the gel portion, which contains $S_{pep}$ as well as tris, glycine and starch, resulted in a production of dark red-brown coloured hydrolysate and the colour could not be removed by passing the hydrolysate, diluted with water and adjusted to pH 2 with HCl, through a Dowex 50H$^+$ column (1 x 5 cm). Analysis of the hydrolysate on the amino acid analyzer yielded numerous peaks representing by-products in addition to amino acids. The amino acid analysis was affected by the presence of the by-products' peaks.

It was found, however, that extraction with 7 M urea of the starch gel portion containing $S_{pep}$ and subsequent separation of the starch by centrifugation gave an urea solution fairly free from soluble starch. Tris and
glycine were removed from the extract, together with urea, by exhaustive dialysis against water. The precipitate was removed from the mixture in the dialysis casing by centrifugation and the supernatant lyophilised. The absence of peptide material in the supernatant was shown by amino acid analysis. Therefore, the whole mixture (including the precipitated residue) in the dialysis casing was brought to dryness by rotary evaporation and the resulting residue was hydrolysed in 6 M HCl. After removal of the humin in the hydrolysate by centrifugation the light brown coloured HCl solution containing amino acids was evaporated. The caramel-like colour of the residue was removed by passage of the acidic aqueous solution of the hydrolysate through a Dowex 50H⁺ column. After washing the column with water amino acids were eluted with 1 M NH₃ and any ammonia removed from the amino acid residue by rotary evaporation.

Table 6.1 shows the amino acid composition of Sₚₑₚ and Sₚ₂ra. The numbers of amino acid residues were calculated from the analytical results on the basis of approximate molecular weight 10,000 for Sₚₑₚ in the light of the possible penetration of Sₚₑₚ through dialysis casing (see 6.2.1.iii). No homocitrullin was detected in either samples. Summation of the number of each amino acid residue in Sₚₑₚ and Sₚ₂ra should theoretically give equal number of respective amino acid in TfD₁II, if Sₚₑₚ is a peptide cleaved from TfD₁II. Table 6.1 shows the presence of fairly large deviation in the number of several amino acid residues. This is probably due to the fact that the procedures of separation to obtain samples for the amino acid analysis of
Table 6.1  Amino Acid Composition of $S_{pep}$ and $S_{para}$.

<table>
<thead>
<tr>
<th></th>
<th>$S_{pep}$</th>
<th>$S_{para}$</th>
<th>TFD2II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp+Asn</td>
<td>9.6</td>
<td>80.5</td>
<td>90.7</td>
</tr>
<tr>
<td>Glu+Gln</td>
<td>10.4</td>
<td>68.5</td>
<td>60.6</td>
</tr>
<tr>
<td>His</td>
<td>0.9</td>
<td>9.3</td>
<td>15.7</td>
</tr>
<tr>
<td>Arg</td>
<td>0.5</td>
<td>20.8</td>
<td>24.1</td>
</tr>
<tr>
<td>Lys</td>
<td>7.4</td>
<td>48.2</td>
<td>61.4</td>
</tr>
<tr>
<td>Gly</td>
<td>17.7</td>
<td>46.7</td>
<td>51.8</td>
</tr>
<tr>
<td>Ser</td>
<td>8.2</td>
<td>43.4</td>
<td>41.1</td>
</tr>
<tr>
<td>Thr</td>
<td>4.2</td>
<td>26.8</td>
<td>34.0</td>
</tr>
<tr>
<td>Ala</td>
<td>9.1</td>
<td>55.4</td>
<td>54.2</td>
</tr>
<tr>
<td>Cys/2</td>
<td>1.5</td>
<td>7.8</td>
<td>19.1</td>
</tr>
<tr>
<td>Met</td>
<td>1.9</td>
<td>10.1</td>
<td>8.1</td>
</tr>
<tr>
<td>Val</td>
<td>6.6</td>
<td>42.6</td>
<td>38.1</td>
</tr>
<tr>
<td>Leu</td>
<td>10.1</td>
<td>53.6</td>
<td>52.5</td>
</tr>
<tr>
<td>Ile</td>
<td>4.6</td>
<td>23.9</td>
<td>18.8</td>
</tr>
<tr>
<td>Pro</td>
<td>4.7</td>
<td>29.2</td>
<td>27.6</td>
</tr>
<tr>
<td>Phe</td>
<td>2.6</td>
<td>17.6</td>
<td>28.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.7</td>
<td>11.5</td>
<td>23.6</td>
</tr>
</tbody>
</table>

The number of amino acid residues was calculated by assuming the molecular weight 10,000 and 64,800 for $S_{pep}$ and $S_{para}$ respectively.
$S_{\text{pep}}$ and $S_{\text{para}}$ are too complex to enable an accurate comparison of the analysis to that of TfD$_2$II.

6.2.3 Peptide Maps of Dialysed, Disulphide Bond Cleaved Proteins

Dialysis of a reduced whole transferrin TfD$_2$ sample against water resulted in absence of $S_{\text{pep}}$ from urea gel electrophoresis pattern (see 6.2.1.iii). This indicates the possibility that some small part (less than 10,000 MW) of the polypeptide chain of transferrin penetrates through the membrane of Visking dialysis casing during dialysis of the reduced transferrin sample.

Dialysed, Reduced Protein. In order to examine such a possibility comparative peptide maps were prepared from two samples of component II, which were differently treated. (Since $S_{\text{pep}}$ appears to be a typical band only for components II and IV, but not for I and III, component II was chosen for this purpose). One sample was reduced with dithiothreitol in 7 M urea. The reaction mixture was at first dialysed against 7 M urea solution and then water. The precipitated protein was recovered by lyophilisation of the whole solution in dialysis casing. The other sample was intact component II. These two samples were heated at 95°C for 10 min and then subjected to the ordinary procedures for peptide mapping (see 2.20.1). As can be seen in Fig.6.3 several peptides are staining lightly in the map (B) of the material which had been dialysed compared to the equivalent peptides in the map (A) of the material prepared without dialysis. The peptides with different staining intensity are marked in the figures.
Figure 6.3  Tryptic peptide maps of TfD$_2$II.  
Top (A) : heat denatured TfD$_2$II was digested with trypsin and the resultant digest was oxidised with performic acid. 
Bottom (B) : TfD$_2$II was reduced and dialysed against 7 M urea and then water. The sample was then subjected to the same treatments as for the above sample. 
Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.2.
Dialysed, Reduced and Carboxymethylated Proteins. Further evidence for the presence of a small peptide, which can permeate through the membrane of the Visking dialysis casing, in the disulphide bonds cleaved component II was obtained from comparative peptide maps of component I and II, where the samples were prepared by dialysing the reduced and carboxymethylated proteins.

As can be seen in Fig. 6.4, several peptides have stained lightly on the map of component II compared to the corresponding ones on the map of component I, as marked in the figures. These peptides were eluted from chromatograms and the amount of Lys or Arg examined. The results are summarized in Table 6.2. Approximately less than half of the amount was recovered from peptides 1, 2, 3, 4 and 6 on the map of component II compared to equivalent peptides on the map of component I. At the same time several peptides which had stained with ninhydrin to equal darkness on the two maps were analysed on the amino acid analyzer as 'standards'. The amino acid contents of the equivalent peptides, however, varied with peptide. The variation in values obtained may be due to the difficulty in extracting the peptide quantitatively from chromatogram, although equivalent peptides should give similar yield. Less than 20% recovery of peptide from paper separations can be expected with the procedures used. Therefore, final interpretation of the results from peptide mapping is left with the pictorial evidence where comparative peptide maps of component II (Fig.6.3) and comparative peptide maps of reduced and carboxymethylated component I and II (Fig.6.4) gave at least
Figure 6.4  Tryptic peptide maps of reduced and carboxymethylated TfD₂I and TfD₂II. Samples were dialysed after reduction and carboxymethylation. Maps were obtained from the soluble tryptic peptide fractions by the same procedures as for Fig. 4.2.
Table 6.2  Lysine or Arginine content of the peptides which were stained with different intensity on comparative peptide maps of reduced and carboxymethylated components I and II (Fig. 6.4).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TfD₂I</td>
<td>3.5 (Lys)</td>
<td>2.8 (Lys)</td>
<td>3.5 (Lys)</td>
<td>5.4 (Arg)</td>
<td>5.0 (Lys)</td>
</tr>
<tr>
<td>TfD₂II</td>
<td>1.3 (Lys)</td>
<td>1.0 (Lys)</td>
<td>1.8 (Lys)</td>
<td>2.1 (Arg)</td>
<td>2.2 (Lys)</td>
</tr>
</tbody>
</table>

The values are expressed in terms of nano moles of residue in a peptide.
two same peptides which have stained with different intensity (Peptide 3 and 4 in Fig. 6.4). The observation that some peptides staining with different intensity in Fig. 6.3 are different from those in Fig. 6.4 may be attributable to the experimental fact that disulphide bonds of peptides were oxidised in Fig. 6.3 whereas they were reduced and carboxymethylated in Fig. 6.4.

Relative staining intensity of peptide 5 is the reverse of those of the other peptides. The peptide was more strongly stained on the map of component II than the equivalent one in the map of component I. This phenomenon may reflect the relative position of peptide 5 in the polypeptide chain of component II. It is reasonable to speculate that the peptide 5 may be located in the polypeptide chain of component II, so that its N- or C-terminus represents the internal N- or C-terminal amino acid residue of component II. Yield of this peptide by tryptic digestion may well be higher from component II than from component I where trypsin has to cleave polypeptide chain at two places to produce the peptide 5.

6.3 DISCUSSION

Urea-starch gel electrophoresis pattern of disulphide bond cleaved transferrin components showed the presence of two peptides in components II and IV, and only one in components I and III. This indicates that the difference between components I and II, and between III and IV is due to an internal cleavage existing in polypeptide chain of components II and IV.
Comparative peptide mapping between intact components II and dialysed, reduced component II; and between dialysed, reduced and carboxymethylated components I and II, provided further evidence for the presence of the internal cleavage in the polypeptide chain of components II and IV. Dialysis of the disulphide bond cleaved component II resulted in the production of several peptides which were stained lightly on the peptide maps compared to equivalent ones on the maps of intact component II and dialysed, reduced and carboxymethylated component I respectively.

The molecular weight of the smaller peptide ($S_{pep}$) is assumed to be approximately 10,000 since the peptide appears to permeate partially through the membrane of the Visking dialysis casing.

In the light of results obtained in the present work, hypothetical structures for components I and II are schematically drawn and shown in Fig. 6.5. In these structures, components I and II (or III and IV) possess exactly the same chemical composition for both amino acid and carbohydrate. The only difference existing between them is the presence of "trypic-like" cleavage in the polypeptide chain of component II. This cleavage, shown in N-terminal region in Fig. 6.5, could be the C-terminal region of component II. This internal cleavage produces an additional amino and carboxy terminus in component II. This might result in a slightly more negative charge for component II compared to component I since an α-carboxy group (pK ca. 2.2) is considered fully negatively charged in normal environment whereas an α-amino group (pK ca. 9.6)
Figure 6.5  Schematic hypothetical structures for component I(III) and II(IV) of bovine transferrin. The internal cleavage shown in the N-terminal region of component II could be in the C-terminal region. The number of disulphide bonds shown in the figures does not represent the actual number.
may be only partially positively charged at pH 7.5, the pH of tris-cacodylic acid buffer system. This slight difference in charge may well be reflected in the mobility difference observed on zone electrophoresis at pH 7.5, where component II moves more rapidly than component I. Tryptic peptide maps would not reveal the difference between the two components, I and II, as long as cleavage in the polypeptide chain of component II is "tryptic-like" one. Such an internal cleavage of peptide should be able to be detected by N-terminal amino acid assay. Only Ala was detected as the N-terminal amino acid of whole transferrin D2 (see 5.2.4). However, this does not exclude possible presence of the internal cleavage. There may be two possible explanations of this: (i) recovery of Ala (7%) is too low to exclude the possible presence of the other N-terminal amino acid. (ii) Ala could be the second N-terminus in component II. For instance, in the two naturally occurring fragments of concanavalin A, both possess Ala as N-terminus (Wang et al., 1971).

While most of the sedimentation equilibrium results suggest that transferrin consist of only a single chain as discussed in GENERAL INTRODUCTION 1.4, they do not by themselves preclude the alternative possibility to a single polypeptide chain structure. It is that transferrin is composed of two polypeptide chains, one of which is a relatively small peptide. The presence of a small molecular weight (5,000–10,000) polypeptide chain in transferrin could possibly escape detection in some sedimentation equilibrium
studies. Richardson et al. (1973) obtained molecular weight 66,400 for bovine transferrin by sedimentation equilibrium in 6 M guanidinium hydrochloride pH 8.0 (cf. 77,500 in phosphate-NaCl buffer pH 7.6).

Thus, in the light of the results obtained in the present work it is concluded that the difference between components I and II, and between III and IV is the presence of an internal cleavage in the polypeptide chain of components II and IV.
1. Bovine serum transferrin was isolated from the blood of animals homozygous for the variants, A and D₂, by gentle procedures involving ammonium sulphate fractionation and column chromatography. Ammonium sulphate fractionation of plasma did not give clear cut separation of transferrin from the other proteins. However, the third (NH₄)₂SO₄ fraction was found to be rich in transferrin and served as starting material for the isolation of transferrin.

Crude transferrin was obtained in large amount by passing the third (NH₄)₂SO₄ fraction through DEAE-Sephadex A-25 column, pH 6.85, at 3°C. Further purification of transferrin was performed by column chromatography on DEAE-Sephadex A-50, pH 7.10, at 20°C. Starch gel electrophoresis pattern of this transferrin preparation showed no presence of other proteins.

2. Complex patterns were obtained for each variant on starch gel electrophoresis in several buffer systems; one in particular tris-cacodylic acid, pH 7.50, was found to have the best resolution and was used in further studies. On the basis of these patterns, in which zones were detected by staining with nigrosin and by autoradiography with Fe⁵⁹, it was concluded tentatively that there are four major and two (or possibly more) minor components in each variant. The four main and two of the minor iron binding fractions were isolated by column
chromatography on DEAE-Sephadex A-50 and found to give rise to single bands in the correct respective positions on zone electrophoresis. Rechromatography of the isolated component did not change the electrophoretic pattern exhibited by non-rechromatographed sample. The fractions were designated I-VI in order of increasing mobility.

3. Differences between component I of the two genetic variants, A and D₂, were examined. Amino acid analysis did not reveal any clear difference in the amino acid compositions of the two variants. However, tryptic and chymotryptic peptide mapping of the proteins showed that at least two amino acid substitutions occur: A:D₂, Glu:Asp and Lys:Arg. In addition a single amino acid deletion was indicated for D₂ (A:D₂, Asp: - ). The presence of these amino acid substitutions and Asp deletion in D₂ was also confirmed by tryptic peptide mapping of cyanogen bromide fragments. These two amino acid substitutions and one amino acid deletion (found in the present work) account for the electrophoretic behaviour and peptide distribution patterns of the two variants. However in view of the molecular size and genetic variation of bovine serum transferrin there may be further difference in the polypeptide chains of the two variants in addition to those found in the present work although definite conclusion must await complete knowledge of the amino acid sequence of bovine transferrin. The presence of three or more differences in amino acid residues in bovine transferrin variant is in contrast to the transferrin variant of human, where only one amino acid substitution has been detected and it is sufficient to explain their electrophoretic mobilities (see 1.5).
Difference in electrophoretic mobilities between A and D2 remained after performic acid oxidation of the proteins, indicating both variants have a similar overall conformation.

No difference was detected between the carbohydrate composition of the whole protein of the two variants. Both variants contain 2 mannose, 2 galactose, 4 glucosamine, 2 sialic acid.

4. Glycopeptides were prepared from whole transferrin A and D2 by digestion with subtilisin and pronase. The two genetic variants yielded glycopeptides with the same amino acid compositions. Amino acid sequence determination of some of the subtilisin glycopeptides yielded the two sequences:

\[
\begin{align*}
\text{CHO} & \\
\text{Asn-Ser-Ser-Leu-Cya} & \\
\text{and} & \\
\text{CHO} & \\
\text{Arg-Asn-Ala-Thr-Tyr} &
\end{align*}
\]

The first sequence is very similar to, but not identical with, one reported by Graham and Williams (1975), but the second sequence is quite different. The pronase glycopeptide 5, Gly-(Cya,Asn,Ser), could overlap with the first sequence. It was found, carbohydrate moieties are linked to the polypeptide chain through glucosamine-asparagine linkage in line with the majority of plasma proteins.
5. The heterogeneity of a single variant was studied. The differences between components I and III, and between II and IV are explained in terms of the difference in sialic acid content. Component III contains three residues of sialic acid per molecule of protein and Component I possesses two residues. The electrophoretic difference between the two components was abolished by treatment of the components with neuraminidase. This was the case also for the relation between component II and IV. However the difference between I and II (and III and IV) can not be explained in this way. Electrophoretic difference between component I and II, and between III and IV remained even after treatment with neuraminidase. Components I and II have the same carbohydrate composition and their carbohydrate moieties are linked in the same way to their respective peptide chains. The tryptic and chymotryptic peptide maps of the two components are identical. The same is also the case for components III and IV indicating that the four major components of bovine transferrin possess the identical amino acid sequence. A possible cause of the difference between components I and II, and between III and IV was sought in the presence of an internal cleavage of the polypeptide chains of some of the components. However, whole transferrin D2 yielded only alanine as N-terminal residue, although the recovery was too low (7%) to draw any definite conclusion with respect to the number of polypeptide chains in bovine transferrin.

6. Components I and II showed different behaviour on urea starch gel electrophoresis after cleavage of
disulphide bonds. With reduced proteins a single band was obtained with components I and III, whereas component II and IV each gave two bands, indicating the presence of an internal cleavage in the polypeptide chain of component II and IV. The presence of an internal cleavage in polypeptide chain of component II and IV resulted in the production of two fragments by the cleavage of the disulphide bonds. Electrophoretic separation in urea gel of the components after oxidation gave similar results. An amino acid composition of the peptide material contained in the extra band was obtained after elution of this band from a gel of the reduced component II.

Tryptic peptide maps of component I and II which had been reduced and carboxymethylated in urea (a procedure which involves dialysis) resulted in the presence of several peptides staining lightly in component II. Similarly peptide maps of component II which had been reduced in urea and dialysed prior to tryptic digestion, showed apparently the same peptide to have been lost when compared with maps of component II which had not been reduced and dialysed from urea then water.

One interpretation of these results suggesting that the difference between components I and II (and III and IV) involves a cleavage of the peptide chain in component II (and IV) to yield a dialysable fragment after reduction (or oxidation) of the disulphide bonds is presented.

The origin of the fragments is not known. They may result from proteolytic cleavage prior to the isolation, analogous to the activation process of chymotrypsinogen A,
where the process starts from the cleavage of the bond between Arg₁₅ and Ile₁₆ by trypsin (Wilcox, 1970). The possibility that these fragments result during preparation is unlikely since all of four major components are observed on starch gel electrophoresis of serum. The presence of unusual linkage in bovine transferrin (e.g. esters) that are cleaved either chemically or enzymatically cannot be excluded.

Recently Spooner et al. (1970) observed strikingly different patterns in bovine foetal transferrins compared with the adult pattern. In foetal transferrins there are only three bands. They correspond to I, III, V in the present work, while in adult cattle the pattern is six banded (I–VI). The gradual change from the three to six banded pattern took place over a relatively short period before term.

In recent years, it has become evident that many physiologically active proteins are synthesized as inactive precursors in the foetal life and that these are subsequently converted in the adult life to physiologically active forms by the selective enzymatic cleavage (limited proteolysis) of peptide bonds. It has been found that virtually all the activation processes require the enzyme-catalyzed cleavage of a unique peptide bond by "limited proteolysis". A review has been recently made by Neurath and Welsh (1976) of the role of proteolytic enzymes in the activation processes of precursors of physiologically important proteins.
Analogous to the above protein activation process, the cause of change of bovine serum transferrin pattern from three to six banded before term may be sought in the limited cleavage of the polypeptide chains of components I, III and V.

Thus, the relationship of the differences among the major four components of a single bovine transferrin variant may be expressed as drawn below schematically on the basis of the results obtained in the present work.

\[
\begin{align*}
\text{proteolytic cleavage} & \quad \text{component I} \quad \rightarrow \quad \text{component II} \\
\text{difference in sialic acid} & \quad \downarrow \quad \uparrow \text{difference in sialic acid} \\
\text{component III} & \quad \rightarrow \quad \text{component IV} \\
\text{proteolytic cleavage} & 
\end{align*}
\]
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